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Applying a Molecular Genetics Approach to Shark Conservation and Management: Assessment of DNA Barcoding in Hammerhead Sharks and Global Population Genetic Structuring in the Gray Reef Shark, Carcharhinus amblyrhynchos.

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Applying a molecular genetics approach to shark conservation and management: Assessment of DNA barcoding in hammerhead sharks and global population genetic structuring in the gray reef shark, *Carcharhinus amblyrhynchos*

By

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General Introduction to Thesis

The globally widespread overfishing of sharks is now well documented, leading to growing concerns about their sustainability if effective management and conservation measures are not urgently implemented (Baum et al. 2003; Dulvy et al. 2008; Ferreti et al. 2008; Hayes et al. 2009). The generally K-selected life histories of the globally fished, large sharks make them highly susceptible to over-exploitation, and there are many examples of rapid population collapses after short periods of intensive targeted fishing. Adding to the exploitation pressure from targeted fisheries is the tremendous volume of shark bycatch that accompanies various fisheries targeting teleosts, especially in pelagic and reef habitats. In addition to direct impacts on shark populations, the overfishing has also led to concerns that rapid declines in shark numbers is likely altering marine ecosystem functioning via disruptions in top-down control due to predator release of prey (Shepherd and Myers 2005; Myers et al. 2007; Polovina et al. 2009).

Development of effective management and conservation measures for sharks has been hampered by the limited information available on their population biology and fisheries. In most parts of the world, shark fisheries remain largely unmanaged. There is almost no recording of the numbers of each species landed partly due to difficulties in identification of landed sharks and their traded body parts. Furthermore, there is little information of the stock structure of most shark species to aid in robust assessments of their population status and trends. There is an urgent need for these types of data to guide management efforts, and genetic approaches are proving increasingly useful in providing this information.

In this context, my thesis examines the development and assesses the comparative utility of a nuclear DNA marker to assist in species identification of sharks by a tool known as DNA Barcoding (Chapter 1). In Chapter 2, I investigate the detailed genetic population structure of a strongly coral-reef associated shark species (*Carcharhinus amblyrhynchos*) by using a combination of mitochondrial sequence and nuclear microsatellite markers.

Chapter 1

Integration of a nuclear marker into DNA barcoding for species identification: application in the hammerhead sharks (Family *Sphyrnidae*)

Abstract

DNA barcoding based on the mitochondrial cytochrome *c* oxidase subunit I (COI) gene sequence is emerging as a useful tool for identifying unknown, whole or partial organisms to species level. However, the application of only a single mitochondrial marker for robust species identification has also come under some criticism due to the possibility of erroneous identifications resulting from species hybridizations and/or the potential presence of nuclear-mitochondrial psuedogenes. The addition of a complementary nuclear DNA barcode has therefore been widely recommended to overcome these potential COI gene limitations, especially in wildlife law enforcement applications where greater confidence in the identifications is essential. In this study, we examined the comparative nucleotide sequence divergence and utility of the mitochondrial COI gene (N=182 animals) and nuclear ribosomal internal transcribed spacer 2 (ITS2) locus (N=190 animals) in the 8 known and 1 proposed cryptic species of globally widespread, hammerhead sharks (family Sphyrnidae). Since hammerhead sharks are under intense fishing pressure for their valuable fins with some species potentially set to receive CITES listing, tools for monitoring their fishery landings and tracking trade in their body parts is necessary to achieve effective management and conservation outcomes. Our results demonstrate that both COI and ITS2 loci function robustly as stand-alone barcodes for hammerhead shark species identification. Phylogenetic analyses

of both loci independently and together accurately place each hammerhead species together in reciprocally monophyletic groups with strong bootstrap support. The two barcodes differed notably in levels of intraspecific divergence, with average intraspecific K2P distance an order of magnitude lower in the ITS2 (0.297% for COI and 0.0967% for ITS2). The COI barcode also showed phylogeographic separation in *Sphyrna zygaena*, *S. lewini* and *S. tiburo*, potentially providing a useful option for assigning unknown specimens (e.g. market fins) to a broad geographic origin. We suggest that COI supplemented by ITS2 DNA barcoding can be used in an integrated and robust approach for species assignment of unknown hammerhead sharks and their body parts in fisheries and international trade.

Keywords: barcoding, hammerhead shark, ITS2, neighbor-joining, fin trade

Introduction

Hammerhead sharks (Carcharhiniformes, Sphyrnidae) are an important resource to global inshore and offshore fisheries (Compagno 1984), particularly with the increased consumption of shark fins since the 1980s (Castro et al. 1999) and the high market values fetched by hammerhead fins (Abercrombie et al. 2005). However, sharks in general have K-selected life histories characterized by slow development, late maturation and low fecundity, making their populations less resilient to intense fishing (Musick et al. 2000; Stevens et al. 2000). All hammerhead sharks utilize parturition grounds in typically mainland coastal bays (Compagno 1984) resulting in concentrations of neonates and young-of-the-year animals in areas easily accessible even to small artisanal fishers. Furthermore, two of the large hammerhead species, the scalloped hammerhead (*Sphyrna lewini*) and the smooth hammerhead (*Sphyrna zygaena*), often form schools that increase their risk of being targeted or caught as by-catch (Compagno 1984). Overfishing and coastal habitat degradation is believed to have led to an estimated 89% decline in the abundance of hammerhead sharks in the Northwest Atlantic since 1986 (Baum et al. 2003).

Five of the currently eight morphologically described Sphyrnidae species are listed by the International Union for Conservation of Nature and Natural Resources (IUCN) as near threatened (*Eusphyra blochii*), lower risk/near threatened (*S. lewini* and *S. zygaena*), lower risk/least concern (*Sphyrna tiburo*) and vulnerable (*Sphyrna tudes*). However, given new information and rising concerns about hammerhead shark population declines, the U.S.A. will officially propose to the CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) Secretariat in 2010

that the three large hammerhead species (*S. lewini*, *S. zygaena* and *S. mokarran*) whose fins fetch premium prices in the global fin markets be granted trade restrictions by listing on CITES Appendix II (M. Shivji, *pers comm*.).

Despite a strong need for informed management and conservation measures due to dwindling populations, monitoring fishery landings and obtaining accurate stock assessments for hammerhead sharks has proven difficult, in part due to species identification problems. Although the family as a whole is easily identified in fisheries due to its characteristic "hammer" head shape, accurately distinguishing juveniles and often even adults to species level is not simple for the three largest and most common fin trade species (*S. lewini*, *S. zygaena*, and *S. mokarran*) (Compagno 1984; Rose 1996). Additional identification complications are encountered when dismembered body parts (e.g. fins, carcasses, meat) are found in fishery landings and trade.

Molecular species identification methods are useful for distinguishing morphologically similar species within the wildlife trade (Pank et al. 2001; Shivji et al. 2002; Chapman et al. 2003; Abercrombie et al. 2005; Clarke et al. 2006; Magnussen et al. 2007). The approach developed by Abercrombie et al. (2005) used the nuclear ribosomal internal transcribed spacer 2 (ITS2) marker in a multiplex PCR format to rapidly distinguish *S. lewini*, *S. mokarran* and *S. zygaena*. However, this method failed to amplify a recently discovered, cryptic hammerhead lineage (Abercrombie et al. 2005; Quattro et al. 2006) which is likely the hammerhead species most closely related to *S. lewini*.

An alternative molecular species identification method known as "DNA Barcoding" is a standardized molecular identification system that has been proposed for

identifying all eukaryotic life forms (Stoekle and Hebert 2008). This method relies on the premise that genetic divergence in the sequence of a standardized DNA fragment corresponds to biological separation of species. For animals, considerable research is occurring to assess the suitability of the cytochrome c oxidase subunit I (COI) mitochondrial gene sequence to provide an array of "barcodes" that acts as a reliable DNA identifier for each species (Hebert et al. 2003). DNA barcodes have now been successfully applied to a broad range of taxa (Waugh 2007), including a recent study on Australian sharks and rays (Ward et al. 2008). Yancy et al. (2007) incorporated DNA barcodes as an additional identification source for 72 species listed in the U.S. Food and Drug Administration's Regulatory Fish Encyclopedia, and then conducted a blind study that accurately identified 60 unknown fish muscle samples with 100% accuracy.

As a mitochondrial DNA (mtDNA) marker, COI holds a number of advantages such as multiple copies per cell allowing easy amplification from even trace samples (Scicluna et al. 2006). The lack of recombination and introns simplify sequence alignment and analysis (Hebert et al. 2003; Saccone et al. 1999). The maternal mode of inheritance in vertebrates results in an effective population size that is one-fourth as large as a nuclear gene, which can make a mitochondrial gene tree closer in similarity to a species tree than a nuclear based gene tree might be (Moore 1995). A growing body of literature has demonstrated that COI is well conserved at the species level in animals, but still maintains sufficient interspecific divergence to allow species to be delineated (Waugh 2007). The standardized, roughly 650 base pair fragment of COI from the 5' end utilized in DNA barcoding is short enough to be amplified and sequenced in single reactions, yet long enough to exhibit the necessary variation. The use of widely

applicable primers (Ivanova et al. 2007) further aids in streamlining and standardizing the DNA barcoding process.

There are, however, disadvantages to the use of COI. The maternal inheritance of mtDNA requires a note of caution as there may be inconsistencies between the analysis of mtDNA and nuclear DNA data. In particular, DNA barcoding is unable to address the possibility of hybrid specimens, as mtDNA would assign all hybrids to the maternal lineage. Concerns have also been raised about the possibility of heteroplasmy and other issues that would lead to contrasting species boundaries indicated by mitochondrial and nuclear genes (Rubinoff 2006). DNA barcoding should be viewed as a gateway to further analysis promoting an integrated approach, as opposed to a definitive end, for species delineation. Therefore, the use of a complementary nuclear barcode marker in addition to the traditional COI barcode would enhance barcoding's utility (Dasmahapatra & Mallet 2006; Rubinoff 2006).

Due to rising concerns about the sustainability of sharks given their high exploitation especially the fin trade, problems with species identification in management contexts, and the increasing regulations being implemented to prevent overfishing, development of a DNA barcode approach to shark identification will be useful (Ward et al. 2008). In an attempt to provide an integrated approach utilizing both mitochondrial and nuclear genome barcodes, this study examines the congruency between species trees generated by the traditional COI DNA barcode and a nuclear marker, in all known hammerhead sharks. The nuclear, ribosomal internal transcribed spacer 2 (ITS2) non-coding region was chosen as the nuclear marker as previous work has demonstrated that ITS2 is highly conserved within sharks but is also sufficiently divergent to allow species

level discrimination (Pank et al. 2001). The ITS2 marker has universal primer annealing sites located in the 5.8S and 28S ribosomal subunit genes flanking the locus (Hillis & Dixon 1991), and has been used in many shark species identification studies (Pank et al. 2001; Shivji et al. 2002; Chapman et al. 2003; Abercrombie et al. 2005; Clarke et al. 2006; Magnussen et al. 2007). Congruent species trees and boundaries between the mitochondrial and nuclear markers would support the overall DNA barcode initiative with a nuclear marker enhancement to the traditional COI barcode for shark species identification.

