UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE TECNOLOGIA E GEOCIÊNCIAS
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PROGRAMA DE PÓS-GRADUAÇÃO EM OCEANOGRAFIA

Population genetics and conservation strategies for the West Indian manatee (*Trichechus manatus* Linnaeus, 1758) in Brazil

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This thesis is dedicated to:

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The one process now going on that will take millions of years to correct is the loss of genetic and species diversity by the destruction of natural habitats.

Edward. O. Wilson

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LIST OF ABBREVIATIONS

- **Organizations**

- ABI - Applied Biosystems
- AQUASIS – Associação de Pesquisa e Preservação de Ecossistemas Aquáticos (Association for Research and Preservation of Aquatic Ecosystems).
- CMA - Centro Nacional de Pesquisa e Conservação de Mamíferos Aquáticos (National Center for Research and Conservation of Aquatic Mammal)
- FWRI - Florida Fish and Wildlife Research Institute
- GEOMARE – Investigação Terrestre e Marinha (Terrestrial and Marine Research)
- IBAMA - Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (Brazilian Institute of Environment and Renewable Natural Resources)
- ICMBio - Instituto Chico Mendes para Conservação da Biodiversidade (Chico Mendes Institute for Biodiversity Conservation)
- IMA - Instituto Mamíferos Aquáticos (Aquatic Mammal Institute)
- LABOMAR – Instituto de Ciências do Mar (Institute of Marine Sciences)
- MMA - Ministério do Meio Ambiente (Environmental Ministry)
- REMANE - Rede de Encalhe e Monitoramento de Mamíferos Aquáticos do Nordeste do Brasil (Brazilian Northeast Stranding Network)
- UERN - Universidade Estadual do Rio Grande do Norte (Rio Grande do Norte State University)
- UF - University of Florida
- UFC – Universidade Federal do Ceará (Federal University of Ceará)
- UFPE – Universidade Federal de Pernambuco (Federal University of Pernambuco)
- UFRPE - Universidade Federal Rural de Pernambuco (Federal Rural University of Pernambuco)
- USGS - United States Geological Survey

- **Laboratory**

- BSA - Bovine serum albumin
- DMSO - Dimethyl sulfoxide
- EDTA - Ethylenediaminetetraacetic acid
- MgCl2 - Magnesium chloride
- NaCl - Sodium chloride
- PCR - Polymerase chain reaction
- SDS - Sodium dodecyl sulfate
- Tris-HCl - Tris(hydroxymethyl)aminomethane hydrochloride

- **Protected Area denominations and categories in Brazil**

- APA - Área de Proteção Ambiental
- ESEC - Estação Ecológica
- MPAs - Marine Protected Areas
- PARNA - Parque Nacional
- REBIO - Reserva Biológica
- RESEX - Reserva Extrativista

- **Genetic**

- Allele - a variant segment of the genetic material
- Bottleneck - reduction in population size that can have major influence on genetic variation (because of the relationship between genetic drift and population size)
- Coefficient of relatedness (r) - a measure of the degree of relatedness between individuals, ranging from - 1.0 (no genes in common, at least over the genetic markers assayed) to + 1.0 (identical twins or clones). In a random mating individuals should have r = 0.0
- Correspondence analysis (CA) - multivariate statistical technique. Provides a means of displaying or summarizing a set of data in two-dimensional graphic.
- d-loop - displacement loop
- dNTP - deoxynucleotide triphosphate

- DNA - deoxyribonucleic acid
- Genetic diversity (expected heterozygosity) - a measure of genetic variation in a population
- Gene flow - movement of genes from one population to another, causing them to become more similar
- Genetic frequencies - the term used in population genetics for allele frequencies
- Genetic drift - a force that reduces heterozygosity by the random loss of alleles (inversely related to population size)
- Genetic migration - primary agent of gene flow
- Genetic markers - any trait used as marker of genetic variation within and among individuals and taxa
- Genotype - the set of DNA variants found at one or more loci in an individual. The information from which genotypes are developed could include allozyme alleles, microsatellite alleles or sequence information
- F-statistics - measure of genetic structure related to statistical analysis of variance (ANOVA)
- F_{IS} - the proportion of the variance in the subpopulation contained in an individual (inbreeding coefficient)
- F_{ST} - the proportion of genetic variation distributed among subpopulations relative to the total genetic variance. This is used as an index for estimating gene flow and population subdivision.
- Homoplasy - convergent evolution - describes the acquisition of the same biological trait in unrelated lineages
- Homozygote - alleles are the same on both chromosomes
- Heterozygote - alleles differ in each chromosome
- H_E - expected heterozygosity - a measure of the expected proportion of heterozygotes in the population based on the principle of Hardy-Weinberg equilibrium
- H_O - observed heterozygosity - the actual proportion of heterozygotes in the population calculated from the dataset.

- HWE - Hardy-Weinberg Equilibrium - a law stating an idealized population of diploid organisms that reproduces sexually in a random fashion with non-overlapping generations. The model population of infinite size experiences no mutation, migration, and selection processes. In population genetic data analysis, this principle states that allele frequencies will remain the same, but genotype frequencies will change over time. Values for *Ho* and *H**E*** are used to test if allele frequencies meet HWE expectations.
- *K* - number of genetic population clusters assigned by the program STRUCTURE.
- Karyotype - the complement of chromosomes that constitute the genetic material of a eukaryote
- MCMC - Markov Chain Monte Carlo - an analog statistical technique used to estimate the probability of distribution for all possible combinations of parameter values in equilibrium distribution (Excoffier and Heckel, 2006).
- Microsatellites - short tandem repeats of nucleotide sequences
- mtDNA - mitochondrial DNA
- nDNA - nuclear DNA
- *N* - number of samples
- N_A - number of alleles
- N_E - effective number of alleles. The measure of allelic diversity that takes into account the homozygosity as well as the number of alleles (Yeh and Boyle, 1997).
- *P*(*ID*) - probability of Identity. Probability of choosing two individuals from a population that has identical genotypes at the loci examined
- *P*(*ID*)*HW* - standard probability of identity assuming sample is in Hardy-Weinberg equilibrium (Waits et al., 2001)
- *P*(*ID*)sib - probability of identity of siblings which accounts for related individuals in the dataset (Waits et al., 2001)
- *PR(X/K)* - Log probability of data
- Phylogeography - study of the patterns of genetic differentiation across landscapes, often involving intraspecific variation and the comparison of patterns across a range of different taxa in the same region

- Primer - short, preexisting single-stranded polynucleotide chain to which new deoxyribonucleotides can be added by DNA polymerase. The primer anneals to a nucleic acid template (DNA of the organism of interest) and promotes copying of the template, starting from the primer site
- *q* estimate - amount of admixture associated with assignment of an individual to a population cluster using the program STRUCTURE. Admixture of individuals within a population corresponds to mixed ancestry
- Random mating - a fundamental simplifying assumption from many population genetics models
- *RST* - estimator of FST that is relatively insensitive to mutation rates and thus more appropriate for rapidly mutating microsatellite loci. RST tends to have a high variance than other measures of population differentiation and is sensitive to violations of the stepwise mutation model
- Sympatric - occurring in the same geographic area
- UPD - Uniparental Disomy - situation where the origin of both members, homologs, of a given chromosome pair in an individual are derived from one parent

- **Brazilian states mentioned in this study**
- AL - Alagoas
- AP - Amapá
- CE - Ceará
- MA - Maranhão
- PA - Pará
- PB - Paraíba
- PE - Pernambuco
- PI - Piauí
- RN - Rio Grande do Norte
- SE - Sergipe

- **Terms used in this study**

- AL-CE - from Alagoas state to east of Ceará state in Brazil
- EAR - Estuary of the Amazon River (mouth of the Amazon River – Pará and Amapá states in Brazil)
- NEB - Northeast coast of Brazil (from Alagoas to Maranhão state)
- NWA - Northwest of the Amazon River estuary (region northwest of the mouth of the Amazon River - from Amapá in Brazil to Venezuela)
- PI-MA - from west of Ceará state to east of Pará state in Brazil
- STM - Santarém region on Pará state.

ABSTRACT

The West Indian manatee (*Trichechus manatus)* and the Amazonian manatee (*Trichechus inunguis*) belong to the mammalian Order Sirenia, classified respectively as critically endangered and as vulnerable in Brazil. Both species have been hunted commercially since the sixteenth century, resulting in a drastic reduction or elimination of manatees in some places. This hunting pressure may have caused isolation of the populations resulting in geographic genetic isolation, with a reduction of their genetic diversity. The objective of this study is to understand the genetic diversity and population structure of the West Indian manatee (*T. manatus*) in Brazil; verify the existence of hybridization between the manatees *T. manatus* and *T. inungus*; define the degree of relatedness of individuals in captivity; and provide strategies for the conservation of the species. In northeastern Brazil, a large number of dependent calves strand on beaches. Many of these calves have been rescued alive and rehabilitated for future release. Previously, the identification of release sites did not take into account the genetic issues. Through this study, it has been possible to identify suitable release sites based on phylogenetic traits of the manatees. The genetic structure of the population was studied using nuclear DNA in the program STRUCTURE, and comparing F_{ST} and R_{ST} values. On the Northeast coast one population was identified, which is subdivided in two subpopulations. A different genetic population was found in the Northwest of the Amazon River estuary (extreme north of Brazil, Guyana and Venezuelan). Manatees from the Estuary of the Amazon River grouped with Amazonian manatees from the Santarém region to form a third population. A total of four manatee Management Units were identified along the Brazilian coast, which should be treated separately for management and conservation needs. Using a combination of molecular markers (mtDNA and nDNA) and cytogenetic analyses the existence of hybridization between *T. manatus* and *T inunguis* was confirmed, although this does not appear to be a frequent occurrence. Some of the dependent calves rescued, and other individuals transferred from irregular captive conditions to the CMA/ICMBio, were kept together and breeding occurred. Maintaining related manatees together can lead to inbreeding. It was possible to establish the degree of relatedness between individuals kept at CMA/ICMBio and verify the extistence of inbreeding. To avoid inbreeding, hybridization, and overpopulation in captivity, it is necessary that males and females be separated immediately, thereby preventing reproduction *ex-situ*. The present study allows us to establish actions for the conservation of the West Indian manatee. Actions that should be prioritized include: protection and restoration of habitat with the creation and implementation of specific protected areas; creation and maintenance of travel corridors that allow gene flow; reduction and elimination of anthropogenic pressures on the species; stopping pointless reproduction in captivity; designing further studies in the north of Brazil to better understand the hybridization and implement the action plan.

Keywords: genetics population of manatees; conservation of aquatic mammals; hybrids between two sirenian species; inbreeding of manatees in captivity.

RESUMO

Os peixes-bois (*Trichechus manatus*) e os peixes-bois amazônicos (*Trichechus inunguis*) são mamíferos pertencentes à Ordem Sirênia e se encontram classificados respectivamente como criticamente ameaçados e vulneráveis de extinção no Brasil. As duas espécies foram caçadas comercialmente desde o século XVI, resultando na eliminação ou na redução drástica dos animais em alguns locais, o que pode ter causado um isolamento de populações resultando num isolamento genético geográfico, com redução da diversidade genética deles. Este estudo objetiva entender a diversidade genética e identificar a estrutura genética populacional do peixe-boi *(T. manatus)* no Brasil, assim como verificar a existência de hibridização entre os peixes-bois *T. manatus e T. inunguis*, definir o grau de parentesco dos indivíduos em cativeiro e prover estratégias para a conservação da espécie. No nordeste do Brasil, um elevado número de filhotes dependentes de peixe-boi encalha nas praias, muitos são resgatados com vida e reabilitados para posterior soltura. Anteriormente, quando da devolução de indivíduos reabilitados à natureza não se considerava as questões genéticas, mas este estudo tornou possível indicar áreas para soltura baseadas em nas características filogenéticas dos peixes-bois. A estrutura genética populacional foi estudada a partir da análise de DNA nuclear, usando o STRUCTURE e comparando os valores F_{ST} e R_{ST}. O estudo encontrou e identificou três populações geneticamente diferentes: a primeira no litoral nordeste, subdividida em duas subpopulações; a segunda no noroeste do estuário do rio Amazonas (extremo norte do Brasil, Guiana e Venezuela); e a terceira no, estuário do rio Amazonas, onde os peixes-bois se agruparam com os amazônicos da região de Santarém. Foram identificadas quatro Unidades de Manejo para os peixes-bois na costa brasileira, que devem ser tratados separadamente devido às diferentes necessidades de manejo e conservação. Usando a combinação de técnicas de marcadores moleculares (mtDNA e nDNA) e de análises citogenéticas foi confirmada hibridização entre o *T. manatus* e o *T. inunguis*, ocorrência que não aparenta ser frequente. Alguns dos filhotes dependentes resgatados e indivíduos em cativeiros irregulares e ilegais foram transferidos para o CMA/ICMBio, onde, mantidos juntos, se reproduziram. A manutenção conjunta de peixes-bois com parentesco pode acarretar em endogamia. Neste estudo foi possível identificar graus de parentescos entre os indivíduos mantidos no CMA/ICMBio, confirmando-se a ocorrência de endogamia. Para evitar endogamia, hibridização e superpopulação em cativeiro, é necessário impedir a reprodução *ex-situ,* com a imediata separação de machos e fêmeas. O presente estudo permitiu estabelecer ações necessárias para conservação do *T. manatus*. Dentre as que devem ser priorizadas se incluem: proteger e recuperar o habitat; criar e implantar unidades de conservação específicas; criar e manter corredores que permitam o fluxo gênico; reduzir e eliminar as pressões antrópicas sobre a espécie; cessar reproduções em cativeiro; intensificar estudos na região norte; aprimorar informações sobre a hibridização e implementar o plano de ação.

Palavras-chave: genética populacional de peixe-boi; conservação de mamíferos aquáticos; hibridização entre duas espécies de sirênios; endogamia de peixe-boi em cativeiro.

Chapter I - General Introduction

I.I - INTRODUCTION

I.I.1 - The Order Sirenia

The West Indian manatee (*Trichechus manatus* Linnaeus, 1758) belongs to the Order Sirenia. The sirenians are the only herbivorous aquatic mammals (Hartman, 1979) currently inhabiting the world, which differs from evolutionary records documenting other aquatic mammals in the fossil record. The current representatives of the sirenians inhabit rivers, estuaries and shallow coastal waters between the tropics of Cancer and Capricorn (Ronald et al., 1978).

De Jong and Zweers (1980) and De Jong and colleagues (1981) suggested that sirenians have a monophyletic origin with the orders Proboscidea and Hyracoidea, by presenting similar biochemical characteristics of the crystalline lens of the eye. Often considered phylogenetic, sirenians are outliers. Despite similarities in adaptations, habitat and body shape, sirenians do not have the evolutionary relationships shared by other marine mammals.

The Order Sirenia is represented by four living species (Marsh and Lefebvre, 1994; Marsh et al., 2011), subdivided into two families: Dugongidae and Trichechidae. The Dugongidae has two recent genera, with one extinct and the other living: the species *Hydrodamalis gigas* Zimmerman, 1780 (Steller's sea cow) which was extinct by 1768, and the species *Dugong dugon* Müller, 1776 (dugong). The family Trichechidae, consists of only one genus, *Trichechus*, which has three known species: *Trichechus senegalensis* Link, 1795 (West African manatee), *Trichechus inunguis* Natterer, 1883 (Amazonian manatee) and *Trichechus manatus* Linnaeus, 1758 (West Indian manatee) (Husar, 1977; Husar, 1978a and b).

Steller's sea cow was discovered in 1741 by Georg Wilhelm Steller (Stejneger, 1884) on the Vitus Bering expedition, which sank in the region of the Bering Sea between Alaska (USA) and Asia (Russia). At the time there were expected to be about 2,000 Steller sea cows according to published accounts presented in Walker (1968). Their distribution was confined to the waters around Bering Island and the Commander Islands in the Bering Sea, an Aleutian chain of islands in the North Pacific. After the loss of their ship, sea cows were hunted to sustain the crew's survival. Due to the ease of their harvest, the species was extinct only 27 years after its discovery (Husar, 1978a and c; Odell, 1991; Reynolds and Odell, 1991; Reep and Bonde, 2006). The Steller's sea cow was the largest specimen of any known species of sirenian, with a length of approximately 8 meters and a weight over 4 tons. It was the only species of the Order Sirenia to adapt to cold waters by increasing body size and mass (Domning, 1997).

Dugongs are similar to manatees in appearance but have a visible difference in the caudal tail, which is more visually similar in shape to dolphins and whales (Marsh et al., 1986). Other differences are less apparent, with adult males retaining incisors, and all dugongs having a pronounced rostral deflection, bending down ventrally to facilitate feeding on the bottom (Eisenberg, 1981). Dugongs are the only sirenian restricted to completely marine waters, and occur along tropical and subtropical coastal waters and islands of the Indian and Pacific oceans between 27°N and 27°S latitude (Marsh and Lefebvre, 1994).

West Indian manatees occur in coastal waters and rivers of the Atlantic region, from northern Florida (USA) along the east coast of Mexico and Central America and northern South America to northeastern Brazil (Marsh et al., 1986). Their current range is between 19°N and 12°S latitude. They also live in coastal waters and estuaries of the Caribbean and West Indies. In Brazil, the species occurs from Amapá state to Alagoas state.

The West Indian manatee consists of two subspecies: *Trichechus manatus manatus* Linnaeus, 1758, (Figure I.1) that occurs in Central and South America, and *Trichechus manatus latirostris* Harlan, 1824, that is restricted to the southeastern United States. This division was proposed by Hatt and Lang (1934) based on anatomical and morphological evidence, but the existence of two subspecies was questioned by Husar

(1978b) who judged the distinguishing characteristics of the subspecies as not sufficient for such subspecies designation. However, Domning and Hayek (1986) through craniometric analysis confirmed the separation of subspecies. This separation has also been confirmed through recent genetic evidence (Hunter et al., 2012).

Figure I.1 – West Indian manatee (*Trichechus manatus*).

Picture: Luciano Candisani

The West African manatee is similar to the West Indian manatee in size and weight and their utilization of similar fluvial and estuarine resource habitats. According to Reeves and colleagues (1992) West African manatees inhabit coastal waters, estuaries, rivers, wetlands and coastal lagoons. It is the least known species among all the sirenia.

The Amazonian manatee (Figure I.2) is distributed throughout the Amazon basin (Domning, 1981a), with distribution being determined mainly by the availability of food resources and avoidance of rough waters and swift currents (Best, 1984). The species is the smallest of the manatees (Coimbra Filho, 1972), reaching only three meters in length and weighing less than 300kg.

Unlike other Trichechids, the Amazonian manatee has no nails on the pectoral flippers, the skin is smooth and its color is dark black. On the ventral surface of the chest, between the pectoral flippers and the lower abdomen they often have an irregular white patch (Marsh et al., 1986). There is variation in individuals with respect to the size, shape, color and extent of this patch (Rosas, 1994; Luna, 2001).

Figure I.2 – Amazonian manatee (*Trichechus inunguis*).

Picture: Fábia Luna – CMA/ICMBio

I.I.2 - Manatee biology

The adult manatee is generally between 2.5 and 4.0 meters in length and weighs between 200 to 600 kg (Husar, 1977) and the pectoral flippers may or may not have nails (Hartman, 1979). There are some differences specific to each species of manatee, like body color and skin texture (gray and rough for West Indian and black and smooth for Amazonian manatees), however morphologically the three manatee species are generally similar. Manatees are generalist herbivores that eat mainly algae, sea grass, leaves of mangroves and freshwater plants.

The preferred species of vegetation most commonly consumed by manatees are: *Gracilaria* sp., *Soliera musciformes*, *Hypnea* sp., *Halodule wrightii*, *Syringodium filiforme*, *Thalassia testudinum*, *Avicennia nitida*, *Laguncularia racemosa*, *Rhizophora mangle*, *Montrichardia arborescens*, *Spartina brasiliensis*, *Eichhornia crassipes*, *Eleocharis interstincta*, *Echinochloa polystachya*, *Paspalum repens*, *Ruppia maritima*, *Hydrilla verticillata, Myriophyllum spicatum, Ceratophyllum demersum* and *Vallisneria neotropicalis* (Best, 1981; Best, 1982a; Paludo, 1997; Colares and Colares, 2002; Castelblanco-Martínez et al., 2009). Manatees are also coprophagous (Hartman, 1979; Best, 1981).

Because they are herbivores, manatees need to consume large quantities of food, consuming each day from 8 to 13% of their body weight (Best, 1981). Therefore, manatees forage six to eight hours a day during feeding bouts (Bertram and Bertram, 1964; Husar, 1977). As many of the plants they eat have high silica content, and likely are ingested with sand, manatees have evolved cyclical replacement of the dentition to replace worn down teeth (Domning and Magor, 1978).

Many West Indian manatees are living in isolated coastal habitats and are rarely found in deep water or out in open ocean (Lefebvre et al., 2001). Deepwater habitats are generally not useful to the herbivorous manatee; they often remain near the shore to facilitate access to food resources (aquatic plants) and to acquire freshwater to drink. A few individuals have been observed traveling in open water, but this is likely directed travel from one habitat type to another.

In Florida there is a seasonal variation in water temperature which causes the manatees migrate to warm water refugia during winter and return to marine ecosystems rich in nutrients for forging during the warmer periods (Rathbun et al., 1985; Rathbun et al., 1990 and Rathbun et al., 1995). This affect results in individuals traveling long distances for migration that puts them into contact with other individuals from other populations (Fertl et al., 2005; Deutsch et al., 2003; Weigle et al., 2001). If breeding between two separate populations occurs, this is usually beneficial by providing gene flow resulting in less genetic differentiation between mixing populations.

In Brazil, the tropical climate fluctuates little and there is no detected migration solely for thermoregulation or other seasonal movement between areas of occurrence of manatees, so therefore it is unlikely that manatees need to travel long distances to survive. This could lead to a reduction in gene flow and more differentiation in population structure when compared to Florida manatees.

Manatees have superior immune systems, resulting in an ability to facilitate wound healing and repair injuries when compared to other animals, despite the lack of marrow in the long bones (Bonde et al., 2004). Accordingly, the manatee immune system appears highly developed to protect against the harsh marine environment and the effects of human-related injury (Bossart et al., 2002). Manatees appear resilient to natural disease and traumatic anthropogenic induced injury (Buergelt and Bonde, 1983; White and Francis-Floyd, 1990; Bossart, 1999; Bonde et al., 2004), which credits their immune system as a reason for their ability to survive in heavily polluted water.

In a study to determine the age of individual manatees based on the count of growth layer groups deposited during development of the tympanic-periotic bone, the Florida manatee with the oldest assigned age was estimated to be greater than 50 years (Marmontel et al., 1990). The average calving interval for Florida manatees is approximately three years with length of dependency varying between different habitat types (Rathbun et al. 1995; Reep and Bonde, 2006). Newborns are generally between 0.80 and 1.60 m in total straight-line length (Marmontel, 1995). The calf stays with the mother on average 1.2 to 2.0 years before weaning (Rathbun et al., 1995).

I.I.3 - Threats to manatees

Unfortunately, in the Americas, manatees have been hunted since before the period of colonization. Intense hunting in certain locations resulted in severe reduction of populations. In Brazil between 1935 and 1954, it is estimated that 4,000-7,000 Amazonian manatees were taken per year for the purpose of commercial exploitation, and approximately 200,000 Amazonian manatees were killed during that 20 year period. (Best, 1982b; Domning, 1982). Domning (1982) described that West Indian manatees also suffered commercial exploitation in Brazil.

Those harvested manatees were rendered for oil and meat, and the hides were used to make machine belts, pulleys and hoses (Best, 1984). Hunting for subsistence still occurs in the Amazon basin and the northern coast of Brazil (Luna et al., 2000; Luna et al., 2008a). This is of major concern, since the number of the West Indian manatees in Brazil is estimated to be only 500-1,000 individuals (Lima, 1997; Luna, 2001; Luna and Passavante, 2010; CMA/ICMBio, in prep.). The species is considered critically endangered by the government of Brazil (Ibama, 2001).

Although the hunting of manatees continues in some places, it has reduced gradually from the sixteenth century through the 1900’s in most of the regions (Hartman, 1979; O’Shea, 1988 and Luna et al., 2008a). However, other factors are affecting manatees, among them: accidental capture in fishing nets (Oliveira et al., 1990), entanglement in or ingestion of fishing gear or debris (Beck and Barros, 1991; Marmontel et al., 1997; Mignucci-Giannoni et al., 2000; de Thoisy et al., 2003; Attademo, et al., 2008), collisions with water vessels (Marmontel et al., 1997; Borges et al., 2007b), crushing in flood control structures (O’Shea et al., 1985), accelerated growth of human activities in the coastal zones resulting in habitat destruction and alteration (de Thoisy et al., 2003), which intensifies the stranding of calves (Parente et al., 2004), and pathogens (Vergara-Parente et al., 2003; Borges, et al., 2007a).

Annually, direct and indirect human-related mortality can account for 33% of all Florida manatee deaths (O'Shea et al., 1985; Bossart, 1999). In the late 1980's, more than 100 manatees were found dead each year in an estimated population of approximately 1,200 individuals. Many deaths were anthropogenic in nature, with boat strikes causing the most human induced mortality (O'Shea et al., 1985). In 1996, 150 manatees died in southwest Florida from brevetoxin exposure (U.S. Marine Mammal Commission, 1996; Bossart et al., 1998). In 2010, 766 manatees died in Florida from natural and anthropogenic causes in an estimated population size of 5,000-6,000 individuals (FWRI, 2012).

These threats, coupled with a low reproductive rate, have caused breaks in the distribution of the manatees, generating small disjunct populations, which may result in the existence of small subpopulations genetically isolated. Small populations, or captive populations, may suffer inbreeding, which reduces genetic diversity of the species and the ability of the immune system to respond to changes, such as an increase in susceptibility to disease. It can also result in physical defects and lower population viability (Sherwin and Moritz, 2000). The reduction of genetic diversity can result in decreased fertility and survival, and compromises the ability of the species to withstand and adapt to environmental changes, such as global warming and sea level rise, thereby enhancing the probability of extinction (Avise, 2004).

When there are breaks in the distribution between areas occupied by the species, these are probably the result of a geographical barrier, and can be a limiting factor for gene flow between individuals and populations. This alone, makes it possible to have small pockets of isolated groups of manatees, since the animals do not disperse far due to their dependence on coastal systems.

I.I.4 - Conservation genetics

Conservation genetics is a tool that evaluates and monitors species and their relatedness (Frankham et al., 2002). Detailed information on the genetic status of an endangered species, like manatees, can assist in developing management plans and recovery actions (Bonde et al., 2012). Genetic studies using mitochondrial and microsatellite DNA have been used to quantify genetic diversity and identify subpopulations, the extent of hybridization, and inbreeding. Reduced genetic diversity can result in a decrease in fecundity and survival, which compromises a species' ability to evolve and endure exposure to environmental change, potentially resulting in extinction (Avise, 2004). Genetic diversity has also been linked to fitness, population size, number of inbred individuals and population persistence. According to Frankham and colleagues (2002) there are four threats to reduction of genetic diversity: (1) the extinction of populations or species, (2) the extinction of alleles, (3) inbreeding reducing heterozygosity, and (4) selection favoring one allele over another.

Small populations, or subpopulations, can suffer from inbreeding and reduced immune system function, which increases susceptibility to disease, fitness and physical defects. Consequently, genetic studies, using mitochondrial and microsatellite DNA, are used to quantify genetic diversity and identify unique populations or regions in need of special protective measures. These studies will facilitate manatee conservation, management and recovery efforts.

I.I.5 - Genetic studies of sirenians

The first Florida manatee population genetic study was conducted in 1988 by McClenaghan and O'Shea on 59 manatees from Florida using 24 allozyme loci, 10 of which were polymorphic with two or three alleles. The study suggested that manatees have equivalent or higher heterozygosity than other mammals. The first sequencing-based study used mitochondrial cytochrome *b* to analyze the Florida population (Bradley et al., 1993). All individuals were determined to have the same haplotype, although the sample size was small and only a 225 base pair (bp) segment was sequenced.

The mitochondrial DNA study that followed (García-Rodríguez et al., 1998) amplified a 410 bp fragment of the control region (D – loop) in eight West Indian manatee geographic populations. In 86 individuals a total of 15 haplotypes were identified. Three lineages constituted three clusters: Florida and the West Indies; the Gulf of Mexico to the Caribbean rivers of South America; and the northeast Atlantic coast of South America. García-Rodríguez and colleagues (2000) developed and characterized primers in an effort to find markers for microsatellite DNA with a higher resolution of genetic variation.

Cantanhede and colleagues (*2*005) addressed the genetic differentiation and geographical population structure in the Amazonian manatee amplifying a 361 bp unit of the mitochondrial DNA control region. Amazonian manatees had more diversity than any of the three proposed West Indian manatee clusters. The high diversity may have been from pooling divergent populations or a very large population size.

Vianna and colleagues (2006a), with 330 individuals from West Indian, Amazonian, West African manatee species, conducted a study using the 410 bp fragment of the mtDNA control region employed by Garcia-Rodriguez and collaborators in 1998. Individuals from 10 countries revealed 20 West Indian, 31 Amazonian, and five West African manatee haplotypes. Three haplotypes were detected for manatees in Brazil (M01, M03, and T; Figure I.3). However, it was not described by state or region to help the conservation plan specific for Brazil. In the analyses the program BARRIER suggested two likely geographic obstructions to migration and potential population division. A geographic separation isolated the Dominican Republic and Puerto Rico from the other West Indian populations and another isolated Guyana and Brazil. The *T. manatus* subspecies split into three distinct clusters with median-joining networks and neighbor-joining trees. This displayed a heterogeneous geographical distribution similar to those proposed by García-Rodríguez and colleagues (1998). Cluster 1 is composed of Florida and the Greater Antilles (or Caribbean); Cluster II includes Mexico, Central

America, and the Caribbean coast of northern South America; and Cluster III comprises the Northeastern coast of South America from Guyana to Brazil.

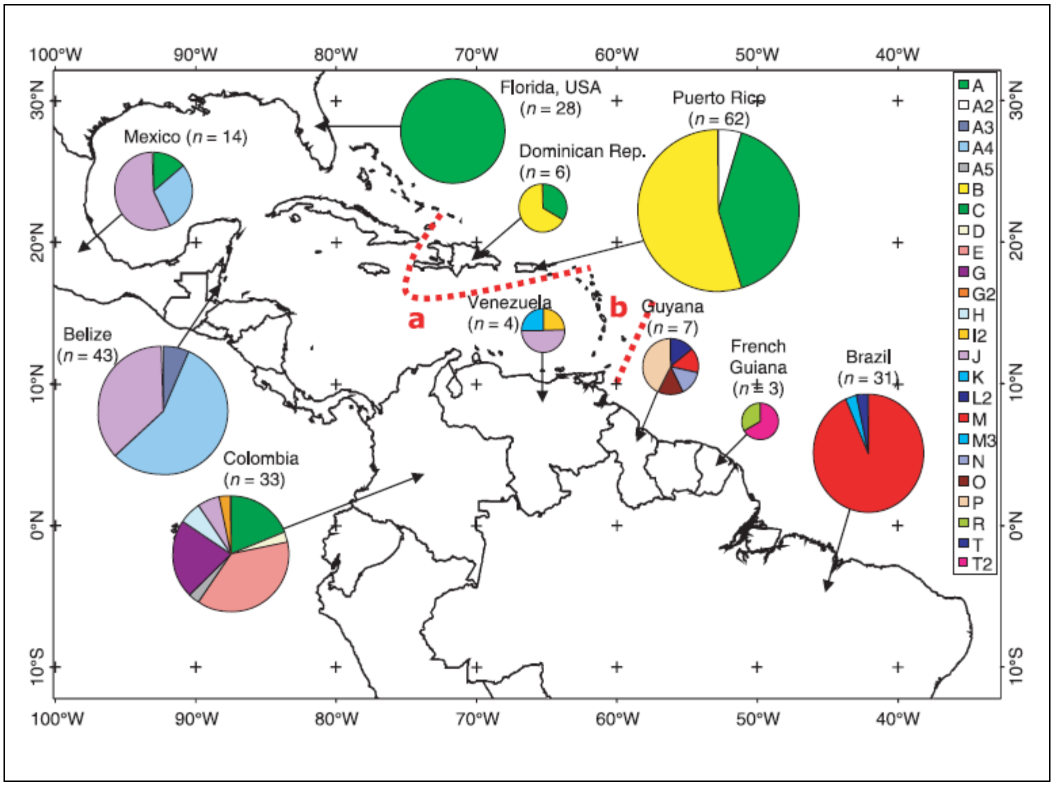


Figure I.3 – Control region mtDNA haplotypes found in *Trichechus manatus* populations suggested by Vianna et al. (2006)

From: Vianna et al. (2006)

Vianna and colleagues (2006a) also examined potential hybrids between *T. manatus* and *T. inunguis* using the mtDNA control region, two microsatellite primers, and cytogenetic techniques. Eight potential hybrids were identified at the mouth of the Amazon River in Brazil and throughout the Orinoco River. One individual had a *T. inunguis* mtDNA control region (haplotype T), but a diploid number of 2N = 50 chromosomes. The authors extrapolated that the number of chromosomes could be the result of at least two generation backcrosses terminating from an F1 hybrid female breeding with a normal male *T. manatus.*

To increase the resolution, additional microsatellite markers were developed (Pause et al., 2007), adding to the microsatellite primers already developed by García-Rodríguez et al. (1998), which were not variable enough to provide individual multilocus genotypes for the Florida manatees.

New studies using the García-Rodríguez and Pause microsatellite primers allowed for better understanding of the genetic structure of the manatees in Puerto Rico, Belize, Florida and Mexico (Kellogg, 2008; Hunter et al., 2010a; Bonde, 2009; Nourisson, 2011; Nourisson et al., 2011; Hunter et al., 2012; Tucker et al., 2012). Additionally, 18 new microsatellite primers and a DNA sex marker were developed for manatees in Florida (Tringali et al., 2008; Lanyon et al., 2009) and additional 17 microsatellite primers were refined for use with dugong and manatee populations (Hunter et al., 2010b). Some of the 17 primers were successfully amplified and appear polymorphic in the manatee. In Brazil, despite the studies cited, there was no genetic structure study that examined the manatee population to date.

In 2011 Ferreira and colleagues developed a study using cytochrome b gene as a useful protocol in forensic genetics against the illegal hunting of manatees, including West Indian and Amazonian manatees. The authors reinforced the potential use of that genetic methodology as a powerful marker for species identification, especially in vulnerably endangered species, such as manatees.

The presence of both *T. m. manatus* and *T. inunguis* around the island of Marajó was detected (Domning, 1981b; García-Rodríguez et al., 1998; Luna, 2001; Luna et al., 2008c). This area is an area of sympatry, especially on the east side of the island, near the towns of Salvaterra and Soure/Pará. As Vianna and colleagues (2006a and b) found a possible hybrid in Brazil, more investigation about this issue needs to be encouraged. Hybridization causes introgression and the introduction of genetic material from other species into a population that may result in a reduction of the size of recipient population through reproductive failure. For these reasons this area of sympatry, which seems to be where hybridization could occur, should be investigated in detail.

I.I.6 - Sirenia conservation in Brazil

Manatees are protected in Brazil through Federal Law: Protection of Wildlife (N° 5197/1967 and Amendment N° 7653/1987) and Environmental Crimes (N° 9605/1998). The West Indian and Amazonian manatees are listed in Appendix I of CITES, the Official List of Threatened and Extinct Species in Brazil (MMA, 2003), and the Brazilian Red Book about the threatened and extinction of fauna (MMA, 2008; da Silva et al., 2008a).

In the beginning of the 1980’s the Brazilian Government decided to focus on the status of the West Indian manatee on the coast of Brazil and created the project called “Projeto Peixe-Boi” (manatee project). At that time the government did not know where the manatees specifically occurred and all their best available information was based solely on historical numbers of animals hunted during the last several years.

To better understand the species in Brazil, expeditions were made along the North and Northeast coast, where fishermen were interviewed about localities where the manatees were observed and important areas that could be protected. In the middle of the 1980’s an office was created in an estuary (Mamanguape River, at Paraíba state), where research to obtain information about the biology and ecology of the species was carried out. After some initial studies, the West Indian manatee was categorized as critically endangered in the country and a calf stranding area was identified along the northeast coast. By the end of the 1980’s the Project had rescued a stranded calf for rehabilitation.

During 1990-1993 the Project team made new expeditions called “Igarakuê” to research and gather more details on the species in all known manatee distribution areas identified by Albuquerque and Marcovaldi (1982). The team interviewed 800 people who were living on the coast, who would have general information about the manatees. During “Igarakuê” some manatees were discovered in inadequate conditions for captivity and also recognized that the problem with stranded calves was growing and considered much greater then thought with just the one individual rescued years before.

A rehabilitation center was built in 1990 on Itamaracá Island in Pernambuco state to receive the manatees held in inadequate conditions and to care for stranded/rescued calves, rehabilitate them and subsequently release the manatees as a strategy to conserve and supplement the population on the coast. After a few years some manatees were completely rehabilitated and were considered releasable. The first release of a West Indian manatee in Brazil occurred in 1994, when two manatees "Astro" and "Lua" were released in Alagoas state (Lima et al., 2005). During 18 years of releases, over 30 manatees have been released into the wild to date.

The "Projeto Peixe-Boi" from IBAMA (actually ICMBio now) began to work with the Amazonian manatee in 2000, also rescuing and aiding dependent calves for rehabilitation and release. In 2007 two Amazonian manatees, "Kika and Hargos", were successfully released in a lake in the Amazon. In 2008, the manatee project changed their actions from principally rescuing manatees and releasing them to focusing on prioritizing research related to veterinary medicine, pathology, genetics, contaminants and other areas to increase our knowledge of manatees, and help improve conservation efforts.

By the 30 year anniversary, in 2010, the "Projeto Peixe-Boi-CMA/ICMBio" team made a plan to capture, with partners, wild native manatees along the area where stranding of calves was prevalent, in order to examine general health parameters and genetics of the population, evaluate habitat and determine migration patterns (with the use of satellite-monitored radio tags and aerial surveys). The first expedition to capture wild manatees was carried out in May of 2012 and coordinated by CMA/ICMBio with the assistance of the USGS Sirenia Project-USA, Geomare-Mexico, Aquasis, Universidade Estadual do Rio Grande do Norte (UERN) and the Instituto Mamíferos Aquáticos (IMA). This was performed as part of an environmental condition established by CGPEG/IBAMA for Petrobras, which signed a contract with CMA/ICMBio and FUNBIO for execution.

In 2011, ICMBio published a National Action Plan for Sirenia in Brazil (ICMBio, 2010; Luna et al., 2011) that covered a term of five years with specific aims to conserve the two species of manatees that occur in Brazil.

I.II - OBJECTIVES

I.II.1 - General objectives of the thesis

This study aims to assess conservation efforts by examining the population genetic structure of the West Indian manatee (*Trichechus manatus*) in Brazil.

Cytogenetic, mitochondrial and microsatellite DNA techniques will be used to elucidate the likelihood of individual relatedness among the animals kept in captivity at CMA/ICMBio, manatee population structure and genetic diversity, and the potential hybridization that will assist in protecting these unique endangered populations of manatees.

I.II.2 - Specific objectives and description of the chapters

- Assess mitochondrial genetic diversity of West Indian manatees in the Northeast of Brazil and establish appropriate areas for release of rehabilitated calves of manatees using genetic criteria (Chapter II);
- Assess nucleotide genetic diversity and population structure of the West Indian manatee in their current range in Brazil (Chapter III);
- Verify the occurrence of hybrids between the West Indian manatee and the Amazonian manatee near the Estuary of the Amazon River (Chapter IV);
- Verify the likelihood of individual relatedness and the occurrence of inbreeding of manatees in captivity at CMA/ICMBio (Chapter V);
- Suggest management measures for the conservation of the West Indian manatee in Brazil (Chapter VI).

I.III - HYPOTHESES

The specific aims of this study will be addressed through the following hypothesis testing.

Hypothesis 1:

- H_0: There are phylogeographic implications that may influence the choice of the release site for rehabilitated manatee calves in Brazil;
- H_1: There are no phylogeographic implications that may influence the choice of the release site for rehabilitated manatee calves in Brazil.

Hypothesis 2:

- H_0: There are different populations of West Indian manatees along the current range in Brazil;
- H_1: There are no different populations of West Indian manatees along the current range in Brazil.

Hypothesis 3:

- H_0: There is occurrence of hybrids between West Indian and Amazonian manatees in Brazil;
- H_1: There is no occurrence of hybrids between West Indian and Amazonian manatee in Brazil.

Hypothesis 4:

- H_0: There is evidence of inbreeding among manatees in captivity at CMA/ICMBio;
- H_1: There is no evidence of inbreeding among manatees in captivity at CMA/ICMBio.

I.IV - BACKGROUND GENETIC RESEARCH

The first Action Plan for Brazilian Aquatic Mammals (Ibama, 1997), for Conservation Measures, considered that one of the steps necessary to enhance the conservation of aquatic mammals is to "*Maximize efforts with high priority for protection of Trichechus manatus manatus the most endangered aquatic mammal in Brazil*". The second version of the Action Plan for Brazilian Aquatic Mammals (Ibama, 2001), proposes in Projects and Priority Actions for the manatee (*Trichechus manatus manatus*) in "*S.6 - develop studies to determine the genetic variability, the possibility of isolated populations and the occurrence of hybrids with the Amazonian manatee*".

Genetic analysis is a tool to make inferences about the biology, behavior, evolution, and life history of species-level populations. It is important to analyze genetic diversity and how it is structured to see if there are different subpopulations of manatees, and then analyze the level of gene flow between the populations.

Genetic studies can assist strategies for conservation, including management of small populations, to avoid inbreeding and genetic depression, loss of genetic diversity and reduced gene flow and hybridization.

The identification of captive individuals that were the product of inbreeding and/or hybridization is important for managers. Knowing this information will prevent the release of related individuals into a population, thereby avoiding genetic problems for the native population. This knowledge may assist in defining areas of release of rehabilitated manatees and individuals born in captivity.

In Brazil there are few genetic studies that have been conducted on manatees: García-Rodríguez and colleagues (1998); Cantanhede and colleagues (2005); Vianna and colleagues (2006a and b); Ferreira and colleagues (2011). The present study is aimed at diagnoses of the population genetic structure of the West Indian manatee in Brazil. This is important as it will serve to establish and direct management actions for healthy genetic exchange and thus lead to development of strategies for conservation of the species.

I.V - DESCRIPTION OF THE AREA

The area covered by this study is predicated on the current range of the species in Brazil (Figure I.3). That area includes the coastal zones form Amapá (AP) state in the North to the northeast coast of the Alagoas (AL) state, which is the current southern limit of the known occurrence of the West Indian manatee in the world (Luna et al., 2008b; Lima, 1997; Luna and Passavante, 2010).

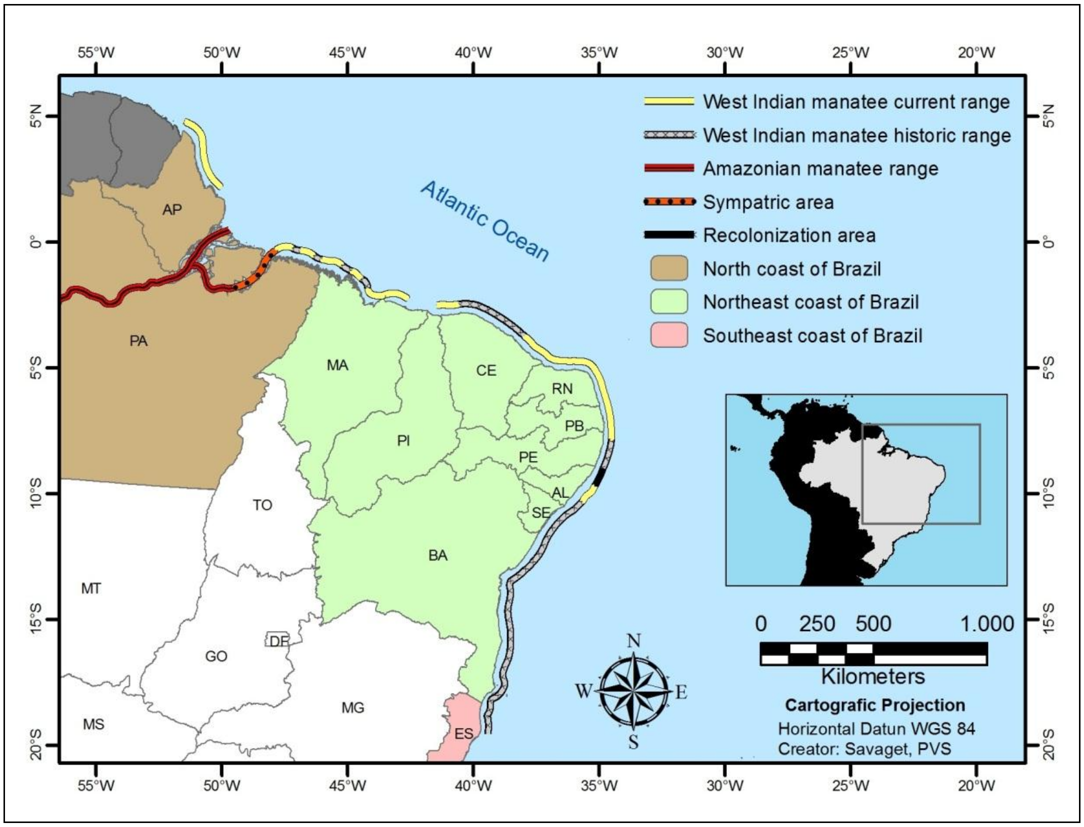


Figure I.3 - Map of research area indicating the range of manatees and the sympatric areas.

Adapted from: Luna and Passavante (2010)

The northeast coast of Brazil has two very different physiognomic characteristics. From Alagoas (AL) to the east of Ceará (CE) the coast is formed of white sandy beaches divided by rocky outcroppings with few small rivers and mangrove estuaries, and edged with numerous sandstone reefs. These reefs were formed by beach sand consolidated by fragments of recrystallized calcareous organisms (Mabesoone and Coutinho, 1970 as cited in Paludo, 1997). They are usually classified as “barrier reefs” that occur parallel to the beach and separated by a strip of sandy submerged areas called “inside sea”. These barrier reefs form a navigation channel for manatees and provide sheltered areas encouraging growth of seagrasses. Some of these beaches are high energy and there are strong breaking waves, especially during the rising tide.

From the west coast of CE, Piaui (PI) and Maranhão (MA), there are more abundant rivers. Preguiça (MA), Parnaíba (Pi), Timonha (PI-CE) and Acaraú (CE) provide a higher fluvial influence on the area and the beaches constitute mudflat ecotypes with some larger mangrove estuaries than those observed in other northeast states (MMA, 1996).

The state of MA is geopolitically part of the northeast coast but that state has a physiogeographic characteristic that is transitional between the north and the northeast of Brazil.

Most of the coast of MA, PA and AP states is formed by mangrove estuaries, consistently vegetated with *Rhizophora* sp., *Laguncularia* sp. and *Avicennia* sp. It also provides dense and tall tree coverage that has an appearance of a coastal forest, with creeks, bays, gulfs and estuaries rich in species diversity.

In part of the states of MA (western edge of the Maranhão Gulf) and PA (eastern edge of the Amazon Gulf) the mangrove forests form a belt up to 30km wide (Goes Filho et.al., 1973, as cited in MMA, 1996). Along the banks of some major rivers the vegetation penetrates inland up to 40km (MMA, 2001 as cited in Luna, 2001). This is a very important area for the occurrence of the West Indian manatee, where the population is probably better preserved.

