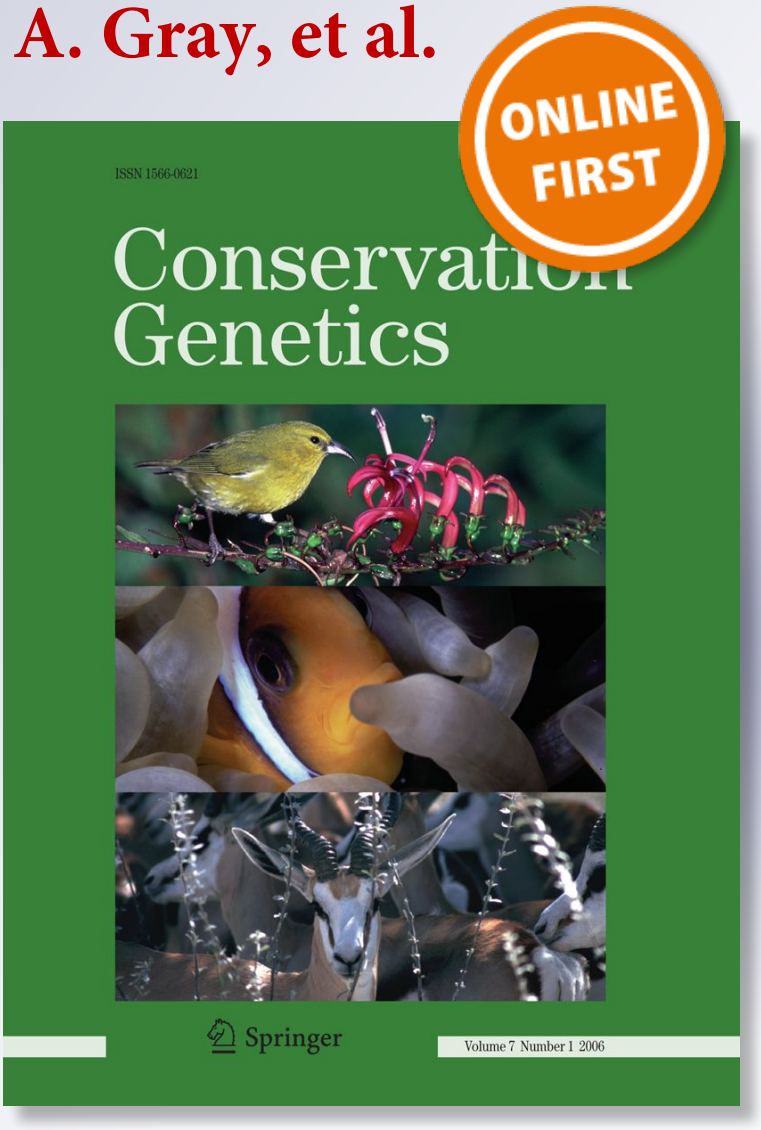
Puerto Rico and Florida manatees represent genetically distinct groups

Margaret E. Hunter, Antonio A. Mignucci-Giannoni, Kimberly Pause Tucker, Timothy L. King, Robert K. Bonde, Brian A. Gray, et al.

Conservation Genetics

ISSN 1566-0621

Conserv Genet
DOI 10.1007/s10592-012-0414-2



Springer

Conserv Genet
DOI 10.1007/s10592-012-0414-2

RESEARCH ARTICLE

Puerto Rico and Florida manatees represent genetically distinct groups

Margaret E. Hunter · Antonio A. Mignucci-Giannoni · Kimberly Pause Tucker · Timothy L. King · Robert K. Bonde · Brian A. Gray · Peter M. McGuire

Received: 10 January 2012 / Accepted: 24 September 2012

Abstract The West Indian manatee (*Trichechus manatus*) populations in Florida (*T. m. latirostris*) and Puerto Rico (*T. m. manatus*) are considered distinct subspecies and are listed together as endangered under the United States Endangered Species Act. Sustained management and conservation efforts for the Florida subspecies have led to the suggested reclassification of the species to a threatened or delisted status. However, the two populations are geographically distant, morphologically distinct, and habitat degradation and boat strikes continue to threaten the Puerto Rico population. Here, 15 microsatellite markers and mitochondrial control region sequences were used to determine the relatedness of the two populations and investigate the genetic diversity and phylogeographic organization of the Puerto Rico population. Highly divergent allele frequencies were identified between Florida and Puerto Rico using microsatellite ($F_{ST} = 0.16$; $R_{ST} = 0.12$ ($P < 0.001$)) and mitochondrial ($F_{ST} = 0.66$; $\Phi_{ST} = 0.50$ ($P < 0.001$)) DNA. Microsatellite Bayesian cluster analyses detected two populations ($K = 2$) and no admixture or recent migrants between Florida ($q = 0.99$) and Puerto Rico ($q = 0.98$). The microsatellite genetic diversity values in Puerto Rico ($H_E = 0.45$; $N_A = 3.9$), were similar, but lower than those previously identified in Florida ($H_E = 0.48$, $N_A = 4.8$). Within Puerto Rico, the mitochondrial genetic diversity values ($\pi = 0.001$; $h = 0.49$) were slightly lower than those previously reported ($\pi = 0.002$; $h = 0.54$) and strong phylogeographic structure was identified ($F_{ST\ \mathrm{global}} = 0.82$; $\Phi_{ST\ \mathrm{global}} = 0.78$ ($P < 0.001$)). The genetic division with Florida, low diversity, small population size ($N = 250$), and distinct threats and habitat emphasize the need for separate protections in Puerto Rico. Conservation efforts including threat mitigation, migration corridors, and protection of subpopulations could lead to improved genetic variation in the endangered Puerto Rico manatee population.

Keywords Microsatellite DNA · Mitochondria DNA · Marine mammal · Endangered species · Distinct population segment · Landscape genetics · Phylogenetics · Conservation genetics

Electronic supplementary material The online version of this article (doi:10.1007/s10592-012-0414-2) contains supplementary material, which is available to authorized users.

M. E. Hunter (✉) · R. K. Bonde
Sirenia Project, Southeast Ecological Science Center, U.S. Geological Survey, 7920 NW 71st Street, Gainesville, FL 32653, USA
e-mail: mhunter@usgs.gov

M. E. Hunter · R. K. Bonde · P. M. McGuire
Aquatic Animal Health Program, College of Veterinary Medicine, University of Florida, PO Box 100136, Gainesville, FL 32610, USA

A. A. Mignucci-Giannoni
Puerto Rico Manatee Conservation Center, Inter American University of Puerto Rico, PO Box 361715, San Juan, PR 00936, USA

K. P. Tucker
Biology Department, Stevenson University, 1525 Greenspring Valley Road, Stevenson, MD 21153, USA

T. L. King
Aquatic Ecology Branch, Leetown Science Center, U.S. Geological Survey, 11649 Leetown Road, Kearneysville, WV 25430, USA

B. A. Gray
Department of Pediatrics, Division of Genetics, College of Medicine, University of Florida, 4800 SW 35th Dr, Gainesville, FL 32608, USA

Published online: 07 October 2012

Introduction

The West Indian manatee (*Trichechus manatus*) populations located in Puerto Rico (*T. m. manatus*) and Florida, USA (*T. m. latirostris*) are recognized as distinct subspecies based on morphological data, and are listed and managed together as endangered under the U.S. Endangered Species Act (U.S. Fish and Wildlife Service 1982; Domning and Hayek 1986; Domning 1994, 2005). Habitat loss and high anthropogenic and natural mortality rates are threats to the species (Mignucci-Giannoni et al. 2000; Lefebvre et al. 2001; Reep and Bonde 2006). Sustained management and conservation efforts in Florida have resulted in population growth and a recommendation to reclassify West Indian manatees from both populations to a threatened or delisted status (U.S. Fish and Wildlife Service 2007). To date, only mitochondrial DNA (mtDNA) data analyses have been used to assess the extent of migration and breeding between the Florida and Puerto Rico manatee populations (García-Rodríguez et al. 1998; Vianna et al. 2006). Furthermore, the Puerto Rico Manatee Recovery Plan was drafted in 1986 (Rathbun and Possardt 1986) and could benefit from an updated threat assessment and conservation plan incorporating genetic data for improved recovery efforts. Microsatellite DNA analyses were conducted here to provide nuclear gene flow estimates for Florida and Puerto Rico. Treating these populations as one unit for management may not be advantageous, as the threats, habitat, population sizes, and recovery efforts differ for the two populations.