Materials and Methods

Shark samples

A total of 190 hammerhead shark species samples were obtained through shark population abundance surveys conducted by the U.S. National Marine Fisheries Service (NMFS) or from qualified shark researchers (Figure 1). All eight morphologically described hammerhead species and the recently genetically described cryptic hammerhead species (Abercrombie et al. 2005; Quattro et al. 2006) were included in the analyses. Samples for DNA analysis were taken as fin clips, muscle, heart, or liver tissue and stored in 95% ethanol at room temperature until processed. Approximately 25mg of tissue was utilized to extract genomic DNA from the samples using the DNeasy Tissue Kit (QIAGEN Inc., Valencia, California). Extracted DNA was stored at -20°C until use.

Laboratory Procedures

Mitochondrial COI locus

A 652 base pair fragment from the 5' region of the COI gene was PCR amplified using a pair of primer cocktails, C_FishF1t1 and C_FishR1t1, as detailed by Ivanova et al. (2007) (Table 1). Cocktail components are modifications of the primers used by Folmer et al. (1994). Each primer was also modified with the M13 tail (Messing 1983) corresponding to its appropriate direction (Table 1).

Each PCR reaction mixture consisted of 6.25µl of 10% trehalose, 3.0µl of ultrapure ddH_2O, 1.25µl of 10X PCR buffer for Platinum® Taq (Invitrogen, Inc.), 0.625µl of 50mM $MgCl_2$, 0.125µl of each primer (10µM), 0.0625µl of 10mM dNTP mix, 0.06µl of Platinum® Taq DNA polymerase (Invitrogen, Inc.), and 0.5-2.0µl of template DNA. PCR amplification reactions were conducted in Eppendorf Mastercycler® gradient thermal cyclers (Brinkmann Instruments, Inc.) The reaction program consisted of 2 min. at 94ºC, followed by 35 cycles of 30s at 94ºC, 40s at 52ºC, and 1 min. at 72ºC. Upon completion of the 35 cycles, the thermal program concluded with 10 min. at 72ºC and then held at 4ºC.

PCR products were visualized on 2% agarose E-gel® 96 plates (Invitrogen, Inc.). PCR products were labeled using the BigDye® Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Inc.). Each cycle sequencing reaction mixture consisted of 5.0µl of 10% trehalose, 0.917µl of ultrapure ddH_2O, 1.917µl of 5X buffer (400mM Tris-HCl ph 9.0 and 10mM $MgCl_2$), 1.0µl of primer (10µM; M13F or M13R), 0.167µl of BigDye® (Applied Biosystems, Inc.), and 1.5µl of PCR product. Bi-directional sequencing reactions were carried out with the M13 primers (Table 1) and resolved using an ABI3730 capillary sequencer.

Nuclear ITS2 locus

An approximately 670 base pair fragment of the ITS2 was amplified using the shark universal primers FISH5.8SF and FISH28SR (Pank et al. 2001). Some *S. lewini* individuals were sequenced using newly designed internal primers (ScHHint131F 5'CTCACTGGCCTAGCCTCCTTG, ScHHint268F 5'GTGGCTCCTCCAGGTAAAG, ScHHint438R 5'ACCCAGCGTGGTGAAGTGTG) along with the existing universal external amplification primers. The 50µl polymerase chain reactions contained 10-25ng extracted DNA, 12.5pmol of each primer, 1x PCR buffer, 40µM dNTP's and 1 unit of HotStart Taq DNA polymerase (QIAGEN Inc.). All PCR reactions were performed on an iCycler (BioRad) thermal cycler. The PCR thermal cycling profile for the amplification was 94°C initial heating for 15 min, followed by 35 cycles at 94°C for 1 min, 65°C for 1min, 72°C for 2 min and a 5 min extension at 72°C. PCR was always run with a negative control (same reaction components minus DNA template). Results of amplification were checked on a 1.2% agarose gel. All products were purified with the QIAquick PCR purification kit using manufacture's protocol (QIAGEN Inc.). A cycle sequencing reaction following standard ABI procedure using BigDye Terminator v3.1 (Applied Biosystems, Inc.) and the amplification primers was performed and products were gel purified using the DyeEx 2.0 Spin Kits (QIAGEN Inc). All sequencing was done on a DNA analyzer 3130 (Applied Biosystems, Inc.).

Sequence Analysis

Bi-directional contig assembly and alignments for COI sequences were done using SeqScape v2.1.1 (Applied Biosystems, Inc.). In total, 182 hammerhead shark sequenced samples were included for COI analysis.

ITS2 sequences were aligned using the sequence editing program GeneDoc v.2.6.002 (available at http://www.nrbsc.org/gfx/genedoc). In total, 190 sequenced samples were included for the ITS2 analysis. A K2P neighbor-joining tree was drawn for both loci using PAUP* v4.0b10 (Swofford 2003) to compare the utility of the COI and ITS2 sequences as barcodes. K2P distances were calculated among and between species by Mega v3.1 for both COI and ITS2 (Kumar et al. 2004).

Locus Tree Congruence

Maximum parsimony analysis was conducted on the COI and ITS2 data sets separately and using the concatenated sequences from both loci to form a total evidence tree using PAUP* v4.0b10 (Swofford 2003). Bootstrap analysis was comprised of 1000 bootstrap replicates of 1000 pseudo-replicates each.

Results

The COI haplotypes and ITS2 genotypes were unique for each of the nine hammerhead species. Both COI and ITS2 sequences grouped every shark individual with its conspecifics in the neighbor-joining (NJ) trees (Figures 2 and 3).

Average K2P intraspecific and interspecific genetic distances for COI and ITS2 are summarized in Tables 2 and 3 and Figure 4. The average intraspecific K2P distance was 0.297% for COI (range: 0-1.94%) and 0.0967% for ITS2 (range: 0-0.083%). *Sphyrna lewini* showed the highest intraspecific sequence divergence of 1.94% at COI. Four other species also showed intraspecific divergence at COI (*S. mokarran*, *S. zygaena*, *S. corona*, and *S. tiburo*)(Table 2). The only species demonstrating intraspecific

divergence in the ITS2 were *S. lewini* (0.004%) and *S. tiburo* (0.083%). The average pairwise interspecific divergence for COI ranged from 3.91% to 11.9% (Table 2), with an overall average of 8.93% across all species. For ITS2 the average pairwise interspecific divergence was almost two times lower (Figure 4), ranging from 0.65% to 7.29% (Table 3), with an overall average of 3.83% across all species.

Maximum parsimony reconstructions of the COI (Figure 5) and ITS2 (Figure 6) datasets yielded similar but non-identical topologies. The COI tree provided some phylogeographic structure for *S. lewini*, which was not evident in the ITS2 tree. As with the NJ trees, no COI haplotypes or ITS2 sequence types were shared between any of the hammerhead species included in the maximum parsimony reconstructions. A total evidence tree is given in Figure 7.

The ITS2 sequences for *S. lewini* had a poly-G tract either 3 G's (in 34 of the sequences, hereafter called sequence type I) or 4 G's (in 2 sequences, hereafter called sequence type II) in length. The remaining 33 of the 69 *S. lewini* ITS2 sequences showed evidence of nucleotide heterogeneity, comprised of sequence type I and II, seen as dual peaks of the same height and intensity in the electropherograms, starting 335 base pairs from the beginning of the fragment.

Discussion

Both COI and ITS2 sequences delineated reciprocally monophyletic groups for each hammerhead species, clearly distinguishing them from each other. These unambiguous groupings indicate that either locus will be sufficient as a stand alone DNA barcode marker. The most notable differences between the two markers were the larger

intraspecific and interspecific K2P distances within COI (Table 2), despite mtDNA evolution rates in sharks being six or seven times slower than in mammals (Martin et al. 1992). More species exhibited intraspecific variation in COI compared to ITS2, and to a greater degree. This intraspecific variation may be advantageous for determining the broad geographic origin of hammerhead products obtained from markets (Shivji 2009). Though the variation seen within COI for *S. zygaena* is small (0.124%), it corresponded to a phylogeographic division between Atlantic and Pacific populations, which was not detectable with the ITS2 sequences. This division could correspond to population genetic structure suggesting a barrier to gene flow between Atlantic and Pacific *S. zygaena*.

The parsimony trees for COI (Figure 5) and ITS2 (Figure 6) do not have identical phylogenetic topologies, but this does not affect their ability to serve as a species identification tool. Because DNA barcoding does not attempt to resolve deeper phylogeny, minor topological differences in species relationships are inconsequential. More importantly, each hammerhead individual sample formed a distinct cluster with its conspecifics. The COI parsimony tree contained sufficient resolution to differentiate two major groups of *S. lewini*, corresponding to the Atlantic and western edge of the Indian Ocean (Madagascar), and the Pacific. The initial COI NJ trees generated by PAUP did suggest a very minor divergence between the Atlantic and Madagascar samples, but this difference was not strong enough to appear in the parsimony tree (bootstrap value <50%).

The cryptic hammerhead lineage formed a sister group to *S. lewini* in both COI and ITS2 parsimony trees, and as monophyletic units these tentatively separate species exhibited the smallest interspecific divergence in COI at 3.91%. An approximately 4% divergence in COI is well beyond the normal, average intraspecific values seen in the

other hammerheads, consistent with the notion (Abercrombie et al. 2005; Quattro et al. 2006) that the cryptic lineage constitutes a separate species. In general compared to other hammerhead sharks, the divergence between the cryptic lineage and *S. lewini* is much lower than that of any other pairwise interspecies comparison, yet still much higher than the very shallow intraspecific values seen throughout the other hammerhead species. Additionally, *S. lewini* had the highest level of intraspecific divergence in COI (1.93%); some previously proposed COI threshold values for species separation (Hebert et al. 2003) might begin to suggest that *S. lewini* should be split into two sub-species. Both genetic divergence scenarios are different interpretations of the same pattern. However, it is known that COI threshold values do not necessarily apply for species delineation in elasmobranchs (Ward et al. 2005), and should therefore be not be used by themselves to propose taxonomic revisions. Furthermore, a COI DNA barcode species definition does not exist, and further morphological and/or ecological evidence, in addition to the genetic evidence, will be required to determine the taxonomic status of the cryptic lineage (Quattro et al. 2006). Nonetheless, DNA barcoding does phenetically differentiate these clusters.

The NJ ITS2 tree (Figure 3) did not produce an evident phylogeographic split within *S. lewini*. However, the pattern of nucleotide heterogeneity within *S. lewini* ITS2 corresponds to a split between ocean basins. For example, all the *S. lewini* that demonstrated sequence heterogeneity were from the Pacific or Indian Oceans, with no heterogeneity observed in the Atlantic samples. The minute divergence between the Atlantic and Indian Ocean groups compared with the Pacific Ocean group, supports the

hypothesis that *S. lewini* dispersal likely occurred from west to east, around South Africa, as suggested by Duncan et al. (2006).

