A Tale of Two Aggregations: Kinship and Population Genetics of Whale Sharks (*Rhincodon typus*) at Shib Habil, Saudi Arabia, and Mafia Island, Tanzania.

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ABSTRACT

A Tale of Two Aggregations: Kinship and Population Genetics of Whale Sharks (*Rhincodon typus*) at Shib Habil, Saudi Arabia, and Mafia Island, Tanzania.

Royale Hardenstine

In a recent global study of whale shark population genetics, aggregations were found to belong to either the Indo-Pacific or Atlantic population. This overview included an aggregation found within the Red Sea near Al Lith, Saudi Arabia, however the Mafia Island, Tanzania, aggregation was not part of the study. Both aggregations have unique aspects with the Saudi Arabian individuals showing sexual parity with no segregation, while recent acoustic results have revealed cryptic residency at Mafia Island. Genetic analysis using 11 microsatellite markers was performed on whale sharks from both locations. A combination of primers sourced from previous studies and newly designed primers were used to compare both aggregations and the individuals within. Samples were collected in the Red Sea for 5 seasons spanning 6 years, and for 2 seasons in Tanzania. Analysis with STRUCTURE showed a lack of significant genetic differences between the two aggregations, confirming that whale sharks in Tanzania are part of the Indo-Pacific population. Kinship analysis using COLONY found two potential pairs of full siblings in Tanzania. One pair had a high probability (.993) of being a full sibling dyad while the other had a lower probability (.357). There were no sibling pairs identified from the Red Sea aggregation. Genetic diversity

was investigated using allelic richness over the 6 seasons at Al Lith, with values showing no significant change. This is in contrast to results that showed a decline in genetic diversity at Western Australia's Ningaloo reef. These differences, however, only highlight the need for genetic diversity studies over longer time periods and at other aggregations within the Indo-Pacific.

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1. Introduction

The whale shark, *Rhincodon typus* Smith 1828, can be found throughout the world's tropical and temperate seas. Their range is generally limited to between 30º N to 30º S (Compagno, 2001), preferring temperatures of 20 - 35º C (Rowat and Brooks, 2012). This range is by no means restricted, with individuals being seen in such extremes as New Zealand (Duffy, 2002) and the Bay of Fundy, Canada (Turnbull and Randell, 2006). They have also been recorded for periods at temperatures as low as 6ºC in the Seychelles (Rowat and Gore, 2007). It therefore, appears that short-term exposure to cold water would not hinder whale shark movements (Rowat and Brooks, 2012). In fact, recent work has suggested that a specialized body plan for thermoregulation allows whale sharks to retain environmentally-derived heat in cooler temperatures, specifically when foraging in deep waters (Meekan et al., 2015).

Reaching up to 14m, this species is the world's largest fish and one of the three species of filter feeding sharks (Pauly, 2002). They are believed to live for approximately 80 years, reaching maturity at the length of approximately 9m, which is estimated to take 25 years (Hsu et al., 2014). Gaining new information on the species is difficult due to the rarity of sighting individuals in the open ocean. Despite these sharks first being described in 1828 there were only 320 records of whale sharks recorded up to 1985 (Wolfson, 1986). This lack of sightings and available data has resulted in limited knowledge about many aspects of their life history. Basic reproductive information such as approximate

litter size, reproductive strategy, and paternal contribution are only known because a pregnant female was fished in Taiwan and has since been used to better understand these aspects of whale shark biology (Joung et al., 1996; Schmidt et al., 2010).

Throughout their range, whale sharks are known to seasonally aggregate in various locations including: Australia (Taylor, 1996), Djibouti (Rowat et al., 2007), Maldives (Riley et al., 2010), Seychelles (Rowat and Gore, 2007), Qatar (Robinson et al., 2013), the Red Sea (Berumen et al., 2014), and Tanzania (Cagua et al., 2015), to name a few within the Indo-Pacific region. These aggregation areas have become hot spots for research on these otherwise elusive sharks. The reliable nature of these aggregations and relative ease of approaching these sharks in the water has greatly improved the amount and kind of data that can now be collected. Along with being a major attraction for interested researchers, many aggregation sites have become hotspots for tourism. In many areas, whale shark tourism can produce economic benefits for the nearby communities. For example, in South Ari Atoll Marine Protected Area, Maldives, direct spending on whale shark tourism was US $7.6 million in 2012 and US $9.4 million in 2013 (Cagua et al., 2014). A common practice in these areas is to encourage tourists to become citizen scientists and contribute their photos for identification studies by submitting them to Wildbook for Whale Sharks (whaleshark.org).

Photo identification of whale sharks is a common method to track individuals, to determine sight fidelity, and to help model populations within aggregations

(Meekan et al., 2006), with many researchers and tourists contributing to an international database at Wildbook for Whale Sharks. It is surprising, however, that the amount of crossover between aggregations has been small. These efforts are most successful when data is successfully shared between all aggregations, especially those within close proximity to each other, because crossover has been seen at sites that are geographically close together (Rowat and Brooks, 2012). There is however only a limited amount of knowledge that can be gained using only sightings data, as this is only a small portion of whale sharks' ecology and movement patterns.

Although tourism can be beneficial in this way, there is also the potential of these activities disturbing the natural behaviors of whale sharks. One such example is Oslob, Cebu, Philippines, where some whale sharks are provided with food. Although the long term effects of this form of tourism cannot immediately be predicted, provisioned individuals are shown to have longer residence times and a higher probability of being resighted than non-provisioned individuals (Araujo et al., 2014). In one case, the maximum residency time of one individual reached 574 days (Araujo et al., 2014). Another aspect would involve disruptive behaviors by tourists or tour boat operators. This can be detrimental to whale sharks because they are generally sighted at the surface while feeding. Negative interactions with tourists could alter their behavior and prevent them from efficiently foraging.

