Green Sea turtle *(Chelonia mydas)*

mtDNA Analysis

A preliminary trial analyzing population genetic structure

CBES 620 Molecular Biology Research Techniques

Tropical Conservation Biology and Environmental Science Program

University of Hawaii at Hilo

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Objective

Determine the genetic structure and origin of Hawaiian green sea turtles *(Chelonia mydas)* by haplotype through mtDNA analysis. Samples for this experiment was generously provided and permitted by Dr. Peter Dutton, Program Leader of the Marine Turtle Genetics Program and George Balazs, Team Leader Zoologist of the Southwest Fisheries Science Center, National Oceanic and Atmospheric Administration (NOAA) USA.

Morphological Systematics and Taxonomic History

Kingdom: Animalia

Phylum: Chordata

Class: Reptilia

Order: Testudines

Family: Cheloniidae

Genus: *Chelonia*

Species: *mydas* (NOAA Fisheries, 2012)

Direct child taxa Subspecies: *Chelonia mydas agassizzi* (Appeltans, et al.)

Linnaeus in 1758 was the first to identify the green marine turtle as *(Testudo mydas)*; with further taxonomic binary nomenclature, Schweigger in 1812 renamed the green turtle as *(Chelonia mydas)*, the Latin identity used presently (Fish and Wildlife Service, 2012). The genus *(Chelonia)* has a controversial taxonomic history that has been debated by researchers for over a century, encompassing the argument of whether

the genus *(Chelonia)* should be split into two groups *(Chelonia mydas)* and *(Chelonia agassizii)* Bocourt (1868), the latter recognized by some authors as *(Chelonia mydas agassizii)* (Pritchard, 1999; Bonin et al., 2004). For the purposes of this report *(C. agassizii)* and *(C. mydas)* will be used. To adequately assess conservation priorities amongst an endangered species, taxonomic rank is critical (Karl & Bowen, 1999). In debate of this taxonomic separation, Pritchard (1999) has proposed three possibilities for *(C. agassizii)*: it is a color morph; a subspecies of *(C. mydas)*; or it deserves its own designation as a full species. Karl and Bowen (1999) argued that while *(C. agassizii)* can be identified at a basic level, morphological tendencies of color and size will vary throughout the distribution of *(C. mydas)* and this genus would be more accurately described at the population level.

Distinct vernacular names for sea turtles will vary depending on the cultural context, geography and political boundaries. Typically, the black turtle *(C. agassizii)* may also be recognized as Eastern Pacific Green Turtle; *Tortuga negra* and *Tortuga prieta* (Spanish); *Tortue verte du Pacifique* (MEDASSET, 2006) and *Tortue noire* (French) (Pritchard & Mortimer, 1999). The green turtle *(C. mydas)* may be commonly named as: *Caguama prieta* and *Tortuga blanca* (México); "yellow turtle" (Galapagos Islands) (Pritchard, 1999); *Tortuga Verde* (Spanish); *Tartaruga-verde* and *Aruanã* (Portuguese); *Tortue comestible, Tortue franche* and *Tortue verte* (French); and *Honu* (Hawaiian) (IUCN, 2012). For a further list of sea turtle common names refer to the World Registry of Marine Species (WoRMS).

Geographically, the green sea turtle *(C. mydas)* is distributed through circumglobal tropical regions (Karl & Bowen, 1999) extending through the Atlantic

Ocean, Indian Ocean, Mediterranean Sea and Pacific Ocean; nesting in over 80 countries, typically in oceans where water temperatures surpass 20°C (Bonin, Devaux, & Dupré, 2006; IUCN, 2004; IUCN, 2012). Northern extensions of the green turtle's distribution may be Northern Ireland, Nova Scotia and southern extensions include northern New Zealand and the coast of Argentina, possibly occupying coastal waters of at least 500 km wide and nonexistent throughout the Pacific coasts of the North, Central and South Americas where *(C. agassizii)* is present (Bonin et al., 2004). The black turtle *(C. agassizii)* is found in the eastern Pacific tropical and temperate regions nesting on sites between the Galapagos Islands and Michoacán and Revillagigedos Islands, México (Chassin-Noria, Abreu-Grobois, Dutton, & Oyama, 2004; Karl & Bowen, 1999). Northern limits of the black turtle may reach British Columbia extending to Chile with the inclusion of the Galapagos at its equatorial boundary (Bonin et al., 2004).