The phylogeographic split in *S. lewini* re-emerged with higher bootstrap values in the maximum parsimony total evidence tree when both COI and ITS2 sequence data sets were combined into one data matrix (Figure 7), The topology of the total evidence tree supports the topology given by the COI data alone, and in turn corroborates the tree derived from the mitochondrial control region for the genus *Sphyrna* by Duncan et al. (2006). There are minor topological differences between the total evidence tree and a recent composite supertree in which the five component trees were based on either morphology, isozymes, or mitochondrial sequence data (Cavalcanti 2007). However, the relative relationship of sister species remain consistent in the total evidence tree. For example, *S. tudes* and *S. tiburo* remain sister species to each other in both this study and the previous ones (Duncan et al. 2006; Cavalcanti 2007).

S. tiburo is the only other hammerhead species to have within species variation in the ITS2, other than *S. lewini*. Chapman and Shivji (unpublished data) have shown distinct haplotypes and a phylogeographic split, using the mitochondrial control region sequence, between *S. tiburo* from the Atlantic (South Carolina coast through the northern Gulf of Mexico) and the western Caribbean (Belize). The COI and ITS2 data corroborate the control region results and, as evident by both COI and ITS2 trees there are at least two *S. tiburo* populations from the Atlantic and Caribbean.

While both COI and ITS2 were effective at placing each hammerhead individual with its conspecifics into discrete clusters for species identification, it is notable that both markers, although from different organelles with ostensibly different evolutionary rates,

also demonstrated complete congruency in their species groupings. Either marker alone would perform equally well in identifying hammerhead sharks and possibly sharks in general, but integrated multigene approaches are now encouraged and advised (Dasmahapatra & Mallet 2006). As fishery and trade regulations for hammerheads accumulate, accurate results that will hold up in law enforcement contexts will be crucial. Supplemental nuclear markers such as ITS2 for DNA barcoding in sharks will play an important role in providing multiple, independent support for species identification.

Table 1. PCR primer cocktail components and corresponding sequences. M13 tails are highlighted.

Primer name		Sequence	
C_FishF1t1 (1:1 ratio)	**VF2_t1**	5'TGTAAAACGACGGCCAGTCAACCAACCACAAAGACATTGGCAC3'	(Ward et al. 2005)
	FishF2_t1	5'TGTAAAACGACGGCCAGTCGACTAATCATAAAGATATCGGCAC3'	(Ward et al. 2005)
C_FishR1t1 (1:1 ratio)	**FishR2_t1**	5'CAGGAAACAGCTATGACACTTCAGGGTGACCGAAGAATCAGAA3'	(Ward et al. 2005)
	FR1d_t1	5'CAGGAAACAGCTATGACACCTCAGGGTGTCCGAARAAYCARAA3'	(Ivanova et al. 2007)
M13F		5'TGTAAAACGACGGCCAGT3'	(Messing 1983)
M13R		5'CAGGAAACAGCTATGAC3'	(Messing 1983)

Table 2. Average inter- and intra-specific K2P distance comparisons for nine hammerhead species for the mitochondrial COI gene. Intra-specific distances are bolded.

	S. lewini	Cryptic	*S. mokarran*	*S. zygaena*	*S. tiburo*	*S. tudes*	*E. blochii*	*S. corona*	*S. media*
S. lewini	**0.01935**								
Cryptic	0.03914	**0**							
S. mokarran	0.09512	0.08602	**0.00010**						
S. zygaena	0.09129	0.08036	0.09288	**0.00124**					
S. tiburo	0.09614	0.08749	0.10938	0.10836	**0.00556**				
S. tudes	0.08860	0.08743	0.11926	0.09929	0.06784	**N/A**			
E. blochii	0.08757	0.08958	0.07719	0.08563	0.11865	0.11690	**0**		
S. corona	0.07568	0.07009	0.10809	0.10210	0.07713	0.07417	0.10577	**0.00044**	
S. media	0.09436	0.09115	0.11336	0.08504	0.06445	0.04819	0.10761	0.07429	**N/A**

N/A: intra-specific genetic distances not calculated since 1 animal only sequenced.

Table 3. Average inter- and intra-specific K2P distance comparisons for nine hammerhead species for the nuclear ITS2 locus. Intra-specific distances are bolded.

	S. lewini	Cryptic	*S. mokarran*	*S. zygaena*	*S. tiburo*	*S. tudes*	*E. blochii*	*S. corona*	*S. media*
S. lewini	**0.00004**								
Cryptic	0.01469	**0**							
S. mokarran	0.05190	0.04324	**0**						
S. zygaena	0.03982	0.03298	0.03640	**0**					
S. tiburo	0.03572	0.02724	0.05105	0.04410	**0.00083**				
S. tudes	0.02635	0.01799	0.04149	0.03467	0.01225	**N/A**			
E. blochii	0.07288	0.06401	0.06576	0.06049	0.07024	0.06046	**0**		
S. corona	0.03311	0.02467	0.04838	0.03809	0.01885	0.00974	0.06751	**0**	
S. media	0.02973	0.02133	0.04494	0.03807	0.01555	0.00648	0.06398	0.01303	**N/A**

N/A: intra-specific genetic distances not calculated since 1 animal only sequenced.

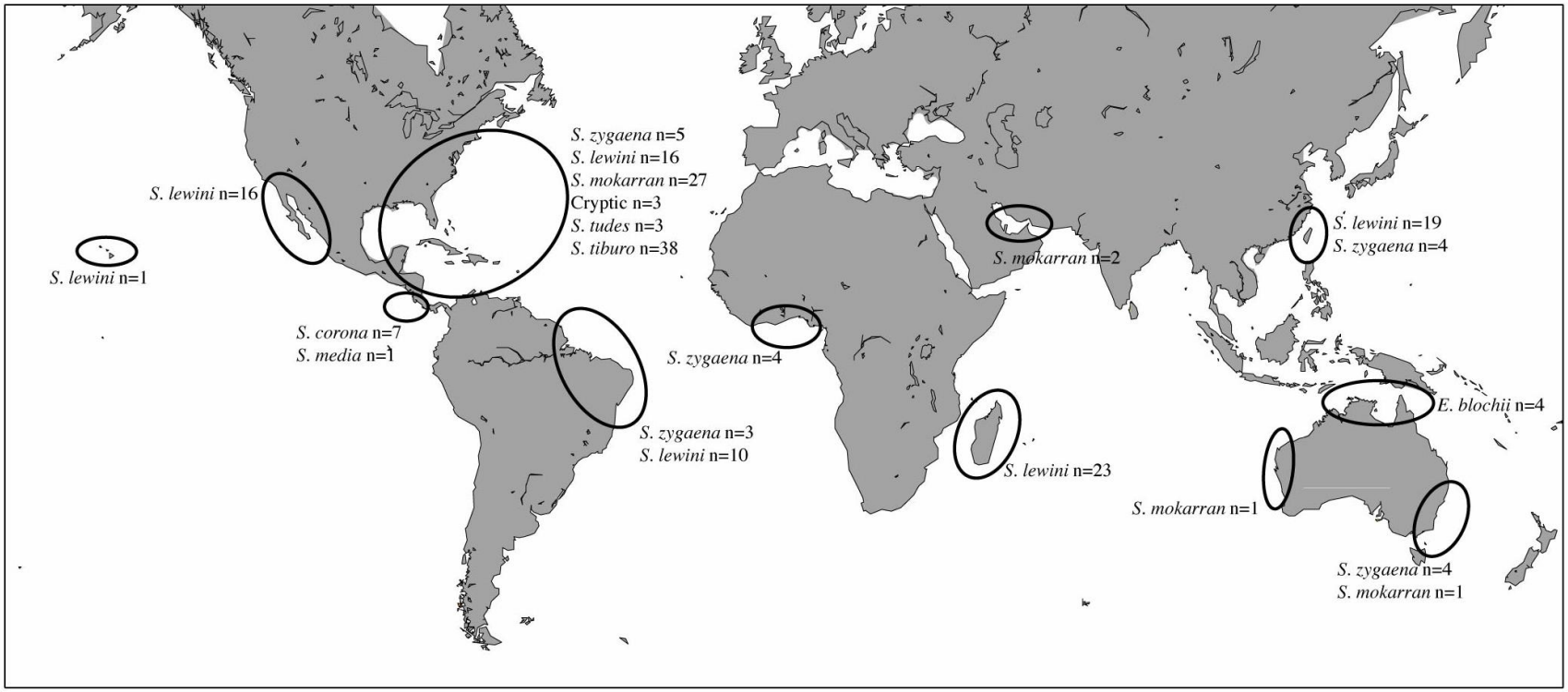

Figure 1. Sample sizes and distribution by region for all nine hammerhead species examined including the cryptic hammerhead.

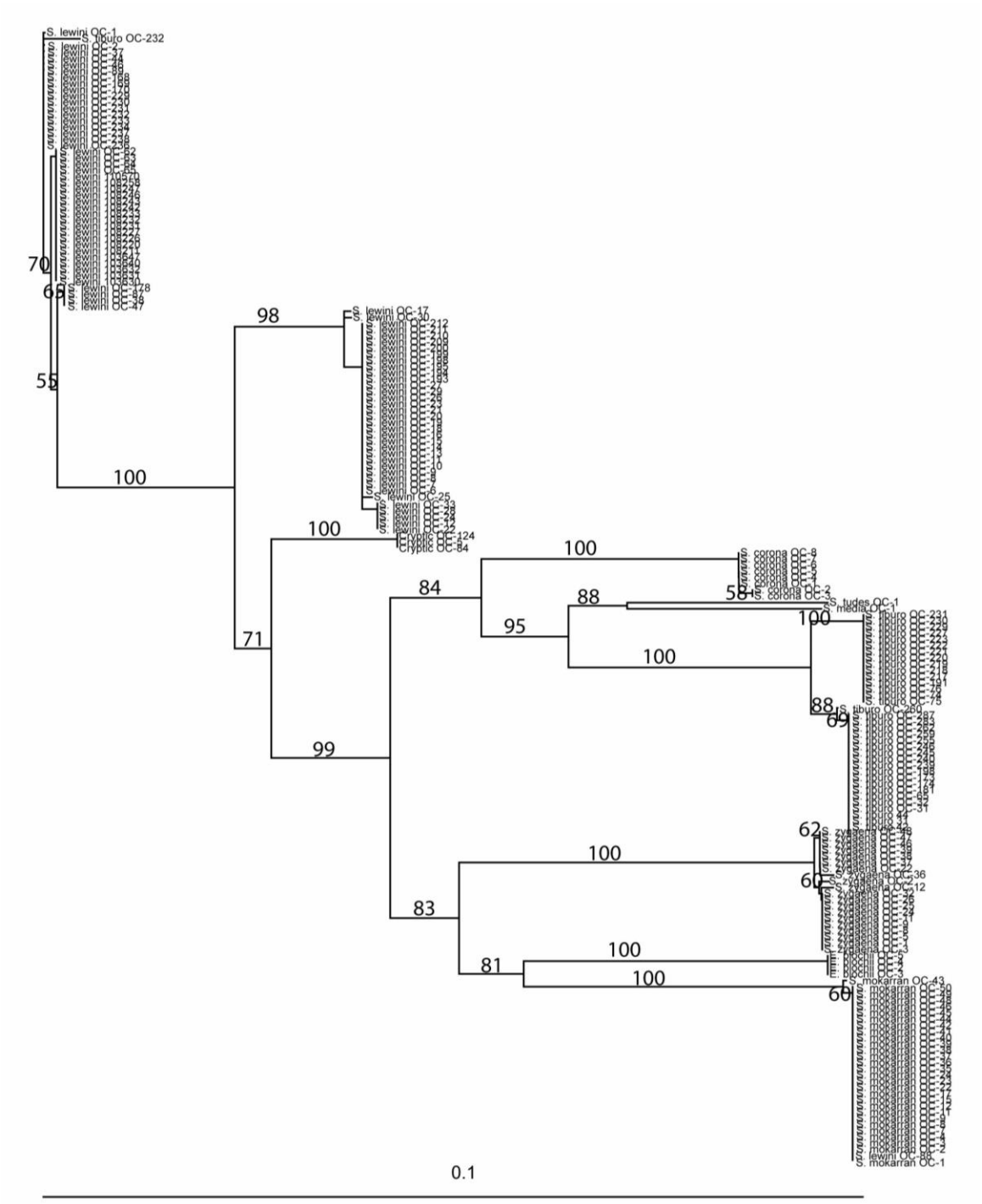

Figure 2. Neighbor-joining tree of all hammerhead COI sequences. Bootstrap values greater than 50 are displayed on the branch.