The manatee population in Puerto Rico is estimated to include 250 (170–360) individuals (Mignucci-Giannoni et al. 2000; Slone et al. 2006). From 1990 to 2006 a carcass recovery program determined that anthropogenic mortality (28.9 % of all mortality) was caused by watercraft strike (19.8 %), poaching (4.1 %), pollution (1.7 %), gunshot (1.7 %), and drowning or entanglement (1.6 %; Bonde et al. 2012). Analogous to Florida, watercraft related mortalities have recently become the leading cause of anthropogenic mortality in Puerto Rico (Bonde et al. 2012; FWRI 2010). Further, a long-term study of Puerto Rico manatee deaths (1864–2006) revealed an overall increase in mortality during the last 30 years of the study, rising an average of 9.6 % per year (SD = 16.9 %; Mignucci-Giannoni et al. unpublished data; Mignucci-Giannoni et al. 2000). Hunting of manatees for sustenance was documented from 1590–1995 and likely contributed to the endangered status of the population (Acosta 1590; Stahl 1883; Durand 1983; Mignucci-Giannoni et al. 2000).

Overall, the West Indian manatee was found to have similar mtDNA haplotype diversity ($h = 0.86$) and greater nucleotide diversity ($\pi = 0.039$) and number of polymorphic sites ($S = 45$) than the Amazon ($h = 0.88$, $\pi = 0.005$, $S = 34$) and West African ($h = 0.93$, $\pi = 0.020$, $S = 15$) manatee species (Vianna et al. 2006). Within Florida, no variation was identified in the mtDNA cytochrome *b* or the control region (Bradley et al. 1993; García-Rodríguez et al. 1998). The single Florida control region haplotype (A01) was also identified in the Puerto Rico population, along with two others, A02 and B01 (García-Rodríguez et al. 1998; Vianna et al. 2006). Since the two populations contain the same haplotype, they may be related through past or present gene flow, or genetic drift in the mitochondrial control region. Alternatively, they may be related through a common ancestor and experiencing incomplete lineage sorting. Indeed, Florida is believed to have been colonized by manatees from the Greater Antillean islands (Cuba, Jamaica, Hispaniola, and Puerto Rico) as they moved northward after the last glacial event, approximately 12,000 years ago (Domning 2005).

A limited number of studies have addressed nuclear DNA (nDNA) diversity and population structure in manatees using microsatellites. The Florida subspecies ($H_E = 0.48$, $N_A = 4.8$; 11 loci) indicated weak differentiation ($F_{ST} = 0.02$ ($P < 0.001$) between the east and west coasts of Florida at 18 loci (Tucker et al. in press). The Belize manatee population ($H_E = 0.46$, $N_A = 3.4$) was genetically divergent from Florida ($F_{ST} = 0.14$ ($P < 0.001$)) at 16 loci and showed evidence of two genetic clusters (Hunter et al. 2010a). At 13 loci, genetic differentiation ($F_{ST} = 0.13$ ($P < 0.05$)) was found between the Mexico population in Chetumal Bay ($H_E = 0.46$, $N_A = 3.0$), the Gulf of Mexico ($H_E = 0.41$; $N_A = 2.7$), and with Florida ($F_{ST} = 0.10$ ($P < 0.05$); Nourisson et al. 2011).

The diversity within Puerto Rico and the genetic relationship with other populations has been calculated using the mtDNA control region; however, no accompanying population structure information was provided (García-Rodríguez et al. 1998; Vianna et al. 2006). Mitochondrial DNA is maternally inherited and provides only information on the female genetic structure of the population at a single marker. Therefore, information from both mtDNA and nDNA markers are used here to address the genetic structure of both sexes and to increase the number of markers evaluating the population structure within Puerto Rico and the relationship with Florida.

Genetic diversity is typically low in small populations and can be quickly eroded during stochastic or demographic events (Sherwin and Moritz 2000; Frankham et al. 2002). Low diversity can also negatively influence fitness, decrease population viability, and increase susceptibility to disease (O'Brien et al. 1983; Roelke et al. 1993). An integrative conservation approach uniting genetic and demographic information is needed to protect the biodiversity available in small manatee populations and to sustain ecological processes and evolutionary lineages. Here, mtDNA control region haplotypes and multilocus microsatellite

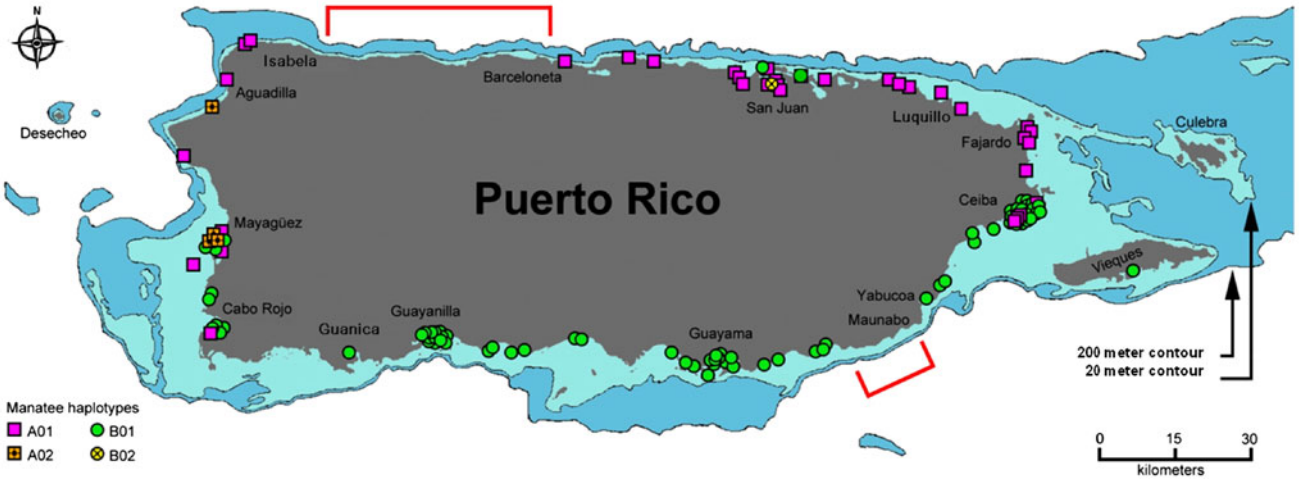


Fig. 1 Puerto Rico bathymetric map with manatee sample locations and assigned mitochondrial haplotypes. Red bars represent near-shore water depths ≥200 meters. Manatee samples were from each of the four coasts: North (Isabela to Luquillo), East (Fajardo to Yabucoa), South (Maunabo to Guanica), and West (Cabo Rojo to Aguadilla). (Color figure online)

genotypes are used to examine the relationship between Puerto Rico and Florida manatees and to address the level of variation and fine-scale genetic structure within the Puerto Rico population. The basic knowledge of migration and breeding levels can help to determine the genetic health of the population and assist with conservation and management decisions.

Materials and methods

Sample collection and DNA extraction

Florida and Puerto Rico manatee blood and dermis tissue were collected from recovered carcasses or during wild manatee health assessments. Additional Puerto Rico samples were collected through the manatee rescue and rehabilitation program. Puerto Rico manatee genomic DNA was isolated using QIAGEN's DNeasy Blood and Tissue kits (Valencia, California) for 112 animals, comprised of 50 males and 62 females. Puerto Rico manatee samples were from each of the four coasts: North (Isabela to Luquillo), East (Fajardo to Yabucoa), South (Maunabo to Guanica), and West (Cabo Rojo to Aguadilla; Fig. 1). Florida tissue DNA extraction techniques included traditional phenol/chloroform methods (Hillis et al. 1996) on carcass samples and QIAGEN's DNeasy Blood and Tissue kits on samples from live manatees. The 95 Florida samples used for the microsatellite analyses were chosen to represent proportionally the four geographically imposed management units (MUs), which are based on threats, available habitat, and usage patterns (U.S. Fish and Wildlife Service 2001; Haubold et al. 2006).

Microsatellite DNA analysis

Puerto Rico manatee samples ($N = 110$) were tested with 18 Florida manatee microsatellite markers and were determined to be polymorphic at 15 loci (García-Rodríguez et al. 2000; Pause et al. 2007). The three additional markers, *TmaE4*, *TmaE26*, and *TmaH23* (Pause et al. 2007; Hunter et al. 2010b) were monomorphic and were not included in the study. Isolated DNA was PCR amplified using: 14 ng DNA, 0.8 mM dNTPs, 1× PCR buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl, 0.001 % gelatin; Sigma Aldrich, Inc., St. Louis, MO), 0.04 units Sigma Jump Start *Taq* polymerase, 0.24 μM each primer and BSA where needed (Table 1). $MgCl_2$ concentrations were 3 mM, except for *TmaH13*, *TmaKb60*, and *TmaSC5,* which required 2 mM. Amplifications were carried out on a PTC-200 thermal cycler (MJ Research, Waltham, MA) using the following conditions: initial denaturing at 95 °C for 5 min, 35 cycles at 94 °C for 30 s, annealing temp for 1 min following Pause et al. (2007), and *TmaA02* (56 °C), *TmaE02*, *TmaE11*, *Tma F14* (58 °C), and *TmaE0, TmaH13* (60 °C), 72 °C for 1 min, final extension 10 min at 72 °C. Fragment analysis was performed on an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA).

