

A review of the key genetic tools to assist imperiled species conservation: analyzing West Indian manatee populations

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Abstract

Managers faced with decisions on threatened and endangered wildlife populations often are lacking detailed information about the species of concern. Integration of genetic applications will provide management teams with a better ability to assess and monitor recovery efforts on imperiled species. The field of molecular biology continues to progress rapidly and many tools are currently available. Presently, little guidance is available to assist researchers and managers with the appropriate selection of genetic tools to study the status of wild manatee populations. We discuss several genetic tools currently employed in the application of conservation genetics, and address the utility of using these tools to determine population status to aid in conservation efforts. As an example, special emphasis is focused on the endangered West Indian manatee (Order Sirenia). All four extant species of sirenians are imperiled throughout their range, predominately due to anthropogenic sources; therefore, the need for genetic information on their population status is direly needed. [JMATE. 2012;5(1):8-19]

Keywords: *Conservation, Management, Genetics, Endangered Species, Manatee*

Introduction

Conservation Genetics and Sirenian Conservation

Conservation genetics (the study of genetic methods and how they relate to biodiversity in populations) can inform managers of the optimal actions needed to conserve these species. Some species have demonstrated successful adaptations to meet environmental challenges through random gene mutations. The natural evolutionary processes of a wildlife population may also be affected by anthropogenic impacts to the environment, as well as the well-intended efforts of wildlife managers (54). Conservation has a broad definition but for our discussion it is defined as preservation of a target species to persist through time. Conservation incorporates a great deal more than basic science, and the availability of data does not automate complex conservation actions. However, society often must make decisions regarding

wildlife populations based upon the best available science. More rigorous and detailed genetic studies will assist managers in making policy decisions that could have long-term consequences for a species. The conservation and recovery of imperiled wildlife may require biologically and legally defensible delineation of management units (MUs), distinct population segments (DPSs), and subspecies and species designations (28, 79). Additionally, threats analyses must justify that smaller segments or portions of a population be designated for fine-scale protective measures.

Genetic data have been instrumental in the determination of listing status for several endangered species (19). The data from genetics, coupled with other demographic sources of information (life history parameters, abundance, distribution, habitat, etc.), are necessary for determining which population units or stocks will benefit from applied management decisions (18). A recent study on the genetic tools used to assess threatened and endangered populations of animals aptly illustrated that when multiple sources of genetic data were used in concert, there was a higher probability for the species to receive protection (19).

Some especially helpful techniques employed in conservation genetics include the use of coding (genes) or non-coding DNA sequences which are used as markers to identify individuals, differentiate populations, provide pedigree information, or examine functional genomic differences. Within the mitochondrial genome, a number of regions have been valuable tools in the field of conservation genetics. Markers such as the control region and cytochrome-b have been implemented in phylogeography and phylogenetic studies. Cytochrome oxidase 1 is used for 'bar-coding' or determining species identity with a single, conserved marker (74). In the nuclear genome, neutral microsatellite loci can provide contemporary information on which landscape and environmental

Received May 9, 2012; Accepted July 28, 2012

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factors are influencing population structure.

High-throughput sequencing technology can identify genes for molecular systematics and functional genomic analysis, as well as enable marker development (52). Examination of functional markers can enhance genetic research by providing information to compare the distinctiveness of individual populations, identify specific genes and their regulation, and examine the organism's response to environmental change.