The mouth of the Amazon River converges with the Pará/Tocantins rivers and forms a large estuary with numerous islands, the main one being Marajó Island located between the mainland coast of Pará and Amapá states (MMA, 1996). West Indian manatees and Amazonian manatees occur in sympatry in this region (Domning, 1981b; Luna, 2001; Luna and Passavante, 2010).

The Amazon River is characterized by an extensively huge volume of water discharge and is influenced by seasonal rainfall and melting of ice caps in the Andes (MMA, 1996). The Amazon River has a discharge of about 220.000m^3/s^{-1} (MMA, 1996) or one trillion $m^3/year^{-1}$ according to Nittrouer and colleagues (1991). This tremendous discharge is responsible for the development of highly dynamic processes occurring at the mouth; providing the establishment of the world's largest estuarine area.

The second river of the Amazon drainage system is the Pará formed by the confluence of a branch of the Amazon River with the river Tocantins. It has a flow rate estimated at 10.000m^3/s^{-1} (Oltman, 1968 as cited in Santos, 2000).

A large discharge in the mouth of the Amazon River causes flooding in extensive areas resulting in erosion phenomena (MMA, 1996). The “pororoca” is another phenomenon that influences this region, generally occurring at the meeting with the high tide flood of the Amazon River, when the strength of the rising sea overcomes the rush of the outflowing river water, lasting two to three days. This phenomenon occurs to the west of the Marajó region in Pará state and continues to Bailique archipelago in Amapá state (Miranda Neto, 1993). All these oceanographic conditions may influence manatee distribution and dispersal, which will be assessed with genetic status.

Chapter II - Phylogeographic implications for release of critically endangered manatee calves rescued in Northeast Brazil

Luna, F. O.; Bonde, R. K.; Attademo, F. L. N.; Saunders, J. W.; Meigs-Friend, G.; Passavante , J. Z. O.; Hunter, M.E. Phylogeographic implications for release of critically endangered manatee calves rescued in Northeast Brazil. **Aquatic Conservation: Marine and Freshwater Ecosystems**, v. 22, p. 665-672, 2012.

II.I - INTRODUCTION

Anthropogenic threats and a low reproductive rate have historically limited West Indian manatee (*Trichechus manatus*) population growth. As a result, the species is classified as vulnerable by the International Union for the Conservation of Nature (IUCN, 2012) and the population in Brazil as critically endangered by the Brazilian government (Ibama, 1989; MMA, 2003; Deutsch et al., 2008; da Silva et al., 2008a).

During Brazilian colonization by Portugal, manatees were severely exploited for sustenance and commercial activities (Domning, 1981b; Domning, 1982). Consequently, the distribution and abundance of the population was considerably diminished, although no census estimates were reported (Whitehead, 1977; Domning, 1981b; O'Shea, 1994).

The current estimate of West Indian manatees in Brazil is approximately 500-1,000 individuals (Lima, 1997; Luna, 2001; Luna et al., 2008b; CMA/ICMBio, in prep.). The northeast regional population is estimated to be 278 individuals (Lima, 1997), not including Maranhão state (MA; Figure I.3). Low genetic connectivity is observed between Brazil and the neighboring manatee populations in French Guiana and Guyana (Vianna et al., 2006a).

In 1967, Brazilian federal laws were enacted to protect manatees from hunting pressures throughout their distribution, from Amapá to Espírito Santo (Whitehead, 1978). Even with these protections, Brazilian manatees are severely threatened in some areas and the subspecies is classified as extinct in the southern portion of the historic range, from Sergipe (SE) to Espírito Santo (Albuquerque and Marcovaldi, 1982; Lima et al., 1992), and along the Alagoas (AL) and Pernambuco (PE) state boundaries (Lima, 1997; Luna, 2001).

Once the hunting pressures were removed, incidental fishing gear entanglement and stranding of dependent calves became the main causes of mortality for the West Indian manatee population in the northeast of Brazil (Paludo, 1998; Oliveira et al., 1990). Lima (1997) suggested that pregnant females were not gaining access to quiet and protected estuarine waters to give birth. Therefore, the calves were born in open water with strong winds and coastal currents, which often resulted in separation from the mother. Critical habitat degradation through urban development and shrimp farm construction has resulted in extensive mangrove deforestation and is attributed to the increase in lone manatee calf strandings (Meirelles, 2008).

Calf strandings occur mostly in Ceará (CE) and Rio Grande do Norte (RN), but also in Paraíba (PB), AL, PE, and MA states. Calves recovered alive are rescued by institutions of the Stranding Network of Northeastern Brazil (REMANE), including Aquasis, UERN, and Rebio Atol das Rocas/Chico Mendes Institute for Biodiversity Conservation (ICMBio). Typically, local fishermen and citizens discover the calves stranded above the surf zone with the umbilicus still attached, suggesting that the calf separated from the cow soon after birth. On average, manatee calves remain with their mothers for two to three years and would be unlikely to survive without rescue (Reep and Bonde, 2006).

The calves are transported (Figure II.1) to the Brazilian National Center of Research and Conservation of Aquatic Mammals (CMA) of the ICMBio in Itamaracá, PE, Brazil. These calves, and those born in captivity since 1997, are reared at CMA until they are old enough to be reintroduced into the wild population. (Figure II.2).

Figure II.2. Manatee calf during transportation by airplane from Ceará state to CMA/ICMBio on Pernambuco state.

Picture: Fábia Luna – CMA/ICMBio

Figure II.2. Manatee rehabilitated transferred to a sea pan to release in the wild.

Picture: Fábia Luna – CMA/ICMBio

Molecular studies can be used to determine appropriate source populations of releasable individuals and to monitor the genetic composition of populations after the release of rehabilitated captives (Sarrazin and Barbault, 1996). Allendorf and Luikart (2007) stress that when working with wild animals, '*genetics should be considered in all reintroductions, introductions, and translocations*'. Genetic diversity, effective population size and reproductive contribution of released individuals can also be estimated using molecular techniques before and after release (Allendorf and Luikart, 2007).

The mitochondrial DNA (mtDNA) control region from rescued calves, calves born in captivity and wild manatee carcasses from all age groups was genetically analyzed in this study. A previous mtDNA control region study identified low haplotype (h = 0.0667) and nucleotide (π = 0.0002) diversity in West Indian manatees in coastal Brazil (Vianna et al., 2006). The study reported only two West Indian manatee haplotypes (M01 and M03) from 30 individuals (excluding a suspected hybrid), with M03 identified in only one sample (Vianna et al., 2006). In that study, no sample or haplotype locations were provided.

Therefore, geographic haplotype frequency and regional genetic variation are addressed here to aid selection of release sites for rehabilitated calves. Manatees from certain localities may be better adapted to specific habitats, vegetation types and seasonal differences, and may have particular migration or behavioral knowledge. Further, genetic swamping or outbreeding depression is possible when individuals from other localities are introduced into areas without their genetic signature (Frankham et al., 2002).

The objective of this study was to characterize the geographic distribution of haplotypes and genetically related subpopulations of manatees in Northeast Brazil and to identify the haplotypes of rescued and rehabilitated manatees. This information will help to determine the appropriate or ideal alternative release locations of rehabilitated and captive-born *T. manatus* calves. Conservation implications will also be discussed for this small, critically endangered manatee population.

II.II - MATERIALS AND METHODS

II.II.1 - Sample collection and DNA extraction

Beginning in 1989, stranded calves were rescued in seven Brazilian states along the northeastern Atlantic coast by CMA and REMANE (Figure I.3). Stranded calves are typically identified by local fishermen and transported to CMA by either airplane or automobile after receiving supportive care from institutions affiliated with the REMANE. Blood and epidermis tissue collected from rescued calves (N=47) were archived by the CMA/ICMBio from 1990 to November 2010.

Additionally, recovered calf carcasses (N=5), adult carcasses (N=10), captive-born calves (N=9), captive adults (N=1), and placenta (N=1) samples were archived, resulting in a total of 73 samples. Vianna and colleagues (2006a) also used samples from the CMA archive, however, the study focused on range-wide species patterns and no sample or location information was provided for the samples from Brazil. Therefore, all available samples and associated geographic information were used for analyses in this study.

The blood samples were preserved in EDTA or lysis solution [10 mM NaCl, 100 mM EDTA, 100 mM Tris (pH 8), and 1% (w/v) SDS]. Muscle and skin tissues were preserved in 70-90% ethanol or in a saturated salt solution buffer [saturated NaCl, 250 mM EDTA (pH 7.5) and 20% v/v DMSO]. Additionally, after observing parturition in the wild, a placenta was collected and preserved in 10% formalin. Brazil manatee genomic DNA was isolated using the Qiagen’s DNeasy Blood and Tissue Kit (Valencia, California) for 71 blood and tissue samples and the one placenta sample, while a single carcass sample was isolated using traditional phenol chloroform techniques, due to the decomposed state of the sample.

II.II.2 - Mitochondrial DNA analysis

Primers from García-Rodríguez and colleagues (1998), CR-4 and CR-5 (Palumbi et al., 1991; Southern et al., 1988) were used to amplify a 410 base pair portion of the mtDNA control region displacement loop. The mtDNA control region was PCR amplified following the techniques described by Hunter and colleagues, 2010a. Briefly, the PCR reaction conditions were as follows: 10 ng DNA, 1 x PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.001% gelatin; Sigma-Aldrich, Inc., St. Louis MO), 0.8 mM dNTP, 3 mM $MgCl_2$, 0.24 µM of each primer, 0.04 units of Sigma Jump Start *Taq*DNA polymerase. The PCR cycling profile was: 5 min at 94°C; then 35 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C; then 10 min at 72°C. Amplified products were purified using the Qiaquick PCR Purification Kit (Qiagen). DNA sequencing was accomplished at the DNA Sequencing Core at the University of Florida, Gainesville, FL with the BigDye terminator protocol developed by Applied Biosystems Inc. using fluorescently labeled dideoxynucleotides. To verify sequences, haplotypes were aligned with manatee sequences obtained from GenBank using the default settings in GENEIOUS 5.3.5 (Drummond et al., 2011).

II.II.3 - Mitochondrial statistical analysis

Estimates of sequence divergence were obtained under the assumptions of the Kimura 2-parameter genetic distance model (Kimura, 1980; Jin and Nei, 1990). The variance distribution was based on haplotype frequencies alone; all haplotypes were treated as equally differentiated. The genetic diversity (*h)*, nucleotide diversity (π), and the number of nucleotide substitutions (*NS*; Nei, 1987; Tajima, 1993) were calculated for each group using GENALEX 6.41 (Peakall and Smouse, 2006) and MEGA 4 (Tamura et al., 2007).

II.III – RESULTS

II.III.1 - Northeast Brazilian manatee populations exhibit strong phylogeographic division and low haplotype diversity

Manatee mtDNA sequences in GenBank (NCBI) were compared with the Brazil samples sequenced for this study. Three haplotypes were identified in 73 individuals: 69 M01, one M03, and three M04 (a previously unidentified haplotype entered into GenBank accession # JX171295). Genetic diversity statistics for the samples as a whole indicate that within Brazil there is a low chance of randomly drawing two different haplotypes (Table II.1). Two polymorphic sites, with 0.49% maximum sequence divergence, and two nucleotide substitutions were identified in the three haplotypes, indicating low levels of nucleotide divergence among haplotypes.

Table II.1 - Population parameters for all *Trichechus manatus* categories: sample size *(N)*, HT (identified haplotypes), *NS* (number of nucleotide substitutions), *h* (haplotype diversity), and π (nucleotide diversity).

Sample category	*N*	HT	*NS*	*H*	π
Stranded live calves*	47	M01,M03, M04	2	0.043	0.00021
Carcasses and live adults	17	M01, M04	1	0.221	0.00054
Captive-born calves	9	M01	0	0	0
Total	73	-	2	0.08	0.00262

*Including a calf released immediately after rescue

The M01 haplotypes (N=69) were all found from Piauí state (PI) to AL, however, it is interesting to note that none were found in MA, the most northern state in the Northeast region of Brazil (Figure II.3). The previously un-identified haplotype, M04 (N=3), was found in one calf and one carcass from MA and in one placenta from PI (Table II.2). The M04 calf from MA is designated as releasable at this time. An M03 calf was also identified from MA, although it was released in AL in 2001 after rehabilitation.

Table II.2 - Identified haplotypes by location of recovery in states of the Northeastern region of Brazil. Captive-born manatees were born at CMA.

Origin of the animal	M01	M03	M04	Total samples
Maranhão (MA)	-	1	2	3
Piauí (PI)	1	-	1	2
Ceará (CE)	21	-	-	21
Rio Grande do Norte (RN)	20	-	-	20
Paraíba (PB)	8	-	-	8
Pernambuco (PE)	4	-	-	4
Alagoas (AL)	6	-	-	6
Captive-born (CMA)	9	-	-	9

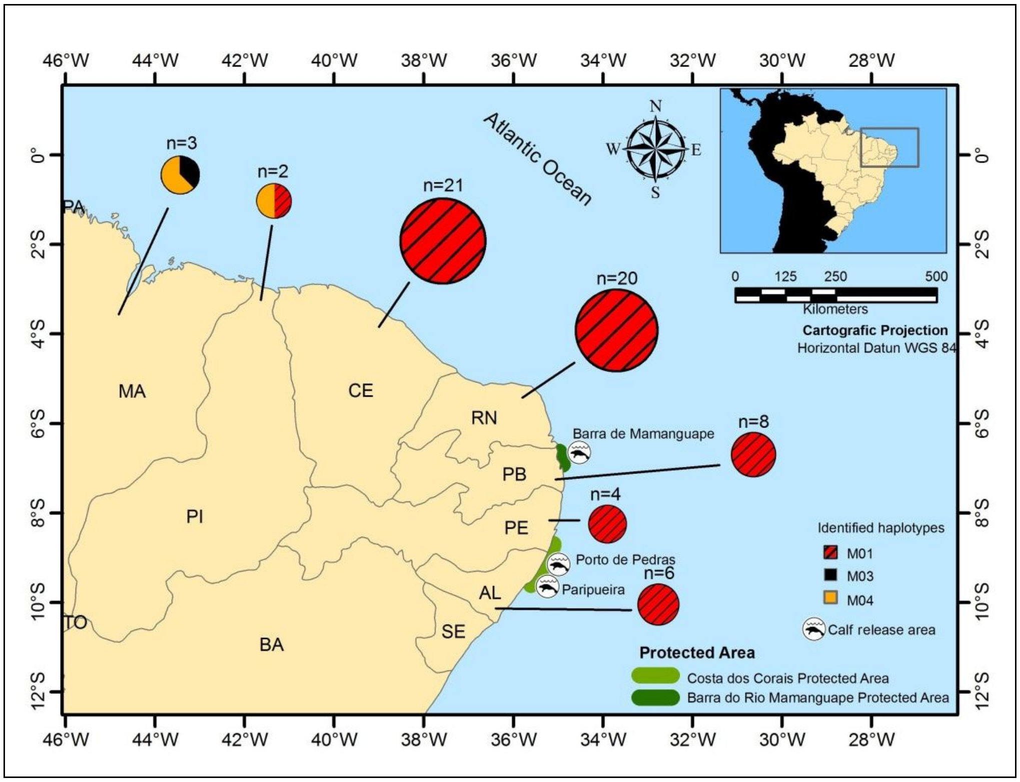


Figure II.3. Map of West Indian manatee haplotype distribution in the Northeastern region of Brazil. Locations of cities where the releases occur are demarcated by the international manatee symbol. The states are as follows, Maranhão (MA), Piauí (PI), Ceará (CE), Rio Grande do Norte (RN), Paraíba (PB), Pernambuco (PE), and Alagoas (AL).

Adapted from: Luna and colleagues (2012)

II.III.2 - Rescue, rehabilitation and release of manatee calves from 1989-2011

From 1989 to February 2011, CMA received 67 rescued calves. After successful rehabilitation, 25 calves have been released in two different National Protected Areas (APAs; ICMBio): APA da Barra de Mamanguape (PB state) and APA Costa dos Corais (AL state; Figure II.3). In addition, following sample collection, a rescued calf was reunited with its mother after the cow was found in the area. The first manatee release event, a male and female pair, known as “Astro” and “Lua”, occurred in 1994 in a third site, Paripueira, AL, which was subsequently designated as a conservation area in 1997 (Lima, 2008). Of the 25 calves released in AL or PB, 22 came from the distant northern states of MA, CE, and RN. Currently, an additional 13 manatees are designated as healthy and releasable. However, one is potentially inbred and a second has an aberrant number of chromosomes (N=50) for the West Indian manatee, suggesting F2 hybridization with an Amazonian manatee (Vianna et al., 2006b). Additional genetic analysis could help to determine their genetic disposition before release. A second group of six calves are candidates for release in the near future.

II.IV - DISCUSSION

The high incidence of dependent calf strandings in Brazil has been reported since 1987, however the rescues began in 1989 (Paludo, 1998; Parente et al, 2004; Meirelles, 2008). The rescue and rehabilitation program makes every effort to return rehabilitated calves to the wild to supplement the small manatee population in Brazil. Conservation biologists typically recommend releasing animals at the site of rescue, or if born in captivity, the location where the mother was captured (Waples and Stagoll, 1997). However, release at the site of rescue is not advised when the area has anthropogenic threats, unsuitable or inappropriate habitat or is not easily accessible for post-release monitoring (Waples and Stagoll, 1997).

II.IV.1 - Considering genetic information in the release of rehabilitated and captive-born manatees

Rescued dependent calves and those conceived in captivity are difficult to release successfully, since they do not have knowledge of obtaining food, water, and shelter in the wild. A few rehabilitated and released manatees had to be rescued a second time by CMA, near the area of release. Since the number of dependent stranded calves continues to grow, captive breeding is not necessary for the conservation of Brazil manatees at this time. However, if the Brazilian manatee population needs supplementation in the future, the development of a captive breeding program and studbook would be necessary to prevent inbreeding. Further, genetic studies using nuclear DNA (nDNA) are needed to aid in the determination of appropriate release candidates. Some captive manatees are suspected to be inbred, or hybridized from West Indian and Amazonian species, and could be reproductively detrimental to the population if released.

Since mtDNA is maternally inherited and does not recombine, it provides a historical perspective of the population and may not provide fine-scale and contemporary genetic sub-structure of the population. Detailed studies using nDNA can help to identify further sub-structuring or subpopulations in manatees, especially in the Northeast Brazil locations where the M01 haplotype was found exclusively (Hunter et al., 2010a). Quantifying relatedness using nDNA markers could better help to determine the most suitable release sites for rehabilitated manatees.

II.IV.2 - Inbreeding depression in isolated Brazilian manatee populations

The extinction of West Indian manatees along the AL and PE state boundary has geographically separated the small AL population from those to the north (Figure I.1). Reintroduction of manatees in this region, specifically in the city of Porto de Pedras, AL, could reconnect the AL population to those in the north, minimize inbreeding depression and establish a continuous manatee population in this area. Establishing a population within this distribution gap could also provide an additional Brazil population to aid in the recovery of the species.

Populations characterized by low diversity, small size and isolation are at risk for inbreeding depression, defined as a reduction in the survival and fertility of the offspring of related individuals, or further reduction of genetic diversity and can become inbred rapidly with little warning (Frankham, 1995; Bijlsma et al., 2000; Frankham et al., 2002). Diversity is considered necessary for adaptation to environmental changes and erosion of the currently low variation could negatively affect the population in the near future (Reusch and Wood, 2007).

II.IV.3 - Genetic swamping and outbreeding depression

The single manatee with the M03 haplotype is presumably the same animal identified by Vianna and colleagues (2006a). This M03 individual was released in 2001 in AL (Lima, 2008), where only M01 has been found in the wild previously. Additionally, many of the other released calves were rescued from the northern states of MA, CE, and the northern coast of RN and may be distinct from the southern populations in AL and PB at nDNA loci.

The release of genetically divergent individuals can result in the genetic swamping of the local populations (Frankham et al., 2002). Genetic swamping occurs when gene flow from dense to sparse populations causes the loss of locally adapted alleles or genotypes (García-Ramos and Kirkpatrick, 1997; Lenormand, 2002). In this case, immigration over time reduces the population fitness and may result in unfit hybrids, ultimately reducing the population size (Allendorf and Luikart, 2007; Roberts et al., 2010). Swamping of local gene pools can also occur when individuals from multiple source populations have interbred and their offspring are released into areas with locally adapted individuals, as has likely occurred with a number of captive-born, released calves (Allendorf and Luikart, 2007).

Nuclear DNA data could help to determine the degree to which interbreeding has occurred. The release of manatees in areas with extirpated populations, such as Porto de Pedras, could reduce the effect of genetic swamping in an established population (Alleaume-Benharira et al., 2006). A reestablished population should be supplemented over time to avoid a genetic founder effect until breeding from the surrounding populations occurs or the population becomes sufficiently large.

Alternatively, outbreeding depression can occur when separate populations adapted to similar environmental conditions interbred. Outbreeding depression results when gene complexes that interact to produce favorable effects are disrupted in the genetic cross (Frankham et al., 2002). Since the Northeast regional population is small and has low mtDNA diversity, release of genetically divergent captive animals into the population could result in a reduction of fitness due to outbreeding depression or genetic swamping. Nuclear microsatellite analyses could help to identify genetically divergent populations to avoid detrimental genetic consequences.

According to Allendorf and Luikart (2007), efforts should be made to release animals in locations with the same haplotypes until nDNA testing is completed. Intraspecific variation is also needed for adaptive potential and could be encouraged by using nDNA data as a guide. The two release locations, AL and PB, were found to contain the M01 haplotype, making it a prudent location to release rehabilitated or captive born M01 manatees at this time (Figure I.1). However, these locations would not be suitable for release of individuals with M03 or M04 haplotypes, such as the MA, M04 calf currently classified as releasable.

However, the majority of the released calves (N=22), with M01 haplotypes have originated from distant locations and may be genetically different from the release site populations at nDNA loci. Animals released in non-natal locations (genetically or environmentally) may be genetically different at nDNA loci and may not be well adapted to the local habitat, vegetation, diseases or seasonal changes (Frankham et al., 2002).

The new M04 haplotype was found in a calf and carcass from MA and in a placenta from PI (Table II.2). Since haplotypes are passed to the offspring from their mothers, both the mother and calf associated with the placenta are expected to be the M04 haplotype, bringing the total to two in PI. It appears that PI could be a mixing zone, with both M01 and M04 haplotypes present.

II.IV.4 - Conservation of West Indian manatees in Northeastern Brazil

The low levels of haplotype and nucleotide diversity of the Northeastern Brazilian manatee population are indicative of small, isolated populations enduring bottlenecks and/or long-term persecution (Jamieson et al., 2006). Current management practices of releasing manatees into areas containing non-related individuals may be detrimental to the population and affect the chances of survival of the rehabilitated animals. Release to extirpated habitat, such as Porto de Pedras, could limit genetic swamping and outbreeding, while connecting the isolated AL group to the northern population. Releasing manatees together to improve their adjustment to the wild may be beneficial, and strong protections against hunting pressures would need to continue.

Further studies focused on nDNA are anticipated to provide more detail on the fine-scale phylogeographic relationships of manatee populations throughout Brazil. Identification of isolated or fragmented populations is important, as lack of nuclear gene flow has been shown to have significant deleterious effects on inbreeding, fitness and population sustainability (Frankham, 1995; Frankham et al., 2002). Further monitoring and conservation efforts will help to protect the critically endangered West Indian manatee population in Brazil.

Chapter III - Nuclear genetic diversity and population structure of the West Indian manatee (*Trichechus manatus*) in Brazil.

III.I - INTRODUCTION

The West Indian manatee (*Trichechus manatus*) is classified as vulnerable by the International Union for the Conservation of Nature (IUCN, 2012). In Brazil it is classified as critically endangered by the Brazilian government (Ibama, 1989; MMA, 2003). Hunting since the Portuguese colonization has severely decreased abundance of the manatee in Brazil (Domning, 1981b; Domning, 1982). Further, the low reproductive rate has limited West Indian manatee population growth in the country.

The actual range is from Alagoas to Amapá states, but with discontinuity in areas between Alagoas, Pernambuco, Ceará (Lima, 1997), Maranhão and Pará (Luna, 2001). The species is extinct in Espírito Santo, Bahia and Sergipe state (Albuquerque and Marcovaldi, 1982; Borobia and Lodi, 1992; Lima et al., 1992; Lima, 1997). (Figure III.1). The total population is estimated to about 500-1,000 individuals (Lima, 1997; Luna, 2001; Luna et al., 2008b; Luna and Passavante, 2010; CMA/ICMBio, in prep.).

Currently hunting has decreased, however, new anthropogenic threats have been increasing for the manatees throughout the country. These threats include accidental death in fishing gear (Oliveira et al., 1990), habitat degradation on the coast and estuaries including effluent from industry, shrimp farms, salt production, sugar cane production, the oil industry and a higher concentration of boats. Also a large number of stranded calves occur in Ceará (CE) and Rio Grande do Norte (RN) states, limiting the growth of the population (Chapter II). In the north coast of Brazil, the ecosystems are more conserved than in the northeast, and the manatee population is likely more preserved (Luna et al., 2008b; Luna and Passavante, 2010).

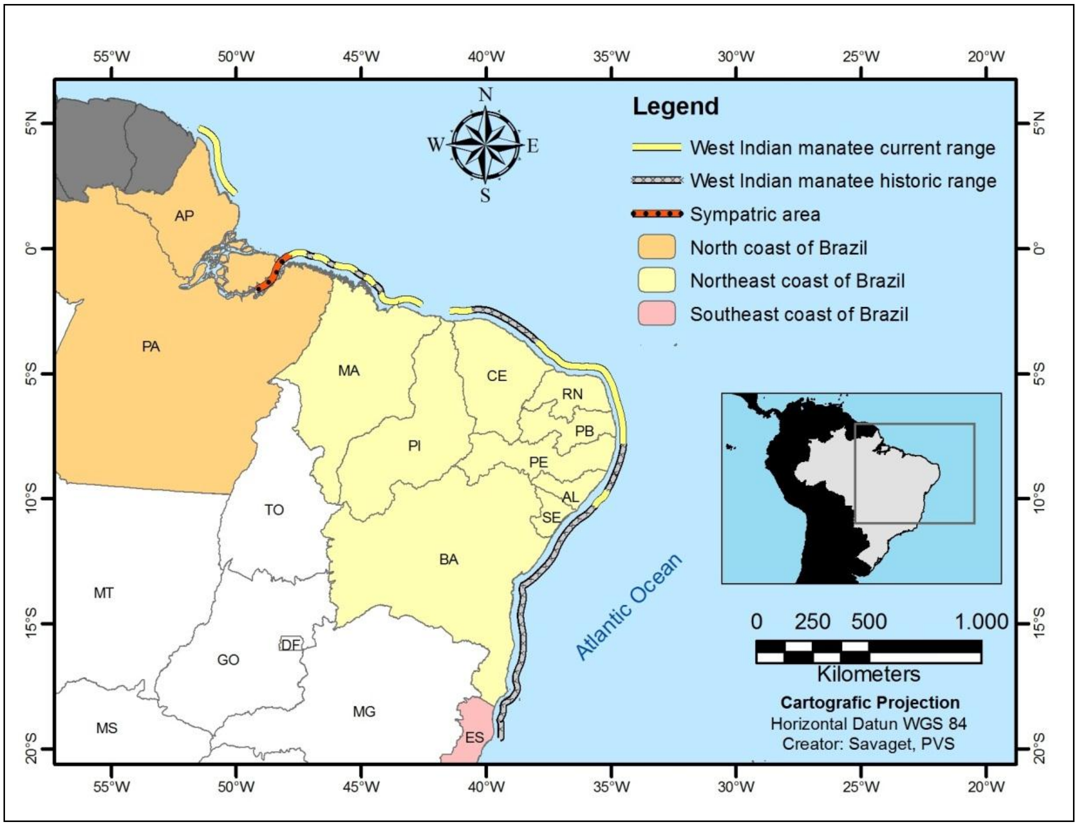


Figure III.1 – Range of the West Indian manatee in Brazil.

Adapted from: Luna and Passavante (2010)

For West Indian manatees low levels of genetic diversity and small population sizes have been observed in previously studied populations in Florida, Puerto Rico, Belize and Mexico (Bradley et al., 1993; García-Rodríguez et al., 1998; Vianna et al., 2006a; Hunter et al., 2010a; Hunter et al., 2012; Nourisson 2011; Nourisson et al., 2011, Tucker et al., 2012), when compared with other fragmented or hunted populations (DiBattista, 2007, Garner et al., 2005). Small populations usually have reduced genetic diversity, which can negatively influence fitness (Roelke et al., 1993), decrease population viability (Sherwin and Moritz, 2000) and increase susceptibility to disease (O’Brien et al., 1983). Demographic and stochastic events can reduce genetic variation and population size very rapidly (Frankham et al., 2002).

Previous genetic studies in Brazil using mitochondrial DNA (García-Rodríguez et al., 1998; Vianna et al., 2006a) and only two microsatellites were used to address hybridization of a single manatee and to define the mtDNA haplotype presence in Brazil with a comparative phylogeography study of manatees throughout their range (García-Rodríguez et al., 1998; Vianna et al., 2006a). However, there is no knowledge about the specific genetic structure and diversity of the entire manatee population distributed along the coast of Brazil.

To protect small populations and improve their conservation, genetic and demographic information are desperately needed. Detailed actions for conservation of the manatees are listed in the National Action Plan for Sirenia in Brazil (ICMBio, 2010; Luna et al., 2011). This study will address fine-scale genetic structure within the Brazilian manatee population using microsatellite markers. This information can be used to update conservation plans thus improving manatee management and preservation in Brazil, describe the genetics of the Brazilian manatee population along it's range, and establish actions based on this information to avoid genetic problems for the species.

III.II – MATERIALS AND METHODS

III.II.1 - Microsatellite DNA amplification and fragment analysis

Samples from 76 manatees from different states of the Northeast coast of Brazil including, Alagoas (AL, N=4), Pernambuco (PE, N=2), Paraíba (PB, N=11), Rio Grande do Norte (RN, N=20), Ceará (CE, N=24), Paui (PI, N=3), Maranhão (MA, N=4); and two states in the North of Brazil, Pará (PA, N=5) and Amapá (AP, N= 3), were analyzed. The five samples from PA and two from AP were geographically collected from the estuary in the mouth of the Amazon River. A third sample ("Poque") from AP was collected north of the Amazon River, near the Brazilian border with French Guiana (Figure III.1). As "Poque" was the only sample from north of the Amazon River, 10 more samples from Guyana (N= 6) and Venezuela (N=4) were added for comparison with the region called "Northwest of the Amazon River estuary - NWA".

Blood or skin tissue from wild carcasses and stranded calves was collected by manatee researchers throughout Brazil. Blood from wild-born captive manatees with known rescue locations was also utilized for this study. Blood and tissue samples were preserved with lysis or tissue buffer respectively: lysis buffer: 100 mM Tris–HCl, 100 mM EDTA, 10 mM NaCl, 1.0% SDS (White and Densmore, 1992); SED tissue buffer: saturated NaCl; 250 mM EDTA pH 7.5; 20% DMSO (Amos and Hoelzel, 1991; Proebstel et al., 1993).

DNA extraction, amplification and fragment analysis were performed at the US Geological Survey, Southeast Ecological Science Center, Conservation Genetics Laboratory in Gainesville, FL, USA. DNA extractions were carried out using the DNeasy Blood and Tissue Extraction Kit (QIAGEN, Valencia, CA, USA). Three samples (a single carcass and two calf samples) were isolated using traditional phenol chloroform technique, due to the state of decomposition of the carcass and the small size of the calf samples. Polymerase chain reaction (PCR) amplifications were performed for the Brazil samples with 16 microsatellite loci previously designed for manatees (Table III.1; García-Rodríguez et al., 2000; Pause et al., 2007). Samples collected from the countries to the northwest of Brazil (Guyana and Venezuela) were analyzed at 12 loci, due to the poor amplification of markers TmaF14, TmaA02, TmaE01 and TmaK01.

Amplifications were conducted in a Biometra UNOII, T-Gradient thermocycler (Biometra, Gottengen, Germany) or on a PTC-100 or PTC-200 (MJ Research, Waltham, MA) thermocycler using the following conditions: 95°C for 5 min, 35 cycles of 95°C for 30 s, with the specific annealing temperature for each primer as listed in Table III.1, 72°C for 30 s, and a final extension at 72°C for 10 min. Amplifications were performed in a total volume of 12.7 uL, with 10 ng target DNA, 1X Sigma PCR Buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl, 0.001% gelatin), 2.5mM of $MgCl_2$, 0.2 mM each dNTP, 0.01U ul^{-1} of Sigma JumpStart Taq polymerase (Sigma–Aldrich, St. Louis, MO, USA), quantity of each primer as specified in Table III.1, and $1mg.ml^{-1}$ bovine serum albumin (BSA) when indicated by García-Rodríguez and colleagues (2000) and Pause and colleagues (2007).

For fragment analysis, the forward primers were labeled with the fluorescent dyes VIC, HEX or 6-FAM for processing and visualization on an ABI 3130xl Automated DNA Analyzer. Fragment data from the PCR products were performed on an Applied Biosystems ABI 3730 Genetic Analyzer and analyzed using GENEMARKER, version 1.5 (Soft Genetics, State College, PA, 2008) to determine allele sizes. Allele sizes were standardized using previously analyzed Florida manatee samples for comparison and data binning.

Table III.1 - Characteristics of the 16 polymorphic microsatellite loci implemented on the Brazilian manatee samples from the Northeast coast. Optimized annealing temperature and primer concentration requirement.

Locus name	Temperature °C	*Primer µM*
TmaSC5	60*	0.118
TmaJ02	60	0.094
TmaKb60	60	0.091
TmaSC13	56*	0.118
TmaE14	56*	0.103
TmaE04	57	0.181
TmaE07	57	0.220
TmaH13	54*	0.099
TmaK01	54	0.107
TmaE01	54	0.134
TmaE08	57	0.069
TmaE11	57	0.089
TmaE26	59	0.098
TmaA02	59	0.122
TmaF14	59	0.071
TmaM79	59	0.086

*Same as published

III.II.2 - Data analysis

CONVERT (Glaubitz, 2004) was used to convert the data into different input file formats. The program STRUCTURE version 2.3.3 (Falush et al., 2007) was used to identify possible subpopulation designations, without an *a priori* assignment of the overall population structure from Brazil. STRUCTURE, a model-based clustering algorithm, infers population structure by probabilistically assigning individuals without any *a priori* geographic or ancestral knowledge to a specific number (K) of clusters (presumably populations). In determining the number of clusters, the algorithm attempts to minimize deviations from Hardy-Weinberg equilibrium.

Simulations were conducted using the admixture model, which assumes that individuals could have some proportion of membership (q) from each of K clusters, leading to the potential identification of recent immigrants. Multiple Markov chains can delineate differences within populations; therefore 11 parallel chains were analyzed for $K = \{1 – 11\}$, with a run-length of 100,000 repetitions of Markov chain Monte Carlo, following the burn-in period of 100,000 iterations. Twenty independent analyses were simulated for each value of K.

The most probable number of populations, K, was determined by calculating ΔK, an *ad hoc* quantity related to the change in posterior probabilities between runs of different K values (Evanno et al., 2005) in STRUCTURE HARVESTER (Earl and vonHoldt, 2011). An assignment value of >0.90 indicates that the individual is highly assigned to the cluster, with little likelihood that it belongs to a different cluster.

All the samples from the Brazilian coast, the estuary in the mouth of the Amazon River and the countries to the northwest of Brazil were analyzed together. Hieratical analyses were used to analyze the resulting sub-clusters from the initial analysis.

The Northeast coast of Brazil was analyzed alone with 16 microsatellites to address the fine-scale genetic structure in this region. Within this region, the LOCPRIOR model was used to uncover cryptic structure by providing priors for the Bayesian assignment process based on the coasts of sample collection and significant F_{ST} values (Hubisz et al., 2009).

To characterize the clusters identified in STRUCTURE, GENALEX version 6.41 (Peakall and Smouse, 2006) was used to calculate the number of alleles (Na), the effective number of alleles (Ne), the heterozygosity observed (Ho) and expected (He) for all samples together and subgroups of samples (Table III.7).

GENALEX also was used to estimate the probability of identity HW $P_{(ID)}$ which assumes Hardy-Weinberg equilibrium and the $P_{(ID)sib}$, which takes into account the possibility of close relatives in the population. The probability of identity ($P_{(ID)}$) calculates the probability that two individuals drawn at random from a population will have the same multilocus genotype by chance.

GENEPOP web version (Raymond and Rousset, 1995) was used to examine for linkage disequilibrium. Departures from the expected genotypic frequencies in Hardy-Weinberg equilibrium (HWE) were tested using the Markov chain method (dememorization 1000, batches 100, and iterations per batch 1000) in GENEPOP (Raymond and Rousset, 1995).

The presence of null alleles was analyzed with MICRO-CHECKER (Oosterhout et al., 2004). The presence of a potential bottleneck was estimated using BOTTLENECK with different parameters to examine mutation-drift equilibrium (Cornuet and Luikart, 1996).

To assess overall genetic differentiation at the population level, GENALEX calculated F_{ST} using the infinite alleles model and R_{ST} using the stepwise mutation model through an analysis of molecular variance (AMOVA).

Correspondence analysis (CA) was conducted in PAST (Hammer et al., 2001) using a genetic distance matrix constructed in GENALEX. CA was used to examine a dissimilarity matrix of pairwise differences between samples and uses eigenvalue analysis to condense the variation between samples into a limited number of dimensions. The maximum amount of variation was plotted at the first axis with less variation in each additional dimension.

III.III - RESULTS

III.III.1 - Population structure

Bayesian methods in the program STRUCTURE using log-likelihood and Δ*K* analyses assigned West Indian manatees to two clusters and Amazonian manatees to a third cluster (*K* = 3; Figure III.2). The highest Δ*K* was for *K* = 3 (Figure III.3 and Table III.2) which captured the majority of the structure obtained from the data (Figure III.2; Falush et al., 2007).

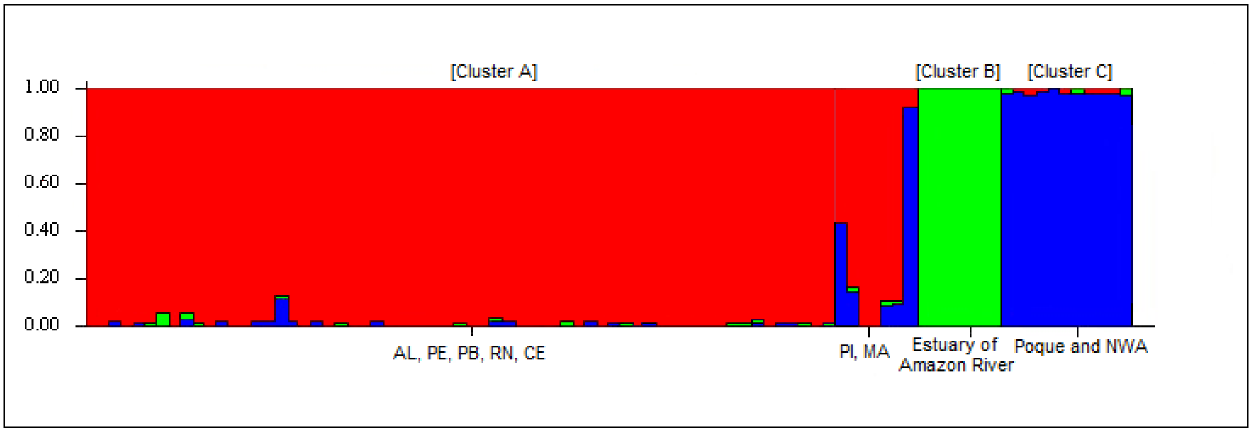


Figure III.2 - Proportions of ancestry for individuals were assessed without *a priori* information using Bayesian clustering via the program STRUCTURE. This graphic represents the best fit of the data, where three population clusters are distinguished (*K*=3).

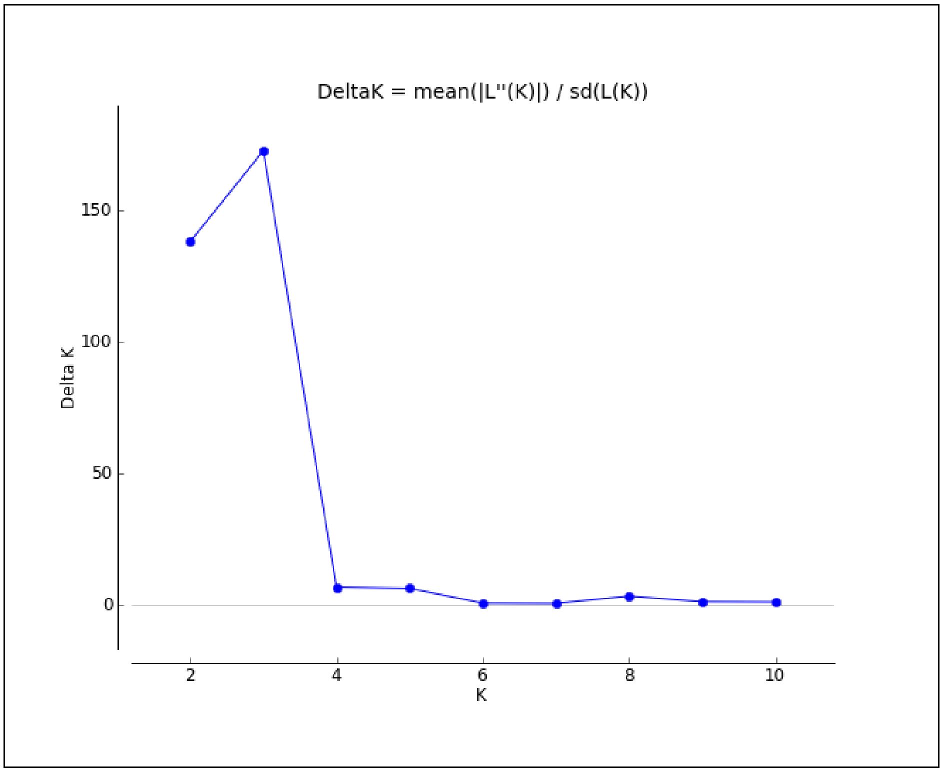


Figure III.3 – Mean DK vs. *K* plots for genotypes obtained from the Brazilian coast.

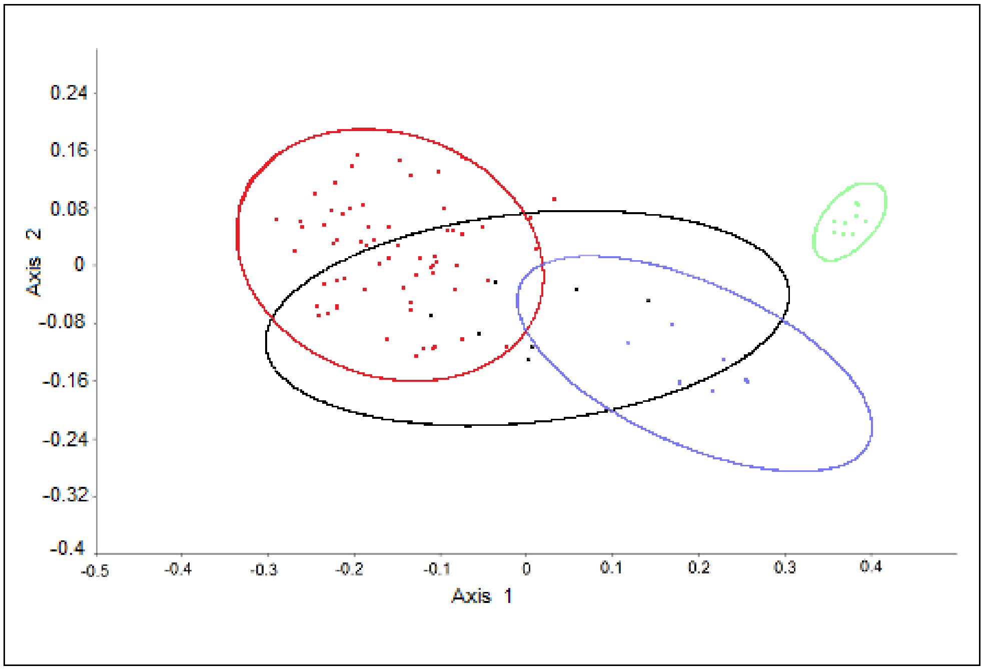


Figure III.4 - Correspondence analysis (CA) of microsatellite genotype data. Northeast coast of Brazil (AL-CE – red; PI-MA - black), Estuary of the Amazon River (green) and Northwest of the Amazon River estuary (blue).

Table III.2 – Delta *K* plot determined in Structure Harvester, to identify the most probable number of populations, *K*, for the West Indian manatee in Brazil. The highest value of delta *K* was obtained for *K*=3.

K	**Reps**	**Mean LnP(*K*)**	**Stdev LnP(*K*)**	**Ln'(*K*)**	**|Ln''(*K*)|**	**Delta *K***
1	21	-2179.790476	0.218872	—	—	—
2	20	-1889.515000	1.181112	290.27576	162.875476	137.900074
3	20	-1762.115000	0.534371	127.400000	92.145000	172.436298
4	20	-1726.860000	1.028745	35.255000	6.730000	6.541953
5	20	-1698.335000	6.897084	28.525000	41.740000	6.051833
6	20	-1711.550000	30.448118	-13.215000	14.69000	0.482460
7	20	-1710.075000	20.301513	1.475000	7.970000	0.392582
8	20	-1700.630000	14.682861	9.4455000	46.0400005	3.135629
9	20	-1737.225000	14.163551	-36.595000	14.270000	1.007516
10	20	-1788.090000	26.785638	-50.865000	24.285000	0.906643
11	20	-1814.670000	29.406303	-26.580000	—	—

The region along the Northeast Brazilian coast including AL, PE, PB, RN, CE, PI, and MA states were identified as a single cluster (Cluster A, red; q = 98.8% (AL-CE) and q = 75.0% (PI-MA); Figure III.2). Within Cluster A, the region containing PI and MA states with some individuals sharing attributes with Cluster C (blue; q = 24.2%) (Table III.2).

Cluster C represents manatees from countries to the northwest of Brazil, and also had a strong assignment value (q =97.8%) (Table III.3). Some individuals from PI and MA states (Cluster A) also had attributes in common with Cluster C (q = 44.0%; q = 14.6%; q = 8.7% and q = 7.8%), and even one individual was strongly assigned to C (q = 92.7%).

Manatees from Cluster B (PA and AP on the Estuary of the Amazon River - green; q = 99.3%) consistently grouped separately from the manatees assigned to Cluster A (Northeast coast of Brazil - AL to MA – red) and Cluster C. The only sample from the Northwest of the Amazon River estuary (“Poque”) was attributed to Cluster C (blue; q = 98.2%). The correspondence analyses (CA) identified three groups overlapping to some degree (red, blue and black; Figure III.4) and one separated from the other (green – Figure III.4). The green are the manatees from the Estuary of the Amazon River and CA indicates that this cluster is totally separated from the others.