The pros and cons of tourism must be taken into account because these interactions can help promote conservation of this enigmatic shark. Whale sharks

are listed as “Vulnerable” on the International Union for Conservation of Nature and Natural Resources (IUCN) Red List with the population trend listed as decreasing (Norman, 2005). The decrease in whale shark numbers is caused by boat strikes, incidental catch, and targeted fisheries (Norman, 2005). There were targeted fisheries for whale sharks found in locations throughout the Indian Ocean and parts of the Pacific. Most of the large fisheries are now closed and whale sharks are protected under Appendix II of the Convention of Migratory Species of Wild Animals (CMS) since 1999 (CMS,1999) and Appendix II of the Convention on International Trade in Endangered Species (CITES) since 2002 (CITES, 2002; Rowat and Brooks, 2012). Unfortunately, this has not stopped the trade of whale shark meat, fins, and other associated products. Fisheries in China continue (Li et al., 2012), which will have an effect on the population size whale sharks.

In order to make informed decisions about whale shark conservation, an effort has been put forth to better understand their genetics from an individual to global scale. Studies using mitochondrial and microsatellite markers to determine genetic connectivity and structure between and within whale shark aggregations have been growing in popularity. Castro et al. (2007) designed mitochondrial markers that were used to begin to understand the global whale shark populations. This study was the first to suggest the possibility that there was two distinct global populations. However, with limited locations and few samples belonging to those locations, a definitive conclusion could not be drawn. A total of 70 samples were used from 10 locations that were grouped into 5 ocean basins

resulting in 44 haplotypes (Castro et al., 2007). Conclusions pointed to at least ocean basin wide connections, but without more samples, global connections of populations could not be ruled out.

Other mitochondrial work was done by Ramirez-Macias et al. in 2007 where they analyzed the population of whale sharks found in the Gulf of California. Using mitochondrial markers on 36 individuals they found 14 haplotypes. This aggregation is home to segregated groupings of juvenile males closer to shore, and mature females in deeper water (Eckert and Stewart, 2001). No significant genetic structure was found within these individuals, however, results from large, mature females suggested the possibility of natal philopatry. Therefore, it would seem the whale sharks aggregating in the Gulf of California are a single population (Ramirez-Macias et al., 2007).

Microsatellite markers are another method that has been used to analyze the whale shark population. A set of primers was designed by Ramirez-Macias in 2008, however, these were never used in an independent study. Another set of primers was used for a second global assessment (Schmidt et al., 2009). Although the study suggested one global population, the results were presented with caution due to low sample size and few locations globally (Schmidt et al., 2009).

A global assessment of whale shark population genetics was published by Vignaud et al., in 2014. This study is the largest genetic study of whale sharks to date, including samples from over 600 individuals. These individuals were

sourced from 9 locations spanning multiple ocean basins including Red Sea, Indian Ocean, Pacific, and Atlantic samples. Results from this study confirmed early mitochondrial results that suggested two global populations (Castro et al., 2007; Vignaud et al., 2014). High genetic structure was found between the Atlantic and Indo-Pacific individuals, meaning if the populations do occasionally mix, it is not enough to counter act genetic drift. Results also suggested a historic population expansion. However, a recent reversal in this trend could be underway, as the study also showed declines in genetic diversity over 6 years at Ningaloo Reef, Australia (Vignaud et al., 2015).

While the recent global evaluation of whale shark population genetic structure included samples of individuals from the Saudi Arabian Red Sea, the Tanzanian aggregation was not included. Due to the lack of genetic structure found between the aggregations in areas including the Red Sea, Djibouti, and Mozambique (Vignaud et al., 2014), it is expected that individuals sampled in Tanzania would also be grouped into this larger Indo-Pacific population. By comparing analysis of genetic structure between Tanzania and the Red Sea to results from Vignaud et al. (2014), the aim is to confirm that individuals at the Tanzanian aggregation are part of the Indo-Pacific population.

Both the Tanzanian and Saudi Arabian aggregations have their own unique aspects. The acoustic results from these aggregations could not be more different. Based on sightings data alone, they appear to both be seasonal aggregations, with a high season during March through May in Saudi Arabia (Berumen et al., 2014) and during October through February in Tanzania (Cagua

et al., 2015). The acoustic results from Saudi Arabia confirms what sightings data implied: a strong spring-seasonality was recorded, with few detections outside of the season (Cochran, 2014). The Tanzanian aggregation contrarily, was found to have a much different movement ecology. In this case, passive acoustic monitoring showed that whale sharks remained around Mafia Island year round, simply shifting to deeper waters further offshore (Cagua et al., 2015). However, like the vast majority of aggregations within the Indian Ocean, the Tanzanian aggregation shows sexual segregation with the 87.5% of the individuals being sighted in the area identified as males (Rohner et al., 2015). The Red Sea aggregation has approximately equal numbers of males and females (Berumen et al., 2014).

Another method to elucidate the genetic connections between aggregations is to analyze kinship within a population, either through parentage or sibling analyses. At the current time, kinship analysis has not been used within or between aggregations of whale sharks. However, the analysis was used to analyze a subset of 300 embryos found in a female whale shark harpooned in Taiwan (Joung et al., 1996; Schmidt et al., 2010). Over a decade after the initial capture of the female, the paternity of 29 of the embryos was tested; this analysis determined that even individuals at various stages of development were sired by the same male. It should be noted, however, that based on probability analysis there is a possibility that <10% of the approximately 300 pups may have been sired by another male (Schmidt et al., 2010). This suggests that female whale sharks have the capability of storing sperm and fertilizing eggs over time (Rowat

and Brooks, 2012). In this case, there was one parental genotype known because all of the individuals were from the same maternal source, which simplified kinship analysis. In order to determine parentage in wild populations however, individuals from multiple generations and size ranges must be sampled. The majority of whale shark aggregations consist only of juvenile sharks, and many of these aggregations have only been studied for short periods of time with genetic sampling active for even less time or not at all. These factors contribute to parentage analysis on most aggregations not being feasible at this time. Mature females are commonly seen in some areas, such as the Galapagos and Baja Mexico, however, a lack of samples has limited the work that can be completed (Ramirez-Macias et al., 2008; Hearn et al., 2013). Whale sharks are a K-selected species with slow growth and long generation times, therefore study periods to analyze parentage would have to take place over long periods of time, or extensive sampling would have to take place in locations with known adults. However, by using sibling group assignment analyses that do not require parental genotypes, it may be possible to find sibling relationships within or between aggregations. This could begin to give a deeper understanding of movements of juveniles and allow further analysis of aggregation sites that act as nurseries.