Differences in morphological characteristics of *(mydas-agassizii)* have lead systematists to agree that there is a significant difference between the two turtles (Pritchard, 1999). The black turtle is characterized by its domed, heart shaped carapace, marked incurving of the posterolateral shell margin above the hind limbs and dark gray pigmentation on the plastron (Parker, Dutton, & Balazs, 2011; Pritchard, 1999; Chassin-Noria et al., 2004). The head may have scalation, finer and darker than what is found in other species (Bonin et al., 2004). Compared to *(C. mydas)* it has been found to be more petite in size with a slight dorso-ventral expansion (Karl & Bowen, 1999) and females lay smaller clutches of eggs (Chassin-Noria et al., 2004). The green turtle has a light brown to black rounded carapace with thick juxtaposed scutes forming a flat shell and a cream to yellow plastron (Parker et al., 2011; Bonin et al., 2004). Adults can weigh between

140 to 160 kg with exceptionally large individuals surpassing 230 kg in weight (Bonin et al., 2004).

In attempt to decipher the geographic variation of the green turtle, Kamezaki and Matsui (1995) analyzed the skull morphology of individuals spanning across the globe. In carapace and skull lengths, *(C. mydas)* appeared to be smaller in the northern hemisphere compared to the southern hemisphere but skull lengths did not change based on climatic variations. Skull width could not be separated into two geographic groups but showed a climatic inclination for skull width to increase in size in accordance with average sea surface temperature regimes. Even though green turtle skulls found in the Galapagos is unique, supported by canonical discriminant analyses, a single characteristic cannot differentiate these samples from others. Therefore, it was concluded that the eastern Pacific population of *(C. mydas)* be considered as a subspecies *(C. mydas agassizii)* and not as a distinct species (Kamezaki & Matsui, 1995).

Other vertebrate species such as the Red footed Booby *(Sula sula)* and numerous fish species will vary in coloration sympatrically without evidence of gradation amongst the population and not obtain nomenclatural designation due to their ability to reproduce morphologically identical individuals with color morphs, lacking a separation in geography (Pritchard, 1999). The variability of the organism's color morphs may depend on its life stage and environmental exposure. Amongst vertebrate taxas, even if the molecular results are in alignment with zoogeography boundaries, it may not correspond to the phylogenetic relationships within the taxa discussed (Kamezaki & Matsui, 1995). Comparably, the sea turtle genus *(Lepidochelys)* has two distinct subspecies *(L. kempii)* Kemp's ridley and *(L. olivacea)* Olive ridley, which cannot easily be identified

morphologically but can be separated by coloration (Pritchard, 1969), have gained taxonomic recognition based on genetic analysis solely (Karl & Bowen, 1999).

Genetic Evaluation of *(Chelonia mydas)*

Although the separation of the two *(mydas-agassizii)* falls within traditional criteria for morphological divergence and reproductive isolation (Pritchard, 1999), mtDNA analysis for matriarchal phylogeny showed no distinguished evolutionary evidence between the two turtle morphs (Karl & Bowen, 1999). Mitochondrial DNA analysis displayed a low level of genetic variability and was found to have a slow, turtle's pace evolutionary rate compared to other vertebrate species. But in nonturtle species, phylogeographical representation corresponded with genetic separations in turtles (Avise, Bowen, Lamb, Meylan, & Bermingham, 1992; Karl & Bowen, 1999).

The black sea turtle *(C. agassizii)* through molecular analysis was found to be a regional melanistic population within the Pacific clade of *(C. mydas)* (cited in Chassin-Noria, Abreu-Grobois, Dutton, & Oyama, 2004; Dutton, Davis, Guerra, & Owens, 1996; Karl & Bowen, 1999). Molecular phylogeny on sequences of the ND4-Leucine tRNA and control regions of mtDNA showed that *(C. mydas)* within the Atlantic and Pacific Oceans, populations were paraphyletic in reference to the black turtle, deeming the necessity to reevaluate the Pacific *(Chelonia)* populations (Dutton et al., 1996). Genetic analysis based on single-copy nuclear loci for male-mediated gene flow did not show clustering of *(C. agassizii)* (Karl & Bowen, 1999), but levels of diversity of single-copy loci were low with limited inferences (Roberts, Schwartz, & Karl, 2004). Variability of microsatellite loci displayed male-mediated gene flow, which confirms that a genetic divergence between the Atlantic and Pacific populations exists (Roberts et al., 2004).