Table III.3 - Percentage of individuals correctly assigned to one of *K* = 3 initial clusters (A–C) identified by the program STRUCTURE surveyed at 12 microsatellite loci sites. Individuals were assigned to a cluster based on the largest value of *q.*

Groups	*q* value (*K*=3)		
	Cluster A	Cluster B	Cluster C
AL-CE	98.8		
PI-MA	75.0		
EAR		99.3	
NWA			97.8

The samples from the Estuary of the Amazon River grouped in one cluster (B; green) which is strongly genetically divergent from Clusters A and C. At this site, both species occur in sympatry, allowing for the possibility of hybridization (Luna, 2001; Vianna et al., 2006a). This cluster is analyzed separately in more detail in Chapter IV. Information from this case study will allow us to better address *T. manatus* and *T. inunguis* hybridization theories. Also, it will allow us to analyze more details regarding Cluster C with the addition of more samples from the northern part of Brazil and specific areas north of the Amazon River.

In the subsequent part of this chapter, we address the fine-scale genetic structure within the Northeast coast of Brazil, where Cluster A (red) is analyzed alone with 16 microsatellites.

III.III.2 - Population structure analyses within the Northeast coast of Brazil

Hierarchical structure using log-likelihood and Δ*K* analyses addressed the Northeast coast manatee population alone (Cluster A). Two subclusters were identified by STRUCTURE (*K = 2*; Figure III.5): from AL to east of CE (AL-CE; red; q = 88.6%) and from the states of MA and PI (PI-MA, green; q = 90.1%). The highest Δ*K* was for *K* = 2 (Figure III.6 and Table III.4).

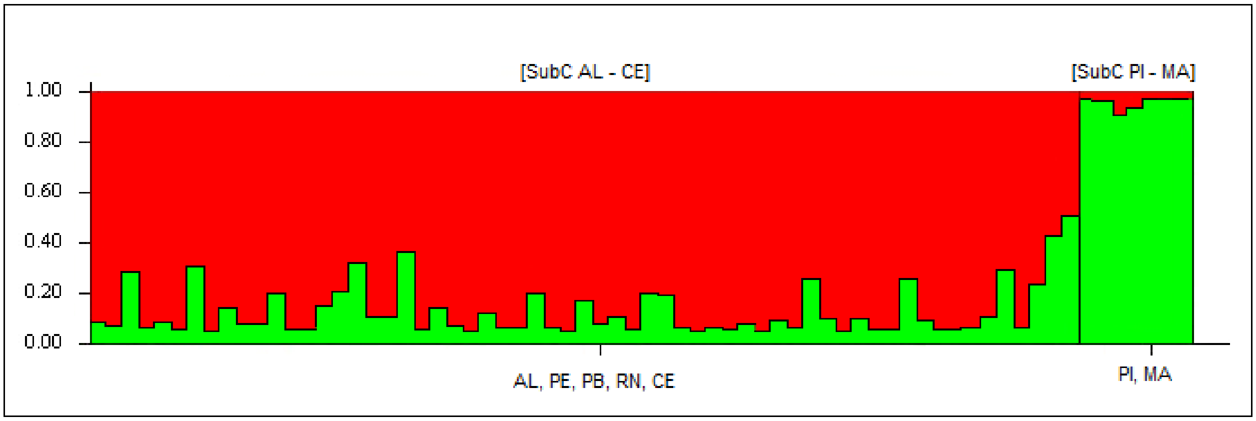


Figure III.5 - Proportions of ancestry for individuals in the Northeast coast of Brazil were assessed without *a priori* information using Bayesian clustering via STRUCTURE. This graphic represents the best fit of the data, where two subpopulation clusters are clearly distinguished (*K*=2). Manatees from PI and MA grouped separately from the manatees from the other states (AL, PE, PB, RN, CE).

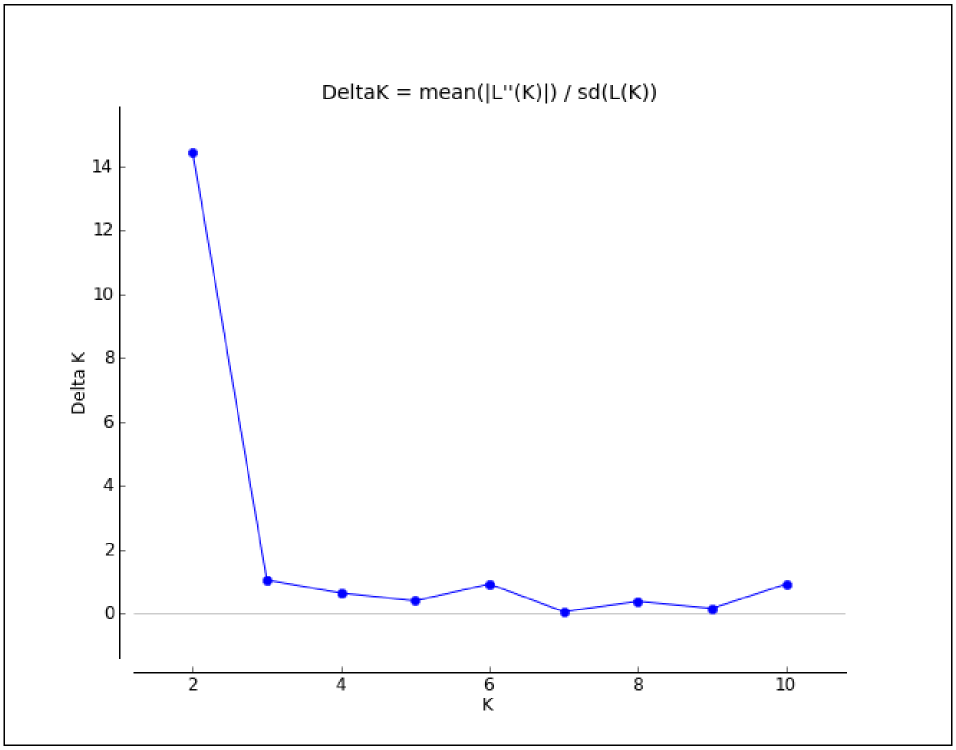


Figure III.6 – Mean D*K* vs. *K* plots for Northeast coast of Brazil manatee genotypes.

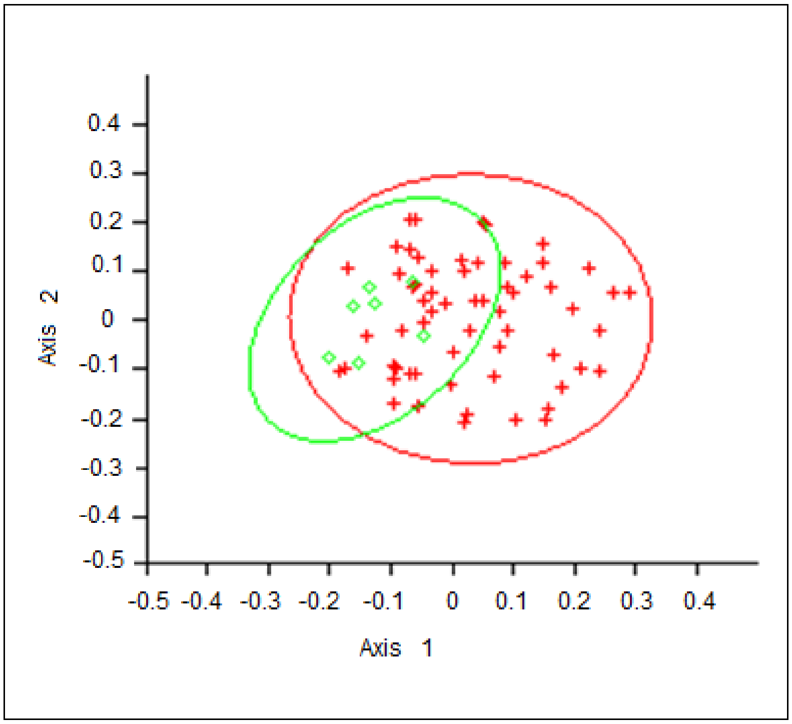


Figure III.7 - Correspondence analysis (CA) of microsatellite manatee genotype data for the Northeast coast of Brazil. AL-CE (green) and PI-MA (red).

Table III.4 - The northeast coast most probable number of manatee populations, *K*, determined by STRUCTURE HARVESTER.

K	Reps	Mean LnP(*K*)	Stdev LnP(*K*)	Ln'(*K*)	|Ln''(*K*)|	Delta *K*
1	20	-1373.38	0.190843	—	—	—
2	20	-1342.44	5.119136	30.945	73.885	14.4331
3	20	-1385.38	43.34537	-42.94	45.14	1.041403
4	20	-1473.46	116.7422	-88.08	74.205	0.635632
5	20	-1487.33	108.8529	-13.875	42.5	0.390435
6	20	-1458.71	72.46247	28.625	66.6	0.919096
7	20	-1496.68	112.5516	-37.975	5.8	0.051532
8	20	-1540.46	171.405	-43.775	64.135	0.374172
9	20	-1520.1	160.7048	20.36	24.785	0.154227
10	20	-1474.95	130.303	45.145	119.56	0.917554
11	20	-1549.37	191.2834	-74.415	—	—

The pairwise F_{ST} and R_{ST} values between AL-CE and PI-MA are presented in Table III.5. Private alleles were identified for AL-CE (N = 13) and PI-MA (N = 5; Table III.6). The correspondence analyses (CA) are presented in Figure III.7.

Table III.5 - Pairwise F_{ST} and R_{ST} values comparing the AL-CE defined (red) and PI-MA (green) manatee subpopulations, generated by GenAlEx 6.2

F-Statistics	Value	P(rand >= data)	9999 permutations- AMOVA- genalex
F_{ST}	0.10	0.000	Significant
R_{ST}	0.30	0.001	Significant

Table III.6 - List of private alleles for AL-CE and PI-MA subpopulations.

Subpopulation	Locus	Private Allele
AL-CE	SC5	139
AL-CE	Kb60	215
AL-CE	SC13	109
AL-CE	SC13	129
AL-CE	E14	250
AL-CE	H13	241
AL-CE	E01	274
AL-CE	E01	276
AL-CE	E08	206
AL-CE	E11	193
AL-CE	E11	197
AL-CE	E26	252
AL-CE	A02	162
PI-MA	J02	238
PI-MA	E14	252
PI-MA	E14	256
PI-MA	K01	194
PI-MA	E11	205

III.III.3 - Genetic diversity within the subclusters

The genetic diversity and differentiation values were calculated for Cluster A identified by STRUCTURE. Subcluster AL-CE, has similar levels of diversity as subcluster PI-MA. Estimates of the mean number of alleles, effective number of alleles, heterozygosity expected and observed, F_{IS} and presence of private for the subcluster AL-CE and subcluster PI-MA are presented in Table III-7.

Table III.7 - Diversity statistics over the 16 microsatellite loci examined for manatees from AL-CE and PI-MA subpopulations in Brazil. The mean number of samples (N), number of alleles (N_A), effective number of alleles (N_E), observed and expected heterozygosity (H_O and H_E, respectively), inbreeding coefficient (F_{IS}), loci out of Hardy-Weinberg equilibrium after Bonferroni correction, linkage disequilibrium after sequential Bonferroni correction (LD), and private alleles for each subpopulation examined: AL-CE and PI-MA.

Population	**AL-CE**	**PI-MA**
N	61	07
N_A	3.00±0.32	2.50±0.29
N_E	1.74±0.18	1.86±0.20
H_O	0.34±0.07	0.33±0.07
H_E	0.34±0.06	0.37±0.06
F_{IS}	0.00558 (not significant)	0.14961(not significant)
HWE after BC	A02, K01,E26, E01	No detection desequilibrium
Bottleneck results	Normal L shape distribution	Normal L shape distribution
LD after Bonferroni	No detection	No detection
Private alleles	13	05

The AL-CE subpopulation has more alleles but less effective alleles than PI-MA subpopulation. The heterozygosity observed is similar (0.33 vs 0.34) for the two populations as the heterozygosity expected is similar, but slightly higher in the PI-MA than in the AL-CE (0.37 vs 0.34) populations.

Monomorphic loci were found for the AL-CE subpopulation (F14, M79), and for the PI-MA subpopulation (M79, F14, A02). After 120 comparisons and a sequential Bonferroni correction, no linkage disequilibrium was observed for any pair (overall $\alpha = 0.05$). Private alleles were found in AL-CE (n=13) and PI-MA (n=5) (Table III-6). All loci were in Hardy–Weinberg equilibrium (HWE) after a sequential Bonferroni correction for PI-MA. Within the AL-CE subpopulation, TmaK01, TmaA02, TmaE01 and TmaE26 were not within HWE. This deviation may be due to cryptic substructuring of the subpopulation (i.e., Wahlund effect), or the presence of null alleles.

In AL-CE, TmaSC5, TmaSC13, TmaE26 and TmaA02 had evidence of null alleles due to heterozygote deficiency. For the PI-MA, no detection was possible by MICROCHECKER because not enough samples were available. The inbreeding coefficient, F_{IS} is not significant for either population. No presence of a bottleneck was detected; both subpopulations have a normal L-shaped distribution.

III.III.4 - Microsatellite genetic information

For the 16 microsatellites used for Cluster A, PIC values ranged from 0.639 for TmaE08 for the most informative marker to 0.056 for TmaA02 for the least informative (Table III-8). Two loci (TmaM79, TmaF14) were monomorphic. HW $P_{(ID)}$ at each locus varies from 0.15 for the most informative locus (TmaE08) to 0.89 for the least informative locus (TmaA02), with the cumulative HW $P_{(ID)}$ for the 16 loci being 1.6E-06. $P_{(ID)sib}$ at each locus varies from 0.44 for the most informative locus (TmaE08) to 0.94 for the least informative locus (TmaA02), with the cumulative $P_{(ID)sib}$ for the 16 loci being 1.9E-3 (Table III-8).

Table III. 8 - Characteristics of the 16 microsatellite loci implemented on the Northeast Brazilian manatee (*T. manatus*) samples (Alagoas to Maranhão). Number of alleles (NA), effective number of alleles (NE), PIC value (PIC), observed and expected heterozygosity (HO and HE), Hardy-Weinberg probability of identity (P(ID) HW) and probability of identity for related individuals (P(ID)sib) and cumulative of P(ID) HW and P(ID)sib, were determined for each loci over the 16 loci starting from the most informative to the least informative.

Locus name	N_A	N_E	*PIC*	H_O	H_E	$P_{(ID)}$ HW	Cumulative $P_{(ID)}$ HW	$P_{(ID)}$sib	Cumulative $P_{(ID)}$sib
TmaE08	5	3.32	0.639	0.565	0.699	1.5E-01	1.5E-01	4.4E-01	4.4E-01
TmaJ02	5	3.001	0.598	0.667	0.667	1.8E-01	2.7E-02	4.6E-01	2.0E-01
TmaSC5	5	2.891	0.584	0.507	0.654	1.9E-01	5.1E-03	4.7E-01	9.5E-02
TmaKb60	4	2.039	0.468	0.507	0.509	2.8E-01	1.4E-03	5.7E-01	5.4E-02
TmaE14	5	2.116	0.423	0.449	0.527	3.3E-01	4.7E-04	5.7E-01	3.1E-02
TmaE11	5	1.758	0.395	0.478	0.431	3.6E-01	1.7E-04	6.2E-01	1.9E-02
TmaK01	3	2.016	0.384	0.913	0.504	3.7E-01	6.2E-05	5.9E-01	1.1E-02
TmaE01	4	1.817	0.379	0.349	0.450	3.7E-01	2.3E-05	6.2E-01	7.0E-03
TmaSC13	5	1.668	0.376	0.291	0.401	3.8E-01	8.9E-06	6.5E-01	4.5E-03
TmaH13	3	1.471	0.277	0.246	0.321	5.1E-01	4.5E-06	7.2E-01	3.2E-03
TmaE07	2	1.348	0.225	0.246	0.258	5.8E-01	2.6E-06	7.7E-01	2.5E-03
TmaE26	3	1.107	0.093	0.072	0.097	8.2E-01	2.2E-06	9.1E-01	2.2E-03
TmaE04	2	1.107	0.092	0.072	0.096	8.2E-01	1.8E-06	9.1E-01	2.0E-03
TmaA02	2	1.061	0.056	0	0.058	8.9E-01	1.6E-06	9.4E-01	1.9E-03
TmaF14	1	1	0	0	0	1.0E+00	1.6E-06	1.0E+00	1.9E-03
TmaM79	1	1	0	0	0	1.0E+00	1.6E-06	1.0E+00	1.9E-03

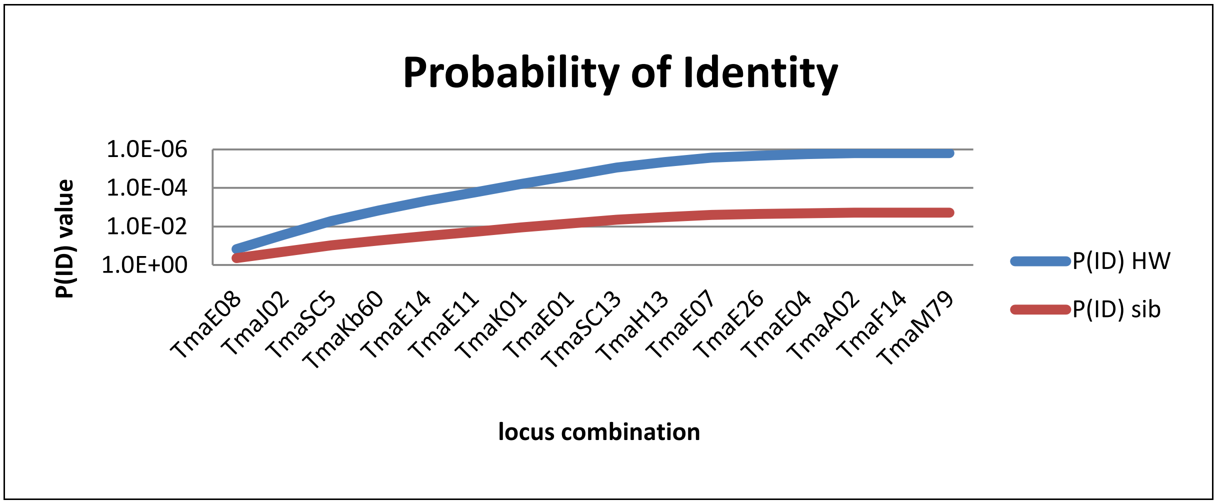


Figure III.8 – Probability of identity for the successive combinations from one locus to 16 loci. P(ID) HW.

The manatee population is not likely composed only of close relatives and therefore, the true value of $P_{(ID)}$ is somewhere in between these two estimates. The individual genetic fingerprint allowed us to distinguish each individual separately.

III.IV – DISCUSSION

III.IV.1 - Population structure

The program STRUCTURE was run for the manatee samples from the Brazilian coast, the Estuary of the Amazon River and from other countries to the northwest of Brazil for comparison. The manatees clustered as follows: *i*) Northeast coast of Brazil (NEB), composed of samples from Alagoas to Maranhão states; *ii*) Northwest of the Amazon River estuary (NWA), composed of samples from Amapá state in Brazil and from Guyana and Venezuela; and *iii*) Estuary of the Amazon River (EAR). However, the correspondence analysis indicated that NEB and NWA had some degree of relatedness, with slight 95% confidence ellipses overlapping. Further, small sample sizes in NWA could bias results and additional samples are needed from this region.

The upper most hieratical structure analysis indicated that the Northeast coast of Brazil (Cluster A – Figure III. 2) has two separate subpopulations (AL-CE and PI-MA – Figure III. 5). The $\ln(Pr(X|K))$ for K=2 value is only slightly less than the value for K=1. Falush and colleagues (2007) suggest that the smallest K is often correct when several values of K give similar estimates of $\ln(Pr(X|K))$. However, some individuals are strongly assigned to one population or another. Falush and colleagues (2007) suggest that if the proportions assigned to each group are asymmetric, then there is a strong indication that real population structure is present. That is especially indicated when the K value captures the majority of the structure in the data and is biologically sensible (Falush et al., 2007). The program STRUCTURE identified two different subpopulations, with asymmetric assignment and the majority of the structure in the data, suggesting that K=2 should be considered.

The AL-CE subpopulation appeared more homogenous and the PI-MA appeared to have some mixing with AL-CE, but also to some degree with Cluster C (from AP, Guyana and Venezuela, on the Northwest of the Amazon River estuary), which are consistent with previous mtDNA analyses (Chapter II). The pairwise F_{ST} value between AL-CE and PI-MA is moderate and significant (F_{ST} = 0.10), and R_{ST} is strong and also significant (R_{ST} =0.30) (Balloux and Goudet, 2002). Pairwise F_{ST} and R_{ST} values presented in Table III.5 also separate the northeast into two subpopulations. The correspondence analyses (CA) followed the pattern found in the Bayesian analyses, genetic differentiation was detected between AL-CE and PI-MA, which is divided in two subpopulations. There is a large discontinuous area that has formed due to recent anthropogenic extinction events, which can separate the manatees in these two areas into two different populations.

The separation in the distribution appears to have decreased the gene flow between the two subpopulations along the northeast coast of Brazil, which seens to be totally separated now. There is no guarantee of the promotion of the gene flow through this area, because of the great distance (~400km) between AL-CE and PI-MA subpopulations, and the establishment of open travel corridors is very difficult, which suggests that the subpopulations will remain separated and two populations will be estabilished. These results indicate the existence of two management units for the northeast coast of Brazil, and four in total on the coast of Brazil, as there are four differents groups delimitated by STRUCTURE, each with different threats and habitat use (Figure III.9).

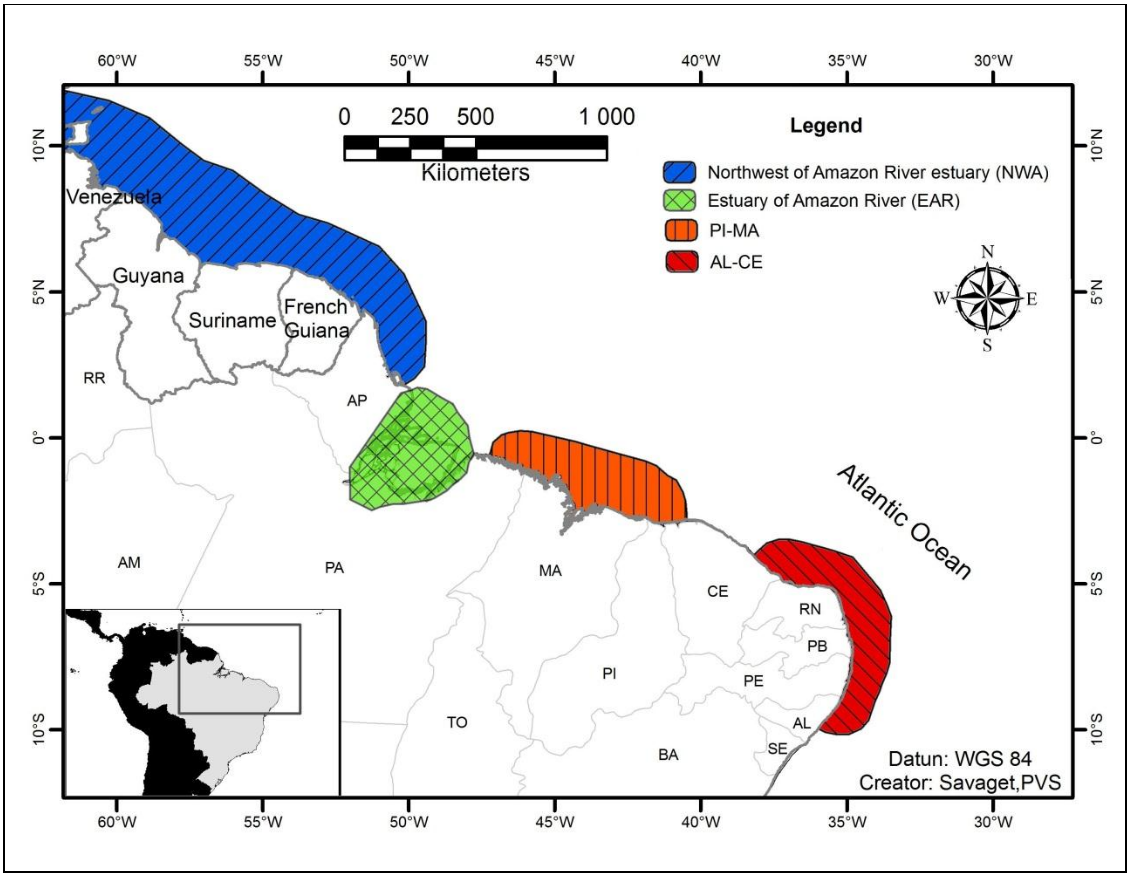


Figure III.9 – Four manatee groups (NWA; EAR; PI-MA; AL-CE) delimitated by STRUCTURE.

The seven samples analyzed from the Estuary of the Amazon River in PA (N = 5) and AP (N = 2) states, cluster together as one population (Cluster B; green – Figure III.2) that is strongly divergent from the samples obtained from the Northeast coast of Brazil (AL to MA states and AP state).

The manatees from the Estuary of the Amazon River represent the region where the Amazonian manatee species occurs sympatrically with the West Indian manatee (Luna, 2001). It was previously hypothesized that hybridization could be occurring between these two manatee species in this area. Chapter IV addresses these questions further by including additional Amazonian manatee samples from other regions for comparative analyses.

Vianna and collaborators (2006a) observed a low genetic connectivity between Brazil and the neighboring manatee populations in French Guiana and Guyana using the mitochondrial control region and a few microsatellite loci. In this study, the only individual that came from the coast of AP was from an area situated on the Northwest of the Amazon River estuary and the sample grouped with the other countries to the north of Brazil (Cluster C, blue; Figure III.2). Some individuals (N=5) from MA and PI state (Subcluster PI-MA) were also assigned to Cluster C (two of which have a strong assignment value) which illustrates connectivity between the PI-MA manatees and the Northwest of the Amazon River estuary population. Additional samples are necessary to address the genetic connectivity in this area.

However, despite the fact that the Amazon River could be a natural barrier, this study identified that some connectivity remains. That supports the theory that some of the West Indian manatees moved southeast and passed the Amazon River to colonize the Northeast coast, as there is higher genetic diversity for manatees in Colombia, followed by Venezuela, Guyana, French Guiana and Brazil (Vianna et al., 2006a). Aditionally, Luna and colleagues (2012) identified more haplotypes in the PI-MA subpopulation (N= 3; M01, M03, M04) versus only a single haplotype found for the AL-CE subpopulation (M01; Chapter II).

The migration from Colombia to Brazil, and from the north coast of Brazil to the northeast of the country (Figure III.10), supports the idea of a founder effect on the southern range of the species, where the genetic diversity is low since their founding. According to Moritz (1995) the comparison between historical and current estimates may identify significant changes to long-term tendencies, thus identifying management unit needs. This information can potentially suggest strategies for declining or fragmented populations which need urgent management and protection measures.

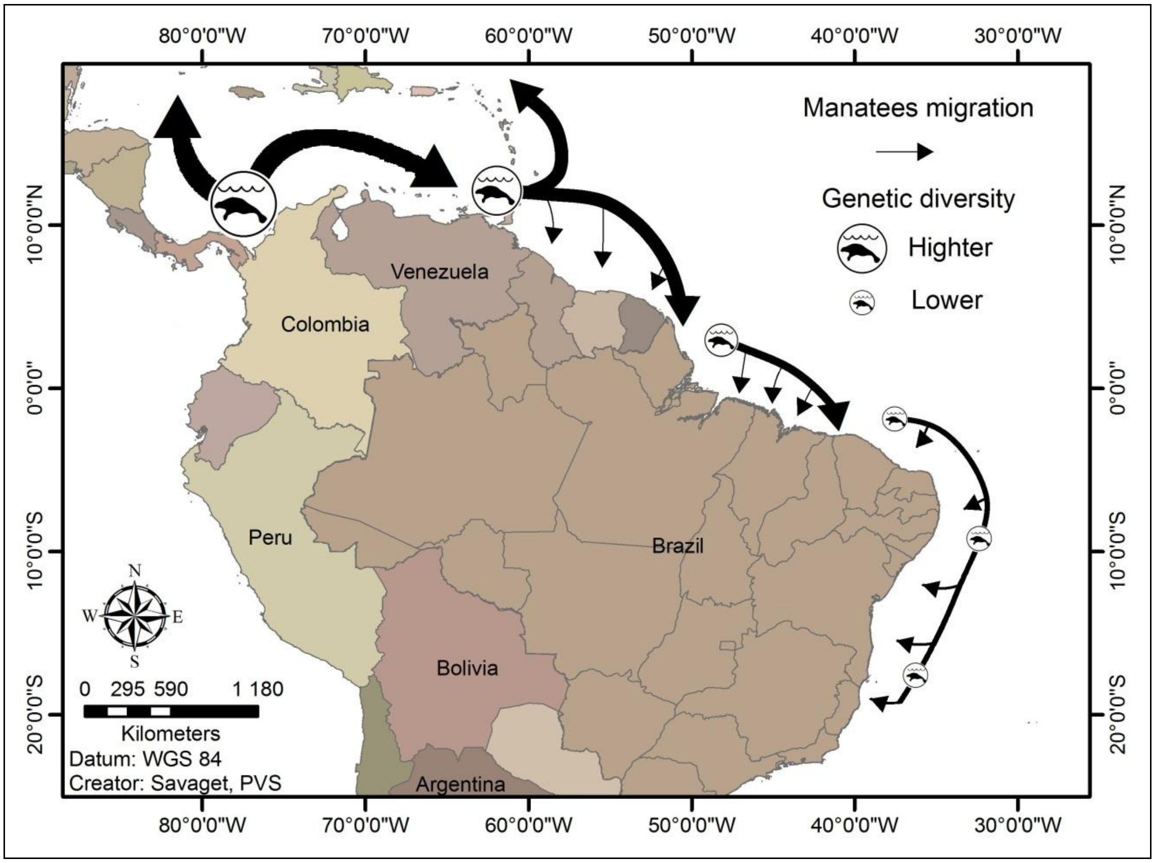


Figure III.10 – Probable road of colonization in Brazil by the West Indian manatee.

In the Northeast coast of Brazil there are two genetic manatee subpopulations, despite the fact that this area is only part of one geopolitical division, and the management of these two subpopulations will require different recovery focus. It is especially important, that in the case of the extinction of one of those two subpopulations, it would not likely be recolonized by the other. This would result in local extinction. The inbreeding coefficient, F_{IS} is not significant for either subpopulation, which does not suggest inbreeding in the population. No presence of a bottleneck was detected and both subpopulations have a normal L-shaped distribution. However, it may be too early to be detected by the program, as the intensive hunting in Brazil may be accelerating the decrease of genetic diversity in the country, which already was low because the founder effect.

According to Frankham and colleagues (2002) an $F_{ST} \geq 0.15$ represents strong genetic differentiation. Other studies have also corroborated this division, suggesting that an F_{ST} in the range of 0-0.05 indicates little differentiation; 0.05-0.15 moderate differentiation; 0.15-0.25 great differentiation; and values above 0.25 have very great genetic differentiation (Balloux and Goudet, 2002; Hartl and Clark, 1997). The significant values for $F_{ST} = 0.10$ and $R_{ST} = 0.31$ between AL-CE and PI-MA indicate genetic differentiation between these subpopulations. The R_{ST} result is larger than the F_{ST}, which indicates mutation has been more responsible for the genetic differences than random drift (Frankham et al., 2002). That indicates that the population was big enough to have more mutation than random drift would allow and is consistent with the theory that manatee populations were much larger prior to hunting during the colonization period, so, even under a founder effect, the genetic diversity was higher and affected by the hunting.

The genetic differentiation values indicate organized structure along the Northeast coast of Brazil, with two genetic clusters. Some mixing was found in PI-MA on the first structure analysis with a K=3 value, but no mixing structure was detected in the analysis of the sub-clusters. Manatees in Brazil do not have seasonal migrations as has been observed in Florida, which reinforces the likely separation of the subpopulations (AL-CE and PI-MA), and enables the identification of two Management Units for the northeast coast of Brazil. The connectivity of the populations and genetic exchange is important for the sustainability of genetic diversity, but appears to be lost due to historical hunting and excessive anthropogenic habitat use and destruction.

Vianna and collaborators (2006) also suggested that “Poque” was a hybrid. The hybridization could lead to a reduction in the number of pure *T. manatus* individuals in the region of French Guiana and Brazil. Reduction of breeding individuals in the population will affect their fitness. Further discussion regarding hybridization will be presented in Chapter IV.

III.IV.2 - Genetic diversity

In general, marine mammal populations have limited population structure and low diversity, influenced by glacial events, founder effects, bottlenecks, anthropogenic mortality, habitat degradation and specialized mate selection and breeding strategies (Waldick et al., 2002; Paster et al., 2004; Roman and Palumbi, 2003)

For manatees, long-distance dispersal ability and opportunities for gene flow can be limited as they must remain close to the shore for food and freshwater (Vianna et al., 2006; Lefebvre et al., 2001). Also, manatees lack specific breeding and calving grounds, unlike other marine mammal populations, which could bring diverse individuals together.

The higher number of alleles found in the AL-CE population when compared to the PI-MA is not consistent with the higher number of haplotypes found in the PI-MA population from the mtDNA data. However, this is likely due to the discrepancy in sample sizes between the two regions and the limited sample size of the PI-MA population.

The genetic diversity within the Northeast coast of Brazil is significantly lower than demographically-challenged mammalian populations (DiBattista, 2007; Garner et al., 2005). It also has less heterozygosity than other manatee populations like Florida, Belize, Puerto Rico and Mexico (Kellogg, 2008; Hunter et al., 2010a; Nourisson 2011; Nourisson et al., 2011; Hunter et al., 2012; Tucker et al., 2012). The genetic diversity measured by N_A is superior to the Gulf of Mexico (GMx) and similar to the Chetumal Bay (ChB) populations in Mexico but lower than other studied manatee populations (Florida, Puerto Rico and Belize) (Table III.9), which means that Brazil's manatee population has one of the lowest genetic diversties among the West Indian manatee distribution.

Table III.9 – Genetic diversity for different manatee populations. Number of samples (N), Number of alleles (N_A), effective number of alleles (N_E), observed and expected heterozygosity (H_O and H_E).

	Brazil AL-CE	Brazil PI-MA	Belize	Mexico ChB	Mexico GMx	Puerto Rico	Florida
N	61	7	118	51	28	113	341
N_A	3.0	2.5	3.1	3.0	2.6	3.9	4.8
N_E	1.74	1.86	2.00	1.99	1.84	1.90	2.08
H_O	0.34	0.33	0.45	0.47	0.44	0.45	0.46
H_E	0.34	0.37	0.45	0.46	0.41	0.45	0.48

The R_{ST} value between AL-CE and PI-MA is much larger than the R_{ST} value between the two subspecies (*T. m. manatus* and *T. m. latirostris*) found between Mexico/Florida; Belize/Florida and Puerto Rico/Florida (Kellogg, 2008; Hunter et al., 2010a; Nourisson 2011; Nourisson et al., 2011; Hunter et al., 2012; Table III.10). However, only seven PI-MA manatee samples were analyzed so more samples are needed from this region.

Table III.10 - Pairwise F_{ST} and R_{ST} values comparing Brazil AL-CE/PI-MA, Mexico ChB/GMx, Mexico/Florida, Belize/Florida, Belize BCC/SLS and Puerto Rico/Florida. (Kellogg, 2008; Hunter et al., 2010a; Nourisson 2011; Nourisson et al., 2011; Hunter et al., 2012).

	Brazil AL-CE/ PI-MA	Mexico ChB/GMx	Mexico ChB/ Florida	Belize/ Florida	Belize BCC/SLS	Puerto Rico/ Florida
R*st*	0.30*	0.11*	0.02	0.08*	0.04*	0.12*
F*st*	0.10*	0.13*	0.09*	0.14*	0.03*	0.16*

* Significant values

The low manatee genetic diversity in the Northeast coast of Brazil can be a combination of a founder effect, as the population defines the southern limit to the range of the West Indian manatee, and to the decrease in abundance of manatees due to intensive historical hunting, added by habitat loss. Despite the severely decreased abundance of the manatee in Brazil since the colonization of the country by the Portuguese, no evidence of a bottleneck was found. However, the population may have gone through a bottleneck, but too recently to be detected with this analysis. As the hunting was very intensive for many years and manatees are a slow reproducing aquatic mammal, there may not have been enough generations to provide evidence of the bottleneck, since only approximately five new manatee generations have occurred since indiscriminate hunting was drastically reduced (Luna and Passavante, 2010).

Demographic and stochastic events can quickly reduce genetic variation and population size in groups with only a few individuals. Small populations also have reduced genetic diversity, which can negatively influence fitness (Roelke et al*.,* 1993), increase susceptibility to disease (O'Brien et al., 1983) and decrease population viability (Sherwin and Moritz, 2000).

Meta-analyses of microsatellite data determined that demographically-challenged mammalian populations have lower genetic variation (H_E = 0.60 and N_A = 6.17-6.59) when compared to undisturbed, healthy populations (H_E = 0.65 and N_A = 8.18; (DiBattista, 2007; Garner et al., 2005)*.* The two Brazilian subpopulations studied here (AL-CE and PI-MA) had lower genetic diversity (H_E = 0.34 and 0.37; N_A = 3.0 and 2.5) than one would expect from disturbed populations experiencing pressure due to pollution, harvesting or habitat fragmentation.

To increase the genetic diversity, long-term protections are needed to allow the population to grow and for genetic mutations and diversity to accumulate. Loss of gene flow can cause the populations to become inbred and therefore lose more diversity. Despite the low genetic diversity, and the reduced population size, no inbreeding was detected in the Northeast manatee populations of Brazil.

III.IV.3 - Management recommendations

The West Indian manatee population in Brazil is estimated to be around 500-1,000 animals (Lima, 1997; Luna, 2001; Luna and Passavante, 2010; CMA/ICMBio, in prep.). The high number of deaths subjected towards manatees has likely forced the populations to remain small, thereby potentially losing genetic diversity. Small inbred populations can limit the effective population size (N*e*), and in turn, genetic diversity.

It has been suggested that a minimum of 500 effective breeders in a population is needed for long-term survival and the prevention of excessive inbreeding (Wright, 1951). This implies that population levels in the upper thousands are needed to maintain evolutionary potential (Franklin, 1980; Lande, 1995). Although no physiological or genetic implications of inbreeding have been identified to date, the population is small and highly susceptible to detrimental genetic effects, so management actions should be conservative.

Due to small population size and low genetic diversity, strong conservation efforts are needed to protect the manatee populations in Brazil. Threats including pollution and loss of habitat, especially with respect to the mangroves, need to be mitigated and impacted areas restored to productive manatee habitat. Long-term exploitation and small population sizes can lower genetic diversity, resulting in decreased fitness, reduced adaptation to environmental change and potentially leading to extinction.

Since manatees have a long generation time (Marmontel, 1995; Rathbun et al., 1995), special habitat requirements (Reynolds et al., 1995) and vulnerability to stochastic events it is imperative that anthropogenic threats be monitored and reduced. The improper use of estuaries that prevents manatee access and limits areas for feeding, drinking freshwater and breeding and parturition are not acceptable.

The rapid growth of these anthropogenic activities reduces the availability of habitats used for breeding and parental care. This loss of habitat results in calves stranding, and has become the main threat to the species in the Northeast coast of Brazil since 1989 (Lima et al., 1992; Lima, 1997; Parente et al., 2004). The impacted areas are predominately found in CE, RN and PB states.

As described in Chapter I the coast of the MA and PI states have better habitat conditions for manatees, including calmer water and more favorable vegetation for the species. However, the loss of habitat has been increasing in the region, which results in calf strandings on the beach, requiring rescue and long-term care for rehabilitation by the CMA/ICMBio team.

In this chapter, two genetically distinct subpopulations were found in Northeast coast of Brazil, which supports the mtDNA result in terms of population division (Chapter II) and reinforces that the stranded calves should not be released in regions with divergent genetic signatures (e.g., AL-CE to PI-MA).

The genetic diversity found in the AL-CE region is low and no substructure was found inside this region. However, as some fragmentation of the manatee distribution has occurred along the coast, there is potential for new genetic substructure or isolated populations. This separation may have already begun but would not be detectable if it is too recent, since one migrant per generation is usually enough to maintain minimal population connectivity (Frankham et al., 2002). Therefore, it is very important to maintain travel corridors to establish connectivity between fragmented populations.

Manatees in AL state are geographically isolated, since the small population is discontinuous with the northern portion of the state. To mitigate the gap in the distribution, and prevent genetic isolation in the area, some captive manatees have been released by the CMA/ICMBio in the federal Costa dos Corais Protected Area where this gap occurs (Figure III.11). To prevent inbreeding and possible further local extinction of the species in AL state, the release of animals into the discontinuous habitat should continue, and also future efforts should focus on releases in the historical range south of PE state.

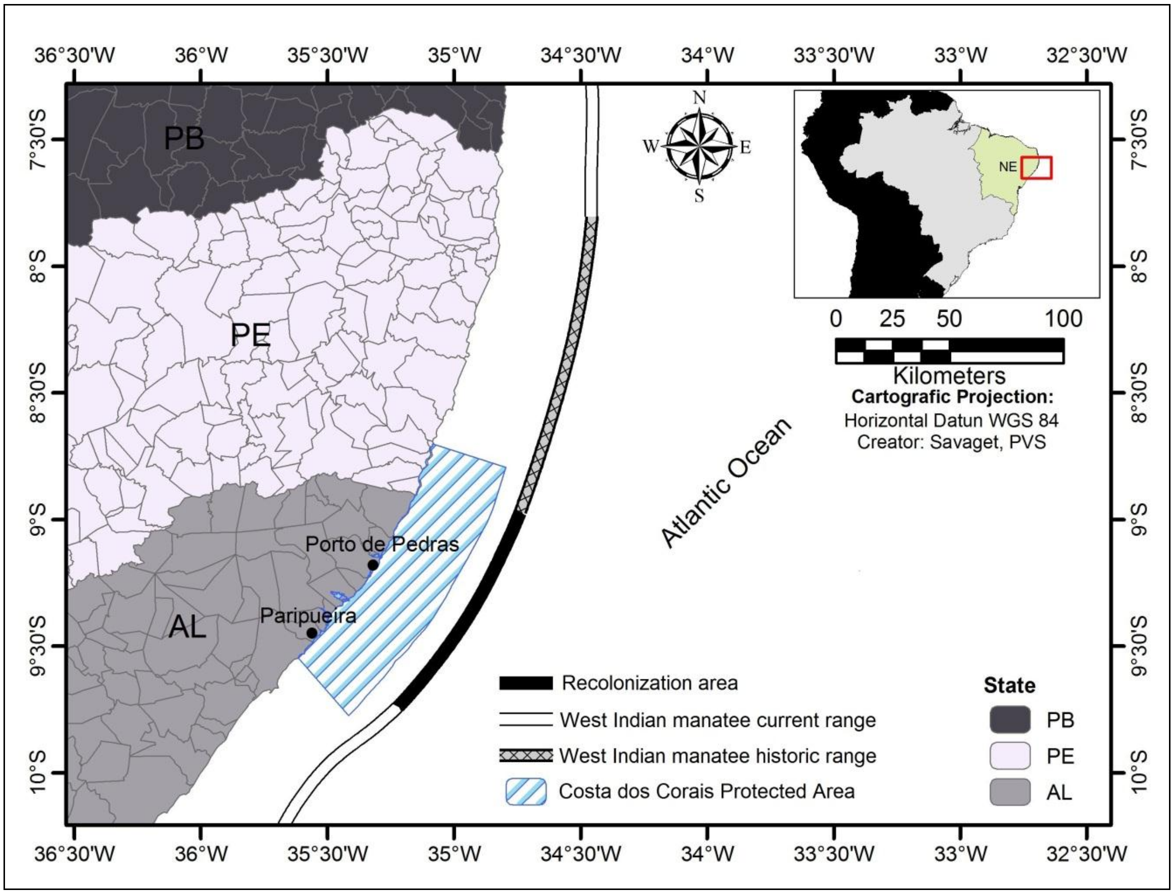


Figure III.11 – Recolonization area in Alagoas state, on northeast coast of Brazil.

The protection of the manatee's habitat is fundamental to the survival of this critically endangered species. In the National Action Plan for Sirenia in Brazil (ICMBio, 2010; Luna et al., 2011), one of the goals is to increase the protection and maintain the quality of the habitat for the species. The effectiveness of the protected areas depends on their management and implementation and more areas need to be created in manatee habitat in Brazil. Attention needs to be taken for the protected areas to be designed to increase the connectivity between the fragmented areas in order to ensure adequate genetic flow.

III.V - CONCLUSION

Two separate subpopulations were found in the Northeast coast of Brazil, one from Alagoas to east of Ceará state and one from west of Ceará, Piauí and Maranhão states. Separate management of the two subpopulations would be beneficial to preserve unique genetic diversity, especially since the manatee habitats and threats are different for the two subpopulations. There is some connectivity between the population in Maranhão, Piauí and west of the Ceará state and the population to the Northwest of the Amazon River estuary.

Despite a long history of intensive hunting, no bottleneck was detected, but it might have occurred too recently and may not be detectable using these tools (Bonde et al., 2012). A founder effect seems to be the likely catalyst for describing the Northeast Brazilian manatee population. Despite the low genetic diversity and the reduced population size, no inbreeding was found, but loss of gene flow through fragmentation areas can cause the populations to exhibit artifacts of inbreeding.

The genetic diversity within the Northeast coast of Brazil is significantly low. To increase the low genetic diversity, long-term protections are necessary to encourage gene flow to these populations. The protection of the habitat and the establishment of travel corridors are fundamental to increase the genetic diversity of manatees in the Northeast coast of Brazil. Additional protected areas need to be created (e.g., east of Ceará state; border between west of Ceará and Piauí; Ilha dos Gatos in Maranhão state; and others), and the protected areas that exist must have effective protection and enforcement, especially in the mangrove ecosystems.

Although these results provide important genetic information, it is necessary to further evaluate the subpopulation structure and the genetic diversity of the species in some regions, like in the northern part of Brazil (Amapá and Pará states) and for the PI-MA subpopulation. Efforts are needed to acquire more samples for further studies from those areas.

Chapter IV – Analysis of hybridization between West Indian and Amazonian manatees using genetic markers and cytogenetic karyotyping

IV.I - INTRODUCTION

In Brazil there are two of the four living species of the Order Sirenia: the West Indian manatee (*Trichechus manatus*) on the coast, and the Amazonian manatee (*Trichechus inunguis*) endemic to the Amazon River. The Amazonian manatee is distributed within all major tributaries and lakes from headwaters in Peru, Colombia, Ecuador and Brazil to the mouth on the Atlantic.

There is no current knowledge about the original extension or distribution of the species in the Amazon, or quantifiable information on areas where the species has become extirpated. Although the species has been massively exploited in Brazil since the pre-colonial period, there is anecdotal information that the species still occurs scattered throughout most of its original range (da Silva et al., 2008b). These pockets of manatees have been reduced in numbers due to intense historic hunting on a commercial scale (Domning, 1982; Best, 1984; Luna, 2008a). For example, in the years 1776-1778 approximately 8,500 manatees were hunted for meat and fat, and in 1886-1898, the equivalent of 6,000 manatees were taken for meat and imported to the mouth of the Amazon River for shipping. Between the years 1935 and 1954, more than 200,000 manatee skins were exported from the Amazon for industrial use (Best, 1982b).

Currently, besides hunting, the Amazonian manatee still faces the destruction and degradation of habitat caused by increased use of the environment. Actually, the species, protected in Brazil since 1967 (Lei n° 5.197/1967), is classified as vulnerable to extinction by the IUCN (2012) and is listed on the Brazilian official endangered species list (Ibama, 1989; MMA, 2003).