Finally, as previously mentioned, a decrease in genetic diversity at Ningaloo Reef, in Western Australia was recorded over the course of 6 years (Vignaud et al., 2014). This was the only site suitable for analysis due to short sampling periods or limited samples from all other locations in the study. Studies

at the Al Lith aggregation began in 2009, with genetic sampling ongoing from 2010 to 2015, the same 6 -year time period used in the Vignaud et al. (2014) analysis. Therefore, it would seem appropriate to compare the genetic diversity found at this location with the results from Australia.

The genetic diversity decline observed in Australia is speculated to be caused by boat collisions and commercial fisheries that previously and to some extent still do target whale sharks (Vignaud et al., 2014). Other threats include bycatch and accidental entanglement. These possibilities are wide spread threats to whale sharks throughout the world's oceans, and their effects would therefore, not be restricted to one specific locality. This could cause a decline in genetic diversity to be seen in whale shark aggregations throughout the Indo-Pacific region, including the Red Sea. For example, a whale shark in a Red Sea study was fatally entangled in a gill net and other sharks in the study exhibited scarring potentially related to outboard motors of local fishing vessels (Cochran, 2014). Therefore, the effects of fisheries and boats could also be affecting individuals occurring at Shib Habil due to the location's proximity to shore and large number of outboard motor boats used in the area.

In addition, satellite tracks from individuals tagged at the Al Lith aggregation, showed some sharks moving into the Indian Ocean, creating a connection between the Indo-Pacific and Red Sea populations (Berumen et al., 2014). This is consistent with the lack of genetic structure found between the aggregations in these locations (Vignaud et al., 2014). It is, therefore, possible

that the Al Lith aggregation may also display a decrease in genetic diversity from 2010 through 2015. If so, this would add evidence in support of a recent reverse in the long-term trend of population expansion found in the global population assessment (Vignaud et al., 2014).

In this study, 11 microsatellite loci were used in order to help determine the population genetic structure between the Red Sea and Tanzanian whale shark aggregations. It is hypothesized that Tanzanian individuals would be a part of the larger Indo-Pacific population by having very little genetic differentiation from the Red Sea individuals. Secondly, kinship analysis was used to find sibling groups within both aggregations. Movements of whale sharks under 3m and adults is poorly understood however finding siblings within or between aggregations could begin to show nurseries for young individuals and breeding habits of adults. Finally, genetic diversity was measured via allelic richness in the Al Lith whale shark population over 6 seasons in order to examine any changes or trends. It is expected that, due to individuals within the Red Sea connecting with the Indian Ocean, the same genetic diversity decline seen in Australia may also be present at the Al Lith aggregation.

2. Methods

2.1. Sample Collection

Samples were collected in two locations Mafia Island in Tanzania and Shib Habil, near Al Lith in, Saudi Arabia (Figure 1). Sampling took place in Saudi

Arabia over 6 seasons starting in 2010 and ending in 2015. The majority of whale shark sightings occur around Shib Habil, a reef near to the shore and near the port for the city of Al Lith, Saudi Arabia. Sampling effort was focused around this reef. The Tanzanian individuals were sampled during two seasons: the late 2012-early 2013 season and late 2013- early 2014 season. At Mafia Island, the majority of whale sharks sightings occur within Kilindoni Bay, located near the largest town on the island Kilindoni (Figure 1). At both locations, samples were collected from free swimming individuals, using Hawaiian sling pole spears fitted with biopsy tips. Following collection, samples were stored in one of two ways; with the majority stored in 70-90% ethanol, depending on what was available in the field. Samples were stored long term in 90% ethanol at -20ºC until DNA was extracted. The final season in Saudi Arabia differed in that, samples were immediately put on ice and put in a freezer upon returning to shore. After dermal issue was removed from subcutaneous tissue, the samples were frozen at -20ºC.

In the field, attempts were made not to sample the same individuals more than once. Photo-identification was used as a method to prevent analysis of the same individual when positive IDs could not be made immediately in the field, or when the whale shark was a new addition to the database. Due to their distinct patterning, individual whale sharks can be identified using an area on their sides that is formed by the top and bottom of their fifth gill slit and trailing edge of the pectoral fin. Photographs of the Saudi Arabian sharks were analyzed using the Interactive Individual Identification System (I3S) (Van Tienhoven et al., 2007), with matches being confirmed by at least two researchers. Individuals from both

the Tanzanian and Saudi Arabian aggregations have been submitted to the online database of whale shark identification photos, “Wildbook for Whale Sharks” at whaleshark.org.