The genus *(C. mydas)* was found to have high frequencies of single nucleotide polymorphisms (SNPs) and homoplasy, possibly a rich source of variable loci within the genome (Roberts et al., 2004; Roden, Dutton & Morin, 2009a). Roden et al., (2009a) designed the first SNP markers for genotyping green sea turtles, which can potentially be used to evaluate populations on a regional basis and connections between populations. Genotyping marine turtles through SNP can be a rapid procedure that can avoid the irregularity of microsatellite genotypes due to the differences in laboratory technologies and scoring techniques (Roden, Dutton, & Morin, 2009b).

The development of tetranucleotide microsatellite loci markers for green turtles will also contribute to the evaluation of individuals and regional populations (Shamblin, Berry, Lennon, Bagley, Ehrhart, & Nairn, 2012). Sequencing complete mitogenomes of all seven species of sea turtles from geographical regimes and limits has shown a variability in the ATP8 gene length and a exceptionally variable site in ND4 by a proton translocation channel in a protein revealing phylogeographic patterns and relationships amongst all sea turtles, illustrating the intricacies of sea turtle diversity, phylogeography and molecular evolution interpretation (Duchene, Frey, Alfaro-Núñez, Dutton, Gilbert, & Morin, 2012). Complete mitogenome analysis is able to provide more consistent evolutionary divergence times than single mitochondrial markers. Positive selection in regions of the genome may be due to environmental adaptation and should be considered in molecular evolutionary processes and phylogenetic development. The occurences of speciation and its connection to geological processes such as the development of the Panama Isthmus that served as a migratory barrier between the Pacific and Atlantic

Oceans and changing global oceanic temperature regimes is key to deciphering the timeline of the evolutionary history of sea turtles (cited in Duchene et al., 2012).

Hawaiian Archipelago: a Regional Designation

On a regional basis *(Chelonia mydas)* of the Hawaiian Islands has been recognized as a single closed genetic stock, endemic to the Hawaiian archipelago (Bowen, Meylan, Ross, Limpus, Balazs, & Avise, 1992). Green sea turtles are distributed through the entire Hawaiian island chain which consists of more than 130 islands, coral reefs and coastal foraging grounds spanning across 2400 km from the Northwest region, Kure Atoll to the Southeast, Hawaii Island (Dutton, Balazs, LeRoux, Murakawa, Zarate, & Martínez, 2008; IUCN, 2012; Chaloupka & Balazs, 2007). Resident female green turtles migrate every few years to French Frigate Shoals (FFS), the main nesting rookery to lay their clutches of eggs on the sandy islands (Balazs & Chaloupka, 2004a).

Harvesting of green sea turtles started in the 19th century during exploratory expeditions in the Northwestern Hawaiian Archipelago. Reduction of turtle stocks was mainly due to nesting habitat destruction and over-exploitation of eggs and nesting female turtles, including a commercial harvest, which persisted from the 1940s to 1970s. The harvesting of adults ended in 1974 when stocks were depleted due to the US Endangered Species Act (cited in Chaloupka & Balazs, 2007). The Hawaiian green sea turtle population has been reportedly on the rise since conservation efforts have been established. Since 1973, annual surveys of turtles nesting at FFS have been orchestrated (Balazs & Chaloupka, 2004a) and populations have been observed to rise and recover since the late 1970s regardless of the regional outbreaks of fibropapillomatosis (disease

associated tumours) and incidental take from Hawaii inshore fisheries (cited in Chaloupka & Balazs, 2007). Social pressures exists surrounding the possibility of opening a selected cultural harvest of green turtles in Hawaii but this topic is still in its early developmental stages. Chaloupka and Balazs (2007) investigated the recovery and harvest potential of Hawaii green sea turtles through Bayesian state-space modeling, a surplus-production model since there was no age class specific harvest and demographic background available. This study found that a restricted cultural harvest may be demographically feasible but stock predictions past 25 years may lead to uncertain population estimates since marine turtles are a long lived species with late sexual maturation (Chaloupka & Balazs, 2007). Based on age specific growth rates estimated age of maturity for four southeastern populations are between 35 - 40 years and in northern populations at Midway potentially > 50 years (Balazs & Chaloupka, 2004b).