One case-in-point of an imperiled species that merits increased attention from conservation genetics is the manatee. Manatees are large, aquatic, generalist herbivores utilizing primarily tropical habitats (Figure 1). The Order Sirenia is represented by two Families, the Trichechidae and the Dugongidae (67). The three extant species in the family Trichechidae have a wide range on both sides of the Atlantic Ocean (Figure 2). The Amazonian manatee (*Trichechus inunguis*) occupies the Amazon River Basin, while the West African manatee (*T. senegalensis*) inhabits nine coastal countries along the Atlantic coast of Africa. The oft-researched West Indian manatee (*T. manatus*) is divided into two subspecies, the Florida manatee (*T. manatus latirostris*) occupying the southeastern United States and the Antillean manatee (*T. manatus manatus*), present throughout the Caribbean and along coastal Central and South America through Brazil. The only extant representative of the family Dugongidae is the dugong



Figure 1: Florida manatee in Kings Bay, Crystal River, Florida. (Photo courtesy of USGS, Sirenia Project).

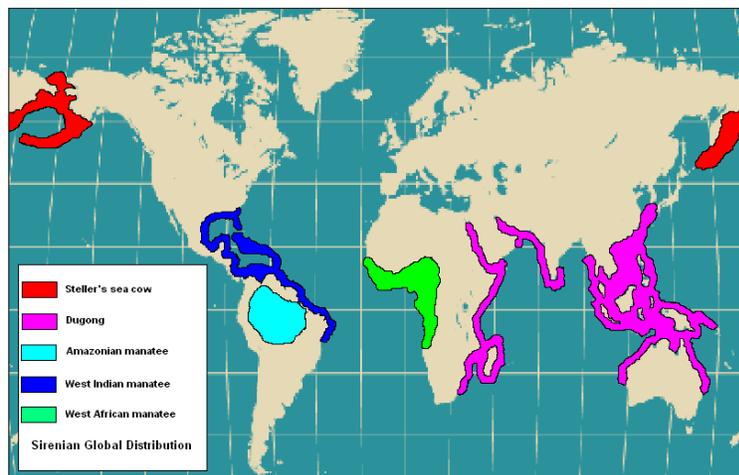


Figure 2: Sirenian global distribution. Red is Steller's sea cow (extinct), pink is dugong, light blue is Amazonian manatee, dark blue is West Indian manatee, and green is West African manatee.

(*Dugong dugong*) which has a more pandemic distribution, occupying much of the tropical waters of the Indian Ocean from East Africa to Australia. A sister taxa in the Dugongidae, the Steller's sea cow, occupied the Bering Sea until it was extirpated in the mid 1700's by fur sealers searching for foodstuffs during voyages (72).

Management and conservation of sirenian populations would benefit from multiple methods of genetic population analyses. Utilizing multiple genetic tools has strengthened the position for implementation of conservation measures in other threatened and endangered species as well (19). Due to their cryptic existence and avoidance of people, manatee behavior outside of Florida is seldom studied. Genetic data could provide insight into behaviors and stochastic demography, including mate selection, reproductive success, and migration patterns. Mitochondrial DNA (mtDNA) sequencing, multi-locus analyses utilizing microsatellites and single nucleotide polymorphisms (SNPs), gene expression analysis, and genomic sequencing are the major techniques currently employed in conservation genetics and could benefit sirenian populations.

Why Study Manatees?

Emigration through migration and dispersal has played an important role in manatees' ability to adapt and respond to survival challenges, and accounts for their broad geographic distribution.



Dispersal is also necessary for gene flow and can increase genetic diversity. West Indian manatees have persisted in environments where more fragile wildlife populations like the Florida panther, Florida red wolf, pallid beach mouse, and Caribbean monk seal have not thrived or have been completely extirpated due to human interactions (38, 80). The Steller's sea cow was forced to extinction by unregulated hunting as early as the middle 1700's (72), thus providing evidence of human impacts on a population with a very limited distribution. Because of legal protections and the increasing appreciation for wildlife by humans, Florida manatees have adapted recently to tolerate and coexist with human populations. Populations of Antillean manatees are much more secretive due to direct threats related to historical and continued hunting (59, 66). The geographical separation of this species has resulted in neutral and likely functional genetic differences between the subspecies (35, 56, 82).

