

## ***Phylogeographic implications for release of critically endangered manatee calves rescued in Northeast Brazil***

FÁBIA O. LUNA<sup>a,b</sup>, ROBERT K. BONDE<sup>c,d</sup>, FERNANDA L. N. ATTADEMO<sup>a,c</sup>, JONATHAN W. SAUNDERS<sup>c</sup>, GAIA MEIGS-FRIEND<sup>c</sup>, JOSÉ ZANON O. PASSAVANTE<sup>b</sup> and MARGARET E. HUNTER<sup>c,d,\*</sup>

<sup>a</sup>*Centro Mamíferos Aquáticos – CMA/ICMBio-MMA, Ilha de Itamaracá, PE, Brazil*

<sup>b</sup>*Departamento de Oceanografia, Universidade Federal de Pernambuco, Recife, PE, Brazil*

<sup>c</sup>*Sirenia Project, Southeast Ecological Science Center, U.S. Geological Survey, Gainesville, FL, USA*

<sup>d</sup>*Department of Large Animal Clinical Sciences, College of Veterinary Medicine, University of Florida, Gainesville, FL, USA*

<sup>c</sup>*Departamento de Medicina Veterinária, Universidade Federal Rural de Pernambuco, Recife, PE, Brazil*

### ABSTRACT

1. The Antillean manatee (*Trichechus manatus manatus*), a subspecies of the West Indian manatee, is a large-bodied marine mammal found in fresh, brackish, and marine habitats throughout the Caribbean Islands and Central and South America. Antillean manatees in Brazil are classified as critically endangered, with a census size of approximately 500 individuals. The population in the Northeast region of Brazil is suspected to have approximately 300 manatees and is threatened by habitat alteration and incidental entanglement in fishing gear.

2. A high incidence of dependent calf strandings have been identified near areas of altered critical manatee habitat. The majority of the calves are neonates, discovered alive, with no potential mothers nearby. These calves typically require human intervention to survive.

3. Since 1989 the calves have been rescued (N=67), rehabilitated, and released (N=25) to supplement the small wild manatee population. The rescued calves, and those born in captivity, are typically, not released to their rescue location, mainly for logistical reasons. Therefore, phylogeographic analyses can help to identify related populations and appropriate release sites.

4. Here, mitochondrial DNA analyses identified low haplotype ( $h=0.08$ ) and nucleotide ( $\pi=0.0026$ ) genetic diversity in three closely related haplotypes. All three haplotypes (M01, M03, and a previously unidentified haplotype, M04) were found in the northern portion of the region, while only a single haplotype (M01) was represented in the south. This suggests the presence of two genetic groups with a central mixing zone. Release of rehabilitated calves to unrelated populations may result in genetic swamping of locally adapted alleles or genotypes, limiting the evolutionary potential of the population.

5. The small population size coupled with low genetic diversity indicates that the Northeast Brazil manatee population is susceptible to inbreeding depression and possible local extinction. Further conservation measures incorporating genetic information could be beneficial to the critically endangered Brazilian manatee population. Published 2012. This article is a U.S. Government work and is in the public domain in the USA.

Received 11 August 2011; Revised 19 March 2012; Accepted 21 April 2012

KEY WORDS: coastal; estuary; mangrove; genetics; endangered species; reintroductions; mammal; urban development; aquaculture

### INTRODUCTION

Anthropogenic threats and a low reproductive rate have historically limited West Indian manatee

(*Trichechus manatus*) population growth. As a result, the Antillean manatee subspecies (*T. m. manatus*) is classified as endangered by the International Union for the Conservation of

\*Correspondence to: M. Hunter, Sirenia Project, Southeast Ecological Science Center, U.S. Geological Survey, Gainesville, FL, USA. E-mail: mhunter@usgs.gov

