SIRENIAN CONSERVATION GENETICS AND FLORIDA MANATEE
(*Trichechus manatus latirostris*) CYTOGENETICS

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2008
To my mother, Mary Sudholt Kellogg, for her unwavering support and encouragement and instilling scholarly excellence in all I do.
ACKNOWLEDGMENTS

I would like to thank all the exceptional people who made this dissertation possible. My mentor, Dr. Peter McGuire has provided steadfast support, encouragement, and expert editorial advice. I thank him for providing me with an extensive and multi-discipline scientific education. I will always consult the life lessons he has so generously bestowed upon me. I also greatly appreciate the numerous national and international fieldwork and scientific conference opportunities. Mr. Robert Bonde has been extremely munificent with his immense sirenian knowledge. I appreciate all of the time he spent discussing and editing my work. This dissertation would not be possible without the samples and collaborations he has acquired throughout the past 30 years. I am grateful to have such wonderful mentors and role models. Dr. Kimberly Pause provided the foundation on which my population genetics knowledge is based. She has generously assisted with every aspect of this dissertation and has been a wonderful friend. Mr. Sean McCann has enthusiastically contributed to this project, providing the assistance I needed to complete it. Many thanks to Ms. Ginger Clark and the UF ICBR Genetic Analysis Laboratory for providing extensive technical and writing assistance and laboratory space for this project.

Ms. Cathy Beck, Ms. Susan Butler, Mr. Jim Reid, and the USGS Sirenia Project staff have been generous with their time and have graciously included me in many wonderful manatee captures. I am very thankful to partake in such exciting work with the experts in the field. The USGS provided funding for this project and the samples collected under the Sirenia Project permit (USFWS Wildlife Research Permit MA791721/4).

I would like to thank my committee members, Drs. Roger Reep, Roberto Zori, Timothy King, and Lynn Lefebvre, for providing guidance and editorial advice. Drs. Charles Courtney, Ruth Francis-Floyd, and the UF Aquatic Animal Health Program have been extremely generous
in providing financial support for this project and wonderful academic and fieldwork opportunities.

I would like to thank Drs. Roberto Zori, Thomas Dennis, and Mr. Brian Gray, and the UF Cytogenetics Laboratory staff for providing copious technical support and for taking time out of their important work and busy schedules for the manatee projects. I am also greatly indebted to Ms. Melanie Pate in Dr. John Harvey’s Laboratory for her knowledge, time, and equipment. She is a wonderful and patience teacher. Additionally, Ms. Linda Green and Ms. Diane Duke in the ICBR hybridoma core have provided expert assistance and technical skills. Lastly, I would like to thank Dr. William Farmerie and his laboratory for support, advice, and superior knowledge in the molecular field.

I am grateful to the collaborators that made this work possible. I thank Dr. Antonio Mignucci-Giannoni (Environmental Research) for the samples and his assistance with the Puerto Rico study. Dr. James Powell (The Sea to Shore Alliance) and Ms. Nicole Auil-Gomez (Wildlife Trust) provided samples and assistance with the Belize study. Drs. Janet Lanyon, Damien Broderick, Jennifer Ovenden, and Ms. Helen Peereboom (University of Queensland) provided samples, primers, and statistical analysis for the dugong microsatellite study. Drs. Roscoe Stanyon (University of Florence), Sandra Burkett, and Mr. Gary Stone (National Cancer Institute) provided the Zoo-FISH reagents, conducted cytogenetic experiments, and a great place to stay in Italy! Additionally, I thank them for their immense editorial assistance and superb scientific understanding.

I am ever indebted to my friends and family for all of their encouragement and support during the last four years. I thank Mr. Charles Hunter, the love of my life, for his support, excellent editorial suggestions, and always providing a willing ear to listen and a keen mind to
discuss science (and for cooking dinner the last two months!). Many thanks to my Melbourne Beach Sudholt family, Grandma, Marty, Ginny, Lois, Richard, and James, for providing unending prayers, love, and the courage to keep going; they have been my support-system through this process. I thank Ms. Meagan Beresford, a wonderful friend, for her encouragement and unwavering faith in my work. Always follow your dreams; they will transpire! I thank my brother, Mr. E. Harrison Kellogg, for his kindness, support, and wisdom beyond his age. Finally, I thank my mother, Ms. Mary S. Kellogg, whom this dissertation is dedicated to; she has instilled in me a deep love of learning, a spirit of perseverance (perfection), and a desire for academic excellence that were the foundation for this degree. Her unwavering support, encouragement, and faith have tremendously contributed to the completion of this dissertation. I thank her for inspiring the fortitude I needed to succeed!
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The threatened West Indian manatee is a slowly reproducing aquatic mammal, whose small, isolated populations are negatively impacted by habitat destruction and anthropogenic mortality. Long-term exploitation and small population sizes can lower genetic diversity, resulting in decreased fitness, reduced adaptation to environmental change, and potentially lead to extinction. Consequently, genetic studies, using microsatellite and mitochondrial DNA, were implemented to quantify the genetic diversity and identify unique populations or regions in need of protection. These studies will facilitate manatee conservation, management, and recovery efforts.

The West Indian manatee is composed of the Florida (*Trichechus manatus latirostris*) and Antillean manatee (*T. m. manatus*) subspecies. The Florida and Antillean Puerto Rico manatees are managed as a single endangered population under the U.S. jurisdiction of the Endangered Species Act. A recent status review suggested that the species be downlisted to threatened, primarily due to the recovery of the Florida population. In this study, the Florida and Puerto Rico populations were determined to be genetically distinct, and in conjunction with the differing habitats, threats, and population sizes, it is recommended that each population be managed separately.
Genetic studies of the Belize and Puerto Rico manatee populations detected reduced variation and subtle genetic structure, suggesting limited vagility and the potential for unique populations. The conservation of the subpopulations and delineation of corridors could maintain and potentially improve the low genetic diversity. Moreover, Belize is believed to be a source population, where adequate protection could lead to increased expatriation and repopulation of exploited regions.

To improve sirenian population genetic analyses, dugong (*Dugong dugon*) and manatee microsatellite primers were compared and the most informative loci for each group were selected. A panel of 11 dugong and 13 manatee cross-species and species-specific markers obtained better results utilizing fewer primers, improving time and cost effectiveness.

Finally, a cytogenetic study applied Zoo-FISH techniques to investigate the cross-species homology and evolutionary relationship of human and Florida manatee chromosomes. The clade Paenungulata was supported, linking manatees, hyraxes, and elephants and confirming their assignment to the Afrotheria super-order. Furthermore, Afrotheria, Xenarthra, or a combination of both was supported as the basal eutherian super-order.
Manatees and dugongs compose the Order Sirenia, and are large, herbivorous, obligate aquatic mammals. Early European explorers mistook these animals for mermaids. The order was named ‘sirenia’ when authors confused mermaids (half-women, half-fish) with the sirens of Greek mythology (half-women, half-birds). Sirens are sea nymphs who lured ancient mariners to their doom on dangerous rocks with mesmerizing songs. The name ‘manatee’ was either derived from the Latin word *manatus*, meaning “having hands,” as their pectoral fins resemble human hands, or from the Carib word *manati*, meaning udder, and referring to the mammary glands located beneath their flippers. The name ‘dugong’ was derived from the Malay word *duyung*, meaning “lady of the sea.”

Evolving from terrestrial quadrupeds, the order Sirenia originated near Africa 45-50 million years ago (Mya), during the Middle Eocene. The most primitive sirenian known to date, *Prorastomus* (48 -37 Mya), was discovered in Jamaica. The four-legged amphibious creature possessed adaptations still present in modern sirenians. These morphological characteristics include dense rib bones and an ancient fifth premolar, lost in the Cretaceous by all other mammals. The most recent common ancestor to extant Sirenians, *Protosiren* (37-33 Mya) possessed a modern sirenian skeleton that lacked hind limbs (Domning 1981b; Domning 1982b; Domning 2001).

The order is currently represented by five species in two families, Dugongidae and Trichechidae. In the family Dugongidae, the subfamily Dugonginae appeared in the Oligocene (33.9-23 Mya). Dugongidae contains two modern species, the extant dugong, and the extinct Steller’s sea cow. The dugong (*Dugong dugon*) inhabits tropical and subtropical regions in the
Indian and Pacific Oceans. Its closest living relative, the Steller’s sea cow (*Hydrodamalis gigas*) was hunted to extinction by 1768 (Stejneger 1887), only 27 years after its discovery by modern European humans. It lived in subpolar regions and fed on marine algae. Fossil records indicate that the adult sea cow was 25 feet in length, had flukes seven to eight feet long, and weighed more than 8,000 pounds.

The other Sirenian family, Trichechidae, comprises the genus *Trichechus* speculated to have evolved from the extinct *Ribodon* genus (5.3-3.6 Mya). The two genera have supernumerary molars, an adaptation for a grass diet, which are replaced horizontally throughout life (Domning 1982b; Domning 2001). Trichechids originated in South American lagoons and expanded into the Caribbean in the Late Pliocene to Early Pleistocene.

The family Trichechidae comprises three living species of manatee, the West Indian, Amazonian, and West African manatee. The West Indian manatee, *Trichechus manatus*, includes two subspecies, the Florida manatee, *T. m. latirostris*, and Antillean manatee, *T. m. manatus*. The Florida manatee is located throughout the coastal areas of the southeastern United States. The Antillean manatee is found in the Caribbean, Mexico, Central America and South America to the northeast coast of Brazil. West Indian manatees are distributed throughout rivers, estuaries, and marine environments. The Amazonian manatee, *T. inunguis*, is the only exclusively freshwater trichechid, and is restricted to the Amazon basin. The West African manatee, *T. senegalensis*, resides on the west coast of Africa from Senegal to Angola. Morphologically, manatees are similar. However, *T. manatus* tends to be larger and *T. inunguis* has a white pigmentation patch on its chest or abdomen and does not have nails on its pectoral fins.
Sirenia’s closest phylogenetic relatives are the orders Proboscidea, elephants, and Hyracoidea, hyraxes. Together, these orders form the clade, Paenugulata (De Jong & Zweers 1980; Kellogg et al. 2007). Sirenians and proboscidians diverged approximately 50-60 Mya (Rainey et al. 1984).

**Manatee Life History**

Manatees are large, long lived, and slowly reproducing aquatic mammals. They have a lifespan of up to 60 years. Upon adulthood, Florida manatees reach an average of 2.7 m (9 ft) and weigh between 400-545 kg (900-1,200 lb), with females of comparable age generally being larger. A maximum of 4.0 m (12 ft) and 1,772 kg (3,900 lb) has been recorded.

Female manatees become sexually mature between the ages of 3-5 and produce a calf every 2-5 years. Gestation is about 13 months and a calf may remain dependant on its mother for two years. Newborn calves have an average mass of 27 kg (60 lbs) and are 1.2-1.37 m (4-4.5 ft) long. Due to the low reproductive rate of the species, the human inflicted mortality rate may exceed the population’s ability to grow (Bossart 1999). The cow-calf pair is the main social unit for the species. Other social groups are transient and include mating herds and warm-water aggregations (Reynolds III & Odell 1991).

Extant sirenians inhabit tropical and subtropical regions and are the only strictly aquatic herbivorous mammals. West Indian and West African manatees are adapted to shallow water in fresh, brackish, or marine environments. The Amazonian manatee is limited to fresh water habitats. Water depths between 0.9-2.1 m (3-7ft) are preferred and manatees rarely dive deeper than 6.1 m (20ft). Most manatees migrate seasonally, between winter warm-water sites and summer distribution areas. Tracking studies and photo identification have indicated that some Florida manatees annually migrate long distances (Deutsch et al. 2003). Tracked manatees have ranged up to 850 km (Reid et al. 1991).
A diet constrained by low calorie aquatic plants may contribute to the species’ limited cold
tolerance. Manatees cannot maintain sufficient body heat in cool water and are restricted to
temperatures above 17-20°C (Glaser & Reynolds III 2003). To optimize the limited vegetation
 calories and minimize energy expenditure, a low metabolic rate has evolved. The metabolic rate
is 15-22% of what is expected for similar sized terrestrial animals (O'Shea & Reep 1990).
Sirenians have evolved large body sizes to process substantial quantities of food for sufficient
energy production.

The manatee diet consists of seagrasses in marine and estuarine systems, (*Syringodium
filiforme*, *Thalassia testudinum*, *Halodule wrightii*) and various submerged and floating fresh
water vegetation, (*Vallisneria americana*, *Ceratophyllum demursum*, *Hydrilla verticillata*,
*Myriophyllum spicatum*, *Ruppia maritime*, *Potamogeton pectinatus*, *Elodea canadensis*). The
fibrous diet along with unintentionally consumed substrate quickly erodes the teeth and has
selected for a supernumerary molar dental adaptation, otherwise known as hind-molar
progression (Domning 1982b). The molars wear down and are stimulated to move forward in
the jaw. Bone material from the socket in front of each tooth is replaced on the back of the tooth,
effectively moving the teeth forward. New molars erupt in the back of the row approximately
every year and manatees can produce an unlimited number of teeth in their lifetime (Domning
1983). This form of molar replacement is seen only in one other species, the Nabarlek rock
wallaby (*Peradorcas concinna*; Sanson 1980). The retention of five premolars, as opposed to
three in most herbivores, is a synapomorphic trait linking all present day sirenians (Domning
1994).

Manatees have the capability to remember the precise locations of resources. An
abnormally thick cerebral cortex is used for higher order information processing and long-term
memory storage (Purves et al. 2001; Reep & Bonde 2006). The size and layering patterns are comparable to carnivores and primates (Marshall & Reep 1995; Reep et al. 1989). The large cerebral cortex may be used to store the detailed locations of fresh water, aquatic plant beds, and winter warm water refuges. Manatees have strong winter site fidelity and predictable patterns of movement over great distances, indicating remarkable memorization skills (U.S. Fish and Wildlife Service 2001).

Manatees have poor vision, which is in part due to highly vascularized corneas (Bauer et al. 2003; Harper et al. 2005). Additionally, the majority of their time is spent in turbid water, which severely limits the use of eyesight. To compensate for reduced vision, manatee sensory systems have adapted to the aquatic environment. Instead of relying on eyesight, sensory tactile hairs on the face and body are used to identify vibrations and movements in the water column (Reep et al. 2002). This is analogous to the lateral line sensory organ in fish. The manatee is one of the few mammals to have tactile hairs on the body.

To feed easily on submerged vegetation, manatees are negatively buoyant, sinking at less than 10 m, while positively buoyant at the surface (Kipps et al. 2002). Dense rib bones provide a ballast or weight, allowing them to remain submerged.

The dense osteosclerotic rib bones lack marrow, except at the tips (Clifton et al. 2008). This is unusual, as most mammals have high concentrations of marrow in their long bones. Marrow is present in the manatee skull, vertebrae, and sternum. Hematology studies indicate that manatees have low numbers of white blood cells in comparison to domestic species. Manatees have both large and small lymphocytes, monocytes, and basophils. They do not have typical neutrophils, but instead pseudoeosinophils or heterophils, whose granules stain pink with the Wright-Giemsa stain. Elephants and hyraxes also have cells with similar staining
characteristics, linking the species (Schalm 1965). Manatees have large but low numbers of red blood cells, prolonging the rate of oxygen diffusion during a dive (Medway et al. 1982).

Despite the lack of marrow in the long bones, manatees have a superior immune system, wound healing, and repair process compared to other animals (Bonde et al. 2004). The manatee immune system appears highly developed to protect against the harsh marine environment and the effects of human-related injury (Bossart et al. 2002). They appear resilient to natural disease and traumatic human-related injury (Bonde et al. 2004; Buergelt & Bonde 1983; White & Francis-Floyd 1990). In fact, Bossart points to the immune system as a reason for their ability to survive in heavily polluted water (1999). Manatees are relatively immune to infectious agents, and show no clinical signs to infection by pseudorabies virus, San Miguel sea lion virus type 1, and equine encephalitis, porpoise and dolphin morbilliviruses (Duignan et al. 1995; Geraci et al. 1999).

**West Indian Manatee Conservation**

All three manatee species are considered vulnerable by the International Union for Conservation of Nature and Natural Resources (IUCN Thornback & Jenkins 1982). *T. manatus* and *T. inunguis* are listed in Appendix I and *T. senegalensis* is listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). The manatee was the first marine mammal to receive legal protection from hunting, granted by Florida in 1893. Additionally, the species received protection from the Endangered Species Preservation Act in 1967 and the Marine Mammal Protection Act in 1972. The U.S. Federal government granted the Florida manatee endangered status in 1973, due to excessive mortality and small population size (U.S. Fish and Wildlife Service 1979).

Manatees have been hunted throughout their habitat, exterminating or severely reducing some populations. Records from 1542 reveal that indigenous people hunted Amazonian
manatees for their meat and hides to make shields, shoes, or canoes (Best 1984). From 1935-1954, approximately 200,000 Amazonian and West Indian manatees were poached in Brazil (Best 1982; Domning 1981a; Domning 1982a). It is estimated that 4,000-7,000 Amazonian manatees were killed per year. The carcasses were rendered for oil and meat, and the hides were used to make machine belts, pulleys, and hoses (Best 1984). Currently, the *T. manatus* population in Brazil has approximately 500 individuals and is considered critically endangered by the Brazilian Action Plan for Aquatic Mammals (IBAMA 2001; Lima 1997; Luna 2001). The hunted Florida manatee population was reduced gradually from the 16th century through the early 1900’s, with the largest reduction in the 1800’s by European settlers (Hartman 1974).

Although no longer hunted, in 2006 417 Florida manatees died from natural and anthropogenic causes in an estimated population size of 3,000-4,000 individuals (Florida Fish and Wildlife Research Institute 2007b). Recent human-related manatee mortality has been caused by: crushing in flood control structures (O'Shea *et al.* 1985), entanglement or ingestion of debris or fishing gear (Beck & Barros 1991; de Thoisy *et al.* 2003; Marmontel *et al.* 1997; Mignucci-Giannoni *et al.* 2000) blunt or sharp trauma from collision with water vessels (Marmontel *et al.* 1997), and habitat destruction and alteration (de Thoisy *et al.* 2003).

Annually, direct and indirect human-related mortality can account for 33% of all Florida manatee deaths (Bossart 1999). In the late 1980’s, more than 100 manatees were found dead each year in a population of approximately 1,200 individuals. Many deaths were anthropogenic in nature, with boat strikes causing the most mortality (O'Shea *et al.* 1985). To decrease the number of human-related mortalities, manatee sanctuaries and boat slow speed zones were implemented (U.S. Fish and Wildlife Service 1996). Studies on life history and patterns of
seasonal movements and distributions provided information for management plans and habitat regulations (Deutsch 2000; Deutsch et al. 2003; O'Shea & Hartley 1995; Rathbun et al. 1990).

Many manatee populations have remained small due to a variety of natural mortality. Natural mortality is most frequently due to neonatal complications, cold weather stress (Bossart et al. 2003), and brevetoxicosis from red tide (Bossart et al. 1998). These events often lead to a large number of manatee deaths. In 1996, 150 manatees died from brevotoxicosis alone (U.S. Marine Mammal Commission 1996).

Integrative approaches are needed to investigate, manage, and monitor the extensively threatened Trichechus family. The conservation of manatees will require multi-discipline approaches including biological, ecological, and genetic data. In particular, conservation genetics can assist with identifying populations, genetic substructure, and quantifying immigration.

### Conservation Genetics

Conservation biology is the study of organisms, communities, and ecosystems directly or indirectly affected by human activities (Soule 1985). Within the discipline of conservation biology, genetics has been a powerful tool to quantify genetic variation and protect and manage threatened species (Frankham et al. 2002). Molecular studies can assist in protecting populations that are small or experiencing environmental change (Groves et al. 2002; Raven & Wilson 1992).

A major tenet of conservation genetics is the preservation or improvement of genetic diversity to allow greater amplitude for adaptation, speciation, or evolution. Reduced genetic diversity can result in a decrease of fecundity and survival and compromises a species’ ability to evolve and endure environmental change, potentially resulting in extinction (Avise 2004). Genetic diversity has been linked to fitness, population size, number of inbred individuals, and
population persistence. Small populations can suffer from inbreeding and reduced immune system variation, which increases susceptibility to disease, e.g., the cheetah (*Acinonyx jubatus*; O'Brien 1994), fitness and physical defects, e.g., the Florida panther (*Felix concolor coryi*; Roelke *et al.* 1993), and overall lower population viability (Sherwin *et al.* 2000).

Conservation genetics can identify unique populations and address issues of low variation. Substantial genetic variation, the degree varying from species to species, is essential for population vitality and persistence (O'Brien 1994). An extended period of low population abundance, or near extinction of the population, must occur for a substantial loss of genetic variation. Bottlenecks result in severe reductions in genetic variation due to loss of individuals, possibly resulting in inbreeding depression. During inbreeding depression, fitness may often decline in populations that recover more slowly or have reductions in heterozygosity. Low heterozygosity may be due to the buildup of mildly deleterious alleles and lethal heterozygous recessive alleles (Amos & Rubinsztein 1996; Westemeier *et al.* 1998). Delays in population recovery can cause further losses in fitness. Both theory (Nei 1975) and experiment (Miller & Hedrick 2001) have suggested that the impacts of severe bottlenecks may be minimized with rapid population recovery.

Conservation genetics can also assist in forensic applications, defining subspecies, understanding species’ biology, identification of management units, and the resolution of taxonomic uncertainties. Management regulations often rely on classifications of subspecies or distinct population segments (DPS) for supervision or special protections. A DPS is defined as a reproductively isolated or evolutionary significant population. Subspecies inhabit a unique habitat or geographic range from other members of the species and do not normally exhibit reproductive isolation. Therefore subspecies are distinguished by habitat and genetic differences
Another term, evolutionary significant unit, indicates genetically differentiated populations within a species and has been criticized for ignoring behavioral adaptation, such as mating rituals or habitat use, which can create a reproductive barrier before molecular changes occur (Crandall et al. 2000).

Genetic factors that can be addressed in conservation biology include the management of small populations, inbreeding and outbreeding depression, loss of genetic diversity, and the reduction of gene flow. Many wild populations have experienced substantial and long-term population losses. Glacier movement and strong hunting pressure can cause marine mammal population reductions. For example, northern elephant seal harvesting reduced the species to approximately 10-30 individuals in 1860. Small inbred populations can limit the effective population size ($N_e$) and in turn genetic diversity. The effective population size is the number of individuals in an idealized population that would have the same genetic properties as observed in a real population (Wright 1931). The effective population size is typically smaller than the actual population because of unequal sex ratios, fluctuation in population sizes, and variation in progeny numbers. Not all individuals contribute to the next generation equally. For instance, highly inbred individuals are genetically the same and count as fewer effective individuals to provide unique genotypes in the next generation.

**Molecular Markers**

**Mitochondrial and microsatellite DNA**

Genetic markers typically used in conservation genetics include chromosomes, allozymes, and mitochondrial and microsatellite DNA. Mitochondrial and microsatellite DNA are commonly implemented in population genetics due to their variability, ease of use, and high mutation rate. Non-invasive techniques can be used to acquire small samples of tissues for polymerase chain reaction (PCR) DNA amplification.
Circular mitochondrial DNA (mtDNA) molecules can assist in defining taxonomic relationships and can differentiate among populations within a species (Frankham et al. 2002). The maternally inherited mitochondrial genome provides female migration and lineage patterns. The cytochrome $b$ and displacement loop sequences in the control region are commonly used to distinguish haplotypes or sequence differences. Inferences are most accurate when the genetic diversity is statistically analyzed below the ordinal level. Haplotypes outside of the order may or may not follow the pattern of mutation observed within the lineage.

Microsatellites, or short tandem repeats (STR), are nuclear polymorphic loci with 2-6 base-pair (bp) repeat units. In most species, the markers provide enough variability to distinguish individuals from each other with high probability. Many loci are investigated to create a unique genotype for each individual. The majority of microsatellite loci are ‘selectionally neutral’ and base-pair mutations are not constrained by functional demands of natural selection, allowing for more variation.

Replication slippage is the primary mutational mechanism that creates microsatellites. During replication, the DNA polymerase enzyme may stutter, or the DNA strands may be displaced and realign incorrectly, leading to an insertion or deletion of repeat units (Ellegren 2000). The stepwise mutation model (Ohta & Kimura 1973) is the simplest model and assumes that the microsatellite expands or contracts by one repeat unit each mutation. This gain and loss of repeats can lead to a homoplastic state, false equality of alleles based on independent mutation to the same size. Therefore, individuals may be similar by state but not by descent. For this reason, statistics were adapted to address relatedness based on the mutational mechanism to achieve a certain state.
Nuclear microsatellite DNA is inherited from both parents and participates in a recombining process. Both parents have two alleles and the offspring randomly inherits one allele from each parent at every microsatellite locus. In a breeding population, the combination of microsatellites will become distinct from other populations.