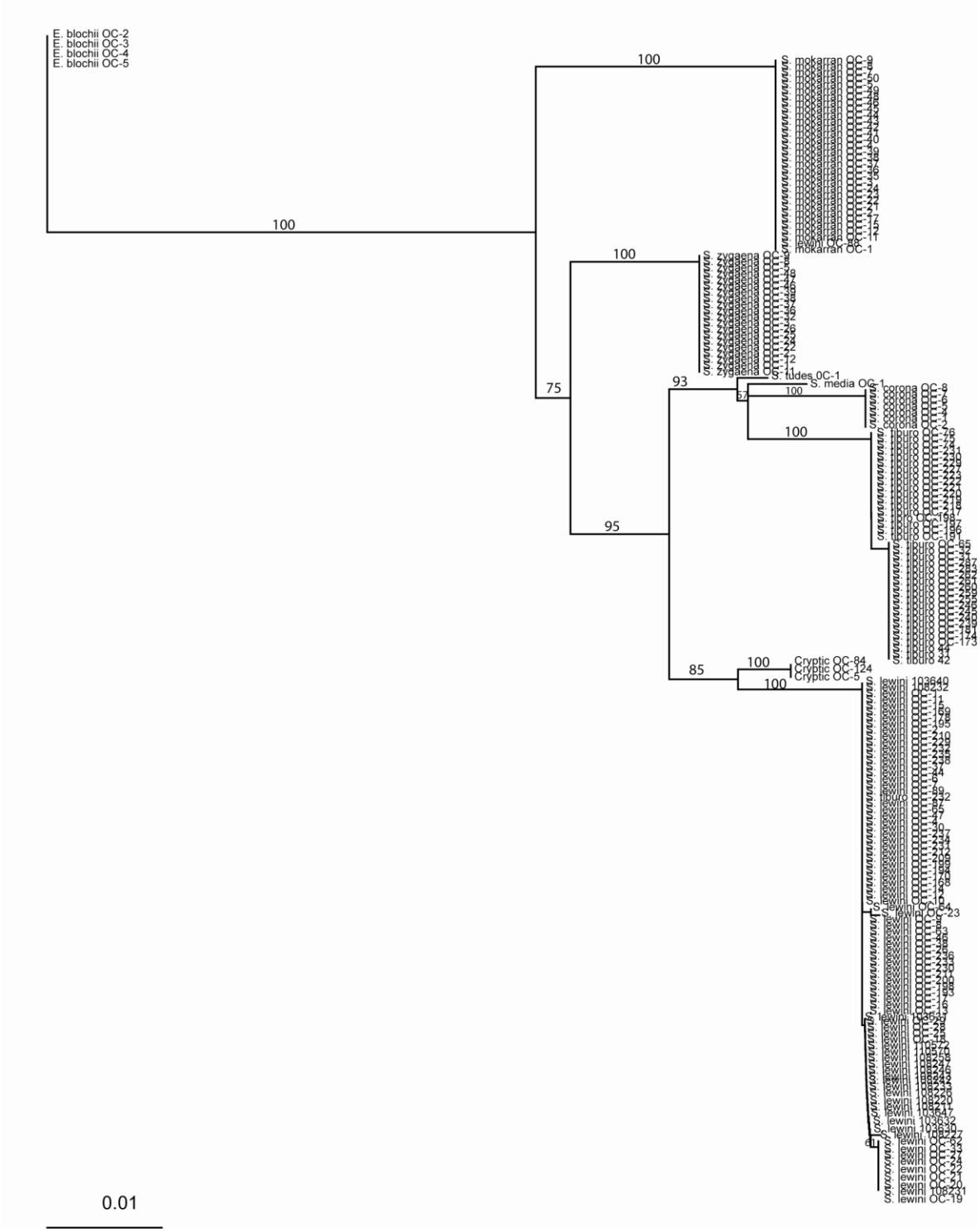

Figure 3. Neighbor-joining tree of all hammerhead ITS2 sequences. Bootstrap values greater than 50 are displayed on the branch.

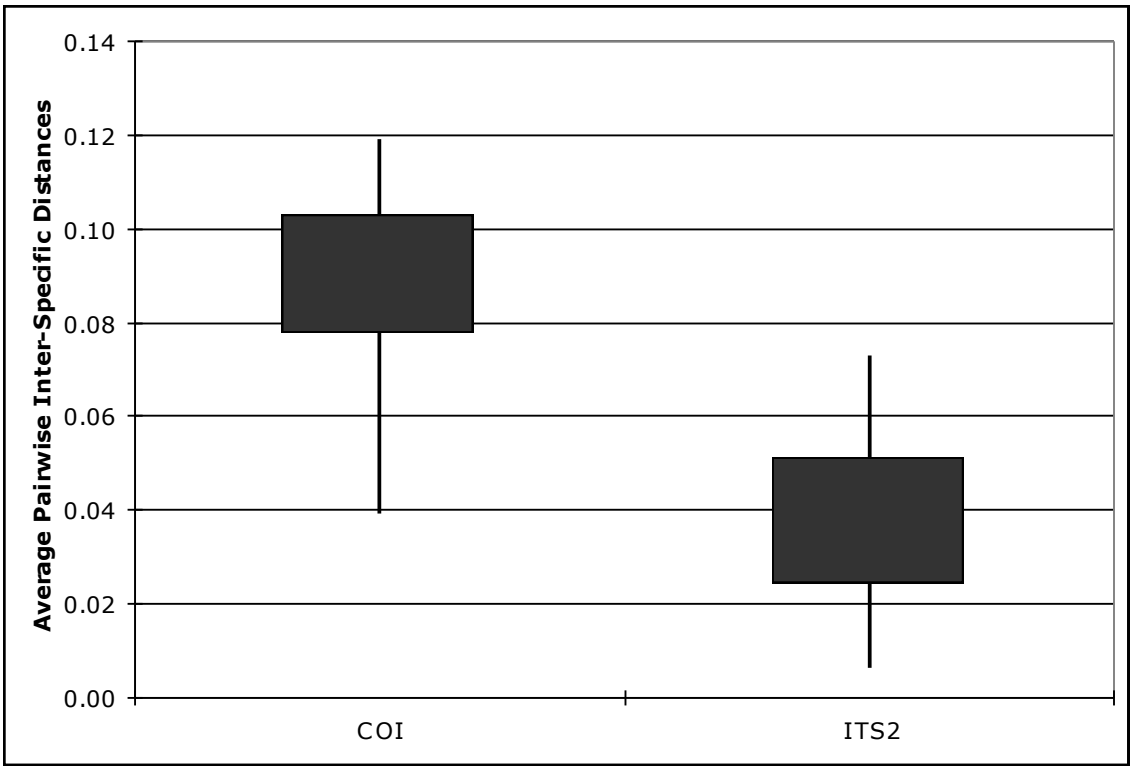

Figure 4. Boxplot comparing the average, pairwise K2P genetic distance between hammerhead species for COI and ITS2. Boxes represent 50% of the data. Whiskers represent minimum and maximum non-outlier values.

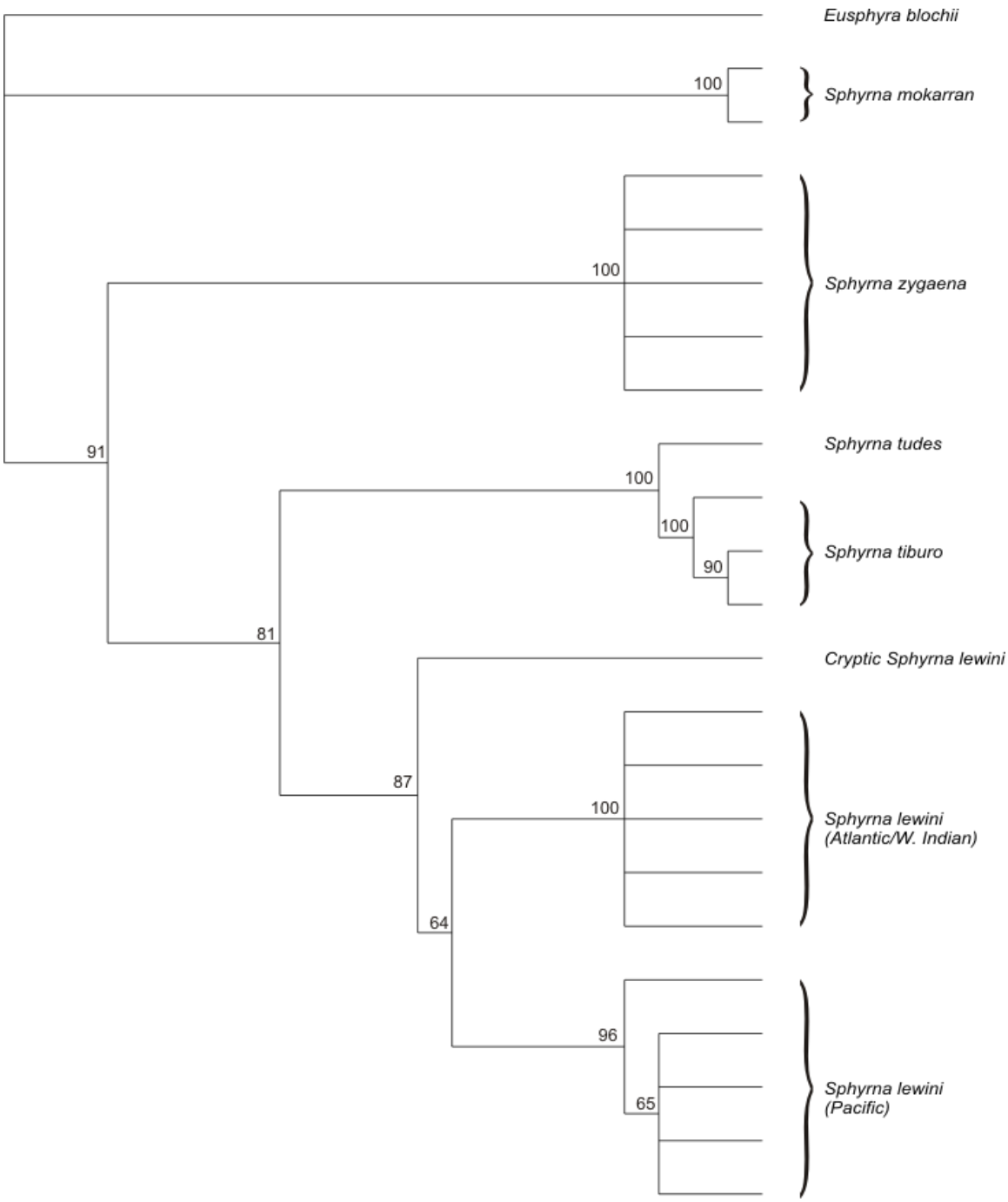

Figure 5. Strict consensus tree of the maximum parsimony analysis of the hammerhead shark COI haplotypes. Bootstrap values are listed at each node (1000 pseudo replicates). Only nodes supported by >50% are shown.