It is likely that all manatee populations have undergone significant fluctuations, due to natural and anthropogenic events, that have resulted in low allelic diversity when compared to other wildlife populations (57, 58). The low genetic diversity observed in previous studies on manatees (23, 36, 56, 62, 77, 82) might have placed limits on their ability to cope with some environmental and anthropogenic changes, but those limitations appear not to have affected fecundity and survival of the Florida subspecies. To achieve a better understanding of the low diversity and how that impacts fecundity between stocks of manatees, efforts should focus on immune and health related functional markers.

The Florida manatee's resilience to anthropogenic and environmental pressures, coupled with the isolation from surrounding manatee populations by distance and open bodies of water, has led to interesting biological responses on the organismal level. For example, Florida manatees respond to cold temperatures by amassing in large numbers at natural and industrial warm water sites. Manatees in large aggregations are in close proximity to one another, increasing the potential for disease transmission through direct contact and coprophagy. These discrete wintering populations can undergo fluctuations in numbers resulting in lower genetic diversity, are vulnerable to potential catastrophic events and may not have the genetic make-up or resilience to

resist an extreme environmental challenge. Any reductions in the population size will likely result in loss of genetic resistance, making them more vulnerable to future perturbations. Understanding the relationship between manatee genetics and manatee behavioral responses and adaptive capabilities will help managers direct the recovery of the species.

The consensus among researchers is that the Florida manatee population has grown in recent decades and this indicates that the manatee population in Florida appears to be doing well. However, evidence documenting a low genetic diversity (23, 36, 56, 62, 77, 82) suggests that the manatee population may not be as fit as predicted based on just the estimated number of individuals. In other mammalian species a lack of allelic diversity has resulted in deleterious effects (22). This was aptly illustrated when the Florida panther population was pressed to the verge of extinction resulting in drastic measures to restore genetic fitness (40). Still, some wildlife populations, such as the northern elephant seal, have been able to thrive despite severe historical genetic bottlenecks resulting in low allelic diversity (33, 71). Whether this will be the case for the Florida manatees has yet to be determined. However, the recent increase in census size is promising for the maintenance of the current genetic diversity, thus providing some adaptive advantage into the future.

Genetic Markers

A host of genetic markers and tools are available to researchers to aid in determining population status. The selection of the appropriate marker depends on several variables and on the animal under investigation. Considerations for proper selection criteria include three primary factors: (1) Is the selected marker (or markers) appropriate to answer the questions at hand? (2) Are there limitations related to sample size and method of preservation? and (3) What are the budget and time restrictions, if any? Many analyses are expensive, but with recent technological advances, the cost has been reduced. The number of genetic analyses available to the research community is great. Here we provide information on the tools that have been employed in the past to better understand manatee populations and offer suggestions on methods to help enhance those studies. Several techniques are discussed in more detail below.

Allozymes

Previously, allozyme analysis (variation of specific enzymes) was performed on the Florida manatee to examine geographic distribution patterns in five areas of Florida corresponding to carcass recovery locations (49). Due to the homozygosity observed in this study, the authors suggested subtle differentiation between each Florida coast population of manatees, with the likelihood of some movement among the five regions sampled.

Mitochondrial DNA

Matrilineal haplotypes from sequenced mtDNA can yield information on phylogenetic and evolutionary processes. The mitochondrial control region has been used consistently for studies of manatee populations (24, 48, 82). A 410 bp segment has been most widely used and no variation in this segment has been detected in Florida manatees analyzed to date. Cytochrome b (cyt b) analysis has also been implemented, and illustrated little variation in the Florida population, but the sample size was small (12). Additional employment of cyt b studies would have significant bearing on our understanding of evolution and lineages between many manatee populations. Greater diversity in Antillean manatee samples has illustrated the potential for use of cyt b to detect phylogeographical units (82). Sequencing other regions of the mitochondrial genome, such as the cytochrome c oxidase 1 (CO1) gene, has also been integrated into the genetic toolbox.