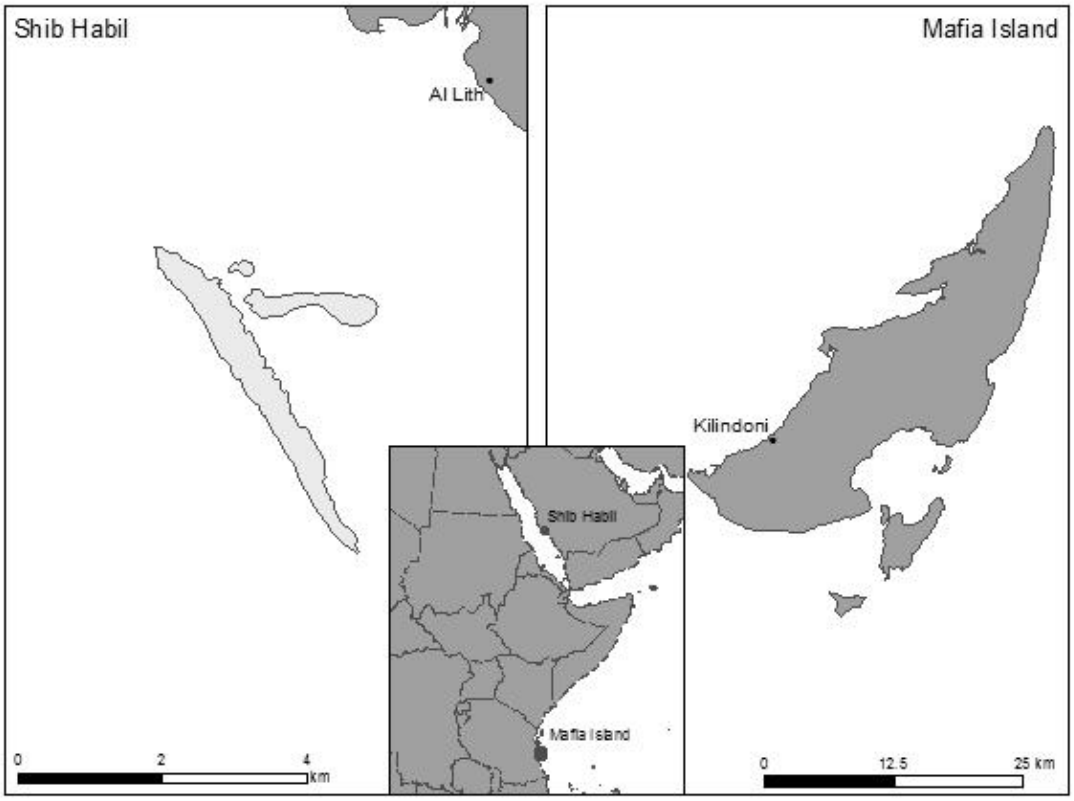

Figure 1: Map showing the location of Shib Habil and Mafia Island in relation to each other (A). Location of Shib Habil with nearest port Al Lith (B) and Mafia Island with Kilindoni, town located on Kilindoni Bay where majority of whale shark sightings occur (C). (Campbell, 2015)

2.2. DNA Extraction and PCR

DNA from skin samples was extracted using one of two kits, either the DNeasy Blood and Tissue Kit (Qiagen Inc.) or the NucleoSpin Tissue Kit (Macherey-Nagel). Both extractions were performed following kit instructions. However, the final elution step for Macherey-Nagel kit was reduced to 100 uL.

An initial pool of 22 markers was available for the microsatellite analysis. Eight of these were sourced from other studies, with 3 (Rty_15, Rty_18, and Rty_38) from Ramirez-Macias et al. (2008) and 5 (Rtyp1, Rtyp,3, Rtyp 4, Rtyp 7 and Rtyp 8) from Schmidt et al. (2009). The remaining 14 primers were designed for this study. These included: Rhin_t_03, Rhin_t_04, Rhin_t_05, Rhin_t_07, Rhin_t_11, Rhin_t_10, Rhin_t_13, Rhin_t_16, Rhin_t_28, Rhin_t_30, Rhin_t_31, Rhin_t_32, Rhin_t_46, and Rhin_t_47.

The mix used for the PCR reactions was as follows: 5 ul of Master Mix (Qiagen Inc.), 3 ul of RNA free water, 1 uL of primer mix, and 1 uL of template DNA. The protocol used for PCR was an initial step at 95ºC for 15 minutes, followed by 35 cycles of 94ºC for 30 sec, 55ºC for 90 sec, and 72ºC for 60 seconds. The final step was 60ºC for 30 minutes. The PCR products were diluted 1:50 with water before being sent for fragment analysis.

2.3. Population Structure Analysis

Microsatellite allele size was read using Geneious 8.1.6 software (Biomatters Ltd.). Following scoring, the Microsatellite Toolkit (Park, 2001) was used to look for duplicate genotypes, in cases where no photo-ID was available. Genepop 4.2 (Rousset, 2008) was used to check for the presence of null alleles, Hardy-Weinburg equilibrium, and linkage disequilibrium. After analyzing these results a final set of 11 primers was chosen for the remainder of the analysis (Table 1); AMOVA, pairwise Fst, private alleles, and expected and observed

heterozygosity analyses were all performed on GenAlEx version 6.4 (Peakall and Smouse 2006).

Table 1: Subset of 11 microsatellite markers used for analysis. Locus names with * are sourced from Ramirez-Macias et al. (2008). Na is number of alleles per locus, observed heterozygosity (Ho), expected heterozygosity (He).

Locus	Size range (bp)	Na	Ho	He	Fis
Rty_18*	175-185	4	0.475	0.451	-0.055
Rty_38*	172-185	7	0.740	0.728	-0.016
Rhin_t_03	247-255	5	0.639	0.645	0.010
Rhin_t_05	220-248	12	0.835	0.863	0.032
Rhin_t_07	264-286	10	0.821	0.831	0.012
Rhin_t_10	144-154	5	0.661	0.667	0.010
Rhin_t_11	143-149	3	0.242	0.239	-0.013
Rhin_t_30	321-336	6	0.075	0.073	-0.028
Rhin_t_31	152-164	4	0.412	0.427	0.036
Rhin_t_32	115-123	5	0.631	0.702	0.101
Rhin_t_47	117-139	7	0.590	0.620	0.047

STRUCTURE (v2.3.4 Pritchard et al., 2000) was run on all individuals from both aggregations in order to determine the most likely number of populations. In this instance the model was run with K values from 1 to 3. The model burn-in was 10,000 iterations, followed by 100,000 iterations after the burn-in period. Each K scenario was repeated 5 times, and results were uploaded to Structure Harvester (Earl and vonHoldt, 2012). Structure Harvester was used in order to determine which K is the most likely population scenario, using Evanno's delta K and averaged maximum likelihood scores. After selecting the most likely K value, STRUCTURE results were uploaded to CLUMPAK (Kopelman et al., 2015), which streamlines processing results from population

structure analysis. CLUMPAK takes results from a single K value to find the optimal alignment based on those results.