The high microsatellite mutational rate provides a contemporary perspective of the population’s unique genetic signature. Statistical analyses are most appropriately conducted with individuals from the same species, geographic location, and evolutionary time. The increased potential for homoplasy and high mutational rates reduces the microsatellite phylogenetic signal throughout evolution. Comparisons across species compound this effect and are not recommended.

**Microsatellite and mitochondrial DNA comparisons**

Microsatellite and mitochondrial DNA data can be compared within a population, but may provide contrasting information. Mitochondrial DNA provides information only from the matrilineal lineage and has a slower mutation rate than that of microsatellites (Frankham et al. 2002). Because of these characteristics, mtDNA data characterize evolutionarily historical patterns of a population and can address phylogenetic relationships among species. Strict geographical partitioning of mtDNA lineages is often found in animal species with low or moderate dispersal abilities (Knoll & Rowell-Rahier 1998). Many marine mammal populations have strong matrifocal genetic structure. Consequently, more population structure is detected at mtDNA than at microsatellite DNA (Hoelzel et al. 2002). Microsatellite data provide information on female and male dispersal among the populations. Therefore, comparing the data from both markers may convey contrasting population structure due to sex-biased dispersal and/or different evolutionary periods being addressed.
The construction of phylogenetic trees can assist in the analysis of populations or species. The construction of a mitochondrial tree to illustrate phylogenetic relationships can be performed within an order, using the most recent common ancestor to root the tree. Alternatively, microsatellites cannot accurately quantify distant evolution between species, and therefore it is inappropriate to include an ancestor or root the phylogenetic tree. Excessive homoplasy is found in microsatellites due to high variation and slipped-strand mis-pairing. The phylogenetic tree affinities of microsatellite alleles can be deduced only from the repeat number. This may be a poor indicator of the true evolutionary relationships among long-diverged alleles. The ultimate consequence of extended periods of evolution with high mutation is deterioration of the phylogenetic signal, limiting microsatellite tree analyses to the recent genetic changes of a single species.

Measuring Mammalian Genetic Differentiation

Haplotype analysis

Molecular studies can quantify genetic variation and assess the evolutionary potential of species with three main statistical measures. The first measure is the number of haplotypes \( (k) \) in a population or species. When comparing the number of haplotypes among populations and studies, a similar sample size, number of populations, and base-pairs is needed. In contrast, the following two measures can address frequencies and averages and can be compared across studies. The first is haplotype diversity \( (h) \), which is a function of the number and frequency of haplotypes within a sample, or the probability that two randomly chosen haplotypes will be different in the sample (Nei & Tajima 1981). The second is nucleotide diversity \( (\pi) \), defined as the heterozygosity at the base pair level, or the average number of nucleotide differences per site in pair-wise comparisons among DNA sequences (Nei 1987). In contrast to haplotype diversity, \( \pi \) accounts for relationships among haplotypes. Depending on the loci and species surveyed, \( h \) is
above 0.5 for animal DNA and π is typically in the range of 0.001-0.020. When comparing among studies, the number of populations and markers are variables that should always be addressed.

**Genotype analysis**

The most extensively implemented technique for addressing genome-wide genetic diversity is the quantification of heterozygosity (H) and allelic diversity (A). However, pooling divergent populations in the analysis will incorrectly inflate the observed diversity. Sample number, number of populations, type of marker, and number of loci must be considered. The observed and expected heterozygosity (H₀ and Hₑ) values are averaged over many loci to characterize genetic diversity for a population or species. Observed heterozygosity is the number of heterozygotes, having two alleles at a locus, divided by the total number of individuals sampled. Expected heterozygosity is the fraction of a population that would be heterozygous if the population mated at random.

The second measure, allelic diversity, is the mean number of alleles per locus averaged over multiple loci. The values are obtained within a species or population and the results can be effectively compared across species or populations.

Heterozygosity and allelic diversity values can be compared over different variables and taxa. Microsatellite variation in threatened species is one of the most powerful and practical means currently available to quantify diversity. Allozymes, different alleles of a gene producing one enzyme and RFLPs, variation in DNA sequence detected by restriction enzyme cleavage, can also be used to measure these variables. Microsatellite genetic diversities do not strongly differ among placental mammalian orders (Garner et al. 2005). In large outbred placental mammal populations, the average microsatellite heterozygosity (Havo) for polymorphic loci is 0.6-0.7 and the average number of alleles per locus (Aave) is 8.8 (DiBattista 2007; Garner et al.
2005). In comparison, endangered species can have up to one-half lower genetic diversity than non-endangered species (Frankham *et al.* 2002). Compared to undisturbed populations, hunted or harvested, and fragmented populations were found to have reduced differentiation ($H_{ave} = 0.5-0.6$, $A_{ave} = 6.9$; DiBattista 2007).

A temporal effect was found in the genetic variation of historically or long-term disturbed populations. Populations with historical disturbances have lower diversity ($A_{ave} = 4.8$) than recent disturbances ($A_{ave} = 7.8$) and long-term disturbances have lower diversity ($A_{ave} = 6.5$) than short-term disturbances ($A_{ave} = 8.2$; DiBattista 2007). Many marine mammal populations experience similar genetic diversity losses from historical and long-term (~200 years) hunting pressures.

**Population Genetic Theory**

Basic theoretical principles create mathematical models of idealized populations and can compare them to natural populations. This comparison provides information on the processes responsible for the detected genetic patterns (Gillespie 1998).

**Hardy-Weinberg Equilibrium**

Hardy-Weinberg equilibrium (HWE) is a law stating that allele and genotype frequencies will reach equilibrium, defined by a binomial distribution, in one generation and remain constant in large, randomly mating populations that experience no migration, selection, mutation, or non-random mating. HWE is an assumption applied in many models and analyses and provides the basis for detecting selection and the effects of inbreeding. The HWE assumptions include large populations, diploid organisms, random mating, sexual reproduction, non-overlapping generations, and negligible migration, mutation, and natural selection. In a natural population, HWE expected and observed allele frequencies are calculated in a chi-squared analysis to determine if deviations are from chance or enduring processes (Frankham *et al.* 2002).
In general, large natural outbreeding populations are in equilibrium. The effects of migration, mutation, selection, and overlapping generations, although violating the model, are minimal in these populations. Alternatively, marine mammal populations often violate the HWE assumptions with appreciable effects. For example, marine mammals are long lived and therefore have lengthy and overlapping generations. Research has indicated that recently killed bowhead whales (*Balaena mysticetus*) still have harpoons in their bodies from the 1790s, which, along with analysis of amino acids, has estimated a life span of 211 years (George *et al.* 1999).

Secondly, elevated marine mammal migration and high dispersal capabilities may violate the model. Annually, adult male sperm whales (*Physeter macrocephalus*) migrate between the poles and tropical waters (Rice 1989a). Third, some marine mammal populations are extremely small, increasing the effects of migration, drift, and natural selection, such as the North Atlantic right whale (*Eubalaena glacialis*), numbering in the 300’s (Waldick *et al.* 2002) and the Puerto Rico Antillean manatee (*T. m. manatus*), estimated to have 250 individuals (Slone *et al.* 2006). Finally, many marine mammals exhibit non-random mating strategies. For example, dugongs (*Dugong dugon*) defend mating territories. Dominant males may mate with all of the females in an area, creating a high proportion of genetically similar offspring (Anderson 1997).

Manatees deviate from the HWE assumptions. Manatees have long and overlapping generation times and most populations are small. They have the potential for long distance migrations between populations. The manatee mating system appears to be promiscuous and random, with females being surrounded by a number of males. However, if only the strongest male succeeds in fertilizing a female, a few dominant males will contribute to the population, constituting a non-random mating system.
Statistical Methods

F-statistics

Numerous population studies address the partitioning of genetic variation within and among subdivided populations (Wright 1951). Differentiation is directly related to the population’s inbreeding coefficient among subpopulations. $F_{ST}$, the fixation index, is the effect of population sub-division due to inbreeding. $F_{ST}$ is calculated from the relationship between heterozygosity and inbreeding. It is the probability that two alleles drawn randomly from a population are identical by descent. High rates of gene flow lower the probability, while low rates increase the probability. Small populations have more potential for inbreeding and usually genetically drift more rapidly than large populations. A value above 0.15 indicates significant divergence between the populations (Frankham et al. 2002).

Computer simulations

Computer simulations provide the means for addressing complex models with many interacting factors. Maximum parsimony, maximum likelihood, Bayesian phylogenetic inference, and neighbor-joining trees are mathematical methods used in computer programs to estimate phylogenies and construct phylogenetic trees.

Maximum parsimony is a non-parametric statistical method, in which no assumptions are made about the frequency distributions of the variables being assessed. The method is ultimately used to create a phylogenetic tree. The correct tree topography has the least number of evolutionary changes between the character states. Parallel and convergent evolution can cause the method to be erroneous because it cannot decipher between the two processes. Secondly, maximum likelihood is a parametric statistical method, which makes assumptions about certain models of character evolution. This method must correctly account for differences in the rate of evolution among characters to estimate phylogenies accurately. A third method, Bayesian
phylogenetic inference, uses maximum likelihood to create a posterior distribution for a parameter generated by multiple alignments. It typically uses the Markov Chain Monte Carlo (MCMC) algorithm, which is a stochastic, non-deterministic repetition of algorithms, where the previous state is irrelevant for predicting the probability of subsequent states. Finally, neighbor-joining is a bottom-up clustering method used in the composition of phylogenetic trees. A distance matrix comparing all the individuals can be made from a variety of measures to create the tree. Nei’s distance, $D_a$, and Calvalli-Sforza and Edwards distance, $D_c$, are the most common measures for microsatellite analyses (Takezaki & Nei 1996). The tree is based on the minimum-evolution criterion and the least total branch length is preferred at each step of the algorithm.

**Application of Conservation Genetics**

**Taxonomic Standings**

Statistical models are used to address evolutionary relationships, resolve taxonomic uncertainties, and define management units. A recognized taxonomically distinct subspecies of the meadow jumping mouse (*Zapus hudsonius preblei*) was recommended for delisting from the U.S. Endangered Species Act (ESA) after morphological and phylogenetic comparisons determined it not to be significantly different from the other four subspecies (Ramey *et al.* 2005). A second study using 21 microsatellite loci and 1380 mtDNA base pairs revealed that *Z. h. preblei* was genetically distinct and confirmed the original taxonomic classification (King *et al.* 2006). Phylogeographical structuring of haplotypes and multilocus genotypes using Bayesian inference and trees, were found for all five subspecies of *Zapus hudsonius*. *Z. h. preblei* appears to have been reproductively isolated from the other subspecies for enough time to be on an independent evolutionary path. The study supported the threatened subspecies status under the ESA.
Although Florida and Puerto Rico manatees share a mitochondrial haplotype, the nuclear data confirm that the two populations are distinct. Florida and Belize manatees also appear to be on reproductively isolated paths. These preliminary data support the subspecies distinction between Florida and Antillean manatees.

**Habitat Fragmentation**

Molecular techniques can assess detrimental genetic effects in severely fragmented populations. Microsatellite markers addressed the effects of habitat fragmentation and loss on nine Florida black bear populations (*Ursus americanus floridanus*; Dixon *et al.* 2007). Small populations were isolated by habitat fragmentation, indicating inbreeding depression. Some Florida populations had extremely low genetic variation, although most were within the range of other North American black bears. Heterozygosity ranged from 0.27-0.71 in nine populations. The analyses compared genetic diversity and population size and found that maintaining bear populations above 200 individuals provided the most genetic variation. Additionally, managing the population as a metapopulation and creating or maintaining corridors assists in maintaining gene flow. Monitoring should be continued to indicate whether a translocation of a genetically distinct bear is needed to rescue a population from negative inbreeding effects.

The populations of Antillean manatees must also be monitored to prevent inbreeding. Many of these populations are small and isolated on islands or coastal refuges. Large-scale coastal construction or dock building could easily fragment the habitat. Specifically, to increase genetic diversity in Puerto Rico, it will be important to preserve the habitat linking the two putative subpopulations and perhaps provide or improve corridors.

**Pedigree Reconstruction**

Observing marine mammal reproduction to obtain information on mating success and lineages is difficult and logistically challenging. Additionally, females may mate with multiple
males. This lack of biological data and ambiguous paternity can be addressed with molecular tools. Male reproductive success was analyzed in the Mexican Pacific populations of humpback whales (*Megaptera novaeangliae*; Cerchio et al. 2005). Maximum likelihood analysis in the program CERVUS (Marshall et al. 1998), examined 13 microsatellite markers. A few highly represented males displaying successful reproductive mating tactics were expected. However, no male was assigned to more than three calves and most males sired only one calf. The lack of dominant males was attributed to previous whaling pressures, creating a young male population with less competitive abilities. Genetic diversity values were robust. The number of alleles ranged from 4-19 with an average of 10.1 and expected heterozygosity ranged from 0.338 to 0.889 with an average of 0.710. Similar studies could assist with understanding manatee reproductive success.

**Forensic Applications**

Molecular techniques were used in a forensic fashion to identify species and sample origin of cetaceans in Asia. The only cetaceans reported to be harvested are Minke whales and dolphins. Sold in unregulated Japanese markets, Minkes are taken in Japan’s program for scientific research and small cetaceans are harvested by the Japanese and incidentally in Korean fishing nets. Endangered species were suspected of being harvested. ‘Whale products’ purchased in markets in Japan and the Republic of South Korea were PCR amplified at the sample collection locations, so that only amplified products were removed from the countries (Baker et al. 2000). Microsatellite analyses determined taxonomic species and at times distinguished regional subpopulations of purchased products.

In the 655 products investigated, DNA sequencing identified 12 species or subspecies of whale, numerous dolphin species, sheep, and horse. Seven of the identified whale species are protected by international whaling agreements dating back to at least 1989. Tissues of Minke
whale from the exploited North Pacific and the protected Sea of Japan were identified. Mix stock estimates and maximum-likelihood methods determined that 31% of the Minke samples originated from the protected area. These results of undocumented/unreported exploitation were then added to a model of population dynamics for the International Whaling Commission.

**Cytogenetic Studies**

Sirenians are phylogenetic outliers and despite similarities in body shape, adaptations, and habitat, manatees and dugongs have no evolutionary relationship with the other major orders of marine mammals. The order’s geographical evolution is not fully understood and phylogenetic relations are based on morphology, limited recent biochemical, and genetic evidence. Sirenians likely originated in Eurasia or Africa and spread into tropical South America by the middle Eocene (45-50 million years ago; Domning 1994; Reynolds III & Odell 1991).

Paleontological evidence suggests that sirenians are members of the super-order Afrotheria, and are grouped with proboscidea (elephants) and hyracoidea (hyraxes) in the clade Paenungulata (De Jong & Zweers 1980). The manatee Zoo-FISH study presented here provides support of the evolutionary relationships within the super-order. Further support of this grouping was provided by cross-species Zoo-FISH within the clade (Pardini et al. 2007).

Additionally, the root of the ancestral eutherian tree has not been identified with certainty. Afrotheria, Xenarhtra or a combination of the two super-orders, contains the most basal placental mammalian karyotype. These classifications are complicated and could be resolved by using cytogenetic techniques.

Chromosome diversity can assist in differentiating species. Species usually differ in the number, shape, and/or banding pattern within their chromosomes. For example, the Chinese and Indian muntjacs (barking deer) morphologically appear to be the same species and are managed as such. However, their chromosomes are dramatically different, the Chinese muntjac having 46
chromosomes and the Indian muntjac males and females having 6 and 7 chromosomes respectively (Ryder & Fleisher 1996).

**Marine Mammal and Manatee Chromosome Investigations**

Previous marine mammal chromosome studies were restricted to conventional Giemsa stained chromosomes on a limited number of individuals (Arnason 1974a; Arnason 1974b; Arnason & Benirschke 1973; Assis et al. 1988; Duffield et al. 1967; Loughman et al. 1970; White et al. 1976b). These studies established the modal diploid number (2N), total number of chromosome arms (fundamental number), and restricted gross morphological features (size and centromere position).

Conventional Giemsa solid-stained chromosome studies, completed on a limited number of individual manatees, established the chromosome number as 2N = 56 for the Amazonian manatee (Trichechus inunguis Loughman et al. 1970) and 2N = 48 for the Florida manatee (White et al. 1976b). Following conventional Giemsa staining, chromosome-banding procedures allowed for the identification of individual chromosome regions. Giemsa and trypsin staining, or GTG-banding, was used to create karyotypes and ideograms for the Amazonian manatee (Assis et al. 1988) and the Florida manatee (Gray et al. 2002). The divergence of the two species occurred from a fusion or fission rearrangement in the homozygous state involving four biarmed autosome pairs in *T. manatus* or eight pairs of acrocentric autosomes in *T. inunguis*. An additional study was conducted on potentially hybridized Amazonian manatees and coastal Brazilian West Indian manatees. The individuals had a mixture of Amazonian and West Indian physical morphological characteristics. It had a *T. inunguis* mtDNA control region (T) haplotype but a diploid number of 2N = 50, intermediate between the two species. The authors extrapolated that the number of chromosomes could result from an F2 backcross of an F1 hybrid...
female breeding with a male *T. manatus*. There are limited additional cytogenetic data published on Sirenians.

**Zoo-FISH**

While analysis of chromosome banding homologies has been used extensively to investigate the evolutionary conservation of chromosomal segments and phylogenetic relationships among various mammalian species, established interspecific banding comparisons are speculative and should be confirmed by direct mapping of DNA sequences to chromosomes (Bielec *et al.* 1997).

Direct comparisons using *in situ* hybridization identifies conserved chromosomal blocks or regions and differentiates between true and false chromosomal banding similarities. Chromosomal banding does not differentiate between true phylogenetic (homologous) similarity and false or convergent (non-homologous) resemblance as seen by cross-species chromosome painting or Zoo- fluorescence in situ hybridization (Zoo-FISH; Stanyon *et al.* 1995). Direct comparisons are useful in identifying large chromosomal blocks of conserved syntenies, especially for species that do not share any morphological or banding pattern similarity or that diverged more than 90 million years ago (Rettenberger *et al.* 1995).

Phylogenetic studies and direct comparative mapping between vertebrate genomes have become possible through the development of comprehensive genome maps for humans and mice. Although the karyotype and ideogram of the Florida manatee have been completed, species comparisons should be made using cross-species chromosome paints or manatee specific sequences. These comparative genomic techniques are being applied to broad groups of animals to facilitate comparative genomic studies at the chromosome level (Bielec *et al.* 1998; Chowdhary *et al.* 1998; O'Brien *et al.* 1997). Zoo-FISH is informative for intraspecific, within the order, and interspecific, outside the order, comparisons. A probe from a species with a
mapped genome, consisting of a complex mixture of sequences, is hybridized to metaphase chromosomes from a different species, identifying the corresponding chromosome regions in both species as being evolutionary "orthologues."

Delineating homologous chromosomal segments among mammalian orders determines the order of genes and serves as the foundation of comparative genetic maps (Bielec et al. 1998). Zoo-FISH studies help to identify conserved chromosomal and neighboring segments, specific rearrangement patterns, major genes in “map-poor” species, and improve the understanding of the evolutionary processes of mammalian genomes through identifying phylogeny, ancestral genomic segments, and human race origins (Chowdhary et al. 1998). Comparative maps provide unique information for studying changes in genome organization and inferring the sequence of rearrangements occurring during mammalian evolution. Additionally, Zoo-FISH provides reliable information on linkage predictions and candidate disease trait and gene identification (Eppig & Nadeau 1995). Genes that are inherited as a single unit, i.e., linked, occur on the same chromosome.

The nature of conserved chromosomal segments represents a powerful suite of phylogenetic data for resolving the precise progression of mammalian evolution (O'Brien et al. 1997). Ancestral chromosomal segment identification will contribute to evaluating the human and manatee genomic divergence.

**Mammalian Zoo-FISH**

Comparative Zoo-FISH studies have delineated extensively conserved chromosome segments in the karyotypes of humans and other distantly related species, including the great apes (Wienberg et al. 1990), pig (Rettenberger et al. 1995), cow (Chowdhary et al. 1996; Hayes 1995; Solinas-Toldo et al. 1995), cat (Rettenberger et al. 1995), horse (Raudsepp et al. 1996) and mink (Hameister et al. 1997). Published comparative molecular cytogenetic data on marine
mammals are limited to the fin whale (Scherthan et al. 1994), harbor seal (Fronicke et al. 1997), and bottlenose dolphin (Bielec et al. 1998). Zoo-FISH comparisons between human (HSA) and harbor seal (Phoca vitulina) identified 31 conserved segments, 79% similarity, and covered the complete autosomal complement and the X chromosome. Zoo-FISH comparisons between human and bottlenose dolphin (Tursiops truncatus) found that all HSA chromosome paints, except the Y probe, hybridized to corresponding chromosomes on Tursiops. All dolphin chromosomes were painted except for the smallest submetacentric pair. Thirty-six segments of conserved synteny were identified (Bielec et al. 1998). Human and manatee chromosomes will be similarly compared to assess the evolutionary conservation between the two distantly related species.

Investigations of the West Indian manatee genome address the composition of phylogenetic relationships. Hybridization of human (Homo sapien; HSA) and West Indian manatee (Trichechus manatus latirostris) chromosomes directly compares the conservation of genomic evolution. Used as a template, the sequenced human genome establishes a preliminary map of the manatee karyotype. The comparative investigation evaluates the degree of syntenic conservation and chromosomal rearrangement. Evaluation of chromosomal rearrangements, such as inversions and translocations, can assist in discerning genome organization (Eppig & Nadeau 1995). Within the conserved segments, human whole chromosome paints also serve as a reliable guide for manatee gene order. Zoo-FISH maps for model animals usually show a 97% agreement with detailed gene mapping data (Chowdhary & Raudsepp 2001).

Manatee Zoo-FISH studies can be used to identify and quantify human conserved chromosome homology as well as evolutionary conservation and phylogenetic relationships within Afrotheria and Paenungulata.
Microdissection

Micro-manipulated dissection or chromosome microdissection is a cytogenetic technique primarily used to identify rearranged segments of abnormal chromosomes. This technique physically isolates whole or partial chromosomes using a microscopic needle. Multiple copies of the isolated DNA are produced through polymerase chain reaction (PCR) amplification. The product is used as a probe to identify homologous segments. Microdissection has been used to link chromosomal aberrations to cancer or inherited genetic disorders and to identify irregularities exhibited by chromosomes of tumor cells (Dennis & Stock 1999).

Varieties of studies use microdissection to produce probes from whole or partial chromosomes. Microdissected manatee probes can identify regions of homology with human chromosomes. This could support the results from the reciprocal experiments using human derived probes. Additionally, microdissected probes may clarify the position and orientation of homologous chromosome segments. West Indian manatee probes could be used to identify evolutionary divergence within the order sirenia and with other related species. This technique could also be used to identify rearrangements found in any aberrant manatee karyotype.

Sirenian Molecular Studies

All extant species of Sirenia are considered ‘vulnerable to extinction’ by conservation organizations. Many conservation efforts have been made to protect and manage West Indian manatees. Throughout Florida and Puerto Rico, steps have been taken to limit hunting and the number of anthropogenically related deaths. Biological studies have illuminated life history characteristics and tracking studies have disclosed movement patterns and habitat use. Genetic studies have elucidated potential population relatedness and structure and can assist in protecting unique populations.
West Indian manatees typically exist in small isolated populations. Small populations tend to have low levels of variation and can easily become inbred, cyclically reducing genetic diversity. This can then reduce fecundity, survival, and a population’s ability to cope with environmental change. Populations with low genetic diversity have a severely compromised ability to evolve and a high extinction risk. Molecular techniques can monitor these risks by quantifying inbreeding and genetic variation in a population.

Genetic markers may assist in identifying migration and defining management units within the species. Distinct or unique populations inhabiting different environments should be managed as separate populations. Manatee dispersal patterns and natal area can be investigated. Knowledge of a population’s genetic signature may lead to the identification of an individual’s original population, especially in the Caribbean where different populations reside on separated islands. Reproductive success in male manatees is difficult to assess because multiple males are involved in mating herds. Microsatellites have the capability to decipher paternity and male reproductive success. Molecular markers may also assist in detecting hybridization of *T. manatus* and *T. inunguis* in Brazil. Finally, nuclear markers have been developed to assign manatee sex (Tringali *et al.* 2008a). This is helpful in the case of badly decomposed carcasses or field conditions in which gender may not be evident.