GeneMarker version 1.5 (Soft Genetics, State College, PA), was used to analyze the microsatellite fragment data. The Florida and Puerto Rico microsatellite fragment analysis was conducted in the same laboratory, using standardized protocols. The Florida samples were PCR analyzed following Pause et al. (2007) and data analysis was conducted as described above. For consistent data scoring and combination of data sets, Puerto Rico analysis was conducted using Florida samples as scoring controls

Table 1 Characteristics of the 15 polymorphic microsatellite loci implemented on 110 Puerto Rico manatee (*T. m. manatus)* samples

Locus name	*BSA*	N_A	N_E	*PIC*	H_O	H_E
TmaA02		2	1.31	0.40	0.20	0.24
TmaE1	+	5	2.29	1.06	0.56	0.57
TmaE02		2	1.77	0.63	0.42	0.44
TmaE7	+	5	1.99	0.84	0.43	0.50
TmaE08		5	1.59	0.72	0.21	0.37
TmaE11		5	3.37	1.34	0.74	0.71
TmaE14	+	5	1.86	0.85	0.38	0.46
TmaF14		2	1.42	0.47	0.29	0.30
TmaH13		4	1.96	0.83	0.52	0.49
TmaJ02		3	1.76	0.72	0.50	0.43
TmaK01		4	1.85	0.72	0.64	0.46
TmaKb60		6	1.93	0.81	0.46	0.48
TmaM79	+	2	1.20	0.31	0.19	0.17
TmaSC5		5	2.25	0.93	0.63	0.56
TmaSC13		4	2.14	0.92	0.61	0.53

Optimized annealing temperature (T_m), addition of *BSA* (0.4 mg/mL), number of alleles (N_A), effective number of alleles (N_E), polymorphism information content (*PIC*), and the observed and expected heterozygosity (H_O and H_E)

Table 2 Microsatellite and mitochondrial genetic differentiation values for geographic regions within Puerto Rico

Geographic region	North	East	Southwest
North	–	0.479	0.537
East	0.026	–	*0.007*
Southwest	0.028	0.024	–

Microsatellite pairwise F_{ST} values (below diagonal) and mtDNA Φ_{ST} values (above diagonal) generated from *T. m. manatus* in three geographic regions in Puerto Rico. Italics indicate a non-significant value ($P \leq 0.007$)

and to standardized fragment scoring calls. A Microsoft Access database was used to store allelic information for all manatee samples. The error rate was determined by repeating PCR and genotyping analyses for 11 % of the individuals.

Microsatellite statistical analysis

The genetic diversity was estimated by the observed (H_O) and expected heterozygosity (H_E), polymorphism information content (PIC), average number of alleles per locus (N_A), and average effective number of alleles (N_E) using GenAlEx 6.41 (Table 1; Peakall and Smouse 2006). GenAlEx 6.41 was also used to calculate the inbreeding coefficient, F_{IS}. Departures from the expected genotypic frequencies in Hardy–Weinberg equilibrium (HWE) were tested using the Markov chain method (dememorization 1000, batches 100, iterations per batch 1000) in Genepop 3.4 (Raymond and Rousset 1995). Additionally, linkage disequilibrium was tested for non-random associations between alleles of different loci. The Markov chain method was implemented and the *P*-values were adjusted using Bonferroni sequential correction for multiple comparisons (Rice 1989). Micro-Checker (Van Oosterhout et al. 2004) was used to identify loci with evidence of null alleles. To assess the degree of relatedness within sample groups, and to limit the chance for sampling bias among closely related groups, the Queller and Goodnight (1989) mean estimator was calculated in GenAlEx 6.41. Relatedness values were calculated for known Florida cow-calf field-identified pairs ($N = 115$), which were not included in the Florida data set. The average value for the highly-related individuals was compared to the average values calculated for Florida and Puerto Rico, and groupings of samples collected on Puerto Rico's North, East, South, West, and the South and West coasts (S&W; Maunabo to Aguadilla) were considered.

To assess overall genetic differentiation at the population level, GenAlEx 6.41 calculated F_{ST} using the infinite alleles model and R_{ST} using the stepwise mutation model. Comparisons of groupings included Florida ($N = 95$) and Puerto Rico ($N = 110$), and within the three regions of Puerto Rico based on a strong haplotype structuring pattern (Table 2). The global F_{ST}, which is the proportion of the genetic variance contained within the population relative to the total genetic variance, was also calculated for Puerto Rico. Within Puerto Rico, Bottleneck 1.2.02 evaluated heterozygote excess under the sign test and mutation-drift equilibrium under the allele frequency distribution test (Piry et al. 1999). Since all but one microsatellite had dinucleotide repeats, the proportions were set in favor of the infinite alleles model (IAM = 95 %; Cristescu et al. 2009). Geographic distances were calculated using GPS waypoints. Monmonier's maximum difference algorithm can identify barriers between groups using geographical distance-corrected genetic distances (Pseudoslopes; Monmonier 1973). Monmonier's maximum difference was applied on a geometric network connecting all the samples by a Delaunay triangulation. The corresponding distance matrix identifies the edges of the triangulation associated with the highest rate of change. Isolates with known geographic sample-collection locations were used in this analysis (Florida, $N = 362$; Puerto Rico, $N = 110$). Principal Component Analysis (PCA) was conducted in Past (Hammer et al. 2001) using a distance matrix constructed in GenAlEx 6.41. PCA 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 as the first axis with less variation in each

additional dimension. LDNe (Waples and Do 2008) was used to estimate N_e with 95 % confidence intervals (CI) following the bias-corrected method of Waples (2006). The single point estimate method removes the downward bias associated with the true N_e being greater than the sample size used to estimate it (Waples 2006).

The program Structure 2.3.3 (Pritchard et al. 2000) was used to identify the genetic relationship and putative ancestral source populations of Florida and Puerto Rico manatees, and the genetic clusters within Puerto Rico. To keep the Florida and Puerto Rico sample sizes similar for this analysis, 95 geographically distributed Florida samples were used. Structure, a model based clustering algorithm, infers population structure by probabilistically assigning individuals without 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 can identify recent migrants by assuming that individuals have some proportion of membership (q) from each of K-clusters. Multiple Markov chains can delineate differences within populations; therefore 10 parallel chains were analyzed for $K = 1$–10, with a run-length of 100,000 repetitions of Markov chain Monte Carlo, following the burn-in period of 100,000 iterations. The most probable number of populations, K, was determined in Structure Harvester (Earl and vonHoldt 2011) by calculating ΔK, an *ad hoc* quantity related to the change in posterior probabilities between runs of different K-values (Evanno et al. 2005). Individual assignment success was recorded as the highest likelihood of assignment (q), and the percentage of individuals in a cluster with $q > 0.90$ was calculated. 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 the within-subset analyses, K was determined by identifying the level of K in which q values produced a consensus-clustering pattern across six independent runs. Within Puerto Rico, along with the admixture model, the Locprior model was used to uncover cryptic structure by providing priors for the Bayesian assignment process based on the region of collection and significant F_{ST} values (Hubisz et al. 2009). The Locprior setting is not biased towards detecting structure when it is not present (Hubisz et al. 2009). For the Locprior model, consensus analyses were performed in Clumpp 1.1.2 (Jakobsson and Rosenberg 2007) on the averaged scores for the inferred K value. Distruct 1.1 was used to visualize the Structure output (Rosenberg 2004).

A landscape-scale exploration analysis of patterns of genetic diversity and structure (genetic landscape shape) was performed using Alleles in Space (Miller 2005) to examine the existence and approximate location of barriers to gene flow between Florida ($N = 362$) and Puerto Rico ($N = 110$). This procedure allows for the graphical representations of genetic distance patterns across landscapes, through interpolation procedures of inter-individual genetic distances. A connectivity network among sample locations was generated based on pairwise geographic distances using the Delaunay triangulation method (i.e. each collection point was connected in a straight line to its nearest neighbors, creating a triangular network with no overlapping lines). The genetic distances between pairs of collection locations were then plotted at the geographic midpoints between collection locations along the network. Genetic distances were on the Z-axis and X- and Y-axes corresponded to geographic locations. Peaks are indicative of areas with high pairwise genetic distance and valleys are indicative of areas of low pairwise genetic distance. Positive peaks show genetic discontinuities or possible barriers to gene flow.