Nature (IUCN), and the population in Brazil is classified as critically endangered by the Brazilian government (IBAMA, 1989; MMA, 2003; Self-Sullivan and Mignucci-Giannoni, 2008). During Brazilian colonization by Portugal, manatees were severely exploited for sustenance and commercial activities (Domning, 1981, 1982). Consequently, the distribution and abundance of the population was considerably diminished, although no census estimates were reported (Whitehead, 1977; Domning, 1981; O'Shea, 1994). The current estimate of Antillean manatees in Brazil is approximately 500 individuals (Lima, 1997; Luna, 2001; Luna *et al.*, 2008). The Northeast regional population is estimated to be 278 individuals (Lima, 1997), not including Maranhão state (MA) to the north (Figure 1). Low genetic connectivity is observed between Brazil and the neighbouring manatee populations in French Guiana and Guyana (Vianna *et al.*, 2006). 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 this protection, Brazilian manatees are severely threatened in some areas and the subspecies is classified as extinct in the southern portion of the range, from Sergipe (SE) to Espírito Santo (Albuquerque and Marcovaldi, 1982; Lima *et al.*, 1992) and along the Alagoas (AL) and Pernambuco (PE) state boundary (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 Antillean manatee population in the Northeast region of Brazil (Oliveira *et al.*, 1990; Paludo, 1998). Lima (1997) suggested that pregnant females are not gaining access to quiet and protected estuarine waters to give birth. Therefore, the calves are born in open water with strong winds and coastal currents, which often results in their separation from their mother. Critical habitat degradation through urban development and