**Sirenian Population and Conservation Genetic Studies**

The first manatee genetic study was conducted on 59 Florida manatees using 24 allozyme loci, 10 of which were polymorphic with two or three alleles (McClenaghan & O'Shea 1988). Allozymes represent different alleles of a gene producing one enzyme. The allozymes had 0.3 % polymorphic loci and 0.0500 mean heterozygosity in Florida. The estimates were determined to be equivalent or higher than estimates for other terrestrial and marine mammals. Genetic homogeneity was found throughout the differentiated regions in Florida and was most likely due
to high rates of gene flow throughout the peninsula. No unique alleles were observed among the regions. The authors did hypothesize that Florida’s genetic diversity was limited by decreased gene flow from other Antillean populations because of strong geographical barriers in the Straits of Florida and the northern Gulf of Mexico (Domning & Hayek 1986).

The McClenaghan and O’Shea (1988) study suggested that manatees have equivalent or higher heterozygosity than other terrestrial and marine mammals, most likely due to the high gene flow across the region. Allozymes are non-neutral gene products, which may experience selection. The diverse variables of the manatee habitat (i.e., salinity, temperature, and red tide) may cause the Florida manatee to have increased heterogeneity. Alternatively, this study may be biased due to the pooling of tissues and degradation of enzymes, which could increase the apparent number of alleles.

The first DNA-based study used mitochondrial cytochrome b to analyze the Florida population (Bradley et al. 1993). All individuals were determined to have the same haplotype, although the sample size was small and only 225 base pairs were sequenced. Sirenia’s evolutionary relationship with proboscideans was supported by an altered amino acid locus that is invariant for 20 other mammal species.

The following mitochondrial DNA study amplified 410 bps of the control region displacement loop in eight West Indian manatee populations (Garcia-Rodriguez et al. 1998). A total of 15 haplotypes was identified in 86 individuals. The pooled populations of the T. manatus species had high diversity $h = 0.839$ and $\pi = 0.04$. A strong division between populations was indicated with significant haplotype frequency shifts. Three lineages were apparent: Florida and the West Indies; the Gulf of Mexico to the Caribbean rivers of South America; and the northeast Atlantic coast of South America. The highest population diversity was in Guyana with suspected
hybrids, \( h = 0.857 \) and \( \pi = 0.044 \). The lowest population diversity excluding Florida, which had no variation, was Puerto Rico with \( h = 0.530 \) and \( \pi = 0.001 \). \textit{T. inunguis} diversity values were \( \pi = 0.005 \) and \( h = 0.875 \) (\( n = 16 \)). Only one haplotype was identified in Florida (A01), possibly due to a recent colonization or bottleneck. This haplotype was also found in Puerto Rico, connecting Puerto Rico to Florida historically.

In an effort to find a marker with a higher resolution of genetic variation, microsatellite DNA primers were developed and characterized (Garcia-Rodriguez \textit{et al.} 2000). Development of microsatellite primers implemented two enrichment techniques for nucleotide repeats; magnetic bead capture and nylon filters. From 61 microsatellite-bearing clones, eight polymorphic primer sets were identified in 50 Florida manatees. An additional three markers were polymorphic for Antillean and Amazonian manatees, while a total of nine was polymorphic for the dugong. The overall level of heterozygosity (0.410) and allelic diversity (\( A = 2-6, A_{AVE} = 2.9 \)) were low. The markers had some of the lowest levels of genetic diversity found in species-specific microsatellites, indicating limited intrinsic diversity, a founder effect, or bottleneck of evolutionary significance.

In a subsequent study, 361 bps of the mitochondrial DNA control region addressed the genetic differentiation and geographical population structure in the Amazonian manatee (Cantanheide \textit{et al.} 2005). A total of 84 Amazonian manatees represented 33 haplotypes (\( \pi = 0.0075, h = 0.909 \)). The majority of haplotypes (\( n = 25 \)) were found in one individual each, while only eight were found in multiple individuals. Amazonian manatees had more diversity than any of the three proposed West Indian manatee clusters. The high diversity may be from pooling divergent populations or a large population size. Subsequently, no genetic bottlenecks were detected.
A phylogenetic analysis showed no difference among Amazonian manatee populations. A nested clade analysis indicated evidence of minimal and long-distance dispersal, but also restricted gene flow among some regions. In essence, the population behaves in a panmictic fashion, but because of the vastness of the range, gene flow is reduced among isolated regions. This molecular signal is attributed to the seasonal migration patterns of distant dispersal during the flood season and the return to a few deeper areas during the low-water season.

The Amazonian manatee has been under protection for the last 40 years and calculations estimate a current population size of 445,000 female effective breeders ($N_{ef}$). Cantanhede et al. (2005) suggested that since 1960, the population has undergone a 95% increase in size due to reduced hunting. This is corroborated by calculations of life span, female reproductive age, and the number of calves produced each year. In 40 years, a single female could produce a lineage with 27 female offspring. Historical and current population size based on $\theta$ agrees with these estimates, increasing from $2.23 \times 10^4 N_{ef} (\theta_0 = 0.337)$ to $4.55 \times 10^5 N_{ef} (\theta = 6.565)$. The statistic $\theta$ uses sequence data to assess genetic difference, similar to $F_{ST}$. Since $\theta = 4N_{e}\mu$, where $\mu$ is the mutation rate, the number of effective breeders can be calculated.

The study also suggests that the genus *Trichechus* is most accurately viewed as four equally divergent lineages. The three *T. manatus* lineages mentioned in Garcia-Rodriguez et al. (1998) are as different from each other as they are from *T. inunguis*. Diverse aquatic biogeographical habitats may have produced the four distinct lineages.

Next, a study was conducted using a 410 bp fragment of the mtDNA control region on 330 individuals from the West Indian, Amazonian, West African, and dugong species (Vianna et al. 2006). Individuals from 10 countries revealed 20 West Indian, 31 Amazonian, and five West African manatee haplotypes. The West Indian manatees had the highest nucleotide diversity ($\pi =$
0.038648), followed by the West African manatee ($\pi = 0.019581$), and the Amazonian manatee
($\pi = 0.005353$). The $T. \textit{inunguis}$ and $T. \textit{manatus}$ values are similar to those reported in Garcia-
Rodriguez \textit{et al.} (1998) and Cantanhede \textit{et al.} (2005). Three haplotypes were observed in Puerto
Rico (A01, A02, and B01) and Belize (A03, A04, and J01). The program \textit{BARRIER} proposed two
geographic obstructions to migration and potential population divisions. A geographic
separation isolated the Dominican Republic and Puerto Rico from the other West Indian
populations and another isolated Guyana and Brazil.

The $T. \textit{manatus}$ subspecies split into three distinct clusters with median-joining networks
and neighbor-joining trees, displaying a heterogeneous geographical distribution. Cluster I was
composed of Florida, Mexico, the Greater Antilles, Central America and the Caribbean coast of
South America; Cluster II contained Mexico and Central America and the Caribbean coast of
South America; and Cluster III was the northeastern coast of South America (Brazil and
Guyana). These are comparable clusters to Garcia-Rodriguez \textit{et al.} (1998). A positive and
significant correlation was found between genetic and coastline geographical distances using a
Mantel test, supporting the idea that manatees migrate along the coasts and rarely in pelagic
ocean. The Florida manatee bottleneck was supported.

The authors concluded that the distribution of $T. \textit{manatus}$ is limited around the warm
waters of the equator and is a result of a stepping stone model of expansion along shallow coastal
waters. The coalescence time for the mtDNA lineages suggest that the Amazon manatee is the
most basal taxon followed by the West African and West Indian manatees. The mtDNA control
region data suggest that the Amazonian manatee is the sister group to the West Indian manatee
cluster I. Therefore, the species would be paraphyletic and not all of the descendants from the
most recent common ancestor would be included in $T. \textit{manatus}$. This is contradictory to the
control region data in Cantanhede et al. (2005), where four equally divergent lineages were identified. Possibly the addition of four West Indian haplotypes related to the Amazonian haplotypes influenced the results.

However, analyses of 615 bps of cytochrome b in the same study suggested that T. inunguis is the basal species and that T. manatus and T. senegalensis were derived from the same marine ancestor. This is in agreement with Domning (1994), making the marine species monophyletic. The authors then extrapolate that since T. inunguis is the most basal lineage, it may be the only surviving species of an ancient lineage adapted to fresh water. More analyses with additional characters are needed to determine the true relationship.

Potential hybrids from T. manatus and T. inunguis were studied using the mtDNA control region, two microsatellite primers and cytogenetic techniques. At the mouth of the Amazon River in Brazil and throughout the Orinoco River, eight potential hybrids were identified. Interspecific hybrids resulting from at least two generation (F2) backcrosses between T. manatus and T. inunguis were documented.

In a study of Australian dugongs, mitochondrial and microsatellite markers addressed migration and population structure (McDonald 2005). Of the 115 individuals analyzed, 52 haplotypes were identified, although only 19 were found in more than one individual. The 492 bps indicated 59 variable sites, $h = 0.96$ and $\pi = 0.029$. A strong separation at the now submerged Torres Straight land bridge indicated population division during the Pleistocene. The lack of homogenization between the two areas indicates female philopatry.

The authors also implemented six manatee microsatellite primers developed by Garcia-Rodriquez et al. (2000) that were highly polymorphic on dugongs. The analyses failed to indicate any geographic population structure across Australia. An analysis of molecular variance
AMOVA indicated high connectivity and gene flow among the regions. An isolation-by-distance pattern was found, most likely due to the large spatial scale of Australia. High allelic diversity may be an indicator of the larger population (100,000 individuals) and great geographic area inhabited by dugongs. Additionally, analyses did not indicate that the population had endured a bottleneck, as suggested in the Florida manatee population.

The lack of concordance between the nuclear and mtDNA results indicate male dispersal. Males travel between the two mtDNA assigned populations and provide the gene flow to homogenize the nuclear DNA. The two mtDNA lineages are interbreeding in the overlapping areas and assist in producing the isolation by distance pattern.

The next study focused on manatee microsatellites. The microsatellite primers developed by Garcia-Rodriguez et al. (1998) were not variable enough to provide individual multilocus genotypes for the Florida manatee. Therefore, to increase the resolution, additional microsatellite markers were developed (Pause et al. 2007). Multiple protocols were used: magnetic-bead capture technology, an unenriched library, and a biotinylated probe enriched library. A total of 10 polymorphic loci was identified with the number of alleles per locus ranging from 2-7 and an average of 4.2 alleles. Heterozygosity ranged from 0.321-0.680 with an average of 0.501. All primers amplified T. inunguis and all but one amplified D. dugon.

To increase the arsenal of polymorphic microsatellite primers on Florida manatees, Tringali et al. (2008b), developed 18 additional primers. A PCR-based isolation of microsatellite arrays (PIMA) method was used. The average number of alleles per locus was 2.5 and ranged from 2-4. Average heterozygosity was 0.34 and ranged from 0.02-0.78.

Lastly, microsatellite primers were developed for the dugong. A biotin-labeled oligonucleotide was used for library enrichment. A total of 32 polymorphic loci was
characterized but only 26 were reported (Broderick et al. 2007). The average number of alleles was 4.88 with a range of 2-10 alleles per locus. The average heterozygosity of 50 dugongs was 0.52, with a range of 0.12-0.84. Of the 26, 22 were polymorphic on one Florida manatee and six were polymorphic on an Asian elephant.

**Belize and Puerto Rico Manatee Conservation Studies**

In the following studies, further analyses will use mitochondrial and microsatellite DNA to gain an understanding of the West Indian manatee. Belize and Puerto Rico manatee populations will be compared to the Florida population. The study will address nearly all of the genetic issues Frankham et al. (2002) identifies in conservation biology, including: species’ biology, forensic applications, inbreeding and outbreeding depression, loss of genetic diversity, reduction of gene flow, management of small populations, defining management units, and the subspecies taxonomic distinction. Additionally, a dugong and Florida manatee cross-species microsatellite primer comparison was made to determine the most informative and efficient panel of markers to aid population studies for both families.

The long life span, elusive behavior, isolated habitat, and the turbid environment of West Indian manatees make it difficult to obtain information on movement and mating patterns. Relative to the long lifespan of the animal, mark-recapture or satellite tracking studies occur over short periods of time. Alternatively, genetic techniques can provide information on measurable parameters such as the movement of individuals and population structure. Comprehensive long-term movement patterns can be inferred from patterns of gene flow. Genetic fingerprinting can assist in the identification of individuals and parentage assignments.

The amount of interbreeding between the adjacent geographical localities of Belize, Florida and Puerto Rico will be quantified and the degree of genetic structuring within the
populations will be assessed. From these data, important ecological inferences and management decisions can be made to enhance the conservation efforts for the West Indian manatee.
CHAPTER 2

COMPREHENSIVE GENETIC INVESTIGATION RECOGNIZES EVOLUTIONARY DIVERGENCE IN THE FLORIDA (*Trichechus manatus latirostris*) AND PUERTO RICO (*T. m. manatus*) MANATEE POPULATIONS AND SUBTLE SUBSTRUCTURE IN PUERTO RICO

Introduction

Manatees (Sirenia: Trichechidae), inhabit tropical and subtropical waters and are the only obligate herbivorous aquatic mammals. The endangered West Indian manatee (*Trichechus manatus*) is found in freshwater rivers, estuarine, and marine environments. The West Indian manatee has two recognized subspecies distinguished by morphological, biological, and ecological data (Domning 1994; Domning 2005; Domning & Hayek 1986). The Florida manatee subspecies (*T. m. latirostris*) is restricted to the southeastern United States and Gulf of Mexico. Small populations of the Antillean manatee subspecies (*T. m. manatus*) exist in the West Indies, Caribbean, Mexico and Central and northeastern South America despite severe past exploitation (Domning & Hayek 1986; Hatt 1933). Low reproductive rate and intrinsic population density make this species particularly vulnerable to human disturbance (Bossart 1999). Ongoing habitat loss and high mortality threaten the future of the populations.

The West Indian manatee is recognized as a vulnerable taxon by the International Union for Conservation of Nature and Natural Resources (IUCN; Thornback & Jenkins 1982). The Florida and Puerto Rico populations are listed and managed together as endangered under the authority of the Endangered Species Act (U.S. Fish and Wildlife Service 1982). Sustained management and conservation efforts have resulted in the recent recommendation of downlisting the populations to a threatened status (U.S. Fish and Wildlife Service 2007). This may not be advantageous as the threats, habitat, population sizes, and needed protections are different for the
two groups. Additionally, the extent of migration and breeding between the two populations has not been thoroughly examined (Garcia-Rodriguez et al. 1998).

The Florida manatee population size is estimated to be 3300 individuals (Florida Fish and Wildlife Research Institute 2007b). The major causes of mortality in Florida include boat strikes (24%; O'Shea et al. 1985), perinatal (24%) and unknown (29%) (based on 6338 necropsies conducted from 1974-2007; Florida Fish and Wildlife Research Institute 2007a). The rate for each mortality cause has remained stable throughout the last 30 years, while known mortalities have increased, possibly a reflection of increasing population size.

The Puerto Rico population is estimated to be about 250 individuals with increasing threats and mortality (Slone et al. 2006). They are coastally marine, dependent on seagrass beds and sources of fresh water (river mouths, run-offs, coastal fresh water holes, water treatment plant outfalls, etc.) but rarely venturing inside the rivers (Mignucci-Giannoni 1989; Rathbun & Possardt 1986). Reported Puerto Rico manatee deaths are increasing an average of 9.6% per year (SD = 16.9%), and were higher during the last 20 years of the study, 1974-1995 (Mignucci-Giannoni et al. 2000). The major cause of mortality was due to human interaction, including net entanglement, boat strike, and poaching (52.2%; 15.6% watercraft), natural (22.2%; 20.0% dependent calves), and unknown (25.6%). Recently, watercraft mortalities in Puerto Rico have almost reached the proportions seen in Florida. An assessment of the genetic health of the population would benefit conservation efforts.

Conservation genetics represents a powerful tool for the assessment and management of threatened species (Frankham et al. 2002). Molecular markers can reveal genetic distinctiveness among or between taxa or populations with subtle or undetectable morphological differentiation, which traditional conservation biology techniques cannot. Genetic examination of animals has
contributed to the understanding of population structure, individual identification, life history traits, patterns of gene exchange, and genealogical or evolutionary relationships (i.e. phylogeny; Avise 2004). Genetic diversity is associated with fitness, population size and persistence, and the number of inbred individuals. Reduced genetic variation in a species can decrease fecundity, the ability to evolve and endure environmental change, and may eventually lead to extinction. Studies of endangered marine mammals, such as the Guadalupe fur seal (Weber et al. 2004) and the North Atlantic right whale (Waldick et al. 2002) have addressed low genetic variation and aided in conservation of genetically unique and endangered populations.

Previous studies of genetic diversity in Florida identified only one matrilineal haplotype, A01. The A01 haplotype was additionally identified in the Puerto Rico population along with two others, A02 and B01 (Garcia-Rodriguez et al. 1998; Rodriguez-Lopez 2004). It has been suggested that since the two populations contain the same haplotype, they may be related and experiencing immigration.

To date, low levels of diversity have been found in genetic studies of the West Indian manatee (Bradley et al. 1993; Garcia-Rodriguez et al. 1998; McClenaghan & O'Shea 1988; Vianna et al. 2006). Within Florida, no variation was identified in the mitochondrial (mt) cytochrome b or control region DNA (Bradley et al. 1993; Garcia-Rodriguez et al. 1998). Other manatee populations also have limited mitochondrial diversity, making it difficult to elucidate detailed geneflow within and between the manatee populations. To improve the resolution of genetic investigations, more variable Florida manatee nuclear microsatellite markers were developed (Garcia-Rodriguez et al. 2000; Pause et al. 2007; Tringali et al. 2008b). These markers allow for examination of population genetic units, evolutionary and taxonomic patterns, and forensic investigations to identify regions of origin (Garcia-Rodriguez et al. 1998). The
robust marker panel successfully provided each analyzed Florida individual with a unique genotype. The Florida population was determined to have low genetic diversity, possibly due to a bottleneck or founder effect (expected heterozygosity = 0.501; average number of alleles = 4.2). A fundamental knowledge of the diversity within Puerto Rico can help to determine the genetic health of the population and assess the frequency of new recruits into the population.

An integrative conservation approach that identifies and sustains ecological processes and evolutionary lineages is needed to protect and manage the biodiversity present in these small populations. The analysis presented here uses mitochondrial control region haplotypes and multilocus microsatellite genotypes to examine the relationship between Florida and Puerto Rico manatees and to address the level of variation and fine scale genetic structure within the Puerto Rico population. The identification and characterization of migration, colonization, and extinction processes (Avise 2004) will assist the conservation of the populations.

**Materials and Methods**

**Sample Collection and DNA Extraction**

Florida and Puerto Rico manatee blood and epidermis tissue were collected from recovered carcasses or during wild manatee health assessments. Additional Puerto Rico samples were collected from manatees in the rescue and rehabilitation program. Puerto Rico manatee genomic DNA was isolated using QIAGEN's DNeasy Blood and Tissue kits (Valencia, California) for 115 animals, 52 males and 63 females. Florida tissue DNA extraction techniques are described by Pause et al. (2007). From the Florida dataset, 96 individuals were randomly chosen, proportionally representing the four geographically imposed management units: Northwest, Southwest, Atlantic, and St. Johns River.
Mitochondrial DNA Analysis

Primers from Garcia-Rodriguez et al. (2000) were used to amplified a 410 base pair portion of the mitochondrial DNA control region displacement loop in 81 Puerto Rican samples, while additional sequences were obtained from Vianna et al. (2006). In total, 115 sequences from Puerto Rico (22 North, 36 East, 37 South, 20 West coast animals) and 96 sequences from Florida were analyzed.

The mitochondrial DNA control region was polymerase chain reaction (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; Palumbi et al. 1991; Southern et al. 1988). The PCR reaction conditions were as follows: 10 ng DNA, 1 x PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.001% gelatin; Sigma-Aldrich, Inc., St. Louis MO), 0.8 mM dNTP, 3 mM MgCl₂, 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, FL with the BigDye terminator protocol developed by Applied Biosystems Inc. using fluorescently labeled dideoxynucleotides. To verify sequences, haplotypes were aligned with manatee sequences located in GenBank using the default 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.

The degrees of differentiation, FST and ΦST, between Florida and Puerto Rico and among Puerto Rico’s geographic regions were calculated using ARLEQUIN 3.1 (Excoffier et al. 2005).
Estimates of sequence divergence used the Kimura 2-parameter genetic distance model (Jin & Nei 1990; Kimura 1980). The variance distribution was based on haplotype frequencies alone; all haplotypes were treated as equally differentiated (FST). Lastly, Tajima’s D of selective neutrality, the number of polymorphic sites, S, number of nucleotide substitutions, NS, the genetic diversity, \( h \), and nucleotide diversity, \( \pi \), were calculated (Nei 1987; Tajima 1993).

**Microsatellite Analysis**

A total of 15 microsatellite loci (Garcia-Rodriguez et al. 2000; Pause et al. 2007) was used for the Puerto Rico samples. The remaining three polymorphic Florida manatee markers, \( TmaE4, TmaE26, \) and \( TmaH23 \) (Pause et al. 2007) were determined to be homozygous in Puerto Rico manatees. Isolated DNA was PCR amplified using: 14 ng DNA, 0.8 mM dNTPs, 1x Sigma PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.001% gelatin), 0.04 units Sigma Jump Start Taq polymerase, 0.24 µM each primer and BSA where needed (Table 2-1). MgCl2 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 (Table 2-1), 72°C for 1 min, final extension 10 min at 72°C. All individuals were successfully amplified at a minimum of 14 loci. Fragment analysis was performed on an Applied Biosystems ABI 3730 Genetic Analyzer. GENE MARKER, version 1.5 (Soft Genetics, State College, PA), was used to analyze the microsatellite fragment data. The microsatellite data for Florida samples were kindly provided for use in this manuscript (Pause et al. 2007). A Microsoft Access database was used to store allelic information.

**Statistical Analysis**

The level of polymorphism was estimated by the observed (\( H_O \)) and expected heterozygosity (\( H_E \)), polymorphic information content and the number of alleles per locus (\( A; \))
Table 2-1) using GENALEX 6 (Peakall & Smouse 2006). 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 & 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 1989b). To assess overall genetic differentiation at the population level, GENALEX 6 calculated $F_{ST}$ using the infinite alleles model and $R_{ST}$ using the stepwise mutation model. Comparisons included Florida and Puerto Rico and Puerto Rico divided into nine categories that could be interchangeably grouped: male (M), female (F), North (N), South (S), West (W), East haplotype A01 (EA), East haplotype B01 (EB), dry (December to May) and wet (June to November) season. Geographic locations were based on sample recovery location, haplotypes, radio tracking data, usage areas, and genetic differentiation. The South group included the B01 individuals from the Southwest corner, as genetic differentiation test indicated that these were not significantly different. EA and EB were combined to east (E) for the male and female geographic comparisons to maintain sample sizes for proper comparisons.

**Cluster analysis using multi-locus genotypes**

The program STRUCTURE 2.2 (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 subdivision within Puerto Rico. 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 assumes that individuals could have some proportion of membership \((q)\) from each of \(K\) clusters, leading to the potential identification of recent immigrants. Multiple Markov chains can delineate differences within populations; therefore three 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 10,000 iterations. The three values for the estimated \(\ln(Pr(X|K))\) were averaged, from which the posterior probabilities were calculated. The \(K\) with the greatest posterior probability \((Pr \approx 1.0000)\) was identified as the optimum number of subpopulations. If the \(\ln(Pr(X|K))\) does not fluctuate strongly, the fewest number of clusters with the greatest \(Pr\) is correct. 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. A 0.90 assignment value indicates that the individual is highly assigned to the cluster, with little likelihood it belongs to a different cluster.

To test the relatedness and degree of Florida and Puerto Rico admixture, the two groups were analyzed together. A subsequent analysis was conducted with a clustered Florida group acting as a divergent population, since only one population was detected when Puerto Rico was analyzed alone. This allowed subtle Puerto Rico population differentiation to be detected by the program.