New sequencing technology that makes mapping of the entire Florida manatee mitogenome possible is providing additional leads for determining genetic variation among subpopulations. Additionally, this information will confer evidence of female gene flow, philopatry, and lineages to compare and contrast with results obtained from nuclear DNA studies. The Antillean manatee mitogenome was sequenced recently and will have value in comparative research among sirenian species (2). Complete mapping of genomic mtDNA in killer whales (*Orcinus orca*), for example, has provided information on variations among populations that have led to discrimination between three ecotypes within the species (53).

Microsatellites

Microsatellites, also known as simple sequence repeats (SSRs), short tandem repeats (STRs), or variable

number tandem repeats (VNTRs), are sequences of di-, tri-, tetra-, or larger tandem nucleotides and are an excellent tool for determining relatedness among individuals and populations. Each microsatellite locus can vary in length among individuals. Microsatellites are neutral markers (not under the influence of selection) passed down from both parents that provide historical and contemporary information on relatedness and population structure. They can also be used to identify individuals in DNA profiling (sometimes referred to as “fingerprinting”). This trait has been particularly beneficial in mark-recapture studies, where genetic samples acquired through time from individuals is matched to life-history observations. Recently, this technique has been applied in the “cradle to grave” tracking concept used in modeling survivability and cause of death. It can also provide additional demographic data, such as age of first reproduction and paternal component. Additionally, microsatellite analyses can identify an individuals’ genetic diversity in relation to health status classification and mortality events (pers. comm. M. Tringali, FWC). Microsatellites are helpful in comparing allelic diversity among populations, determining effective population size, and calculating the size of an original founder population (1, 22).

Microsatellite studies have been conducted extensively on the Florida manatee population to determine spatial structure (23, 62, 78). Highly polymorphic microsatellite loci are difficult to identify in manatee tissue samples. Currently, 36 microsatellite loci have been developed for Florida manatee genotyping, however only a limited number of alleles have been identified (23, 36, 62, 77) and all but two are dinucleotide repeats, increasing scoring difficulty. An additional 17 primers, designed originally for dugong populations, were determined to be polymorphic in Florida manatees as well (36). In the studies conducted on the Florida manatee the average number of alleles per locus has been low ($N_a=2.9$, (23); $N_a=2.8$, (62); $N_a=2.5$, (77); $N_a=4.2$, (78)) when compared to other healthy mammalian populations. This low allelic diversity in the Florida manatee and other manatee populations may decrease the accuracy of population differentiation, pedigree analyses, and individual assignment to populations. The identification of more diverse microsatellite loci may allow for pedigree fingerprinting



studies that are more precise.

Microsatellites have also been used to address whether gene flow in Florida manatees is occurring with other manatee populations (14, 35, 37, 42, 56, 82). Microsatellite studies of other threatened West Indian manatee populations are needed urgently to help determine their relatedness and conservation status.

SNPs

Single nucleotide polymorphisms (SNPs) are nucleotide variations at a single base which can also aid in population genetic studies. SNPs are bi-parentally inherited and can be identified in both intron (noncoding) and exon (protein-coding) regions. The presence and uniqueness of polymorphic loci can yield information on taxonomy and individual identification, and have proven useful in identifying populations with low genetic diversity (83). Furthermore, restriction-site associated DNA (RAD) studies can identify gene sequences and large numbers of individual SNP sites (4). Identification of SNPs from genes could as well provide functional information in addition to detecting subpopulations for demographic analysis, enabling managers to implement more effective conservation measures.