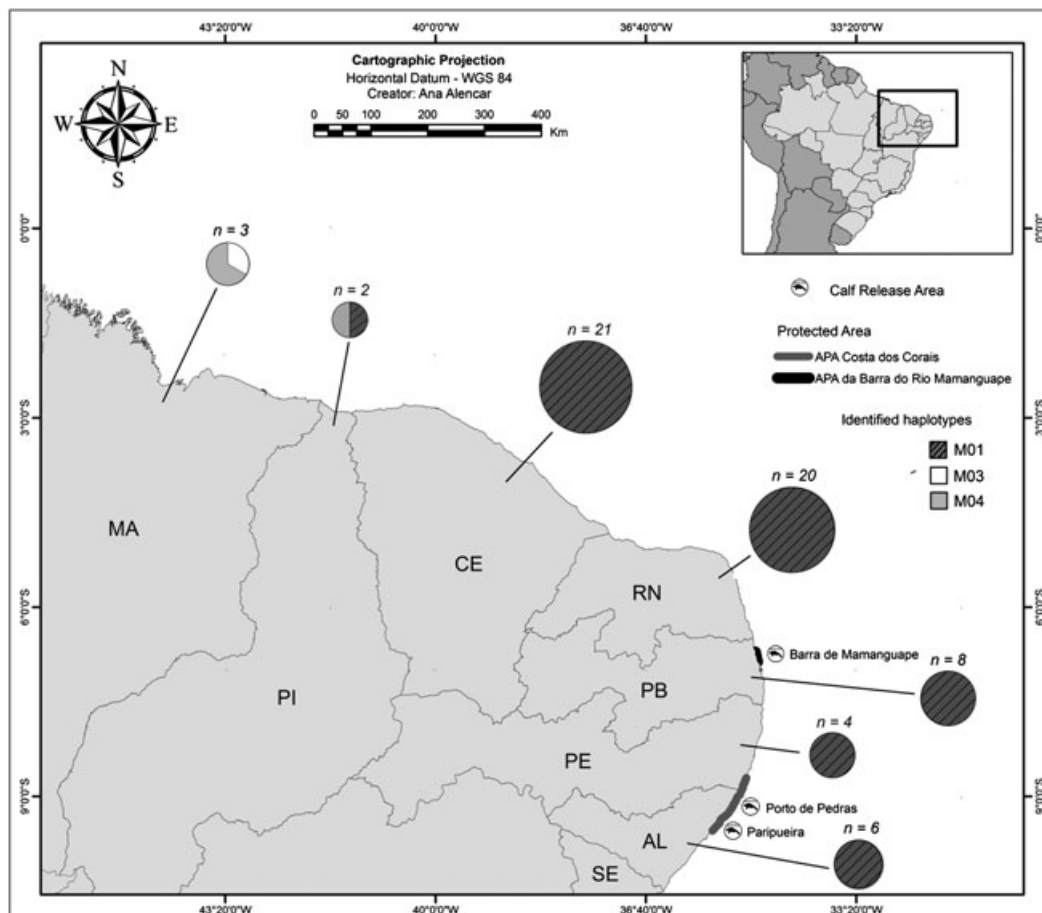


Figure 1. Map of Antillean manatee haplotype distribution in the Northeast region of Brazil. Locations of cities where the releases occur are demarcated by the international manatee symbol. National Protected Areas (APAs) are demarcated by a line. The states are as follows, Maranhão (MA), Piauí (PI), Ceará (CE), Rio Grande do Norte (RN), Paraíba (PB), Pernambuco (PE), and Alagoas (AL).

shrimp farm construction has resulted in extensive mangrove deforestation and is attributed to the increase in 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 (Figure 1). Calves recovered alive are rescued by institutions of the Stranding Network of Northeast 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 2–3 years and would be unlikely to survive without rescue (Reep and Bonde, 2006). The calves are transported 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 can be reintroduced into the wild population.

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 individuals (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 analysed genetically in this study. A previous mtDNA control region study identified low haplotype ( $h=0.0667$ ) and nucleotide ( $\pi=0.0002$ ) diversity in Antillean manatees in coastal Brazil (Vianna *et al.*, 2006). The study reported only two Antillean haplotypes (M01 and M03) from 30 individuals (excluding a suspected hybrid), with M03 identified only once (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 behavioural knowledge. Furthermore, 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. m. manatus* calves. Conservation implications will be discussed for this small, critically endangered manatee population.

## MATERIALS AND METHODS

### Sample collection and DNA extraction

Beginning in 1989, stranded calves were rescued in seven Brazilian states along the Northeast Atlantic coast by CMA and REMANE (Figure 1). Stranded calves are typically identified by local fishermen and are 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 *et al.* (2006) 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 Brazil samples. Therefore, all available samples and associated geographic information were analysed for this study.

The blood samples were preserved in EDTA or lysis solution (10 mmol L<sup>-1</sup> NaCl, 100 mmol L<sup>-1</sup> EDTA, 100 mmol L<sup>-1</sup> 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 mmol L<sup>-1</sup> 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 Qiagen's DNeasy Blood and Tissue kits (Valencia, California) for 71 blood and tissues samples and one placenta

sample, while a single carcass sample was isolated using traditional phenol chloroform techniques, due to the decomposed state of the sample.

### Mitochondrial DNA analysis

Primers from Garcia-Rodriguez *et al.* (2000), CR-4 and CR-5 (Southern *et al.*, 1988; Palumbi *et al.*, 1991) 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 *et al.* (2010). Briefly, the PCR reaction conditions were as follows: 10 ng DNA, 1 × PCR buffer (10 mmol L<sup>-1</sup> Tris-HCl, pH 8.3, 50 mmol L<sup>-1</sup> KCl, 0.001% gelatin; Sigma-Aldrich, Inc., St. Louis MO), 0.8 mmol L<sup>-1</sup> dNTP, 3 mmol L<sup>-1</sup> MgCl<sub>2</sub>, 0.24 μmol L<sup>-1</sup> 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 labelled 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).

### 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 ( $\pi$ ), 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).

## RESULTS

### 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. Of the 73 individuals sampled, 69 were M01, one was M03, and three were M04 (a previously unidentified haplotype (GenBank accession number 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 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.

The M01 haplotypes (N=69) were found from Piauí state (PI) to AL, however, they were not found in MA, the most northern state in the Northeast region of Brazil (Figure 1). The previously unidentified haplotype, M04 (N=3), was found in one calf and one carcass in MA and in one placenta in PI (Table 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.