**Un-rooted neighbor-joining trees**

Neighbor-joining trees based on individual and population genetic distances were used to visualize relationships among populations, subpopulations, and individuals. The Phylogeny Inference Package (PHYLIP; Felsenstein 2004) estimated genetic distances based on pairwise Calvalli-Sforza and Edwards chord distance, \(D_c\). \(D_c\) is based on allele frequencies and provides accurate microsatellite tree topology (Takezaki & Nei 1996). Trees were constructed by
comparing individual genotypes with or without *a priori* partitioning. Comparison of the Florida and Puerto Rico individual genotypes was conducted with MICROSAT, version 1.5d (Minch *et al.* 1997), to create a distance matrix and NEIGHBOR in PHYLIP to produce the tree. To create population trees, *a priori* sub-grouped allele frequencies were subjected to the programs in PHYLIP, using 1,000 replicates with the bootstrap support values at the branching nodes of the tree. The lengths of the branches represented relative genetic distances. Geographic localities were analyzed from Florida and Puerto Rico. Due to the strong geographical haplotype structuring and genetic division identified in Puerto Rico, the genetic distances among the samples from the four geographic regions: N, S, E, and W were compared using a neighbor-joining tree.

**Cytogenetic Analyses**

Giemsa-banded karyotype analyses have only previously been performed on the Florida subspecies (*T. manatus latirostris*). Therefore, to assess cytogenetic differences between the subspecies, banded karyotype analysis was performed on Puerto Rico animals. Sodium heparin vacutainers were used to collect blood and samples were transported as quickly as possible to the laboratory. The cytogenetic analysis followed protocols described by Gray *et al.* (2002).

**Results**

**Mitochondrial Sequence Analysis**

Mitochondrial DNA sequences from the NCBI database were compared with the 81 Puerto Rico samples sequenced for this study, and the previously sequenced Florida and Puerto Rico samples. Within the 115 Puerto Rico individuals, 35 A01, four A02, 75 B01, and one B02, a previously unidentified haplotype, were found (*π* = 0.00132; *h* = 0.48500). This indicates that within Puerto Rico, there is a moderate to low chance of randomly drawing two different haplotypes, but low nucleotide divergence among those haplotypes. Within Florida one
haplotype was observed (A01; \( \pi = 0.00000 \)). Adjusted (net) Florida and Puerto Rico mtDNA sequence divergence estimates were \( \Phi_{ST} = 0.49596 \) (Kimura 2-parameter) and \( F_{ST} = 0.65696 \) (\( P < 0.00001 \)). Three polymorphic sites (0.73%) and three nucleotide substitutions were identified in the four haplotypes.

Within Puerto Rico, Tajima’s \( D = -0.08330 \) (\( P < 0.50200 \)) was not significant (\( P < 0.05 \)) therefore; the null hypothesis of selective neutrality cannot be rejected. Analysis of molecular variance (AMOVA) of data genetic differentiation between the strongly structured haplotypic regions in Puerto Rico was global \( \Phi_{ST} \) and \( F_{ST} = 0.77967 \) and 0.81501 (\( P < 0.00001 \)), respectively. The five Puerto Rico regions had strong haplotype division (Figure 2-1). The north shore was composed primarily of the A01 haplotype with one B01 and one B02 individual identified. When the nuclear genotypes were submitted to assignment testing, the B01 individual was assigned to the south (B01) and the B02 individual was assigned to the east (B01) group. The south shore was composed entirely of the B01 haplotype. A mixture of A01 and B01 were detected along the east coast. A01, B01, and the other closely related haplotype, A02, were located on the west coast. This mitochondrial DNA pattern suggests female specific site-fidelity on the north and south coasts and some movement to the east and west coasts.

**Microsatellite Marker Analysis**

The Puerto Rico population has low levels of nuclear polymorphism (\( H_E = 0.447 \) (0.173-0.708); \( H_O = 0.454 \) (0.191-0.745); \( A = 3.9 \) (2-6)) as compared to the Florida population (\( H_E = 0.480 \); \( A = 5.3 \)) over 18 loci. Additional results for the Florida population can be found in Pause et al. (2008). Within Puerto Rico, three loci deviated from Hardy-Weinberg equilibrium (\( TmaE7 \), \( TmaE08 \), and \( TmaK01 \)) even after a sequential Bonferroni adjustment. The deviation may be due to inbreeding, substructuring of the population (i.e., Wahlund effect), or the presence of null alleles. Two loci (E08 and E14) had evidence of null alleles due to a heterozygote
deficiency. After 105 comparisons and a Bonferroni correction, linkage disequilibrium was not observed (overall $\alpha = 0.05$, $P < 0.001$). The inbreeding coefficient $F_{IS}$ was -0.004 overall and did not suggest inbreeding in the population. Private alleles were detected for Florida (16) and Puerto Rico (13) at low frequency. The error rate was determined by re-genotyping 11% of the individuals. All detected errors were due to inconsistencies in the PCR, fragment analysis, or scoring. Average error among all alleles was 16.6% due to one problematic Puerto Rico sample. No errors were found after that sample was removed from the analysis.

Genetic differentiation among populations was estimated by using pairwise $F_{ST}$ and $R_{ST}$ comparisons. Pairwise $F_{ST}$ and $R_{ST}$ values for Florida and Puerto Rico were 0.163 and 0.119, respectively, and significant ($P < 0.001$). The majority of values among the five geographic groups in Puerto Rico were low but significant (Table 2-2).

The proportion of the genetic variance contained in the Puerto Rico population relative to the total genetic variance, $F_{ST}$, was 0.1008 ($P < 0.01$), indicating moderate differentiation among the populations. Of the 10 pairwise $F_{ST}$ estimates, nine were significant. Moderate but highly significant ($p \leq 0.007$) $F_{ST}$ population structure was identified for N and EA (0.051) and W and EA (0.064). Of the 10 $R_{ST}$ comparisons, five were significant. Significant $R_{ST}$ values indicating moderate to strong structure with W versus N (0.052), EB (0.074), S (0.099), and EA (0.111). It should be noted that the $R_{ST}$ value of adjacent units N and EA was slightly over significance ($P < 0.053$) with moderate differentiation (0.048).

The only significant female $F_{ST}$ comparison with moderate genetic differentiation was E and W (0.051). Significant female $R_{ST}$ values were higher, including S and W (0.085) and E and W (0.096). Male $F_{ST}$ values were low but significantly different between N and E (0.027) and N and S (0.044). No male $R_{ST}$ values were significant.
Significant male and female pairwise F_{ST} comparisons included FE and MS (0.047) and FE and MW (0.063). The significant male and female R_{ST} comparison FW and MS (0.138) indicated strong structure.

**Cluster analysis using multi-locus genotypes**

Bayesian methods in the program STRUCTURE assigned individuals to genetic clusters without *a priori* population designation. The Florida and Puerto Rico manatees analyses had similar fluctuating $\ln(Pr(X|K))$ estimates \{K = 2-8\} with K = 2 being the fewest number of clusters capturing the major structure in the data with little admixture (Pritchard & Wen 2004). The resultant $K = 2$ proportion of each Florida individual having ancestry in Florida was $q = 0.986$ and each Puerto Rico individual having ancestry in Puerto Rico was $q = 0.979$ (Figure 2-4). Florida analyzed alone indicated four clusters (Pause et al. 2008).

The Puerto Rico population analyzed alone showed no delineation of population structure. The results remained mostly unchanged from 1-10 clusters with the lowest value at $K = 1$ cluster (-2576). Since Florida and Puerto Rico were determined to be genetically distant, Puerto Rico was run with a genetically similar Florida cluster serving as a divergent group for comparison. This analysis produced $K = 4$ clusters (-2945), with Florida individuals grouping as one cluster and the Puerto Rico population breaking into 3 clusters. Low $q$ values were detected in each cluster, indicating admixture of these individuals.

The first Puerto Rico cluster, $q = 0.76$, contained 41 individuals, 71% from the N and E. No seasonal or haplotype grouping was detected. The first 18 individuals were 82% N and E with an average $q = 0.91$. Clusters 2 and 3 had low assignment values, $q = 0.48$ and 0.56, respectively, indicating reduced genetic signature or admixture. Cluster 2 contained 45 individuals and was mostly composed of S and W animals (78%) with 69% of its total membership collected during the rainy season. It was mostly composed of B01 haplotypes.
(80%) but did contain the four A02 haplotypes in the W Puerto Rico population, three of which grouped together. The E animals in cluster 2 were all B01. Cluster 3 contained 23 individuals with a majority of N and E individuals (65%) and 70% of its members being collected during the rainy season.

**Un-rooted neighbor-joining trees**

The neighbor-joining method is useful to address evolutionary relationships between populations (Nei 1996). The Florida and Puerto Rico individual neighbor-joining tree represented a robust division between the two subspecies. All individuals were correctly assigned to their respective populations except for two Florida animals. These individuals are migrants or had genotypes similar to Puerto Rico. As these are subspecies and have not been separated for a long evolutionary time, it is likely that individuals may have similar genotypes by chance. Additionally, a large genetic distance separated the Florida and Puerto Rico groups (Figure 2-2). The Florida and Puerto Rico population neighbor-joining tree depicted a strong distinction between the two West Indian subgroups with 100% bootstrap support (not shown).

An individual Puerto Rico genotype tree indicated mostly mixed genotypes and some geographic substructure (not shown). The Puerto Rico population neighbor-joining tree specified two major sub-groups within the island, N clustering with E and S clustering with W (Figure 2-3). The S and W coasts were separated from the N and E coast with 100% bootstrap support. The N and E coasts were separated with 96% bootstrap support.

**Cytogenetic Analyses**

The Giemsa-banded karyotype analysis confirmed that the Puerto Rico manatee has 48 chromosomes. This is in agreement with solid stained West Indian coastal Brazil manatees and banded Florida manatee chromosomes (Assis et al. 1988; Gray et al. 2002; Vianna et al. 2006). The banding pattern was analogous to that observed in the Florida manatee.
Discussion

Temporal separation of the Florida and Puerto Rico manatee populations has resulted in considerable divergence at the nuclear DNA level and separate evolutionary progression. Although a reproductive barrier is most likely extrinsic, the long standing division, minimal migration, and an apparent bottleneck in Florida has resulted in the accumulation of considerable differences between the Florida and Puerto Rico populations. The extent of the Florida and Puerto Rico differentiation is substantial and supported by highly significant findings corresponding to subspecies taxonomic classification and morphological and craniometry distinctions (Domning & Hayek 1986; Mignucci-Giannoni 1996).

The separation is supported by F_{ST} and R_{ST} pairwise values, multi-locus genotype analyses, and un-rooted microsatellite neighbor-joining trees. From these results the Florida and Puerto Rico manatee should be considered, managed, and conserved as two distinct populations with little to no migration and divergent genetic makeup. The small, isolated Puerto Rico population should be managed to protect its genetic diversity, since divergent individuals from Florida are unlikely to join the population. Additionally, significant genetic diversity statistics and strong assignment to the resident populations indicate that the Belize and Mexico population is also separate from the Puerto Rico population (data not shown). This suggests that there is little genetic relationship between these populations in the Western Caribbean.

Florida and Puerto Rico Mitochondrial DNA

Garcia-Rodriguez et al. (1998) and Vianna et al. (2006) also identified one haplotype in Florida (A01) and three of the haplotypes in Puerto Rico (A01, A02, and B01). Colonization and/or a population bottleneck were proposed to explain the limited haplotype diversity found in Florida (Garcia-Rodriguez et al. 1998). The A01 haplotype in both populations suggests a common ancestor or historical relationship and is likely due to the colonization of Florida from
the Greater Antilles approximately 12,000 ya (Domning 2005; Reep & Bonde 2006). To date, Florida does not contain the B01 haplotype, corroborating the limited migration between the populations and the A01 colonization of Florida. It is possible that A01 females on the North coast of Puerto Rico traveled further north and colonized Florida. A01 is unique to northern populations, found also in Mexico and the Dominican Republic. The effective number of mtDNA is 25% of nuclear DNA, indicating that mtDNA is more sensitive to population bottlenecks. Therefore, A01 could have quickly drifted to fixation in Florida.

A clear haplotype pattern was identified in Puerto Rico, suggesting strong female site-fidelity. MtDNA is maternally inherited and is not reflective of gene flow from males. Single haplotypes were detected exclusively on the N and S coasts indicating minimal movement between subpopulations. The mixed haplotypes to the E and W suggest movement of A01 and B01 females into these regions, presumably from the bordering subpopulations. In fact, an A01 male manatee captured on the west coast as part of a radiotelemetry study traveled to the north coast for a short time and then returned to the west coast. On the other hand, a B01 female captured during the same study traveled consistently back and forth between the west and south coasts of the island. Since A02 has not been found in any other population to date, it most likely evolved from A01 descendants in this subpopulation.

The unique pattern of A01 and B01 haplotypes on the N and S coasts, respectively, may be explained by the reduced population size. Small, isolated sub-populations of single haplotypes may have persisted and grown. The S coast may have been founded by B01 animals that moved to or from the E and W coasts followed by divergence to the A01 haplotype. Alternatively, the two coasts may have been founded by the separate haplotypes.
Florida and Puerto Rico Microsatellite Analysis

Genetic diversity statistics, genetic distances, and multilocus Bayesian assignment tests support a strong nuclear divergence between the Florida and Puerto Rico manatee populations. According to Frankham et al. (2002) an $F_{ST}$ of $\geq 0.15$ represents significant genetic differentiation. Other studies have also corroborated this division, suggesting that an $F_{ST}$ in the range of 0-0.05 indicates little differentiation; 0.05-0.15, moderate differentiation; 0.15-0.25, great differentiation; and values above 0.25, very great genetic differentiation (Balloux & Goudet 2002; Hartl & Clark 1997). Therefore, the significant $F_{ST}$ value of 0.163 between Florida and Puerto Rico indicates great genetic differentiation between the populations. The populations have apparently been separated for many years with little recent migration between them. In fact, Vianna et al. (2006), using the program BARRIER 2.2 (Manni et al. 2004), identified a gene flow barrier between Florida and Puerto Rico using mitochondrial DNA.

$F_{ST}$ was 1.4 times larger than $R_{ST}$, indicating that random drift rather than mutation is responsible for the genetic differences (Frankham et al. 2002). Since Puerto Rico is a small population and Florida was likely founded by few individuals from the Greater Antilles, drift could have quickly separated the allele frequencies of the two populations.

The delineation of the populations as shown by the program STRUCTURE 2.2 supports the hypothesis that these two populations have little genetic similarity, with no admixture or recent migration, and should be considered two genetically distinct groups (Figure 2-4). The high proportion of assignment and ancestry of Florida individuals to Florida (0.986) and Puerto Rico individuals to Puerto Rico (0.979) represents strong separation and minimal recent migration between the populations. The similar Puerto Rico genotypes assigned to Florida could be explained by chance, since the genotypes could be similar, immigration or descendants of recent immigrants, or sample processing errors. The individual and population trees from Puerto Rico
and Florida also indicated large genetic separation and little mixing between the two populations with 100% bootstrap support.

**Population Structure and the Environment**

The different habitats in Florida and Puerto Rico may influence the divergence observed in population structures. Although some were significant, the Florida genetic differentiation values did not reach the 0.05 cut-off value, indicating great mixing and weak structure. The largest $F_{ST}$ value was 0.033 and the largest $R_{ST}$ value was 0.027 (Pause *et al.* 2008). Within Puerto Rico, substantial genetic differentiation was reached (e.g., $F_{ST}$ 0.064 and $R_{ST}$ of 0.111).

Peninsular Florida has a temperate climate, with significant seasonal water temperature changes. These changes trigger manatee migration between warm-water refugia in the winter and highly nutritious marine ecosystems in the warmer months (Rathbun *et al.* 1995b; Rathbun *et al.* 1990). Therefore, Florida manatees travel long distances around the peninsula and breed with other individuals throughout the population (Deutsch *et al.* 2003; Fertl *et al.* 2005; Weigle *et al.* 2001). This results in high gene flow and little genetic differentiation or structure in the population.

Alternatively, Puerto Rico is a marine environment with minimal seasonal climate change. The manatees are not required to travel long distances to obtain resources or warm water for their survival. During the period of April 1992 to June 2006, 33 manatees were radio tagged and tracked by USGS-FISC personnel and their collaborators (Slone *et al.* 2006). Manatees were captured on the E coast or in the SW region. The majority of the tracked animals had restricted movement patterns, alternating between seagrass beds and local fresh water sources. Many animals remained in the immediate area of Guayanilla Bay in the SW, or around Ensenada Honda on the E coast. This limited movement decreases gene flow and allows for the formation of subtle population structure. Weak seasonal differences were detected only by the Bayesian
clustering method. Two of the clusters were 70% composed of animals collected during the rainy season.

**Puerto Rico Genetic Diversity and Geographic Division**

Recent meta-analyses of microsatellite data determined that demographically-challenged mammalian populations ($H_E$ of 0.60 and $A = 6.17$-$6.59$) have lower genetic variation than undisturbed, healthy populations ($H_E = 0.65$ and $A = 8.18$; DiBattista 2007). Garner *et al.* (2005), identified similar heterozygosity values (challenged $H_E = 0.5$; healthy $H_E = 0.68$). The Puerto Rico population had lower genetic diversity ($H_E = 0.447$; $A = 3.9$) than disturbed populations experiencing pollution, harvesting, or habitat fragmentation. Expected heterozygosity values were 9% lower for Puerto Rico than Florida, indicating less variation in Puerto Rico. The earliest accounts of Puerto Rico include Tainos, Caribs, and Spaniards using manatee meat as an important food source (Acosta 1590; Stahl 1883). In fact, the Pope declared manatees a type of fish, allowing Spaniards to consume them on Fridays (Reep & Bonde 2006). Manatee hunting and consumption continued until a last record was registered in 1995 (Mignucci-Giannoni *et al.* 2000). The severely reduced genetic diversity in Puerto Rico is likely reflective of the small population size and long-term persecution.

**Geographical genetic structure**

The Puerto Rico population was subdivided by five geographically separated haplotype groups. The small area and documentation of manatees traveling great distances (Reep & Bonde 2006) would have likely caused a homogenous haplotype distribution without habitat barriers. Instead, a strong haplotype separation was detected, indicating habitat barriers. The nuclear DNA subpopulation separation was not as severe, suggesting that animals do travel and breed throughout the population to some degree.
The majority of the geographical subpopulation pairwise F$_{ST}$ and R$_{ST}$ values were low, but significant and consistent with the haplotype pattern, suggesting subtle but detectable geographical structure. Only two pairwise F$_{ST}$ values were above the 0.05 moderate differentiation threshold. The N and EA moderate divergence (0.051) was unexpected as the units are adjacent to one another. A large amount of recreational development has taken place in the NE region, and may have contributed to the separation of the subpopulations. The E and W were significantly different at a moderate level, most likely due to the large distances and barriers to movement between the two coasts.

When calculating significant R$_{ST}$ pairwise values, four had moderate differentiation and six of the 10 were significant. Interestingly, the S and W R$_{ST}$ value was highly divergent (0.099) and significant, while the F$_{ST}$ value was not significant. This could suggest differentiation from mutation as opposed to divergence through allele frequency drift.

When the population was analyzed by geographic location and sex, SM and EF values were significant and moderately divergent, suggesting that only the SF (B01) are traveling and breeding with the E (B01) subgroup and only EM are traveling and breeding in the south. The A01 haplotype is not disseminated by the EF to the S, while the B01 haplotype is moved to the E from the SF. Large and significant R$_{ST}$ values separated the WF from the SM and SF. This again suggests a lack of WF (A01, A02, or B01) traveling S and so neither A01 haplotype is incorporated into the S and only B01 is maintained. Males and females from within one geographical group did not show any significant differentiation, supporting the genetic unity of the subpopulations.

Strong division in the island was indicated by the separation of the NE and SW areas. The distribution of individuals identified through strandings, aerial surveys, and tracking studies
indicate that the S and E are the most inhabited areas on the island. The 1992-2006 tracking studies identified the W and SW and the E and NE coasts as heavy use areas (Slone et al. 2006). The rivers used by manatees to obtain fresh water include the Guanajibo, Guanica, and Guayanilla, on the W and S coasts, and Yauco, and the effluent of the Cape Hart Sewage Treatment Plant on the E coast, corresponding to the heavy use areas. A high incidence of movement between the W and SW coasts was observed from Guanajibo to Guanica, potentially between the fresh water sources. Regions known to have large populations, but not targeted in the tracking studies included Jobos Bay, San Juan, and Luquillo. No radio tagged manatees utilized these areas, which were not far from E and SW capture sites, respectively, supporting the theory that these manatees have small home ranges and rarely travel to these nearby areas.

**Barriers to movement in Puerto Rico**

These analyses indicate subtle population structure, which may be influenced by migration patterns, lack of time for convergence, or poor habitat areas. Few sightings take place in the NW (Rincón to Barceloneta) and in the SE municipality of Maunabo, which are deep close to shore (Figure 2-1, black bars). For example, aerial surveys from 2002-2004 sighted many manatees along the S coast but none in Maunabo. Seagrass beds were detected in these regions, but in waters deeper than 18 ft with higher wave energy. Manatees remain in isolated coastal habits and are rarely seen in open or deep ocean (Lefebvre et al. 2001). Deep water represents poor manatee habitat and possibly hinders manatee movement. A positive and significant correlation was found between genetic and coastline geographical distances using a Mantel test, supporting the idea that manatees migrate along the coasts and rarely move in pelagic waters (Vianna et al. 2006). Because manatees are dependent on freshwater sources and shallow vegetation, they stay close to shore. Manatees feed and rest in shallow water and prefer water depths of three to seven
ft (0.9 to 2.1 m). Deep seagrass beds are unlikely to be foraged and may be located in areas with high-energy wave action.

Another impediment to movement may be high wave action on the NW coast of Puerto Rico. Strong waves and currents likely discourage manatees from traversing these waters. Powell et al. (1981) and Rathbun et al. (1985), using aerial survey techniques, did not detect any animals from the Culebrina River (W coast) to the Manti River (central N coast) where high energy wave action is observed.

The poor and unused manatee habitats appear to prevent a panmictic population from forming, but do not prevent all movement. Manatees can travel great distances (Deutsch et al. 2003; Fertl et al. 2005) and have crossed open ocean (Alvarez-Alemán et al. 2007). A degree of movement throughout the island is corroborated by low genetic differentiation statistics and little geographic population structure detected by STRUCTURE.

**Preservation of the Manatee in Puerto Rico**

Due to the small number of manatees in Puerto Rico, the population must be actively monitored and managed. The population estimate of 250 (ranging from 150-360) is a cause for concern (Frankham et al. 2002). It has been suggested that a minimum of 50 effective breeders (10% of the population) is needed to prevent inbreeding depression needed for long term survival (Wright 1951). Puerto Rico is well below this threshold. This implies that population levels in the upper hundreds to thousands are needed to maintain evolutionary potential (Franklin 1980; Lande 1995). Although no physiological or genetic implications of inbreeding have been identified to date, the population is small and highly susceptible to detrimental genetic effects.

Demographic and stochastic events can quickly reduce genetic variation and population levels in groups with few individuals. Small populations also have reduced genetic diversity, which can negatively influence fitness (Roelke et al. 1993), increase susceptibility to disease
(O'Brien et al. 1983), and decrease population viability (Sherwin et al. 2000). Immigration is most likely low, limiting the potential genetic diversity that could supplement the population. The islands close to Puerto Rico (i.e., Cuba, Jamaica, Hispaniola) are thought to have small or no remaining manatee populations to provide additional individuals. The high genetic differentiation values suggest that immigration is minimal from the larger Florida, Belize, or Mexico manatee populations.

Since manatees have a long generation time (Marmontel 1995; Rathbun et al. 1995a), special habitat requirements (Reynolds et al. 1995), and vulnerability to stochastic events such as cold stress and red tide (O'Shea et al. 1991), it is imperative that anthropogenic threats that cause manatee mortality be monitored and reduced.

Increased human impacts and the high rate of development throughout Puerto Rico have strongly affected the environment. Humans tend to colonize regions that are excellent manatee habitat, such as protected and shallow bays with access to fresh water. Watercraft traffic and human presence has increased in bays where manatees previously sought food, freshwater, and protected areas for rest and giving birth. The Puerto Rico manatee population has a history of being hunted and is therefore wary of humans. They may not utilize resources near areas of high human activity. Boat traffic increases proportionally to the human population and poses the largest anthropogenic mortality threat to the Puerto Rico manatee population (Mignucci-Giannoni et al. 2000). If the fast pace of human colonization and habitat destruction continues in Puerto Rico, manatees may be left with little sustainable habitat and no place to go.