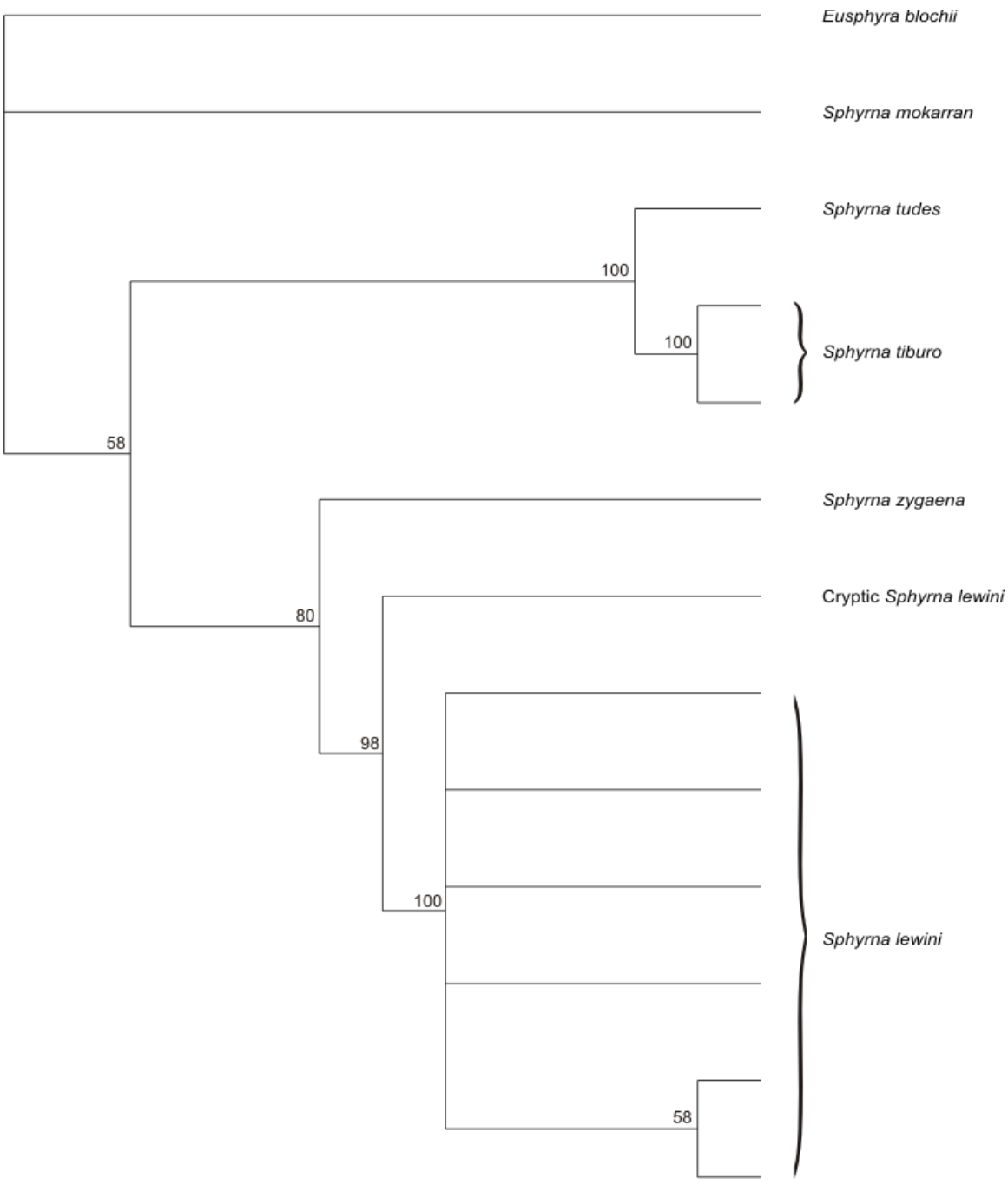

Figure 6. Strict consensus tree of the maximum parsimony analysis of the hammerhead shark ITS2 sequence types. Bootstrap values are listed at each node (1000 pseudo replicates). Only nodes supported by >50% are shown. Gaps were treated as a fifth character state.

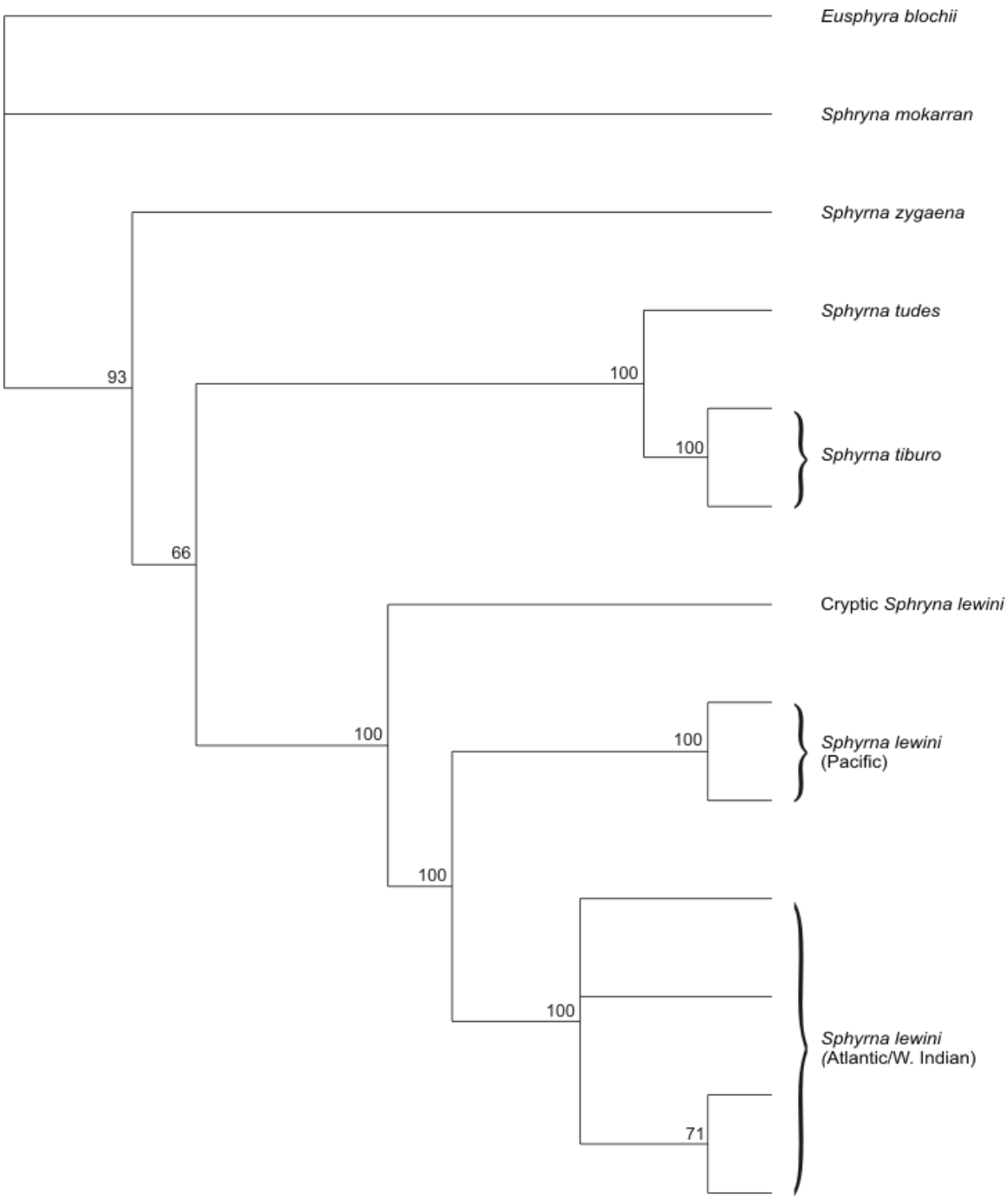

Figure 7. Strict consensus tree of the total evidence maximum parsimony analysis of the hammerhead shark concatenated COI and ITS2 sequences. Specimens corresponding to the unique ITS2 haplotypes were used. Bootstrap values are listed at each node (1000 pseudo replicates). Only nodes supported by >50% are shown. Gaps are treated as a fifth character state.