Mitochondrial DNA analysis

Primers from García-Rodríguez et al. (2000) were used to amplify a 410 base pair portion of the mtDNA control region displacement loop in 58 Puerto Rico samples. Vianna et al. (2006) previously published Puerto Rico control region sequences for the population, but did not include the geographic location of sample collection. Therefore, the samples and associated location, haplotype, and demographic information are included here. Puerto Rico individuals from both studies ($N = 112$; 22 North, 35 East, 37 South, and 20 West coast) and Florida individuals ($N = 28$) were analyzed. The mtDNA control region was PCR amplified with primers developed from regions of 100 % homology between cow and dolphin sequences (heavy strand primer, CR-5, and light strand primer, CR-4; Southern et al. 1988; Palumbi et al. 1991). The PCR reaction conditions were as follows: 10 ng DNA, 1× 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. PCR cycling profile: 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, Florida, USA with the BigDye terminator protocol developed by Applied Biosystems (Foster City, CA) using fluorescently labeled dideoxynucleotides. To verify sequences, haplotypes were aligned with manatee sequences located in GenBank using the default setting in Sequencher 4.5 (Gene Codes Corporation, Ann Arbor, MI). Control region fragments were sequenced in the 5′–3′

heavy-strand orientation. Finally, representatives from each haplotype and any ambiguous sequences were sequenced in the 3′–5′ direction to ensure the accuracy of nucleotide designations. Novel and rare sequences were sequenced in both directions at least twice to validate the sequence.

Mitochondrial statistical analyses

The degrees of differentiation, F_{ST} and Φ_{ST}, between Florida ($N = 28$) and Puerto Rico ($N = 112$) and among Puerto Rico's geographic regions were calculated using Arlequin 3.1 (Excoffier et al. 2005) and using an AMOVA in GenAlEx 6.41 (Peakall and Smouse 2006). Estimates of sequence divergence used the Kimura 2-parameter genetic distance model, to allow for differences between transition and transversion rates (Kimura 1980; Jin and Nei 1990). Within Puerto Rico, mtDNA differentiation estimates were calculated for the three geographic regions. The regions were treated separately based on distinct haplotype patterns and significant genetic diversity at nDNA loci (Table 2). Finally, Tajima's D of selective neutrality, the number of polymorphic sites (S), number of nucleotide substitutions (NS), genetic diversity (h), and nucleotide diversity (π) were calculated (Nei 1987; Tajima 1993).

Cytogenetic analyses

Giemsa-banded karyotype analyses were previously performed on Florida manatees (Gray et al. 2002). Therefore, to assess cytogenetic differences between the subspecies, banded karyotype analyses were performed on Puerto Rico manatees using trypsin and Giemsa staining (GTG) methodology following Gray et al. (2002). Sodium heparin Vacutainer tubes were used to collect blood and samples were transported as quickly as possible to the laboratory.

Results

Microsatellite DNA analysis

The Puerto Rico population was found to have similar, but lower levels of nDNA diversity ($H_E = 0.45$ (0.17–0.71); $H_O = 0.45$ (0.2–0.74); $N_A = 3.9$ (2–6); $N_E = 1.9$ (1.2–3.4)) compared to the Florida (Tucker et al. in press). Puerto Rico had a slightly higher average number of alleles than manatees from Belize and Mexico (Table 1; Hunter et al. 2010a; Hunter et al. 2010b; Nourisson et al. 2011). Within Puerto Rico, *TmaE7*, *TmaE08*, and *Tma*K01 deviated from Hardy–Weinberg equilibrium even after a sequential Bonferroni adjustment. This deviation may be due to inbreeding, the presence of null alleles, or cryptic substructuring of the population (i.e., Wahlund effect). Two loci, *TmaE08* and *TmaE14*, had evidence of null alleles due to a heterozygote deficiency. After 105 comparisons and a sequential Bonferroni correction, linkage disequilibrium was not observed for any pair (overall $\alpha = 0.001$, $P < 0.005$). The inbreeding coefficient ($F_{IS} = -0.004$) did not suggest inbreeding in the population. Private alleles were detected for Florida ($N = 18$) and Puerto Rico ($N = 16$). All samples amplified at a minimum of 14 loci. No scoring errors were identified after repeating PCR and genotyping analyses for 11 % of the 110 samples used in the microsatellite DNA analyses.

Sampling bias in the form of a disproportionately high number of closely related individuals within any single group was not found. When highly related cow-calf pairs were analyzed, the average relatedness value (r) was 0.21. However, within both Florida and Puerto Rico population data sets, $r = -0.01$, suggesting that neither of the sample-groups were highly related internally. Further, when Puerto Rico was analyzed by region, all values were less than zero (North $= -0.04$, East $= -0.04$, South $= -0.04$, West $= -0.08$, and South and West together $= -0.02$), suggesting that overall the individuals in the sample groups were not closely related.

Great genetic differentiation was found between Florida and Puerto Rico ($F_{ST} = 0.163$ and $R_{ST} = 0.119$ ($P \leq 0.001$); Wright 1978; Hartl and Clark 1997; Balloux and Lugon-Moulin 2002; Frankham et al. 2002). Global $F_{ST} = 0.101$ ($P \leq 0.01$) for Puerto Rico and indicated moderate differentiation among the subpopulations. When Puerto Rico samples were separated by coast, all comparisons were significant except for the South and West coasts ($F_{ST} = 0.002$, $P \leq 0.32$). When these samples were combined and compared to the North and East coasts, all three pairwise F_{ST} estimates were significant (Table 2). Puerto Rico was not found to be in mutation-drift equilibrium and bottleneck signatures were detected for the IAM ($P = 0.004$) and stepwise mutation model (SMM, $P = 0.023$), but not for the two-phase model ($P = 0.072$). The allele frequency distribution test remained in a normal L-shape distribution, perhaps due to the bottleneck occurring too recently to detect (Cristescu et al. 2009).

Monmonier's maximum difference algorithm analysis detected a single barrier in the Florida and Puerto Rico data. The PCA identified separation between Florida and Puerto Rico on the first and second axis with 90 % of the variation and in the first and third axis with 80 % of the variation (Fig. 2). Similar results were obtained using different weighting parameters. The genetic landscape shape analysis indicated a large barrier to gene flow located directly between the Florida and Puerto Rico populations (Inset, Fig. 2). For allele frequencies ≥ 0.05, $N_e = 51.9$ (CI = 37.7–74.5) for the Puerto Rico samples.

Bayesian methods in the program Structure assigned Florida and Puerto individuals to two genetic clusters

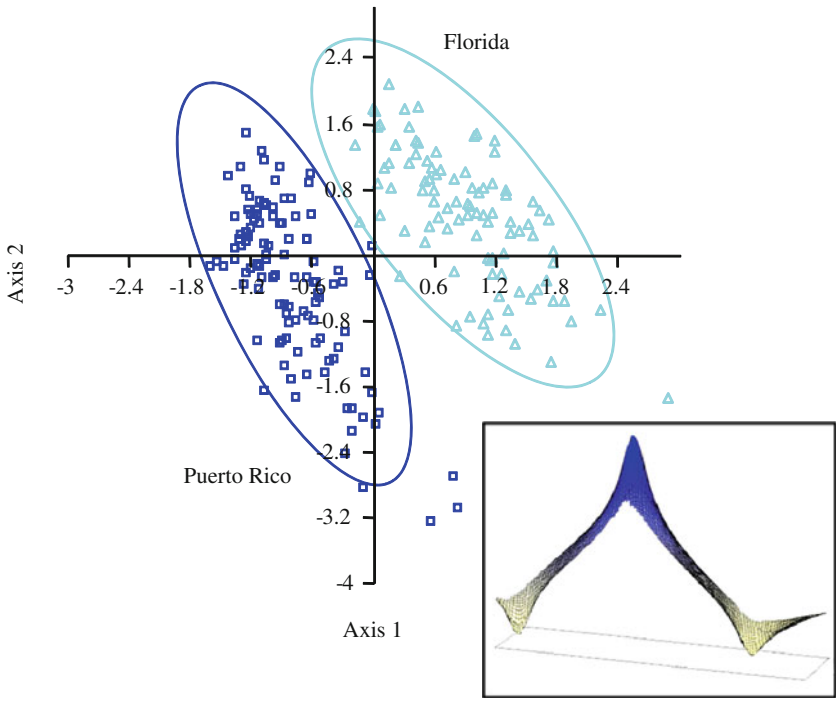


Fig. 2 Two-dimensional principle component analysis of microsatellite genotype data for Florida (*triangles*) and Puerto Rico samples (*squares*). Ellipses are 95 % confidence intervals for each collection. *Inset* contains genetic landscape shape interpolation analysis using genetic and geographic distances. Left and right valleys are Florida and Puerto Rico genotypes, respectively, indicating areas of low genetic distance. The positive peak (*blue*) between the populations indicates genetic discontinuity and a barrier to gene flow. (Color figure online)

($K = 2$) using log-likelihood and ΔK analyses (Figs. 3 and 4). The $\ln(Pr(X|K))$ were similar, with all estimates being much greater than $K = 1$. $K = 2$ captured the majority of the structure in the data with the fewest clusters and little admixture (Fig. 4; Pritchard et al. 2007). After $K = 2$, STRUCTURE continued to break Florida down into equally admixed clusters. The resultant $K = 2$ proportion of each Florida individual having ancestry in Florida was $q = 0.99$ (dark cluster) and each Puerto Rico individual having ancestry in Puerto Rico was $q = 0.98$ (light cluster; Fig. 3).