### 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 1). 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

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

Sample category	$N$	HT	$NS$	$h$	$\pi$
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.

Table 2. Identified West Indian manatee haplotypes by state of recovery in the Northeast 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

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 had an aberrant number of chromosomes for the West Indian manatee ( $N=50$ ), suggesting F2 hybridization with an Amazonian manatee (Vianna *et al.*, 2006). 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.

## 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 programme 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).

### 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 programme and studbook would be necessary to prevent inbreeding. Furthermore, 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 (Vianna *et al.*, 2006).

Since mtDNA is maternally inherited and does not recombine, it typically 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 sub-populations in manatees, especially in the Northeast Brazil locations where the M01 haplotype was found exclusively (Hunter *et al.*, 2010). Quantifying relatedness using nDNA markers could better help to determine the most suitable release sites for rehabilitated manatees.

### Inbreeding depression in isolated Brazilian manatee populations

The extinction of Antillean manatees along the AL and PE state boundary has geographically separated the small AL population from those to the north (Figure 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 from inbreeding depression, or further reduction of genetic diversity and can become inbred rapidly with little warning (Frankham, 1995; Bijlsma *et al.*, 2000; Frankham *et al.*, 2002). Inbreeding depression is defined as a reduction in the survival and fertility of the offspring of related individuals. 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).

### Genetic swamping and outbreeding depression

The single manatee with the M03 haplotype is presumably the same animal identified by Vianna *et al.* (2006). 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 re-established 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 favourable 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 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 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.

### Conservation of Antillean manatees in Northeast Brazil

The low levels of haplotype and nucleotide diversity of the Northeast 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 Antillean manatee population in Brazil.

#### ACKNOWLEDGEMENTS

The genetic analysis was conducted under the USFWS Wildlife Research Permit MA791721, issued to the USGS, Sirenia Project. Tissue samples were obtained for lab analyses utilizing Brazilian CITES export permits 09BR003661/DF and 10BR005242/DF and USA CITES import permits 08US808447/9 and 10US06625A/9. Funding for this project was provided by the ICMBio/MMA, US Geological Survey (USGS), Society of Marine Mammology and the University of Florida. We would like to thank the Brazil Manatee Project (Projeto Peixe-Boi - CMA/ICMBio) which provided access to genetic samples, logistical support and permission to collaboratively work under their Brazil Biodiversity Institute (ICMBio) and research permits SISBIO-ICMBio 19.204/2009 and 24.473/2010 and the USGS Sirenia Project for providing laboratory facilities to conduct the genetic analysis. The authors would like to thank Alexandre Sanchez, and the REMANE (stranding network of Northeast Brazil, specially Aquasis, UERN and Rebio Atol das Rocas/ICMBio), for their work rescuing and handling the stranded manatees, and also for providing samples for the study. The authors are grateful to Ana Alencar for producing the map. We would like to thank James Reid, USGS, for his assistance with the collaboration. Any use of trade, product, or firm names is for descriptive purposes only and does not imply endorsement by the US Government.