To assist the survival of the Puerto Rico Antillean manatee population, the Puerto Rico Manatee Recovery Plan (Rathbun & Possardt 1986) must be updated and implemented by law enforcement, rescue and rehabilitation groups, and cooperative multiagency agreements to assess
and reduce threats. Specifically, boat and jet-ski speed and traffic in manatee use areas must be evaluated, regulated, and enforced to reduce mortality and encourage manatee utilization. Enforcement must be accompanied with a multimedia outreach campaign to educate the boat and coastal community. The SE and NW corners of the island should remain open to allow gene flow and promote genetic diversity between the subpopulations. Additionally, scientific research on life history traits and recurrent population surveys are needed to monitor the population status in the quickly changing Puerto Rico environment. An active and continued, science, conservation, and management effort will help to ensure the preservation of the Puerto Rico manatee population.
Table 2-1. Characteristics of the 15 polymorphic microsatellite loci implemented on the Puerto Rico manatee (T. m. manatus) samples

<table>
<thead>
<tr>
<th>Locus name</th>
<th>Tm(°C)</th>
<th>BSA</th>
<th>A</th>
<th>N&lt;sub&gt;E&lt;/sub&gt;</th>
<th>PIC</th>
<th>H&lt;sub&gt;O&lt;/sub&gt;</th>
<th>H&lt;sub&gt;E&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>TmaA02</td>
<td>56</td>
<td>2</td>
<td>1.319</td>
<td>0.407</td>
<td>0.209</td>
<td>0.242</td>
<td></td>
</tr>
<tr>
<td>TmaE1</td>
<td>55</td>
<td>+ 5</td>
<td>2.323</td>
<td>1.065</td>
<td>0.571</td>
<td>0.570</td>
<td></td>
</tr>
<tr>
<td>TmaE02</td>
<td>58</td>
<td>5</td>
<td>1.766</td>
<td>0.625</td>
<td>0.418</td>
<td>0.434</td>
<td></td>
</tr>
<tr>
<td>TmaE7</td>
<td>56</td>
<td>+ 5</td>
<td>1.996</td>
<td>0.841</td>
<td>0.420</td>
<td>0.499</td>
<td></td>
</tr>
<tr>
<td>TmaE08</td>
<td>60</td>
<td>5</td>
<td>1.581</td>
<td>0.714</td>
<td>0.205</td>
<td>0.368</td>
<td></td>
</tr>
<tr>
<td>TmaE11</td>
<td>58</td>
<td>5</td>
<td>3.419</td>
<td>1.355</td>
<td>0.745</td>
<td>0.708</td>
<td></td>
</tr>
<tr>
<td>TmaE14</td>
<td>56</td>
<td>+ 5</td>
<td>1.840</td>
<td>0.837</td>
<td>0.369</td>
<td>0.457</td>
<td></td>
</tr>
<tr>
<td>TmaF14</td>
<td>58</td>
<td>2</td>
<td>1.427</td>
<td>0.476</td>
<td>0.295</td>
<td>0.299</td>
<td></td>
</tr>
<tr>
<td>TmaH13</td>
<td>60</td>
<td>4</td>
<td>1.984</td>
<td>0.843</td>
<td>0.527</td>
<td>0.496</td>
<td></td>
</tr>
<tr>
<td>TmaJ02</td>
<td>62</td>
<td>3</td>
<td>1.754</td>
<td>0.714</td>
<td>0.500</td>
<td>0.430</td>
<td></td>
</tr>
<tr>
<td>TmaK01</td>
<td>58</td>
<td>4</td>
<td>1.849</td>
<td>0.722</td>
<td>0.640</td>
<td>0.459</td>
<td></td>
</tr>
<tr>
<td>TmaKb60</td>
<td>62</td>
<td>6</td>
<td>1.923</td>
<td>0.808</td>
<td>0.464</td>
<td>0.480</td>
<td></td>
</tr>
<tr>
<td>TmaM79</td>
<td>54</td>
<td>+ 2</td>
<td>1.209</td>
<td>0.315</td>
<td>0.191</td>
<td>0.173</td>
<td></td>
</tr>
<tr>
<td>TmaSC5</td>
<td>60</td>
<td>5</td>
<td>2.264</td>
<td>0.933</td>
<td>0.636</td>
<td>0.558</td>
<td></td>
</tr>
<tr>
<td>TmaSC13</td>
<td>56</td>
<td>4</td>
<td>2.129</td>
<td>0.921</td>
<td>0.613</td>
<td>0.530</td>
<td></td>
</tr>
</tbody>
</table>

Optimized annealing temperature (T<sub>m</sub>), BSA requirement (0.4 mg/mL), number of alleles (A), effective number of alleles (N<sub>E</sub>), polymorphic information content (PIC), and the observed and expected heterozygosity (H<sub>O</sub> and H<sub>E</sub>) for the Puerto Rico T. manatus manatus population

Table 2-2. Pairwise F<sub>ST</sub> (above diagonal) and R<sub>ST</sub> values (below diagonal) generated from a survey of 15 microsatellite loci from T. m. manatus in five geographic regions in Puerto Rico. Italics indicate statistically significant values

<table>
<thead>
<tr>
<th>Geographic region</th>
<th>North</th>
<th>East A01</th>
<th>East B01</th>
<th>South</th>
<th>West</th>
</tr>
</thead>
<tbody>
<tr>
<td>North</td>
<td>0.051</td>
<td>0.025</td>
<td>0.031</td>
<td>0.038</td>
<td></td>
</tr>
<tr>
<td>East A01</td>
<td>0.048</td>
<td>0.029</td>
<td>0.037</td>
<td>0.021</td>
<td></td>
</tr>
<tr>
<td>East B01</td>
<td>0.009</td>
<td>0.014</td>
<td>0.030</td>
<td>0.064</td>
<td></td>
</tr>
<tr>
<td>South</td>
<td>0.030</td>
<td>0.016</td>
<td>0.007</td>
<td>0.015</td>
<td></td>
</tr>
<tr>
<td>West</td>
<td>0.052</td>
<td>0.111</td>
<td>0.074</td>
<td>0.099</td>
<td></td>
</tr>
</tbody>
</table>
Figure 2-1. Puerto Rico bathymetric map with location and mitochondrial haplotype assignment of captured or recovered manatees. Black bars represent water depths of ≥100 fathoms close to shore.
Figure 2-2. Florida (orange) and Puerto Rico (blue) individual neighbor-joining tree, depicting nearly 100% correct assignment of individuals to their resident population and a large genetic distance between the populations (arrow)
Figure 2-3. Puerto Rico un-rooted neighbor-joining tree, depicting a separation of the South and West from the North and East (100% support) and a North and East separation (96% support).

Figure 2-4. Summary plot of $q$ estimates generated by the sequential cluster analysis of the program STRUCTURE performed on the Florida and Puerto Rico $T. manatus$ genotypes.
The West Indian manatee (*Trichechus manatus*) is an endangered aquatic mammal found throughout the southeastern United States, Caribbean, and Central and South America. All West Indian populations are classified as vulnerable to extinction (1982) by the International Union for Conservation of Nature (IUCN 2007). Two subspecies are recognized, the Florida manatee (*T. m. latirostris*) and the Antillean manatee (*T. m. manatus*). The Antillean manatee population in Belize, located south of Mexico and north and east of Guatemala, is the largest and most stable population in the subspecies’ range (Auil *et al.* 2007). Early legislation limited harvesting and encouraged conservation of the imperiled population throughout Belize.

Extensive hunting decimated many of the Antillean manatee populations and continues in many countries even today (Lefebvre *et al.* 2001; Smethurst & Nietschmann 1999). The historical Mayan people consumed a large amount of manatee meat and utilized many tissues in ceremonial activities, as indicated by examination of bone middens at archeological sites (Gann 1911; Thompson 1939). Records from the voyages of Christopher Columbus introduced European society to the Caribbean manatee, and during the 1700s and 1800s, the Spaniards and indigenous people severely exploited the species for sustenance. By 1936, the population decline was so severe that Belize introduced Manatee Protection Ordinances to preserve the population (McCarthy 1986). Currently, the manatee is listed as endangered by the Belize Wildlife Protection Act of 1981, Part II, Section 3(a) (Auil 1998). A Manatee Recovery Plan was written in 1998, requesting information on habitat use and movement patterns to aid in the development of conservation policies for the protection of the population.
Direct threats, a long generation time, and environmental impacts from the burgeoning human population limit manatee population growth. Manatees inhabit highly developed coastal regions, including fresh water rivers, brackish lagoons, and marine habitats (Lefebvre et al. 2001). Therefore, anthropogenic harm and mortalities are a result of watercraft collision, development and habitat destruction, incidental net and fishing gear entanglements, and recently, tourism activities (Auil et al. 2007). Increasing residential and industrial coastal development can quickly destroy manatee habitat through dredging, agricultural contamination, sewage and industrial effluents, and mangrove and seagrass destruction. Harm to seagrass, a major food source for manatees, occurs through pollution, sedimentation, siltation, and secondarily through mangrove destruction. This environmental damage has a direct impact on manatee food sources and thus population size. Additionally, despite the 1930s protections, imprudent poaching continues in Belize to this day (Bonde & Potter 1995).

Causes of manatee deaths in Belize are similar to those imposed on most manatee populations. A study from 1996-1998 identified perinatal (32%) and human related mortalities involving watercraft (16%) and poaching (20%) as the most common causes of death (Auil 1998). Calves made up 44% of the moribund manatees evaluated. A more recent study identified watercraft (17%) as the most common cause of death, aside from undetermined (2004-2007; Auil et al. 2007).

Belize manatee abundance and distribution has been evaluated using extended-area aerial survey methods developed by Packard (1985). The greatest number of individuals counted during an aerial survey was 338 in 2002 (Auil 2004). High use areas were determined to be the Cayes adjacent to Belize City, the Belize River, Southern Lagoon, Placencia Lagoon, Corozal Bay, Indian Hill Lagoon, and the Port Honduras area including Deep River and Seven Hills.
Lagoon. A five-year survey (1997 and 1999-2002) of manatee abundance in Belize indicated an overall slightly negative trend in the number counted per year, although variation in seasons and survey routes make conclusions difficult (Auil 2004).

Capture of wild manatees for health assessment began in 1997 in an effort to provide information on the health status, age class, reproduction, distribution, and genetic structure of the Belize population. Since that time, 118 unique animals (50% female and 50% male) have been captured in 213 health assessments (55% female and 45% male). The study locations include the high use areas of Belize City Cayes, a reef lagoon system off Belize City, the Southern Lagoon system, two large brackish inland lagoons, and more recently Placencia Lagoon, located 75 miles south of the Southern Lagoon system.

The Belize City Cayes (BCC) are utilized by the tourist industry and experience heavy boat traffic from Belize City, the largest port in the country. Sightseeing, fishing, SCUBA diving, and recreational watercraft activities take place in the marine waters surrounding the Cayes. The majority of carcass samples used in this study were recovered near the Belize River mouth, an area of high watercraft activity, or south of the river, possibly caught in the ocean current.

The Southern Lagoon system includes Southern (SL) and Northern (NL) Lagoons, collected by Main Creek, a narrow 2km waterway. It is located in a remote region, less developed than the Cayes. Travel between the Belize City Cayes and Southern Lagoon can be accomplished using the Sibun or Belize Rivers from Belize City or through the Bar River connecting Southern Lagoon with the Caribbean coast (Figure 3-1).

In 1989, Southern Lagoon had the highest aerial survey count in Belize, with 55 manatees (O'Shea & Salisbury 1991). While the population is not heavily affected by watercraft, it is
potentially impacted by salinity changes due to increased rainfall, influencing food abundance for the manatee. Placencia Lagoon (PL) is a long, narrow lagoon 24km in length and 1-2 m deep, protected by the Placencia Peninsula. Placencia is a popular tourist destination, with a large amount of coastal development negatively affecting the environment. Manatees here are also affected by agricultural runoff and shrimp farm discharge, as the sub-aquatic vegetation quantity and possibly quality has changed.

Since the Belize manatee population is largest in the Wider Caribbean (Auil 2004; Quintana-Rizzo & Reynolds III 2007), it is postulated that Belize could provide genetic diversity and potentially assist in the recovery of populations to the south. The adjacent Antillean manatee population in Mexico, which is also protected, is the second largest. Little is known of the abundance or distribution of manatees in the other Central American populations, although they are considered elusive and thought to be rare (Quintana-Rizzo & Reynolds III 2007). Manatees can travel great distances and could easily travel to and utilize resources among surrounding countries. In fact, a radio tagged individual was documented to travel from Mexico to Stann Creek, near PL, where the tag broke free (Morales-Vela et al. 2007). It is therefore essential that the manatee population in Belize be protected to allow for growth and supplementation of other populations. Future studies of genetics and immigration analyses could identify the degree of movement and breeding occurring with other populations.

Previous mitochondrial DNA studies identified three haplotypes in Belize, A03, A04, and J01, but could not address fine scale population structure (Vianna et al. 2006). Presented here are genetic analyses evaluating movement of manatees in the Belize City Cayes and Southern Lagoon system, high use areas of Belize. Mitochondrial and microsatellite DNA were used to elucidate the genetic diversity, relatedness, and population structure of three regions in Belize.
Subpopulations that are determined to be divergent should be protected to preserve the unique genetic diversity. Additionally, corridors should be maintained to encourage the exchange of diversity among regions. The Belize Antillean population will also be compared to the Florida population (*T. m. latirostris*), to address the genetic relationship between the subspecies.

**Materials and Methods**

**Sample Collection and DNA Extraction**

Manatee blood and/or epidermis tissue were collected from recovered carcasses or during wild manatee health assessments. Genomic DNA was isolated using QIAGEN’s DNeasy Blood and Tissue kits (Valencia, California). Florida manatee sample DNA extraction techniques are described by Pause *et al.* (2007). Within the Florida dataset, 96 individuals were randomly chosen, proportionally representing the four demographically imposed management units: Northwest, Southwest, Atlantic and St. Johns River.

**Mitochondrial DNA Analysis**

Primers and PCR parameters from Garcia-Rodriguez *et al.* (1998) amplified a 410 base pair portion of the mitochondrial DNA control region displacement loop for 116 individuals, 101 live captures and 15 carcasses. Mitochondrial DNA is maternally inherited and reflects the movement patterns of only females. The mitochondrial DNA control region was polymerase chain reaction (PCR) amplified with primers developed from 100% homology of cow and dolphin sequences (heavy stand primer: CR-5 and light strand primer CR-4) after Southern *et al.* (1988) and Palumbi *et al.* (1991). The PCR reaction conditions were as follows: 10 ng DNA, 1 x Sigma PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.001% gelatin; 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 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, a representative from each haplotype and any ambiguous sequences were sequenced in the 3’-5’ direction to ensure the accuracy of nucleotide designations.

The degree of differentiation, $F_{ST}$ and $\Phi_{ST}$, between Florida and Belize and between the BCC and SL groups in Belize were calculated using ARLEQUIN 3.1 (Excoffier et al. 2005). Estimates of sequence divergence used the Kimura 2-parameter genetic distance model (Jin & Nei 1990; Kimura 1980). The variance distribution was based on haplotype frequencies alone; all haplotypes were treated as equally differentiated ($F_{ST}$). Lastly, Tajima’s D of selective neutrality, the number of polymorphic sites, $S$, number of nucleotide substitutions, $NS$, the genetic diversity, $h$, and nucleotide diversity, $\pi$, were calculated (Nei 1987; Tajima 1993).

**Microsatellite Analysis**

A total of 16 polymorphic microsatellite primers (Garcia-Rodriguez et al. 2000; Pause et al. 2007) was PCR amplified from 122 individuals. The study included 88 from the SL system (12 NL, 76 SL), 21 from BCC, 2 from PL and 11 carcasses. The Southern Lagoon system was analyzed both without division and separated as SL and NL. Three NL individuals were also captured once in SL. Two of these were identified in NL multiple times.

PCR conditions were 14 ng DNA, 0.8 mM dNTPs, 1x Sigma PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.001% gelatin), 0.04 units Sigma Jump Start Taq polymerase, and 0.24 $\mu$m each primer. MgCl$_2$ concentrations were 3 mM, except for $TmaH13$, $TmaKb60$, and $TmaSC5$,
which required 2 mM. BSA was added as indicated in Table 3-1. 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 (Table 3-1), 72°C for 1 min, final extension 10 min at 72°C (Pause et al. 2007). Fragment analysis was performed on an Applied Biosystems ABI 3730 Genetic Analyzer. GENEMARKER, version 1.5 (Soft Genetics, State College, PA) was used to analyze the microsatellite fragment data. All individuals amplified at 14 or more loci. The Florida data were kindly provided for use in this manuscript (Pause et al. 2008). A Microsoft Access (Microsoft Corp., Redmond, WA) database was developed for storage of allelic information.

Statistical Analysis

The level of polymorphism was estimated by the observed (H_{O}) and expected heterozygosity (H_{E}) and the number of alleles per locus (A) using GENALEX 6 (Peakall & Smouse 2006). Departures from the expected genotypic frequencies in Hardy-Weinberg equilibrium (HWE) were tested using the Markov chain method (dememorization 10000, batches 100, iterations per batch 5000) in GENEPOP 4.0 (Raymond & Rousset 1995). Additionally, linkage disequilibrium was tested for non-random associations between alleles of different loci. The Markov chain method was used and the P-values were adjusted using Bonferroni sequential correction for multiple comparisons. To assess overall genetic differentiation at the population level, GENALEX 6 calculated F_{ST} using the infinite alleles model and R_{ST} using the stepwise mutation model. Comparisons included Belize and Florida, and NL, SL, and BCC. The analyses within Belize may be biased due to the large proportion of SL system individuals (67.8%). Comparisons with PL were limited, due to the small sample size.
Cluster analysis using multi-locus genotypes

The program STRUCTURE 2.2 (Pritchard et al. 2000) was used to identify the genetic subdivision within Belize and the genetic relationship and putative ancestral source populations of Belize and Florida manatees. 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 assumes that individuals could have some proportion of membership \(q\) from each of \(K\) clusters, leading to the potential identification of recent immigrants. Multiple Markov chains can delineate differences within populations, therefore three 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 10,000 iterations. The three values for the estimated \(\ln(Pr(X|K))\) were averaged, from which the posterior probabilities were calculated. The \(K\) with the greatest posterior probability \(Pr \approx 1.0000\) was identified as the optimum number of subpopulations. 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.

In the Florida and Belize STRUCTURE analysis, an increasing number of clusters were identified in Florida. To allow subpopulations to be determined, Belize was analyzed with a genetically similar Florida cluster. Contrasting a diverse cluster with the Belize population allows subtle structure to be elucidated.
Un-rooted neighbor-joining trees

Un-rooted neighbor-joining trees based on individual and population genetic distances were used to visualize relationships among populations, subpopulations, and individuals. The Phylogeny Inference Package (PHYLIP) estimated genetic distances based on pairwise Calvalli-Sforza and Edwards chord distance, $D_C$ (Felsenstein 2004). $D_C$ is based on allele frequencies and provides accurate microsatellite tree topology (Takezaki & Nei 1996). Trees were constructed by comparing individual genotypes with or without a priori partitioning. Comparison of the Belize and Florida individual genotypes was conducted with MICROSAT, version 1.5d (Minch et al. 1997), to create a distance matrix and Neighbor in PHYLIP to produce the tree. To create population trees, a priori sub-grouped allele frequencies were subjected to the programs in PHYLIP. Support values were determined by 1,000 bootstrap replicates, indicated at the branching nodes of the trees. The lengths of the branches represented relative genetic distances. Florida and the NL, SL, and BCC Belize regions were analyzed.

Cytogenetic Analyses

Giemsa-banded karyotype analyses have only previously been performed on the Florida subspecies \( T. manatus latirostris \); Gray et al. 2002). Therefore, to assess cytogenetic differences between the subspecies, banded karyotype analysis was performed on Belize manatees. Sodium heparin vacutainers® were used to collect blood and samples were transported as quickly as possible to the laboratory. The cytogenetic analysis followed protocols described by Gray et al. (2002).

Results

Mitochondrial Sequence Analysis

The A04 (4) and J01 (15; 79%) haplotypes were identified in the Belize City Cayes individuals. The Southern Lagoon system had A04 (52; 65%), J01 (22) and the only A03 (6)
haplotypes. Placencia had one A04 and one J01 individual. Recovered carcasses consisted of
A04 (9) and J01 (6) haplotypes. MtDNA sequence divergence estimates were $h = 0.030602$ and
$\pi = 0.53430$. Within Belize, Tajima’s $D = 4.11764 (P < 1.0000)$ was not significant ($P < 0.05$)
therefore; the null hypothesis of selective neutrality cannot be rejected. Genetic differentiation
estimates between BCC and SL were $F_{ST} = 0.07818 (P < 0.04505)$ and $\Phi_{ST} = -0.03632 (P <
0.71171)$. Twenty-eight polymorphic sites (6.8%) and nucleotide substitutions were identified in
the three haplotypes.

Within Florida one haplotype was observed (A01; $\pi = 0.00000$). Belize and Florida
mtDNA sequence divergence estimates were $\Phi_{ST} = 0.30208$ (Kimura 2-parameter) and $F_{ST} =
0.62640 (P < 0.00001)$.

**Microsatellite Marker Analysis**

The sixteen nuclear microsatellite markers had lower levels of polymorphism ($H_E = 0.455
(0.238-0.755); H_O = 0.455 (0.157-0.745); A = 3.4 (2-6); Table 3-1) than the Florida population
over 18 loci ($H_E = 0.480; A = 5.3$). Additional results for the Florida animals can be found in
Pause et al. (2008). In Belize, $TmaKb60$ and $TmaSc13$ had evidence of null alleles due to a
heterozygote deficiency. The null alleles in those loci may have caused the deviation from
Hardy-Weinberg equilibrium, even after a sequential Bonferroni adjustment. After 120
comparisons and a Bonferroni correction, linkage disequilibrium was observed between $TmaE1$
and $TmaE14$ (overall $\alpha = 0.05, P < 0.05$). The inbreeding coefficient $F_{IS}$ was 0.012 overall,
suggesting slight inbreeding in the population. Private alleles were detected for Florida (23) and
Belize (15) at low frequency. The error rate was determined by re-genotyping 11% of the
individuals. No errors were detected.

Genetic differentiation among populations was estimated using pairwise $F_{ST}$ and $R_{ST}$
comparisons, which were significant, although low (Table 3-2). $F_{ST}$ and $R_{ST}$ between the BCC

85
and SL system were, 0.029, 0.038, respectively and significant. Pairwise $F_{ST}$ and $R_{ST}$ values for Belize and Florida were 0.141 and 0.082, respectively, and significant ($P < 0.001$).

**Cluster analysis using multi-locus genotypes**

The **STRUCTURE** Bayesian assignment test identified two highly divergent populations, Belize (98.1%) and Florida (98.6%), with little admixture, $\ln(PD)_{AVE} = -5804$ (Figure 3-2). The Belize assignment percentages were reduced due to two individuals, TMBZ-004 and 099, with partial assignment to Florida (33.0% and 41.1%). A value above 40% indicates that the individual’s parents may have originated in Florida. TMBZ-099 was an A04 male from SL.

**STRUCTURE** identified only one population when Belize was analyzed alone, $\ln(PD)_{AVE} = -3011.7$. However, when Belize was analyzed with a $q$-clustered Florida group, two Belize clusters were detected $\ln(PD)_{AVE} = -3552.53$ (Figure 3-3). Florida animals were strongly assigned to Florida (97.1%). The Belize clusters had lower assignment values, 83.3%, and 79.9% and contained 52 and 35 SL individuals, respectively. The first Belize cluster contained the majority of the NL inhabitants (83.3%). The second cluster contained the majority of the BCC residents (80.9%). Again, TMBZ-004 and 099 were assigned to Florida, reducing the Belize assignment values. TMBZ-004 was weakly assigned to Florida (42.4 %), while TMBZ-099 was strongly assigned to Florida (82.6%), indicating a similar genotype by chance or a strong genetic relationship to the chosen Florida group.

**Un-rooted neighbor-joining trees**

The individual neighbor-joining tree identified Belize and Florida as two separate populations (Figure 3-4). Within the tree, two Belize groups contained three Florida animals. One of those clusters included the two Belize individuals that were assigned to Florida by **STRUCTURE**. The genetic distance separating the Belize and Florida populations was modest. In the Belize individual neighbor-joining tree, the BCC individuals were in groups of two to five
scattered among the SL individuals (Figure 3-4). The population tree identified strong division between NL and SL/BCC (100%). SL and BCC were also separated 90% (Figure 3-5).