Gene Expression

Functional genomics applies to the parts of the genome that are actively expressing genes. These processes result in protein production and relate to the ability of an individual to respond to biochemical input, as well as environmental and health challenges. Examination of gene expression and regulation in manatees can provide insight into health, reproductive fitness, and early disease detection. Serial analysis of gene expression (SAGE) is an approach for analyzing the observed variation in gene expression among individuals and populations (81). Understanding manatee immune genes, susceptibility to disease, wound healing, bone resorption, dental or other adaptations, and response to cold or hazardous algal blooms will inform researchers and managers so they may better monitor population health status. Also, examination of major histocompatibility complex (MHC) genes can increase our understanding of population differences, genetic diversity, disease defense, mate selection, and

reproductive fitness (7, 64). The variations in gene expression among individuals and populations can also provide information on demographics and adaptive responses.

Behavioral responses to density-dependent pressures may affect breeding success, reproduction, dispersal, and disease susceptibility, which can be tracked using genetics. Information such as this can help us gauge the cumulative impacts of climate change (75). Using advances in health assessment techniques will allow scientists to develop predictive modeling programs and study target animal response, which will enable a more informed approach for management.

The development of manatee specific genetic assays can provide information on the levels of gene expression of manatees encountering various environmental stressors, such as red tide (10), cold stress (11), and exposure to pollutants (73). Research to detect gene expression can utilize special microarray chips with unique sequences. These chips are coated with several thousand cDNA probes and when linked with a sample will generate a signal if hybridization occurs. Gene expression research becomes even more pressing as the manatee population in Florida responds to a reduction in available warm water sites with reduced spring flows or accessibility and the imminent closing of power plants which provide artificial winter refugia for much of the Florida manatee population (43, 69). It is uncertain when artificial sources of warm water currently available for Florida manatees' use will finally disappear, but it will most likely happen within the next couple of decades (43).

Use of field tests could benefit diagnostic studies during wild manatee health and risk assessments. For example, a manatee caught in 2004 in Port of the Islands, Florida, suffered from a massive internal infection undetected at the time of capture. Recently, tools have been designed for diagnosticians to detect inflammation in manatees using levels of serum amyloid A (SAA), but assays are expensive and must be performed at a later time in the lab (29). The manatee captured in 2004 was released with a radio tag but, unfortunately, died one month later. Had real-time information on the acute level of SAA been available in 2004 and during the examination of the manatee in the field, that manatee could have been taken to a

rehabilitation facility for treatment (30). Additionally, real-time assessment could be utilized to benefit the entire local population by informing researchers of links to potential diseases as well as removing individuals that may be carriers of disease.

Genomics

Many evolutionarily important species genomes are currently being sequenced by large-scale initiatives such as the Genome 10K Project, the Broad Institute and the Beijing Genomics Institute (31). The complete Florida manatee whole genome has recently been shotgun sequenced by the Broad Institute Genome Assembly and Analysis Group (13). Having this total genome coverage is extremely beneficial for the species for collaborative research opportunities and allows research teams to detect trends in gene expression and comparisons with other species. Genome-wide association studies (GWAS), which identify genetic links to physical or immune traits, could be important in detecting trends in variability between genetic traits and disease susceptibility or possibly adaptive potential.

Chromosomes

Individual chromosome homologues for the Florida manatee (*Trichechus manatus latirostris*) were identified using primary chromosome banding techniques prepared from T- and B-cell peripheral blood lymphocyte cell cultures established from six individuals (27). A standard banded karyotype was constructed, for both sexes, based on the G-banded chromosome pattern (27). The previously published modal chromosome number of 48 (47, 84, 85) was confirmed for this species, whereas, karyotype divergence can be observed in the Amazonian manatee which supports a model chromosome number of 56 (3). Digital imaging methods were subsequently employed and individual homologues were identified by unique G-banding patterns and chromosome morphologies. Characterization of additional cytogenetic features of this species by supplemental chromosome banding techniques, C-banding (constitutive heterochromatin), Ag-NOR staining (nucleolar organizer regions), and DA/DAPI staining was also performed. Cytogenetic features, including chromosome morphology and banding patterns, of *T. manatus latirostris* were described. The

karyotype has also been employed in evolutionary and taxonomic studies of sirenians through zoo-Fluorescent In Situ Hybridization (zoo-FISH) on chromosomes from other species (41, 42, 60) confirming their affiliation to the Paenungulata and Afrotheria groups.