#### REFERENCES

- Albuquerque C, Marcovaldi GM. 1982. Ocorrência e distribuição do peixe-boi marinho no litoral brasileiro (SIRENIA, Trichechidae, *Trichechus manatus*, Linnaeus, 1758). In *Proceedings of the First Simpósio Internacional sobre a utilização de Ecossistemas Costeiros: Planejamento, Poluição e Produtividade* 27, Brazil.
- Alleaume-Benharira M, Pen IR, Ronce O. 2006. Geographical patterns of adaptation within a species' range: interactions between drift and gene flow. *Journal of Evolutionary Biology* 19: 203–215.
- Allendorf FW, Luikart G. 2007. *Conservation and the Genetics of Populations*. Wiley-Blackwell: Malden, MA.
- Bijlsma R, Bundgaard J, Boerema AC. 2000. Does inbreeding affect the extinction risk of small populations? Predictions from *Drosophila*. *Journal of Evolutionary Biology* 13: 502–514.
- Domning DP. 1981. Distribution and status of manatees *Trichechus* spp. near the mouth of the Amazon River, Brazil. *Biological Conservation* 19: 85–97.
- Domning DP. 1982. Commercial exploitation of manatees *Trichechus* in Brazil c. 1785–1973. *Biological Conservation* 22: 101–126.
- Drummond AJ, Ashton B, Buxton S, Cheung M, Cooper A, Duran C, Field M, Heled J, Kearse M, Markowitz S, *et al.* 2011. Geneious v5.4. 2011. <http://www.geneious.com> [09 February 2011].
- Frankham R. 1995. Inbreeding and extinction – a threshold effect. *Conservation Biology* 9: 792–799.
- Frankham R, Ballou JD, Briscoe DA. 2002. *Introduction to Conservation Genetics*. Cambridge: Cambridge University Press.
- García-Ramos G, Kirkpatrick M. 1997. Genetic models of rapid evolutionary divergence in peripheral populations. *Evolution* 51: 21–28.
- García-Rodríguez AI, Moraga-Amador D, Farmerie W, McGuire P, King TL. 2000. Isolation and characterization of microsatellite DNA markers in the Florida manatee (*Trichechus manatus latirostris*) and their application in selected sirenian species. *Molecular Ecology* 9: 2161–2163.
- Hunter ME, Auil-Gomez NE, Bonde RK, Powell J, McGuire PM. 2010. Low genetic variation and evidence of limited dispersal in the regionally important Belize manatee. *Animal Conservation* 13: 592–602.
- IBAMA. 1989. Lista oficial das espécies da fauna brasileira ameaçada de extinção. *Portaria* N°. 1522, 19/12/1989.
- Jamieson IG, Wallis GP, Briskie JV. 2006. Inbreeding and endangered species management: is New Zealand out of step with the rest of the world? *Conservation Biology* 20: 38–47.
- Jin L, Nei M. 1990. Limitations of the evolutionary parsimony method of phylogenetic analysis. *Molecular Biology and Evolution* 7: 82–102.
- Kimura M. 1980. A simple method for estimating evolutionary rate of base substitution through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* 16: 111–120.
- Lenormand T. 2002. Gene flow and the limits to natural selection. *Trends in Ecology & Evolution* 17: 183–189.
- Lima RP. 1997. Peixe-Boi Marinho (*Trichechus manatus*): distribuição, status de conservação e aspectos tradicionais ao longo do litoral nordeste do Brasil. Master's Degree dissertation, Universidade Federal de Pernambuco, Brazil.
- Lima RP. 2008. Distribuição espacial e temporal de peixes-bois (*Trichechus manatus*) reintroduzidos no litoral nordestino e avaliação da primeira década 1994–2004 do Programa de reintrodução. PhD thesis, Universidade Federal de Pernambuco, Brazil.
- Lima RP, Paludo D, Soavinski RJ, Silva KG, Oliveira EMA. 1992. Levantamento da distribuição, ocorrência e status de conservação do peixe-boi marinho (*Trichechus manatus*, Linnaeus, 1758) no litoral nordeste do Brasil. Peixe-boi. *Coletânea de Trabalhos de Conservação e Pesquisa de Sireníos no Brasil - MMA/IBAMA* 1: 47–72.
- Luna FO. 2001. Distribuição, Status de Conservação e Aspectos Tradicionais do peixe-boi marinho (*Trichechus manatus manatus*) no litoral norte do Brasil. Master's Degree dissertation, Universidade Federal de Pernambuco, Brazil.
- Luna FO, Lima RP, Araújo JP, Passavante JZO. 2008. Status de conservação do peixe-boi marinho (*Trichechus manatus*