Appendix A. Alignment of all unique ITS2 sequence types. Dots indicate identical sequence to top sequence. Slew, *Sphyrna lewini*; Cryptic, cryptic hammerhead; Smok, *Sphyrna mokarran*; Szyg, *Sphyrna zygaena*; Stib, *Sphyrna tiburo*; Stud, *Sphyrna tudes*; Eblo, *Eusphyra blochii*; Scor, *Sphyrna corona*; Smed, *Sphyrna media*

```
                          20                  40                  60
SlewOC1    : GACAATCAATCGCACTTTGCTGTTTT-CTGAGCGGCAAAGAGCGCGGCTGGGGTGTCGCAGAGGTGCTGT :  69
SlewOC19   : ........................-........................................... :  69
SlewOC23   : ........................-........................................... :  69
SlewOC62   : ........................-........................................... :  69
Slew103631 : ........................-........................................... :  69
Slew108227 : ........................-........................................... :  69
CrypOC5    : ........................-........................................... :  69
SmokOC2    : ......................C..-...T..A..........T.........T...A........... :  69
SzygOC1    : ......................C..-...T......................C................ :  69
Stib31     : ......................C..-........................................... :  69
StibOC74   : ......................C..-........................................... :  69
EbloOC2    : ......................C..-...T..A.....................................A. :  69
Stud0C1    : ......................C..T........................................... :  70
ScorOC1    : ......................C..-.C.....................................C..... :  69
SmedOC1    : ......................C..-........................................... :  69

                 80                 100                 120                 140
SlewOC1    : CCTCTCTGTCCCCC--TAAGTGCAGACTCTGAGTAATCCGCGTCGGAGAGATTGACCCGCTCCCTCACTG : 137
SlewOC19   : ............--.............................................................. : 137
SlewOC23   : ............--.............................................................. : 137
SlewOC62   : ............--.............................................................. : 137
Slew103631 : ............--.............................................................. : 137
Slew108227 : ............--.............................................................. : 137
CrypOC5    : ............--.............................................................. : 137
SmokOC2    : ............--..........C.A.................................................. : 137
SzygOC1    : ............--..........C.A.................................................. : 137
Stib31     : ............--.............................................................. : 137
StibOC74   : ............--.............................................................. : 137
EbloOC2    : ............--...........A...C.................................G.. : 137
Stud0C1    : ............--.............................................................. : 138
ScorOC1    : T.-....C.GT...CC.............................................................. : 138
SmedOC1    : ............--.............................................................. : 137

                         160                 180                 200
SlewOC1    : GCCTAGCCTCCTTGGGGTCGCCGGCACGGCTGTCATCAGGTTGCCAGAGAAAAAATGTGACTGCCACGCT : 207
SlewOC19   : ..................................................................... : 207
SlewOC23   : ..................................................................... : 207
SlewOC62   : ..................................................................... : 207
Slew103631 : ..................................................................... : 207
Slew108227 : ..................................................................... : 207
CrypOC5    : ..................................................TG..--......----- : 200
SmokOC2    : ...C..............................................TG..--.......... : 205
SzygOC1    : ...C..............................................TG..--.......... : 205
Stib31     : ...C.............................................C.....TG..--.......... : 205
StibOC74   : ...C.............................................C.....TG..--.......... : 205
EbloOC2    : ...C......T.....................................T.G..TG..--..--------A : 197
Stud0C1    : ...C.............................................C.....TG..--.......... : 206
ScorOC1    : ...C.............................................C.....TG..--.......T... : 206
SmedOC1    : ...C.................................G.........C.....TG..--.......... : 205

                 220                 240                 260                 280
SlewOC1    : GCCGGGACCCTGTGTGCCTTCCGTTT-GGCTTGTGCCCAGGGGT---CGTATCTCTGTCGATTTGTGGCT : 273
SlewOC19   : ........................-..............---...................... : 273
SlewOC23   : ........................-..............---...................... : 273
SlewOC62   : ........................-..............---...................... : 273
Slew103631 : ........................-..............---...................... : 273
Slew108227 : ........................-..............---...................... : 273
CrypOC5    : ---......G.......T........-..............---...................... : 263
```

Appendix A continued

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SmokOC2    : .........G.................-......G......A.-.---..................... : 270
SzygOC1    : .........G................A-....C.G.........GGGT..................... : 274
Stib31     : .........G...T..G...--..----------..........---T..................... : 260
StibOC74   : .........G...T..G...--..----------..........---..................... : 260
EbloOC2    : ....C....G............T...T..............-.---.....................T.. : 263
Stud0C1    : .........G...T..G...--..----------..........---..................... : 261
ScorOC1    : .........G...T..G...--..----------..........---..................... : 261
SmedOC1    : .........G...T..G...--..----------..........---.............C........ : 260

                            300                 320                 340
SlewOC1    : CCTC--CAGGTAAA-GGATCCGCTTTGCTGGACTGGTTGGTTC-TGGCACTG--AGTG-AGAGAG----C : 332
SlewOC19   : ....--........-.............................-........--....-......----. : 332
SlewOC23   : ....--........-.............................-........--....-......----. : 332
SlewOC62   : ....--........-.............................-........--....-......----. : 332
Slew103631 : ....--........-.............................-........--....-......----. : 332
Slew108227 : ....--........-.............................-........--....-......----. : 332
CrypOC5    : ....--........-............T...C...........-........--....-......----. : 322
SmokOC2    : ....--..C.....-.....T......T...C.G.......C.-.T......GC.C..-..T...AGAG. : 335
SzygOC1    : ....--..C.....A............T...C...........-.T......GC.C..-..T...AG--- : 337
Stib31     : ....TC........-............T...C...........-.T......GC.C..-..T.G.AGAG. : 327
StibOC74   : ....TC........-............T...C...........-.T......GC.C..-..T.G.AGAG. : 327
EbloOC2    : ....--..C.....A............T...C........A.C.C.T......--....-......AG--. : 326
Stud0C1    : ....TC........-............T...C...........-.T......GC.A..G..T...AGAG. : 329
ScorOC1    : ....--........-............T...C...........-.T...G..GC.C..-..T...AGAG. : 326
SmedOC1    : ....TC........-............T...C...........-.T......GC.C..-..T...AGAG. : 327

                  360                 380                 400                 420
SlewOC1    : AGAGGG----------TCTGGCCTTCCGCCAGGTTGGCCTTTCCCCTCGTTCTGT-AACACCTTTGCGTT : 391
SlewOC19   : ......----------.....................................-.............. : 391
SlewOC23   : ......----------.....................................-.............. : 391
SlewOC62   : ......---------G.....................................-.............. : 392
Slew103631 : ......---------G.....................................-.............. : 392
Slew108227 : ......----------.....................................-.............. : 391
CrypOC5    : ......----------.......................-............-.............. : 380
SmokOC2    : ....T.ACTCGCTCTG..........T....A..........-.....A......-...........AC. : 403
SzygOC1    : ------ACTCGCTATG..........................-.....A......T...........A.. : 400
Stib31     : ....T.ACTCGCTGTGC.........T......C........-....T.......-...........A.. : 395
StibOC74   : ....T.ACTCGCTGTGC.........T......C........-....T.......-...........A.. : 395
EbloOC2    : ....T.ACTCGCTCTGG.A...TG..T.........C.T..--.A...A......C-..........A.. : 393
Stud0C1    : ......ACTCGCTGTGC.........T......C............-.........-...........A.. : 397
ScorOC1    : ....T.ACTCGCTCTGC.........T......C............-.........-...........A.. : 394
SmedOC1    : ......ACTCGCTGTGC.........T......C............-.........-...........A.. : 395

                            440                 460                 480
SlewOC1    : GCATTCGA-CCCGCC-AGCACTGGTTT-CCGTTACTGTGGAAGTGCAGACACAC--TTCACCACGCTGGG : 456
SlewOC19   : ........-......-...........-..................T.........--.............. : 456
SlewOC23   : ........-......-...........-.................G.........--.............. : 456
SlewOC62   : ........-......-...........-..................T.........--.............. : 457
Slew103631 : ........-......-...........-..................N.........--.............. : 457
Slew108227 : ......C.-......-...........-..................N.........--.............. : 456
CrypOC5    : ........-......-...........-........A............T.....--.............. : 445
SmokOC2    : .....T..-......-...........-....................T.....--.............. : 468
SzygOC1    : ........-......-...........-..C..................G.....--.............. : 465
Stib31     : ........-...CA.C...........-.......G.............T.....--.............. : 461
StibOC74   : ........-...CA.C...........-.......G.............T.....--.............. : 461
EbloOC2    : ..G..AA.--...CG.C...........-..C..................T.....AC...G.......... : 460
Stud0C1    : ........C...A..A-...........-....................T.....--.............. : 463
ScorOC1    : ........C...A..AGC......G..T.........C...........T.....--.............. : 462
SmedOC1    : ........C...A..A-T.........-....................T.....--.............. : 461

                  500                 520                 540                 560
SlewOC1    : TTACCTGGAAGAGTTGATGTGCCGAGCCCGGTCCGTGTGCTGAGTGCTGTGAGGGCACACGGCAGTGCCT : 526
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Appendix A continued

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SlewOC19    : ...................................................................... : 526
SlewOC23    : ...................................................................... : 526
SlewOC62    : ...................................................................... : 527
Slew103631  : ...................................................................... : 527
Slew108227  : ...................................................................... : 526
CrypOC5     : ...T..................................................T....T.......... : 515
SmokOC2     : ...T.......................................................T.......... : 538
SzygOC1     : ...T......................................................GT.-----.... : 530
Stib31      : ...T.............................T......G..................T.......... : 531
StibOC74    : ...T.............................T......G..................T.......... : 531
EbloOC2     : ..CT...A..........................C........................T.......... : 530
Stud0C1     : ...T.......................................................T.......... : 533
ScorOC1     : ...T.......................................................T.....C.... : 532
SmedOC1     : ...T.......................................................T.......... : 531


                                580                 600                 620
SlewOC1     : TGTACGTTCTGTGTGTGTCCAGTGCCTGTGCATCCTCTGCGCAGCACGCCCACGCATTGCGTGCAGCTAC : 596
SlewOC19    : ...................................................................... : 596
SlewOC23    : ...................................................................... : 596
SlewOC62    : ...................................................................... : 597
Slew103631  : ...................................................................... : 597
Slew108227  : ...................................................................... : 596
CrypOC5     : ..................-.................................T................. : 584
SmokOC2     : ................---....................T..........-.T................. : 604
SzygOC1     : ..................-...............................-.T................. : 598
Stib31      : ................---...............................-.T................. : 597
StibOC74    : ................---...............................-.T................. : 597
EbloOC2     : ..................-...............................-.T...............C. : 598
Stud0C1     : ................---...............................-.T................. : 599
ScorOC1     : ................---...............................-.T................. : 598
SmedOC1     : ................---.................................T................. : 598


                      640                 660                 680                 700
SlewOC1     : GTGTGCCTGCAGCCCTCGATGGTGCCTGAGACCGGCCGGCCACACAGCACCCCGCT--TGTGCTGCCTTC : 664
SlewOC19    : ........................................................--............ : 664
SlewOC23    : ........................................................--............ : 664
SlewOC62    : ........................................................--............ : 665
Slew103631  : ........................................................--............ : 665
Slew108227  : ........................................................--............ : 664
CrypOC5     : ........................................................--............ : 652
SmokOC2     : .............G.....AT...................................--............ : 672
SzygOC1     : ...................A...................................A--............ : 666
Stib31      : ....................................................T...TG............ : 667
StibOC74    : ....................................................T...TG............ : 667
EbloOC2     : ...................A......C.............................--............ : 666
Stud0C1     : ........................................................TG............ : 669
ScorOC1     : .......................................................ATG............ : 668
SmedOC1     : ........................................................TG............ : 668


SlewOC1     : TGT : 667
SlewOC19    : ... : 667
SlewOC23    : ... : 667
SlewOC62    : ... : 668
Slew103631  : ... : 668
Slew108227  : ... : 667
CrypOC5     : ... : 655
SmokOC2     : ... : 675
SzygOC1     : ... : 669
Stib31      : ... : 670
StibOC74    : ... : 670
EbloOC2     : ... : 669
Stud0C1     : ... : 672
```

Appendix A continued

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ScorOC1     : ... : 671
SmedOC1     : ... : 671
```