2.4. Kinship Analysis

The software COLONY Version 2.0 (Jones and Wang, 2010) was run using genotype data from all individuals to screen the population for sibling relationships. This software has the ability to use individual genotypes with multiple loci to assign sibling and half sibling groups. This is done using the maximum likelihood method (Jones and Wang, 2010). The Saudi Arabian and Tanzanian individuals were analyzed together as one population with default parameters, medium run, full-likelihood analysis, assuming polygamy for males and females, and without parental genotype data (following Schmidt et al., (2010)). Along with COLONY, KINALYZER (Berger-Wolf et al., 2007) was used to create potential sibling groups among individuals from the two aggregations. This program is used to reconstruct sibling groups from a single generation without knowledge of the parental data. In this way, it is good for biological datasets where either samples of parental DNA are lacking for one or both of the parents. The software reconstructs full sibling groups, using Mendelian laws of inheritance (Berger- Wolf et al., 2007). The analysis further confirmed the full sibling dyads assigned by COLONY. KINALYZER was used to analyze all individuals as one population grouping individuals from both Tanzania and Saudi Arabia together. After viewing initial results, each location was analyzed independently to assist in evaluating sibling groups assigned within each aggregation.

2.5. Genetic Diversity

Genetic diversity was determined through the rarefaction method using the Allelic Diversity Analyzer (ADZE) version 1.0 (Szpiech et al., 2008). The rarefaction method is designed to analyze allelic richness and private alleles in populations with varied sample sizes because for these analyses sample size can have a significant effect on results. ADZE was used to determine the genetic diversity of both the Al Lith and Tanzanian populations individually. Further, the 5 seasons of samples from Al Lith, from which genetic samples were available were run independently to examine the genetic diversity from 2010 to 2015. In this case, all seasons were run with g=7, due to a low sample size in one season. Here “g” represents standardized sample size, therefore, “g” is the number of individuals randomly chosen as a subsample from each population, or in this case season, for analysis. This was followed up with a second analysis comparing only the 2010 and 2015 seasons, in order to allow for a larger sample size (g=19).

Table 2: Indices of genetic diversity from this study highlighted in grey. Locations followed by * are from Vignaud et al. (2014).

	N	Mean number of allele over loci	Expected Heterozygocity (He)	Observed Heterozygocity (Ho)	Allelic richness
Red Sea	**84**	**5.82**	**0.57**	**0.55**	**5.30**
Red Sea*	46	7.28	0.65	0.67	4.60
Djibouti*	89	9.14	0.65	0.50	4.82
Seychelles*	20	6.00	0.66	0.67	4.57
Tanzania	**74**	**5.46**	**0.57**	**0.56**	**5.07**
Mozambique*	26	6.64	0.64	0.67	4.52
Ningaloo*	128	8.57	0.65	0.62	4.49
Gulf of California*	47	7.14	0.63	0.62	4.37
Isla Holbox*	50	5.71	0.60	0.58	3.95

3. Results

3.1. Population Structure Analysis

After removing samples that were known duplicates due to photo-ID and those found using microsatellite toolkit, a final number of 158 individuals with 74 from Tanzania and 84 from the Red Sea remained. For the Al Lith aggregation there was initially 102 samples, 11 duplicates were found through photo-identification methods. Seven more were removed based on genetic analysis, three were unknowns and the other four were most likely misidentifications of individuals in the field. There was an initial pool of 137 samples from Tanzania, however, nearly half (63) of the samples were removed based on genetic analysis. Table 2 shows a comparison between the Vignaud et al. (2014) results and those found within this study. Mean number of alleles over loci for the Red Sea fell within the range of all locations, with a value of 5.82. This is lower than the mean of 7.28 that was previously reported, for the Red Sea (Vignaud et al., 2014). The Tanzanian number of alleles over loci falls below the other values at 5.46. Although expected heterozygosity values for both locations fell below the previous findings, the observed heterozygosity remained within the range of those results. There were no distinct private alleles found in either population, with no private allele frequency over 0.025. Genotypic differentiation between Tanzania and Saudi Arabia was significant ($p = 0.02539$). The pairwise Fst value of 0.0028 between the populations was significant ($p=0.030$).

STRUCTURE results showed no structure between the two populations, indicating K=1 as the most reasonable estimate. When viewed with K=2 in a diagram (Figure 2) no difference can be seen between the two populations. This suggests that both the Red Sea and Tanzania are part of the same population.

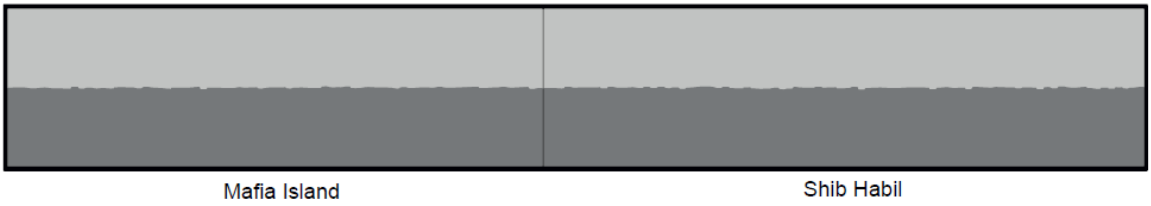

Figure 2: Structure plot where K=2, showing lack of genetic differentiation between the two aggregations.

3.2. Kinship Analysis

Analysis using COLONY, including both individuals from Tanzania and Saudi Arabia resulted in two sibling pairs being detected. Both potential sibling pairs were individuals sighted at the Tanzanian aggregation (Table 3) with all four individuals being identified on Wildbook for Whale Sharks. The first sibling pair had a high probability (0.993) of being a full sibling dyad. A second pair MF032 and MF419 was given a low probability (0.357) of being siblings. Both of these individuals have been acoustic tagged twice at the Tanzanian aggregation. The first tag deployments began in late 2012 and both individuals were sighted in November 2014 without tags. Both individuals were then re-tagged in early December 2014.