Future studies include determining the karyotype of the West African manatee and the potential for hybridization between West Indian manatees and Amazonian manatees at the mouth of the Amazon River in Brazil (48, 82) will be very important for understanding sirenian evolution. Knowledge of interspecific and intraspecific comparisons of chromosomes between sirenian karyotypes and ideograms will be beneficial for future studies determining genome mapping, human (HSA) region definitions, and banding characteristics.

Implications for Manatee Conservation

Genetic Samples

Current genetic analyses of many manatee populations in the Trichechidae are incomplete because of inadequate sample size. Manatee sample collection has evolved from opportunistic collection of carcasses and stranded manatees to live animal biopsy. Live manatee tissue sampling techniques have improved from the use of cattle ear notchers applied to tail margins of free-swimming manatees (USGS unpublished data), dermal skin scrapers (15), biopsy needles attached to long poles which are employed to sample many manatees at aggregation sites in winter (16), and health assessment captures. The collection of fecal samples has also led to innovative genetic applications in very remote areas of sirenian distribution where sample collection is extremely difficult (55, 76). However, use of fecal material has limitations because the possibility of contamination, collection of multiple samples from the same individual, and a lack of DNA in the sample can cause difficulty in data analysis. There are new technologies aimed at utilization of fecal samples to assess hormone and stressor levels (pers. comm. I. Larkin, UF). Certainly, additional samples influenced by environmental/endogenous factors obtained from remote areas throughout the range of manatees and analyzed with current genetic methods will be useful. Once a cache of samples is available, a host of options for

genetic analyses exists.

Implementing Molecular Tools in Conservation Genetics

Currently, no standards or guidelines are in place to assist researchers and managers with the appropriate selection of genetic tools to study the status of wild populations (19). As each population of wildlife is unique and warrants different considerations, standardization among species is problematic. The information gained through additional genetic analyses, however, would greatly enhance the possibilities for preservation of the populations of imperiled populations.

The discreteness (amount) and significance (type) of genetic analysis must be appropriate for the questions to be addressed (19). Microsatellites have been useful for delimiting population differences in many species (17, 26, 39, 61, 65, 70). Species that have been isolated for long periods of time generally require smaller data sets or fewer markers for analysis, whereas recent separation of populations (such as that which has happened in the Florida manatee subspecies) usually requires more samples and the application of multiple genetic techniques (19). Generally, multiple genetic markers versus a single type of marker are favored, but care should be exercised when selecting the appropriate set of markers. Studies have demonstrated that a more robust assessment is obtained when more markers are employed (46). Neutral markers, versus genes that code for adaptive variation under selection in the environment, change more quickly, making them good indicators of population uniqueness (34, 50, 51). Care must be taken to ensure that the genetic data are used in conjunction with existing ecologic, geographic, morphologic, and life history data sets (28).

Technologies and tools for molecular analyses continue to evolve and many currently are applied to understand the Florida manatee population. Allozymes, mtDNA, and microsatellites demonstrated little to no genetic differences among the four existing Florida manatee MUs (12, 25, 49, 63, 82), however recent analyses using new spatial statistical techniques are suggesting differences among these four MUs, but more specifically the populations inhabiting the east and west coasts of Florida (pers. comm. M. Tringali, FWC). This difference between the coasts also was supported when

the Crystal River manatee population in northwest Florida when compared to the rest of Florida and cluster analysis identified two distinct groupings (8). When samples were compared between the Florida and Belize manatee populations, very distinct structure was observed (35), as expected, since the two groups represent subspecies. Further, the Endangered Species Act in the U.S. only recognizes the West Indian manatee throughout its range as one all-inclusive group for legal protection (79), thereby raising questions as to whether recovery of a population in one region (e.g., Florida) implies recovery at the species level of another isolated population, and subspecies, elsewhere (e.g., Puerto Rico; 37). Thus, one benefit of genetic scrutiny may be in assisting with the development of separate management strategies by responsible agencies for each subspecies or distinct population.