Recently, in August 2012, the Hawaiian population of *(C. mydas)* IUCN Red List criteria and status was revised from "endangered" to a "species of least concern" (IUCN, 2012). The recognition as a Regional Management Unit complies with the IUCN Red List assessment criteria for being defined as an independent subpopulation (Wallace et al., 2010). Some concerns are expressed regarding the delisting of this ancient species due to the risk associated with the isolation of the endemic Hawaiian population and its vulnerability to environmental degradation and climatic variation.

Genetic Stock of Hawaii Green Sea Turtles

Evaluation of stock composition through stock mixture analysis at five feeding ground sites has proven that the Hawaiian population is composed of one genetic stock originating from the FFS, with a mean estimate of 99.9%. From mtDNA sequences, six

haplotpes have been identified amongst 788 green sea turtles sampled throughout the Hawaiian Islands from nesting and feeding ground populations and Hawaii strandings, with three turtles with haplotypes not found at FFS. This suggests that Hawaii is sometimes frequented by turtles from the eastern and western Pacific Oceans. Even though Hawaiian *(C. mydas)* populations have been identified to be a genetic distinct population, stock compositions at foraging grounds within the Hawaiian Archiepelago have not been defined. Populations are often characterized by rookery or clusters of adjacent rookeries in a geographical area (Dutton et al., 2008). The potential for exposure to mixed genetic stocks is high because foraging grounds may spand over grand georgraphical areas. Individuals from FFS, one rookery will disperse to various foraging grounds (Dutton et al., 2008). Only a few Hawaii green turtles have been recorded beyond the Hawaiian Islands: Japan (1), Phillipines (1) and Marshal Islands (1) (IUCN, 2012). Recent studies have shown that foraging ground aggregations are composed of mixed stock origins varying at each location (Dutton et al., 2008).

Materials and Methods

Field sampling. MtDNA control region sequences were taken from frozen tissue samples and anal swabs from live turtles. Tissue samples (5) were collected from frozen turtles in freezer storage retrieved by the University of Hawaii at Hilo (UHH) Sea Turtle Stranding Program. Small square samples approximately 6 x 6 mm and 2 - 4 mm in depth were collected using a 6 mm chisel, small hammer and tweezers. Samples were taken from the neck region to ensure accessibility to soft tissue and uniform collection methods on all turtles. Samples were weighed and placed in plastic test tubes with 20%

Dimethyl sulfoxide (DMSO) saturated with salt for storage (Dutton & Balazs, 1995). The Marine Turtle Stranding Program at UHH is responsible for collecting sea turtles that have stranded in the Hilo vicinity. Turtle corpses are stored in the UHH freezer until they are flown to Oahu for necropsies conducted by NOAA officials. On a yearly basis George Balazs facilitates green sea turtle live captures for tagging (PIT tag and flipper tags (2)) at Punalu'u, Big Island with the UHH Marine Option Program. During turtle morphometric sampling procedures, anal swabs were collected using Whatman Omni swabs. The swabs were air dried and stored in individual paper sleeve at room temperature until they were used for DNA extraction.

Laboratory analysis. For laboratory processes see Dutton et al., (2008). Notable differences involve using the DNeasy 96 Blood and Tissue Kit for purification of total DNA. PCR amplification of mtDNA was completed by using the primers HDCM2 and LTCM2 designed to target 488' bp at the 5' end of the control region of the mitochondrial genome. One notable difference from following Dutton et al., (2008) lab procedures, is the template DNAs were amplified in 10 µl and 20 µl instead 50 µl as recommended. The samples amplified with 10 µl did not transpire in the electrophoresis in a 2% agarose gel stained with ethidium bromide but the samples with 20 µl were successful and showed up in the stain perfectly. After the DNA extraction, there was no notable difference in genetic yield between the two types of samples, when tissue samples (5) and anal swabs (3) were run through the Nanodrop.

Sequence analysis. The sequences from the identified mtDNA haplotypes were blasted for relative nomenclature using the National Center for Biotechnology Information (NCBI), Basic Local Alignment Search Tool (BLAST) website. Sequences were aligned

with *(C. mydas)* mtDNA sequence designations on the Southwest Fisheries Science Center NOAA website using Molecular Evolutionary Genetics Analyses 5.1 (MEGA) software.