Table 3: Sibling pairs, from Tanzanian aggregation, predicted by COLONY including; sample name, photo-ID, number of sightings recorded on Wildbook for Whale Sharks, and acoustic tag numbers.

Probability	Sibling	Wildbook ID	Sex	Sightings	Accoustic Tag
0.993	MF033	TZ-067	M	12	-
	MF066	TZ-096	M	1	-
0.357	MF032	TZ-035	M	35	15803/32685
	MF419	TZ-053	M	23	28063/61242

KINALYZER results grouped all 158 individuals from both Saudi Arabia and Tanzania into 45 sibling sets. There were no individuals that stood alone and all individuals were grouped with at least one other individual. Of these 45 groupings 3 were restricted to individuals that were only from Tanzania and 5 were restricted to individuals only sighted in Saudi Arabia. The remaining 37 sets of siblings contained individuals from both aggregations.

When populations were analyzed separately, not all of the same individuals were grouped together. When the Red Sea was independently run, the 84 individuals were placed into 26 sibling pairs and again all individuals were grouped with at least one other individual. When compared to the full population results 2 pairs of individuals remained grouped together, and 2 sets holding three individuals each remained in a sibling group. For the separate Tanzanian analysis, the 75 individuals were placed into 23 sibling sets. Out of these, 7 pairs of individuals were grouped as siblings in both the full population and location restricted analysis. One of these pairs included MF 032 and MF 419, which were also paired in both KINALYZER runs. However, MF033 and MF066 were only

paired as siblings in the analysis involving Tanzania and the Red Sea grouped together as one large population.

3.3. Genetic Diversity

Genetic diversities for both aggregations were similar, with the Red Sea showing slightly more diversity with an allelic richness of 5.30 compared to 5.07 in Tanzania. These results are compared to those found in Vignaud et al. 2014 in Table 2. For the Al Lith whale sharks each season was treated as a separate population and genetic diversity was compared between years. When all 5 seasons were included, the allelic richness showed little variation (Table 4). Due to low numbers of samples in 2012 and 2013, the allelic richness analysis was re-run excluding the season from 2011 through 2015 (Table 4). These results suggest the genetic diversity at the Al Lith aggregation has remained constant from 2010 through 2015.

Table 4: Temporal genetic diversity results determined by allelic richness from the Saudi Arabian aggregation for a period 6 years, including samples from 5 seasons. ***** indicates results where only 2010 and 2015 were analyzed.

Shib Habil	2010	2011	2012	2013	2015
N	30	26	9	7	19
Allelic Richness	3.04	3.01	2.93	3.04	3.16
Allelic Richness*	4.30	-	-	-	4.32

4. Discussion

By analyzing whale shark aggregations genetically, conclusions concerning connections of aggregations can begin to be drawn in a way that sightings data alone cannot provide. In this study, no significant genetic structure was found between the Red Sea and Tanzania, confirming that the latter is a part of the larger Indo-Pacific population. The kinship analysis using COLONY revealed two potential pairs of full siblings found at the Tanzanian aggregation, while none were found within the Red Sea. This difference can potentially be attributed to the ecological behaviors at each location. Finally, the Red Sea aggregation did not show a decline in genetic diversity over a period of 6 years, like the one seen at Ningaloo Reef, Australia.

Photo-identification was used as a method to rule out individuals that may have been sampled twice. However, because in some cases photos were not available, genetic analysis was used to find sequences that were most likely the same individual. Known duplicate results were initially kept in the analysis to confirm that individuals that had been sampled more than once were detected by this method. A large number of samples from Tanzania were removed through genetic analysis because sharks had been sampled multiple times. For this aggregation, photo-IDs are only analyzed using Wildbook for whale sharks, and meta-data that identified individuals was received while genetic analysis ongoing. Therefore, the genetic analysis was relied on for removal of duplicate samples.

In one case, two individuals that had been identified as different sharks at the Al Lith aggregation by I3S software and researchers were found to be one individual resighted 4 seasons later, based on microsatellite results. This misidentification was mostly due to one of the photos being of poor quality and at a bad angle, a common weakness of photo-identification. Upon closer inspection, the photo-ID was confirmed. This individual was previously tagged in 2011, however, when it was tagged during the 2015 season no evidence of the tag, tether, or scarring was present.

4.1. Population Structure Analysis

The structure diagram shows there is very little genetic structure between the two aggregations, and they therefore form one population. There is no significant variation within microsatellite analyses that differentiates the Tanzanian and Saudi Arabia aggregations into separate populations. An Fst of 0.0028 was found between the two aggregations which was significant (p= 0.030). When Fst values reach 1.0, the two subpopulations under analysis would be considered genetically different. An Fst value of 0.0 is considered a homogeneous population, showing very little genetic differences (Balloux, 2002). Therefore, although these results are significant, the low Fst value found between the populations, represents a lack of genetic differentiation. This was expected considering the global survey included areas geographically close to Tanzania including: Seychelles, Maldives, Mozambique, and Djibouti. There was little structure shown between these aggregations and individuals that were

included from the Red Sea previously (Vignaud et al., 2014), therefore, it is not surprising that the Tanzanian and Red Sea individuals would be genetically similar. Individuals previously identified in Mozambique have been sighted based on photo identification at the Mafia Island aggregation (Rohner et al., 2015). Therefore, known connections between these aggregations were previously found and the homogeneous nature of the genetic results was expected.

4.2. Kinship Analysis

The COLONY results suggested two sibling pairs in Tanzania. However, there were no sibling pairs proposed in Al Lith. One of the sibling pairs was supported with a high probability (0.993). The sibling pair of MF032 and MF419 had the lowest probability (0.357). One potential explanation for this result is these two individuals may be a half-sibling pair. In some cases where assignment probabilities are low, the pair may still be siblings or could related in a different manner (Blouin, 2003). These sibling pairs represent the first evidence of sibling relationships documented within the whale shark population.