Geographic information currently is used when selecting release sites for captive born and rehabilitated manatees, to ensure that manatees from one area of Florida, or whose parentage is geographically identified, are not inappropriately introduced into another area. Presently, Florida manatees typically are not relocated intentionally from one coast to another, although some exceptions have occurred in the past (USFWS FMT meeting notes, USFWS Captive Manatee Database, USGS files). Generally, captive manatees are released to populations that have similar characteristics, with the expectation of preserving beneficial local adaptations. In some cases reduced genetic diversity could have positive impacts on the resilience of local populations to face perturbations. For example, manatees may be better suited to certain environmental or habitat characteristics, such as the strong influence of red tide on west coast manatees which may have resulted in locally-adapted gene complexes. Relocating manatees from other areas can result in outbreeding depression where the offspring are poorly suited to the habitat and the ability to produce important gene complexes. Alternatively, breeding with relocated individuals can result in genetic swamping, where the new genetic signature quickly become fixed in the population, replacing the locally-adapted alleles.

Information on manatee effective population size in Florida could be useful for integration into the Core Biological Model (CBM) for determining population viability (68). The CBM is a useful tool for assessing

manatee population status based upon current understanding of annual variability in survival and reproductive rates, demographic stochasticity (including changes in effective population size), effects of changes in warm-water availability, and catastrophes (69). As we are aware, a reduction in effective population size is a loss of genetic diversity (1, 22), as has been observed in the Florida manatee; this loss of diversity in populations may result in loss of the ability to adapt to environmental changes (20, 21). Therefore, greater effort could usefully be given to improve the effective population size in the manatee. As the human population continues to grow, impacts on the environment and available resources becomes even greater, creating a need for rigorous scientific information on affected wildlife species (67).

Recent genetic findings have illustrated that there is low allelic diversity in the manatee population in Florida (62) which is likely the result of a founder event that occurred during the Pleistocene epoch. Additionally, Florida manatees have a low effective population (N_e) (78). This raises concerns for the well-being of the manatee population as a whole in Florida, as many policies regarding their protection are based primarily on estimated total population size. Additional studies are needed to examine population fitness and the impacts of inbreeding in the Florida manatee. Studies have shown that once a population drops below inbreeding thresholds, detrimental effects can occur during stochastic change (22). Continued examination of genetic markers would be very informative for determining the resiliency of manatee populations to perturbations leading to dramatic population fluctuations. These population increases and declines have occurred in the resident manatee population as a whole in Florida during periods of existence with humans (59). Genetic markers can also be utilized to determine evolutionary lineages with predictions of founder population size and subsequent coalescent time calculation dating back to the event (5).

Presently, manatees with distinct scarring are photographed (6) and that information is used in mark-recapture modeling efforts throughout Florida (44, 45). Integration of genetic-identification of individuals with conventional photo-identification technology will provide additional sources for capture-recapture analyses. These additional genetic tools will facilitate the

identification of individuals independent of typical scarring features presently used and essential to confirm identities and lineages. Genetic tools would be useful for studies of other trichechid and dugongid populations as well. The benefits from engaging conservation genetics tools could strengthen important conservation decision processes. Scientific information is the primary component to facilitate effective conservation practices. The future direction of genetic research will utilize sequencing modalities, implement large volumes of data, and will design tools to gauge the health and fitness of individuals within all populations of sirenians. New initiatives to embrace genetic health and risk assessment on wild sirenian populations may give researchers a more effective way of assessing populations and gauging recovery status (9). Such new information will be useful to managers for implementation in manatee management and protection strategies world-wide.

Disclaimer

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