- manatus* Linnaeus, 1758) no Brasil. *Revista Brasileira de Zootecias* **10**: 145–153.
- Meirelles ACO. 2008. Mortality of the Antillean manatee, *Trichechus manatus manatus*, in Ceará State, north-eastern Brazil. *Journal of the Marine Biological Association of the United Kingdom* **88**: 1133–1137.
- MMA. 2003. Lista oficial das espécies da fauna brasileira ameaçada de extinção. *Instrução Normativa N°03/2003*.
- Nei M. 1987. Genetic distance and molecular phylogeny. In *Population Genetics and Fishery Management*. Ryman N, Utter F (eds). University of Washington Press: Seattle; 193–223.
- Oliveira EMA, Langguth A, Silva KG, Soavinski RJ, Lima RP. 1990. Mortalidade de peixe-boi marinho (*Trichechus manatus*) na costa nordeste do Brasil. In *Proceedings of the Fourth Reunião de Trabalho de Especialistas em Mamíferos Aquáticos da América do Sul*. Valdivia, Chile; 191–196.
- O'Shea TJ. 1994. Manatees. *Scientific American* **271**: 66–72.
- Paludo D. 1998. Estudos sobre a ecologia e conservação do peixe-boi marinho *Trichechus manatus manatus* no nordeste do Brasil. *Série Meio ambiente em debate* 22: IBAMA, Brazil.
- Palumbi S, Martin A, Romano S, McMillan WO, Stice L, Grabowski G. 1991. *Simple Fools Guide to PCR, version II*. University of Hawaii: Honolulu, HI.
- Parente CL, Vergara-Parente JE, Lima RP. 2004. Strandings of Antillean manatees, *Trichechus manatus manatus*, in northeastern Brazil. *The Latin American Journal of Aquatic Mammals* **3**: 69–75.
- Peakall R, Smouse PE. 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology* **6**: 288–295.
- Reep RL, Bonde RK. 2006. *The Florida Manatee: Biology and Conservation*. University Press of Florida: Gainesville, FL.
- Reusch TBH, Wood TE. 2007. Molecular ecology of global change. *Molecular Ecology* **16**: 3973–3992.
- Roberts DG, Gray CA, West RJ, Ayre DJ. 2010. Marine genetic swamping: hybrids replace an obligatory estuarine fish. *Molecular Ecology* **19**: 508–520.
- Sarrazin F, Barbault R. 1996. Reintroduction: challenges and lessons for basic ecology. *Trends in Ecology & Evolution* **11**: 474–478.
- Self-Sullivan C, Mignucci-Giannoni A. 2008. *Trichechus manatus* ssp. *manatus*. In IUCN 2010. IUCN Red List of Threatened Species. Version 2010.4. www.iucnredlist.org [11 February 2011].
- Southern SO, Southern PJ, Dizon AE. 1988. Molecular characterization of a cloned dolphin mitochondrial genome. *Journal of Molecular Evolution* **28**: 32–42.
- Tajima F. 1993. Measurement of DNA polymorphism. In *Mechanisms of Molecular Evolution: Introduction to Molecular Paleopopulation Biology*, Takahata N, Clark AG (eds). Japan Scientific Societies Press/Sinauer Associates Inc: Tokyo/Sunderland; 37–59.
- Tamura K, Dudley J, Nei M, Kumar S. 2007. *MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0*. *Molecular Biology and Evolution* **24**: 1596–1599.
- Vianna JA, Bonde RK, Caballero S, Giraldo JP, Lima RP, Clark A, Marmontel M, Morales-Vela B, de Souza MJ, Parr L, et al. 2006. Phylogeography, phylogeny and hybridization in trichechid sirenians: implications for manatee conservation. *Molecular Ecology* **15**: 433–447.
- Waples K, Stagoll C. 1997. Ethical issues in the release of animals from captivity. *Bioscience* **42**: 115–121.
- Whitehead PJP. 1977. The former southern distribution of new world manatees (*Trichechus* spp.). *Biological Journal of the Linnean Society* **9**: 165–189.
- Whitehead PJP. 1978. Registros antigos da presença do peixe-boi do Caribe (*Trichechus manatus*) no Brasil. *Acta Amazônica* **8**: 497–506.