KINALYZER was used as a secondary method to confirm potential sibling pairs, because for this study, the program appears to have greatly over-estimated the number of sibling relationships. The analysis in which both Tanzania and the Red Sea were grouped as one population resulted in all 158 individuals divided into 45 sibling groups. These results suggest that all of the individuals involved in the study had at least one other sibling within this population. However, based on the COLONY results, this appears to be an

incorrect result. When Tanzania was analyzed individually, there were no individuals that were not grouped. The same was found when the Red Sea individuals were analyzed as a single population. However, when the single aggregation analyses were compared with the results from the two aggregations grouped as one population, not all of the sibling groupings remained the same. Therefore, these results should be taken with caution.

When run on populations with a known pedigree, KINALYZER could be used to reconstruct the pedigree despite some missing alleles. In this case at least, results from this program alone cannot used to draw conclusions about sibling pairs. One potential problem is the software is designed to identify the sibling groups out of a single generation. It is difficult to determine exactly what the generation time of whale sharks is, especially with lack of biological data. However, the whale sharks encountered at Shib Habil are all considered to be immature based on length and clasper morphology in males. (Berumen et al., 2014; Cochran, 2014) Considering these results and short time period of 6 years in comparison to a life expectancy of up to 80 years (Hsu et al., 2014), these individuals are most likely members of the same generation. However, at least one mature male shark has been encountered at the Tanzanian aggregation (Rohner et al., 2015). In one case, the mature male was 876cm, but immature males larger than this mature individuals were also sighted (Rohner et al., 2015). It would be unlikely that these smaller mature males would father any of the other individuals within this study, making the assumption that all individuals within the study belong to the same generation likely.

Another possible factor that could affect the effectiveness of KINALYZER is the number of alleles per locus. The mean number of alleles per loci was approximately 5.5 for both populations. Therefore, there may have not been enough alleles within each locus to sufficiently differentiate non-siblings, causing the program to incorrectly overestimate the number of sibling groups. In general, it is recommended that 15 to 20 loci be used discriminating full siblings from unrelated dyads (Blouin, 2003). In this study, a total of 11 loci were suitable for use after initial analysis, this smaller number in available loci, may have also skewed results causing false assignments of sibling pairs in KINALYZER. Considering both of these possible influences, additional loci with more alleles should be considered for continued analysis of possible siblings pairs.

Additionally, COLONY provides multiple options before analyses are begun that include adding values for genotyping error, choosing polygamy or monogamy, and addition of any known parental genotypes. KINALYZER, on the other hand is an online program where data can be uploaded and results are then sent to the user. Therefore, the researcher using the program has much less control over the parameters and analyses being performed. It would therefore appear that COLONY is a better tool for analysis of this type of sibling data in particular and for this reason, those results were more likely to accurately reflect the wild population. With the aforementioned caveats under consideration, the KINALYZER results were used only to confirm those from COLONY. When both Tanzania and the Red Sea were analyzed as a single population one of the sibling pairs determined by COLONY were also grouped as full siblings by

KINALYZER. Both sibling dyads supported by COLONY were confirmed by KINALYZER when Tanzania was analyzed without the inclusion of individuals from the Red Sea.

Although both of the aggregations may serve as nurseries, the differences in the ecologies of both regions may play a role in this pattern. As previously stated, no sibling pairs found in Al Lith according to COLONY. Overall, individuals spend an average of 10 days (Cochran, 2014) at this aggregation, making Shib Habil a short-term destination. In addition, outside of the months of March, April, and May, there were few acoustic detections, showing that individuals leave the immediate area (Cochran, 2014), dispersing throughout the Red Sea and on occasion moving into the Indian Ocean (Berumen et al., 2014). The movements at the Tanzanian aggregation are different, with acoustic data showing detections throughout the year, implying residency at this aggregation site (Cagua et al., 2015). The two individuals that had the lowest probability results (MF 032 and MF419) were both acoustic tagged twice. Both showed extreme residential behavior around Mafia Island, with acoustic detections recorded throughout the year. The largest gap in the time series is found in the approximately 5 month period from early 2014 to December 2014 where both lost their first tags. This was confirmed by photo-ID sightings of both sharks in 2014 without tags. More complete analysis of the acoustic records for these two individuals is underway, in order to determine if they may be associating, performing similar movement patterns, or utilizing the same habitats throughout the area.

Based on mitochondrial genetic analysis, natal philopatry in whale sharks has been hypothesized to occur in the Gulf of California (Ramirez-Macias et al., 2008). This behavior has been documented in multiple species of sharks through either genetic sampling, tagging, tracking, or a combination of the previously mentioned techniques (Hueter et al., 2004). The nurse shark, *Ginglymostoma cirratum*, a member of the order Orectolobiformes (to which the whale shark also belongs), was found to exhibit philopatry in the Dry Tortugas (Pratt and Carrier, 2001). Another well-known example includes the lemon shark, *Negaprion brevirostris,* which was studied for 20 consecutive cohorts in Bimini, Bahamas. A number of females faithfully give birth at the same site, and six females from an early cohort returned after 14-17 years to give birth (Feldheim et al., 2013). If female whale sharks are philopatric, this could potentially result in related individuals being found at the same aggregation. Mafia Island, could be near a location where females routinely return to have young, off shore in deeper water. If Mafia Island is near a location where females regularly return give birth, the Tanzanian aggregation could serve as in important nursery.

Schmidt et al. (2010), used both COLONY and KINALYZER to establish paternal parentage of embryos from a deceased female shark, so it was already clear that these individuals were siblings. Therefore, these programs were used to help elucidate whether multiple males had contributed genetically to the pups. In that case, the population was closed and these analytical methods performed well, confirming that a single male had sired the litter (Schmidt et al., 2010). However, the Red Sea and Tanzanian population are quite open with

connections throughout the Indo-Pacific. This connectivity introduces a large number of paternal and maternal possibilities.