**Cytogenetic Analyses**

The Giemsa-banded karyotype analysis confirmed that the Belize manatee has 48 chromosomes. This is in agreement with solid stained West Indian coastal Brazil manatees and banded Florida manatee chromosomes (Assis *et al.* 1988; Gray *et al.* 2002; Vianna *et al.* 2006). The banding pattern was analogous to that observed in the Florida manatee.

**Discussion**

In a study of Caribbean manatee populations, O’Shea and Salisbury (1991) concluded that “Belize remains one of the last strongholds for the species in this part of the world.” Suitable habitat and reduced poaching has made Belize the largest manatee population in the region (Auil 2004). Furthermore, until recently the human population has remained small, limiting environmental destruction and habitat fragmentation.

Minimal genetic difference was detected within Belize, indicating migration and breeding throughout the subpopulations. The STRUCTURE program identified two groups, SL with BCC and SL with NL, when Belize was analyzed with a genetically divergent Florida cluster. Additionally, SL and BCC manatees were less genetically differentiated than NL and BCC manatees, even though the later are in closer proximity to one another. This suggests that animals traveling from BCC to the Southern Lagoon system (SL and NL) spend more time breeding in SL than in NL and may use the Bar River to a greater extent than the Sibun or Belize Rivers to the north. High human activity may discourage Sibun and Belize River use and the rivers should be protected as manatee habitat to allow geneflow between BCC and NL. NL and SL were not statistically different from each other, indicating that these lagoons comprise a breeding population.
The BCC and SL subpopulations had disparate proportions of haplotypes. The majority of haplotypes in the BCC was J01 (79%) and A04 in SL (65%), possibly indicating female site-fidelity and reduced movement between the BCC and SL. Haplotypes have been analyzed in Florida and from Mexico to Brazil, and the A03 haplotype has only been found in SL (Vianna et al. 2006). The lack of A03 in other regions suggests that A03 females remain in the population in which they were born, although more samples need to be analyzed from BCC and the rest of the region. The haplotype may have spontaneously mutated from A04 and has since remained in isolation in SL.

The individual TMBZ-099 was a male 41% related to Florida. Males are sighted in the summer at breaks in the Belize Barrier Reef along the BCC, most likely utilizing the habitat seasonally, or waiting for receptive females. These males are thought to travel a great distance, potentially allowing for movement between countries and a mechanism for geneflow between subspecies (Self-Sullivan et al. 2003). Radio-tagged males were also more likely to move away from SL (Powell et al. 2001).

Source and Sink

The sheltered environment, extensive food, and fresh water provide excellent habitat for Belize manatees. The country has a wide shelf, with a large, relatively shallow reef lagoon (>1-25m) and no appreciable current or tidal flow. The barrier reef runs parallel to the coastline for 220-250km, providing boughs and lagoons as manatee sanctuaries and feeding grounds (McField et al. 1996). Shallow, calm water is ideal habitat for manatees to forage on superficial seagrass beds and travel in search of resources or reproductive opportunities.

The highly suitable Belize habitat allows the population to expand, potentially repopulating the surrounding countries. However, poaching could remove expatriated animals before they reproductively contribute to the population. A genetic study of other populations
could assess whether individuals are traveling and reproducing outside of Belize. The populations outside of Belize are small and may require supplementation to persist (O'Shea & Salisbury 1991).

**Tracking**

A total of 42 manatees has been radio tracked using a VHF, GPS, or UHF transmitter (Auil et al. 2007). One male tagged with a radio transmitter in SL traveled north to Mexico and returned one month later. Manatees tagged in Mexico have traveled directly to SL (~200km) and Placencia (~50km further south), indicating possible knowledge of the coastline and travel across country boundaries. An animal tagged in Mexico traveled directly to SL through the Bar River to participate in mating herds (Auil et al. 2007)

This type of lengthy migration, possibly searching for fresh water, reproductive opportunities, and high-quality food, is common in other manatee populations, such as Florida. Of the four manatees tagged in the BCC, three stayed within a 15-mile radius of the capture site. The majority of manatees tagged in the Southern Lagoon system remained in SL and had strong site-fidelity to the lagoon system. SL has an upwelling spring, “manatee hole,” which helps support the large population found there and its available fresh water may attract manatees traveling along the coast. The spring is a 10m depression with upwelling water that can reach high temperatures (33 °C). Manatees (up to 20 at a time) actively utilize this depression for thermoregulation when the temperature in the lagoon drops below 26°C (Auil et al. 2007). Only a few individuals traveled to NL, perhaps because SL has better-suited resources than NL, with more quantity and variation of vegetation (Auil et al. 2007).

**Mainland versus Island Habitats**

The Belize manatee population structure reflects the Florida manatee population. These populations are located on the mainland of continents, allowing manatees to travel great
distances as compared to island geography where travel is more restricted. Mainland manatee habitat is typically uninterrupted habitat with few barriers and protected waters, allowing increased movement and geneflow and reducing population structure. The average heterozygosity and number of alleles are lower in Belize than those estimated for Florida. This is likely due to long-term hunting pressures and the smaller population size of the Belize manatees.

Alternatively, manatees on the island of Puerto Rico contend with narrow continental shelves and steep drop-offs into deep ocean trenches. The North-West and South-East coasts of Puerto Rico have extremely deep water close to shore. This represents poor manatee habitat, with high wave action and deep seagrass beds. Slight reproductive barriers were identified, allowing for genetic differentiation and subpopulations to form within Puerto Rico (Kellogg et al. 2008).

A meta-analysis of microsatellite data determined that demographically-challenged mammalian populations affected by historical or long-term harvesting, fragmentation, or pollution have lower genetic variation (\(H_E\) of 0.5 to 0.6 and \(A = 6.9\)) than undisturbed, healthy populations (\(H_E = 0.6\) to 0.7 \(A = 8.8\); DiBattista 2007; Garner et al. 2005). Belize and Puerto Rico have much less genetic diversity than the reported average demographically-challenged populations. This may be intrinsic to the populations, or reflect the severe persecution. Belize and Puerto Rico also have similar genetic diversity, although Belize has a slightly higher expected heterozygosity and lower number of alleles. Belize is a larger population allowing for greater genetic diversity however, historical and long-term exploitation may have severely reduced that diversity. The Belize population will need significant evolutionary time to attain pre-harvesting diversity levels.
Tropical versus Temperate Climates

Florida manatees are at the northern limit of their range and must seek warm-water springs or basins during the winter (Deutsch et al. 2003; Fertl et al. 2005). In the warm months, they can travel extensively in search of marine seagrass and reproduction opportunities. The migration causes extensive mixing and little genetic differentiation to occur within the population. Alternatively, studies indicate that precipitation levels affect Belize manatee distribution (Gibson 1995). The tropical climate in Belize fluctuates little and generally, manatees do not migrate for thermoregulation. In the dry months, animals are more abundant in rivers, most likely to drink freshwater. During wet months, individuals are plentiful in the cayes, were food is more nutritious and fresh water is not difficult to find on the surface or from flowing rivers. Like Florida, the large amount of admixture produces little genetic difference between the Belize regions.

Current Population and Future Directions

Although Belize is the largest Antillean population, it is still under pressure from anthropogenic threats. From 1977 to 1991, the population size appeared stable and calves were consistently 7% of the population, indicating good recruitment and a healthy, possibly increasing, population. However, a more recent study (1997-2002) found an overall negative trend in manatee numbers. The population may be declining from escalating anthropogenic threats, such as development, agricultural run-off and boat traffic (Auil 2004). The long generation time, changing environmental hazards, and close proximity to human activity could quickly erode Belize manatee genetic diversity and ultimately the population size. Moreover, the genetic diversity within Belize is significantly lower than demographically-challenged mammalian populations or the Florida population, potentially due to long-term and severe persecution.
Improving the reporting, recovery, and examination of injured or dead manatees could provide additional distribution, life history, and cause of death data. Enforcement of laws and increasing protections in the impacted regions could reduce the number of anthropogenic deaths. The construction of additional formal sanctuaries, habitat with no-entry or no-fishing areas, is needed. All nets, except for cast nets, should be banned to prevent entanglement. Furthermore, manatee tours have economic value (about $80 per person) and the growing tourism industry could physically harm or disrupt manatee behavior if not conducted properly, making regulations necessary for this industry (Auil 2004).

No wake zones and water vessel operation speed zones in shallow manatee habitat are needed to decrease boat strikes. A study in 1991 indicated that boat strikes were rare (O'Shea & Salisbury 1991). However, studies beginning eight years later identified boat scars on more than half (55.6%) of the individuals caught off Belize City, while only 16.8% of the individuals in Southern Lagoon had scars at the initial capture (Auil et al. 2007). The increasing incidence of boat strikes near Belize City indicates the direct harm increased boat traffic could have on the population.

The evaluation and reduction of habitat degradation is critical to the survival of this elusive aquatic mammal. Pollution and effluents from industry and development should be highly monitored and regulated. Damage to seagrass beds by shrimp trawlers and shrimp farms must be monitored and reduced. The nitrification from shrimp farm effluent can cause extensive algae blooms affecting seagrasses. The alga provides food for a marine snail, which is an intermediate host of a respiratory fluke (Cochleotrema cochleotrema) known to parasitize manatee lungs. Manatees in Placencia Lagoon can carry large loads of the fluke and present at capture with mucoid discharge, possibly related to an infection (Auil et al. 2007). Additionally, the algal
bloom compromises the quality and growth of the seagrass. The relationship of nitrification, snail, and algae densities on seagrass, and increased manatee parasite loads must be investigated in this region.

Enforcement of guidelines and regulations by Belize and neighboring countries’ government and institutions is essential to the preservation of manatee populations. Several local non-governmental organizations have strong local and national manatee education programs. These should be empowered to continue educating the Belizean people (O'Shea & Salisbury 1991). Collaboration among the governments of Guatemala, Honduras, and Belize is needed to reduce poaching in the Gulf of Honduras. Since manatees were over-harvested in Guatemala (Janson 1980), poaching has increased in southern Belize rivers by Guatemalan hunters (Bonde & Potter 1995; McCarthy 1986; Wright et al. 1959). In 1995, investigations documented a large amount of hunting in Port Honduras and identified 11 separate butchering sites where the meat is most likely transported to Guatemala to be sold (Bonde & Potter 1995). There are similar reports of poaching along the Mexican border, even the area is protected by both countries. To assist conservation, DNA analysis of the suspected meat could identify the country of origin and illegal cross-border transport. Additional actions in Belize are needed to limit this activity.

To avoid further loss of genetic diversity in Belize manatees it is essential to preserve and increase the genetic variation and number of individuals. An increase in the population size may also assist in the repopulation of other countries. Quantifying the degree of migration and diversity using genetic tools is recommended to clarify the role Belize plays in maintaining the subspecies in the Wider Caribbean.

The foresight of the Belize government and the actions by Belize residents and organizations has allowed the Belize manatee population to become the largest in the Wider
Caribbean, potentially expatriating to other countries. However, the population is increasingly threatened by human activities. Proper protections and continued monitoring are needed to ensure the sustainability and expansion of the Belize Antillean manatee population.
Table 3-1. Characteristics of the 16 microsatellite loci implemented on Belize manatee (T. m. manatus) samples

<table>
<thead>
<tr>
<th>Locus name</th>
<th>$T_m$</th>
<th>BSA</th>
<th>$A$</th>
<th>$N_E$</th>
<th>$PIC$</th>
<th>$H_O$</th>
<th>$H_E$</th>
</tr>
</thead>
<tbody>
<tr>
<td>TmaA02</td>
<td>56</td>
<td>2</td>
<td>1.55</td>
<td>0.54</td>
<td>0.308</td>
<td>0.355</td>
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</tr>
<tr>
<td>TmaE1</td>
<td>55</td>
<td>+</td>
<td>4</td>
<td>2.631</td>
<td>1.123</td>
<td>0.608</td>
<td>0.62</td>
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<td>TmaE02</td>
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<td>+</td>
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<td>1.471</td>
<td>0.623</td>
<td>0.298</td>
<td>0.32</td>
</tr>
<tr>
<td>TmaE08</td>
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<td>2.967</td>
<td>1.123</td>
<td>0.705</td>
<td>0.663</td>
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</tr>
<tr>
<td>TmaE11</td>
<td>58</td>
<td>5</td>
<td>4.087</td>
<td>1.482</td>
<td>0.76</td>
<td>0.755</td>
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<td>TmaE14</td>
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<td>4</td>
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<td>1.022</td>
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<td>TmaE26</td>
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<td>5</td>
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<td>TmaF14</td>
<td>58</td>
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<td>60</td>
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<td>1.541</td>
<td>0.536</td>
<td>0.355</td>
<td>0.351</td>
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<tr>
<td>TmaJ02</td>
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<td>2</td>
<td>1.93</td>
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<tr>
<td>TmaK01</td>
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<td>0.529</td>
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<tr>
<td>TmaKb60</td>
<td>62</td>
<td>6</td>
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<tr>
<td>TmaM79</td>
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</tr>
<tr>
<td>TmaSC5</td>
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<td>1.611</td>
<td>0.708</td>
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</tr>
<tr>
<td>TmaSC13</td>
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<td>1.312</td>
<td>0.429</td>
<td>0.157</td>
<td>0.238</td>
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</table>

Optimized annealing temperature ($T_m$), BSA requirement (0.4 mg/mL), number of alleles ($A$), effective number of alleles ($N_E$), polymorphic information content ($PIC$), and the observed and expected heterozygosity ($H_O$ and $H_E$) for the Belize T. manatus manatus population

Table 3-2. Pairwise $F_{ST}$ (above diagonal) and $R_{ST}$ values (below diagonal) generated from a survey of 16 microsatellite loci in T. m. manatus in three geographic regions in Belize. Statistically significant values are in italics

<table>
<thead>
<tr>
<th>Geographic region</th>
<th>NL</th>
<th>SL</th>
<th>BCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>NL</td>
<td>0</td>
<td></td>
<td>0.042</td>
</tr>
<tr>
<td>SL</td>
<td>0</td>
<td></td>
<td>0.026</td>
</tr>
<tr>
<td>BCC</td>
<td>0.021</td>
<td>0.033</td>
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Figure 3-1. Geographic map of Belize with the three study sites inset, A) Belize City Cayes, B) Northern (blue) and Southern (green) Lagoon, and C) Placencia Lagoon
Figure 3-2. Summary plot of $q$ estimates generated by the sequential cluster analysis of the program STRUCTURE performed on the Belize and Florida $T. manatus$ genotypes.

Figure 3-3. Summary plot of $q$ estimates performed on the $q$-sorted Florida group and Belize $T. manatus$ genotypes, indicating two Belize clusters.
Figure 3-4. Belize (green) and Florida individual un-rooted neighbor-joining tree, depicting a genetic separation between the populations (blue bar)
Figure 3-5. Belize and Florida un-rooted neighbor-joining tree, depicting a separation of the NL from BCC and SL (100% support) and a BCC and SL separation (90% support)
CHAPTER 4
CROSS-SPECIES COMPARISON OF AUSTRALIAN DUGONG AND FLORIDA MANATEE MICROSATELLITE LOCI AND THE CHARACTERIZATION OF HIGHLY INFORMATIVE MARKER-PANELS

Introduction

The Australian dugong (*Dugong dugon*) and Florida manatee (*Trichechus manatus latirostris*) are species in the Order Sirenia, threatened by anthropogenic mortality and habitat degradation. Long generation times and small, fragmented populations make this order highly susceptible to human exploitation. Currently, all extant sirenian species are considered vulnerable to extinction on a global scale (International Union for Conservation of Nature and Natural Resources (IUCN 2007)). The Florida manatee, located throughout the waters of the southeastern United States, is listed as federally endangered and estimated to have a population of approximately 3300 individuals (USFWS 2007). Because of their slow reproductive rate, annual mortality may exceed the population’s ability to produce a sufficient number of new recruits (Bossart 1999). Dugongs are found in the tropical Indian and Western Pacific Oceans, with the greatest population concentration in the marine waters of northern Australia. A 2001 aerial survey estimated 14,061 ± 2,314 dugongs in the Torres Straight region. The urban coast of Queensland, Australia, sustains a smaller population of approximately 2200 dugongs (Marsh 2006).

Conservation genetics is a useful tool to evaluate and monitor threatened species (Frankham *et al.* 2002). Detailed information on the present genetic status of threatened populations can assist with the development of comprehensive long-term management and protection plans. Molecular genetic studies can identify breeding populations, track migration of individuals and populations, and assist in modeling adult survival and reproductive rates.
Reduced genetic diversity in a species can decrease fecundity, compromise the ability to evolve or endure environmental change, and may ultimately result in extinction (Avise 2004).

Microsatellites, short tandem repeats of nuclear DNA, are highly polymorphic co-dominant markers used to determine the genetic state of small populations (Cerchio et al. 2005; Coltman et al. 2007; Dixon et al. 2007), especially those with limited genetic variation. Typically, identification of microsatellites for each new species demands considerable time, effort, and cost. Cross-species comparisons can identify polymorphic loci, so that fewer species-specific markers are needed for robust studies (Chbel et al. 2002; Huang et al. 2005; Maudet et al. 2004; Nguyen et al. 2007).

To aid conservation, dugong (Broderick et al. 2007) and Florida manatee (Garcia-Rodriguez et al. 2000; Pause et al. 2007) microsatellite markers have been developed for population and pedigree analyses. In an effort to increase the number of available primers, this study assesses the cross-species transferability and efficiency of the dugong and manatee primers. Using this information, the most effective marker-panels are compiled, in which identification of individual animals is achieved for each population.

**Materials and Methods**

The species-specific and cross-species amplification and polymorphism of 25 manatee and 32 dugong microsatellites was tested across four study groups; i) manatee samples amplified with manatee primers, ii) dugong samples amplified with dugong primers, iii) dugong samples amplified with manatee primers and iv) manatee samples amplified with dugong primers. Of those loci that amplified, the overall usefulness, such as polymorphic information content, probability of identity, and effective number of alleles were assessed among 98 dugongs collected from individuals on the northeast coast of Australia and 91 manatees representative of the four current management units identified in the state of Florida (USFWS 2007).
A total of 30 dugong and 21 manatee polymorphic microsatellites was examined on dugong and manatee samples. Development and amplification of the loci followed protocols previously described (Broderick et al. 2007; Garcia-Rodriguez et al. 2000; Pause et al. 2007). Additionally, five microsatellite loci were employed; DduE06, DduD02 DduG06 and DduH13, (Broderick et al. 2008) and TmaH23 used the same conditions described in Pause et al. (2007). Polymerase chain reaction (PCR) parameters used for cross-species amplification were the same as those published, with slight modification to annealing temperature, $T_m$, and MgCl$_2$ concentration. The MgCl$_2$ concentration for the dugong primer-manatee sample set was 3mM. The MgCl$_2$ concentration for the manatee primer-dugong sample set was 2mM, except for TmaK01 (1.5mM) and TmaKb60 (3mM).

The number of alleles ($A$), observed and expected heterozygosities ($H_O$ and $H_E$) and adherence to Hardy-Weinberg equilibrium (HWE) were assessed using ARLEQUIN, version 3.1 (Schneider et al. 2000). The effective number of alleles ($N_E$; Kimura & Crow 1964) was calculated using POPGENE, version 1.32 (Yeh & Boyle 1997). MICROCHECKER, version 2.2.3 (Van Oosterhout et al. 2004) tested for the presence of null alleles at a 95% confidence interval. Linkage disequilibrium was analyzed by GENEPop, version 3.2 (Raymond & Rousset 1995) and the polymorphic information content (PIC) was tested using CERVUS (Kalinowski et al. 2007). GENECAP (Wilberg & Dreher 2004) calculated the probability of identity ($P_{ID}$), which is the probability of two individuals drawn at random from a population will have the same genotype at the loci assessed (Paetkau & Strobeck 1994), and a related more conservative statistic for calculating $P_{ID}$ among siblings ($P_{ID\text{~sib}}$; Evett & Weir 1998). As wild populations consist of both related and unrelated individuals, the actual probability that a pair of individuals in a given population will have the same genotype depends on the degree of relatedness in that population.
and therefore lies between the two extremes of $P_{(ID)}$ and $P_{(ID)\text{si}b}$ (Waits et al. 2001). Sample size is a critical parameter in sample-resample studies. The probability that two genotypes match by chance among $n$ samples is approximately $1-(1-P_{(ID)})^n$ (Evett and Weir 1998, p 243) and is known as the shadow effect (Mills et al. 2000). The shadow effect is exacerbated in large populations because the number of pairwise comparisons increases exponentially with sample size.

The most informative dugong and manatee markers were selected based on PIC scores and $N_E$. Combinations of the highest-ranking loci were analyzed until $P_{(ID)}$ values indicated that all individuals in the population have unique genotypes. Documented population sizes may be underestimated (Marsh et al. 2004) and intrapopulational breeding through long distance travel is possible (Fertl et al. 2005). Therefore, for the dugong and manatee $P_{(ID)}$ analyses inflated population sizes of 20,000 and 4,500 were used, respectively. For comparison, $P_{(ID)}$ values were also calculated for the most informative species-specific primers. The number of primers that achieved similar $P_{(ID)}$ values as the cross-species and species-specific panels are reported.

**Results**

The transferability of dugong and manatee derived microsatellites confirmed the conservation of primer sites in the sirenian genome. Twenty-six dugong primers were used with the dugong samples and 17 were used with the manatee samples. Thirteen of those were used on both species. Twelve manatee primers were used with the dugong samples and 18 were used with the manatee samples. Seven of those were used on both species.

Of the 25 manatee primers tested, 23 (92%) produced PCR product in the dugong. Of those that amplified, 11 (48%) were polymorphic. Of the 32 dugong microsatellite primers tested, 27 (84%) yielded PCR product in the manatee. Of those that amplified, 17 (53%) were polymorphic. The resultant combined primer values for each species are reported in Tables 4-1
and 4-2. The mean heterozygosity ($H_e$), number of alleles ($A$), polymorphic information content (PIC), and effective number of alleles ($N_e$) are reported for various combinations of dugong and manatee primers (Table 4-3). The most informative dugong and most informative manatee panels achieved a similar $P_{ID}$ as the combined panels. The $P_{ID}$ for each study group and the most informative marker-panels are reported in Table 4-4. The level of variability at each species-specific microsatellite locus in this study was comparable to those formerly reported.

**Amplification of Manatee Loci in Dugong**

All 11 polymorphic manatee microsatellites used with the dugong samples were determined to be in Hardy-Weinberg equilibrium. The number of alleles per locus ranged from 2 to 10. The single locus observed heterozygosities ranged from 0.071 to 0.734. Linkage disequilibrium was not identified after 55 pairwise comparisons. No evidence of null alleles was observed. The mean proportion of individuals typed was 0.84.

The eight manatee primers that amplified both dugong and manatee samples displayed more variation in the dugong samples. Manatee primers, $TmaE4$, $TmaE7$ and $TmaKb60$ identified larger $A$, $N_e$, and PIC in the dugong than in the manatee samples (Tables 4-1 and 4-2). $TmaKb60$ identified considerably more alleles in the dugong than in the manatee samples (7 vs. 3).

**Amplification of Dugong Loci in Manatee**

The 17 polymorphic dugong microsatellites used with the manatee samples were determined to be in Hardy-Weinberg equilibrium after a sequential Bonferroni correction. The number of alleles per locus ranged from 2 to 5 and the single locus observed heterozygosities ranged from 0.044 to 0.593. Linkage disequilibrium was not identified after 136 pairwise comparisons. Evidence of null alleles was observed in $DduC09$ and $DduF07$. The mean proportion of individuals typed was 0.982.
The 13 dugong primers that amplified both species identified equal or fewer alleles in the manatee than in the dugong (Tables 4-1 and 4-2). The dugong primers, *DduE08*, *DduF06*, and *DduF07*, identified equal *A* and greater *N*<sub>E</sub> than in the dugong samples. A greater PIC was observed for *DduE08* and *DduF07* in the manatee samples.