Following the log-likelihood $\ln(Pr(X|K))$ data analysis procedures, STRUCTURE identified $K = 1$ for the Puerto Rico population analyzed alone (Pritchard et al. 2007). Based on the strong mtDNA structure and significant F_{ST} values, the LOCPRIOR model was run with the North, East, and S&W groups. The log-likelihood was greatest for $K = 2$ ($\ln(Pr(X|K)) = 2{,}519$), with $K = 1$ being slightly less ($\ln(Pr(X|K)) = 2{,}526$; Fig. 4). Further, $K = 2$ had asymmetric proportions of assignment and was identified in the ΔK analyses as having the greatest second-order rate of change in $\ln(Pr(X|K))$; Figs. 3 and 4). Pritchard et al. (2007), suggested that the smallest K is often correct when several values of K give similar estimates of $\ln(Pr(X|K))$. However, the authors go on to say that if some individuals are strongly assigned to one population or another, and if the proportions assigned to each group are asymmetric, then there is a strong indication that real population structure is present. This is especially the case when the K value captures the majority of the structure in the data and is biologically sensible (Pritchard et al. 2007). The graphical results are presented for $K = 2$, with assignment to the light cluster recorded as North ($q = 0.56$), East ($q = 0.66$), South ($q = 0.88$), and West ($q = 0.89$). This structuring corresponds to the genetic differentiation between the North and East (N&E; Isabela to Yabucoa) and S&W groups identified by the mtDNA and F_{ST} values (Fig. 3).

Mitochondrial DNA analysis

Mitochondrial DNA sequences from the NCBI database were compared with the 58 Puerto Rico samples sequenced for this study, and the previously sequenced Florida and Puerto Rico samples (García-Rodríguez et al. 1998; Vianna et al. 2006). Of the resultant 112 Puerto Rico individuals, the haplotypes consisted of A01 ($N = 34$), A02 ($N = 4$), B01 ($N = 74$), and B02 ($N = 1$), a previously unidentified haplotype (GenBank Accession number: JX564997; Table 3). The mtDNA genetic diversity values ($\pi = 0.001$; $h = 0.49$) indicated that within Puerto Rico, there is low nucleotide divergence among the four haplotypes as they differed by only one or two base pairs. Within Florida, only the A01 haplotype was observed ($\pi = 0.000$). Adjusted

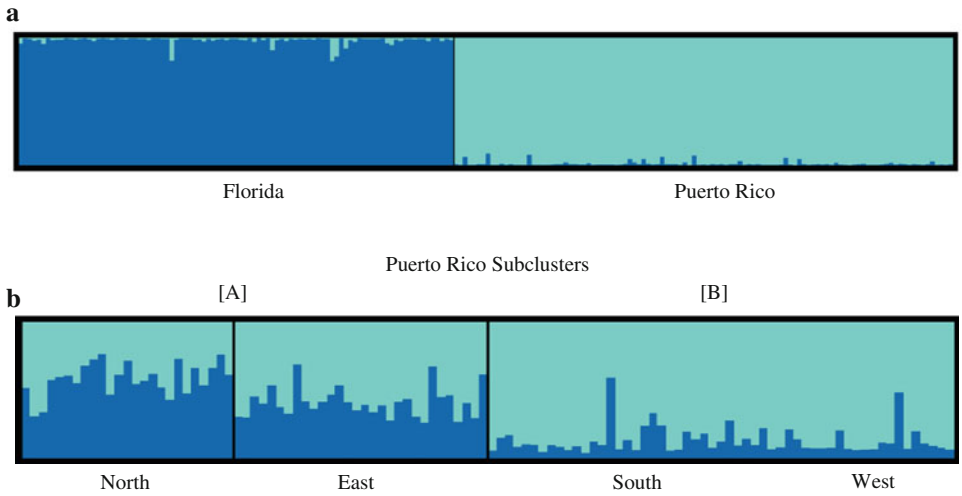


Fig. 3 Summary plot of q estimates generated by the sequential cluster analysis using the program STRUCTURE and 15 microsatellite loci. a.) Florida (*dark cluster*, $q = 0.99$) and Puerto Rico (*light cluster*, $q = 0.98$) manatee genotypes ($K = 2$). b.) Sequential analysis of the Puerto Rico genotypes indicating two subclusters ($K = 2$), [A] North & East (*dark cluster*, $q = 0.39$) and [B] South & West (*light cluster*, $q = 0.89$) subpopulations. Each individual is represented by a single vertical line, broken into colored segments and proportional to the membership in each of the K clusters. (Color figure online)

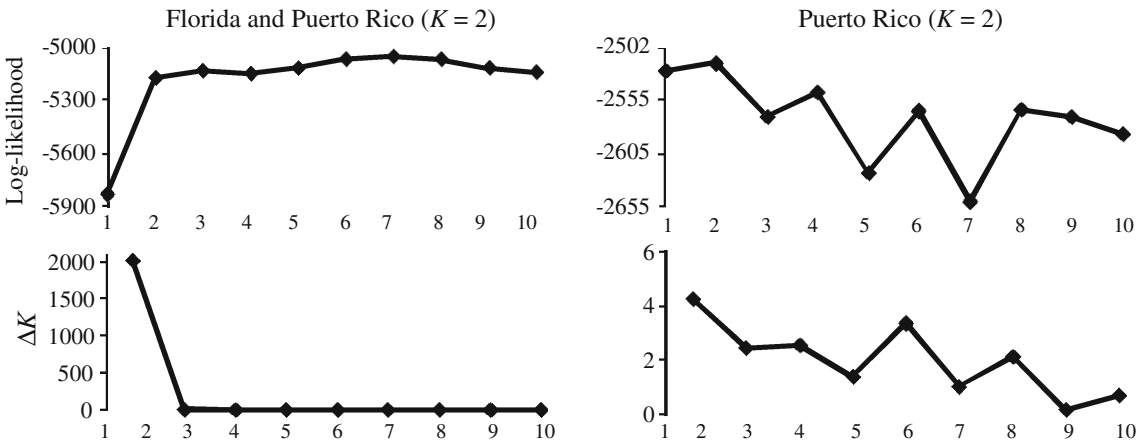


Fig. 4 Mean likelihood (*upper*) and ΔK vs. K (*lower*) plots for Florida and Puerto Rico genotypes and Puerto Rico genotypes alone

Florida and Puerto Rico mtDNA sequence divergence estimates were $\Phi_{ST} = 0.50$ (Kimura 2-parameter) and $F_{ST} = 0.66$ ($P < 0.0001$). Three polymorphic sites (0.73 %) and three nucleotide substitutions were identified in the four haplotypes (Table 3).

Within Puerto Rico, Tajima's D $= -0.08$ ($P < 0.5$) was not significant ($P < 0.05$); therefore, the null hypothesis of selective neutrality cannot be rejected. The Puerto Rico regions had strong haplotype division and Φ_{ST} values, including a $\Phi_{ST\ \text{Global}} = 0.46$ ($P < 0.0001$; Fig. 1 and Table 2). The North coast was composed primarily of the A01 haplotype with two B01 and one B02 individuals identified (Fig. 1). The South coast was composed entirely of the B01 haplotype. A mixture of A01 and B01 was detected along the East coast. A01, B01, and the other

Table 3 Summary of mitochondrial haplotype diversity in Puerto Rico indicating the number of variable sites and number of haplotypes by geographic region

Haplotype	Number of samples	Variable sites			Haplotypes by region			
		78	246	251	North	East	South	West
A01	34	T	C	C	19	9	0	6
A02	4	C	C	C	0	0	0	4
B01	74	T	C	T	2	25	38	9
B02	1	T	T	T	1	0	0	0
Haplotype diversity					0.11	0.13	0	0.29

Mitochondrial control region amplification of 410 basepairs on 112 *T. m. manatus* samples from the North, East, South, and West coasts. The variable site locations are denoted by the basepair position within the sequence

closely related haplotype, A02, were found on the West coast. When the nDNA genotypes were subjected to assignment testing, the North B01 individuals were assigned to the South B01 group, and the B02 individual was assigned to the East B01 group.