These current results allow analysis of sibling relationships within two whale shark aggregations, potentially giving more information about the ecology of neonates and individuals under 3m. In order to successfully draw strong kinship conclusions in the future, more samples from both aggregations, as well as, from other locations in the Indo-Pacific are necessary. Furthermore, work including analysis of half-sibling relationships and the use of additional genetic markers would add confirmation to future studies investigating kinship.

4.3. Genetic Diversity

The allelic richness of the two aggregations were found to be 5.30 in the Red Sea and 5.07 at Mafia Island. These values are slightly higher than those found in Vignaud et al. 2014, where the allelic richness ranged from 3.95 at Isla Holbox, Mexico to 4.82 in Djibouti. The Red Sea allelic richness from that study was 4.60, the second highest in the study (Vignaud et al., 2014). The differences seen in these values between the two studies are most likely attributed to differences in sample size. This is especially true when considering the lowest number of samples from one location, which would dictate the number of individuals subsampled from each population based on the rarefaction method. Another explanation for the differences could be that the microsatellite loci chosen for analysis were not consistent between the two studies. There was, however, a slight difference in the allelic richness between Tanzania and the Red

Sea, with the latter showing a higher value. One reason for this could be the longer sampling time period in the Red Sea that led to a larger number of samples being collected. Another consideration could be the ecologies of the two locations, because the Tanzanian population appears to have many individuals returning to and remaining at that location. The Red Sea aggregation has individuals that are resighted, but the individuals generally spend less time at the aggregation, and show no residency. With this higher turnover, this aggregation could be sampling from a larger pool of individuals.

Allelic richness analysis was not undertaken for the Tanzanian individuals on a temporal scale because samples in this study only covered two seasons. Therefore, it would not be possible to draw conclusions on population trends without at least a third season, to accurately determine if any change was in effect. However, with continued sampling in that location, changes in genetic diversity could be analyzed for that region.

Within the Saudi Arabian aggregation, genetic diversity was analyzed over a span of 6 years, where genetic samples were available for 5 seasons. These results showed that the genetic diversity of the Al Lith aggregation has held steady from 2010 through 2015. This trend differs from the slight decrease in genetic diversity seen at Ningaloo Reef, Western Australia, where allelic richness dropped from 4.52 in 2010 to 4.29 in 2012 (Vignaud et al., 2014). This decline was further substantiated by mitochondrial data that also showed a trend of lower genetic diversity for all years from 2007 through 2012. The results from this study

are only the second to be found concerning genetic diversity between seasons within an aggregation.

The differences in trends have a strong possibility of being caused by location, as both of these analyses are only a snapshot of a particular aggregation within a larger population. Juvenile and adult whale sharks have few if any predators, including great white sharks *Carcharodon carcharias* (Fitzpatrick et al., 2006) and killer whales *Orcinus orca* (O'Sullivan and Mitchell, 2000). Neither of these species are known to commonly occur in the Red Sea, with the majority of sightings believed to be mis-identifications or extremely rare. Therefore, the individuals that inhabit the Red Sea would be under less pressure from predators. Other sharks are under immense fishing pressure within the Red Sea (Spaet and Berumen, 2015), and their reduced numbers could indirectly prevent attacks on very small juveniles. Western Australia does have large species of sharks present, and scarring or missing parts of fins were seen in Ningaloo sharks that would suggest predation by great white sharks (Fitzpatrick et al., 2006).

Secondly, despite heavy fishing pressure along the Saudi Arabian coast of the Red Sea, the fisheries consist of mainly hand lining from small outboard motor boats, making bycatch of whale sharks rare. There is no targeted fishery for whale sharks within the Red Sea, although the accidental death of an individual occurred due to entanglement in a gill net (Cochran, 2014). Australia is geographically closer to areas such as the South China Sea, where active

fisheries previously and to some extent continue to exist (Li et al., 2012). The Ningaloo aggregation could be suffering diminishing genetic diversity due to this pressure on previous and current generations. However, analyses at both locations should be considered with some caution, as the changes in genetic diversity is considered for only a period of 6 years. This time period, as previously mentioned would not span an entire generation of sharks, and could therefore be affected by sightings of individual sharks. There could also be seasonal fluctuations in the individuals and overall number of sharks that arrive at these aggregations, therefore biasing the results. Finally, the usual sexual segregation of aggregations should be considered, since many are strongly dominated by juvenile males. In order to truly track and analyze genetic diversity within the Indo-Pacific region, annual sampling should occur at all aggregations, as well as follow up studies to continue to monitor genetic diversity.

5. Conclusion

The results from this study suggest, as expected, that Tanzania is genetically part of Indo-Pacific whale shark population. The genetic similarities between aggregations throughout the Indo-Pacific, now including Tanzania, emphasize the importance of multi-national conservation measures. The lack of temporal change in genetic diversity found in the Red Sea is a localized result that cannot be used to indicate healthy whale shark populations elsewhere. The decrease seen in Australia could serve as an early warning sign for the rest of

the Indo-Pacific, especially aggregations near continued fishing pressure. With these two opposing results found, investigation of other aggregations could help paint a clearer picture for whale shark populations. Therefore, analysis of genetic diversity should be performed at locations with samples spanning multiple years. Finding more about sibling connections throughout the Indo-Pacific could further aid in conservation of the species, by locating important strongholds and nurseries for juveniles. However, these results only involve two aggregations; only one of which was show to have full siblings, allowing limited conclusions to be made. Further work with mitochondrial markers would allow for a more detailed comparison with the current global analysis of whale shark genetics. Also, the use of more sensitive genetic markers could be useful to begin to compare aggregations on a finer scale. However, in order for future work to be successful, cooperation of researchers, institutions, and countries where these sharks aggregate is essential to determine effective conservation measures for the species.

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