Amazonian manatees live in freshwater and are present in lakes and rivers, but can also be found close to the coast in areas within Pará state, Brazil (Domning, 1981b; Luna, 2001). Manatees are not territorial and generally perform annual migrations (Best, 1982a; Best, 1984). West Indian manatees are also known to be present along the coast of Pará state, in the mouth of the Amazon River. The complex estuary in Pará state, with several islands, is a sympatric area for the two manatee species and it is believed that hybridization may occur there (García-Rodríguez, 1998; Luna, 2001). Luna (2001) and Luna and Passavante (2010) mention that the areas of the possible hybridization would likely be near the east side of the Marajó Island (near Salvaterra and Soure) and along the opposite side of the island near the mainland of the State of Pará (Figure IV.1), and strongly suggest that genetic investigations of the hybridization in this area is warranted.

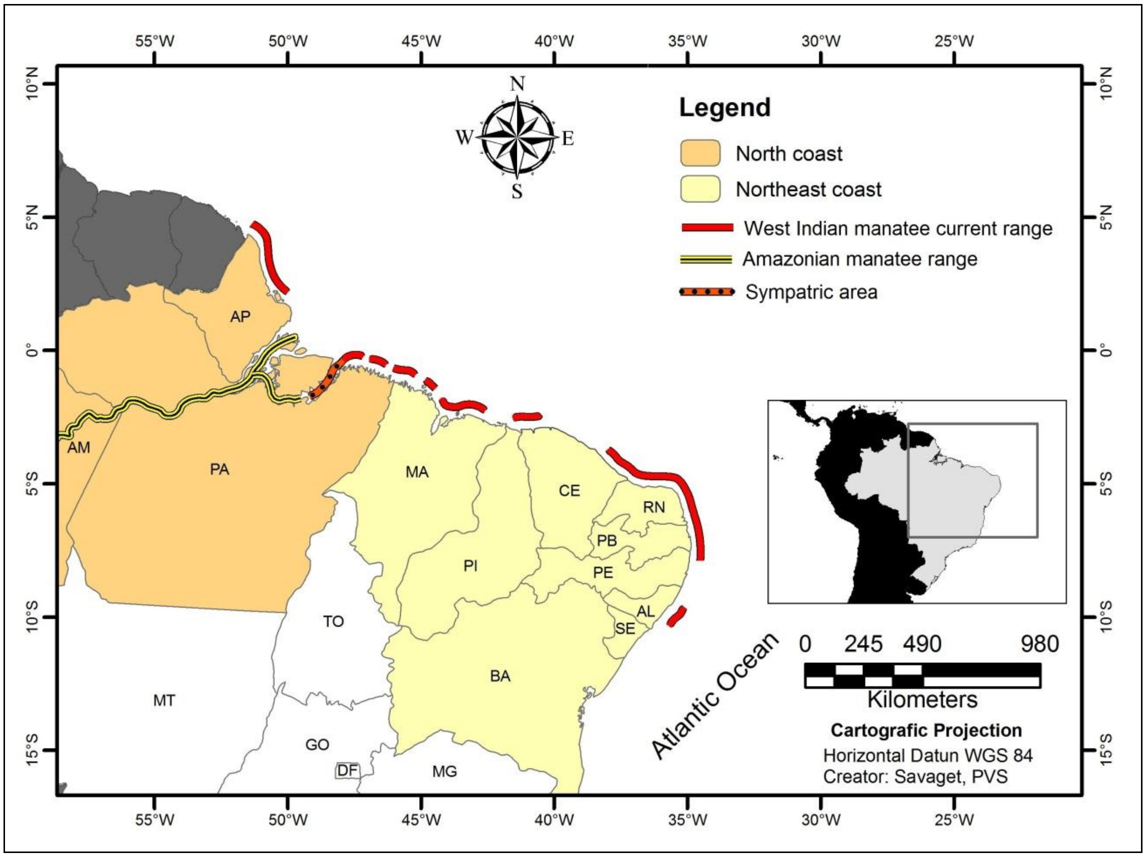


Figure IV.1 – Map of manatee occurrence in Brazil – with sympatric area marked in red/black.

Adapted from: Luna and Passavante (2010)

Genetic studies conducted with samples from manatees from Brazil and Guyana suggested the existence of hybrids between West Indian and Amazonian manatees (García-Rodríguez et al., 1998; Cantanhede et al., 2005; Vianna et al., 2006a and b).

Mechanisms are characteristic in cases of reproductive isolation and are generally believed to be strong enough to prevent the existence of large-scale, successful hybridization between populations. However, in the case of the manatees it is difficult for the researcher to identify hybrids where their interspecific characters can be confusing to discern when making inferences for interspecific comparison using physiology, morphology and genetic attributes. Typically, an increase in the gene exchange between species has been reported, as described for several species by Lehman and colleagues (1991), and specifically for wild mice (Ferris et al., 1983), water frogs (Spolsky and Uzzell, 1984), sunfish (Warn and Saunders, 1984), tree frogs (Advise and Lamb, 1986), deer (Carr et al., 1986), voles (Tegelstrom, 1987), whales (Berubé and Aguilar, 1998), wolves and coyotes (Pilgrim et al., 1998), ducks (Muñoz-Fuentes et al., 2007), bears (Preuß, et al., 2009), pinnipeds (Goldsworthy et al., 2009), mouse lemurs (Hapke et al., 2011), warblers (Hansson et al., 2012). In some of these cases, mtDNA was documented to be transferred across boundaries between species in one or both directions.

The mtDNA genome is frequently used for studies to detect hybridization, where some associated changes relative to geographic areas can be associated. Unlike nuclear alleles that change rapidly through recombination during reproduction, a female's mtDNA genotype can be inherited without disruption or rapid changes and can therefore increase in frequency in a population through future generations without additional hybridization.

A diagnostic mtDNA test detected the introgressive hybridization between wolves and coyotes (Pilgrim et al., 1998). Lehman and colleagues (1991) and Roy and colleagues (1994) identified directional inheritance, affecting wolves but not coyotes. Repeated hybridization between wolves and coyotes has led to the introgression of several coyote mtDNA genotypes into wolf populations. But no coyotes have been found with the wolf genotype. This uni-directional transfer of mtDNA genes is maternal and suggests initial mating of male wolves with female coyotes. As the mtDNA is maternally inherited, the offspring that bred with wolves will backcross with the coyote mtDNA, fixing into the wolf population.

Pilgrim and colleagues (1998) observed that wolves and coyotes coexist in some regions, and hybridization seems unlikely as long as habitat is preserved and wolf numbers are maintained. They identified the increased occurrence of hybridization because of ongoing anthropogenic habitat destruction, as the habitat in hybrid zones is being altered from virgin forest to agriculture use. For wolves and coyotes the hybridization has occurred in areas where deforestation and a conversion to agriculture have taken place (Lehman et al., 1991; Wayne, 1996). In other areas of sympatry, conversion to agriculture is slow or nonexistent; therefore no wolves appear to possess the coyote mtDNA (Lehman et al., 1991; Wayne, 1996).

Cytogenetic tools have also been used in the study of hybridization between species, with the analyses of specific chromosome numbers and morphology of typed individuals. These studies also permit the inference that hybrids can probably be capable of reproduction, but in some cases will not. Jorge and colleagues (1976) reported that animals with more divergent chromosome sets can produce male hybrids with a complete set of sterile germ cells that can be inferred to be secondary to failure during meiotic pairing. They gave the example of meiotic activity that is frequently seen in the testes of mules and hinnies, where spermatozoa are not usually formed because the first meiosis cannot be successfully completed.

In another study, hybrid dogs (*Canis familiaris*) and gray wolves (*C. lupus*) produced fertile offspring in captivity and sometimes in the wild (Mech, 1970; Bibikov, 1982; Boitani, 1982; Lehman et. al., 1991). Hybrids between dogs and coyotes (*C. latrans*) are also fertile and are occasionally found in the wild, being recognized by distinct morphological and behavioral traits (Mengel, 1971).

The two manatee species that occur in Brazil have physical differences. Amazonian manatees do not have nails on the pectoral flippers, measure up to three meters in length and can weigh up to 450 kg, have thick and smooth skin, dark gray to black in color, and generally have a pink or white patch on the ventral region of the body. All these attributes are characteristic to Amazonian manatees and can be used for individual identification to species, however it has been discovered that some individuals do not have the patches on their ventrum (Rosas, 1994; Luna, 2001). West Indian manatees have 3-4 nails on each pectoral flipper, can measure 3-4 meters in length and attain an average weight of 600 kg (Husar, 1978b), skin is thick and rough and the color may vary from gray to brown. West Indian manatees usually do not have patches on the body, nevertheless pink and white patches have been observed on some West Indian manatees in Florida, Puerto Rico, Belize, Mexico and Brazil (Silva Jr et al., 2008; Nourisson, 2011 Nourisson et al., 2011; R. Bonde and C. Beck, personal communication; Luna, personal observation).

A possible manatee hybrid could have some physical characteristics of both species. However, that is not easy to be determined and can be difficult to document. The manatee “Poque” (captive at CMA/ICMBio since 1993; Figure IV.2) is smaller than the other manatees of the same age at CMA/ICMBio, has only three nails instead of four (number most common to date observed in wild West Indian manatees in Brazil; Figure IV.3), and two small markings on his ventrum (Figure IV.4). For all these similar characteristics for a long time the CMA/ICMBio team suspected that “Poque” was a hybrid. In 2006, Vianna and collaborators analyzed “Poque’s” karyotype and revealed an abnormal number of chromosomes ($N = 50$), different from the West Indian manatee ($n=48$) and the Amazonian manatee ($n=56$).

Figure IV.2 – The manatee named “Poque”.

Picture: Jeorge Gregório – CMA/ICMBio

Figure IV.3 – Pectoral flipper with three nails on “Poque”.

Picture: Alisson Cavalcanti – CMA/ICMBio

Figure IV.4 – Two small markings on ventrum of "Poque".

Picture: Fábia Luna – CMA/ICMBio

The National Action Plan for Sirenia in Brazil (ICMBio, 2010; Luna et al., 2011) established the strategic actions necessary to promote studies on genetic variation, gene flow and hybridization of the West Indian manatee (*Trichechus manatus*). The objective of the present study is to examine the evidence and determine the likelihood of hybrids between West Indian and Amazonian manatees in Brazil; especially in the Estuary of the Amazon River group.

The seven manatees examined from the Estuary of the Amazon River did not cluster with other West Indian manatees from the NEB (Chapter III). As those manatees are from the sympatric area (Luna, 2001; Luna and Passavante, 2010) and potentially located in the hybridization mixing zone, this furthers more intensive investigation. The morphological data suggested that they all had Amazonian manatee physical characteristics, but for this analysis they were not previously classified as Amazonian manatees due to their original geographic location of recovery in the sympatric area.

IV.II – MATERIAL AND METHODS

IV.II.1 – Sample collection and DNA extraction.

There were seven manatee samples from the Estuary of the Amazon River (EAR, N = 7), where five were from Pará state and two from Amapá state. To test for the potential of hybridization between West Indian and Amazonian manatees in this region, samples were included from three other areas along the border with the Amazon estuary: Northeast coast of Brazil (NEB; N= 21), Santarém River region (STM; N = 14) and the Northwest of the Amazon River estuary (NWA; N = 11; including the captive manatee "Poque"). This represents samples from all geographic locations within the area of study (Figure IV.5), as well as contains samples from both manatee species.

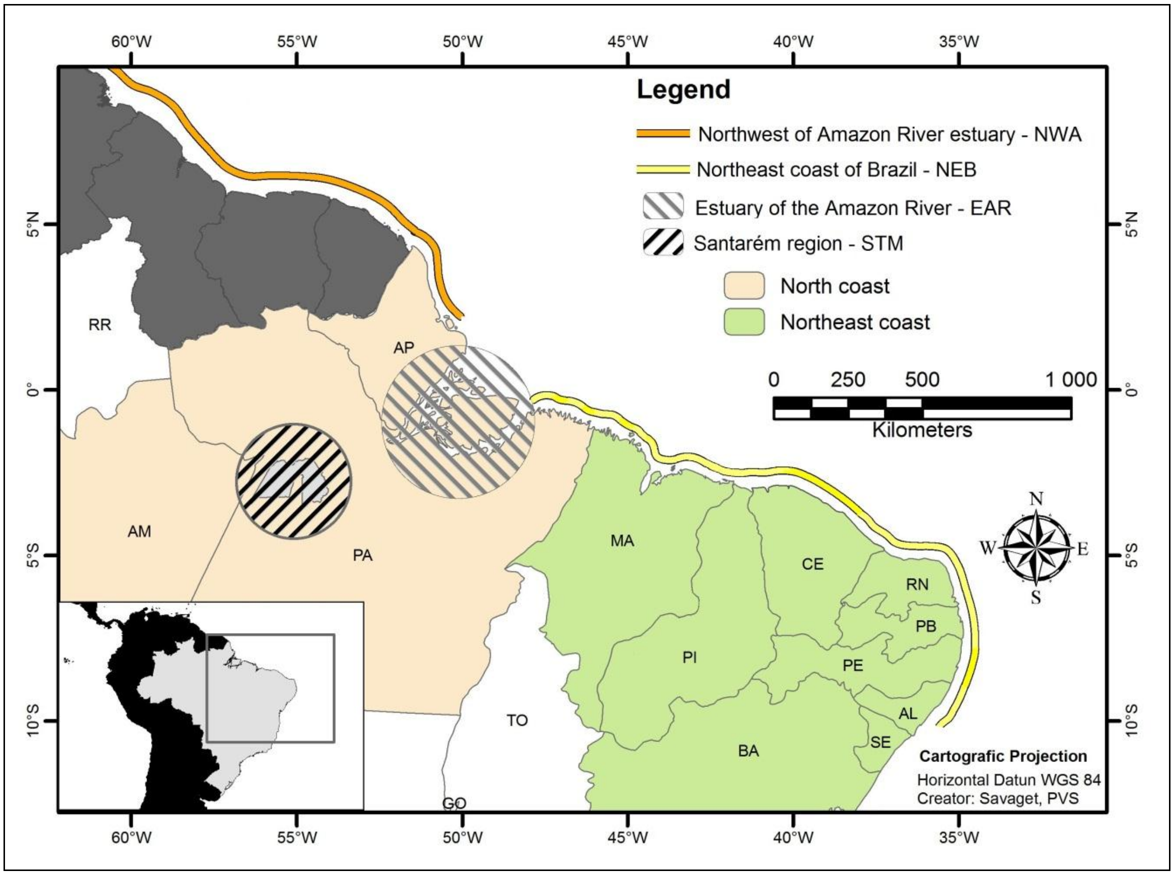


Figure IV.5 – Map of the area. Estuary of the Amazon River (EAR) and the three neighboring areas with manatee populations of two species: West Indian manatee - Northwest of the Amazon River estuary (NWA) and Northeast coast of Brazil (NEB); Amazonian manatee - Santarém region (STM).

Blood or skin tissue from wild carcasses and stranded calves (dependent calves stranded in NEB and orphans that lost their mothers through illegal harvesting in the EAR and STM) was collected during manatee research projects conducted throughout Brazil (Figure VI.6). Blood from captive manatees with known location of rescue were also utilized for this study.

Figure IV.6 – Tissue sample collecting (a stillborn manatee – note ventral patches).

Picture: Fábia Luna – CMA/ICMBio

Blood and tissue samples were preserved with lysis or tissue buffer respectively: lysis buffer: 100 mM Tris–HCl, 100 mM EDTA, 10 mM NaCl, 1.0% SDS (White and Densmore, 1992); SED tissue buffer: saturated NaCl; 250 mM EDTA pH 7.5; 20% DMSO (Amos and Hoelzel, 1991; Proebstel et al., 1993). DNA extraction, amplification and fragment analyses were performed at the US Geological Survey, Southeast Ecological Science Center, Conservation Genetics Laboratory in Gainesville, FL, USA. DNA extraction was carried out using the DNeasy Blood and Tissue Extraction Kit (QIAGEN, Valencia, CA, USA). Three samples (a single carcass and two calves) were isolated using traditional phenol chloroform technique, due to the decomposed state of the carcass at time of sampling and the small size of the samples from the calves.

IV.II.2 - Mitochondrial DNA analysis

Primers described by García-Rodríguez and colleagues (1998), CR-4 and CR-5 (Palumbi et al., 1991; Southern et al., 1988) were used to amplify a 410 base pair portion of the mtDNA control region displacement loop for the samples from the EAR, NEB, STM and for “Poque”. The mtDNA control region was PCR amplified following the techniques described by Hunter and colleagues (2010a) and Luna and colleagues (2012). Briefly, the PCR reaction conditions were as follows: 10 ng DNA, 1 x PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.001% gelatin; Sigma-Aldrich, Inc., St. Louis MO), 0.8 mM dNTP, 3 mM $MgCl_2$, 0.24 µM of each primer, 0.04 units of Sigma Jump Start *Taq* DNA polymerase. The PCR cycling profile was: 5 min at 94°C; then 35 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C; then 10 min at 72°C.

Amplified products were purified using the Qiaquick PCR Purification Kit (Qiagen). DNA sequencing was accomplished in the DNA Sequencing Core at the University of Florida, Gainesville, FL with the BigDye terminator protocol developed by Applied Biosystems Inc. using fluorescently labeled dideoxynucleotides. To verify sequences, haplotypes were aligned with manatee sequences located in GenBank using the default settings in GENEIOUS 5.3.5 (Drummond et al., 2011).

IV.II.3 - Microsatellite DNA amplification and fragment analysis

Polymerase chain reaction (PCR) amplifications were performed for all samples with 16 microsatellite loci previously optimized for manatees (Table IV.1; García-Rodríguez et al., 2000; Pause et al., 2007). Samples collected from Santarém region and the countries in the Northwest region of Brazil were analyzed with only 12 loci, due to the poor amplification of primers TmaF14, TmaA02, TmaE01 and TmaK01.

All amplifications were performed in a Biometra UNOII, T-Gradient thermocyclers (Biometra, Gottengen, Germany) or on a PTC-100 or PTC-200 (MJ Research, Waltham, MA) thermocycler using the following conditions: 95°C for 5 min, 35 cycles of 95°C for 30 s, with the specific annealing temperature for each primer as listed in the Table IV.1, 72°C for 30 s, and a final extension at 72°C for 10 min.

Amplifications were performed in a total volume of 12.7 uL, with at least 10 ng target DNA, 1X Sigma PCR Buffer (10 mM Tris– HCl, pH 8.3, 50 mM KCl, 0.001% gelatin), 2.5mM of $MgCl_2$, 0.2 mM each dNTP, 0.01U ul^{-1} of Sigma JumpStart Taq polymerase (Sigma–Aldrich, St. Louis, MO, USA), quantity of each primer as specified in Table IV.1 and 1mg.ml^{-1} bovine serum albumin (BSA) when indicated by García-Rodríguez and colleagues (2000) or Pause and colleagues (2007).

For fragment analysis, the forward primers were labeled with the fluorescent dyes VIC, HEX or 6-FAM for processing and visualization on an ABI 3130xl Automated DNA Analyzer. Fragment data from the PCR products were analyzed using GENEMARKER, version 1.5 (Soft Genetics, State College, PA, 2008) to determine allele sizes. Allele sizes were standardized using previously analyzed Florida samples for comparison and appropriate assignment to bins.

Table VI.1 - Characteristics of the 16 microsatellite loci utilized on manatee samples from the Northeast coast of Brazil, the Estuary of the Amazon River and the Santarém region. Optimized annealing temperature and primer PCR requirement.

Locus name	*Temperature* °C	*Primer µM*
TmaSC5	60*	0.118
TmaJ02	60	0.094
TmaKb60	60	0.091
TmaSC13	56*	0.118
TmaE14	56*	0.103
TmaE04	57	0.181
TmaE07	57	0.220
TmaH13	54*	0.099
TmaK01	54	0.107
TmaE01	54	0.134
TmaE08	57	0.069
TmaE11	57	0.089
TmaE26	59	0.098
TmaA02	59	0.122
TmaF14	59	0.071
TmaM79	59	0.086

*Same as published

IV.II.4 - Data analysis

CONVERT (Glaubitz, 2004) was used to convert the data into different input file formats. The program STRUCTURE version 2.3.3 (Falush et al., 2007) was used to identify possible subpopulation designations, without an *a priori* assignment of the overall population structure from Brazil. STRUCTURE, a model-based clustering algorithm, infers population structure by probabilistically assigning individuals without any *a priori* geographic or ancestral knowledge to a specific number (*K*) of clusters (presumably populations). In determining the number of clusters, the algorithm attempts to minimize deviations from Hardy-Weinberg equilibrium.

Simulations were conducted using the admixture model, which assumes that individuals could have some proportion of membership (*q*) from each of *K* clusters, leading to the potential identification of recent immigrants. Multiple Markov chains can delineate differences within populations; therefore 11 parallel chains were analyzed for $K = \{1 – 11\}$, with a run-length of 100,000 repetitions of Markov chain Monte Carlo, following the burn-in period of 100,000 iterations. Twenty independent analyses were simulated for each value of *K*.

The most probable number of populations, *K*, was determined by calculating ΔK, an *ad hoc* quantity related to the change in posterior probabilities between runs of different *K* values (Evano et al., 2005) in STRUCTURE HARVESTER (Earl and vonHoldt, 2011). An assignment value of >0.90 indicates that the individual is highly assigned to the cluster, with little likelihood that it belongs to a different cluster.

In Chapter Ill, all samples from the Brazilian coast were analyzed. Since Cluster B (green) is strongly genetically divergent from Clusters A (red) and C (blue), and the samples from this cluster are from the Estuary of the Amazon River (EAR) where two manatee species occur, this cluster was separated for the analyses used in this chapter.

To address the fine-scale genetic structure within the Estuary of the Amazon River (EAR), Cluster B (Chapter III) was analyzed for 12 microsatellites, with the individuals sampled from the Northwest of the Amazon River estuary (NWA); Northeast coast of Brazil (NEB; composed of the subpopulation AL-CE and PI-MA – Chapter III), Santarém region (STM) and the captive manatee named “Poque”.

To characterize the clusters identified in STRUCTURE, GENALEX version 6.41 (Peakall and Smouse, 2006) was used to calculate the number of alleles (*Na*), the effective number of alleles (*Ne*), the heterozygosity observed (Ho) and expected (He) for all samples lumped together and for identified subgroups of samples (Table IV.5).

GENALEX also was used to estimate the probability of identity HW $P_{(ID)}$ which assumes Hardy-Weinberg equilibrium and $P_{(ID)sib}$, which takes into account the possibility of close relatives in the population. The probability of identity ($P_{(ID)}$) calculates the probability that two individuals drawn at random from a population will have the same multilocus genotype by chance.

Correspondence analysis (CA) was conducted in PAST (Hammer et al., 2001) using a genetic distance matrix constructed in GENALEX. CA was used to examine a dissimilarity matrix of pairwise differences between samples and uses eigenvalue analysis to condense the variation between samples into a limited number of dimensions. The maximum amount of variation was plotted at the first axis with less variation in each additional dimension.

The manatee "Poque", suspected of being a hybrid (Vianna et al., 2006b), was analyzed independently using GENECLASS2 (Piry, 2004) and WHICHRUN version 4.1 (Banks and Eichert, 2000) to determine the degree of assignment to the genetic clusters identified in STRUCTURE.

IV.II.5 - Cytogenetic analysis

Sodium heparin vacutainer blood tubes were used to collect blood from captive West Indian and Amazonian manatees at CMA/ICMBio. Twelve manatees were sampled and five were used in this study, as they presented different characteristics from the others: "Poque" (the suspected hybrid), "Bela" ("Poque's" calf – Chapter V), "Folião" (a West Indian manatee from AL-CE states which has a small ventral belly patch), Ana (*T. inunguis* from STM region – used to compare with the other manatees) and Vitória (manatee from EAR - morphology of Amazonian manatee, was described to have very little nails when rescued, but currently has no nails; K. Legatzki, personal communication). The vacutainers of blood were wrapped with plastic film and chilled during transportation to the University of Florida Diagnostic Lab, Gainesville, FL, USA for cytogenetic processing.

Manatee blood cultures were prepared following standard sample culturing techniques, with modifications which included incubation at manatee body temperature (36°C), the use of a colcemid-ethidium bromide cocktail solution as a mitotic arresting agent and a prewarmed (36°C) KCl hypotonic solution (0.075 M). Metaphase spreading was performed following standard cytogenetic protocols. Manatee metaphase cells were assessed by phase microscopy and adjustments were made to accommodate drying times affecting chromosome spreading and cytoplasm density. Chromosome banding procedures G–banding and Q-banding were performed following protocols described by Gray and colleagues (2002).

IV.II.6 – Investigation of the origin of “Poque”

“Poque”, the captive manatee, is physically different when compared to other West Indian manatees housed at CMA/ICMBio and clusters with the NWA samples (Chapter III). He is suspected to be a West Indian/Amazonian manatee hybrid and was rescued as a result of illegal activity. For these reasons it was decided to make an investigation to explore the roots of his origin.

Technical documents from “Projeto Peixe-Boi”, maintained since the beginning of the program in the 1990’s, were inspected. These documents provided transcripts of interviews made by the investigators of the team in Amapá state during the “Igarakuê” expeditions. Some principal contacts documented by the team in the 90’s were also listed, and the “owner” of “Poque” (while temporarily held illegally in captivity in Oiapoque) was interviewed to gather more information for this study.

IV.III – RESULTS

IV.III.1 - Population structure

Bayesian methods in the program STRUCTURE using log-likelihood and Δ*K* analyses assigned West Indian manatees to one cluster (red) and Amazonian manatees to a second cluster (green; *K* = 2; Figure IV. 7 and 8; Table IV.2).

Manatees from the Estuary of the Amazon River (EAR) cluster with the manatees from Santarém area – STM (green; q = 99.0% (EAR) and q = 99.4% (STM); Figure IV.7) and consistently grouped separately from other manatees from the coast. The samples from the Northeast coast of Brazil (NEB) and the samples from the Northwest of the Amazon River estuary (NWA) were identified as a cluster (red; q = 99.4% (NEB) and 97.6% (NWA); Fig IV-7).

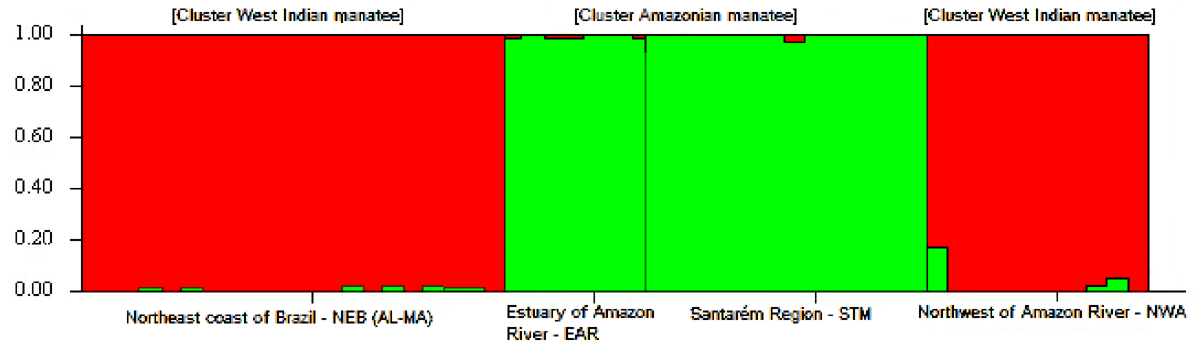


Figure IV.7 - Proportions of ancestry for individuals were assessed without *a priori* information using Bayesian clustering via the program STRUCTURE. This graphic represents the best fit of the data, where two population clusters are distinguished (*K*=2). Manatees from the Northeast coast of Brazil consistently grouped separately from the manatees from the Estuary of the Amazon River (EAR) and from Santarém region (STM). Manatees from the Northwest of the Amazon River estuary (NWA) cluster together with those from the Northeast coast of Brazil (NEB).

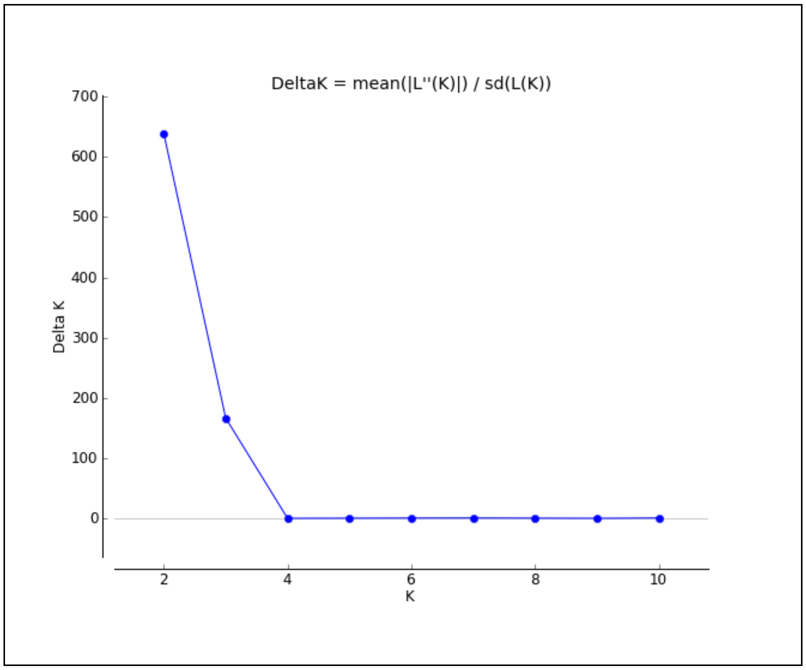


Figure IV.8 – Mean DK vs. *K* plots for the four areas (NEB, EAR, NWA and STM) genotypes.

Table IV.2 - The Northeast coast of Brazil most probable number of populations, *K*, determined by STRUCTURE HARVESTER.

K	Reps	Mean LnP(*K*)	Stdev LnP(*K*)	Ln'(*K*)	|Ln''(*K*)|	Delta *K*
1	20	-1800.5	0.702982	—	—	—
2	20	-1386.85	0.516568	413.645	329.83	638.503
3	20	-1303.04	0.745001	83.815	124.025	166.4763
4	20	-1343.25	22.14682	-40.21	1.105	0.049894
5	20	-1384.56	53.20273	-41.315	14.695	0.276208
6	20	-1411.18	73.22885	-26.62	35.595	0.486079
7	20	-1402.21	44.82997	8.975	27.205	0.606849
8	20	-1420.44	59.1213	-18.23	20.9	0.353511
9	20	-1417.77	62.8108	2.67	5.245	0.083505
10	20	-1420.34	30.22316	-2.575	17.35	0.574063
11	20	-1440.27	33.4361	-19.925	—	—

The program STRUCTURE identified two different populations with ln(*Pr*(*X*|*K*)) with a best fit *K*=2. The correspondence analyses (CA) identified two populations (West Indian manatee in red and Amazonian manatee in green and black; Figure IV.9 and 10).

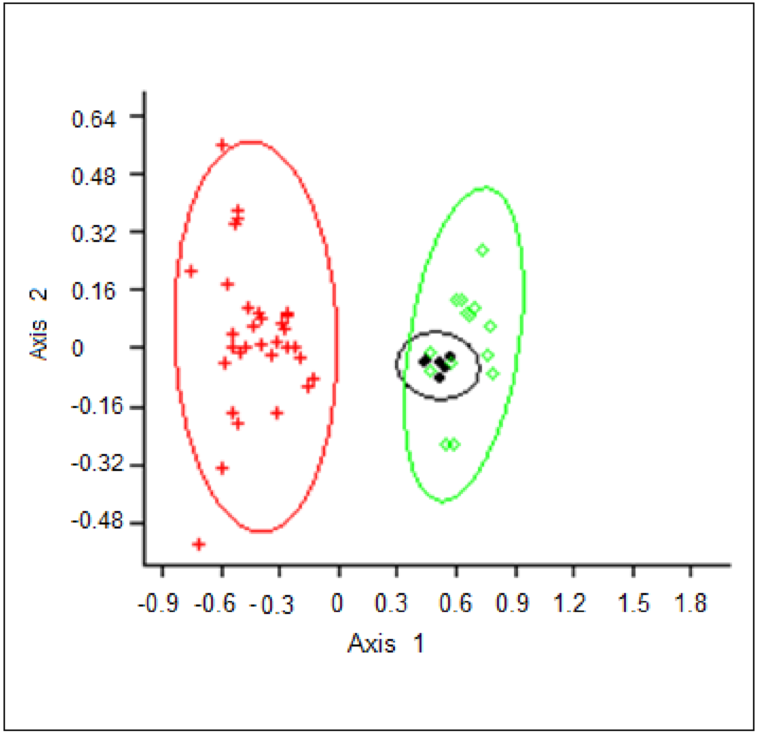


Figure IV.9 - Correspondence analysis (CA) of microsatellite manatee genotype data for West Indian manatees (red) and manatees from the STM region (green) and from the Estuary of the Amazon River (EAR; black).

Private alleles were identified for manatees from the Estuary of the Amazon River (N= 13; Table IV.3).

Table IV.3 - List of private alleles in each manatee group.

Locus	Private Allele	Group presenting this allele
SC5	137	EAR
Sc13	123	STM and EAR
E08	230	STM
J02	224	STM and NWA
J02	228	STMA and NWA
E04	250	NWA
E11	199	NWA
Kb60	211	unique to Poque

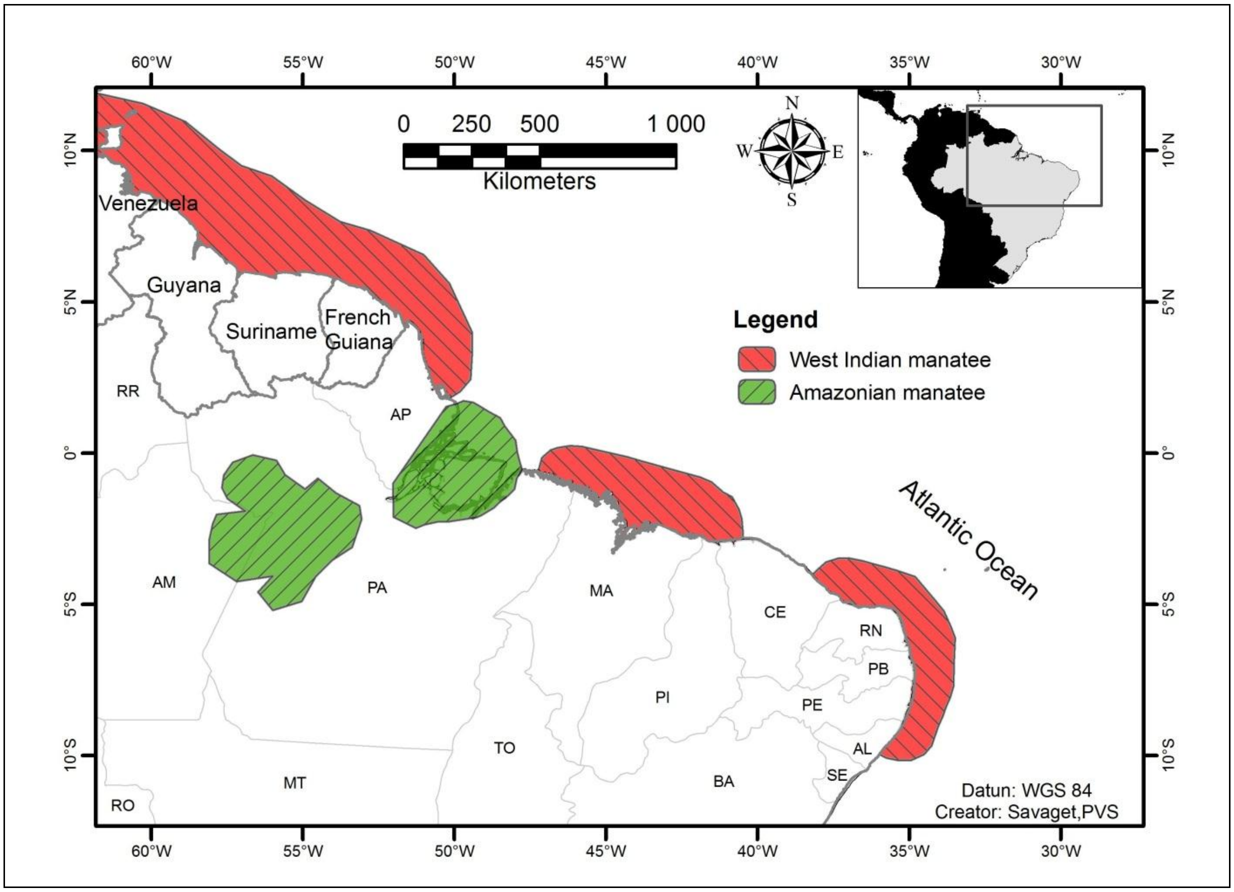


Figure IV.10 – Manatees from the Estuary of the Amazon River (EAR) cluster with the Amazonian manatees from the Santarém region (STM), and the populations along the coast (Northeast coast of Brazil – NEB, and Northwest of the Amazon River estuary – NWA) cluster together as West Indian manatees.

The captive manatee “Poque” was attributed 83.2% to the West Indian manatee cluster (red) and had an assignment of 16.8% to the Amazonian manatee cluster according to STRUCTURE results when compared to West Indian manatee samples from the Northeast coast of Brazil (AL-MA) and from the Northwest of the Amazon River estuary (NWA); and with Amazonian manatee samples from the Santarém region (STM) with the Estuary of the Amazon River (EAR) samples. “Poque” was attributed to the Northwest of the Amazon River estuary population (NWA) by GENECLASS2 (36.2%) and by WHICHRUN (Table IV.4).

Table IV.4 - Attribution of “Poque” to the Northwest of the Amazon River clusters by STRUCTURE, GENECLASS2 and WHICHRUN software programs. Estuary of the Amazon River (EAR); Northwest of the Amazon River estuary (NWA) and Northeast coast of Brazil (NEB) with two subpopulations: Alagoas to Ceará states (AL-CE) and Piauí - Maranhão states (PI-MA).

			STRUCTURE Q value			GENECLASS2 results								
Individual	Sampling population	STRUCTURE assignment	AL-MA	EAR	NWA	most probable population	score for pop1	other population	score for pop2	other population	score pop3	other population	score pop4	WHICHRUN Attribution
Poque	Amapá/AP	NWA	0.004	0.013	98.3	NWA	0.36	AL-CE	0	PI-MA	0	EAR	0	NWA

IV.III.2 - Mitochondrial DNA analysis

Manatee mtDNA sequences of the Brazil manatee samples were compared in this study. Within the West Indian manatee population there were three identified haplotypes: M01, M03 and M04 (Chapter II). The previously identified haplotype (T01) for the suspected hybrid reported by Vianna and colleagues (2006a) was found and confirmed for the animal named “Poque” (T01). The haplotype T01 was found for five manatees from the Estuary of the Amazon River. New haplotypes were identified for the manatees from the Estuary of the Amazon River and also for the Santarém region.

IV.III.3 - Genetic diversity within the study groups

The genetic diversity and differentiation values were calculated for cluster assignment using the five sample groups of manatees. Estimates of the mean number of alleles, effective number of alleles, heterozygosity expected and observed, for each population (AL-CE; PI-MA; EAR; NWA and STM) are presented in Table IV.5.

Table IV.5 - Diversity statistics over the microsatellite loci examined for manatees from AL-CE, PI-MA, EAR, STM and NWA sample groups. The mean number of samples (N), number of alleles (N_A), effective number of alleles (N_E), observed and expected heterozygosity (H_O and H_E, respectively).

Pop	**AL-CE**	**PI-MA**	**EAR**	**STM**	**NWA**
N	61	7	7	14	11
Na	3.00±0.32	2.50±0.29	3.25±0.34	4.00±0.39	3.19±0.37
Ne	1.74±0.18	1.86±0.20	2.47±0.27	2.40±0.24	2.41±0.28
Ho	0.34±0.07	0.33±0.07	0.53±0.09	0.39±0.07	0.43±0.07
He	0.34±0.06	0.37±0.06	0.51±0.06	0.52±0.05	0.47±0.07

The manatees from the Estuary of the Amazon River (EAR) have more alleles than the other three groups of West Indian manatees, but less than the Amazonian manatee group from the Santarém region (STM). The manatees from the EAR have a higher effective number of alleles than all the other manatee populations analyzed.

The heterozygosity observed for the manatees from the EAR is higher than all the other manatee populations analyzed. The heterozygosity expected is similar to the manatees from STM (0.51 vs. 0.52), and higher than the three populations of West Indian manatees.

Monomorphic loci were found for the NEB samples (AL-CE and PI-MA; TmM79). After 120 comparisons and a sequential Bonferroni correction, no linkage disequilibrium was observed for any pair (overall $\alpha = 0.05$). Private alleles were found in NWA (n=2), EAR (n=1), STM (n= 1), and were used to identify a unique allele in the manatee named “Poque”. There were three alleles shared between STM and other areas (EAR = 1; NWA = 2). All loci were in Hardy–Weinberg equilibrium (HWE) after a sequential Bonferroni correction for PI-MA in NEB and for NWA. For populations within the Northeast coast (AL-CE), TmaE26 was not within HWE, as well as TmaE11 from STM. This deviation may be due to the presence of null alleles.

In the EAR and STM groups, TmaE14, TmaE01 and TmaE11 had evidence of null alleles. In the AL-CE states, TmaSC5, TmaSC13, TmaE26 and TmaA02 had evidence of null alleles due to heterozygote deficiency. For the PI-MA and NWA, no detection was possible by MICROCHECKER because not enough samples were available.

IV.III.4 - Microsatellite genetic information on the EAR manatee

For the 16 microsatellites used for the all manatee samples from EAR the PIC value ranged from 0.703 for TmaJ02 for the most informative marker to 0.083 for TmaE07 for the least informative (Table IV-6). No loci were monomorphic. HW $P_{(ID)}$ at each locus varies from 1.0E-01 for the most informative locus to 8.4E-01for the least informative locus, with the cumulative HW $P_{(ID)}$ for the 16 loci being 1.4E-10. $P_{(ID)sib}$ at each locus varies from 4.1E-01 for the most informative locus to 9.2E-01 for the least informative locus, with the cumulative $P_{(ID)sib}$ for the 16 loci being 5.2E-05 (Table IV.6).

Number of samples analyzed, number of alleles, effective number of alleles, PIC, heterozygosity observed Ho and expected He, $P_{(ID)sib}$ and HW $P_{(ID)}$ results for each allele and cumulative values over the 16 loci starting from the most informative to the least informative, are presented in Table IV-6.

Enough variation over the 16 loci was observed to identify each manatee of the EAR with a unique multilocus genotype with a probability of identity between $P_{(ID)sib}$ = 5.2E-05 and HW $P_{(ID)}$ = 1.4E-10 (Table IV.6). Values for the microsatellites can be compared with the one found for the same 16 microsatellites for the West Indian manatee in Chapter III. Markers used in this study are more variable and informative for the Amazonian manatee than for the West Indian manatee. This is somewhat surprising given that they were developed in West Indian manatees and microsatellites typically have more alleles in the species for which they were developed. However, it is believed that the Amazonian manatee has a much greater population size than West Indian manatee (Cantanhede et al. 2005).

Table IV.6 - Characteristics of the 16 polymorphic microsatellite loci implemented on the EAR samples. Number of alleles (N_A), effective number of alleles (N_E), PIC value (*PIC*), observed and expected heterozygosity (H_O and H_E), Hardy-Weinberg probability of identity (P(ID) HW) and probability of identity for related individuals (P(ID)sib) and cumulative of P(ID) HW and P(ID)sib, were determined for each loci over the 16 loci starting from the most informative to the least informative.

Locus name	N_A	N_E	*PIC*	H_O	H_E	$P_{(ID)}$ HW	Cumulative $P_{(ID)}$ HW	$P_{(ID)}$sib	Cumulative $P_{(ID)}$sib
TmaJ02	8	3.738	0.703	0.750	0.733	1.0E-01	1.1E-02	4.1E-01	1.7E-01
TmaSC5	8	3.604	0.689	0.650	0.723	1.1E-01	1.1E-01	4.2E-01	4.2E-01
TmaE26	4	3.447	0.657	0.500	0.710	1.4E-01	1.1E-09	4.3E-01	1.4E-04
TmaE11	6	3.113	0.621	0.136	0.679	1.6E-01	9.4E-09	4.5E-01	3.7E-04
TmaSC13	5	3.093	0.618	0.591	0.677	1.6E-01	3.3E-04	4.5E-01	3.8E-02
TmaK01	4	3.028	0.608	0.944	0.670	1.7E-01	9.2E-07	4.6E-01	2.7E-03
TmaH13	4	2.898	0.596	0.545	0.655	1.8E-01	5.4E-06	4.7E-01	5.9E-03
TmaKb60	7	2.557	0.582	0.667	0.609	1.8E-01	2.0E-03	4.9E-01	8.4E-02
TmaE08	6	2.333	0.536	0.524	0.571	2.2E-01	5.9E-08	5.2E-01	8.1E-04
TmaE04	4	2.258	0.503	0.389	0.557	2.5E-01	3.6E-05	5.3E-01	1.4E-02
TmaE01	5	1.958	0.461	0.278	0.489	2.9E-01	2.7E-07	5.8E-01	1.6E-03
TmaM79	4	1.856	0.395	0.316	0.461	3.6E-01	1.4E-10	6.1E-01	5.2E-05
TmaA02	2	1.994	0.374	0.316	0.499	3.8E-01	4.0E-10	5.9E-01	8.5E-05
TmaE14	4	1.539	0.329	0.227	0.350	4.4E-01	1.5E-04	6.9E-01	2.6E-02
TmaF14	2	1.105	0.09	0.000	0.095	8.2E-01	7.7E-09	9.1E-01	3.3E-04
TmaE07	2	1.095	0.083	0.000	0.087	8.4E-01	3.0E-05	9.2E-01	1.3E-02

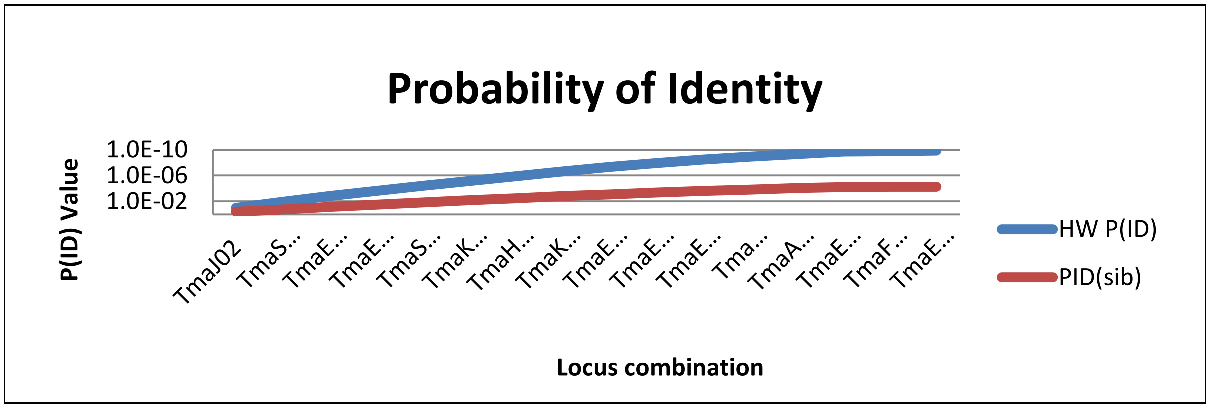


Figure IV.11 – Probability of identity for the successive combinations from one locus to 16 loci for the EAR manatee.