Appendix B. Sequence alignment of 652bp from the 5' end of COI haplotypes . Dots indicate identical sequence to top sequence. Slew, *Sphyrna lewini*; Cryptic, cryptic hammerhead; Smok, *Sphyrna mokarran*; Szyg, *Sphyrna zygaena*; Stib, *Sphyrna tiburo*; Stud, *Sphyrna tudes*; Eblo, *Eusphyra blochii*; Scor, *Sphyrna corona*; Smed, *Sphyrna media*.

```
                          20                  40                  60
SlewOC1    : CCTTTACCTAATTTTTGGTGCATGAGCAGGAATAATTGGAACAGCCCTAAGTCTTTTAATTCGAGCTGAA :  70
SlewOC6    : ..................................................C.................. :  70
SlewOC12   : ..................................................C.................. :  70
SlewOC17   : ..................................................C.................. :  70
SlewOC25   : ..................................................C.................. :  70
SlewOC30   : ..................................................C.................. :  70
SlewOC38   : ..................................................................... :  70
SlewOC62   : ..................................................................... :  70
Slew110570 : ..................................................................... :  70
CrypOC5    : ..................................................................... :  70
SmokOC1    : ............................T...G..................................... :  70
SmokOC43   : ............................T...G..................................... :  70
SzygOC1    : ............................T...G....G..............C.................. :  70
SzygOC2    : ............................T...G....G..............C.................. :  70
SzygOC12   : ............................T...G....G..............C.................. :  70
SzygOC22   : ............................T...G....G..............C.................. :  70
SzygOC36   : ............................T...G....G..............C.................. :  70
Stib31     : ......T......................................................... :  70
StibOC76   : ......T......................................................... :  70
StibOC260  : ......T......................................................... :  70
StibOC232  : ..................................................................... :  70
StudOC1    : ......T......................................................... :  70
EbloOC2    : .......T.G.....................G....T..T.......................... :  70
ScorOC1    : ..........................................T........C..............G :  70
ScorOC2    : .......T..................................T........C..............G :  70
SmedOC1    : ......T..................................................C.................. :  70

                    80                 100                 120                 140
SlewOC1    : CTTGGACAACCAGGCTCTCTTTTAGGAGATGATCAGATTTATAATGTAATTGTAACTGCCCACGCTTTCG : 140
SlewOC6    : ..............A..................................................... : 140
SlewOC12   : ..............A..................................................... : 140
SlewOC17   : ..............A..................................................... : 140
SlewOC25   : ..............A..................................................... : 140
SlewOC30   : ..............A.....................................C.............. : 140
SlewOC38   : ..................................................................... : 140
SlewOC62   : ..................................................................... : 140
Slew110570 : ..................................................................... : 140
CrypOC5    : ..............A..................................................... : 140
SmokOC1    : .....G........A..C.....................C..............C.............. : 140
SmokOC43   : .....G........A..C.....................C..............C.............. : 140
SzygOC1    : .....G........A......C..........C.....................C.............. : 140
SzygOC2    : .....G........A......C..........C.....................C.............. : 140
SzygOC12   : .....G........A......C..........C.....................C.............. : 140
SzygOC22   : ..............A......C..........C.....................C.............. : 140
SzygOC36   : ..............A......C..........C.....................C.............. : 140
Stib31     : .....G........T.....................................................T. : 140
StibOC76   : .....G........T.....................................................T. : 140
StibOC260  : .....G........T.....................................................T. : 140
StibOC232  : ..................................................................... : 140
StudOC1    : .....G........T.....C........C.................................T. : 140
EbloOC2    : ..............A.....C.....................................C..T.......... : 140
ScorOC1    : ........G........C..................................................... : 140
ScorOC2    : ........G........C..................................................... : 140
SmedOC1    : .....G........T..................C.....................C...........T. : 140
```

Appendix B continued

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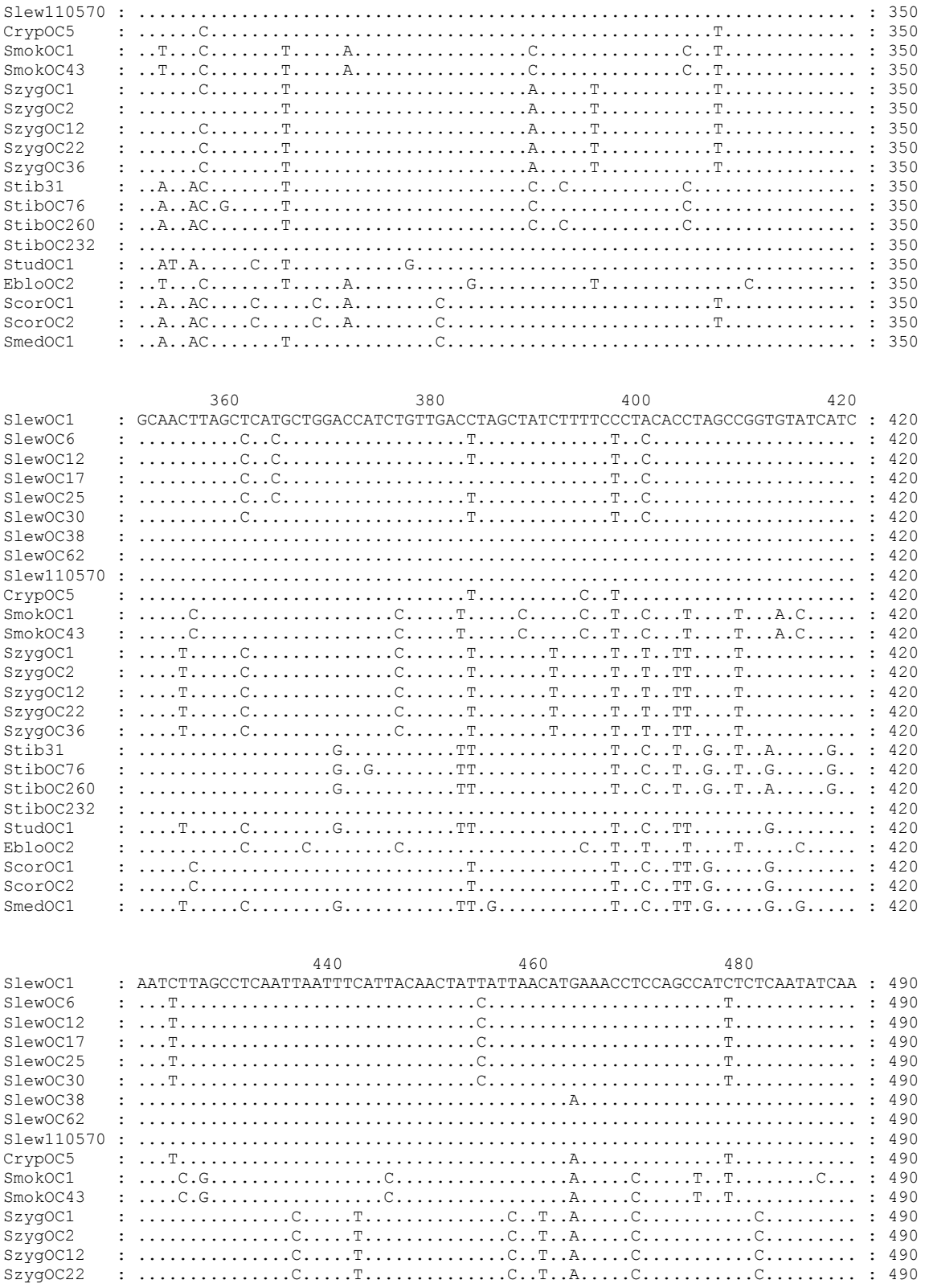
                          160                 180                 200
SlewOC1    : TAATAATCTTTTTCATAGTTATACCAATTATAATTGGTGGTTTTGGGAATTGGCTCGTGCCTTTAATAAT : 210
SlewOC6    : ......................G.................................A..T........... : 210
SlewOC12   : ......................G.................................A..T........... : 210
SlewOC17   : ......................G.................................A..T........... : 210
SlewOC25   : ......................G.................................A..T........... : 210
SlewOC30   : ......................G.................................A..T........... : 210
SlewOC38   : ..................................................................... : 210
SlewOC62   : ..................................................................... : 210
Slew110570 : ..................................................................... : 210
CrypOC5    : ......................G.....................C.....C..A..A..T........... : 210
SmokOC1    : .............T..G..A..G.............................A..A..T........... : 210
SmokOC43   : .............T..G..A..G.............................A..A..T........... : 210
SzygOC1    : .............T........G.....C...........C..C........AT.A..T........... : 210
SzygOC2    : .............T........G.....C...........C..C........AT.A..T........... : 210
SzygOC12   : .............T........G.....C...........C..C........AT.A..C........... : 210
SzygOC22   : .............T........G.....C...........C..C........AT.A..T........... : 210
SzygOC36   : .............T........G.....C...........C..C........AT.A..T........... : 210
Stib31     : .............T..............C...........C.....A..C..A..G..T........... : 210
StibOC76   : .............T.........................C.....A..C..A..G..T........... : 210
StibOC260  : .............T..............C...........C.....A..C..A..G..T........... : 210
StibOC232  : ..................................................................... : 210
StudOC1    : .............T.....A.....................C...........A..A..T........... : 210
EbloOC2    : ...............G.....................................A..T...C........ : 210
ScorOC1    : .............T.....................................A..A..T..C........ : 210
ScorOC2    : .............T.....................................A..A..T..C........ : 210
SmedOC1    : .............T.........................C...........A..A..T........... : 210

                   220                 240                 260                 280
SlewOC1    : TGGTGCGCCAGATATGGCCTTCCCACGAATAAACAACATAAGCTTTTGACTTCTTCCACCATCATTCCTT : 280
SlewOC6    : ............C.........T.............T............C..G..................... : 280
SlewOC12   : ............C........T...........T............C..G..................... : 280
SlewOC17   : ............C........T...........T............C..G..................... : 280
SlewOC25   : ............C........T...........T............C..G..................... : 280
SlewOC30   : ............C........T...........T............C..G..................... : 280
SlewOC38   : ..................................................................... : 280
SlewOC62   : ..................................................G..................... : 280
Slew110570 : ..................................................G..................... : 280
CrypOC5    : ............C.....................T............C..............T......... : 280
SmokOC1    : ......A........A..T..............T............C.....................T... : 280
SmokOC43   : ......A........A..T..............T............C.....................T... : 280
SzygOC1    : ......A.....C.....................T........T..C.....................T... : 280
SzygOC2    : ......A.....C.....................T........T..C.....................T... : 280
SzygOC12   : ......A.....C.....................T........T..C.....................T... : 280
SzygOC22   : ......A.....C.....................T........T..C.....................T... : 280
SzygOC36   : ......A.....C.....................T........T..C.....................T... : 280
Stib31     : ......A.....C.....................T............C..G..C.................. : 280
StibOC76   : ......A.....C.....................T............C..G..C.................. : 280
StibOC260  : ......A.....C.....................T............C..G..C.................. : 280
StibOC232  : ..................................................................... : 280
StudOC1    : ......A.....C.....................T............C..G.................T... : 280
EbloOC2    : ...C..A.....C..A..................T..............G.................T... : 280
ScorOC1    : ......A.....C.....................T............C..G..................... : 280
ScorOC2    : ......A.....C.....................T............C..G..................... : 280
SmedOC1    : ......A.....C..................G..T............C..G.................T... : 280