Results

From the eight mtDNA samples of *(C. mydas)*, anal swabs (3) and tissue samples (5), two haplotypes CmP1 and CmP3 were determined. Haplotypes CmP1 represented (62.5%) and CmP3 (37.5%) of total samples. The samples from the foraging grounds at Punalu'u were found to consists of one CmP3 and two CmP1 haplotypes. From five of the Hawaii stranding turtles frozen tissue samples, two out of five turtles were haplotypes CmP3 to CmP1 respectively.

Table 1. Sequenced results for the mtDNA analysis of Hawaii green sea turtles *(C. mydas)* anal swabs (AS1 - 3) samples from Punalu'u, Big Island and frozen tissue samples (T1 - 5) of stranded turtles in the Hilo vicinity collected by the UHH Marine turtle stranding program. The DNA mentioned is the one bp change that occurred at site # 190 after the forward primer.

No.	Sample	DNA bp	Haplotype
1	AS1	T	CmP 1
2	AS2	T	CmP 1
3	AS3	C	CmP 3
4	T1	T	CmP 1
5	T2	T	CmP 1
6	T3	T	CmP 1
7	T4	C	CmP 3
8	T5	C	CmP 3

Discussion

Dutton et al., (2008) identified three haplotypes most commonly found amongst foraging grounds in the Hawaii Islands, CmP1 (64%), CmP3 (15%) and CmP2 (10%). Only one haplotype CmP1 and 11 CmP3 have been identified at the eastern Pacific rookery Revillagigedos, México and not found at any western Pacific rookeries. Haplotype CmP2 has only been identified at the FFS breeding site (Dutton et al., 2008). The haplotypes identified in this study CmP1 and CmP3 are turtles from Hawaii foraging grounds possibly: Pala'au, Kiholo, Kane'ohe Bay, Midway or Punalu'u, since there is limited movement of individuals between foraging grounds (Dutton et al., 2008), it is likely that the turtles sampled at Punalu'u solely forage in this area. Green turtles strandings and turtles found in foraging grounds in the Hawaiian Islands orignate from the north central Pacific nesting stock, which convinces the argument that this population is of a defined regional management unit (Wallace et al., 2010) unique to other populations in the Pacific Basin. Foraging grounds may be visited by turtles from other genetic stocks, evident by the three turtles found at foraging grounds with a genetic composition, haplotype different from what is found at FFS. Mixed stock analysis confirmed that individuals from Hawaii foraging grounds originate from the FFS nesting stocks, mean estimated stock mixtures and standard deviations for foraging grounds and strandings are (0.999, SD = 0.002) for FFS and (0.001, SD = 0.002) for Revillagigedos, México respectively (Dutton et al., 2008). Turtles with morphological characteristics that resemble *(C. agassizii)* and haplotypes typically belonging to the eastern Pacific populuations have been identified in Hawaiian foraging grounds, one as a stranding and the other found at Pala'au (Dutton et al., 2008). North of the Hawaiian Islands turtles

with the central Pacific morphotype have been found to be distributed in these areas and south of Hawaii, turtles of eastern Pacific morphotypes were detected. One turtle with the haplotype CmP3 captured in the longline fisheries was found to have morphological characterisitcs of an eastern Pacific green turtle (Parker et al., 2011).

Conclusions

Green marine turtles *(Chelonia mydas)* have such a vast geographic range, it is not surprising that the green turtle has developed phylogenetic morphs and regional identities within its populations. A geographical overlap between the justified *(C. mydas)* and *(C. agassizzi)* morphs as well as the occurrence of individuals displaying morphological characterisitcs resembling either morphs is not unusual when considering other species have proven the capabilities to exhibit such qualities with similar geographical circumstances. The wide expansion of the Pacific Ocean separating the Hawaiian Archiepelago and the North American continent creates a geographical void where the two morphs may interact and cross over, possibly breeding and mixing genetic stocks. The *(C. mydas)* and *(C. agassizzi)* once thought to be allopatric species may share a part of their life stage from their juvenile years into their adulthood as two sympatric species, without the occurrence of interbreeding for the most part due to an evolutionary historical event which caused reproductive isolation between the two. Utilizing AFLP fragment isolation or genotype sequencing techniques to produce random sequences for SNPs with the reference to the complete mitogenome as a baseline within and between regional management units (Wallace et al., 2010) may be ideal in deciphering evolutionary events as well as genetic variations between populations.

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