Cytogenetic analyses

The Giemsa-banded karyotype analysis confirmed that the Antillean manatee in Puerto Rico has 48 chromosomes (Supplemental Fig. 1). This is in agreement with the Florida manatee banded chromosome number (Gray et al. 2002) and the coastal West Indian Brazil manatee chromosome number determined by solid staining (Assis et al. 1988; Vianna et al. 2006). The banding pattern of Puerto Rico manatees was analogous to that observed in the Florida manatee (Gray et al. 2002).

Discussion

Puerto Rico and Florida manatees are distinct populations

The geographic separation and lack of gene flow between the Puerto Rico and Florida manatee populations has resulted in an accumulation of considerable genomic differences. The microsatellite divergence supports the recognized taxonomic subspecies classification (Domning and Hayek 1986) and the need for separate Puerto Rico and Florida management plans and conservation efforts (Mignucci-Giannoni 1996). The significant nuclear genetic differentiation ($F_{ST} = 0.16$) and large number of private alleles between Florida and Puerto Rico indicates great genetic differentiation between the populations (Wright 1978; Hartl and Clark 1997; Balloux and Lugon-Moulin 2002; Frankham et al. 2002). The mtDNA $\Phi_{ST} = 0.50$ value between Florida and Puerto Rico was slightly higher than that reported by Vianna et al. (2006; $\Phi_{ST} = 0.41$), perhaps due to the identification of additional rare haplotypes in Puerto Rico. Corresponding to the gene flow barrier between Florida and Puerto Rico, the B01 mtDNA haplotype has not been found in Florida to date, despite the strong prevalence of B01 in Puerto Rico. Between Florida and Puerto Rico, the microsatellite value was lower than the mtDNA $F_{ST} = 0.66$ value, suggesting female philopatry and male-biased dispersal (Prugnolle and de Meeus 2002). However, other factors can also lead to higher F_{ST} values for mtDNA as compared to nDNA, such as the larger effect of drift on uniparentally-inherited haploid loci (Handley and Perrin 2007). Further, the higher microsatellite mutation rate has also been shown to increase heterozygosity, resulting in decreased F_{ST} values (Balloux et al. 2000).

The mtDNA diversity levels found here were reduced as compared to other West Indian manatee populations (Vianna et al. 2006). Vianna et al. (2006) found that the manatee populations at the extreme ends of the species-range, such as Florida, Brazil, Puerto Rico, and the Dominican Republic, had the lowest polymorphic values. The authors attributed this finding to founder effects as manatees colonized higher latitudes following glacial events. The bottleneck detected in Puerto Rico was likely a result of fluctuations in population size due to environmental or anthropogenic impacts after founding event(s). Bottlenecks and the founder effect are considered extreme mechanisms of genetic drift. Drift occurs more quickly in small populations and can result in loss of heterozygosity and allelic diversity (Allendorf and Luikart 2007).

Genetic support for Puerto Rico as a distinct evolutionary lineage

The level of genetic differentiation between the Puerto Rico and Florida manatee populations warrants support for Puerto Rico as a distinct evolutionary lineage with separate management considerations. Vianna et al. (2006), used mtDNA sequences in the program Barrier 2.2 (Manni et al. 2004) to identify a gene flow barrier isolating Puerto Rico and the Dominican Republic from Florida, the Lesser Antilles, and South America. Immigration to Puerto Rico from outside sources is most likely low, since the nearby islands are believed to have biologically insignificant manatee populations (Jamaica and the Dominican Republic), or none at all (Guadeloupe, Haiti and the Virgin Islands; Quintana-Rizzo and Reynolds 2007). Genetic supplementation from the western Caribbean populations (e.g., Cuba and Central America) is not expected (Lefebvre et al. 2001; Quintana-Rizzo and Reynolds 2007), especially since the Florida and Belize manatee populations were shown to have no detectable nDNA admixture (Hunter et al. 2010a).

The isolated location, small size, and low genetic diversity of the Puerto Rico manatee population increases its susceptibility to rapid population decline and local extinction. The population estimate of 250 individuals is a cause for concern. However, the N_e was found to be 52, which is greater than expected for a population estimate of this size (Frankham et al. 2002). It has been suggested that at least 50 genetically effective breeders ($\sim$500 individuals) are needed to prevent inbreeding depression for short-term population survival (Wright 1951; Frankham et al. 2002). Yet, other research has also suggested that population levels in the upper hundreds to thousands are needed to maintain evolutionary potential (Traill et al. 2010).

Phylogeographic population structure in Puerto Rico

The large phylogeographic divergence between the N&E and S&W groups supports separate subpopulations within Puerto Rico. The strong mtDNA haplotype pattern is unusual for highly vagile mammalian populations, especially in the small geographic area of Puerto Rico (Deutsch et al. 2003; Fertl et al. 2005; Alvarez-Alemán et al. 2010). Distinct haplotypes were detected almost exclusively on the North and South coasts, indicating minimal movement between the regions and strong female philopatry. The mixed haplotypes of the East and West coasts suggest movement of A01 and B01 females into these regions, presumably from the North and/or South. Since the rare A02 haplotype was found exclusively on the West coast, it most likely evolved from A01 descendants on that coast and has not yet dispersed.

The phylogeographic division was also supported by the movement patterns of manatees tracked from 1992 to 2006 (Slone et al. 2006). The majority of the 33 radio-tagged manatees had restricted movement-patterns, remaining in close proximity to their capture site and alternating between seagrass beds and local fresh water sources. This limited movement may decrease gene flow and allow for the formation of genetic population structure.

The haplotype pattern in Puerto Rico is consistent with strong female philopatry. However, the weak nDNA Bayesian clustering results also suggest some degree of male-biased dispersal among the coasts. Male dispersal was documented when a radio-tracked A01 male manatee moved from the West to the North coast and back again (Slone et al. 2006). The presence of the extremely low frequency B haplotypes in the North could also indicate male-biased dispersal. For example, a mating herd with one A01 female and four males with one B01, one B02, and two A01 haplotypes was sampled after the group was struck and killed by a barge in San Juan, Puerto Rico.

Conserving regional diversity in the Puerto Rico manatee population

Puerto Rico manatees have lower microsatellite diversity than the average values reported for demographically challenged mammalian populations (DiBattista 2007; Garner et al. 2005; also see discussion in Hunter et al. 2010a). The low diversity may be intrinsic and/or influenced by the small census size, isolated location, and long-term anthropogenic impacts. Since diversity is considered necessary for adaptation to diseases and environmental changes, the low diversity may increase the extinction risk for Puerto Rico manatees (Reusch and Wood 2007). Genetic diversity can be increased through genetic mutation and immigration from divergent populations (Frankham et al. 2002). However, immigration from divergent populations, such as Florida, is unlikely under the current climatic conditions.

The Florida manatee population had higher microsatellite diversity than Puerto Rico (Tucker et al. in press). This may be due the Florida census size being an order of magnitude greater than Puerto Rico, resulting in more reproductive and mutation events. Florida has also had strong management protections and less history of severe hunting (O'Shea 1988; O'Shea et al. 2001). On the other hand, four related haplotypes have been identified in Puerto Rico, while only a single haplotype has been detected in Florida. The fixation of the A01 haplotype is likely due the founding of Florida from the Antillean islands.

To ensure the sustainability of the genetically divergent N&E and S&W Puerto Rico subpopulations, separate management plans concentrating on distinct threats within those regions would be the most beneficial. Providing protected travel corridors between the distinct regions could encourage natural gene flow to increase genetic mixing and improve diversity. Further, preserving the two genetically distinct populations separately could protect against future bottlenecks and loss of diversity, and help to lower the risk of extinction for the population overall.

Environmental features and manatee behavior may limit gene flow between the N&E and S&W subpopulations. West Indian manatees are rarely seen in open or deep ocean waters and typically remain close to shore to access freshwater sources and feed in shallow water (0.9–2.1 m; Lefebvre et al. 2001). The Northwest (Isabela to Barceloneta) and Southeast (Maunabo) areas of the Puerto Rico coast have narrow, insular shelves and high-energy wave action, potentially discouraging manatee movement (Fig. 1; red bars). Powell et al. (1981) and Rathbun et al. (1985), using aerial survey techniques, did not observe any individuals between the Culebrina River (West coast) to the Manatí River (central North coast; Fig. 1). The discontinuity in habitat may influence movement patterns, as seen with the radio tracking data, and corresponds to the N&E and S&W genetic subpopulation division. Further, manatees may be less likely to travel long distances or through deep water, since they are dependent on the limited freshwater resources within their home range. Historically, individuals of different haplotypes may have founded the Puerto Rico coasts and remained relatively isolated thereafter. Alternatively, mutations may have occurred following isolation and the resultant haplotypes drifted to fixation.