IV.III.5 - Cytogenetic analysis

It was possible to obtain the karyotype of the five manatees for this study. The karyotype of each manatee with the chromosomes divided in distinctive morphological groups can be observed in the figures for each manatee; “Poque” (Figure IV.12), “Bela” (Figure IV.13), “Folião” (Figure IV.14), “Ana” (Figure IV.15) and “Vitória” (Figure IV.16). Upon examination it is apparent that there are differences between the karyotypes depicted for “Poque” when compared to the other four manatees (*T. manatus* and *T. inunguis*).

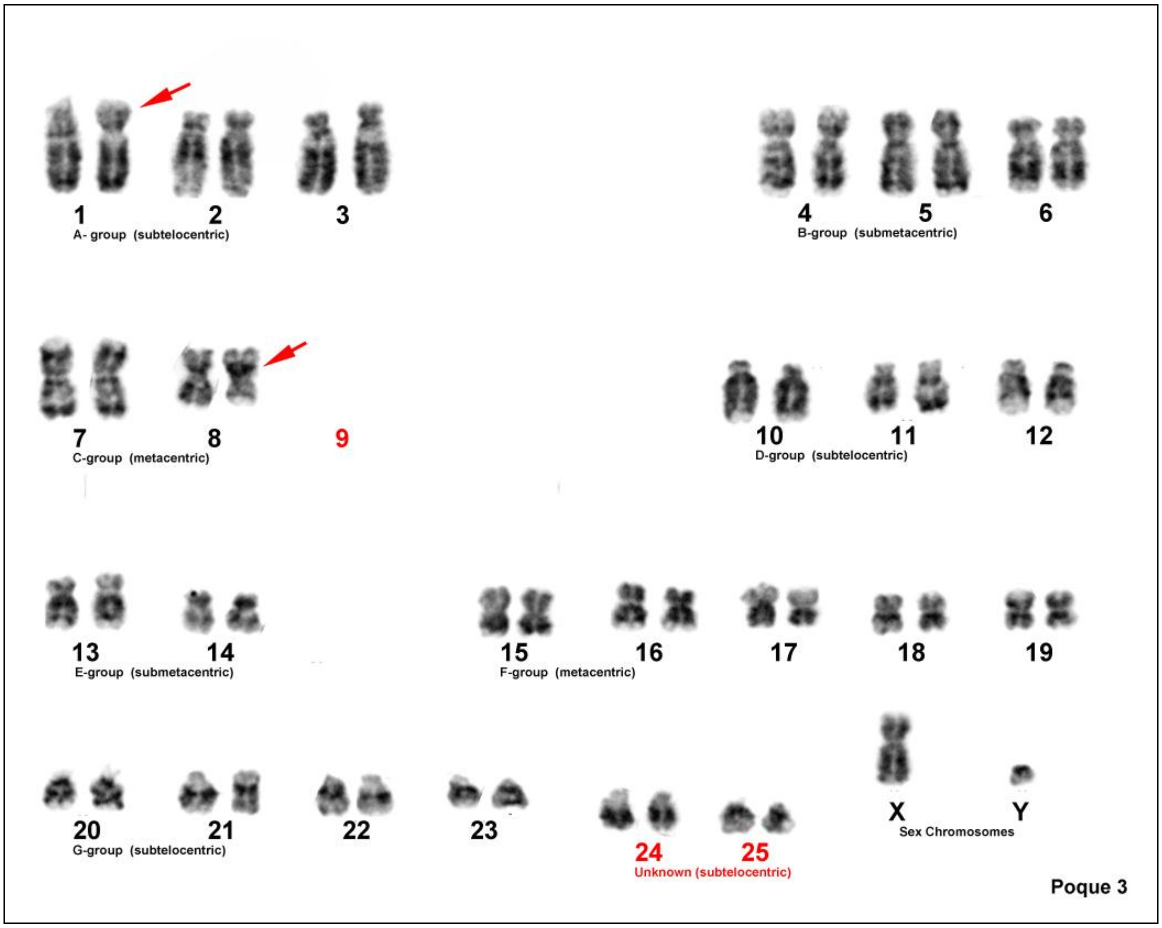


Figure IV.12 - “Poque” karyotype. Note the missing chromosome #9, and the addition of two chromosome pairs #24 & #25. The arrows at chromosomes #1 & #8 point to structural differences in the chromosomes presented as homologs to warrant pairing, but they are morphologically different.

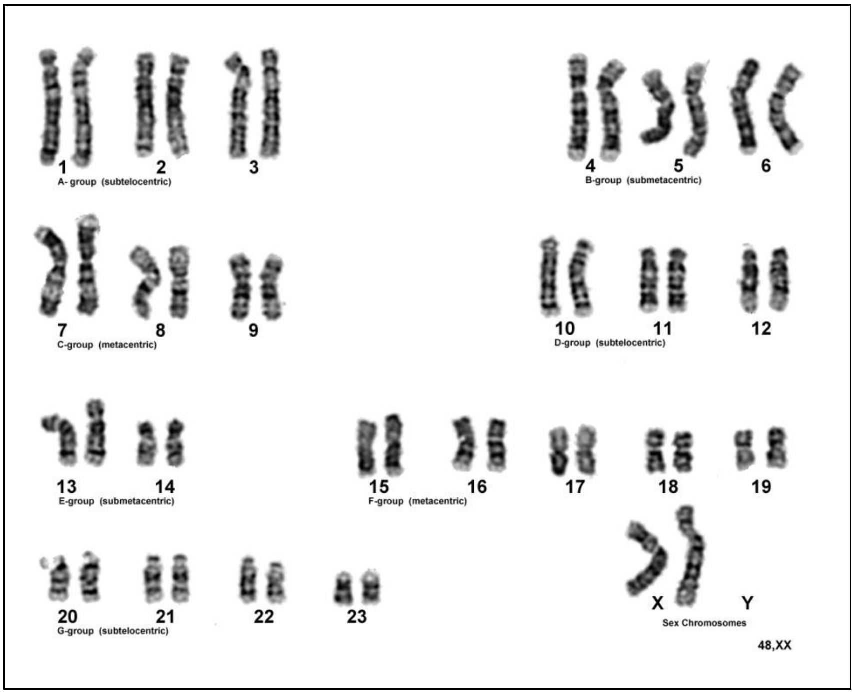


Figure IV.13 - “Bela” karyotype (*T. manatus)*.

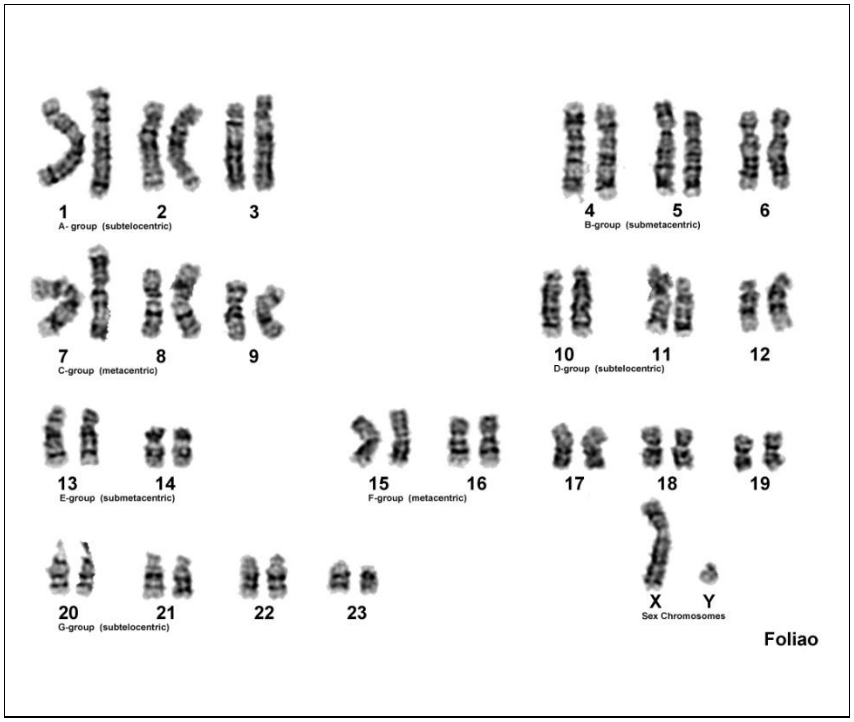


Figure IV.14 – “Folião” karyotype (*T. manatus*).

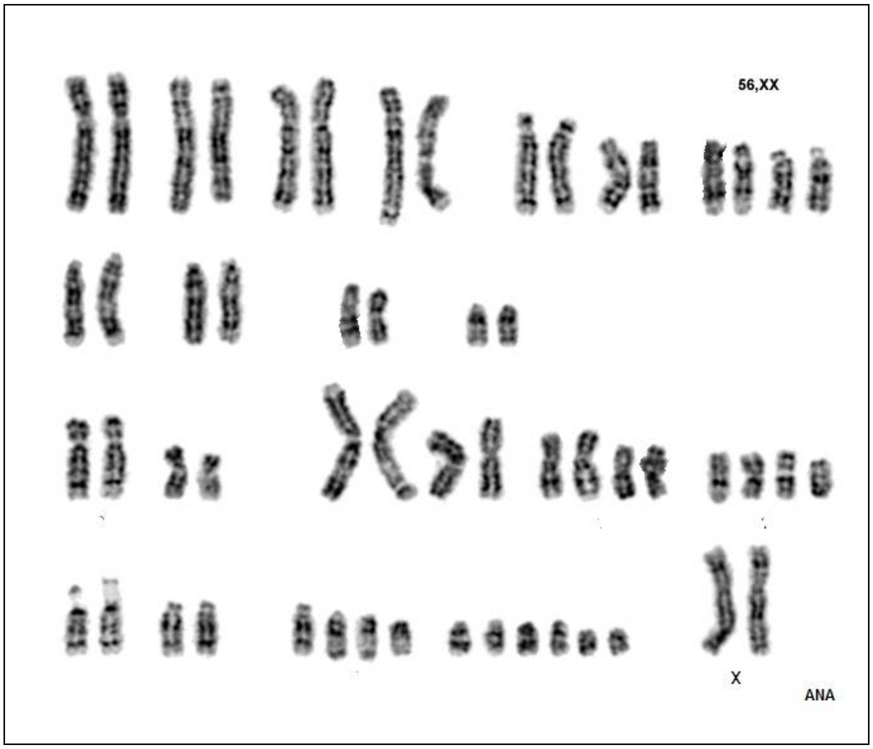


Figure IV.15 – "Ana" karyotype (*T. inunguis*).

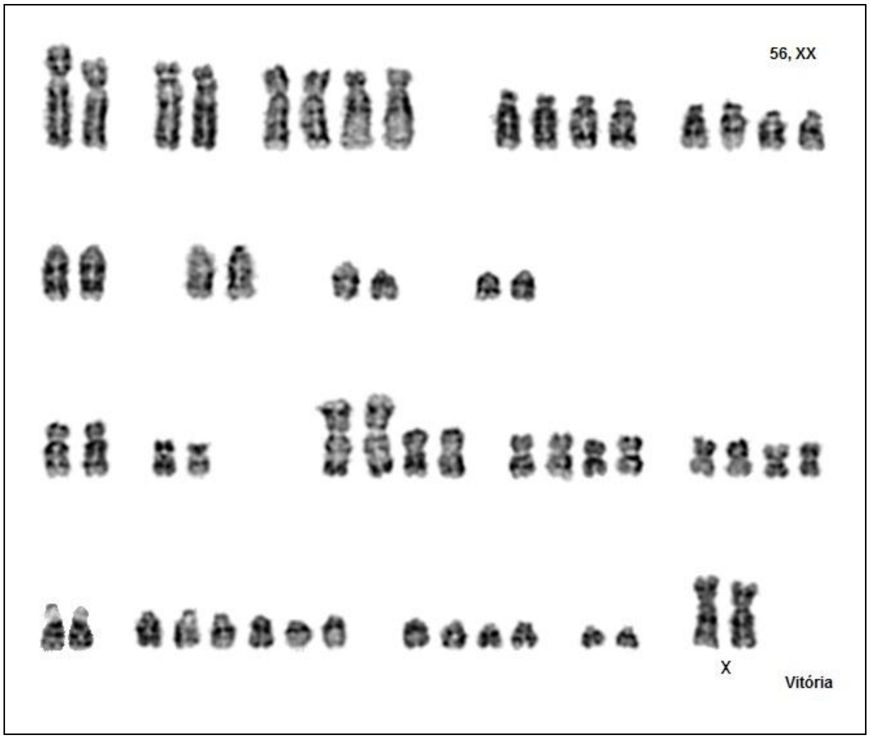


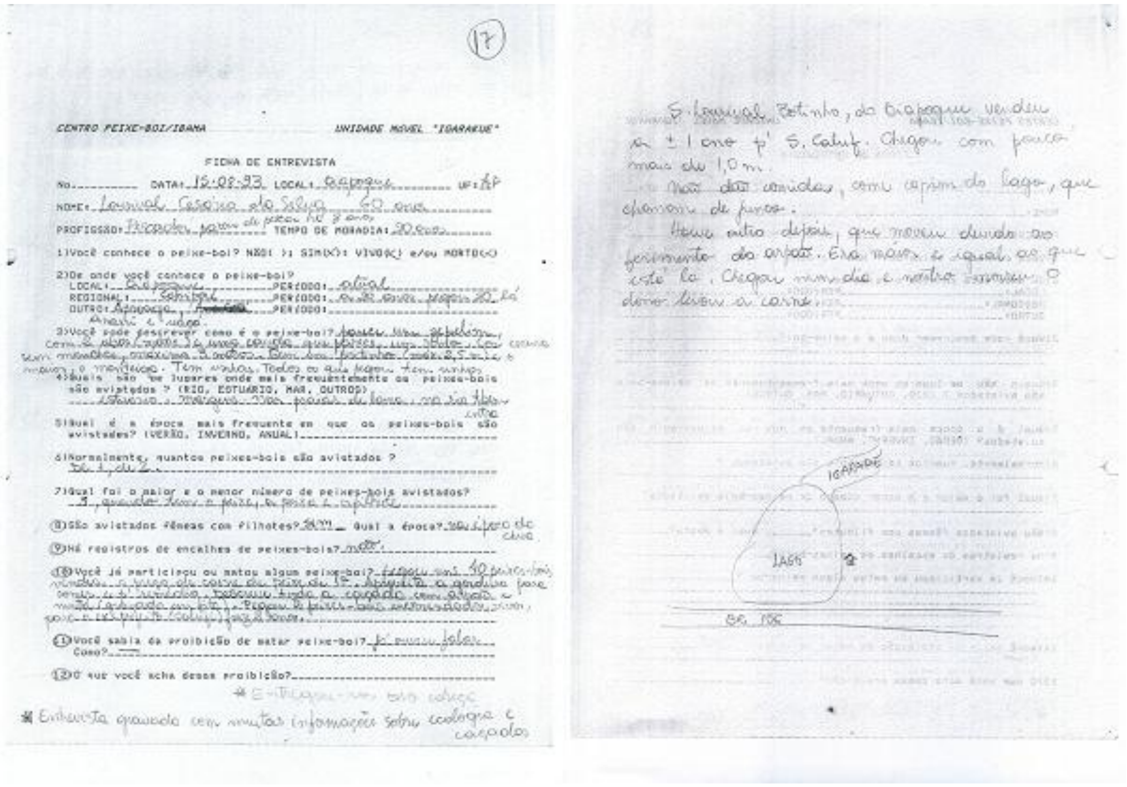
Figure IV.16 – "Vitória" karyotype (*T. inunguis*).

The manatees “Bela” and “Folião” have a normal typically *T. manatus* karyotype, with the chromosomes divided in the seven groups (Figure IV.13 and IV.14). The manatees “Ana” and “Vitória” have a normal typically *T. inunguis* karyotype when compared to Assis and colleagues (1988; Figure IV.15 and IV.16). However, it was not possible to divide the chromosomes into the seven proposed groups for “Poque”, which has a modal number of 50 chromosomes.

According to B. Gray (personal communication), “Poque’s” chromosome 1 shows a different position in the centromere versus those observed in the West Indian manatee. Additionally, the morphology looks like chromosome #1 of the Amazonian manatee. His karyotype also included two pairs of G-grouped sized chromosomes, most likely subtelomeric. This pair was assigned as two extra pairs of subtelecentric chromosomes (#24 and #25), but the karyotype was missing the chromosome #9 pair corresponding with the *T. manatus* karyotype. Also, structural differences were found in the chromosomes presenting as homologs at the chromosome #1 and chromosome #8 positions where they are morphologically different (Figure IV.12).

IV.III.6 - Information on the origin of “Poque”

At CMA/ICMBio documents were obtained that related to the rescue of “Poque”. He was discovered by the “Project” through the “Igarakuê” expeditions conducted in the northeast and north coast of Brazil in 1993. During interviews with local people from Amapá state (Figure IV.17) it was discovered that “Poque” was illegally held with another manatee under inadequate conditions in Oiapoque city along the border with French Guiana. The researchers from the “Project” at the time of the interview asked local people about how these manatees were obtained after it was confirmed that the two manatees were in captivity. When the team went back to Amapá state to rescue the manatees and transfer them to the CMA/ICMBio facility, they discovered that one had died. The live remaining manatee was “Poque”.



(17)

CENTRO PEIXE-BOI/IBAMA UNIDADE MOVEL "IGARAKUE"

FICHA DE ENTREVISTA

No.________ DATA: 15.08.93 LOCAL: Oiapoque UF: AP

NOME: Lourival Cesário da Silva 60 anos

PROFISSÃO: Pescador, parou de pescar há 8 anos TEMPO DE MORADIA: 20 anos

1)Você conhece o peixe-boi? NÃO( ); SIM(X); VIVO(X) e/ou MORTO( )

2)De onde você conhece o peixe-boi?
LOCAL: Oiapoque PERÍODO: atual
REGIONAL: Sobradinho PERÍODO: há 30 anos, parou há 20 anos
OUTRO: Amapá, [illegible] PERÍODO:
Anauí e [illegible].

3)Você pode descrever como é o peixe-boi? [illegible]

4)Quais são os lugares onde mais frequentemente os peixes-bois são avistados? (RIO, ESTUÁRIO, MAR, OUTROS) estuário, mangue, [illegible]

5)Qual é a época mais frequente em que os peixes-bois são avistados? (VERÃO, INVERNO, ANUAL)

6)Normalmente, quantos peixes-bois são avistados?
De 1, de 2.

7)Qual foi o maior e o menor número de peixes-bois avistados?
3, [illegible]

⑧São avistados fêmeas com filhotes? Sim Qual a época? [illegible]

⑨Há registros de encalhes de peixes-bois? não.

⑩Você já participou ou matou algum peixe-boi? [illegible]

⑪Você sabia da proibição de matar peixe-boi? Já ouviu falar.
Como? —

⑫O que você acha dessa proibição?

* Entregou-nos [illegible]

* Entrevista gravada com muitas informações sobre ecologia e caçadas

S. Lourival Betinho, do Oiapoque vendeu
a ± 1 ano p/ S. Caluf. Chegou com pouco
mais de 1,0 m.
Nós dão comidas, como capim do lago, que
chamam de junco.
Houve outro depois, que morreu devido ao
ferimento do arpão. Era maior e igual ao que
está lá. Chegou num dia e noutro morreu. O
dono levou a carne.

Figure IV.17 – Notes taken during the "Igarakuê" interviews with local people who describe "Poque's" captive conditions while illegally held in captivity.

For this study the owner of the facility was contacted and interviewed. He was asked information about the origin of "Poque" and he indicated that the captive manatee was born in the wild. The mother was hunted by a fisherman and the calf (Poque) was sent to his facility. "Poque" measured 1.20m in length when he arrived at the property. This is very important information as in Amapá state there exists the occurrence of both species of manatees, *T. manatus* and *T. inunguis*. This evidence documents that "Poque's" origin is from the wild population.

IV.IV – DISCUSSION

IV.IV.1 - Population structure

The 14 samples from STM represent Amazonian manatees. They were collected from calves that became orphaned, likely because their mothers were hunted (Luna, 2007). The orphan calves were founded stranded in rivers near Santarém city, around 1,000km distant from the Estuary of the Amazon River. The 21 samples from the NEB are from the coast in areas father than 500km to the east of the Estuary of the Amazon River. Consequently, the samples from NEB are genetically *T. manatus* and the samples from STM are genetically *T. inunguis*, which permits comparison of genotypes of the samples provided from the estuary, perhaps lending clues about possible hybridization.

The samples from the Northwest of Amazon River were included as they are from a population near the estuary that can supplement manatee samples obtained from the estuary area. Six samples were from Guyana (N= 6), four from Venezuela (N=4) and one sample from Amapá state (N = 1; animal named “Poque”). The samples from the NWA are not known to be genetically pure *T. manatus* individuals, as there exists some possibility of hybridization that has been mentioned and reported from this area (García-Rodríguez, 1998; Vianna, 2006a).The use of samples from the three areas mentioned above were made possible for comparison with the seven individuals from the estuary area.

STRUCTURE separated the manatees from the four populations (NEB, EAR, NWA and STM) into 2 clusters (Figure IV.7). The clusters aptly defined separation between the two manatee species (*T. manatus* and *T. inunguis*). The 11 manatees from the NWA (including “Poque”) grouped with West Indian manatees from the NEB. “Poque” appeared with a very low assignment probability (q=0.013%) to the manatees from the EAR group. The manatees from NWA shared a rare allele with STM, and “Poque” had alleles in common with the Amazonian manatees analyzed. However, despite the alleles being the same, this can be caused by homoplasy.

IV.IV.2 - Genetic diversity within the groups

The manatees grouped as *T. inunguis* (EAR and STM; H_E = 0.0.51 and 0.52; N_A = 3.25 and 4.0, respectively) had higher genetic diversity than the manatees grouped as *T. manatus* (AL-CE; PI-MA and NWA; H_E = 0.34; 0.37 and 3.19; N_A = 3; 2.5 and 3.19, respectively). Also, more private alleles were identified for the Amazonian manatees when compared to West Indian manatees. The population from the NEB (AL-CE and PI-MA) had the lowest genetic diversity. These manatees have been experiencing pressure due to pollution and habitat fragmentation, and live in much more disturbed habitat than manatees in the other populations. Nevertheless, the other three populations have been also suffering due to illegal harvesting, which can rapidly decrease genetic diversity.

All the populations had low genetic diversity when compared with the average for mammals. Meta-analyses of microsatellite data determined that demographically-challenged mammalian populations have lower genetic variation (H_E of 0.60 and N_A = 6.17-6.59) when compared to undisturbed, healthy populations (H_E = 0.65 and N_A = 8.18; (DiBattista, 2007; Garner et al., 2005*).*

Results from STRUCTURE illustrate that all manatees identified as Amazonian manatees physically, regardless of where they were sampled, do not show the presence of mixed individuals that could be explained by hybridization. Research using others methodologies were needed to confirm the existence of hybridization between the two species, as the use of just the program STRUCTUREis not sufficient to diagnose the hybridization. .

IV.IV.3 – Mitochondrial and Microsatellite genetic information.

The study of hybridization through conserved mtDNA is a very good tool and needs to be utilized, as the mtDNA is maternally inherited and does not change rapidly between generations. However, evidence obtained from mtDNA analyses can be used to determine if a hybridization event has happened only if it occurs with a female of a different species from the offspring's phenotype. Mitochondrial DNA does not pass from the male to offspring, so it can only be useful in some applications to provide clues about the origin of the mother. The nuclear DNA does not remain unchanged over multiple generations, and as it is also paternally inherited and is recombined, it is not as useful as the mtDNA to detect hybrids.

The diversity is lower in the EAR than the STM, which may be due to more samples from the STM region then from the EAR. The mtDNA was not accessed for NWA, except for "Poque" who has the haplotype T01 (Chapter II), which aligns with Amazonian haplotypes, instead of West Indian manatee haplotypes. Five Amazonian manatees from EAR had T01 haplotype, whereas in the Northeast coast of Brazil all manatees from AL to CE states had a single haplotype M01, and from PI to MA states had the haplotypes M03 and M04 (Chapter II; Luna et al., 2012).

IV.IV.4 - Cytogenetic analysis

The application of a primary chromosome banding technique can reveal unique or distinctive homolog banding features and can provide accurate and consistent identification of specific chromosomes, chromosomal regions or rearrangements for many marine mammals. Gray and colleagues (2002), detailed the chromosomes of the West Indian manatee utilizing primary chromosome banding techniques (G- and Q-banding), and based on chromosome banding patterns and measurements proposed a standard karyotype and ideogram for the species.

The morphology of the West Indian manatee chromosomes is different from the Amazonian manatee. Assis and colleagues (1988) proposed fission and/or fusion type rearrangements of acrocentric chromosomes as a mechanism responsible for the karyotype divergence between the Amazonian manatee (n = 56) and the West Indian manatee (n = 48).

Assis and colleagues (1988) assigned a fundamental number (FN) of 82 for the Amazonian manatee, based on the description of 14 bi-armed and 13 acrocentric autosome pairs, and implied an FN=78 for the West Indian manatee, based on the use of similar terminology employed in a previously published karyotype of the West Indian manatee, which described 16 bi-armed and 7 acrocentric autosome pairs (White et al., 1976).

According to Gray and colleagues (2002), the evolution of the morphological karyotype differences observed between these two species may be best explained by the occurrence of a variety of both interchromosomal and intrachromosomal rearrangement mechanisms such as inversions, whole-arm translocations, tandem fusions, etc. A hybrid probably will have a mix of those different karyotypes of the parents.

In this study, the analysis of the five different manatee (*Poque, Bela, Folião, Ana* and *Vitória*) chromosome karyotypes was possible, which permits us to do a comparison between the karyotype of those specific manatees and the karyotype of the two manatee species (*T. manatus* and *T. inunguis*). The karyotype of the West Indian manatees would be divided into seven distinctive group morphologies (Groups A-G), with three subtelocentric, two submetacentric, and two metacentric, as described by Gray and colleagues (2002).

Ordering within chromosome groups follow Gray and colleagues (2002), which was determined by relative total length, reading from largest to smallest from left to right. The sex chromosomes are depicted separately. For this study the manatees Ana (Figure IV.15) and Vitória (Figure IV.16) have a preliminary G-banded karyotype. Their chromosomes have been temporarily arranged (grouped) by morphology. The detail of the Amazonian manatee karyotype is being developed by Gray and colleagues (in prep.).

As showed at figures IV.13 and IV.14, the manatees “Bela” and “Folião” have a normal typically *T. manatus* karyotype, despite the fact that “Bela” is identified as offspring of “Poque” (see details in Chapter V), and “Folião” has a white belly patch. On the figures IV.15 and IV.16, is possible to see that the manatees “Ana” and “Vitória” have a normal typically *T. inunguis* karyotype, despite the fact that “Vitória” may have had nails when rescued (around one month old). Despite a limited number and poor quality of metaphase cells obtained from “Poque’s” karyotype preparations, a consistent G-banding pattern has emerged, and it was not possible to divide the chromosomes into the seven proposed groups for “Poque”. Karyotype data for “Poque” confirms a modal number of 50 chromosomes as previously described by Vianna and colleagues (2006a and b).

However, the previous study did not analyse the morphology of the chromosomes. As seen in the results, according to B. Gray (personal communication), structural differences were found in “Poque’s” chromosomes #1 and #8 positions where they are morphologically different from West Indian manatee homologous chromosomes. Also his karyotype included two extra pairs of subtelecentric chromosomes which look like chromosomes of the Amazonian manatee (#24 and #25); additionally, he was missing the chromosome #9 pair corresponding with the *T. manatus* karyotype. Despite all those differences, “Poque’s” karyotype appears stable. It suggests that in some moment during the multiplication of the cells the chromosomes were rearranged for a total of 25 chromosome pairs with a stable status. This allows for the successful division (mitosis) of the cells and has enough homology to enable “Poque” to survive cell division.

IV.IV.5 - Origin information for "Poque": a case study

At CMA/ICMBio documents were identified regarding the rescue of a young manatee during the "Igarakuê" expeditions in 1993. Researching K-7 interviews made in the region during the expeditions, it was discovered that "Poque" was with another manatee during illegal confinement (perhaps for only one day) in Oiapoque city, along the border with French Guiana. The researchers from the investigating "Project" at the time asked and confirmed that originally there were two captive manatees but one had died prior to rescue by the team. The survivor was a wild born calf that was later named "Poque" and taken to a rehabilitation facility in Itamaracá in PE state.

In Amapá state there exists both species of manatees, *T. manatus* and *T. inunguis*. The owner of the facility that originally housed "Poque", was interviewed and it was determined that "Poque" was born in the wild prior to capture. The reason this information is important is to help determine if he was an offspring of artificial mating while in captivity where they could have forced breeding. This is common practice in parts of the Amazon region (the coast of Amapá is part of the Amazon region), where manatees are illegally held captive as pets and are encouraged to breed. In this specific case, where both species occur in the region, it was possible that the owner of the facility could have put together a male of a West Indian manatee and a female of an Amazonian manatee. Also, generally manatees in captivity breed with ease, where it would be possible to artificially promote mating between very dissimilar individuals, which could result in artificial hybridization.

However, according to the owner of the facility (W. Caluf, personal communication) "Poque" was born in the wild prior to being brought into captivity. He explained that the mother was hunted by a fisherman and the calf was brought to his facility. "Poque", a male, arrived in good condition and was approximately 1.20m in total body length. The hypothesis of "Poque" being a hybrid began during the 1990's, when he was rescued from illegal captivity in Amapá state. Some morphological characteristics were the reason why the CMA/ICMBio team had assumed that "Poque" could be a hybrid. The history passed on after his arrival was: "*Poque is smaller than* *the other captive manatees at the CMA/ICMBio facility, he has two very small white patch markings on the skin (assumed to be depigmentation) on its belly and he has only three nails on each pectoral flipper"* (Figures IV.2; IV.3 and IV.4).

Although, after scrutinizing those physical characters, it raises some questions. Reynolds and Odell (1991) described the West Indian manatee as having three to four nails on each flipper. Also, some small West Indian manatee dependent calves stranded in the Northeast coast of Brazil and were rescued by REMANE (Brazilian Northeast stranding network) with a white or light pink patch on their belly: *Rosinha, Pintado, Branca, Telinha, Joana* and *Folião* (Figure IV.18). Additionally, a stillborn (second calf of Xica – Figures IV.6 and IV.19) and one captive alive born manatee (named "Sereno") at the CMA/ICMBio facility each has a belly patch. The stranded dependent calves were originally from CE, RN and PB states, which are more than 1,000km distant to the Estuary of the Amazon River, in PA state.

Figure IV.18 – Folião; note: pink patch on the belly.

Picture: Jeorge Gregório – CMA/ICMBio

Figure IV.19 – Stillborn at the CMA/ICMBio facility; note patches on the belly and ventral tail.

Picture: Fábia Luna – CMA/ICMBio

Nourisson (2011) mentioned that fourteen manatees (28%) captured in Chetumal Bay, Mexico, had a small pink mark (depigmentation) on the skin of their bodies (either belly or flipper) that looked similar to the patch commonly reported on the Amazonian manatee (*Trichechus inunguis*). However, these manatees in Mexico are West Indian manatees, far distant to any Amazonian manatee populations. Also, this chest blaze has been observed in West Indian manatees from Belize, Puerto Rico and Florida (C. Beck and R. Bonde, personal communication).

Additionally, even with the documentation of many other manatees with patches, after 20 years it was recently discovered that the two patches on “Poque” are not inherited, but were scars resulting from likely inflicted when he was young. According to C. Beck (personal communication), “Poque’s” two small symmetrical patches are clearly identified as healed wounds resulting in scar tissue. Likely the healed scars from a previous injury as they are only partially re-pigmented and are surrounded by fibrous scar tissue, different in texture from the surface of the adjacent skin (Figure IV.20, attention in the red arrows)

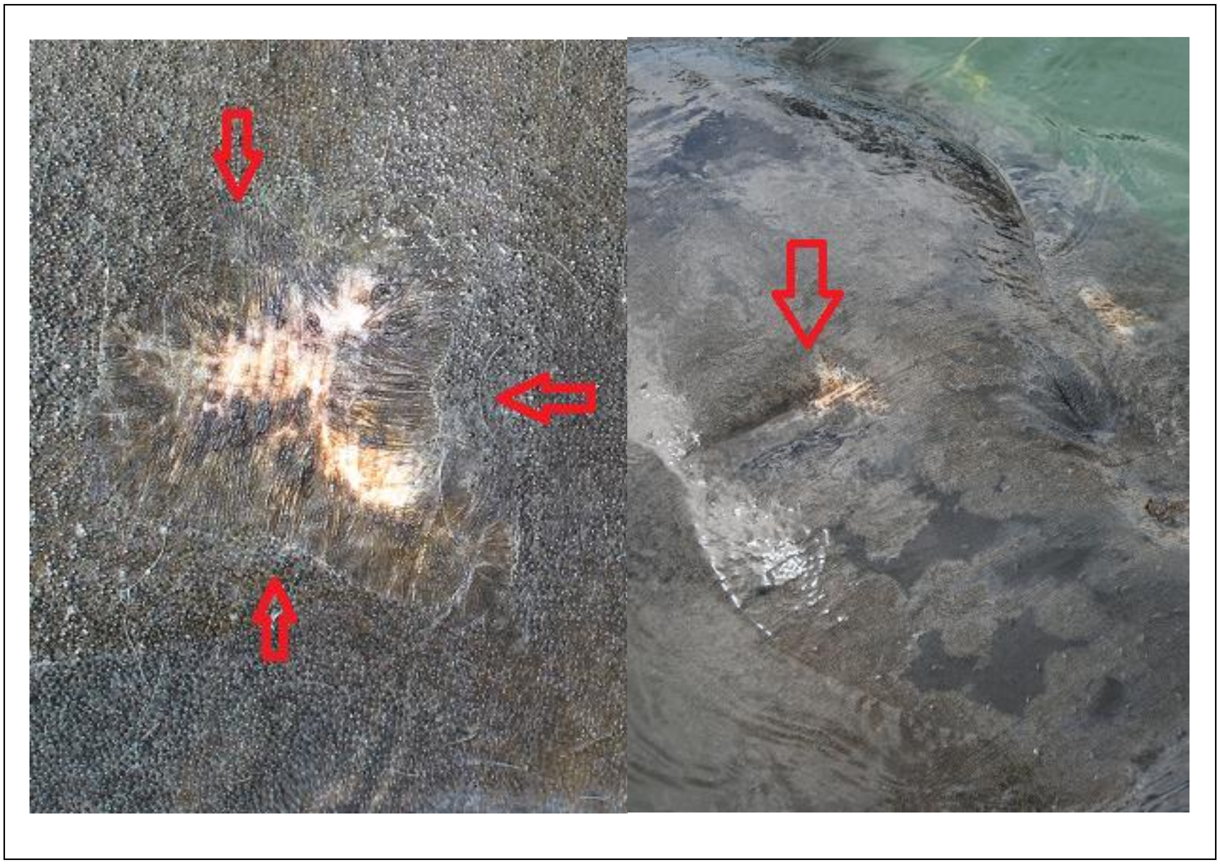


Figure IV.20 – Patches on "Poque"; note: the arrows depict the patch area as deeper and with characteristics of a healed scar.

Pictures: Fábia Luna - CMA/ICMBio and Margaret Hunter

Vianna and colleagues (2006a) bring the discussion of the fact of "Poque's" aberrant number of 50 chromosomes that could be a result of a hybridization between an Amazonian and a West Indian manatee, with an F2 cross back hybridization. They suggested that the occurrence of the T01 haplotype found in "Poque" occurred by a maternal lineage of an Amazonian manatee mother. As the mtDNA is maternally inherited, it would indicate that the haplotype of "Poque" more closely matches the

Amazonian haplotypes, even if an F2 generation of an Amazonian female successfully reproduced with a West Indian manatee male. The result would generate a female hybrid calf, which could possibly reproduce again with another West Indian manatee male. In this case “Poque” would have a phenotype consisting mostly of the West Indian manatee, as he has.

It is generally known from other species that an F1 hybrid can successfully reproduce, but not an F2 hybrid (Lehman et al., 1991; Pilgrim et al., 1998), because the animal is usually sterile. Jorge and colleagues (1976) studied hybridization between the kudu and eland, and discovered the sterility of the hybrid offspring, which was unexpected because of the generally similar karyotype structure of the eland and kudu. That similarity is illustrated with the ten autosomal pairs and the sex chromosomes that are believed to be identical in structure because they pair with the same banding patterns that have been documented. In these cases, the hybrids had an absence of germ cells and the authors could not explain why. It appears that they were expecting this for fertile individuals as found in other hybrids of the domestic horse and the Przewalski horse, where the parental chromosome sets are less divergent and fertility is feasible (Koulischer and Frechkop, 1966; Short et al., 1974).

Hansson and colleagues (2012) detected hybridization, but no evidence for backcrossing between great reed warblers (*Acrocephalus arundinaceus*) and clamorous reed warblers (*Acrocephalus stentoreus*) and suggested that hybrids of these two species are either infertile or their progeny unviable. Short and colleagues (1974) observed that male and female hybrids between the Przewalski horse (*Equus przewalskii*) and the domestic horse (*E. caballus*) are fertile, unlike all other known interspecific equine hybrids in which meiosis is almost totally arrested during prophase. Their study, with chromosome banding, illustrated a close homology between the karyotypes of two species of horses, even with different numbers of chromosomes: Przewalski horse (2n= 66) and domestic horse (2n= 64). They also described that this occurred during a translocation, transforming four acrocentric chromosomes of *E. przewalskii* into two metacentric chromosomes in *E. caballus*. A parental genetic background was sufficiently similar to permit normal gametogenesis and a regular segregation from the trivalent of the F1 hybrid at meiosis. The significance of this is discussed in relation to the evolutionary potential of the Equidae.

The chromosomes of the horse (*E. caballus*), the donkey (*E. asinus*) and the hybrid mule were also studied by Trujillo and colleagues (1962). They described the diploid chromosome number 64 for the horse and 62 for the donkey, which have chromosomes that are completely different morphologically. The mule has a number of chromosomes equal to 63. For the authors, the disparity between the chromosomes is satisfactory by understanding that there is sterility in the male hybrid, but does not explain the occasional fertile female able to produce several offspring with no typical *E. asinus* traits.

"Poque" also has a number of chromosomes (n=50), between the 48 chromosomes from the West Indian manatee and the 56 chromosomes of the Amazonian manatee. Records indicate that "Poque" has been the father of two calves born in captivity (one stillborn Figures IV.6 and IV.16; and one alive named "Bela or Belinha" – see Chapter V) at the CMA/ICMBio facilities. Both of these offspring were from reproduction with two different West Indian manatee females.

According B. Gray (personal communication), two different situations could explain the two extra chromosomes found in "Poque". The first is that "Poque" is a hybrid and the chromosomes suffer a rearrangement in order to carry out important and vital genetic information. This could explain the loss of chromosome #9 and the addition of two smaller extra pairs found in "Poque". The two smaller pairs #24 and #25 are about half size of the original chromosome #9 reported for West Indian manatees. This rearrangement could permit the balance between all chromosomes and result in his survival. The second option for "Poque" is that he could have a double trisomy, or a pair of chromosomes that broke apart during meiosis. In both cases "Poque" would have a chromosome number equal to 50. In humans there are some genetic diseases that occur in chromosomes, such as trisomy 21 or Down's syndrome, that have similar rearrangements. If "Poque" has a genetic disorder like that it could explain why he is smaller than the other captive West Indian manatees at CMA/ICMBio. However, “Poque” has been in captivity most of his life and his small size may be due to artificial rearing. On the other hand, if “Poque” is a hybrid, then his rearing in captivity may have helped him survive the hardships of surviving naturally in the wild. In an *ex-situ* situation, it is possible that he would not have survived the rigorous challenges of life in the wild.

“Poque’s” karyotype is missing the chromosome #9 pair corresponding to a “normal” *T. manatus* karyotype, and includes two additional pairs of G-grouped sized chromosomes (#24 and #25, most likely subtelomeric) that are not believed to belong or conform to the “normal” *T. manatus* karyotypes prepared to date. The two pairs are arbitrarily assigned as pairs #24 and #25 in Figure IV.11, and they are typically reported for *T. inunguis*. There are also structural differences in the chromosomes presented as homologs at the chromosome #1 and chromosome #8 positions (Figure IV.11). These include a significant G-banding homology to warrant pairing, but they are morphologically different. All these differences may vary when compared to karyotype features of *T. inunguis* and *T. manatus.* In addition, it was suspected that there may be other *T. inunguis* chromosomes present which are homologous and/or indistinguishable (by G-banding) from those of *T. manatus*. (B. Gray, personal communication).

“Poque” clustered with the Northwest of the Amazon River estuary (NWA) population by STRUCTURE and attributed likeness of 0.34 to that population when analyzing genotypes with GENECLASS. WHICHRUN also attributed “Poque” to the NWA population. In the nDNA study he had a unique allele and his mtDNA haplotype was T01, described by Vianna and colleagues (2006) as similar to Amazonian manatee haplotypes. In this study five more manatees from the NWA had the haplotype T01, all are morphologic Amazonian manatees and cluster as one group with this species. Table IV.7 reports the genetics techniques used in this study and the results found for all the populations of manatees studied, including “Poque’s” results.

Table IV.7 - Genetic analyses for the manatee populations and for “Poque”. AL-CE (Alagoas to Ceará states); PI-MA (Piauí and Maranhão states); NWA (Northwest of the Amazon River estuary); EAR (Estuary of the Amazon River) and STM (Santarém region).

Individual/ Population	Morphology	Haplotype	Chromosomes	Locus/Private alleles
AL-CE	*T. manatus*	M01	n = 48	-
PI-MA	*T. manatus*	M01; M03;M04	n = 48	-
NWA	*T. manatus*	_	N=48[1]	E04/250; E11/199
EAR	*T. inunguis*	T01*; V#; T#;; S#; R#	n=56	SC5/137
STM	*T. inunguis*	R#; S#; T#	n=56	E08/230
Poque	*T. manatus*	T01	n = 50[2]	Kb60/211

* Five manatees from EAR were identified to have T01 haplotype; # New haplotype; letter denotes haplotype most closely associated with the new sequence, number not assigned for this study.; [1-] Not assessed in this study - number of chromosomes for West Indian manatee described by Gray et al. (2002) for Florida and Hunter et al. (2012) for Puerto Rico. [2] – Chromosome number appears mixed between West Indian manatee and Amazonian manatee. Obs: Number of chromosomes for Amazonian manatee was described by Assis et al. (1988) and match with the manatees from EAR and STM regions from this study.

The result these genetic analyses confirm that “Poque” is a hybrid. As he has reproduced while in captivity, it would be more probable that he is an F1 hybrid, with a different chromosome recombination during the meiosis process where the chromosomes of a hybrid are reorganized to be able to carry out important and vital genetic information to ensure survival and achieve balance inside the cell. This could be the cause for a stillbirth of one of “Poque’s” offspring. It is suspected that the mother was able to grow the fetus during pregnancy, but the fetus was not able to survive outside the mother's body. As “Bela” appears to have a “normal” West Indian karyotype (48 chromosomes; Figure IV.12), she may have had a combination of loss and rescue of chromosomes, and a reorganization of the chromosomes or an uniparental disomy (UPD). Future studies are necessary to address specifics about the karyotype of “Bela” describing all the morphological aspects. This study will also benefit and provide information about “Poque’s” karyotype.

FISH techniques can be used to localize specific DNA segments geographically into specific chromosomal loci (Pinkel et al., 1988 as cited in Gray et al., 2002). Interspecific and intraspecific comparisons of chromosomal homologies through FISH techniques, commonly known as zoo-FISH, are now being applied in studies at the chromosomal level (Kellogg et al., 2007; Pardini et al., 2007; Bielec et al., 1998 as cited in Gray et al., 2002). The use of those painting techniques would be very helpful in discovering more about these chromosome rearrangements. With these results we will be able to comprehend the hybridization, what mechanisms are necessary to better understand the sympatric areas and examine the relationship between the two species to improve plans for the conservation of both manatee species in Brazil.

IV.IV.6 - Hybridism and management recommendations

For the manatees from the Estuary of the Amazon River (n = 7), STRUCTURE clustered the Amazonian manatees from the Santarém region as a one population. That means that manatees found in the estuary and analyzed in this study are very likely Amazonian manatees. They cluster with the 14 manatees found in the Santarém region, 1,000km away from the estuary. The manatees from the estuary likely have some genetic differences from the manatees from STM due to the large geographic distance between the locations.

However, as the other two groups added represent West Indian manatees, a different species, they strongly clustered with STM as a single related group. Additionally, in a phylogenetic study Cantanhede and colleagues (2005) identified a high level of gene flow resulting in phenotypic and genetic variations among different populations of Amazonian manatees in the Amazon River. Incidentally, they found no geographic structuring with a large panmictic population.

The results reported here illustrate the EAR as a possible region commonly occupied more by Amazonian manatees than by West Indian manatees. The influence of the Amazon River in the area is very strong and most times of the year the salt water is diluted and the area has very low levels of salinity (Santos, 2000). This may influence the majority of occurrences of the Amazonian manatee in this region and the reduced presence of West Indian manatees (which in Brazil lives mostly in the sea and visit freshwater only to drink).

Based on all the genetic techniques used in the present study, "Poque" is a hybrid, which is the only one animal in the entire study, but, the limited number of samples from the EAR (n = 7) leaves open the possibility that West Indian manatees use the area more and hybrids would be detected with more samples. However, hybridization does not appear to be frequent, and in the region, there are no reports of stranded dead calves or fetuses that could be derived from spontaneous abortions due to complications expected during reproduction between hybrids. This results in a reduced population growth rate, since some pregnancies do not result in viable offspring, which is a problem for the conservation of both species.

The mating strategies used by manatees have been reported to consist of a mating herd in which larger individuals would have an advantage for breeding (Reynolds et al., 2004). Nevertheless, manatees have a strong sex drive and commonly try to mate with all individuals (male or female) or even objects in the water. Therefore, it would not be surprising if a male of one species attempted to mate with a female of another species.

The West Indian manatee is bigger than the Amazonian manatee. So, in the event of a mating between the two species, it would be more likely for a male West Indian manatee to breed with a female Amazonian manatee than the contrary, as may have been the case with "Poque's" parents. Also, male manatees are generally looking for a female in estrus and the abundance of Amazonian manatees in the Estuary of the Amazon River seems to be higher than the occurrence of West Indian manatees. The abundance of the Amazonian manatee is not known for sure, but it is certainly much greater than the abundance of the West Indian manatee in Brazil (which is currently estimated to be about 500-1,000 individuals; Lima, 1997; Luna, 2001; Luna and Passavante, 2010; CMA/ICMBio, in prep.).

However, in general during speciation, natural barriers have acted to avoid hybridization. It may be possible that mating events between the two species occurs, but pre-meiotic (before the egg and sperm meet) mechanisms exist that result in termination of pregnancy. Additionally, post-meiotic barriers can occur, with the fetus not surviving and abortion terminating the pregnancy. In the case of hybridization between the wolf and the coyote, these preventative mechanisms occur with hybrids only after anthropogenic habitat alteration and a decrease of the wolf population occurs, even if the two species have been coexisting in the region before.

In the case of the manatee, nothing is known about hybridization and it is difficult to define the influence of the condition of the habitat on the potential for hybridization. However, if the habitat along the coast of Brazil continues to be used by humans and depredated it can force the West Indian manatee to seek other areas, even into the river as the species has the ability to live in exclusively in freshwater habitats. In Florida some manatees spend 100% of their time in freshwater systems (Reep and Bonde, 2006).