**Most Informative Markers: Dugong and Manatee Primers Combined**

**Dugong samples**

The 37 polymorphic dugong and manatee loci were sorted by PIC and *N*<sub>E</sub>. Overall, the dugong primers were more informative than the manatee primers in the dugong samples (Table 4-3). The top 11 loci produced a *P*(ID)<sub>sib</sub> estimate of 5.15 x 10^{-05}, in which unrelated individuals could be identified in a sample of N=1.9 x 10^4. After 55 pairwise comparisons, linkage disequilibrium was observed between *TmaA04/DduB01*, *TmaA09/DduB02*, and *TmaA09/DduE04*. When compared to the dugong primers alone, the mean PIC and *N*<sub>E</sub> increased by 0.200 and 1.329, respectively. In comparison, it required two additional (N=13) dugong-specific primers to obtain a similar *P*(ID) estimate (*P*(ID)<sub>sib</sub> 2.93 x 10^{-05}; HW *P*(ID) 7.68 x 10^{-12}), although less informative results were achieved at the other parameters. The resultant mean values for all of the tested marker-panels were lower than those for the combined primer set.

**Manatee samples**

The 35 dugong and manatee loci that amplified manatee samples were sorted by PIC and *N*<sub>E</sub>. Overall, the manatee primers were more informative than dugong primers in the manatee samples tested (Table 4-3). The top 13 loci produced a *P*(ID)<sub>sib</sub> estimate of 1.39 x 10^{-04}, in which unrelated individuals could be identified in a sample of N = 7000. Linkage disequilibrium was not observed after 78 pairwise comparisons. The mean PIC and effective number of alleles increased by 0.21 and 0.88, respectively, when compared to the manatee primers alone. In comparison, it required two additional (N=15) manatee-specific primers to obtain a similar *P*(ID)
estimate ($P_{ID,sib} \ 1.54 \times 10^{-4}$; $HW \ P_{ID} \ 4.77 \times 10^{-5}$), although less informative results were achieved at the other parameters. The resultant mean values for all of the tested marker-panels were lower than those for the combined primer set.

**Discussion**

The most informative markers from the manatee and dugong primer sets produced more sensitive panels than the species-specific primers alone in both the dugong and manatee samples (Table 4-3). These primer-panels incorporated the fewest markers (decreasing cost and improving time effectiveness) while identifying individuals within the current population size estimates. When compared to the most informative species-specific primers, the combined primer sets produced higher mean heterozygosity, number of alleles, effective alleles, and polymorphic information content, and needed fewer primers to achieve similar $P_{ID}$ values. The Hardy-Weinberg disequilibrium observed in the dugong and manatee samples likely resulted from finite population sizes and the increased potential for sampling related individuals in our dataset.

**Cross-Species Amplification**

Overall, the manatee primers performed better than the dugong primers in the cross-species studies. Therefore, when the dugong and manatee primer-sets were combined, the dugong samples had the greatest improvement and fewer loci were needed to obtain a lower $P_{ID}$. Of the 13 dugong primers that amplified in both species, three had a higher $N_E$ and two had a higher PIC in the manatee samples as compared to the dugong samples. Of the nine manatee primers that amplified both species, six performed equally or better for all parameters in the dugong samples. The dugong has a larger population sizes and greater habitat distribution and diversity than the West Indian manatee. These circumstances can lead to an increase in genetic diversity (DiBattista 2007; Garner *et al.* 2005).
Conservation Implications

The characterization of the most informative marker-panels for the Australian dugong and Florida manatee greatly enhances the genetic tools for conservation of sirenians around the world. Molecular markers can identify individuals and provide information on life history, disease processes, and population sizes in conservation studies. Pedigree analyses can detect successful breeders and assist with predicting annual reproductive rates (although, markers for pedigree studies should take into account the effect of null alleles). Genetic studies can also assist in identifying intrapopulational differentiation and genetically unique groups of animals that would benefit from increased protection.

Although the primer-sets presented here may not be optimal for all dugong and manatee populations, these panels are valuable during the initial phase of genetic analyses. For example, recent sightings of dugongs thought to be regionally extinct in Okinawa, Japan have raised concerns for managers. The implementation of highly informative markers could help identify whether the population is unique and isolated or if individuals are traveling from adjacent populations. Microsatellite studies of the Antillean, Amazonian and West African manatees in Central and South America and West Africa could shed light on the connectivity and relatedness among the populations. Identification and protection of source populations could lead to increased expatriation of individuals. Additionally, movement probabilities, adult survivorship, and reproductive rates could assist with population status modeling (Tringali et al. 2008b). The employment of more robust markers in sirenian population genetics studies should greatly facilitate conservation efforts for the recovery of dugongs and manatees around the world.
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The number of alleles ($A$), observed and expected heterozygosity ($H_O$ and $H_E$), polymorphic information content ($PIC$), and effective number of alleles ($N_E$) are reported. The top 11 primers achieved a $P_{ID}$ estimate for accurate individual identification.
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<td>0.468</td>
<td>0.357</td>
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<tr>
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<td>24</td>
<td>TmaF14</td>
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<td>3</td>
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<tr>
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<tr>
<td>29</td>
<td>DduF11</td>
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<td>0.306</td>
<td>0.258</td>
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<tr>
<td>30</td>
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<td>2</td>
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<td>0.262</td>
<td>0.226</td>
<td>1.352</td>
</tr>
<tr>
<td>31</td>
<td>TmaH23</td>
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<td>0.146</td>
<td>0.134</td>
<td>1.169</td>
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<tr>
<td>32</td>
<td>DduA07</td>
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<td>0.133</td>
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<tr>
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<td>0.044</td>
<td>0.043</td>
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</tbody>
</table>

The number of alleles ($A$), observed and expected heterozygosity ($H_O$ and $H_E$), polymorphic information content ($PIC$), and effective number of alleles ($N_E$) are reported. The top 13 primers achieved a $P_{ID}$ estimate for accurate individual identification.
### Table 4-3. *Dugong dugon* and *Trichechus manatus latirostris* marker-panel summaries

<table>
<thead>
<tr>
<th></th>
<th>(A)</th>
<th>(H_O)</th>
<th>(H_E)</th>
<th>(PIC)</th>
<th>(N_E)</th>
</tr>
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<tr>
<td><strong>Dugong samples</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DP</td>
<td>4.920</td>
<td>0.523</td>
<td>0.548</td>
<td>0.501</td>
<td>2.598</td>
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<td>4.727</td>
<td>0.467</td>
<td>0.477</td>
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<td>AP</td>
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<td>0.506</td>
<td>0.518</td>
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<tr>
<td>ID</td>
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<td>0.655</td>
<td>0.677</td>
<td>0.635</td>
<td>3.338</td>
</tr>
<tr>
<td>IP</td>
<td>7.636</td>
<td>0.710</td>
<td>0.739</td>
<td>0.701</td>
<td>3.927</td>
</tr>
<tr>
<td><strong>Manatee samples</strong></td>
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<td></td>
</tr>
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<td>DP</td>
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<td>0.341</td>
<td>0.387</td>
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<td>1.930</td>
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<td>IM</td>
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<td>0.510</td>
<td>0.527</td>
<td>0.459</td>
<td>2.287</td>
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<tr>
<td>IP</td>
<td>4.690</td>
<td>0.548</td>
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<td>0.533</td>
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</tr>
</tbody>
</table>

Dugong primers (DP), manatee primers (MP), all dugong and manatee primers together (AP), the most informative dugong (ID) and manatee (IM) specific primers, and the most informative primer-panels for the dugong and manatee samples combined (IP). Averages are reported for the number of alleles (\(A\)), observed and expected heterozygosity (\(H_O\) and \(H_E\)), polymorphic information content (\(PIC\)), and effective number of alleles (\(N_E\)).

### Table 4-4. Sibling (\(P(\text{ID})_{\text{sib}}\)) and Hardy-Weinberg equilibrium (\(HW P(\text{ID})\)) probability of identity values for the four study groups; dugong and manatee primers PCR amplified on dugong and manatee samples

<table>
<thead>
<tr>
<th>Primer set</th>
<th>(P(\text{ID}))</th>
<th>Dugong samples</th>
<th>Manatee samples</th>
</tr>
</thead>
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<tr>
<td><strong>Dugong primers</strong></td>
<td>Sib</td>
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<td>8.90E-04</td>
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<td></td>
<td>HW</td>
<td>4.69E-09</td>
<td>4.93E-07</td>
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<tr>
<td><strong>Manatee primers</strong></td>
<td>Sib</td>
<td>7.10E-04</td>
<td>9.03E-05</td>
</tr>
<tr>
<td></td>
<td>HW</td>
<td>7.24E-09</td>
<td>1.62E-09</td>
</tr>
<tr>
<td><strong>Most informative primer-set</strong></td>
<td>Sib</td>
<td>5.15E-05</td>
<td>1.39E-04</td>
</tr>
<tr>
<td></td>
<td>HW</td>
<td>1.24E-11</td>
<td>2.75E-09</td>
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CHAPTER 5
CHROMOSOME PAINTING IN THE MANATEE STRONGLY SUPPORTS AFROTHERIA
AND PAENUNGULATA

Introduction

Recently the molecular based approaches of super-ordinal grouping of extant eutherians (Afrotheria, Euarchontoglires, Laurasiatheria, and Xenarthra) has gained popularity (Murata et al. 2003; Murphy et al. 2001b; Springer et al. 2003). However, one of the four proposed super-orders, Afrotheria, is controversial because it unites morphologically distinct species of African placentals (golden moles, tenrecs, otter shrews, elephant shrews, aardvarks, hyraxes, elephants, and sirenians). Within Afrotheria, sirenians, elephants, and hyraxes form a clade called Paenungulata. There is little morphological or paleontological evidence that provides support for Afrotheria (Stanhope et al. 1998). A movable snout was hypothesized as a synapomorphic trait, but this feature is apparently not homologous across different afrotherian lineages (Whidden 2002). More recently, it was proposed that aspects of placentation could provide a synapomorphy for this assemblage (Carter et al. 2006; Carter et al. 2004). Some outstanding issues in higher eutherian phylogenomics include the exact root of the placental tree, the relationships within the super-ordinal clade Laurasiatheria (moles, hedgehogs, shrews, bats, cetaceans, ungulates, pangolins, and carnivores), and resolving the trichotomy of sirenians, elephants, and hyraxes (Murphy et al. 2004).

Sirenia and Hyracoidea are the two afrotherian orders remaining to be investigated with molecular cytogenetic techniques. In this paper, the chromosome painting of the Florida manatee (Trichechus manatus latirostris) is reported. These data should be a valuable addition to our understanding of afrotherian relationships and the eutherian ancestral karyotype.
The Florida Manatee

The endangered Florida manatee is a subspecies of the West Indian manatee (*Trichechus manatus*) in the order Sirenia. Sirenians are often considered phylogenetic outliers. Despite similarities in adaptations, habitat, and body shape, they have no evolutionary relationship with the other orders of marine mammals. Extant sirenians are the only herbivorous marine mammals and live in fresh, brackish, or marine habitats dispersed along tropical and subtropical environments.

Previous Cytogenetic Reports on Manatees

Solid stained chromosome studies were completed on a limited number of individual manatees, establishing the chromosome number as 2N = 48 for the Florida manatee (White *et al.* 1976a; White *et al.* 1977) and 2N = 56 for the Amazonian manatee (*Trichechus inunguis*; Loughman *et al.* 1970). Following solid staining, chromosome-banding procedures allowed for the identification of individual chromosome regions. Giemsa and trypsin staining, or GTG-banding, was used to create karyotypes and ideograms for the Florida manatee (Gray *et al.* 2002) and the Amazonian manatee (Assis *et al.* 1988).

Comparisons of chromosome painting data provide an independent test of the contrasting hypotheses on mammalian evolution and phylogeny. The research presented here clarifies the phylogenetic position of the manatee and tests the validity of the radical taxonomic assemblage known as Afrotheria. The results are then compared to other chromosome painting data in Afrotheria. In light of the findings, the relationships within Afrotheria and the alternative organizations of the ancestral eutherian karyotype are assessed.

Methods

Chromosome preparations of a male Florida manatee (*Trichechus manatus latirostris*, TMA) were obtained from peripheral blood mononuclear cells (PBMCs) and primary fibroblast
cartilage cell culture. Cells were cultured in RPMI 1640 (Hyclone) supplemented with 20% fetal bovine serum (FBS), L-glutamine (0.01%) and gentamicin (25 μg/ml). PBMCs were incubated in-vivo using phytohemagglutinin (PHA, 0.25 mg/mL) as a mitotic stimulant for 72 to 96 hr at 36°C in 5% carbon dioxide, 95% air, and 100% relative humidity. Routine procedures were used for chromosome preparations. We followed the chromosome nomenclature as previously published (Gray et al. 2002) pairing and grouping chromosomes by banding patterns, relative lengths and morphology.

Human chromosome paints were obtained as previously described by chromosome flow sorting followed by degenerate oligonucleotide primed PCR amplification (Stanyon et al. 1999; Telenius et al. 1992). Paints were labeled with either biotin-dUTP, digoxigen-dUTP (both from Roche Applied Science) or Spectrum Orange-dUTP (Vysis).

Interspecific in-situ hybridizations of Florida manatee chromosomes with human probes were performed with 300 to 500 ng of each biotin-labeled probe, 10 μg of human Cot-1 DNA and 5 μg of ssDNA. The mixture was precipitated and dissolved in 13–15 μl of hybridization mixture (formamide 50%, dextran sulfate 10%, 2 × SSC). Direct labeling with Spectrum Orange followed a Nick Translation protocol (Vysis) using 1 μg of each amplified human DNA probe, 0.2 mM Spectrum Orange and 25 μg each of human and manatee Cot-1 DNA (Applied Genetics Laboratories, Inc.). The mixture was precipitated and dissolved in 10 μl distilled water. Approximately 300 ng of probe from this mixture were dissolved in 10.5 μl Hybrizol VII (Q-BIOgene) and 0.75 μg each of human and manatee Cot-1 DNA.

The labeled probe mixture was denatured at 80°C for 10 min and reannealed at 37°C for 90 min before hybridization. Slides were aged at 37°C for 30 min followed by dehydration in a room temperature 70, 80, 90, and 100% ethanol series. The DNA was denatured in 70%
formamide/2 × SSC, at 65°C for 90–120 s, and quenched in an ice-cold ethanol series. Hybridization was carried out in a humidity chamber at 37°C for five days. Post-hybridization washes followed standard procedures at 40°C. Biotin detection was performed with avidin-conjugated FITC (Vector) for 45 min at 37°C. Counterstaining was performed with DAPI (0.8 ng/μl) for 10 min and the slides were mounted with antifade (100 mg p-phenylenediamine in 80 ml glycerine, 20 ml PBS, pH 8).

Analyses were performed under a Zeiss Axiophot 2 or Axioskop fluorescence microscope coupled with a CCD camera (Photometrics), and images were captured with the Smart Capture software (Digital Scientific Inc.).

Results

Examples of human chromosome paints (HSA) hybridized to manatee (TMA) metaphase chromosomes are shown in Figure 5-1. Synteny was found intact in nine (4, 5, 6, 9, 11, 14, 17, 18, and 20) of the 22 human autosomal and X chromosomes (Figure 5-2). Two hybridization signals were evident on separate manatee chromosomes for ten human chromosomes (1, 7, 8, 10, 12, 13, 15, 16, 21, and 22). The human 19 paint hybridized to three TMA chromosomes (2, 12, and 14). Human chromosomes 2 and 3 were highly fragmented in the manatee genome and painted four and five chromosomes, respectively (Table 5-1). Due to the small signals involved and the quality of the metaphases, it was more difficult to assign the hybridization pattern for these two chromosomes. Human chromosome paint 12 provided three signals on TMA 7, most likely due to an inversion. Chromosome paints with pericentromeric signals on both arms of the same chromosome were considered as one signal. Centromere areas on the manatee karyotype were not hybridized. The Y chromosome was the only human probe that failed to provide a signal in the manatee. Altogether, the human autosomal chromosome paints and the X chromosome paint delimited a total of 44 homologous segments in the manatee genome. Human
chromosome paints hybridized to 20 (15 unique) segments in the manatee genome: 1/15, 1/19, 2/3 (twice), 3/7 (thrice), 3/13, 3/21, 5/21, 7/16, 8/22, 10/12 (twice), 11/20, 12/22 (thrice), 14/15, 16/19, and 18/19.

**Discussion**

The painting map of the manatee genome was compared with results published on other Afrotheria taxa: aardvark, elephant, elephant shrew, and golden mole (Fronicke *et al.* 2003; Robinson *et al.* 2004; Svartman *et al.* 2004; Yang *et al.* 2003). An assessment of the associations found in each taxa are shown in Table 5-1. All species have eight associations in common (1/19, 3/21, 5/21, 7/16, 10/12, 12/22, 14/15, and 16/19). Five of these associations are considered ancestral to all eutherians by most proposals (3/21, 7/16, 12/22 twice, 14/15, and 16/19). It appears that the associations 1/19 and 5/21 can be used to link afrotherian species (Fronicke *et al.* 2003; Robinson *et al.* 2004; Svartman *et al.* 2004; Svartman *et al.* 2006). These associations provide cytogenetic support, in agreement with molecular studies, that Afrotheria is a natural clade.

New chromosome painting data in Xenarthra (anteaters, sloths, and armadillos) are also informative towards the ancestral eutherian karyotype. Of the four species studied, *Tamandua tetradactyla*, *Choloepus didactylus*, *C. hoffmanii*, and *Dasypus novemcinctus* (Svartman *et al.* 2006; Yang *et al.* 2006), only the anteater has a 1/19 association. It is not likely that this association is homologous to Afrotheria, because the anteater has the most highly rearranged karyotype known in Xenarthra (Svartman *et al.* 2006).

The manatee data indicate that the association 10/12/22 is most likely ubiquitous throughout Afrotheria. A combination HSA10p/12p/22q and a single HSA10q were found in the aardvark and elephant karyotypes (Fronicke *et al.* 2003; Yang *et al.* 2003). An apparently identical association was later found in the elephant shrew and golden mole (Robinson *et al.*
2004). The question is whether this association is a third cytogenetic landmark for the Afrotheria clade, or instead should be considered part of the ancestral eutherian karyotype.

The entire 10/12/22 association appears to be present in clades I, Afrotheria, and IV, Laurasiatheria, only partially present in clade II, Xenartha (10/12), and absent in clade III, Euarchontoglires (primates, rabbits, rodents, tree shrews, and flying lemurs). Carnivores have a homologous 10/12/22 association to Afrotheria, as demonstrated by reciprocal chromosome painting (Graphodatsky et al. 2002; Nie et al. 2002). Eulipotyphla (shrews, solenodons, moles, hedgehogs, and Nesophontes) also have the 10/12/22 association (Yang et al. 2006; Ye et al. 2006). Chromosome painting data in Xenartha show that a 10/12 association is present in the armadillo (D. novemcinctus; Svartman et al. 2006). To date, the 10/12 association has been found in three of the four eutherian mammal clades. Yet, there is no reciprocal painting in Xenartha to prove that the 10/12 association is truly homologous to that found in Afrotheria. Several hypotheses can be developed with different implications if Afrotheria or Xenartha is considered basal. If Afrotheria is basal, the occurrence of 10/12/22 in clades I and IV would suggest that this association is part of the ancestral eutherian karyotype with a subsequent, independent loss in clades II and III. The occurrence of the 10/12/22 association in clades I and IV could be considered a phylogenetic link. Alternatively, the association could have been independently acquired in the two clades. If Xenartha is basal, this association could have originated in Afrotheria and was then lost in clade III.

Association 3/13 was found in the manatee, elephant and elephant shrew. However, there are no reciprocal painting data between human and manatee or human and elephant shrew. Therefore, it is not possible to confirm that the 3/13 association is homologous (involves the same segments of both chromosomes 3 and 13). In view of the afrotherian molecular data, this
association was independently derived in the Macroscelidae (elephant shrews) and Paenungulata phylogenetic lineages (Murphy et al. 2004).

Support for the Tethytheria and Paenungulata Assemblage

Before the advent of molecular studies, some morphologists placed sirenians, elephants, and hyraxes under Ungulata. Elephants and sirenians were grouped together in Tethytheria, while hyraxes were placed in Phenacodonta along with perissodactyls (McKenna 1975). Results in molecular studies are inconsistent and fail to resolve the Paenungulate trifurcation (Murphy et al. 2004) and some data do not support Tethytheria (Amrine-Madsen et al. 2003; Murphy et al. 2001a; Murphy et al. 2001b; Waddell & Shelley 2003). Mitochondrial genome analyses do support Tethytheria, but exclude Hyracoidea (Murata et al. 2003). SINE insertion data produced incongruent phylogenetic relationships within Paenungulata, most likely due to a rapid divergence from a highly polymorphic last common ancestor (Liu & Miyamoto 1999).

The chromosome mapping data strongly support Tethytheria (Sirenia and Proboscidea) and implies support for the clade Paenungulata (Sirenia, Proboscidea, and Hyracoidea). There appear to be four derived associations linking elephants with manatees: 2/3, 3/13, 8/22, and 18/19. HSA 4/8p was not present in the manatee and may represent a derived trait of Paenungulata. Both publications on the elephant indicate that this association is also lacking (Fronicke et al. 2003; Yang et al. 2003). It is possible that the 4/8 association went undetected in our study, as well as in elephants. Although, the widespread occurrence of the 4/8 association in all mammals, outside of elephants and most primates, lends credence to its inclusion in the ancestral eutherian karyotype. It would be useful to test these hypotheses with rock hyrax chromosome painting data.
Branching Order in Afrotheria

The branching order within Afrotheria has not reached a consensus. Some authors have viewed Macroscelidae, the elephant shrews, as the most basal and early divergent order within Afrotheria (Murphy et al. 2001a; Springer et al. 1999). However, Murphy et al. (2001b) placed the triumvirate of sirenians, elephants, and hyraxes (Paenungulata) as basal, verified by additional molecular data (Kullberg et al. 2006; Murata et al. 2003; Springer et al. 2003). It is difficult to determine which order is most basal because sirenians and elephants, like other afrotherian species, have fairly derived karyotypes.

According to Robinson et al. (2004), associations 2/8, 3/20, and 10/17 link elephant shrews, golden moles/tenrecs and aardvarks. Only the association 2/8 is present in all three. Recently, the association 2/8 was also found in anteater (T. tetradactyla), sloth (Choloepus didactylus), and pangolin (Manis javanica; Svartman et al. 2006; Yang et al. 2006). Associations 3/20 and 10/17 are lacking in golden moles/tenrecs. Murphy et al. (2004) proposed that the associations 3/20 and 10/17 were probably lost in golden moles/tenrecs. No reciprocal painting was done in elephant shrews or golden moles/tenrecs and it is therefore unknown if these associations are actually homologous. There is weak cytogenetic evidence linking elephant shrews and golden moles/tenrecs. An alternate hypothesis might be a sister relationship between aardvarks and elephant shrews. Perhaps a rapid divergence in elephant shrews, golden moles/tenrecs, and aardvarks also resulted in limited phylogenetic signals for these chromosome associations.

The Root of the Eutherian Tree

Although the super-order assemblies appear well established, the most basal position on the eutherian tree has not been determined with certainty (Delsuc et al. 2004; Murphy et al. 2001a; Murphy et al. 2001b). Afrotheria and Xenarthra are the two oldest eutherian clades and
probably emerged from the Southern Hemisphere in excess of 100 million years ago (Eizirik et al. 2001; Springer et al. 2004). Molecular dating and biogeography have provided evidence that crown-group Eutheria may have their most recent common ancestry in the Southern Hemisphere (Gondwana; Springer et al. 2004). The other two clades (Laurasiatheria and Euarchontoglires) can be grouped as Boreoeutheria (Liu et al. 2001).

There are currently three hypotheses for the root of the eutherian tree. Most discussions from molecular studies place emphasis on either Afrotheria or Xenarthra as the most basal clade (Douady et al. 2002a; Murphy et al. 2001b). A third hypothesis states that the ancestral eutherian karyotype is a combination of both clades. This hypothesis cannot be completely ruled out and is preferred in some studies (Douady et al. 2002b; Kriegs et al. 2006). However, the suite of derived chromosomal associations found in all studied Afrotheria argues against the hypothesis that a combination of the two clades is basal to the eutherians.

Recently, a report on retroelements gives support for the hypothesis that Xenarthra is the sister group to all other placentals (Nishihara et al. 2005). Indeed, new cytogenetic comparisons show that the proposed ancestral eutherian karyotype is essentially conserved in Xenarthra, specifically in the two-toed sloth (Choloepus hoffmanii; Svartman et al. 2004). These two studies should be given attention because both take into consideration rare genomic events in which convergence is particularly limited. The conserved xenarthran karyotype may well be indicative of their phylogenomic position among eutherians. However, an essential point is that all reconstructions of the ancestral eutherian karyotype are preliminary until a relevant outgroup is studied with chromosome painting. A taxonomically rich array of species supported by appropriate out-groups is vital to the reconstruction of mammalian genome evolution. The deficiency of comparative chromosome painting data between eutherians and marsupials is a
severe limitation on attempts to delineate the mammalian ancestral genome. The analyses of other afrotherians, xenarths, and marsupials may clarify these unresolved questions.