Natural manatee gene flow may also be restricted by coastal anthropogenic colonization and habitat destruction (Lefebvre et al. 2001). Within Puerto Rico, this could result in a smaller, fragmented, less diverse or less sustainable population. Manatees seek food, freshwater, and sheltered areas for rest and parturition in coastal habitat. Recently, a

large amount of development and increased recreational activity has taken place in coastal areas, especially in the Northeast and Southwest regions. Strandings of dead manatees from 1990 to 2006 ($N = 121$) occurred mostly in the North (25.6 %), East (18.2 %), and South (39.7 %) coasts, identifying these areas for threat mitigation (Mignucci-Giannoni et al. unpublished data, Bonde et al. 2012). Puerto Rico manatees were recently subjected to hunting pressure and are typically wary of humans. Therefore, they do not often utilize resources near areas of high human activity, such as commercial and recreation zones with high watercraft traffic (O'Shea et al. 1991; Mignucci-Giannoni et al. 2000; Quintana-Rizzo and Reynolds 2007). Providing areas with limited human presence along travel corridors and the preservation of habitat in the Southeast and Northwest corners of the island could help to encourage dispersal between the N&E and S&W subpopulations and allow for an increase in genetic diversity.

Along with separate management considerations from Florida, an updated and implemented Puerto Rico manatee recovery plan and cooperative multiagency agreements to mitigate threats and reduce mortality could improve the conservation of the population (Rathbun and Possardt 1986). Development of manatee Marine Protected Areas (MPAs) and the regulation and enforcement of boat and personal watercraft speed and traffic zones could reduce mortality and encourage utilization of manatee habitat. Currently, boat traffic poses the largest anthropogenic mortality threat to manatees in Puerto Rico and increases proportionally with the human population (Mignucci-Giannoni et al. 2000). As the environment changes in Puerto Rico, further genetic research along with systematic studies on health, habitat use, and response to stressors and toxins will assist in preserving the endangered Puerto Rico manatee.

Acknowledgments This work was conducted under the USFWS Wildlife Research Permit MA791721, issued to the USGS, Sirenia Project. Manatee rescue, carcass salvage, and specimen collection in Puerto Rico were conducted under USFWS permits PRT-770191, and under a cooperative agreement with Puerto Rico's Department of Natural and Environmental Resources. All samples were provided by the USGS, Sirenia Project and Caribbean Stranding Network. Funding for this project was provided by the USGS, the University of Florida College of Veterinary Medicine Aquatic Animal Health Program, the National Science Foundation and the Commonwealth of Puerto Rico Legislature. We would like to thank James Reid (USGS) and the reviewers for their guidance and critical review. We also would like to thank Ginger Clark, Sean McCann, and the Genetic Analysis Laboratory of the University of Florida for assistance with laboratory analysis. We gratefully acknowledge the assistance of Caribbean Stranding Network members and volunteers in the rescue, salvage, and necropsy of the manatee carcasses. Any use of trade, product, or firm names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

References

Acosta J (1590) Historia natural y moral de las Indias, en que se traten las cosas notables del cielo, y elementos, metales, plantas y animales de ellas: y los ritos y ceremonias, leyes, govierno y guerras de los Indios, Sevilla, España

Allendorf FW, Luikart G (2007) Conservation and the genetics of populations. Blackwell Publishing, Malden

Alvarez-Alemán A, Beck CA, Powell JA (2010) First report of a Florida manatee (*Trichechus manatus latirostris)* in Cuba. Aquat Mamm 36:148–153

Assis MFL, Best RC, Barros RMS, Yonenagayassuda Y (1988) Cytogenetic study of *Trichechus inunguis* (Amazonian manatee). Revista Brasileira De Genetica 11:41–50

Balloux F, Lugon-Moulin N (2002) The estimation of population differentiation with microsatellite markers. Mol Ecol 11: 155–165

Balloux F, Brunner H, Lugon-Moulin N, Hausser J, Goudet J (2000) Microsatellites can be misleading: an empirical and simulation study. Evolution 54:1414–1422

Bonde RK, Mignucci-Giannoni AA, Bossart GD (2012) Sirenian pathology and mortality assessment. In: Hines E, Reynolds J, Aragones L, Mignucci-Giannoni AA, Marmontel M (eds) Sirenian conservation: issues and strategies in developing countries. University Press of Florida, Gainesville

Bradley JL, Wright SD, McGuire PM (1993) The Florida manatee-cytochrome-b DNA sequence. Mar Mamm Sci 9:197–202

Cristescu R, Sherwin WB, Handasyde K, Cahill V, Cooper DW (2009) Detecting bottlenecks using BOTTLENECK 1.2.02 in wild populations: the importance of the microsatellite structure. Conserv Genet 11:1043–1049

Deutsch CJ, Reid JP, Bonde RK, Easton DE, Kochman HI, O'Shea TJ (2003) Seasonal movements, migratory behavior, and site fidelity of West Indian manatees along the Atlantic Coast of the United States. Wildlife Monographs 1–77

DiBattista JD (2007) Patterns of genetic variation in anthropogenically impacted populations. Conserv Genet 9:141–156

Domning DP (1994) A phylogenetic analysis of the Sirenia. Proc San Diego Soc Nat Hist 29:177–189

Domning DP (2005) Fossil Sirenia of the West Atlantic and Caribbean region. VII. Pleistocene *Trichechus manatus* Linnaeus, 1758. J Vert Paleontol 25:685–701

Domning DP, Hayek LC (1986) Interspecific and intraspecific morphological variation in manatees (Sirenia: *Trichechus*). Mar Mamm Sci 2:87–144

Durand J (1983) Ocaso de sirenias: esplendor de manatíes. Fondo de Cultura Economica, Mexico

Earl D, vonHoldt B (2011) STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conserv Genet Resour 4:359–361

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol Ecol 14:2611–2620

Excoffier L, Laval G, Schneider S (2005) Arlequin ver 3.0: an integrated software package for population genetics data analysis. Evolutionary Bioinformatics Online 1:47–50

Fertl D, Schiro AJ, Regan GT, Beck CA, Adimey N, Price-May L, Amos A, Worthy GAJ, Crossland R (2005) Manatee occurrence in the northern Gulf of Mexico, west of Florida. Gulf Caribb Res 17:69–94

Frankham R, Ballou JD, Briscoe DA (2002) Introduction to Conservation Genetics. Cambridge University Press, Cambridge

FWRI (2010) *Manatee Mortality*. http://research.myfwc.com/features/view_article.asp?id=7324. Accessed 19 July 2010

García-Rodríguez AI, Bowen BW, Domning DP, Mignucci-Giannoni AA, Marmontel M, Montoya-Ospina RA, Morales-Vela B, Rudin M, Bonde RK, McGuire PM (1998) Phylogeography of the West Indian manatee (*Trichechus manatus*): how many populations and how many taxa? Mol Ecol 7:1137–1149

García-Rodríguez AI, Moraga-Amador D, Farmerie WG, McGuire PM, King TL (2000) Isolation and characterization of microsatellite DNA markers in the Florida manatee (*Trichechus manatus latirostris*) and their application in selected sirenian species. Mol Ecol 9:2161–2163

Garner A, Rachlow JL, Hicks JF (2005) Patterns of genetic diversity and its loss in mammalian populations. Conserv Biol 19:1215–1221

Gray BA, Zori RT, McGuire PM, Bonde RK (2002) A first generation cytogenetic ideogram for the Florida manatee (*Trichechus manatus latirostris*) based on multiple chromosome banding techniques. Hereditas 137:215–223

Hammer Ø, Harper DAT, Ryan PD (2001) PAST: paleontological Statistics Software Package for Education and Data Analysis. Palaeontologia Electronica 4:9

Handley LJL, Perrin N (2007) Advances in our understanding of mammalian sex-biased dispersal. Mol Ecol 16:1559–1578

Hartl DL, Clark AG (1997) Principles of population genetics. Sinaur Associates Inc., Sunderland

Haubold EM, Deutsch C, Fonnesbeck C (2006) Final biological status review of the Florida manatee (*Trichechus manatus latirostris*). Final report, Florida Fish and Wildlife Conservation Commission, St. Petersburg

Hillis DM, Mable BK, Larson A, Davis SK, Zimmer EA (1996) Nucleic Acids IV: Sequencing and Cloning. In: Hillis DM, Moritz C, Mable BK (eds) Molecular systematics, 2nd edn. Sinauer Associates, Inc., Sunderland

Hubisz M, Falush D, Stephens M, Pritchard J (2009) Inferring weak population structure with the assistance of sample group information. Mol Ecol Resour 9:1322–1332

Hunter M, Auil-Gomez NE, Tucker KP, Bonde RK, Powell J, McGuire PM (2010a) Low genetic variation and evidence of limited dispersal in the regionally important Belize manatee. Anim Conserv 13:592–602