The Amazon River has a discharge of about 220,000m^3/s^{-1} (MMA, 1996) or one trillion m^3/year^{-1} according with Nittrouer and colleagues (1991) and flows into the Atlantic Ocean in an expansive estuary around 120km^2. The freshwater from the complex of the Amazon River estuary can reach as far as French Guiana, as the Guiana currents move freshwater towards the north (Figure IV.21). So, an Amazonian manatee from the Estuary of the Amazon River can also be forced to seek other areas with fewer disturbances by people, boat traffic, etc. They may choose to leave the habitat in this region, and move out of the estuary, following the river water flowing north along the Amapá coast, as long as the water is not too salty for them.

Additionally, in Amapá state there are some rivers supplying more freshwater along the Atlantic coast, which may also help to keep Amazonian manatees able to travel between the mouths of those rivers, in areas with little influence of salt water. Best and Teixeira (1982) note that apparently only West Indian manatees occur at Amapá coast, however, if records are accurate, “Poque” had been with his mother at the time of their capture near the border of French Guiana, which agrees that Amazonian manatees can go out of the Amazon basin, as described by Domning (1981b), or even may sometimes be found in salt water, as suggested by Domning (1981b).

“Poque’s” similar mtDNA inherited from his mother is described for Amazonian manatees (during this present study the mtDNA analyses five Amazonian manatees were identified with the T01 haplotype). The protection of suitable manatee habitat is essential to avoid the forced movement of manatees that have to leave their natural range in search of new habitats, increasing the possibilities of reproduction between both species and raising the rate of potential hybridization.

“Poque” is originally from Oiapoque, up in the Northwest of the Amazon River estuary, that appears to be part of the sympatric area, and which has been bigger than the region originally described. Therefore, the range of the sympatric area probably goes from the east of Marajó Island on Pará state to Oiapoque in Amapá state, or even farther into other countries, including French Guiana. This may more adequately define the possible hybridization zone (Figure IV. 21). It is highly recommended that additional research is conducted in this area with detailed health assessments of manatees and radio tagging with GPS system technology to better understand movements of both species in the north of Brazil.

The habitat of both species of manatees needs to be protected to avoid increasing the probability of hybridization, which can have a detrimental impact on the existing populations, especially as both species are currently at risk of extinction. The impacts can be more dramatic for the vulnerable fragmented populations of the West Indian manatees, especially in the Northwest of the Amazon River estuary. The same is true for the fragile population of the Amazonian manatees that are located in this region.

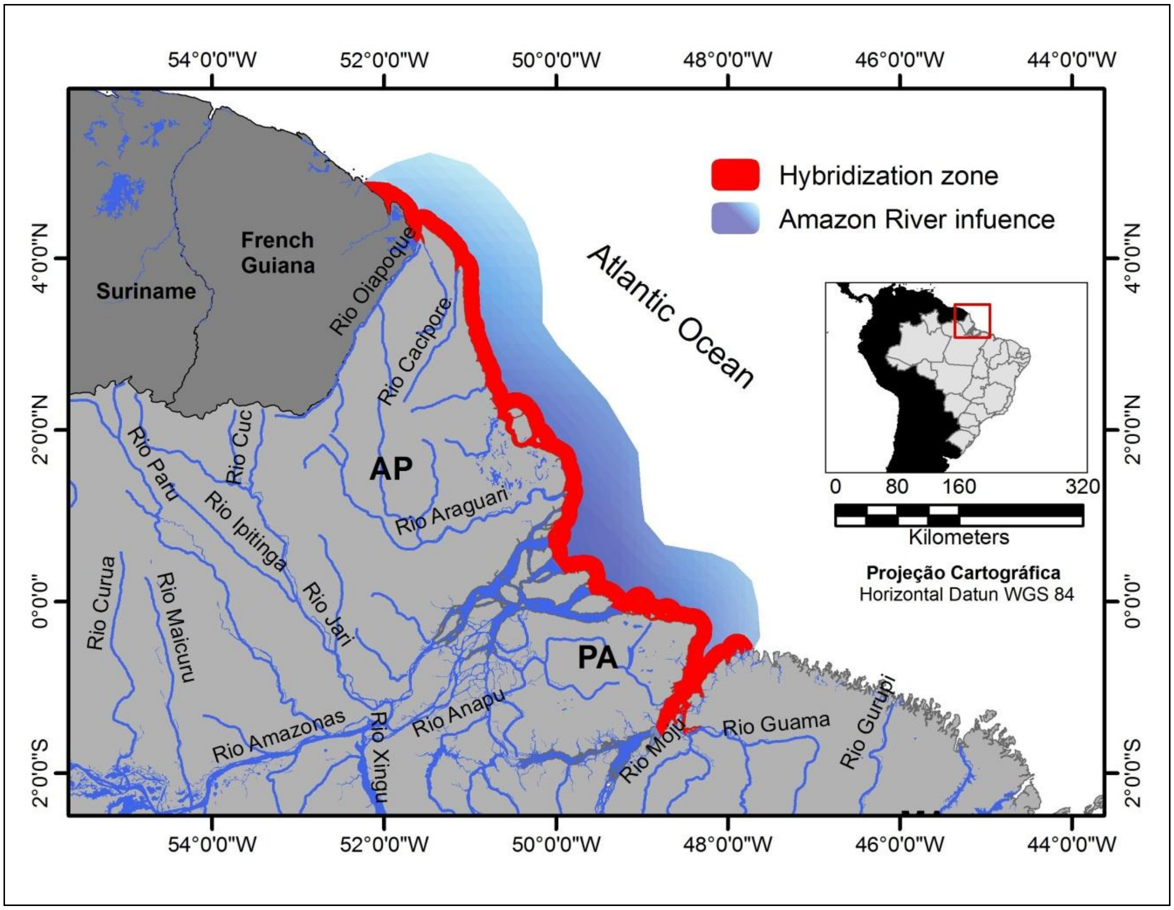


Figure IV.21 – Area of the influence of the Amazon River and the potential hybridization zone.

IV.V - CONCLUSIONS

The presence of hybridization between *T. manatus* and *T. inunguis* in the wild is confirmed at Oiapoque region, on Amapá state, but does not appear to be frequent. This issue warrants further investigation in order to define specific actions necessary to manage and conserve the two species of manatees in Brazil.

It is very important to acquire more samples and conduct more analyses on the manatees from Amapá (AP) and Pará (PA) states in order to better understand the extent of hybridization between the two species of manatees that occur in Brazil.

A single method of genetic study is not enough to diagnose a hybrid. What is necessary is the use of several genetic studies, such as mitochondrial DNA to check the maternal lineage; microsatellite DNA for comparing shared alleles of the individual with each species, examining for private alleles, and use of detailed cytogenetic analyses to investigate chromosomes.

A cytogenetic study applying the Zoo-FISH technique is recommended to investigate the cross-species homology of chromosomes for “Poque” and “Bela” to learn more about hybridization and chromosome rearrangement.

A potential increase in hybridization with the degradation of existing manatee habitat is of concern for both species, especially since they are at risk of extinction. A high occurrence of hybrids could have a detrimental impact on the survival of both species. Their habitats need to be protected and it is necessary to implement the National Action Plan for Sirenia in Brazil and provide Protected Areas along the north coast of Brazil.

Chapter V – Pedigree of manatees in captivity at CMA/ICMBio and the existence of inbreeding

V.I - INTRODUCTION

Overall, the information regarding pedigree relationships and reproduction in manatees is based primarily on photo-identification studies conducted in Florida. The identification of the father is not possible in the wild population using photo-identification techniques. Some new genetic studies have focused on developing methods to identify the father of offspring (M. Davis, personal communication; Pause, 2007; Nourisson, 2011).

The manatee breeding system is described as being promiscuous (Rathbun et al., 1995; Anderson, 2002); where target females are observed in estrous with consorting males in pursuit of her. It is through this event that the receptive female will likely mate with several males. Reynolds and colleagues (2004) suggest that male manatees may exhibit sperm competition. In captivity, observations at the CMA/ICMBio facility indicate that receptive females copulate with all adult males available in the same captive pool.

This may explain why the identity of the mother is usually known in some of the facilities where breeding occurs, whereas the exact identity of the father may be unknown. However, it is very important to know the identity of both parents of captive born calves and the parents’ relatedness in order to avoid potential inbreeding. Genetic studies are currently the only possible way to know which male is the father of each calf where multiple males are housed together.

In captivity, it is well known that manatees breed and reproduce relatively easily, often leading to successful births. Inbreeding can be a problem if the calves are not separated from their parents when they reach sexually maturity. For example, in Europe, there are several captive manatees that have been on public display since

1962. The first three wild manatees were caught in Suriname and transferred to Amsterdam, in the Netherlands. Between 1976 and 1977 three additional manatees were caught in Guyana and transferred to Nurnberg, Germany. Those manatees were allowed to breed in captivity resulting in a total of 58 offspring identified in a published studbook of West Indian manatees held in captivity in Europe (Fersen, 2011).

In August of 2011, there were 38 manatees in captivity, resulting from four of the six first caught manatees mentioned before, distributed in nine facilities in Europe (33 animals), and two facilities outside of Europe (Singapore and Yashima, where five animals were transferred from European facilities). It appears that the breeding allowed to occur in Europe was to maintain a captive population without concern for the issues related to inbreeding. Of the three manatees from Suriname, one died approximately four years after capture, and one of the manatees from Guyana died in 1977, leaving four able to breed. Currently only two of these individuals from the wild survive and are continuing to breed in captivity at both aquariums; the remaining breeders are all offspring. The disturbing note is that in this *ex-situ* population, including all facilities cited above, there have only been a couple of founders contributing to the lineage.

According to the studbook of these captive manatees (Fersen, 2011), a female from Guyana had 13 calves, 10 of them sharing the same father, the male from Guyana that had survived. A female calf offspring of this couple had eight calves from related males (and possibly her father). It is unknown how many of her siblings she mated with, but in at least one case it is known that the father was her full sibling brother. These familial matings result in a high degree of inbreeding within the group and eventually sterility. Additionally, this female gave birth to some stillborn calves and calves that were successfully born but died at a very young age, likely due to the high level of inbreeding. Another female offspring of the pair from Guyana had at least two calves with a full sibling brother. The 2nd generation captive born manatees at these facilities had a high level of relatedness, and several pregnancies occurred between full and half sibling partners, which resulted in a high level of inbreeding.

As was previously discussed in Chapters I, II and III, inbreeding will reduce the genetic diversity of individuals. This can reduce the ability of the immune system to respond to changes, increase susceptibility to disease, result in physical defects and lower population viability (Sherwin and Moritz, 2000).

The reduction of genetic diversity can also result in decreased fertility and survival, and compromises the ability of the species to withstand and adapt to environmental changes, enhancing the likelihood of extinction (Avise, 2004). The preservation of genetic diversity is fundamental to any population, especially in captivity where regulation by responsible managers is needed. For managed captive programs, maintaining the genetic diversity will increase the likelihood of the animals adapting to new environments and will preserve future management options like the possibility of being returned to, or released into, a wild population.

Actually, to maintain an *ex-situ* population in these facilities it is necessary to add new, unrelated individuals from the same subspecies to ensure genetic diversity. To increase the genetic variability and health of the population it is vital that when searching for breeding partners for transfer to facilities, managers select candidates with similar genetic origins, and habitat adaptation, but non-related.

The preservation of genetic diversity can be achieved by optimizing the number of founders, maintaining demographic stability, equalizing family size and regulating founder’s contributions. The reproduction of captive manatees must not be allowed until the relatedness-value (r) between potential breeding pairs is known. Also, the facilities must be prepared to manage the captive born manatees, providing extra pools if necessary to ensure separation of sexually active juveniles and adults from their parents and the siblings or relatives.

In the case of release of captive born manatees, individuals must be very healthy and the genetic pedigree must be known to avoid inbreeding in the wild, which could be promoted by these released animals. Exchanging genetic material (through animals or gametes) with natural populations or within other captive facilities when possible could be a way to avoid inbreeding with a limited number of breeders (Ralls and Ballou, 1986). All facilities need to be prepared to accept the consequences before allowing the animals to breed. When it's necessary, maximizing the selection of appropriate mates for breeding with non-related individuals could ensure a healthier resulting progeny.

The captive-born manatees at CMA/ICMBio are part of an *ex-situ* population that is in exhibition for public education and awareness to promote manatee preservation. As there are more manatees than is necessary for education purposes, some of them potentially could be released in discontinuous areas to encourage admixture with neighboring populations and encourage use of travel corridors. A good example of areas to release healthy manatees is between Alagoas (AL) and Pernambuco (PE) states in Brazil. The genetic data are very important to make informed decisions about these releases (Luna et al., 2012). Hopefully, this study will provide information to help guide the decisions about those releases and to maintain a healthy captive population. This information is very important to strengthen the conservation of the manatees in Brazil.

In Brazil, the number of founders in the captive population of West Indian manatees is six (two males and four females); a number very similar to European facilities. Three of these founders were stranded dependent calves rescued on the beach by CMA/ICMBio and its partners. Two were stranded calves maintained in illegal facilities and one was captured in the wild when the mother was hunted in Amapá state. All were transferred as juveniles or adults to the CMA/ICMBio facility (Figure V.1). All of these animals were maintained in the same pools together, both males and females, without any reproduction plan.

In 2008, a meeting at CMA/ICMBio occurred with consulting manatee researchers and managers, where it was decided that it was necessary to have a detailed genetic study of the pedigree of each manatee in captivity at the CMA/ICMBio facility. This information could be used to improve the management of captive animal populations throughout Brazil. A goal of the National Action Plan for Sirenia in Brazil strives for the improvement of the management of wild populations. It also highlights the necessity to have more studies to determine the feasibility of captive breeding for manatees and evaluate if a future reproduction program is necessary to assist the wild West Indian manatee populations in Brazil (Luna et al., 2011).

The manatees at CMA/ICMBio bred since 1996 with no reproduction plan and no genetic information to help select the appropriate breeding pairs, and have resulted in potential inbreeding cases. Several calves were born (Figure V.2) and there is no knowledge about the true identity of their fathers. The knowledge about the relationship between these manatees is very important to make informed management decisions at CMA/ICMBio facilities.

Pedigree studies provide information about estimated rates of gene flow into a population. These studies also adjust frequency estimates for presence of relatedness in a healthy population, provide estimates of the total number of breeders in a population and gauge the reproductive success among individuals. Additionally, pedigree programs can analyze inbreeding in captive populations (Nielsen et al., 2001; Blouin, 2003), which is very important to minimize or avoid potential problems.

The objective of this chapter is to provide a study using microsatellites to assess the pedigree reconstruction of CMA/ICMBio adult manatees, a released female named “Lua” with her two possible calves and all captive born individuals. This information will permit managers to make decisions about the future of the captive manatees, such as: a separation of manatees to avoid mating between related individuals; selection of appropriate facilities to house manatees; determine if a captive breeding program is necessary; etc. The main purpose of this study is to avoid inbreeding and improve the conservation of the species.

Figure V.1 – CMA/ICMBio facility with the manatees in captivity.

Picture: Fábia Luna – CMA/ICMBio

Figure V.2 - Female manatee and her captive born calf at CMA/ICMBio.

Picture: Fábia Luna – CMA/ICMBio

V.II - MATERIAL AND METHODS

Samples from Chapter III have been used in this study, including all captive adult manatees maintained at CMA/ICMBio. The captive born calves born prior to July of 2011 were included in this study. Blood and tissue were acquired and preserved using methods described in Chapter II to determine pedigrees. Also, two dead stranded calves, found in the area of a released female adult after visual observations of her giving birth in the wild, were also included in the study. A total of 20 animals was used for pedigree reconstruction.

Data regarding sex, age class at arrival to CMA/ICMBio, age class at the time of sample collection, and any available relationship and pedigree information are presented in Table V.I.

Table V.I - Data for CMA/ICMBio captive West Indian manatees with their name, sex, age class at arrival at CMA/ICMBio and at sampling time, available relationship information and actual situation. *Males maintained for at least a period in captivity and can be the father of some or all the calves. **Calves found dead on the beach near where the female Lua had been seen.

Name	Sex	Age class/Arrived CMA	Age class/ Sampling time	Available relationship information	Actual Situation
Sereia	F	Stranded calf	Adult	Mother of Carla, Sheila and Sereno	Captive at CMA/ICMBio In Itamaracá/PE
Xica	F	Adult (rescued from other captive facility)	Adult	Mother of Chiquito, Chiquinho and 1 abortion (Fxica2)	Captive at CMA/ICMBio facility Itamaracá/PE
Xuxa	F	Juvenil (rescued from other captive facility)	Adult	Mother of Tamunha and Luiz Gonzaga	Captive at CMA/ICMBio facility Itamaracá/PE
Marbela	F	Stranded calf	Adult	Mother of Fmarbela	Captive at CMA/ICMBio facility Itamaracá/PE
Poque	M	Juvenil (rescued from other captive facility)	Adult	Can be father of all calves captive born *	Captive at CMA/ICMBio facility Itamaracá/PE

Name	Sex	Age class/Arrived CMA	Age class/ Sampling time	Available relationship information	Actual Situation
Netuno	M	Stranded calf	Adult	Can be father of all calves captive born *	Captive at CMA/ICMBio In Itamaracá/PE
Assú	M	Stranded calf	Juvenile	Can be father of some calves captive born *	Captive at CMA/ICMBio In Porto de Pedras/AL
Sheila	F	Captive born	Adult	Calf of Sereia, twin of Carla and mother of one stillborn (Fsheila)	Captive at CMA/ICMBio facility Itamaracá/PE
Carla	F	Captive born	Adult	Calf of Sereia, twin of Sheila and mother of 2 calves (Fcarla1 – died in captive and FCarla2)	Captive at CMA/ICMBio facility Itamaracá/PE
Sereno	M	Captive born	Juvenile	Calf of Sereia, brother of Carla and Sheila, can be father of some calves	Captive at CMA/ICMBio facility Porto de Pedras/AL
2FiXica	-	Captive stillborn	Fetus	Calf of Xica (dead)	CMA/ICMBio genetic bank
Chiquinho	M	Captive born	Juvenile	Calf of Xica	CMA/ICMBio genetic bank (died after release)
FMar	M	Captive born	Juvenile	Calf of Marbela	CMA/ICMBio genetic bank
FCarla	M	Captive born	Juvenile	Calf of Carla	CMA/ICMBio genetic bank
Tamunha (FXuxa)	M	Captive born	Juvenile	Calf of Xuxa	Released at AL state
FSheila	-	Captive stillborn	Fetus	Calf of Sheila (dead)	CMA/ICMBio genetic bank
Bela (2FiCarla)	F	Captive born	Calf	Calf of Carla (2nd calf)	Captive at CMA/ICMBio facility Itamaracá/PE
Lua	F	Stranded calf	Adult (released in 1994)	Probably mother of two dead stranded calves	CMA/ICMBio genetic bank
Maraca	M	Dead stranded calf	Calf carcasses**	Probably calf of Lua (released in 1994)	CMA/ICMBio genetic bank
Flua	-	Calf stranded died	Calf carcasses**	Probably calf of Lua (released in 1994)	CMA/ICMBio genetic bank

V.II.1 - Genetic analysis

All the samples from the population study (Chapter III) were used in this chapter in order to calculate allele frequencies. The 20 samples were analyzed using the 16 microsatellites cited previously (Chapter III and IV) and for another 18 microsatellite primers (Tringali et al., 2008) using the conditions developed by M. Davis (personal communication); resulting in a total of 34 markers used to address the pedigree of the captive West Indian manatees in Brazil (García-Rodríguez et al., 2000; Pause, 2007; Tringali et al., 2008). The samples were run at the Conservation Genetics Laboratory at USGS.

V.II.2 - Pedigree reconstruction analysis

Three estimator software packages were employed. ML-RELATE (Kalinowski et al., 2006) and COANCESTRY (Wang, 2011) were used to establish the relatedness coefficient between each of the pairs from the 20 individuals and to identify specific relationships. To confirm the suspected fathers of the calves, CERVUS 3.0 (Kalinowski et al., 2006) was used.

The software ML-RELATE was used to reconstruct the relationship, on a blind test without *a priori* knowledge of the known pedigree for the captive manatees, which produced a list of relationships within 0.05 significance using 1000 simulations and calculating the relatedness value (r). The software was run for all 34 microsatellite markers.

CERVUS has the possibility to separate individuals by potential parents and offspring, using likelihood ratios, which are calculated allowing for the possibility that the genotypes of parents and offspring may be mistyped. It determines via simulation the level of confidence of the parentage assignment by giving the result of the potential parents for each offspring, but does not report relatedness values for all the animals. CERVUS was used to assign parents to their offspring using genetic markers.

The software COANCESTRY uses seven different estimators, two maximum likelihood and five moment estimators to measure the relatedness coefficient between pairs of individuals. It also takes into account the nine identical by descent (IBD) modes, which are all of the possible relationships between two individuals. The relatedness coefficient (rxy) represents the proportion of genes identical by descent between two individuals. According to Wang (2011) the reliability and accuracy of the seven estimators depends on the markers and population structure of the data. In this case, with many polymorphic microsatellite loci, the likelihood estimators based on the software TRIOML, WANG, LYNCHLI and DYADML are the most robust (M. Davis, personal communication).

For the program COANCESTRY a test comparing all 34 microsatellite markers to a subset of 15 microsatellite markers was conducted for accuracy. The program was run for all 20 samples using 34 microsatellite loci. Since the 20 samples were run with more loci than the population studies in Chapter III, it was not possible to calculate allele frequencies for the additional loci. Therefore, the 20 samples were run in two simulations with 15 primers to assess whether the frequencies were relatively similar to the large sample size used in Chapter III. In one simulation the allele frequencies were generated from the 20 samples while in the other the allele frequencies were calculated from all samples available from the population study (Chapter III).

V.II.3 - Geographic analysis

The initial captive population at CMA/ICMBio originated from wild rescued manatees. They were dependent calves stranded on beaches along the northeast coast of Brazil (as shown in Chapter II). The geographic locations of the stranded manatees were analyzed to determine the possible relationships among these sites. The date and locality where the manatees were stranded was either known or obtained from the archived database at CMA/ICMBio. The GPS location was acquired and plotted onto a map to illustrate sample locations.

V.III – RESULTS

Of the 34 available manatee microsatellites, seven (Tma-H13, Tma-F14, Tma-A02, Tma-M79, Tma-L14, TmaL15 and Tma-L17) were monomorphic for the 20 samples, resulting in a total of 27 microsatellites useful for the captive manatee study.

Results of the pedigree reconstruction and relatedness values for the captive manatees and for the released adult manatee "Lua" for the ML-RELATE program are presented in Table V.2 and Table V.3.

Results from COANCESTRY: relatedness value (r); relatedness coefficient (r_{xy}); estimates range from Δ_7 and Δ_8; and the relationship deduced from rxy, Δ_7, and Δ_8 values for the 20 animals utilizing 27 microsatellites loci, as seven were monomorphic, are presented in Table V.4. The program COANCESTRY was also run with 15 microsatellite loci using the allele frequencies of the full population from Chapter III or allele frequencies generated only from the 20 samples. The analysis using the 20 samples and 27 microsatellite loci resulted in the most refined data, and was the one considered for this study.

Table V.2 - Pedigree reconstruction for captive and released manatees using ML-RELATE. The unrelated relationships are not highlighted. The parent's offspring (PO) are highlighted in red; the full sibling (FS) in yellow, and the half sibling (HS) in blue.

Sereia	-																			
Lua	FS	-																		
Xica	U	U	-																	
Xuxa	HS	HS	U	-																
Poque	U	U	U	U	-															
Marbela	U	U	HS	U	U	-														
Sheila	PO	HS	U	U	U	U	-													
Carla	PO	U	U	U	U	U	FS	-												
Assú	U	HS	U	FS	U	U	U	U	-											
FXica2	U	U	HS	U	PO	U	U	U	U	-										
Maracá	HS	PO	HS	U	U	HS	U	U	U	U	-									
FXica3	U	U	PO	U	U	U	U	U	U	U	U	-								
FMarl	U	U	U	U	U	PO	U	U	HS	U	U	FS	-							
FCarla	U	PO	U	HS	U	U	U	U	FS	U	U	U	FS	-						
Tamun.	U	U	U	HS	U	U	U	U	HS	U	U	HS	FS	FS	-					
FLua	U	PO	U	U	U	U	U	U	HS	U	U	U	HS	HS	HS	-				
FSheila	U	U	U	U	U	U	FS	FS	U	U	U	FS	HS	U	HS	U	-			
Bela	U	U	U	U	PO	U	U	U	U	HS	U	U	U	U	U	U	U	-		
Netuno	U	U	U	U	U	U	PO	PO	U	U	U	PO	HS	U	HS	U	FS	U	-	
Sereno	PO	U	U	U	U	U	PO	FS	U	U	U	HS	U	U	HS	U	FS	U	PO	-
	Sereia	Lua	Xica	Xuxa	Poque	Marbela	Sheila	Carla	Assú	FXica2	Maracá	Chiquinho	FMarbela	FCarla	Tamunha	FLua	FSheila	Bela	Netuno	Sereno

Table V.3 - Pedigree relatedness values for captive and released manatees using ML-RELATE. Zero means individuals are not related. Based on genetic relatedness tests and knowledge about the animals, the cells highlighted in yellow, indicate pairs that could be good candidates for eventual captive breeding efforts.

	Sereia	Lua	Xica	Xuxa	Poque[1]	Marbela	Sheila	Carla	Assú	2FXica*	Maracá*	Chiquinho*	FMar*	FCarla*	Tamunha	FLua*	FSheila*	Bela	Netuno	Sereno
Sereia	_																			
Lua	0.44	_																		
Xica	0	0	_																	
Xuxa	0.14	0.22	0.07	_																
Poque[1]	0	0	0	0	_															
Marbela	0.02	0.01	0.28	0	0	_														
Sheila	0.5	0.17	0	0	0	0	_													
Carla	0.5	0.11	0	0	0	0	0.8	_												
Assú	0	0.23	0	0.41	0	0.02	0	0	_											
2FXica*	0	0	0.3	0	0.5	0	0	0	0	_										
Maracá*	0.31	0.5	0.13	0.13	0	0.27	0.02	0.14	0	0.05	_									
FXica3*	0	0	0.5	0	0	0	0.09	0	0	0	0	_								
FMar*	0	0	0.06	0	0	0.48	0	0	0.29	0	0	0.29	_							
FCarla*	0.12	0.5	0.06	0.31	0	0.05	0	0	0.37	0	0.06	0	0.46	_						
Tamun.	0	0.03	0.02	0.29	0	0	0	0	0.29	0	0	0.21	0.36	0.76	_					
FLua*	0.14	0.5	0	0	0	0	0.03	0	0.29	0	0.1	0	0.14	0.18	0.32	_				
FSheila*	0	0	0	0	0	0	0.62	0.42	0	0	0.09	0.47	0.19	0.09	0.11	0.06	_			
Bela	0	0	0	0	0.5	0	0	0	0	0.23	0	0	0	0	0	0	0.06	_		
Netuno	0	0	0	0	0	0	0.5	0.5	0	0	0	0.52	0.28	0.09	0.13	0.01	0.66	0.1	_	
Sereno	0.5	0	0	0	0	0	0.51	0.57	0.08	0	0	0.25	0.07	0	0.27	0.15	0.38	0	0.5	_

*Aborted or dead animals; **"Poque" is not allowed to breed (Chapter IV).

Table V.4 - Pedigree reconstruction for captive manatees using maximum likelihood from ML-Relate and Co-Ancestry. Only results with a proposed relationship by one of the programs are shown. Ind1 and Ind2: Identification name of the two animals in a proposed relationship; 0.05 R: other possible relationships significant at 0.05; R: relationship with the likelihood proposed by ML-Relate; LnL(R): Log likelihood of R; Delta Ln(L): the degree that the highest likelihood is above other possible likelihoods (to LnL(R)); 9999 indicates that the relationship is not possible; U: unrelated; HS: half sibling; FS: full sibling; PO: parent offspring; Rxy: relatedness coefficient by Coancestry; Δ7: estimates of Δ7 value; Δ8: estimates of Δ8 value; R* the deduced relationship (interpretation of both programs); Obs: observations for pedigree results; RE: animals related (but relationship not identified).

Individual		**ML-Relate**							**Co-Ancestry**			**Interpretation**	**Observation**
Ind1	**Ind2**	**0.05 R**	**R**	**LnL(R)**	**Delta Ln(L)**				**Rxy**	**Δ_7**	**Δ_8**	**R***	**Obs**
					U	**HS**	**FS**	**PO**					
Lua	Sereia	HS, FS	0.4416	-50.79	2.62	1.04	-	9999	0.413	0.2065	0.4129	FS	Both found in the wild from a near place (see Figure V.2)
Xuxa	Sereia	U, HS	0.145	-67.48	0.22	-	2.70	9999	0.1359	0	0.2718	First cousin	Both found in the wild from a near place (see Figure V.2)
Xuxa	Lua	U, HS	0.2158	-61.97	0.80	-	2.57	9999	0.2283	0	0.4566	HS	Both found in the wild from a near place (see Figure V.2)
Marbela	Xica	HS, FS	0.2827	-65.49	2.03	-	1.20	9999	0.2543	0.0808	0.3471	HS	Both found in the wild in areas distant around 200km
Marbela	FCarla	U, HS	0.0465	-58.04	-	0.81	3.52	9999	0.0851	0.0067	0.1568	Second cousin	Marbela has some relatedness with the Father of FCarla
Sereia	Flua	U, HS	0.1403	-51.63	-	0.61	2.55	9999	0.1087	0.108	0	RE	Sereia is strongly related to FLua mother

Individual		**ML-Relate**							**Co-Ancestry**			**Interpretation**	**Observation**
Ind1	**Ind2**	**0.05 R**	**R**	**LnL(R)**	**Delta Ln(L)**				**Rxy**	**Δ_7**	**Δ_8**	**R***	**Obs**
					U	**HS**	**FS**	**PO**					
Sereia	FCarla	U,HS	0.1216	-52.36	-	1.35	3.58	9999	0.1064	0.104	0	RE	Sereia is the grandmother of FCarla
Sereia	FSheila	U, HS	0	-50.26	2.35	0.73	1.80	-	0.5	0	1	PO	Sereia is the grandmother of Sheila
Carla	Sereia	PO	0.5	-50.53	4.15	1.45	1.36	-	0.5	0	1	PO	Sereia is the mother of Carla
Carla	Sheila	Twin	0.8008	-39.54	9.41	5.44	-	2.46	0.7949	0.7949	0	Twin	Born in captivity. Twin
Carla	Lua	U, HS, FS	0.1132	-49.74	-	0.04	1.61	9999	0.1028	0.0932	0.0191	RE	Lua is strongly related to Carla's mother (Sereia)
Assú	Lua	U, HS	0.231	-54.42	0.89	-	2.69	9999	0.2404	0	0.4808	HS	Both found in the wild from a near place (see Figure V.2)
Assú	Xuxa	FS	0.406	-66.00	3.60	1.38	-	9999	0.3809	0.3809	0	RE	Both found in the wild from a near place (see Figure V.2)
Assú	Marbel	U, HS	0.0169	-65.92	-	1.44	6.67	9999	0.0527	0	0.1054	Second cousin	Both found in the wild in areas distant around 200km
FXic2	Xica	HS, FS, PO	0.303	-71.26	1.84	-	2.28	0.6	0.2911	0.0349	0.5124	HS	Xica is the mother of FXica2
FXic2	Poque	PO	0.5	-86.22	13.56	4.44	5.73	-	0.5	0	1	PO	Poque is the father. He is hybrid (Chapter IV). FXica2 is stillborn
Maraca	Sereia	U, HS, FS	0.3063	-57.76	1.35	-	0.66	9999	0.252	0.0777	0.3486	HS	Sereia is strongly related to Maraca's mother (Lua)
Maraca	Lua	PO	0.5	-52.61	1.51	0.38	2.03	-	0.5	0	1	PO	Lua is confirmed to be the mother of Maraca
Maraca	Xica	U, HS	0.132	-64.31	0.06	-	3.64	9999	0.1488	0	0.2977	First cousin	Wild Maraca's father probably is from the same state as Xica
Maraca	Marbela	HS, FS	0.2707	-62.87	1.91	-	1.50	9999	0.274	0.0262	0.4955	HS	Maraca is related to Xica and Xica is related to Marbela.

Individual		**ML-Relate**							**Co-Ancestry**			**Interpretation**	**Observation**
Ind1	**Ind2**	**0.05 R**	**R**	**LnL(R)**	**Delta Ln(L)**				**Rxy**	**Δ$_7$**	**Δ$_8$**	**R***	**Obs**
					U	**HS**	**FS**	**PO**					
Maraca	FLua	U, HS	0.1041	-54.15	-	1.81	4.32	9999	0.0987	0.0787	0.04	Second cousin	Half Sibling both found dead on the beach in 2003 and 2009
Chiq.	Xica	PO	0.5	-55.60	4.07	1.28	1.96	-	0.5	0	1	PO	Xica is the mother of Chiquinho
FMarb	Marbela	HS, FS, PO	0.4805	-55.76	2.84	0.62	1.55	-	0.5	0	1	PO	Marbela is the mother of FMarb
FMarb	Assú	U, HS, FS	0.2895	-53.81	1.00	-	0.74	9999	0.2811	0.0792	0.4037	HS	Assú is the father of FMarb (by Cervus).
FMarb	Xica	U, HS	0.057	-56.43	-	0.51	2.98	9999	0.0966	0.0151	0.1628	Second cousin	Xica is related in some way with Fmarb's mother (Marbela)
FMarb	Chiquin	HS, FS, PO	0.284	-49.54	1.27	0.42	-	0.64	0.2717	0.2346	0.0742	HS	-
FMarb	FCarla	HS, FS, PO	0.4615	-43.89	3.05	1.53	-	0.69	0.4145	0.414545	0	RE	FMarbela has the same father than FCarla (Assú by Cervus)
FMarb	FLua	U, HS, FS, PO	0.1443	-46.16	0.04	-	1.86	0.58	0.2001	0	0.4002	HS	The father of FLua can be related to the mother of FMarb. They are from areas around 300km apart
FMarb	FSheila	U, HS, FS, PO	0.1865	-47.44	0.45	-	1.13	0.44	0.1202	0	0.2404	First cousin	-
FCarla	Lua	HS, FS, PO	0.5	-48.70	1.29	0.69	0	0.61	0.5	0	1	PO	Lua is strongly related to Carla's grandmother (Sereia)
FCarla	Assú	HS, FS	0.3705	-53.16	1.10	0.05	-	9999	0.3432	0.2751	0.1361	RE	Assú is the father of FCarla (by Cervus)
FCarla	Xica	U, HS	0.065	-55.87	-	2.25	5.43	9999	0.05	0	0.1001	Second cousin	-

Individual		**ML-Relate**							**Co-Ancestry**			**Interpretation**	**Observation**
Ind1	**Ind2**	**0.05 R**	**R**	**LnL(R)**	**Delta Ln(L)**				**Rxy**	**Δ_7**	**Δ_8**	**R***	**Obs**
					U	**HS**	**FS**	**PO**					
FCarla	FSheila	U, HS, FS	0.0933	-47.33	-	0.17	2.02	1.36	0.0792	0.0709	0.0165	Second cousin	Both are calf of the twin sisters
Tam	Xuxa	U, HS, FS	0.2893	-57.74	1.19	-	0.75	9999	0.2894	0.1007	0.3774	HS	PO (relationship not well identified by software: 2 mismatch)
Tam	Assú	U, HS, FS	0.2924	-50.59	0.89	-	0.62	9999	0.2522	0	0.5044	HS	Assú is related in some way to the mother of Tam (Xuxa)
Tam	Chiquin	U, HS, FS, PO	0.2095	-47.81	0.43	-	1.06	0.38	0.1311	0	0.2621	Second cousin	-
Tam	FMarb	HS, FS, PO	0.3632	-42.89	2.11	0.97	-	0.51	0.3574	0.2653	0.1841	RE	-
Tam	FCarla	FS	0.7573	-38.59	6.33	3.52	-	1.5	0.67	0.5945	0.151	RE	The grandmother of FCarla (Sereia) has some relation to the mother of Tam (Xuxa)
Tam	Marbel	U, HS	0	-54.23	-	1.25	4.74	9999	0.018	0	0.036	Second cousin	-
FLua	Assú	HS, FS	0.2933	-50.21	2.00	-	0.87	9999	0.3024	0.0268	0.5512	HS	Assú has some relatedness with the mother of FLua (Lua)
FLua	Lua	PO	0.5	-43.62	3.02	1.18	1.55	-	0.5	0	1	PO	Lua is confirmed to be the mother of FLua (stranded in the wild)
FLua	FCarla	U, HS, FS	0.1763	-45.48	0.17	-	1.05	9999	0.0721	0	0.1442	Second cousin	Grandmother of Fcarla (Sereia) has strong relation to the mother of FLua (Lua)
FLua	Tam	U, HS, FS, PO	0.3216	-44.90	0.57	-	1.44	0.16	0.3576	0	0.7152	HS	The mother of Tam (Xuxa) is related to FLua's mother (Lua)
FSheila	Sheila	HS, FS, PO	0.6236	-43.91	3.68	1.73	-	0.52	0.4891	0.4551	0.068	PO	Sheila is the mother of FSheila

Individual		ML-Relate							Co-Ancestry			Interpretation	Observation
Ind1	**Ind2**	**0.05 R**	**R**	**LnL(R)**	**Delta Ln(L)**				**Rxy**	**Δ_7**	**Δ_8**	**R***	**Obs**
					U	**HS**	**FS**	**PO**					
FSheila	Carla	HS, FS, PO	0.4207	-46.97	2.68	1.25	-	0.55	0.372	0.372	0	FS	Sisters. The father of FSheila is also the father of Carla (Netuno). Carla also is twin with Sheila - the mother of FSheila
FSheila	Chiquin	HS, FS, PO	0.4698	-48.90	2.30	0.68	-	0.05	0.3579	0.2211	0.2736	RE	Brothers. The father of FSheila is the father of Chiquinho (Netuno).
FSheila	FMarb	U, HS, FS, PO	0.1865	-47.44	0.45	-	1.13	0.44	0.1202	0	0.2404	First cousin	-
Bela	Poque	PO	0.5	-85.12	18.28	6.23	5.74	-	0.5	0	1	PO	Poque is hybrid (Chapter IV) and father of Bela (she needs to be studied more)
Bela	FXica2	HS	0.2278	-80.87	1.83	-	2.92	9999	0.2255	0	0.451	HS	Half sibling - father (Poque).
Bela	Carla**	U,HS	0	-45.15	2.29	0.89	1.76	-	0	0	0	-	Relationship not detected in COANCESTRY with 27 primers – detected only with 15 primers
Bela	Netuno	U, HS	0.0998	-69.75	-	1.44	4.83	9999	0.0718	0.0718	0	RE	Netuno is grandfather of Bela
Netuno	Sheila	HS, FS, PO	0.5	-45.74	3.35	1.16	1.50	-	0.5	0	1	PO	Netuno is the father of Sheila
Netuno	Carla	HS, FS, PO	0.5	-48.83	2.32	0.65	1.47	-	0.5	0	1	PO	Netuno is the father of Carla
Netuno	Chiquin	PO	0.5	-48.86	3.85	1.39	0.90	-	0.5176	0.0352	0.9648	PO	Netuno is the father of Chiquinho
Netuno	FMarb	U, HS, FS	0.2843	-45.14	0.74	-	1.48	025	0.2377	0.2377	0	RE	-

Individual		**ML-Relate**							**Co-Ancestry**			**Interpretation**	**Observation**
Ind1	**Ind2**	**0.05 R**	**R**	**LnL(R)**	**Delta Ln(L)**				**Rxy**	**Δ_7**	**Δ_8**	**R***	**Obs**
					U	**HS**	**FS**	**PO**					
Netuno	FSheila	FS, PO	0.663	-43.33	6.46	2.73	-	0.11	0.6238	0.3628	0.522	FS	Netuno is the father and grandfather of FSheila. FSheila (stillborn) was high inbreed level
Netuno	Tam	U, HS, FS	0.1348	-49.17	0.03	-	1.68	9999	0.0935	0.0642	0.0586	RE	Netuno is the father of Tamunha (by CERVUS)
Sereno	Sereia	PO	0.5	-49.23	3.98	1.38	1.63	-	0.5	0	1	PO	Sereia is the mother of Sereno
Sereno	Sheila	U, HS, FS, PO	0.508		1.60	0.46	0.38	-	0.4521	0.0422	0.8198	FS	Full sibling.
Sereno	Carla	HS, FS, PO	0.5677	-46.41	3.08	1.10	-	0.14	0.3702	0.3702	0	FS	Full sibling.
Sereno	Chiquin	U, HS, FS, PO	0.2488	-50.57	0.46	-	1.03	0.38	0.2257	0.0414	0.3685	HS	Half sibling - father (Netuno)
Sereno	Tam	U, HS, FS, PO	0.2657	-47.12	0.41	-	1.38	0.41	0.2373	0	0.4747	HS	Half sibling – father (Netuno)
Sereno	FSheila	HS, FS	0.3762	-46.33	1.79	0.71	--	9999	0.3306	0.2138	0.2336	RE	Sereno is uncle and half sibling of FSheila.
Sereno	Netuno	PO	0.5	-48.81	2.70	0.97	2.35	-	0.5	0	1	PO	Netuno is the father of Sereno

**R= 0.3919 for the run with 15 primers with allele frequency from the full population.

The geographic position of each parent-offspring at CMA/ICMBio and the released animal Lua, was compared to better understand the relationships between the manatees previously rescued from the wild. The Table V.5 and the Figures V.2 and V.3 show that information.

Table V.5 - Rescue date, location and coordinates of the rescue site of the stranded calves that are now adults in captivity at CMA/ICMBio and the released manatee named Lua.

Individual	Rescue date	Origin (Local/State)	Geographic location	
			Lat	Long
1 - Xica*	January 1963	Ponta de Pedras/PE	-7.617804°	-34.807386
2 - Sereia	December 1990	Barro Preto/CE	-3.947917°	-38.271187°
3 - Netuno	January 1991	Sagi/RN	-6.464221°	-34.972448
4 - Lua	February 1991	Morro Branco/CE	-4.151611°	-38.113442°
5 - Xuxa*	December 1992	Barra de Sucatinga/CE	-4.252381°	-38.007941°:
6 - Marbela	May 1994	Pipa-Timbaú do Sul/RN	-6.227300°	-35.048972°
7 - Assú	February 2000	Praia Retiro Grande/CE	-4.642210°	-37.533687°
8 - Poque*	1993 – captive	Oiapoque/AP	3.843348°	-51.832279°

* Rescued from captivity, but originaly from a wild population.

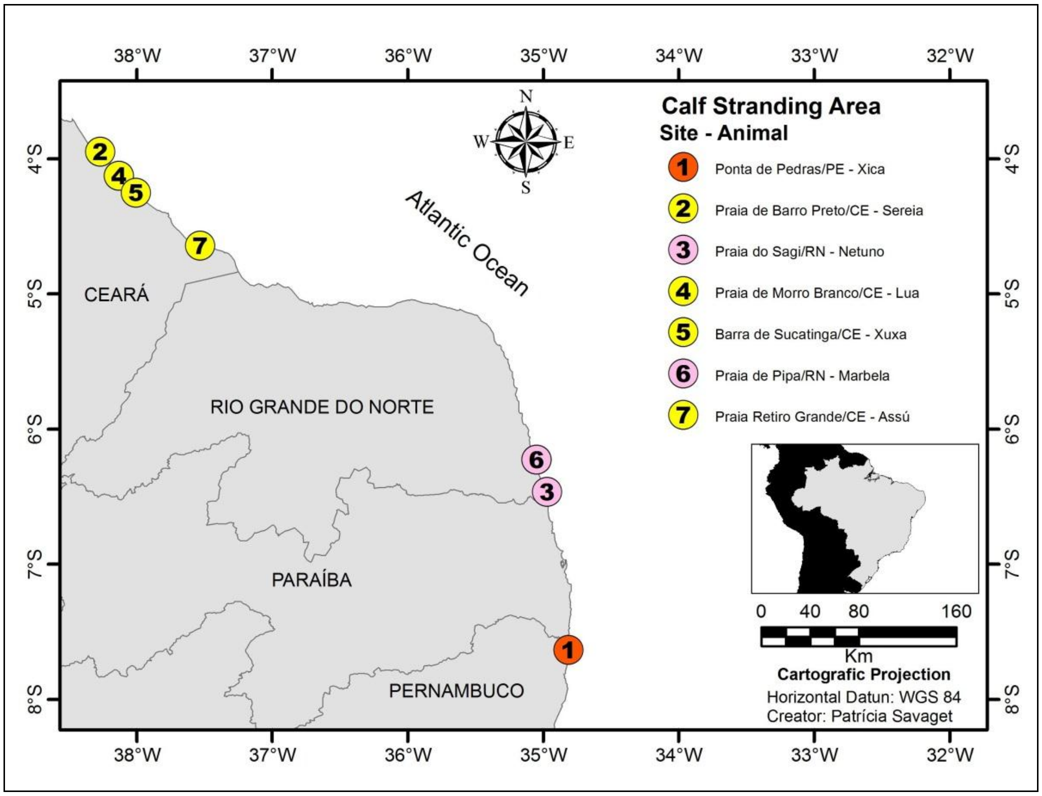


Figure V.2 – Map with the calves’ stranding locations.

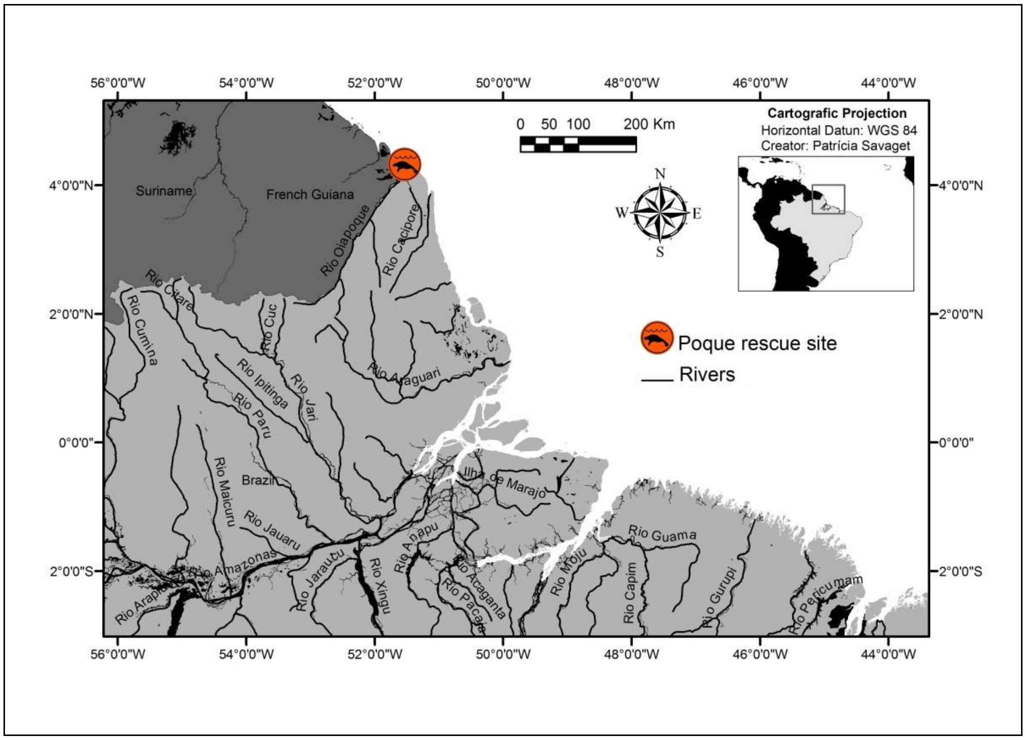


Figure V.3 – “Poque’s” rescue location at Oiapoque, Amapá state.