**Conclusions**

The chromosome painting data presented here leave little doubt that Tethytheria is a clade within Afrotheria and implies support for the Paenungulata assemblage. Recent retroposon data also confirmed Paenungulata, but could not resolve the phylogenetic relationships among elephants, sirenians and hyraxes (Liu & Miyamoto 1999). It is generally appreciated that characters with high evolutionary rates provide good phylogenetic resolution. Afrotherian karyotypes demonstrate high rates of chromosome evolution and numerous derived inter-chromosomal rearrangements link elephants and manatees. It is therefore likely that additional chromosome painting in rock hyraxes could shed light on the divergence sequence and resolve the Paenungulata trichotomy.
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The taxa in the first, left column: AEK = ancestral eutherian karyotype [14, 16, 40]. The second column list the 2n, diploid numbers for each species and the remaining columns refer to signals found for each human chromosome. The number in brackets refers to higher number of hybridization signals due to pericentric inversions.
Figure 5-1. Examples of hybridizations in the manatee a) human 12, b) human 13, c) human 14 in green and 15 in red d) human 17 in green and 18 in red
Figure 5-2. The karyotype of the manatee is shown to the left and the color-coded ideogram to the right (modified from Gray et al. 2002). Manatee chromosomes are numbered below and human chromosome homology is shown laterally.
CHAPTER 6
CONCLUSION

Marine Mammal Population Genetics

Genetic techniques have assisted in quantifying and comparing the genetic diversity and dispersal ability of marine mammals. Some marine mammals have global distribution, like sperm whales, while others are more restricted, like the Puerto Rico manatee. The extent and pattern of dispersal has a large effect on population structure. High mobility and less restrictive aquatic boundaries are conducive for large, panmictic marine mammal populations. However, most marine mammal species develop fine-scale population structure due to social relationships and resource availability (Hoelzel 1998). Multiple genetic stocks in the same geographic area, especially with high human activity, like fisheries, can lead to difficulties in monitoring and conserving specific lineages. This complicates the identification of management units and temporal, spatial, and genetic factors must be taken into account for the conservation of a population.

Many marine mammal species have similar demographic characteristics, including generally large body size, hydrodynamic body shapes, modified appendages and various thermoregulatory adaptations. Additionally, sirenians and cetaceans have long life spans and generation times, producing limited offspring in their lifetime and investing a great deal of time and energy into their young. Marine mammals have different mechanisms for reproduction and parturition. Breeding sites and suitable habitat are limiting factors that affect population structure. Many species travel to one location to breed and then disperse great distances to feed. This may restrict the gene flow, as only certain groups breed together. Resources, predation, and thermal factors affect reproductive population boundaries.
Environmental and anthropogenic factors strongly affect manatee and other marine mammal population structure and often limit genetic diversity. For example, direct harvesting and habitat destruction are anthropogenic disturbances that often result in substantial reductions in population size and loss of genetic variability (genetic bottlenecks). The Caribbean monk seal (*Monachos tropicalis*), the Japanese sea lion (*Zalophus japonicus*), and Steller’s sea cow (*Hydrodamalis gigas*) were hunted to extinction and many populations remain dangerously small, like the Northern Atlantic right whale (*Eubalaena glacialis*; Hoelzel *et al.* 2002). Additionally, Pleistocene glacial events may have limited marine mammal habitat and affected the population structure, as seen in the hooded seal, harbor porpoise, and manatee populations (Coltman *et al.* 2007; Garcia-Rodriguez *et al.* 1998; Tolley *et al.* 2001; Vianna *et al.* 2006).

The following discussion will examine and compare the findings of marine mammal genetic investigations with the manatee results discussed previously. To understand better genetic diversity and population structure in the aquatic environment, the mechanism of population decline, severity, recovery, and current genetic state of other marine mammal populations will be discussed and compared to the Belize, Florida, and Puerto Rico manatee populations.

The average mammalian mitochondrial (mtDNA) haplotype diversity is \( h \geq 0.5 \) and nucleotide diversity, \( \pi = 0.001-0.020 \), depending on the loci and species surveyed (Frankham *et al.* 2002). The Florida manatee population has no mitochondrial diversity and Puerto Rico has low diversity \( (h = 0.48500, \pi = 0.00132) \). Belize has more variation \( (h = 0.53430, \pi = 0.030602) \), due to a high number of nucleotide differences among haplotypes. Disturbed populations (harvested or fragmented) were determined to have lower microsatellite genetic diversity \( (H_E = 0.60; A = 6.17-6.59) \) than non-disturbed populations (DiBattista 2007). The
manatee populations surveyed here have lower expected heterozygosity and allelic diversity, respectively, than the average disturbed mammalian population: Florida (0.48, 5.3), Belize (0.455, 3.4), and Puerto Rico (0.447, 3.9). The Florida and Belize populations appear to have high gene flow and little genetic separation among geographic regions. Meanwhile, Puerto Rico has strong mitochondrial DNA structure and more nuclear subdivision than the other populations.

**Within Population Variation**

**Population Bottlenecks**

Long-term hunting pressures have severely affected manatees and other marine mammal species. For example, the polygynous northern elephant seal population was reduced to approximately 10-30 individuals from 1810-1860 (Hoelzel et al. 1993). In 1922, elephant seals received protection and have recovered from the bottleneck to 150,000 individuals (Stewart et al. 1994). Historical diversity was determined to be extremely high in 179bp of the mtDNA control region. In five pre-bottleneck bone samples, four haplotypes were found with high $h$ (0.9) and $\pi$ (0.0065). The high variation may be an artifact due to the representation of multiple generations in the analysis.

In 149 contemporary samples, one population with low genetic diversity was indicated. In 300bp, only two divergent haplotypes were found, $h = 0.41$ and $\pi = 0.0066$ (Weber et al. 2002). Consistent with the severity of the population bottleneck, the loss of haplotypes in the northern elephant seal is substantial.

Although the population rapidly increased, a severe bottleneck, natural climatic cycles, or multiple population crashes as a consequence of persistent harvesting by native peoples before modern exploitation in 1810 may have limited the genetic diversity. Additionally, genetic diversity in highly polygynous species is strongly affected by a limited number of reproducing
males and respectively the decreased heterozygosity. The effective population size is lowered when all offspring from a harem are related.

Manatees are not polygynous, but severe bottlenecks, persistent exploitation, and a long generation time have reduced the diversity to the levels observed in the elephant seals. The Florida population had lower and the Puerto Rico populations had equivalent diversity. Once reduced, a long generation time can limit the speed with which population size, and more importantly, genetic diversity can recover. The manatee studies investigated an additional 110bp of the same marker, which is a significant increase when analyzing sequence data, giving strong support to the results of the manatee studies.

In an analogous example, Hawaiian monk seals (Monashus schauinslandi) were hunted to near extinction in the 1800’s. An estimated population of 50 animals survived the bottleneck. Unfortunately, after a partial recovery to 3,000 individuals, a second decline occurred from 1950-1970, reducing the population by 50%.

The first 303bp of the control region and 56bp of the proline tRNA gene were amplified in the mitochondrial genome. The combined 359bp identified three haplotypes in 50 individuals ($\pi = 0.0006$), with 86% of the individuals having one haplotype (Kretzmann et al. 1997). This indicates low variation and a homogenous population due to no genetic subdivision and a severe reduction of individuals and genetic diversity. A slow recovery and additional population declines reduced the diversity further.

Another Hawaiian monk seal study analyzed 27 microsatellite loci developed in related species (Kretzmann et al. 2001). Cross-species microsatellite application usually leads to less detected polymorphism than studies with species-specific primers. Only three of the primers were polymorphic, each having two alleles, $H = 0.396$. Reflective of the mtDNA data, the five
Hawaiian Islands populations indicated little to no nuclear genetic structure. Nevertheless, three primers are not typically statistically significant to obtain accurate genetic diversity values.

The Belize, Florida, and Puerto Rico manatee populations also have reduced variation at ≥15 loci. This may be due to climatic or anthropogenic bottlenecks or is intrinsic to the populations. Both the Florida and monk seal studies looked at n = 50 individuals. However, only one haplotype was detected in Florida and the seals had three. Belize and Puerto Rico manatees have three haplotypes, but more individuals were investigated (≥115), increasing the likelihood of identifying an additional haplotype. Bones from historical pre-bottleneck manatee specimens may contain additional haplotypes and should be investigated.

**Gene Flow in Island and Mainland Populations**

Molecular markers provide information on the amount and extent of gene flow among populations and habitats. The Belize and Florida manatees live in mainland habitat and have less genetic geographical structure than the manatees in the island habitat of Puerto Rico. The island has areas of very deep water close to shore, producing poor manatee habitat, and potential barriers to movement. Similarly, molecular analyses of mainland and island populations of southern elephant seals identified strong differences in the genetic diversity between the adjacent mainland and island habitats (Hoelzel *et al.* 2001).

Mainland seal nucleotide diversity was low (0.003), suggesting a female founding event or recent bottleneck. One mainland maternal lineage was indicated, since two of the haplotypes are derivable from the third by a single base-pair change. Alternatively, nucleotide diversity was high in the 24 island haplotypes (0.023), possibly due to extensive immigration from the surrounding network of islands. The island and mainland haplotypes were not derivable from each other and therefore maximum likelihood and neighbor-joining trees indicated no female migration between the populations.
However, little genetic separation was seen between the two habitats ($F_{ST} = 0.025$) with four microsatellite loci. Mainland and island heterozygosity was 0.5750 and 0.6241 while allelic diversity was 5.1 and 5.4, respectively. The similar microsatellite heterozygosity and low genetic diversity value ($F_{ST}$) represents frequent male migration and breeding, homogenizing the nuclear DNA. Alternatively, no female migration between the two populations was indicated, since the mtDNA haplotypes were unrelated. The use of four loci lack statistical power and may have biased the result.

The two habitats also indicated morphological differentiation. The mainland pups are larger, possibly due to more abundant resources, while the island animals have a significantly larger number of vibrissae (perhaps due to different foraging habitat).

The non-related mainland and island haplotypes indicated that unrelated females founded the populations. However, a low $F_{ST}$ value suggests a contemporary relationship between the populations through male migration. In contrast, the Florida manatee population shares the A01 haplotype with the Puerto Rico island population, but the $F_{ST}$ between the populations is high, suggesting strong differentiation and little contemporary migration.

The mainland and island habitats produce similar genetic signals in the seals and manatees. The Puerto Rico island habitat has more mtDNA diversity than the closest mainland population, Florida. The smaller nucleotide diversity in the Florida mainland environment may be due to a founding event, limited supplementation from surrounding populations, or human persecution exterminating haplotypes. Additionally, Puerto Rico may have more diversity from Caribbean and South America immigration and/or geographic barriers within the island, allowing divergence over time. Belize and Florida show less population structure than the island habitat of Puerto Rico, possibly due to uninterrupted habitat and large dispersal capabilities. Florida
manatees follow the seal’s mainland trend of having larger body sizes, although in the manatee this is due to adaptations for thermo-regulation.

**Among Population Variation and Gene Flow**

Molecular markers can test relatedness, gene flow, and population structure among geographic regions to understand better population differentiation. Harvesting, habitat selection, fragmentation, colonization, and/or genetic drift can generate intraspecific structure among populations.

**Moderate Variation and Gene Flow**

Florida and Puerto Rico manatees are managed together as one population. Although the same haplotype (A01) is found in both populations, its presence does not support a contemporary genetic relationship. Therefore, the degree of nuclear relatedness was tested to determine whether they are the same population or should be managed independently. Furthermore, the mtDNA and microsatellite results were compared to address male and female movement patterns.

In a similar study, three Steller sea lion (*Eumetopias jubatus*) populations were tested for migration and relatedness (Hoffman *et al.* 2006). An mtDNA study of 238 bp, 145 haplotypes in 1,500 individuals, indicated separate eastern, western, and Asian groups in the North Pacific (Baker *et al.* 2005). Strong barriers to gene flow were observed among the three stocks and diversity values within the individual rookeries were high ($h = 0.9164; \pi = 0.00967$). The high variation is consistent with other marine mammals that have not encountered severe bottlenecks from exploitation.

A subsequent nuclear DNA study detected two homogenous populations as opposed to three, combining the western and Asian groups. Over 700 animals were analyzed at 13 microsatellite loci (Hoffman *et al.*, 2006). The average number of alleles was 7.9 with a range of
Expected heterozygosity ranged from 0.237-0.843. Slatkin’s linearized $F_{ST}$ (Slatkin 1995) genetic distance matrixes and trees identified two stocks with weak intraspecific structure. Cavalli-Sforza and Edward’s chord distance, $D_c$ (Cavalli-Sforza & Edwards 1967), generated a second genetic distance matrix and tree again identifying two distinct clades of eastern and western stocks with short branch lengths and low levels of differentiation. The resultant phylogenetic tree topography from the two distance matrixes agreed, indicating robust analyses, although bootstrap significance values were not reported. Populations with close geographic proximity clustered together. A Mantel test examined associations between Slatkin’s linearized $F_{ST}$ and straight-line geographic distance among rookeries. An isolation-by-distance pattern was found when the stocks were analyzed together. Isolation-by-distance occurs in subdivided populations via random genetic drift when subpopulations exchange genes at a rate dependent upon the geographical distance. No correlation between genetic and geographic distance was found when stocks were analyzed individually, indicating little subdivision within stocks.

A Bayesian cluster analysis using STRUCTURE 2.1 (Pritchard et al. 2000) tested genetic relationships without a priori designation of geographical locality. Again two weakly separated clusters were identified, similar to the tree groupings, but with some individuals not grouping with their natal areas.

In summary, the mtDNA data specified three stocks, strong female sight fidelity, and a significant east-west split. The east-west separation is also found in harbor seals (Westlake & O’Corry-Crowe 2002) and sea otters (Cronin et al. 1996) and possibly represents a historical structure imposed by the Pleistocene glacier. Conversely, the microsatellite data indicated two populations and less east-west differentiation. The STRUCTURE plot and neighbor-joining trees showed minimum differentiation between the two clusters, suggesting genetic mixing. The
combined results from the mtDNA and microsatellite markers indicate male dispersal and strong philopatry in females. Females may stay within one of the three populations while the males move considerably between the western and Asian regions and moderately between the east and west. Only large tables with pair-wise comparisons among every population were presented. Reporting the averaged $F_{ST}$ and $R_{ST}$ values between the stocks would have assisted in quantifying the genetic differentiation.

Steller sea lions inhabit the North Pacific Rim with a nearly consistent distribution. The historical population was estimated at a quarter of a million animals. The contemporary population has decreased to a little over 100,000, with the smallest population around 13,000 (Calkins et al. 1999). Although the decline is dramatic, these numbers are much larger than other populations of endangered mammals. The potential for a large effective population size and intact genetic variation is high.

All sea lion genetic diversity estimates were higher than those for manatees. Sea lions have large populations, continuous habitat, and a high reproductive rate, while West Indian manatees have small, patchy, and often isolated populations. Belize and Florida are the largest West Indian manatee populations, numbering approximately 1,000 and 3,000, respectively. Small and erratically spaced populations lead to fewer effective breeders, less genetic variation, and limited dispersal and supplementation capabilities. The nuclear division identified between the Florida and Puerto Rico manatees was greater and more conclusive than the sea lion population division.

Both mtDNA and nuclear markers support a geographical population division within the sea lion and Puerto Rico populations. Additionally, the sea lion and Puerto Rico manatee populations have similar male/female movement patterns. Females express strong site fidelity as
indicated by the mtDNA divisions, while the nuclear DNA indicates less of a population separation, suggesting male dispersal among the populations. Because Florida has no mtDNA diversity, the nuclear and mtDNA comparison cannot be made. Microsatellite $F_{ST}$ and neighbor-joining trees identified high gene flow and low genetic differentiation within the Florida, Belize, and Steller sea lion populations. This is most likely due to the continuous habitat and lack of barriers to dispersal.

**Panmictic Populations**

Molecular markers assist in evaluating gene flow among sub-populations or geographical regions within a population. For example, microsatellites detected high amounts of geneflow in the Florida manatee population. Similarly, the world’s hooded seal populations appear to breed in a panmictic manner, although, the two populations and four breeding herds are geographically separated by large distances (Coltman *et al.* 2007). Over 900 bp were sequenced containing cyt $b$, tRNA, and the control region. A total of 105 haplotypes was observed in 123 individuals, with only 12 identified more than once. The variation was high, with haplotype diversity approaching 1.0 in all populations and nucleotide diversity being 0.023. The authors reported that $\pi = 0.023$ was low, although the same value was considered high in southern elephant seals and values of 0.00967 and 0.00660 were reported as low in Steller sea lion and northern elephant seal studies, respectively (Baker *et al.* 2005; Hoelzel *et al.* 2001; Hoelzel *et al.* 1993). The authors may have compared $\pi$ to an unidentified population or used a different scale of relative values. The high values suggest rapid growth allowing the development of many haplotypes. The mtDNA haplotype tree indicated limited geographical structuring and an even diffusion of haplotypes.

In the same study, microsatellite markers investigated 300 hooded seals at 13 polymorphic loci. The heterozygosity ranged from 0.29-0.91 ($H_{ave} = 0.68$) and the number of alleles ranged
from 5-15 (A_{ave} = 12). A high level of genetic variation was observed in both pooled and individual subpopulations. Pair-wise comparisons of genetic differentiation (F\textsubscript{ST}) were at most 0.0009, representing little to no differentiation. A Bayesian cluster analysis using STRUCTURE was performed and only one population was identified. Although considerable genetic variation was detected with the nuclear and mtDNA markers, all of the variation was within, rather than among the subpopulations.

The limited diversity among the herds may be due to recent re-colonization from one population after the last glacial period. A study of harbor porpoises (Phocoena phocoena) also showed a genetic and geographic re-colonization pattern that may have been influenced by the last glacial epoch (Tolley et al. 2001). The high heterozygosity is expected for species that breed on packed ice, as the unstable habitat does not facilitate natal site-fidelity or highly polygynous mating systems. Each hooded seal population was recommended for protection to conserve the variation and allow for the development of genetic differences.

The study lacked analyses that would have increased the robustness of the results. A neighbor-joining tree indicating one population would have strengthened the hypothesis of little geographical structuring. Additionally, the results from STRUCTURE could have been improved with the addition of genotypes from a divergent population for comparison, as was done with the manatee analyses. If the data were available, a regression of F\textsubscript{ST} and straight-line geographic distance could have detected differentiation in the more distant populations.

The results of the hooded seal study corroborate the structure detected in Florida manatees. Florida manatees also move throughout their range, encouraging a high degree of breeding among the members of the population and limiting diversity. Similarly, only one Florida manatee population was identified by STRUCTURE. The Florida population also had a low global
F_{ST} value (0.00735). The manatee F_{ST} is not as low as the seals (0.0009) because some east-west manatee substructure is detected in the winter. The high seal heterozygosity indicates a large and genetically stable population while, the reduced manatee diversity may reflect the limited population sizes.

**Conclusions**

Overall, marine mammal populations have low diversity and limited population structure, influenced by bottlenecks, habitat degradation, founder effects, glacial events, anthropogenic mortality, and specialized mate selection and breeding strategies. Florida, Belize, and Puerto Rico manatees have lower mtDNA and microsatellite genetic diversity than many marine mammal populations. West Indian manatee populations have endured historical and long-term persecution. Most of the populations are small, erratically distributed, and isolated. Manatees must remain close to the shore for food and freshwater and few individuals have been sighted traveling in pelagic waters, limiting dispersal and gene flow. Additionally, manatees lack specific breeding and calving grounds, unlike other marine mammal populations, which could bring diverse individuals together, although in Florida long distance dispersal and breeding are observed.

The high degree of gene flow and migration in Florida and Belize manatees is possibly due to regularly distributed resources and few gene flow barriers in the habitat. Therefore, the population has not been partitioned into strongly divergent subpopulations. The Florida individuals have only one haplotype, produced by a bottleneck or founder event, which may have also reduced the founding nuclear diversity.

The three Puerto Rico haplotypes are derived from one another, producing low nucleotide diversity. Perhaps the founding females were separated by barriers and genetically diverged. Matrilinéal site fidelity has remained strong in Puerto Rico, effectively separating the island at
the north-west and south-east regions. Limited fresh water and poor habitat may have created subtle genetic subdivision at mtDNA and microsatellite markers. More division is seen with mtDNA than microsatellite markers, suggesting female site fidelity and male dispersal. Similarly, the Belize population has different haplotype proportions in the Drowned Cayes and Southern Lagoon.

Many marine mammal populations have experienced strong harvesting pressure and other anthropogenic threats. Sirenians are still hunted throughout the world and recently other factors, such as water vessel strikes and habitat destruction, have increased mortality. The serious anthropogenic threats experienced by sirenians may be due to overlapping habitat use. Manatees inhabit the coastal waters that are ideal for human colonization. Recent protections have assisted in slowly ameliorating these threats. However, high mortality has forced the manatee populations to remain small and grow slowly, potentially stunting genetic diversity.

A similarity was seen between the mainland/island populations of elephant seals and manatees. Greater diversity was identified in the island habitats, owing to a greater capacity for divergence and more immigration potential from genetically differentiated populations.

As mentioned, many marine mammal populations have been strongly influenced by recent glacial epochs. Northern manatee populations may have been reduced by the Wisconsin glacial period. When the glacier receded, manatee habit became available in the northern limits of their range. This allowed a series of founding events, with manatees using the Caribbean islands as stepping-stones. Alternatively, if manatees were present in the northern limits, the population size may have decreased with the cold water and then expanded as the glacier melted. In either scenario, the founding Florida and Puerto Rico manatee population was most likely small and may have contained limited genetic diversity.
The reported manatee diversity was lower than other marine mammal and demographically-challenged populations. Nevertheless, small manatee populations with reduced diversity continue to thrive and grow. No significant immunological or reproductive barriers have been identified to date that would suggest a lack of fitness. However, immunological and functional genetic studies could address immunological function and aid in identifying imperiled populations or manatees with reduced fitness. These studies could also assist overall manatee health assessment and improve treatment.

Antillean manatee populations must be protected from harm and persecution to allow for genetic diversity to increase. The degree of genetic diversity is correlated with a number of individuals and overall health in a population. The genetic diversity within these populations should be monitored to detect changes over time. Furthermore, additional West Indian manatee populations should be genetically analyzed to identify cross-border breeding and populations with reduced variation or risk for inbreeding. The ‘hybrid zone’ between the Amazonian and West Indian manatee ranges should be analyzed with nuclear markers to facilitate management of the species. Ultimately, population viability will depend on the quality of habitat and reduction of anthropogenic threats. Cooperation of legislation, education, and enforcement entities are needed to ensure the sustainability of manatee populations for the future.
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BIOGRAPHICAL SKETCH

Margaret E. Kellogg was born in Boulder, CO, in 1981. She spent her early years in Ward, a small mountain town, and Tucson, AZ, before moving to the plains outside of Boulder. She graduated from Niwot High School in 1999. She earned her B.S. in microbiology with minors in chemistry and plant molecular and cellular biology from the University of Florida in 2003.

While at the University of Florida, Margaret worked with Jacqueline Wilson in the U.S. Virgin Islands, investigating coral reef fish behavioral interactions. She also worked in a research laboratory under the tutelage of Dr. Karen Koch, studying genomic DNA cell-wall mutations in maize. She began working with manatees in 2003, volunteering under Dr. Iskande Larkin, studying reproductive hormones.

In 2004, she was accepted to graduate school under the mentorship of Dr. Peter McGuire in the College of Veterinary Medicine. She was a teaching assistant for the University of Florida, College of Veterinary Medicine Large Animal Anatomy (2004) and SEAVET I (2008) courses. She has participated in many manatee captures, health assessments, and necropsies throughout the state of Florida and Belize.

She has attended many workshops including Applied Conservation Genetics, hosted by the U.S. Fish and Wildlife Service at the National Conservation Training Center, Shepherdstown West Virginia and taught by Drs. Fred Allendorf and Tim King, leading experts in the conservation genetics field.

She has received the University of Florida Named Presidential and Gritner Fellowships. Additionally, she was awarded the Bonde/Reep Manatee Conservation Prize for her conservation genetics work with manatees.