Hunter M, Broderick D, Ovenden JR, Tucker KP, Bonde RK, McGuire PM, Lanyon JM (2010b) Characterization of highly informative cross-species microsatellite panels for the Australian dugong (*Dugong dugon*) and Florida manatee (*Trichechus manatus latirostris*) including five novel primers. Mol Ecol Resour 10:368–377

Jakobsson M, Rosenberg NA (2007) CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. Bioinformatics 23:1801–1806

Jin L, Nei M (1990) Limitations of the evolutionary parsimony method of phylogenetic analysis. Mol Biol Evol 7:82–102

Kimura M (1980) A simple method for estimating evolutionary rate of base substitution through comparative studies of nucleotide sequences. J Mol Evol 16:111–120

Lefebvre LW, Marmontel M, Reid JP, Rathbun GB, Domning DP (2001) Status and biogeography of the West Indian manatee. In: Woods CA, Sergile FE (eds) Biogeography of the West Indies: patterns and perspectives, 2nd edn. CRC Press, Boca Raton, pp 425–474

Manni F, Guerard E, Heyer E (2004) Geographic patterns of (genetic, morphologic, linguistic) variation: how barriers can be detected by using Monmonier's algorithm. Hum Biol 76:173–190

Mignucci-Giannoni AA (1996) Marine mammal strandings in Puerto Rico and the United States and British Virgin Islands. University of Puerto Rico, Doctoral Dissertation

Mignucci-Giannoni AA, Montoya-Ospina RA, Jimenez-Marrero NM, Rodriguez-Lopez MA, Williams EH, Bonde RK (2000) Manatee mortality in Puerto Rico. Environ Manage 25:189–198

Miller M (2005) Alleles In Space (AIS): computer software for the joint analysis of interindividual spatial and genetic information. J Hered 96:722–724

Monmonier M (1973) Maximum-difference barriers-Alternative numerical regionalization method. Geogr Anal 5:245–261

Nei M (1987) Genetic distance and molecular phylogeny. In: Ryman N, Utter F (eds) Population genetics and fishery management. University of Washington Press, Seattle, pp 193–223

Nourisson C, Morales-Vela B, Padilla-Saldivar J, Tucker KP, Clark A, David Olivera-Gomez L, Bonde R, McGuire P (2011) Evidence of two genetic clusters of manatees with low genetic diversity in Mexico and implications for their conservation. Genetica 139:833–842

O'Brien SJ, Wildt DE, Goldman D, Merril CR, Bush M (1983) The cheetah is depauperate in genetic variation. Science 221:459–462

O'Shea TJ (1988) The past, present, and future of manatees in the southeastern United States: realities, misunderstandings, and enigmas. In: Odom RR, Riddleberger KA, Ozier JC (eds) Proceedings of the third Southeastern Nongame and endangered wildlife symposium. Georgia Department of Natural Resources, Game and Fish Division, Social Circle, pp 184–204

O'Shea TJ, Rathbun GB, Bonde RK, Buergelt CD, Odell DK (1991) An epizootic of Florida manatees associated with a dinoflagellate bloom. Mar Mamm Sci 7:165–179

O'Shea TJ, Lefebvre LW, Beck CA (2001) Florida Manatees: perspectives on populations, pain, and protection. In: Dierauf LA, Gulland FMD (eds) Handbook of marine mammal medicine, 2nd edn. CRC Press, Boca Raton, pp 31–43

Palumbi S, Martin A, Romano S, McMillan W, Stice L, Grabowski G (1991) Simple Fools Guide to PCR, version II. University of Hawaii, Honolulu

Pause KC, Nourisson C, Clark A, Kellogg ME, Bonde RK, McGuire PM (2007) Polymorphic microsatellite DNA markers for the Florida manatee (*Trichechus manatus latirostris*). Mol Ecol Notes 7:1073–1076

Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol Ecol Notes 6:288–295

Piry S, Luikart G, Cornuet JM (1999) BOTTLENECK: a computer program for detecting recent reductions in the effective population size using allele frequency data. J Hered 90:502–503

Powell JA, Belitsky DW, Rathbun GB (1981) Status of the West Indian manatee (*Trichechus manatus*) in Puerto Rico. J Mammal 62:642–646

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155:945–959

Pritchard JK, Wen X, Falush D (2007) Documentation for structure software: version 2.2. University of Chicago, Chicago, p 1–36

Prugnolle F, de Meeus T (2002) Inferring sex-biased dispersal from population genetic tools: a review. Heredity 88:161–165

Queller DC, Goodnight GK (1989) Estimating relatedness using genetic markers. Evolution 43:258–275

Quintana-Rizzo E, Reynolds J III (2007) Regional management plan for the West Indian manatee (*Trichechus manatus*). Caribbean Environment Programme, United Nations Environment Programme, CEP Technical Report, Kingston

Rathbun GB, Possardt E (1986) Recovery plan for the Puerto Rico population of the West Indian (Antillean) manatee (Trichechus manatus manatus Linnaeus, 1758). U.S. Fish and Wildlife Service, Atlanta

Rathbun GB, Carr N, Carr T, Woods CA (1985) The distribution of manatees and sea turtles in Puerto Rico, with emphasis on

Roosevelt Roads Naval Station. National Technical Information Service PB86-1518347AS.Springfield

Raymond M, Rousset F (1995) GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. J Hered 86:248–249

Reep RL, Bonde RK (2006) The Florida manatee: biology and conservation. University Press of Florida, Gainesville

Reusch TBH, Wood TE (2007) Molecular ecology of global change. Mol Ecol 16:3973–3992

Rice WR (1989) Analyzing tables of statistical tests. Evolution 43:223–225

Roelke ME, Martenson JS, Obrien SJ (1993) The consequences of demographic reduction and genetic depletion in the endangered Florida panther. Curr Biol 3:340–350

Rosenberg NA (2004) *Distruct*: a program for the graphical display of population structure. Mol Ecol Notes 4:137–138

Sherwin WB, Moritz CM (2000) Managing and monitoring genetic erosion. In: Young A, Clarke G (eds) Genetics, demography and the viability of fragmented populations. Cambridge University Press, Cambridge, pp 9–34

Slone DH, Reid JP, Bonde RK, Butler SM, Stith BM (2006) Summary of West Indian manatee (*Trichechus manatus*) tracking by USGS-FISC Sirenia Project in Puerto Rico. USGS-FISC, Gainesville

Southern SO, Southern PJ, Dizon AE (1988) Molecular characterization of a cloned dolphin mitochondrial genome. J Mol Evol 28:32–42

Stahl A (1883) Fauna de Puerto-Rico. Clasificacion sistématica de los animales que corresponden á esta fauna y catálogo del gabinet zoológica del doctor A. Stahl en Bayamón. Boletin Mercantil, Puerto Rico, p 248

Tajima F (1993) Measurement of DNA polymorphism. In: Takahata N, Clark AG (eds) Mechanisms of Molecular Evolution. Introduction to Molecular Paleopopulation Biology. Japan Scientific Societies Press, Sinauer Associates, Inc, Tokyo, Sunderland, p. 359

Traill L, Brook B, Frankham R, Bradsaw C (2010) Pragmatic population viability targets in a rapidly changing world. Biol Conserv 143:28–34

Tucker KP, Hunter ME, Bonde RK, Austin JD, Clark AM, Beck CA, McGuire PM, Oli MK (2012) Genetic diversity and minimal population substructure in the endangered Florida manatee: implications for conservation. J Mamm 93:6

U.S. Fish and Wildlife Service (1982) Endangered and threatened wildlife and plants (50CFR 17.11 and 17.12). U.S. Government Printing Office, Washington

U.S. Fish and Wildlife Service (2001) Florida manatee recovery plan (*Trichechus manatus latirostris*). Third Revision, United States Fish and Wildlife Service, Atlanta

U.S. Fish and Wildlife Service (2007) West Indian manatee (*Trichechus manatus*) 5-year review: summary and evaluation. United States Fish and Wildlife Service, Atlanta

Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. Mol Ecol Notes 4:535–538

Vianna JA, Bonde RK, Caballero S, Giraldo JP, Lima RP, Clark A, Marmontel M, Morales-Vela B, De Souza MJ, Parr L, Rodriguez-Lopez MA, Mignucci-Giannoni AA, Powell JA, Santos FR (2006) Phylogeography, phylogeny and hybridization in trichechid sirenians: implications for manatee conservation. Mol Ecol 15:433–447

Waples RS (2006) A bias correction for estimates of effective population size based on linage disequilibrium at unlinked gene loci. Conserv Genet 7:167–184

Waples RS, Do C (2008) LDNE: a program for estimating effective population size from data on linkage disequilibrium. Mol Ecol Resour 8:753–756

Wright S (1951) The genetical structure of populations. Annals of Eugenics 15:323–354

Wright S (1978) Evolution and the genetics of populations. volume 4. Variability within and among natural populations. The University of Chicago Press, Chicago