The results reported by CERVUS, a proposed father for each calf and the management decision recommended in light of this new information or the final outcome, are presented on Table V.6.

Table V.6 - Result of the fathers identified by CERVUS and decision for management.

Offspring ID	Known Mother	Father proposed by CERVUS	Decision for management/final outcome
Sheila	Sereia	Netuno	Not for breeding program at CMA/ICMBio; transfer or release
Carla	Sereia	Netuno	Not for breeding program at CMA/ICMBio; transfer or release
FXica2	Xica	Poque	Stillborn
Chiquinho	Xica	Netuno	Died after released
FMarbela (FMar)	Marbela	Assú	Died
FCarla	Carla	Assú	Died
Tamunha (Tam)	Xuxa	Netuno	Released
FSheila	Sheila	Netuno	Stillborn
FCarla2 (Bela)	Carla	Poque	Not releasable; transfer to another facility
Sereno	Sereia	Netuno	Releasable

V.IV - DISCUSSION

Almost all the manatees in captivity at the CMA/ICMBio indicated a level of relatedness. Some of them have a high level of relatedness, as the number of founders at CMA/ICMBio was small and reproduction between related offspring was allowed to occur. The presence of a dominant, breeding male ("Netuno"), who fathered several calves which resulted in some half sibling relationships in the captive population, greatly increased the relatedness in the captive population.

A few inconsistencies were observed in the pedigree reconstruction using MLRELATE, such as “Xica” being a half sibling with her 2^{nd} calf – HS value (r = 0.3), but MLRELATE also gave the possibility of being PO at the 0.05 significance value. For “Carla” and “Bela” the attributed relationship was HS (Delta Ln(L) = 0.89) with a relatedness value of r = 0, but it is known that they are the actual parent-offspring (PO) as “FCarla” was born in captivity and the birth was observed. Because of the differences of likelihoods observed between MLRELATE and the known true relationship, the software COANCETRY was used to verify the results and to increase accuracy by using different programs for comparison. COANCESTRY reported the relatedness coefficient estimates (rxy) and estimates of Δ_7 and Δ_8 (Table V.4). Potentially, additional microsatellites are needed to add more power to the analyses.

COANCESTRY used the allele frequencies generated from the 20 captive manatees only, for the 34 microsatellite loci (27 usefull) used in this study, as no allele frequency data for the other 18 microsatellite primers were available for the full population. The software was also run with data from 15 microsatellite loci (with allele frequencies generated from the 20 captive animals and from the full population) for comparison. COANCESTRY identified some different values when run with 15 and 27 microsatellite loci. Those differences are probably due to the fact that fewer loci are compared between the individuals when COANCESTRY was run only with 15 primers. It was found that with fewer loci used, fewer comparisons were made between the samples, and the relatedness coefficient value was larger. Nevertheless, the relatedness coefficient value was higher when run for 15 primers with the allele frequencies of the full population than when run with 15 primers only using the 20 animals to generate allele frequencies. Both were slightly higher than when 34 primers (7 monomorphic) were used, which was the smallest value reported by the software.

The animals were compared with each other to detect the presence of likely parent-offspring, full sibling and half sibling relationships. In COANCESTRY, to minimize the risk of inflating the number of animals with artificial relatedness, the data from the run with 34 primers (27 usefull) was chosen for this study, which had the value considered the most conservative with respect to the relatedness between the animals, as the value was the smallest. However, even with different values found in the three different runs, it did not change the relationship proposed between each pair of animals, except for “Carla” and “Bela”, (r = 0 with 34 primers and r = 0.3919 with 15 primers and allele frequencies from the full population) which was also not consistent using MLRELATE. “Bela” is described to be “Poque’s” offspring and needs to be studied more in order to adequately address the hybrid issue between the West Indian manatee and the Amazonian manatee (see Chapter IV).

Another calf was not considered as an offspring of its known mother with the software. However, that may be due to mismatches between the mother and calf genotypes at one or more loci (“Tamunha” and “Xuxa”; r = 0.28). Those mismatches could result in a genotyping error or a new mutation in the calf.

The female “Sereia” gave birth to twins in 1997, and the male “Netuno” was determined to be the father. The twins were females (“Sheila” and “Carla”) and still live in captivity at the CMA/ICMBio facility. Both have also reproduced while in captivity. “Carla” had two live calves born and “Sheila” had one stillborn. “Sereia” also gave birth to “Sereno” (2004) and “Ariel” (2008). At that time, there were three more females (“Xica”, “Xuxa” and “Marbela”) able to reproduce in the same pools, as were three adult males (“Netuno”, “Poque” and “Assú”). Sereno, the male captive born in 2004, was kept with the females until his transfer to Alagoas state in 2010. It is possible that he was reproductively mature prior to his separation from the females, and may have fathered a calf in captivity.

Analyzing the relationship between the 20 manatees, some management decisions should be adopted to avoid increasing the inbreeding potential at CMA/ICMBio and also within the local populations due to the potential impact of captive manatees released into the wild.

To avoid inbreeding in captivity, the CMA/ICMBio is releasing some of the captive born calves as there are not enough pools to adequately maintain separation of related siblings. The area of Porto de Pedras in AL was chosen especially as a potential release site. At this area, there are efforts by CMA/ICMBio to reconnect a separated population in the southern range of the species as was previously suggested in Chapter III.

If captive born manatees (four full siblings consisting of three sisters and one brother) were not released, at least six animals (including their parents with high relatedness would remain in captivity and should not be allowed to breed. Additionally other half sibling captive born calves also reside in the same facility.

If wild animals have close relationships with others *in situ* and if the population is very small, the risk of inbreeding is high. In an *ex-situ* population, despite the effort to avoid inbreeding, separating captive born calves from their parents is the best strategy with some animals removed from captivity and returned to the original wild population. The pedigree reconstruction illustrates that some manatees rescued and brought into CMA/ICMBio were related in the wild (“Sereia” and “Lua” = FS; “Xuxa” and “Assú” = FS; “Sereia” and “Xuxa” = HS; “Lua” and “Xuxa” = HS; “Lua” and “Assú” = HS; “Xica” and “Marbela” = HS). Figure V.2 is a map that shows the area where each animal was originally rescued and it is possible to see that “Sereia”, “Lua” and “Xuxa” are from areas very near to one another. Perhaps they had related parents in the wild. “Marbela” and “Xica” also displayed some relatedness, as they are half siblings and the distance between their original rescue location is larger than the distance between “Sereia”, “Lua” and “Xuxa”, but it is still possible with the ability of manatees to migrate great distances, well over 200km.

If manatees were related in the wild and added to the captive population at CMA/ICMBio or released in Porto de Pedras in AL, they possibly could increase the relationship between them and their offspring (increasing also the inbreeding).

It was identified that several HS relationships exist between offspring and some adults: “Sereia” and “Maraca” (1st calf of “Lua”); “Xica” and “Maraca”, “Marbela” and “Maraca”; “Assú” and “Flua” (2nd calf of “Lua”); “FCarla” and “FLua”; “Tamunha”

(“FXuxa”) and “FLua”; “Tamunha” and “Sereno”; “FXica 2” (2nd calf of “Xica”) and “Bela” (“FCarla 2”); “Chiquinho” (“FXica3”) and “Tamunha”).

“Assú” was a calf rehabilitated and released into the wild, but was lost when he went out into the sea. He was eventually rescued and brought back to the same pool with other manatees. “Assú” bred with “Carla” (U); and “Xuxa” with “Netuno” (U), whose calves (“FCarla” and “Tamunha-FXuxa”) were highly related as FS, and it was known that “Assú” was previously related with “Xuxa” (FS) and “Carla” is calf of “Netuno” (PO). Also “Lua” was strongly related with “Sereia” (FS). “Lua” is an animal that was released and lives in AL state. The calves and grandcalves of “Sereia” (*Sheila; Carla; FSheila; FCarla, Bela*) have a relatedness with “Lua” and her calves (“Maraca” and “FLua”).

As the number of manatees released in the Porto de Pedras (AL) is increasing, the objective is to connect the isolated southern group of manatees with the Pernambuco group in the north, but to do so while avoiding inbreeding with wild animals. It is extremely necessary that the new group produced by the releases is genetically healthy and as unrelated as possible. To avoid a high number of related individuals, the release of sibling manatees needs to occur in multiple areas. In addition, Porto de Pedras (AL) has a limited capacity of space, food, etc., and the release of captive manatees there will be reduced in the near future.

Stranded calves rescued by REMANE and related with animals released at Porto de Pedras (AL) should be released at the original rescue area, as conservation biologists typically recommend releasing animals at or near the site of rescue (Waples and Stagoll, 1997). For the near future the institutions from REMANE, in the manatee calf stranding areas, should be prepared to rehabilitate manatees rescued by their teams, and as will be necessary, release the animals at the original rescue site. By doing this it would logistically be easier than transporting manatees for great distances for rehabilitation prior to re-release, sometimes as far at 500km away. However, in the near future, when the capacity to support more rehabilitated manatees is reduced at Porto de Pedras (AL), there will be a need for an additional facility and possibly another rehabilitation site elsewhere.

The animal “Astro” was released in Alagoas state and migrated to Sergipe state. He has survived for more than 10 years where he resides in an estuary with suitable conditions for other manatees. However, to date, no other manatees have been identified in this area. The release of more manatees in the Sergipe state will rebuild a historical occurrence area and increase the actual range of distribution. Also, the possibility of females breeding with “Astro” can contribute to creating a new group at this site. Currently, “Astro” is alone with no possibility to breed and is not contributing to the future of the wild manatee population. So, it is possible that captive born individuals and one or two adults related with captives at CMA/ICMBio facility could be released at the Sergipe site in order to establish a viable founder group in that area.

One of the twins (either “Sheila” or “Carla”), for example, are animals that could potentially be released with “Astro”, beginning a new group and avoiding any chance to inbreeding at the CMA/ICMBio facility. However, if for any reason they cannot be released, they need to be transferred from CMA/ICMBio to one of the other facilities.

The release or transfer of the captive born manatees, such as the ones released in Alagoas state, is important to guarantee no further occasional or accidental inbreeding, as the parent and their offspring will be totally separated. One of the largest problems to manage the West Indian manatee in Brazil is the great number of calves in rehabilitation which need to be released (Chapter II) and the identification of new release sites as necessary to support the carrying capacity at all the facilities.

Nevertheless, the rehabilitated calves can be a strategy to increase wild populations, to establish a connection between groups or to create a new group in the historical range. However, there are some difficulties with monitoring calves after release into the wild. The expense to pay for satellite radio tracking, biologists and veterinarians to provide monitoring of health and behavior, vehicle use, fuel, etc. is not available in many areas. In Brazil, there are many dependent calves to be released, and to avoid additional breeding in captivity, more efforts should be directed towards release and monitoring strategies.

The captive born manatees two or more years of age must be separated from their parents and other related offspring into different pools. The age at first reproduction for female manatees can occur around 3-4 years of age and male spermatogenesis has been known to start at 2-3 years of age (Hernández et al., 1995; Rathbun et al., 1995).

To avoid overpopulation of captive manatees as has happened in Mexico (Nourisson, 2011) and a high level of inbreeding as in European facilities, captive breeding in Brazil should be prohibited at this time. Currently, the recommendation is to maintain males and females totally separately at CMA/ICMBio for now. In the event wild population numbers severely decline in the future and there are no rescued calves available to release, there may be a need to supplement the population. At that point, reproduction programs could be assessed to determine whether controlled breeding is needed to prevent extinction of the population.

For a future breeding program to be developed some fundamental questions need to be addressed. The first recommendation is to know the pedigree of each manatee in the captive population to avoid inbreeding and to make the best choices for genetic pairings. Table V.3 reports (in yellow) the manatees that are the unrelated (U) that could be used for mate selection based on the genetics. However, the health, age, behavior of each animal and the number of reproduction attempts between each couple needs to be considered.

“Xica” is the oldest captive female manatee at CMA/ICMBio, ~50 years old. She gave birth to the first West Indian manatee calf born in captivity in Brazil in 1996 (“Chiquito”). Afterward, she had 3 more pregnancies, two aborted (“FXica2” in 2002, father = “Poque”; and “FXica4”, in May 2012) and one survived to become a juvenile (“Chiquinho or FXica3”; in 2007; father = “Netuno”) before she was released into the wild at AL state. Unfortunately, the calf, “Chiquinho” did not survive once released into the wild. There is no evidence of reproductive senescence in geriatric manatees and females can reproduce at least until their 40’s. There is a previous known case of a female in captivity having a calf at 43 and 48 years of age (Marmontel, 1995). “Xica” was ~45 years old when she gave birth to “Chiquinho” in 2007.

“Xica” stranded as a dependent calf in Pernambuco state in 1963; she was maintained for a long time in an inappropriate facility, which wasn’t deep enough for her and she developed physical problems with her back. She was transferred to the CMA/ICMBio facilities in the beginning of the 1990’s, when she was around 30 years old. “Xica” is currently a geriatric manatee. It has been confirmed in humans that an increase of genetic disease and mutation is more likely to occur when parents are advanced in age (example, Down’s syndrome). No data are available for manatees with respect to this issue, but it is prudent to practice caution. Therefore, it would be advisable not to use “Xica” in an active breeding program.

“Netuno” is a male that has reproduced with all four females that were founders of the captive population at CMA/ICMBio. However, “Xica” needs to be excluded by her age, “Sereia” already had four calves with “Netuno” and more calves as full siblings should be avoided as an increase in more related individuals would be a burden on the program. “Xuxa” and “Marbela” are the best options and most suitable candidates to successfully breed with “Netuno”, however, caution should be advised to not over breed these pairs, since “Netuno’s” genotype is highly represented in the captive population already. A future program needs to have more than one reproductive male. As “Netuno” already reproduced many times, it’s recommended that he be excluded from continuing to breed in order to avoid further impacts of founder effect and make efforts to try to seek and identify other suitable male candidates.

“Netuno” cannot breed with “Sheila” and “Carla” as their relationship was identified as parent-offspring, and they must be kept separate from him. The recommendation is to transfer “Sheila” and “Carla” to another facility or release the twins, with no more chance of mating with “Netuno”. Also, to control the increase of “Netuno’s” genotype at CMA/ICMBio and avoid inbreeding on that *ex situ* population, “Netuno’s” release or transfer to another facility would be helpful, if possible.

As “Sheila” and “Carla” have a very similar genotype (potential monozygotic twins) and are full sibling (FS) with “Sereno” (mother = “Sereia”; father = “Netuno”) and Half sibling (HS) with “Tamunha” (calf of “Netuno”) both released in AL state, the twins could be released in Sergipe state as discussed above, although release into separate locations is recommended, since they are highly related. It’s very important and necessary to transfer those animals from the CMA facility in Itamaracá in PE state as soon as possible. If other factors do not permit the release of those animals they must be transferred to a facility that is capable of adequately holding manatees in captivity.

“Assú” is a potential reproductive male, as he is unrelated with “Marbela”, “Xica” (not available) and “Sereia”. However he cannot reproduce with “Xuxa”, as they are related from the wild originally. Currently, “Assú” is in a sea pen in Porto de Pedras in AL state waiting to be released into the wild as a strategy of ICMBio. However, in a future reproduction program, if necessary, he can return to captivity to be part of the program if it is decided to be in the best interest for conservation of the species.

“Poque” has unique alleles for the captive population as he is from another geographic area, as was identified in Chapter III. That reason was enough for “Poque” to be allowed to reproduce previously with the population at CMA/ICMBio consisting of manatees from the AL-CE population (Chapter III). However, “Poque” is a hybrid between *T. manatus* and *T. inunguis* (Chapter IV) and therefore should not breed with any female from either species to avoid forced hybridization.

It appears that Poque had successfully bred at least two times with females at CMA/ICMBio. The 2nd calf of Xica was a stillborn and “Poque” was the father. The calf had patches on his ventrum – Figure IV.19 – Chapter IV). As there was a shortage of pool space he was housed with a few manatees in the pool at CMA/ICMBio. Therefore, “Poque” was placed in with some adult females where he got them pregnant. “Poque” also is identified to be the father of the 2nd calf (“Bela”) of “Carla” from this occasion. Vianna and colleagues (2006a) suggested that “Poque” was a F2 hybrid. At the time, it was suspected that “Poque” would be sterile.

With new genetic information on manatee hybridization between Amazonian and West Indian manatees, the recommendation is to immediately separate “Poque” from all adult females and not allow him to breed or be released. Additionally, more studies using extra genetic markers and new software programs are necessary to provide more information about “Bela”, identified as “Poque’s” calf. She apparently is healthy and interacting with the other manatees, and has typical *T. manatus* chromosomes (number = 48; Chapter IV). “Bela” does not appear to have any physiological problems, but the decision if she can be released or bred needs to be taken into consideration only after some specific studies are conducted on her. For now, she must be separated from mature males. She is a potential animal that can help the dependent calves during their rehabilitation, or could be transferred to another facility to avoid breeding which would undoubtedly lead to increased inbreeding and forced hybridization. Additionally, “Bela” is an F2 captive born and has a high relationship with the other manatees housed at CMA/ICMBio (parents-offspring, grandmother and grandfather and other half siblings).

The mother of “Bela”, called “Carla”, is one of the twins that were captive born and gave birth to another animal (“1st calf of Carla” or “FCarla”) with the father “Assú” that appeared to be compatible and genetically cleared for breeding. The other twin, “Sheila”, had one stillborn (“FSheila” - stillborn) in September of 2008. The stillborn was attributed to “Netuno” and “Sereno” as a full sibling by MLRELATE. “Netuno” is the father (PO) of “Sheila” and “Sereno”, which are full siblings. Analyzing the difference of likelihood between the proposed relationship and PO are really small (0.11) for “Netuno”, who was identified as the father of “Sheila’s” calf by CERVUS. The program MLRELATE also identified that it is not possible for “Sereno” to be the father as it reported a value of 9999 (which indicates that it is not possible because of the probability that one of her loci does not have any of his alleles).

“FSheila” is a product of one reproduction between manatees with a high relatedness and very close relationship between her parents, as the mother and the father are calf and parent offspring. It is a case of high-related inbreeding, and it may be the reason that “Sheila” had the stillborn. Some species have the ability to avoid inbreeding, but they generally lose that ability when they are held in confinement during captivity.

"Xuxa" had a calf ("Tam-FXuxa"), which the father identified by CERVUS as "Netuno". She also had another calf in April 2012 ("Luiz Gonzaga" - not included on this study). "Marbela" had a calf in December 2007, but the animal died and "Assú" was the father identified by CERVUS.

"Lua" was a young female manatee released in the wild in 1994 by CMA/ICMBio after rehabilitation. Nine years after release she gave birth at Maracá Beach, in Pernambuco state. "Lua" and the calf were monitored as it was the first released female known to give birth in the wild. However, the calf disappeared and was thought to have stranded like "Lua". A dead carcass was found a short time later near the location where she was residing (Lima et al., 2005). Some years later in 2009, she had a second calf ("FLua"), but this one also disappeared and another calf carcass was found near her position again. The calf carcass was recovered by CMA/ICMBio. In 2012 she has been observed with a 3rd calf, which is likely healthy; the calf has been with her more than 6 months and it appears that "Lua" may have learned how to successfully care for her calf. Maternal instinct in manatees is not known but suspected to be similar with most mammals, where the first time mothers do not have the experience to properly care for their calves. After gaining experience, mothers get more proficient at rearing their offspring (Reep and Bonde, 2006).

It was assumed that the two calf carcasses were both calves of "Lua". In fact, genetic analyses with the determination of pedigree could confirm this supposition. The two calf samples were run against Lua and MLRELATE attributed PO between "Lua" and the two calves. The softwares CERVUS and COANCESTRY also calculated that "Lua" was the mother of the two calves.

"Sereia" and "Lua" were highly related (FS). "Sereia" stranded in Barro Preto in CE in December of 1990 and "Lua" in Morro Branco in February of 1991. Those two females could be the calves of the same father, or both mother and father were related.

Also, there is a low possibility that they can be fraternal twins, where in this case one calf was first stranded and less than 3 months later the other calf also stranded (Table V.5).

The animals “Xuxa” and “Assú” were also thought to be related. “Xuxa” was in captivity a long time before “Assú” was born and stranded in the wild (Table V.5). However, it is possible that they have related parents as the area of recovery was near to each other.

V.V - CONCLUSIONS

The reproduction between parent-offspring and their calves was found in one case, but the mother had had an abortion of the 2nd generation calf (stillborn), that could have been a natural way to avoid a high level of inbreeding.

Relatedness between almost all captive manatees and some of the released manatees were found. It is a concern as there is a high possibility in the future for successful reproduction between FS and HS to occur. It is necessary to separate these individuals immediately at CMA/ICMBio facility to avoid any chance of inbreeding.

All manatees that are releasable into the wild should be released to avoid a large captive population, thus promoting inbreeding in captivity. The animals from captivity (adult and captive born) that have some relatedness with others released in AL state should be released into a different area, such as Sergipe.

Some of the captive manatees that cannot be released for any reason and have relatedness issues with others must be transferred to other facilities to avoid any possibility of inbreeding in the future.

Many stranded rehabilitated calves have been released in Porto de Pedras/AL. However, related wild manatees should be released to their original rescue site if possible, thereby avoiding the potential for encouraging an artificial inbreeding group by releasing non-related individuals.

As there are already many rehabilitating calves that need to be rehabilitated and released, it is not necessary to start a captive breeding program at this time. Doing so, would only contribute more manatees to the already crowded system, and require additional release and monitoring effort in the future. Further, captive-born manatees have been shown to not be as successful as wild born manatees in Florida. Additional effort and money would be more useful if used for releasing the current captive manatee population and performing research to advance the knowledge regarding conservation of the species and designation of critical habitat. Efforts should be especially focused on improving habitat to reduce the number of stranding calves.

In the future, if the manatee wild population in Brazil decreased to a critical level, or the connection between groups is compromised leading to isolation and possible local extinction, then a reproductive program in captivity may be able to be integrated into management efforts to aid and supplement existing populations.

To date, it is necessary to immediately separate males from females in order to ensure that occasional or accidental breeding in captivity at CMA/ICMBio is stopped. Manatees breed easily in captivity, which soon leads to overcrowding, inbreeding and an unhealthy population.

The pedigree reconstruction using genetics can detect which manatees are related, and determine the extent of relatedness. This alone will be useful information to assist future management strategies and planning. More research is needed to understand the complexities of pedigree efficiency and the deleterious costs of excessive inbreeding. Similar studies are being conducted on Florida manatees at the University of Florida and USGS Sirenia Project (M. Davis, personal communication) and on Mexican manatees at ECOSUR and GEOMARE (C. Nourisson, personal communication).

Chapter VI – General Discussion and Conclusions

According to Bonde (2009), understanding the relationship among manatee genetics and manatee behavioral responses and adaptive capabilities will help managers direct the recovery of the species. This thesis is a product of an effort to have the scientific knowledge necessary to suggest management measures for conservation of the endangered *Trichechus manatus* in Brazil. The major part of this research effort focuses on a detailed genetic investigation to understand genetic diversity, population structure and the current genetic state of the *T. manatus* along the coast of Brazil, with suggestions to help the conservation of the species.

Frankham and colleagues (2002) suggested that applying genetic concepts should be a priority of conservation biology. His first two priorities were to establish genetic management of wild species at risk, especially for fragmented populations, and manage captive populations of endangered species, to minimize loss of genetic diversity and genetic adaptation in captivity.

This thesis addresses genetic diversity of the *T. manatus* in Brazil using both mtDNA and nDNA genetic markers and cytogenetic applications. Four general subjects have been investigated in this present study: *i)* the mitochondrial genetic diversity in the West Indian manatee (*T. manatus*) in northeastern Brazil (Chapter II); *ii)* the nuclear genetic diversity and population structure of the West Indian manatee (*T. manatus*) along their current range in Brazil (Chapter III); *iii)* the occurrence of hybridization between the *Trichechus manatus* and *Trichechus inunguis* (Chapter IV); and *iv)* the individual relatedness and occurrence of inbreeding of manatees in captivity at CMA/ICMBio (Chapter V).

The following discussion will examine and compare the findings for the different genetic investigations that focused on Brazilian manatees along the coast of the country. This discussion will also present suggestions outlined in previous chapters and suggest management actions that may help to improve difficult conservation efforts to protect this endangered species.

VI.I - Manatee dependent calves and genetic decision to release them

In northeastern Brazil, births of calves have been occurring in challenging environments, instead of calm mangrove areas typically preferred by manatees. This is likely due to the environmental degradation and destruction of the manatee habitat along the coast. The area is heavily occupied by humans and is under pressure of anthropogenic threats, such as oil exploration, no regulations to safely use fishing nets to prevent entanglement, strong tourism seasons with high increases of boats and people, activities heavily using the mangrove areas for aquaculture farming and the impacts of the sugar cane industry located near the coast.

Manatees prefer quiet, protected and calm embayments, which are typically not highly used by humans. The coast along the east of Ceará and west of Rio Grande do Norte states has high energy beaches, with waves and strong currents. These dynamic areas are less adequate for manatees that, through necessity, had to adapt a different behavior strategy to survive. Unfortunately, young calves do not have this ability and therefore suffer the consequences by often stranding along the beaches. It is believed that a large number of young calves get separated from their mothers during their first weeks of life and end up stranding on these beaches unable to get back out into the sea. This is a serious problem for their survival and thus for the conservation of manatees in general.

To minimize the impacts on the population, many of these calves have been rescued alive by a Brazilian Northeast Stranding Network (REMANE) and rehabilitated at CMA/ICMBio facilities for future release. During the early years of the program, the objective of those released manatees was to return the animals to the wild. However, the previous identification of release sites did not take into account the genetic consequences of translocating rehabilitated individuals back into the wild. The first hypothesis of the study was true for H_0, since it was found that there are phylogeographic implications that influence the choice of the release site for rehabilitated manatee calves in Brazil. Through this study, phylogeographic analyses resulting in specific identification of genetic haplotypes along the northeastern coast of Brazil, allow the management team to indicate release sites based on genetic similarity.

This study encourages the release of manatees in the north of Alagoas state and/or south of Pernambuco state, which is necessary for providing a travel corridor that will promote the connection between the manatees populations from Pernambuco with the populations located in Alagoas. This marks the actual southern limit of West Indian manatee range in Brazil. It is important not to repeat the extinction process that has already occurred in Espírito Santo (ES), Bahia (BA) and Sergipe (SE) states (Albuquerque and Marcolvaldi, 1982; Lima, 1997). Additionally, increasing the range and providing more habitat for use by manatees, will allow for the population to grow and reduce the chance of extinction of the species.

Therefore, manatees released in those currently extinct states can promote the recolonization of the southern historical range. An example of the possibility of recolonization occurred in Sergipe state, where one manatee named “Astro”, was released in Alagoas state and migrated over 350km south to Mosqueiro in Sergipe state. The released manatee still lives there after 14 years (Foppel and Ferrari, 2012). This new area, for manatees, was discovered and utilized by “Astro” and is defined as suitable and appropriate for manatees. It promotes survival of released manatees with ample needs, such as food, freshwater, limited boat traffic, calm water and the absence of heavy use by humans.

Nevertheless, only one manatee moved into this new area which would not lead to a recolonization unless other manatees locate to this area. As this region is in the historical manatee range and “Astro” confirmed its actual potential for providing suitable habitat, it could be utilized and encouraged by releasing more manatees into this area to create a population. Therefore, it is recommended that CMA/ICMBio release some of their genetically fit captive born or rehabilitated manatees into this area to encourage population stability.

The release of genetically divergent individuals can result in the genetic swamping of local populations and can eventually cause the loss of locally adapted alleles or genotypes (García-Ramos and Kirkpatrick, 1997; Lenormand, 2002; Frankham et al., 2002). According to Allendorf and Luikart (2007), efforts should be made to release individuals into locations with the same genetic haplotype signatures. In Maranhão state, the haplotypes have been identified as M03 and M04, and therefore the calves rehabilitated from this area should be released back into this state as well. The release of those manatees into other southern states should not be allowed as that would result in genetic swamping and could have deleterious effects on the resident population.

Swamping of local gene pools can also occur when individuals from multiple source populations have interbred and their offspring are released into areas with locally adapted individuals (Allendorf and Luikart, 2007). Therefore, manatee calves rehabilitated or captive born should not be released into Paraíba state since the population is small and has low genetic diversity. Only manatees that originated from areas near the release site should be considered for release there.

Animals released in non-natal locations (genetically or environmentally) may not be well adapted to the local habitat, vegetation, diseases or seasonal changes (Frankham et al., 2002). In this regard, even considering the importance of releasing manatees to connect populations and recolonization areas of historical occurrence, the release of individuals into the rescue site of origin still remains the most appropriate action for ensuring population stability.

VI.II - Population structure, genetic diversity and strategies for conservation of manatees on the coast of Brazil

Multiple genetic programs were used in this study to assign manatees with high nuclear DNA similarity to populations and address the population genetic structure of the West Indian manatee on the coast of Brazil. Analyzing manatee samples from the entire coast of Brazil, with additional samples collected from Guyana and Venezuela, the manatees was first divided in three populations: *i*) Northeast coast of Brazil (NEB); *ii*) Northwest of the Amazon River estuary (NWA), composed by Amapá state from Brazil plus Guyana and Venezuela; and *iii*) Estuary of the Amazon River (EAR), which includes all the manatees sampled in the mouth of Amazon River.

In a subsequent fine-scale analysis of the NEB, there was find genetic differentiation and two subpopulations were identified by STRUCTURE: one subpopulation consisting of individuals that occur in the Ceará to Alagoas states and the other subpopulation composed of individuals in the states of Maranhão and Piauí. The second hypothesis of the study was true for H_0, since it was found that there are different groups of *Trichechus manatus* along the current range in Brazil. A total of four manatee groups were delimited by STRUCTURE, with four management units for the coast of Brazil.

Small sample sizes were used for the PI-MA samples and should be increased in the future for better genetic resolution. The fine-scale study of the EAR identified the manatees from the mouth of the Amazon River as Amazonian manatees. These samples clustered with Amazonian manatees sampled from the Santarém region located about 1,000km upstream of the mouth of the river. This confirms that the manatees found by CMA/ICMBio and IBAMA in the estuary or near the sea, are truly Amazonian manatees as has been described morphologically.

Nevertheless, no hybrids were found among those manatees sampled, but only a few samples were available for this study. More samples from this area, including manatees with morphological characters attributed to both *T. inunguis* and *T. manatus*, should be collected. The presence of hybridization is discussed in section VI.3 of this chapter, as well as in Chapter IV.

The low level of genetic diversity found for manatees from Brazil can increase the extinction risk since diversity is considered necessary for adaptation to diseases and environmental changes (Reusch and Wood, 2007). The limited genetic diversity in Brazil is a combination of a founder effect (as the population defines the southern limit of the range of the West Indian manatee), long-term anthropogenic impacts, such as commercial hunting since the sixteenth century, other anthropogenic impacts mostly with the use and degradation of their habitat, and intrinsic impacts influenced by the small population size (less than 1,000 individuals).

Even with the decrease in the abundance of manatees due to hunting during colonization and earlier in the 1900's (Luna and Passavante, 2010), no evidence of a bottleneck has been detected. However, this is of concern as manatees in Brazil have likely gone through a series of bottleneck events, according to population size records. The bottleneck was not detected by the genetic software likely due to the manatee's low reproduction rate, with only five new manatee generations occurring since the high level of hunting was decreased. The actual anthropogenic impacts have resulted in serious problems, such as stranding of dependent calves (from 1989 to February 2011, N=67) that occurred along the northeast coast, mostly in Ceará (CE) and Rio Grande do Norte (RN) states, which are areas that will require special protection in order to mitigate the impacts on manatees.

Both manatee subpopulations (AL-CE and PI-MA) in the northeastern of Brazil have very little mitochondrial and nuclear genetic diversity, and as they also have a small population size, they are very sensitive to stochastic events which will result in less potential adaptive response than that provided by a larger population. Although most studied manatee populations are not impacted by disease due to an efficient immune system, lack of heterozygosity could result in individuals with immune depression or lower reproduction rates (Bonde et al., 2004).

The West Indian manatee population structure found in Brazil identified at least two genetically distinct populations, with one divided into two subpopulations. The lack of gene flow through emigration is of concern in the event of continued loss of individuals. Studies initiated to learn more about manatee dispersal using telemetry will allow researchers to monitor the actual dispersal rates of manatees between populations.

As West Indian manatees require food, freshwater and refuge areas for rest and parturition along coastal habitats, it is necessary to implement strategies for their conservation and habitat needs in Brazil. It is also necessary to devise different management plans and strategies to ensure the conservation of the species based on their genetic relatedness and habitat protection. The subpopulation in AL-CE has less mtDNA genetic diversity than PI-MA, but has similar levels of nDNA diversity. Two populations (NWA and EAR) and one subpopulation (PI-MA) occupy similar habitats that are more preserved and therefore have less human impact.

Hunting is still occurring and rapid development of the country is increasing as people move along the coast. In areas with a preserved habitat and no hunting pressure, long-term protection of the manatee population should be encouraged to help increase genetic diversity through mutation and gene flow. The conservation plans must guarantee the end of hunting and provide appropriate protection of suitable habitat through the issuance by land managers of permits or licenses for use of resources along the coast.

The habitat for the manatee from AL-CE is highly degraded, with an intense human occupation of the coastline and with increased anthropogenic activities, such as, housing, tourism, sugar cane farming, salt mining, aquaculture, fishing, oil extraction, etc. Many of the historical estuaries do not exist anymore, and the remaining ones are not available to manatees, so use in this area is highly compromised. This region consists of poor habitat and low manatee genetic diversity (just one mtDNA haplotype). Therefore, the survival of the subpopulation is more challenged and, more immediate and effective conservation action is urgently needed. The conservation plan should contemplate the recuperation of manatee habitat with mangrove recovery (especially in CE and RN, where calves are stranding), regulation of fishing activities (as manatees have been known to entangle and die in fishing nets), reduction of pollution in rivers (where they have been documented to have chemical pollutants from industries, agriculture and sewage waste), and guaranteeing travel corridors between the manatee population groups to discourage the continued fragmentation of critical habitat.

The manatee's habitat in PI-MA is geographically situated between the north and northeastern regions of Brazil. This study enables us to identify this area as having some genetic connection with two other West Indian manatee clusters. However, the migration of individuals from other populations appears limited. Therefore, with evidence of a fragmented manatee distribution (like Alagoas - Pernambuco), or local manatee extinction (like in Sergipe), the introduction of manatees into those areas might be a good solution to provide connectivity between the fragmented groups and result in an increase in local genetic diversity.

It is extremely strategic and necessary to reduce the extinction risk for manatees by developing and implementing Marine Protected Areas (MPAs) that identify important manatee habitats (Table VI.1, Figure VI.1; Paludo and Langguth, 2002; Luna et al., 2008b and c; Luna and Passavante, 2010; Silva et al., 2011; Luna et al., 2011) and provide regulation of the use of those areas with travel corridors that could reduce anthropogenic threats for the species through enforcement.

Table VI.1 – Strategic regions to be considered for protection of important habitats for manatees. Listed by state, geographic region, genetic population and location.

State	Geographic Region	Genetic population	Location
AL and PE	Northeast	(AL-CE)	Costa dos Corais Protected Area
PE	Northeast	Travel corridors	From Costa dos Corais Protect Are ato Resex Acaú-Goiana
PE	Northeast	(AL-CE)	Resex Acaú – Goiana region
PB	Northeast	(AL-CE)	Barra de Mamanguape Protected Area
RN	Northeast	(AL-CE)	Sagi beach region
RN	Northeast	(AL-CE) – travel corridors	From Sagi beach to Touros/RN
RN and CE	Northeast	(AL-CE)	From Touros/RN to Aquiraz/CE (very high number of dependent calves stranding and of bycatch of manatee)
PI	Northeast	(PI-MA)	Cajueiro da Praia region
PI and MA	Northeast	Travel corridors	From Cajueiro da Praia/PI to Ilha do Gato/MA
MA	Northeast	(PI-MA)	Ilha do Gato region/Guarapiranga (Tubarão Bay)
MA	Northeast	(PI-MA)	Guarapiranga/Raposa (São Luís Island)
MA	Northeast	(PI-MA)	Alcântara; Resex de Cururupú and Carutapera region (recesses of MA).
PA	North	(PI-MA)	Viseu, Bragança, Algodoal; Maracanã regions (recesses of PA).
PA	North	Estuary of the Amazon River (EAR)	Resex de Soure and Salvaterra (Ilha de Marajó - mouth of Amazon River)
PA	North	Estuary of the Amazon River (EAR)	Vigia, Colares, Mosqueiro to Abaetetuba (mainland - mouth of Amazon River)
AP	North	Northwest of the Amazon River estuary (NWA)	From Sucurijú to Oiapoque (including Parna Cabo Orange and Esec Maracá-Jipioca)

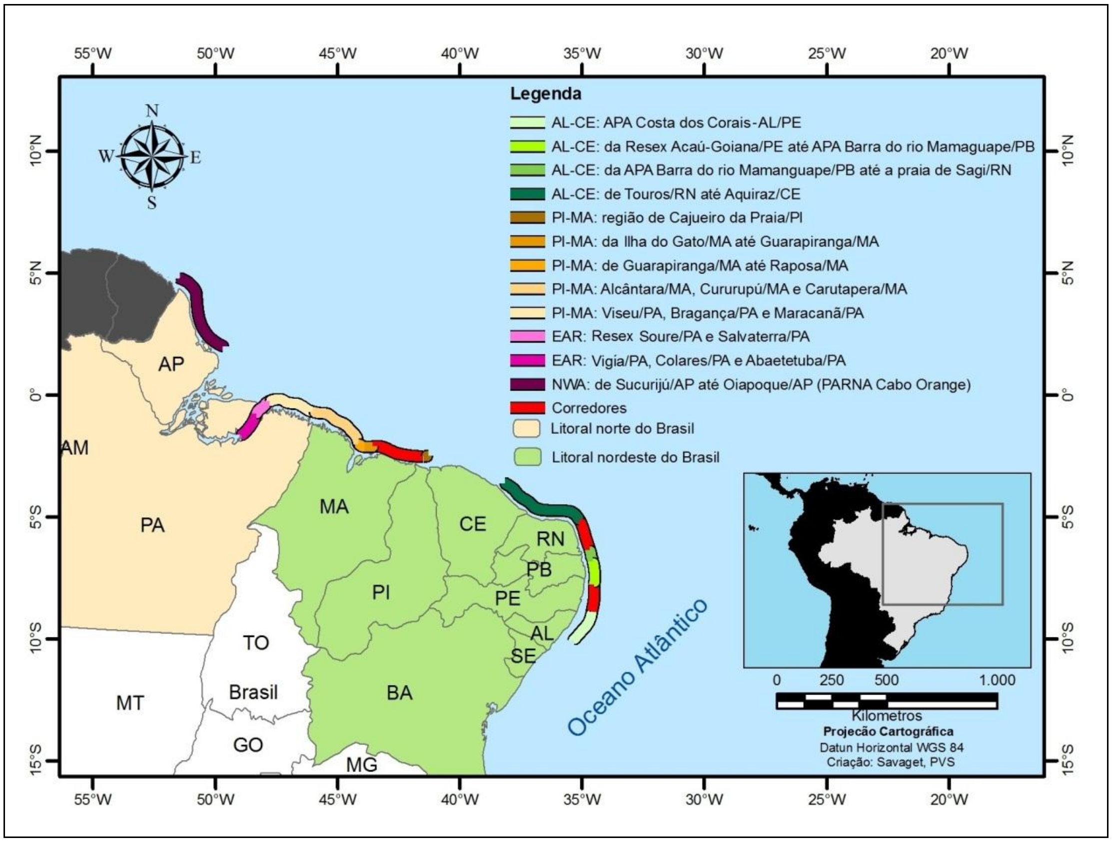


Figure VI.1 – Strategic regions along the Brazilian coast to be protected, recognized as important critical habitats and identifying needs for connectivity by providing travel corridors for manatees.

VI.III - Hybridization between *T. manatus* and *T. inunguis* at the Estuary of the Amazon River and implications for their conservation

The region of the Estuary of the Amazon River, specifically the Marajó Island region, is defined as a sympatric area for the *Trichechus manatus* and *Trichechus inunguis*, and is identified potentially as a hybridization zone between the two species. The present study, using a combination of molecular (mtDNA and nDNA) and cytogenetic analyses has identified the existence of a hybrid individual, however he was not from the previously classified sympatric area. The manatee is from Oiapoque city in AP state, in the Northwest of the Amazon River estuary (NWA), along the border with French Guiana. He was brought into captivity and illegally held captive in a private pool.

The third hypothesis of the study was true for H_0, since the occurrence of a hybrid between West Indian and Amazonian manatees in Brazil was found; The presence of this hybrid caught from the wild population indicates that the potential hybridization zone is larger than suggested before and range from the Estuary of the Amazon River (EAR) to Amapá or farther, even to French Guiana, on the Northwest of the Amazon River estuary (NWA). This is an area where the freshwater of the Amazon River mixes with the seawater of the Atlantic Ocean.

The hybridization is a concern as both species are at risk of extinction. However, hybridization does not appear to be frequent, since only one individual has been implicated to date to be a hybrid, and there is no record in the region of a large number of stranded calves or aborted fetuses which could be derived from processes of miscarriages one would expect of hybrids. More investigation is warranted on the subject of potential hybridization and what managers can do to control the frequency of hybridizing between species. A high occurrence of hybrids could have a detrimental impact on the survival of both species.

VI.IV - Captive manatee inbreeding and recommendation about *ex-situ* reproduction

The pedigree study presented in Chapter V provided valuable information about the degree of relatedness of manatees in captivity at the CMA/ICMBio facilities. The 34 microsatellite markers available for analyses of nuclear DNA allowed the reconstruction of a pedigree of the majority of captive West Indian manatees in Brazil. However, despite the high resolution of the microsatellites for the identification of manatees, the pedigree software and the microsatellites used were not sufficient to determine precise pedigree relationships of all captive manatees. To obtain a higher confidence level for manatee pedigree information, some new investigations need to be employed, like the creation of new and more precise pedigree software. However, general relatedness, as well as some specific relationships, was identified between captive manatees. It was possible to achieve the objectives of the identification of the occurrence of inbreeding in the facilities resulting from mating that was previously allowed.

Hypothesis 4 of the study was true for H_0, since inbreeding among manatees in captivity at CMA/ICMBio was identified. The results of this study enabled to suggest the immediate prohibition of manatee reproduction in captivity. This requires that the institution of CMA/ICMBio does not continue to allow breeding in captivity in order to maintain a healthy captive population. To avoid more inbreeding, artificial hybridization and overcrowding in captivity, it is necessary to separate males and females at all the facilities.

The actual captive population would be too large to manage properly if no captive born manatees were released into the wild. Therefore, it is necessary to reduce the number of captive manatees at the CMA/ICMBio facility and it is recommended that appropriate captive born and adult manatees continue to be released.

In the future, a captive breeding program should be initiated only if some wild manatee groups are at risk of extinction and have little or no reproductive capability in the wild. In that case, no inbred manatee calves should be included in the program and care should be taken to ensure that the best breeding pairs are selected. In this hypothetic captive breeding program it is highly recommended that the selection of mates be based on the degree of relationship of the manatees. The information from this study can be used for that purpose and all future rescued manatees should be genotyped upon entry into captivity. However, as some of the existing captive manatees have already bred several times, leading to various levels of relatedness between the offspring, they should be allowed to reproduce again only if absolutely necessary. If the establishment of a manatee captive breeding program becomes necessary in the future, it may be necessary to bring in unrelated breeders to avoid inbreeding. Nevertheless, a reproduction program in captivity is not recommended at this time unless there is evidence that the wild population has stopped breeding or is in severe threat of extinction.

VI.V - General conclusions

The population structure along the Brazil coast contains two manatee species, *T. manatus* e *T. inunguis*; hybridization in an area from Marajó Island, in Pará state, to the coast of Amapá state. For *T. manatus* there are historic range, and recolonization area in Alagoas state, where dependent calves rehabilitated have been released by CMA/ICMBio, wich also has proceeded with releases in Paraíba state (Figure VI.2).

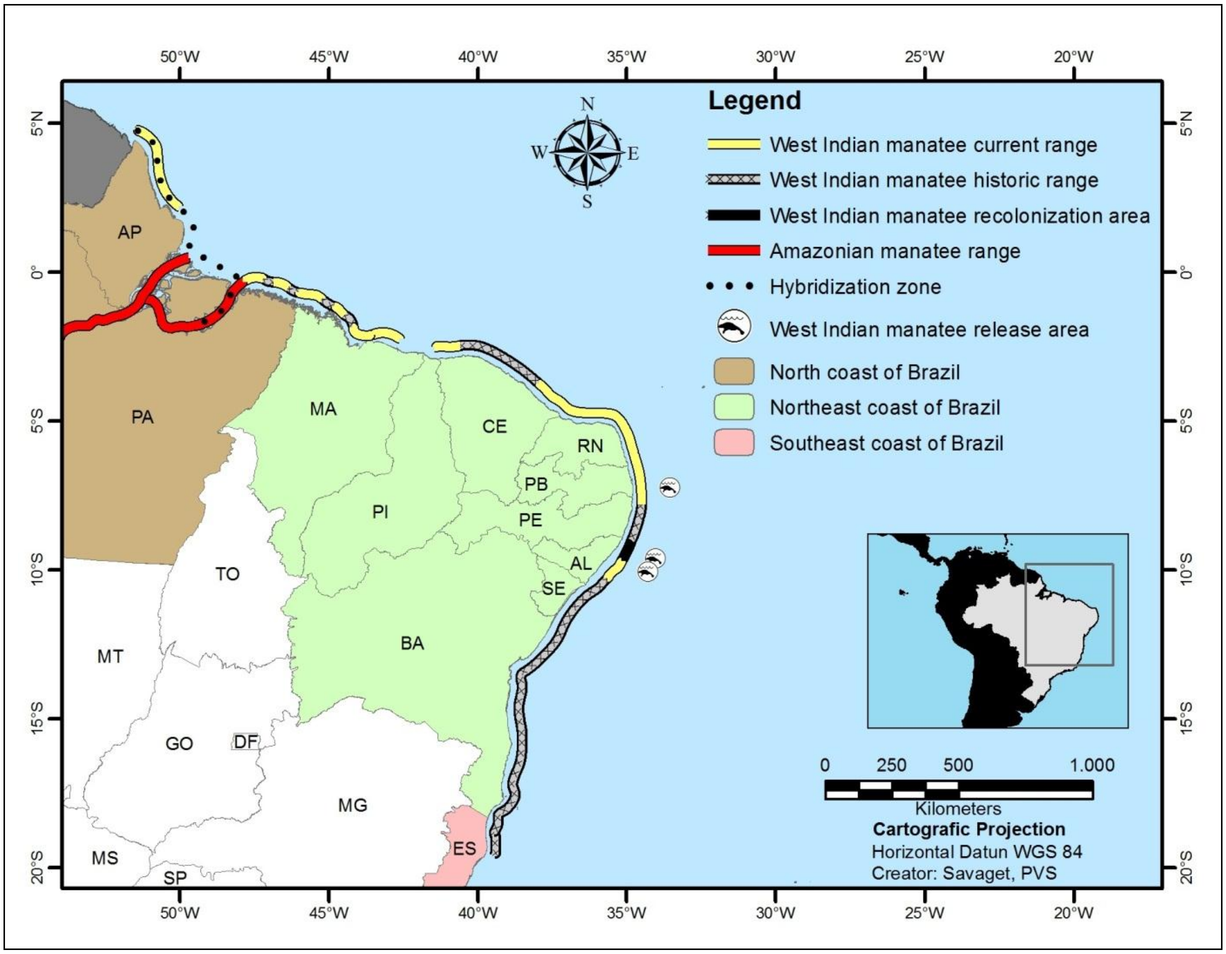


Figure VI. 2 - Manatees on the coast of Brazil, areas used by CMA/ICMBio to release *Trichechus manatus*, West Indian manatee (*T. manatus*) recolonization area, potential hybridization zone between *T.manatus* and *T. inunguis*.

A total of four management units were identified. Within the West Indian species there are three management units, with two genetically differentiated populations: Northwest of Amazon River estuary (NWA) and Northeast coast of Brazil (NEB). The NEB was subdivided further into two different subpopulations (AL-CE and PI-MA; Figure VI.2). The fourth management unit called Estuary of Amazon River (EAR), mainly composed of the amazonian species. In general, all populations and groups have a small size and low genetic diversity. Those facts alone increase their susceptibility to rapid population decline and possible local extinction. Small sample sizes were used in some cases, therefore, additional samples are needed in the future

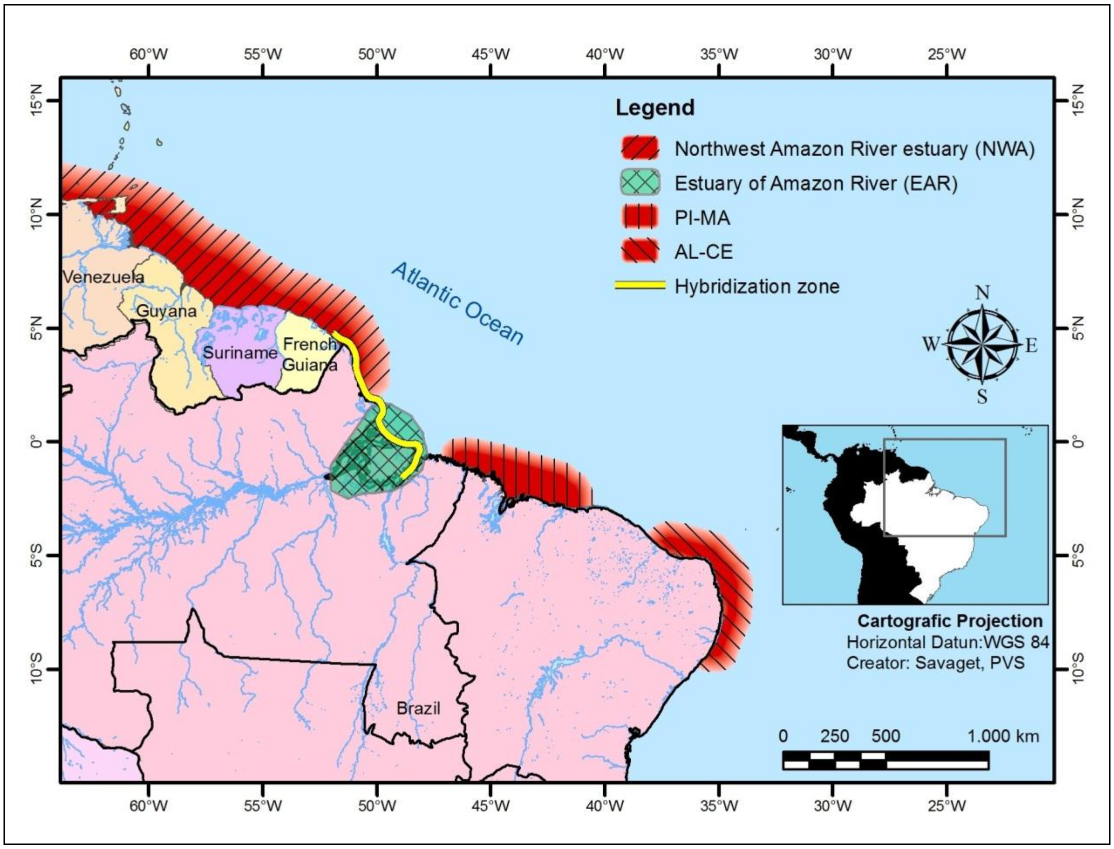


Figure VI.3 – Four management units, two manatee species and potential hybridization zone identified.

To help guarantee the sustainability of the genetically divergent populations found in Brazil (AL-CE, PI-MA; NWA), all with different threats in each region, each management unit should be treated specifically, and the preparation of actions apart is the most appropriate strategy to ensure their conservation in the country. Moreover, providing travel corridors is important because it could encourage natural gene flow allowing for the occurrence of genetic mixing and increasing diversity. Preserving populations genetically may help to protect the species as a whole against the future loss of diversity and thereby reduce the risk of inbreeding and extinction.

The individuals from Alagoas to Ceará states have only one haplotype (M01). As a population with low genetic diversity has a low capacity to adapt, that low diversity will increase the risk of extinction of the manatees in all those states, especially in case of a stochastic or catastrophic events like a red tide, an epizootic outbreak, climate change, sea level rise, hurricanes, etc. Therefore, it is very important to reduce the threats to manatees, especially in some of these regions identified as strongholds or source populations.

The release of successfully rehabilitated stranded dependent calves in the original area should be a priority, but they also can be released in Alagoas and Sergipe states in areas where there are no manatees currently. That will allow for a connection of the Alagoas state individuals with individuals from Pernambuco state, since the release in Porto de Pedras/AL has resulted in recolonization in the region,belonging to the cluster AL-CE, as well as allow for supplemental reintroduction of manatees into Sergipe state, where the species has been recorded as extinct.

However, because of the high number of stranded dependent calves, especially at Ceará (CE) and Rio Grande do Norte (RN) states, it is necessary to have at least one rehabilitation center in this area to accommodate these individuals. This will lessen the demands of logistics and transportation of successful candidates selected for release into this area, as they will be housed and prepared for release closer to their origin of rescue.

It is necessary to acquire more biological information about the manatees in Pará (PA) and Amapá (AP), but also from Piauí (PI) and Maranhão (MA) states. The assessment of wild manatees, through capture and release health assessments, is very important and should be encouraged as this is a great tool for monitoring the health and genetic fitness of the species in Brazil.

The existence of hybrids in the wild has been identified, but additional studies are necessary to delimit the actual hybrid zone and understand the issues of hybridization with respect to the conservation of both species of manatees. This information will guide management actions to prevent genetic sympatric degradation between the two species.

Inbreeding was detected in captive manatees. Male and female manatees must be separated at CMA/ICMBio, and to avoid more inbreeding this captive population, must be prohibited from breeding. Reproduction in captivity shall be approved in the future only if the number of calves born in the wild is inbred and known to be decreasing to low levels and the population would benefit from recruits supplemented by the captive manatee population. However, this program should only be necessary as a final resort to save a local population.

Cooperation of legislation, education and enforcement entities are needed to ensure the sustainability of manatee populations for the future. Enforcement of laws and increasing protections in the impacted regions of Brazil could reduce the number and the impact of anthropogenic deaths.

In summary, manatee populations must be protected to increase genetic diversity, which is correlated with the number of individuals and the general health of the population. Genetic diversity within these populations should be monitored to detect changes through time. Population viability depends of the quality of the habitat, and the reduction of anthropogenic threats is necessary to ensure the sustainability of manatee populations into the future. The implementation of the National Action Plan for Sirenia in Brazil shall be one of the tools and should be a priority for developing government strategies necessary for the protection of this fragile endangered species.

Capítulo VI – Discussão Geral e Conclusões (Português)

De acordo com Bonde (2009), compreender a relação entre a genética, as respostas comportamentais e as capacidades de adaptação dos peixes-bois ajudará os gestores a recuperar a espécie. Esta tese é produto do empenho em se obter o conhecimento científico necessário para sugerir medidas de gestão para conservação do peixe-boi (*Trichechus manatus*) ameaçado no Brasil. A maior parte do esforço da pesquisa teve como foco uma detalhada investigação genética para entender a diversidade genética, a estrutura da população e o corrente estado genético do peixe-boi ao longo da costa do Brasil, com sugestões para ajudar na conservação da espécie.

Frankham e colaboradores (2002) sugeriram que a aplicação de conceitos genéticos deve ser uma prioridade da biologia da conservação. Suas duas primeiras prioridades eram estabelecer manejo genético de espécies naturais em risco, especialmente das populações fragmentadas, e manejar populações cativas de espécies ameaçadas de extinção, para minimizar a perda da diversidade genética e a adaptação genética em cativeiro.

Esta tese descreve a diversidade genética do peixe-boi (*T. manatus*) no Brasil baseada na aplicação de marcadores genéticos de DNA mitocondrial e de DNA nuclear, bem como na citogenética. Quatro assuntos gerais foram investigados no presente estudo: i) a diversidade genética mitocondrial do peixe-boi (*T. manatus*) no Nordeste do Brasil (Capítulo II); ii) a diversidade genética nuclear e a estrutura genética populacional do peixe-boi (*T. manatus*) ao longo de sua área de distribuição no Brasil (Capítulo III); iii) a ocorrência de hibridação entre os peixes-bois *T. manatus e T. inunguis* (Capítulo IV), e iv) o grau de relação entre os peixes-bois e a ocorrência de endogamia em cativeiro no CMA/ICMBio (Capítulo V).

A discussão a seguir, será baseada na análise comparativa das diferentes investigações genéticas realizadas com peixes-bois ao longo da costa do Brasil, e apresentará sugestões de manejo e ações, já descritas nos capítulos anteriores, que podem ajudar no incremento dos esforços de conservação e proteção dessa espécie ameaçada.

VI.I – Filhotes dependentes de peixe-boi e decisão genética para soltura

No Nordeste do Brasil, o nascimento de filhotes tem ocorrido em ambientes dinâmicos em vez dos calmos mangues normalmente preferidos pelos peixes-bois. Isto é devido à degradação ambiental e à destruição do habitat do peixe-boi ao longo da costa litorânea, decorrentes do adensamento demográfico e da crescente pressão de ameaças antropogênicas, como: exploração de petróleo; pesca predatória, falta de regulamentação para uso de redes de pesca de forma que evite o emalhe; assoreamento dos estuários, aumento do turismo com elevado trânsito de embarcações e fluxo de pessoas; incremento de atividades que utilizam intensamente as áreas de mangue a exemplo da aquicultura; agricultura e os impactos da indústria de cana-de-açúcar localizada perto da costa.

Os peixes-bois preferem águas de enseadas tranquilas, protegidas, calmas e não muito utilizadas por seres humanos. A costa do litoral leste do estado do Ceará e oeste do estado do Rio Grande do Norte é formada por praias de alta energia, com ondas e correntes fortes. Estas áreas dinâmicas são menos adequadas para peixes-boi que, por necessidade, tiveram de adaptar uma estratégia de comportamento diferente para sobreviver. Infelizmente, os peixes-bois jovens não têm a capacidade de adaptação bem desenvolvida e, portanto, sofrem consequências, como o encalhes nas praias.

Um grande número de filhotes jovens se separa de suas mães durante as primeiras semanas de vida e acabam encalhando nas praias, sendo incapazes de voltar para o mar. Esse é um problema sério para a sobrevivência do filhote e, em consequência, para a conservação da espécie na região.

Para minimizar os impactos sobre a população, muitos desses filhotes têm sido resgatados com vida pela Rede de Encalhes e Monitoramento de Mamíferos Aquáticos no Nordeste do Brasil (REMANE) e reabilitados nas instalações do Centro Mamíferos Aquáticos - CMA/ICMBio para posterior soltura. Durante os primeiros anos do programa, o objetivo da soltura dos peixes-bois era devolver os animais à natureza. No entanto, a prévia identificação dos locais de soltura não levava em conta as consequências genéticas da translocação e soltura na natureza dos indivíduos reabilitados.

A hipótese 1 do estudo foi verdadeira para H_0, uma vez que se verificou que há implicações filogeográficas que podem influenciar a escolha da área de soltura de filhotes reabilitados de peixes-bois no Brasil. Através deste trabalho, as análises filogeográficas permitiram a identificação genética dos haplótipos existentes ao longo da costa do nordeste do Brasil e possibilitaram a indicação de locais de soltura baseados na similaridade genética.

O estudo indica e incentiva a soltura dos peixes-bois no norte do estado de Alagoas e/ou sul de Pernambuco, o que é importante para o estabelecimento de um corredor que promoverá a ligação entre a população de peixe-boi de Pernambuco com a população localizada em Alagoas, vez que esta área compõe o limite sul de ocorrência do peixe-boi (*T. manatus*) no Brasil e no mundo. É importante que não se repita o processo de extinção que já ocorreu nos estados do Espírito Santo (ES), Bahia (BA) e Sergipe (SE; Albuquerque e Marcolvaldi, 1982; Lima, 1997). Além disso, o aumento da área de distribuição e a oferta de mais habitat para uso pelos peixes-bois permitirá o crescimento da população e reduzirá a chance de extinção da espécie.

Portanto, a soltura de peixes-bois nos estados em que a espécie atualmente se encontra extinta pode promover a recolonização em locais mais ao sul, onde historicamente existia a ocorrência da espécie. Um exemplo de que é possível a recolonização ocorreu no estado de Sergipe, onde um peixe-boi chamado “Astro”, que foi solto no estado de Alagoas, migrou mais de 350km para o sul e chegou na praia do Mosqueiro, no estado de Sergipe, onde vive há 14 anos (Foppel and Ferrari, 2012). Essa nova área foi descoberta e é utilizada por “Astro” e se revela como adequada e apropriada para peixes-bois. A área pode promover a sobrevivência de peixe-bois devolvidos ao habitat natural, já que possui condições de suprir as necessidades amplas dos indivíduos, tais como: comida, água doce, reduzido tráfego de embarcações, águas calmas, com baixa densidade demográfica e baixa utilização por humanos. Como, no entanto, “Astro” foi o único peixe-boi que migrou para aquela área, não se pode classificá-la como uma recolonizada, a menos que se tenham novas migrações ao longo do tempo.

Como o local se insere numa região de ocorrência histórica da espécie, a experiência de “Astro” confirma que a área apresenta potencial efetivo para existência de peixes-bois nativos. Adicionalmente, como provê habitat adequado, a área poderia ser utilizada para as próximas solturas, objetivando formar uma nova população. Portanto, se recomenda que o CMA/ICMBio promova a soltura de alguns peixes-bois, nascidos ou reabilitados em cativeiro e geneticamente aptos, na área do “Astro” para incentivar o estabelecimento de uma nova população.

A soltura de indivíduos geneticamente divergentes pode resultar no *swamping* genético de populações e, eventualmente, causar a perda de alelos ou genótipos adaptados localmente (García-Ramos e Kirkpatrick, 1997; Lenormand, 2002; Frankham et al., 2002). De acordo com Allendorf e Luikart (2007), esforços devem ser feitos para que indivíduos sejam soltos em locais com a mesma assinatura genética haplotípica. Os haplótipos encontrados no estado do Maranhão foram identificados como M03 e M04, portanto, os filhotes reabilitados dessa área devem para ela voltar. A soltura desses peixes-bois em outros estados mais ao sul não deverá ser mais permitida, uma vez que resultaria em um *swamping* genético, podendo causar efeitos deletérios em populações residentes.

O *swamping* de um pool gênico local também pode ocorrer quando indivíduos de diferentes populações reproduzem e seus descendentes são soltos em áreas onde os indivíduos são localmente adaptados (Allendorf and Luikart, 2007). Portanto, os filhotes de peixes-bois reabilitados e os nascidos em cativeiro não devem ser soltos no estado da Paraíba, uma vez que essa população é pequena e tem baixa diversidade genética. Somente peixes-boi que originaram de áreas próximas ao local de soltura deverão ser considerados aptos para liberação nesse estado.

Animais soltos em locais diferentes ao de origem (geneticamente ou em relação ao habitat) talvez não se adaptem bem a nova vegetação, doenças locais ou alterações sazonais do habitat (Frankham et al., 2002). Nesse sentido, mesmo considerando a importância de se soltar peixes-bois em locais que possibilite conectar populações ou recolonizar áreas de ocorrência histórica, a soltura de indivíduos no local original onde foram resgatados continua sendo a ação mais apropriada para garantir a estabilidade populacional.

VI.II – Estrutura populacional, diversidade genética e estratégias para conservação do peixe-boi na costa do Brasil

Diversos programas genéticos foram utilizados neste estudo para, através da alta similaridade de DNA nuclear, atribuir os peixes-bois às populações, descrevendo a estrutura genética populacional do peixe-boi na costa do Brasil. Analisando amostras de peixes-bois ao longo da costa do Brasil, e amostras adicionais da Guiana e Venezuela, os peixes-bois foram primeiramente agrupados em três populações: *i*) Costa nordeste do Brasil (NEB); *ii*) Noroeste do estuário do rio Amazonas (NWA), composto pelo estado do Amapá/Brasil, a Guiana e a Venezuela; e *iii*) Estuário do rio Amazonas (EAR), que inclui os peixes-bois amostrados na boca do rio Amazonas.

Numa subsequente análise detalhada na região NEB, foi encontrada diferenciação genética e duas subpopulações foram identificadas pelo STRUCTURE: uma população constituída por indivíduos que ocorrem nos estados do Ceará até Alagoas e outra subpopulação composta por indivíduos dos estados do Maranhão e Piauí. A segunda hipótese para este estudo foi verdadeira para H_0, uma vez que foram encontradas três diferentes grupos de peixe-boi (*T. manatus*) ao longo da área de ocorrência da espécie no Brasil. Um total de quatro grupos de peixes-bois foi delimitado pelo STRUCTURE para o litoral do Brasil, onde há quatro unidades de manejo de peixes-bois.

Um tamanho amostral pequeno foi utilizado para o grupo PI-MA e deve ser aumentado no futuro para melhor resolução genética. O estudo detalhado da região EAR identificou os peixes-bois amostrados na boca do rio Amazonas como pertencentes à espécie amazônica. Essas amostras agruparam com peixes-bois amazônicos amostrados em Santarém, região localizada cerca de 1.000km distante da boca do rio Amazonas. Isso confirma que os peixes-bois encontrados pelo CMA/ICMBio e pelo IBAMA no estuário ou próximo ao mar são peixes-bois Amazônicos, como previamente descritos morfologicamente.

Não foram encontrados híbridos dentre os peixes-bois amostrados, no entanto, poucas amostras foram utilizadas no estudo. Maior número de amostras provenientes dessa área, incluindo peixes-bois com características morfológicas atribuídas ao *T. manatus* e ao *T. inunguis* deve ser adquirida. A hibridização é discutida no item VI.3 do presente capítulo, bem como no Capítulo IV.

O baixo nível de diversidade genética encontrado para os peixes-bois do Brasil pode aumentar o risco de extinção, uma vez que a diversidade é considerada necessária para adaptação a doenças e mudanças ambientais. (Reusch and Wood, 2007). A limitada diversidade genética no Brasil é resultante da combinação do efeito fundador (já que a população se encontra no limite sul da área de ocorrência do peixe-boi), de impactos antropogênicos por longo prazo como a caça comercial desde o século dezesseis, e outros relacionados principalmente com o uso e a degradação de seu habitat, além de impactos inerentes ao pequeno tamanho populacional (menos de 1.000 indivíduos).

Mesmo com o decréscimo da abundância de peixes-bois devido a caça durante a colonização e início dos anos 1900 (Luna e Passavante, 2010), não foi detectada evidência de efeito gargalo. Contudo, de acordo com os recordes de tamanho populacional, essa é uma preocupação em relação aos peixes-bois do Brasil, uma vez que eles aparentemente diminuíram devido a uma série de eventos de efeito gargalo. O efeito gargalo não foi detectado pelos programas genéticos, provavelmente devido à baixa taxa reprodutiva dos peixes-bois, uma vez que somente cinco novas gerações de peixes-bois ocorreram desde que a caça intensa foi reduzida. Os atuais impactos antropogênicos resultaram em sérios problemas, como o encalhe de filhotes dependentes (de 1989 até fevereiro de 2011, N=67) que ocorre predominantemente nos estados do Ceará (CE) e Rio Grande do Norte (RN), áreas que requerem proteção especial com o objetivo de mitigar os impactos aos peixes-bois.

As duas subpopulações de peixes-bois no nordeste do Brasil (AL-CE e PI-MA) possuem baixa diversidade genética nuclear e mitocondrial, apresentando também um tamanho populacional pequeno, sendo muito sensíveis a eventos estocásticos, que resulta em um menor potencial adaptativo do que o encontrado em populações grandes. Embora a maioria das populações de peixes-bois estudadas não seja impactada por doenças devido a um sistema imunológico eficiente, a falta de heterozigosidade pode resultar em indivíduos com imunodepressão ou baixa taxa reprodutiva (Bonde et al., 2004).

A estrutura populacional do *T. manatus* encontrada no Brasil identificou pelo menos duas populações geneticamente distintas, sendo uma delas subdividida em duas subpopulações. A falta de fluxo gênico através de emigração é motivo de preocupação em casos de perdas contínuas de indivíduos. Estudos iniciados para compreender mais sobre a dispersão de peixes-bois, utilizando a telemetria, permitirá aos pesquisadores monitorar as atuais taxas de dispersão de peixes-bois entre populações.

Como os peixes-bois (*T. manatus*) precisam de comida, água doce e áreas ao longo da costa que sirvam como refúgios para descanso e para os partos das fêmeas, é necessário implementar estratégias para conservação dos habitats necessários no Brasil. É imprescindível também a elaboração de planos de gestão delineando estratégias para garantir a conservação da espécie com base nas relações genéticas e na proteção do habitat. A subpopulação AL-CE apresentou diversidade genética do DNA mitocondrial menor que a subpopulação PI-MA, mas apresentou níveis similares de diversidade do DNA nuclear. Duas populações (NWA and EAR) e uma subpopulação (PI-MA) ocupam habitats similares que são mais preservados e sofrem menor impacto humano.

A caça continua ocorrendo e o desenvolvimento do país está elevando rapidamente a ocupação e o uso da área costeira. Assim, nas áreas de habitat preservado e sem pressão de caça, a proteção por longo prazo das populações de peixes-bois deve ser incentivada para ajudar a aumentar a diversidade genética através de mutação e fluxo gênico. Os planos de conservação devem garantir o fim da caça e fornecer proteção apropriada aos habitats adequados, mediante a emissão, pelos gestores, das licenças ou autorizações de utilização dos recursos ao longo da costa.

O habitat dos peixes-bois de AL-CE é altamente degradado, com intensa ocupação humana da linha da costa e o aumento das atividades antropogênicas, como: habitação, turismo, cana-de-açúcar, salinas, aquicultura, pesca, extração de petróleo, etc. Muitos estuários antigos não existem mais e os remanescentes não estão disponíveis para os peixes-bois, uma vez que estão altamente comprometidos pelo intenso uso da área. Essa região é constituída de habitats pobres e os peixes-bois apresentam baixa diversidade genética (apenas um haplótipo de mtDNA). Assim, a sobrevivência da subpopulação é bastante desafiadora e exige ações imediatas e efetivas de conservação.

O plano de conservação deve contemplar: promover a minimização da fragmentação dos habitats dos peixes-bois, o restabelecimento de habitats com a recuperação dos manguezais (especialmente nos estados do CE e RN, onde filhotes dependentes encalham), a regulamentação das atividades de pesca (já que peixes-bois tem se emalhado e morrido em redes de pesca), a redução da poluição nos rios (que têm sido diagnosticados como poluídos por resíduos químicos de indústrias, da agricultura e de esgotos), e garantir corredores de deslocamento e migração entre grupos e populações de peixes-bois.

O habitat dos peixes-bois no PI-MA é geograficamente situado entre as regiões norte e nordeste do Brasil. Este estudo permite identificar que os peixes-bois dessa área apresentam alguma conexão com os dois outros grupos de peixes-bois do litoral do país. Contudo, a migração de indivíduos de outras populações parece ser limitada. Portanto, com a evidência de fragmentação na distribuição dos peixes-bois (como entre Alagoas e Pernambuco), ou extinção local deles (como em Sergipe), a introdução da espécie nessas áreas pode ser uma boa solução para promover a conectividade entre os grupos fragmentados e resultar no aumento da diversidade genética local.

É extremamente estratégico e necessário reduzir o risco de extinção dos peixes-bois através do desenvolvimento e implementação de Áreas Marinhas Protegidas (MPAs) em habitats identificados como importantes para os peixes-bois (Tabela VI.1, Figura VI.1; Paludo e Langguth, 2002; Luna et al., 2008b e c; Luna e Passavante, 2010; Silva et al., 2011; Luna et al., 2011) e promover a regulamentação do uso dessas áreas, o estabelecimento de corredores e a redução de ameaças antropogênicas para a espécie, através da fiscalização.

Tabela VI.1 – Regiões estratégicas a serem consideradas para proteção de importantes habitats de peixes-bois. Listado por estado, região geográfica, população genética e local.

Estado	Região Geográfica	População Genética	Local
AL e PE	Nordeste	(AL-CE)	APA Costa dos Corais
PE	Nordeste	Corredor	Da APA Costa dos Corais até a Resex Acaú – Goiana
PE	Nordeste	(AL-CE)	Resex Acaú – Goiana
PB	Nordeste	(AL-CE)	APA Barra de Mamanguape
RN	Nordeste	(AL-CE)	Região da praia do Sagi
RN	Nordeste	(AL-CE) – corredor	Da praia do Sagi até Touros/RN
RN e CE	Nordeste	(AL-CE)	De Touros/RN até Aquiraz/CE (alto número de encalhes de filhotes dependentes e de peixes-boi capturados em redes de pesca)
PI	Nordeste	(PI-MA)	Região de Cajueiro da Praia
PI e MA	Nordeste	Corredor	De Cajueiro da Praia/PI até Ilha do Gato/MA
MA	Nordeste	(PI-MA)	Região da Ilha do Gato/Guarapiranga (Baía do Tubarão)
MA	Nordeste	(PI-MA)	Guarapiranga/Raposa (Ilha de São Luís)
MA	Nordestet	(PI-MA)	Alcântara; Resex de Cururupú e região de Carutapera (reentrâncias do Maranhão).
PA	Norte	(PI-MA)	Região de Viseu, Bragança, Algodoal; Maracanã (reentrâncias do Pará).
PA	Norte	Estuário do rio Amazonas (EAR)	Resex de Soure e Salvaterra (Ilha de Marajó – boca do rio Amazonas)
PA	Norte	Estuário do rio Amazonas (EAR)	Vigía, Colares, Mosqueiro até Abaetetuba (boca do rio Amazonas)
AP	Norte	Noroeste do rio Amazonas (NWA)	Região de Sucurijú até Oiapoque (incluindo Parna Cabo Orange e Esec Maracá-Jipioca)

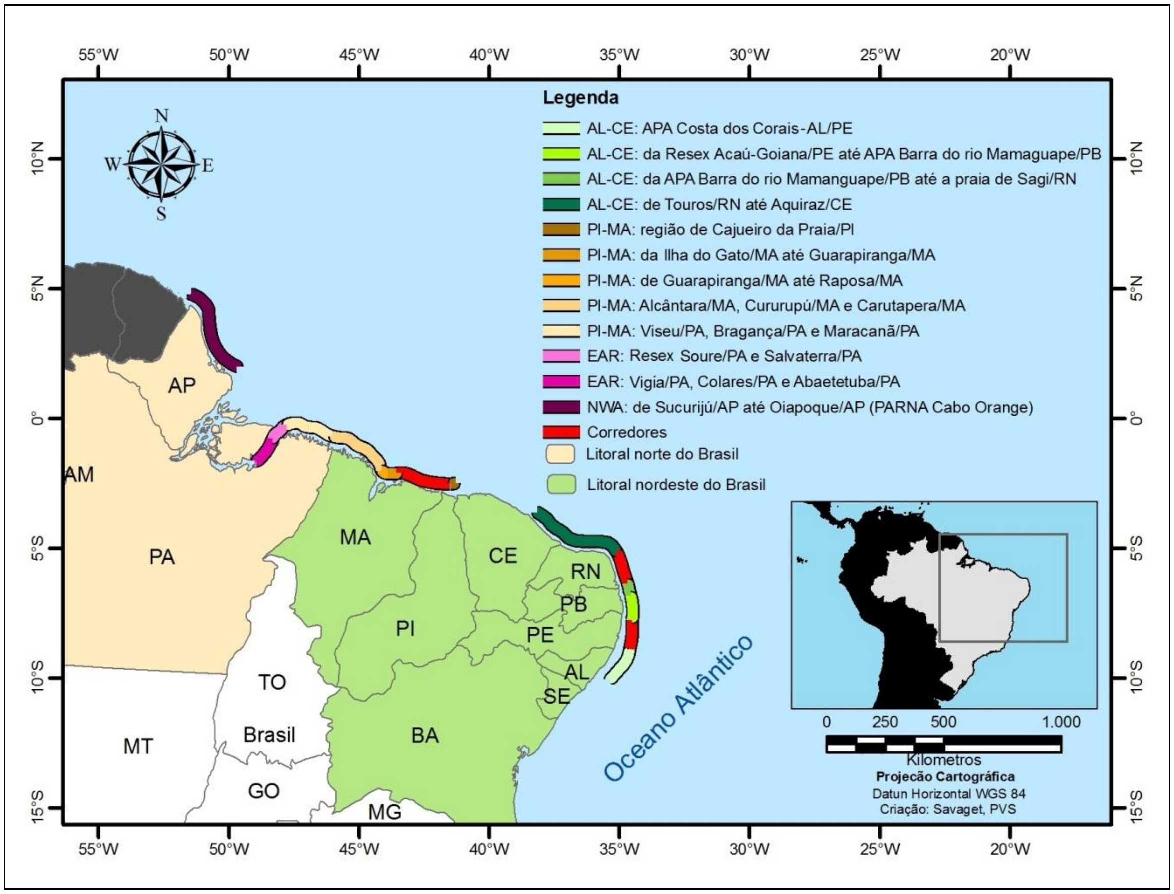


Figura VI.1 – Regiões estratégicas para serem protegidas ao longo da costa brasileira, reconhecidas como habitats críticos importantes e identificação áreas necessárias de conectividade entre peixes-bois, onde se deve estabelecer corredor de deslocamento.

VI.III – Hibridização entre *T. manatus* e *T. inunguis* no estuário do rio Amazonas e implicações para conservação.

A região do estuário do rio Amazonas, especialmente a região da ilha de Marajó, é definida como área de simpatria entre o peixe-boi (*T. manatus*) e o peixe-boi amazônico (*T. inunguis*), e é identificada como potencial zona de hibridização entre as duas espécies. O presente estudo, usando a combinação de análises moleculares (mtDNA e nDNA) e citogenéticas identificou a existência de um indivíduo híbrido, o qual não foi originário da área descrita previamente como de simpatria. O peixe-boi é originário da cidade do Oiapoque no estado do Amapá, no noroeste do estuário do rio Amazonas (NWA), ao longo da borda com a Guiana Francesa. Ele foi levado para cativeiro e foi ilegalmente mantido em uma piscina privada.

A terceira hipótese deste estudo é verdadeira para H_0, uma vez que foi encontrada a ocorrência de um híbrido entre o *T. manatus* e o *T. inunguis* no Brasil. A presença desse híbrido, capturado de uma população selvagem indica que a potencial zona de hibridização é mais ampla do que a sugerida anteriormente, com o alcance desde o estuário do rio Amazonas (EAR) até o Amapá, ou mais distante, como a Guiana Francesa, no noroeste do estuário do rio Amazonas (NWA). Essa é uma área onde a água doce do rio Amazonas se mistura com a água do mar do Oceano Atlântico.

A hibridização é preocupante, uma vez que duas espécies se encontram em risco de extinção. Contudo, a hibridização não aparenta ser frequente, uma vez que apenas um indivíduo foi identificado até a presente data como híbrido e não há registros na região de um grande número de filhotes encalhados ou de abortos de fetos, que pudessem ter derivado de processos relacionados à hibridização. É necessária realização de mais investigações sobre a hibridização, bem como sobre o que os gestores podem fazer para controlar a frequência da hibridização entre as espécies. A alta ocorrência de híbridos pode ter um impacto prejudicial para a sobrevivência das duas espécies.

VI.IV – Endogamia de peixes-bois em cativeiro e recomendações sobre a reprodução *ex-situ.*

O estudo do pedigree presente no Capítulo V forneceu informações valiosas sobre o grau de parentesco dos peixes-boi mantidos no cativeiro do CMA/ICMBio. Os 34 marcadores microssatélite disponíveis para análise de DNA nuclear de peixe-boi permitiu reconstruir o pedigree da maioria dos peixes-bois cativos do CMA/ICMBio. Contudo, apesar da alta resolução dos microssatélites para identificação dos peixes-bois os programas de pedigree e os microssatélites usados não foram suficientes para determinar precisamente as relações de parentesco de todos os peixes-bois em cativeiro. Para obter um maior nível de confiança na informação do pedigree dos peixes-bois, algumas novas investigações devem ser empregadas, como a criação de novos e mais precisos programas para pedigree. Contudo, o parentesco geral, bem como algumas relações específicas, foi identificado entre os peixes-bois em cativeiro. Os objetivos foram atingidos, uma vez que foi possível identificar a ocorrência de endogamia nas instalações do CMA/ICMBio, resultantes de acasalamentos previamente admitidos.

A quarta hipótese para esse estudo foi verdadeira para H_0, uma vez que foi identificada endogamia nos peixes-bois em cativeiro no CMA/ICMBio. Os resultados deste estudo permitem sugerir a proibição imediata de reprodução de peixe-boi em cativeiro. Isso requer que a instituição (CMA/ICMBio) não mais permita a reprodução em cativeiro com o intuito de manter uma população em cativeiro saudável. Ademais, para evitar novas endogamias, hibridizações artificiais e superpopulação em cativeiro, faz-se necessário separar machos e fêmeas em todos os recintos do CMA.

A atual população em cativeiro é grande e há elevada dificuldade para manejar apropriadamente os indivíduos. Essa população seria ainda maior se alguns dos peixes-bois nascidos em cativeiro não tivessem sido soltos na natureza. Portanto, é necessário reduzir o número de peixes-bois cativos no CMA/ICMBio e se recomenda que filhotes nascidos em cativeiro e alguns adultos continuem sendo soltos na natureza.

No futuro, um programa de reprodução em cativeiro pode ser implementado, mas somente caso haja populações ou grupos naturais de peixes-bois que estejam em risco de extinção face à pequena ou nenhuma capacidade reprodutiva na natureza. Neste caso, filhotes de peixes-bois não endogâmicos devem ser incluídos no programa, com o cuidado de se ter certeza de que os melhores pares reprodutores foram escolhidos.

Na hipótese da implementação de um programa de reprodução em cativeiro, é altamente recomendável que a seleção dos casais seja baseada nos graus de relação genética dos peixes-bois. As informações geradas no presente estudo podem ser utilizadas para esse propósito e todos os peixes-bois resgatados futuramente devem ser genotipados quando chegarem ao cativeiro. Contudo, como vários dos peixes-bois mantidos atualmente em cativeiro do CMA/ICMBio já reproduziram várias vezes, instituindo vários níveis de relação entre os pais e seus filhotes, a reprodução deles somente deverá ser permitida novamente em casos absolutamente necessários.

Caso futuramente se torne necessário o estabelecimento de um programa de reprodução em cativeiro, deve-se incluir reprodutores não relacionados para evitar endogamia. Atualmente não é recomendado um programa de reprodução em cativeiro, e o mesmo só deve ser iniciado caso haja evidências de que as populações naturais não estão se reproduzindo ou se encontrem realmente em risco crítico de extinção.

VI.V – Conclusões Gerais

A estrutura populacional dos peixes-bois ao longo da costa contempla duas espécies, *T. manatus* e *T. inunguis*, com uma área de hibridização que se estende da ilha de Marajó, no estado do Pará, ao litoral do estado do Amapá. Para o *T. manatus* se verificam área de ocorrência histórica, bem como uma área de repovoamento no estado de Alagoas, onde filhotes dependentes reabilitados têm sido soltos pelo CMA/ICMBio, que já procedeu com soltura também no estado da Paraíba (Figure VI.2).

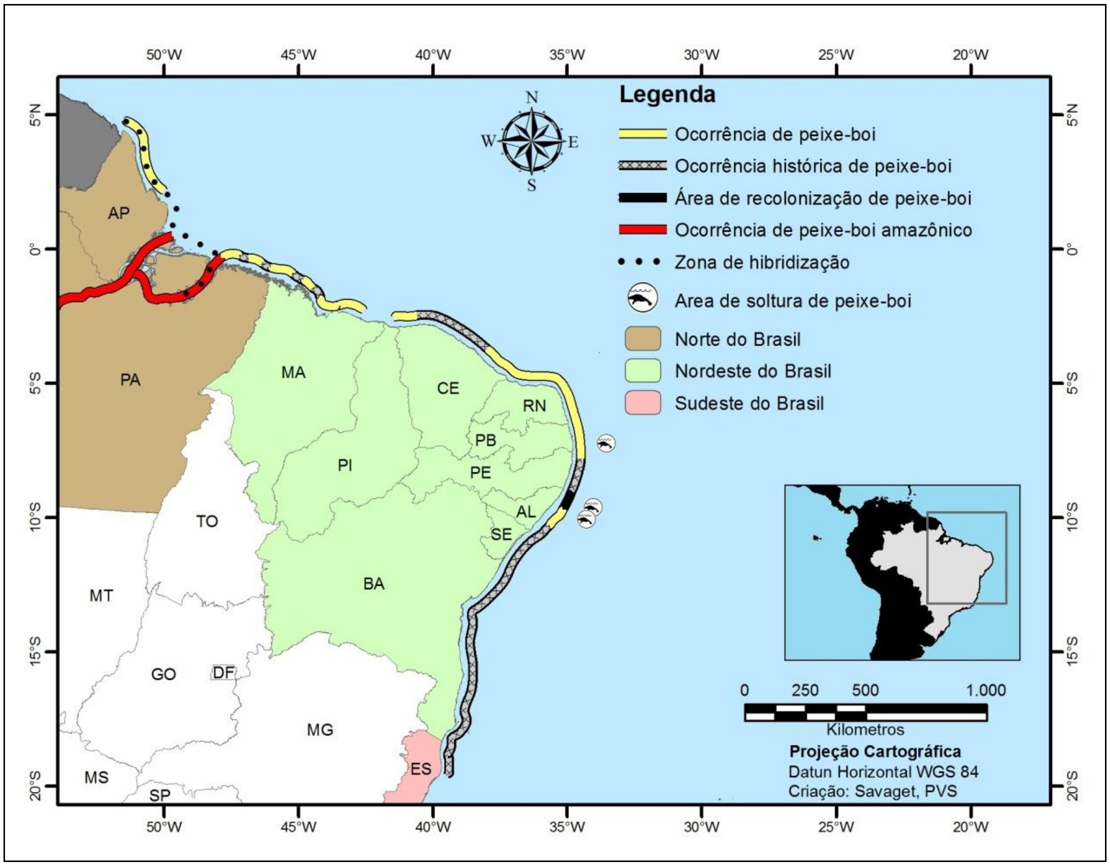


Figura VI. 2 – Ocorrência de peixes-bois na costa do Brasil, áreas utilizadas pelo CMA/ICMBio para soltura de filhotes de *Trichechus manatus*, área de repovoamento de peixe-boi (*T. manatus*), potencial zona de hibridização entre *T.manatus* e *T. inunguis*.

Um total de quatro unidades de manejo foi identificado. Para o peixe-boi *T. manatus* foram estabelecidas três unidades de manejo, com duas populações geneticamente diferenciadas: Noroeste do rio Amazonas (NWA) e nordeste do Brasil (NEB). A população do nordeste é subdividida em duas diferentes subpopulações (AL-CE and PI-MA;). A quarta unidade de manejo é formada por outra população denominada Estuário do Rio Amazonas (EAR), composta pela principalmente espécie amazônica (Figure VI.3). De modo geral, todas as populações e subpopulações possuem um tamanho pequeno e baixa diversidade genética. Esses fatos, por si sós, aumentam a susceptibilidade de rápido declínio populacional e a possibilidade de extinção local. Em alguns casos foi utilizado um pequeno número amostral, assim, amostras adicionais serão necessárias para pesquisas futuras.

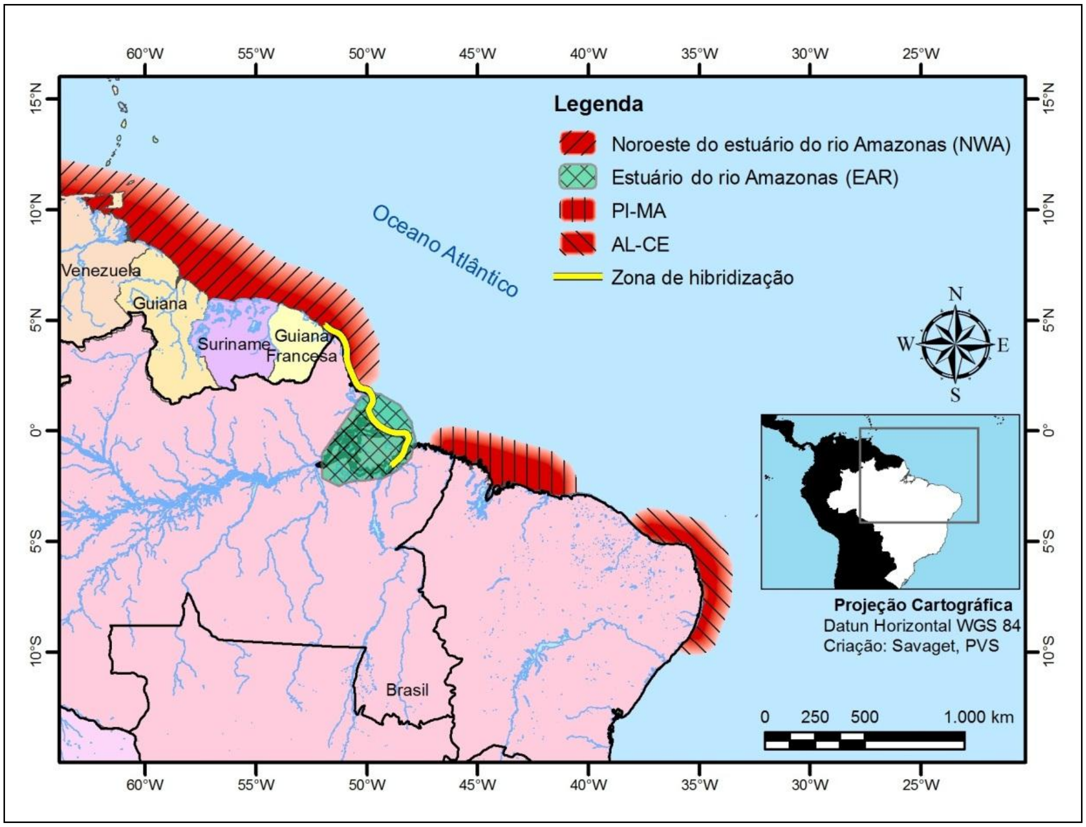


Figura VI.3 – Quatro unidades de manejo, duas espécies de peixes-bois e a potencial zona de hibridização identificada.

Para auxiliar a garantir a sustentabilidade da diversidade genética das populações encontradas no Brasil (AL-CE, PI-MA; NWA), com diferentes problemas em cada região, cada unidade de manejo deve ser tratada especificamente, e a elaboração de planos de ação separados é a estratégia mais apropriada para garantir a conservação dos peixes-bois no país. Além disso, prover corredores de migração é importante porque isso pode encorajar um fluxo gênico natural permitindo que ocorra uma mistura genética e o aumento da diversidade. Preservar geneticamente as populações ajudará a proteger a espécie como um todo contra a futura perda da diversidade e, dessa forma, a reduzir o risco de endogamia e de extinção.

Os indivíduos dos estados de Alagoas ao Ceará apresentaram apenas um haplótipo (M01). Como a população com baixa diversidade genética tem pouca capacidade de adaptação, essa baixa diversidade aumentará o risco de extinção dos peixes-bois nesses estados, especialmente no caso de eventos estocásticos ou catastróficos como maré vermelha, um surto epizoótico, mudanças climáticas, aumento do nível do mar, furacões, etc. Portanto, é muito importante reduzir as ameaças aos peixes-bois, especialmente nas regiões identificadas como áreas de abrigo e reprodutivas de populações.

A soltura de filhotes dependentes que encalharam e que foram reabilitados com sucesso deve ocorrer prioritariamente no local de origem, podendo também ocorrer nos estados de Alagoas e Sergipe, em áreas onde atualmente não existem populações naturais de peixes-bois. Fato que permitirá a conexão dos indivíduos de Alagoas com indivíduos de Pernambuco, uma vez que a soltura em Porto de Pedras/AL tem propiciado uma recolonização na região, pertencente ao grupo AL-CE, assim como a soltura em Sergipe resultará no recolonização de peixes-bois no estado, onde a espécie foi registrada como extinta.

Contudo, devido ao alto número de encalhe de filhotes dependentes, especialmente nos estados do Ceará (CE) e Rio Grande do Norte (RN), é necessário que haja pelo menos um centro de reabilitação na área para acomodar esses indivíduos. O que atenuará a demanda de logística e transporte de candidatos selecionados a serem soltos com sucesso nessa área, assim como, eles serão mantidos e preparados para serem soltos mais próximo ao local de origem em que foram resgatados.

É necessário adquirir mais informações biológicas sobre os peixes-bois nos estados do Pará (PA) e Amapá (AP), mas também nos estados do Piauí (PI) e Maranhão (MA). O acesso a peixes-bois na natureza, através da captura e soltura para verificar a saúde populacional é muito importante e deve ser incentivada, uma vez que se trata de uma excelente ferramenta para monitorar a aptidão física, a saúde e genética da espécie no Brasil.

A existência de híbrido na natureza foi identificada, mas são necessários estudos adicionais para delimitar a atual zona de hibridização e melhor entender a problemática da hibridização em relação à conservação das duas espécies de peixes-bois. Essa informação irá orientar ações de manejo para prevenir a degradação genética entre as duas espécies simpátricas.

A endogamia foi detectada nos peixes-bois em cativeiro. Machos e fêmeas devem ser separados no CMA/ICMBio, para inibir a ocorrência de endogamia na população cativa, assim como, deve ser proibida a reprodução em cativeiro, a qual somente deve ser permitida no futuro se os filhotes nascidos na natureza forem endogâmicos e o número de nascimentos for pequeno e a população se encontre em decréscimo. Dessa forma, a pequena população se beneficiaria com a suplementação de indivíduos originários de população cativa. Contudo, o programa só deve ser implementado no caso comprovado de necessidade real e extrema de salvar uma população local.

A cooperação entre legislação, educação e fiscalização é necessária para assegurar a sustentabilidade das futuras populações de peixes-bois. Aplicação mais incisiva das leis e o aumento da proteção em regiões impactadas no Brasil podem reduzir o número e os impactos das mortes causadas por questões antropogênicas.

Em resumo, as populações de peixes-bois devem ser protegidas para aumentar a diversidade genética, a qual está correlacionada com o número de indivíduos e com a saúde geral da população. A diversidade genética dessas populações deve ser monitorada para detectar mudanças ao longo do tempo. A viabilidade da população depende da qualidade do habitat e da redução das ameaças antropogênicas, para que se possa garantir a sustentabilidade das populações de peixes-bois no futuro. A implemantação do Plano de Ação Nacional para Conservação dos Sirênios no Brasil deve ser prioridade, o qual deve ser utilizado como uma das ferramentas do governo para desenvolver estratégias necessárias para a proteção dessa frágil e ameaçada espécie.

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