                          300                 320                 340
SlewOC1    : CTCCTCTTAGCTTCCGCTGGGGTAGAAGCTGGAGCAGGTACTGGCTGAACAGTTTACCCTCCATTAGCTA : 350
SlewOC6    : ......C.....................................................T.............. : 350
SlewOC12   : ......C.....................................................T.............. : 350
SlewOC17   : ......C.....................................................T.............. : 350
SlewOC25   : ......C.....................................................T.............. : 350
SlewOC30   : ......C.....................................................T.............. : 350
SlewOC38   : ..................................................................... : 350
SlewOC62   : ..................................................................... : 350
```

Appendix B continued



Appendix B continued

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SzygOC36   : ..............C.....T..............C..T..A.....C...........C......... : 490
Stib31     : ...TC....T.....C.....T.....................A.....C........T..C..G..C... : 490
StibOC76   : ...TC....T.....C.....T.....................A.....C........T..C..G..C... : 490
StibOC260  : ...TC....T.....C.....T.....................A.....C........T..C..G..C... : 490
StibOC232  : ..................................................................... : 490
StudOC1    : ...TC....T.....C.....T........C...........A.........T....T..C......... : 490
EbloOC2    : ....................T..C...........C.....A.....C.....T.....C......... : 490
ScorOC1    : ...T.................T.....................A..............T..C.....C... : 490
ScorOC2    : ...T.................T.....................A..............T..C.....C... : 490
SmedOC1    : ...T.....T.....C..C..T.....................A.....C...T.......C......... : 490

                    500                 520                 540                 560
SlewOC1    : ACACCATTATTTGTTTGATCCATTCTTGTAACTACTATCCTACTTCTCCTCTCACTTCCAGTTCTCGCAG : 560
SlewOC6    : ........................C.............................A..............T.... : 560
SlewOC12   : ........................C.............................A..............T.... : 560
SlewOC17   : ........................C.............................A..............T.... : 560
SlewOC25   : ........................C.............................A.....C........T.... : 560
SlewOC30   : ........................C.............................A..............T.... : 560
SlewOC38   : ..................................................................... : 560
SlewOC62   : ..................................................................... : 560
Slew110570 : ..................................................................... : 560
CrypOC5    : ...........C........T..C.....................................T.... : 560
SmokOC1    : ..............C.....T..................T...........T...........C..T.... : 560
SmokOC43   : ..............C.....T..................T...........T...........C..T.... : 560
SzygOC1    : ......C.............T..C..............TT.........................T.... : 560
SzygOC2    : ......C.............T..C..............TT.........................T.... : 560
SzygOC12   : ......C.............T..C..............TT.........................T.... : 560
SzygOC22   : ......C.............T..C..............TT.........................T.... : 560
SzygOC36   : ......C.............T..C..............TT.........................T.... : 560
Stib31     : ..G.................T..C.....G........T..G..A.....T..C..C........T.... : 560
StibOC76   : ..G.................T..C.....G...........G..A.....T..C..C........T.... : 560
StibOC260  : ..G.................T..C.....G........T.....A.....T..C..C........T.... : 560
StibOC232  : ..................................................................... : 560
StudOC1    : ....................T..C...........C........C........C...........T.... : 560
EbloOC2    : .....C..............T..C..............T...........T..............T.... : 560
ScorOC1    : ....................T..C................T.G..C........C...........T.... : 560
ScorOC2    : ....................T..C................T.G..C........C...........T.... : 560
SmedOC1    : ....................T..C..............T.....C........C...........T.... : 560

                          580                 600                 620
SlewOC1    : CAGGAATTACAATATTACTCACAGATCGTAACCTTAATACTACATTCTTTGATCCTGCAGGGGGAGGAGA : 630
SlewOC6    : ............................C.......................................... : 630
SlewOC12   : ............................C............C............................... : 630
SlewOC17   : ............................C.......................................... : 630
SlewOC25   : ............................C.......................................... : 630
SlewOC30   : ............................C.......................................... : 630
SlewOC38   : ..................................................................... : 630
SlewOC62   : ..................................................................... : 630
Slew110570 : ..................................................................... : 630
CrypOC5    : .C.........................C...........C.....................A........ : 630
SmokOC1    : ....................T........C.....................C........A........ : 630
SmokOC43   : ....................T........C.....................C........A........ : 630
SzygOC1    : ....G..............T.................................C.....A........ : 630
SzygOC2    : ....G..............T.................................C.....A........ : 630
SzygOC12   : ....G..............T.................................C.....A........ : 630
SzygOC22   : ....G..............T.................................C.....A........ : 630
SzygOC36   : ....G..............T.................................C.....A........ : 630
Stib31     : ........................C..C.............................A........ : 630
StibOC76   : ..............C..........C..C.............................A........ : 630
StibOC260  : ........................C..C.............................A........ : 630
StibOC232  : ..................................................................... : 630
StudOC1    : ..............................................T..............A........ : 630
EbloOC2    : ...................T.....C..C.............................A........ : 630
ScorOC1    : ........................C...........C.....................A..G..... : 630
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Appendix B continued

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ScorOC2    : .......................C..........C.....................A..G..... : 630
SmedOC1    : ............C............C.................C..C........A..G..... : 630


                   640
SlewOC1    : TCCAATCCTTTATCAACACTTA : 652
SlewOC6    : ........................ : 652
SlewOC12   : ........................ : 652
SlewOC17   : ........................ : 652
SlewOC25   : ........................ : 652
SlewOC30   : ........................ : 652
SlewOC38   : ........................ : 652
SlewOC62   : ........................ : 652
Slew110570 : ........................ : 652
CrypOC5    : ...........C......... : 652
SmokOC1    : ......T..........T... : 652
SmokOC43   : ......T..........T... : 652
SzygOC1    : ......T.............. : 652
SzygOC2    : ......T.............. : 652
SzygOC12   : ......T.............. : 652
SzygOC22   : ......T.............. : 652
SzygOC36   : ......T............T : 652
Stib31     : ................C.. : 652
StibOC76   : ................C.. : 652
StibOC260  : ................C.. : 652
StibOC232  : .....c.T..........C.. : 652
StudOC1    : C........C.....G...C.. : 652
EbloOC2    : ................T... : 652
ScorOC1    : ................C.. : 652
ScorOC2    : ................C.. : 652
SmedOC1    : C................C.. : 652
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Chapter 2

Global population genetic structure and comparative genetic diversity of the coral reef associated gray reef shark (*Carcharhinus amblyrhynchos*) assessed by mitochondrial control region sequences and nuclear microsatellite DNA analysis

Abstract

The gray reef shark (*Carcharhinus amblyrhynchos*) is an Indo-Pacific, coral reef associated species that likely plays an important role as apex predator in maintaining the integrity of coral reef ecosystems. Populations of this shark have declined substantially in some parts of its range due to over-fishing, with recent estimates suggesting a 17% decline per year on the Great Barrier Reef (GBR). Currently, there is no information on the population structure or genetic status of gray reef sharks to aid in their management and conservation. We assessed the genetic population structure and genetic diversity of this species by using complete mitochondrial control region sequences and 15 nuclear microsatellite markers. Gray reef shark samples (n=305) were obtained from 10 locations across the species' known longitudinal Indo-Pacific range: western Indian Ocean (Madagascar), eastern Indian Ocean (Cocos [Keeling] Islands, Andaman Sea, Indonesia, and western Australia), central Pacific (Hawaii, Palmyra Atoll, and Fanning Atoll), and southwestern Pacific (eastern Australia – Great Barrier Reef). The mitochondrial and nuclear marker data were concordant in most cases with population-based analysis showing significant overall structure (ϕ_{ST} = 0.27906 ($p<0.000$); F_{ST} = 0.071 ± 0.02), and significant pairwise genetic differentiation between nearly all of the

putative populations sampled (i.e., 9 of the 10 for mitochondrial and 8 of the 10 for nuclear markers). Individual-based analysis of microsatellite genotypes identified at least 5 populations. The concordant mitochondrial and nuclear marker results are consistent with a scenario of very low to no appreciable connectivity (gene flow) among most of the sampled locations, suggesting that natural repopulation of overfished regions by sharks from distant reefs is unlikely. The results also indicate that conservation of genetic diversity in gray reef sharks will require management measures on relatively local scales. Our findings of extensive genetic structuring suggests that a high level of genetic isolation is also likely to be the case in unsampled populations of this species.

Keywords: Gray reef shark, control region, microsatellites, population, connectivity

Introduction

Rapidly declining populations of many shark species worldwide due to overfishing and habitat degradation is now amply documented, resulting in widespread calls for urgent implementation of improved management and conservation measures (Baum et al. 2003; Myers et al. 2007; Dulvy et al. 2008; Hayes et al. 2009). A fundamental and long-standing paradigm of informed management and conservation of fishes is the requirement for robust knowledge about their population (stock) structure and genetic diversity (Hauser and Carvalho 2008). However there have been only a few studies on shark population structure to date (e.g., Heist and Gold 1999; Schrey and Heist 2003; Keeney et al. 2005; Duncan et al. 2006; Keeney and Heist 2006; Castro et al. 2007; Chapman et al. 2009), and none on sharks that are major components of coral reef ecosystems. Despite the importance of this information, research on shark population structure has been limited in part because many species that are heavily fished have extensive geographic distributions, making collection of spatially appropriate sample sizes for robust genetic analysis logistically difficult.

The gray reef shark (*Carcharhinus amblyrhynchos*) is a primarily coastal and insular, coral-reef habitat associated species endemic to the Indo-Pacific, with a longitudinal distribution ranging from the western Indian Ocean to the Central Pacific (Compagno et al. 2005). Its preferred habitat appears to be reef drop-offs around atolls and shallow lagoons (McKibben and Nelson 1986). Other preferred habitat of the gray reef shark includes clear and unpolluted water and unpopulated coastal areas (i.e. the northwestern Hawaii Islands (NWHI)) (Wetherbee et al. 1997). These sharks are one of the most common apex predators on Indo-Pacific reefs, and based on their large size (up

to 255 cm total length) and often high biomass in these habitats assumed to play a major ecological role in modulating coral reef community dynamics (Dulvy et al. 2004; Stevenson et al. 2007; Sandin et al. 2008).

The extent of gray reef shark fishing through most of their distribution is unknown. However, there is evidence of heavy exploitation on small islands in the Pacific Ocean, where their fins are increasingly found in local markets (D. McCauley, Stanford University, and C. Duffy, New Zealand Department of Conservation, personal communications). Comparative surveys of apex predators, including gray reef sharks, at human inhabited (experiencing fishing pressure) and uninhabited (minimal to no fishing pressure) Line Islands have shown a dramatic reduction in apex predators on reefs of the former (Stevenson et al. 2007; DeMartini et al. 2008). A recent survey of reef sharks inhabiting the Great Barrier Reef (GBR) in Australia suggested that gray reef (and whitetip reef) shark populations are declining most notably in "allowed fishing" zones where sharks are being fished directly off the reef (Robbins et al. 2006). The probability of continued population decline on the GBR was estimated at 100% for the gray reef shark with a median population decline of 17% per year (Robbins et al. 2006). This study also estimated that if current fishing pressure on the GBR continues, the abundance of gray reef sharks would be reduced to 0.1% in twenty years, and population rebound, would require fishing mortalities to be decreased to half its current level.

In contrast, some other islands and atolls in the Indo-Pacific including the uninhabited and un-fished NWHI and Palmyra Atoll still exhibit high levels of gray reef shark abundance (Freidlander and DeMartini 2002; Stevenson et al. 2007). It has been suggested that these relatively pristine coral reef ecosystems can serve as good baseline

models for gauging the comparative status of other coral reef ecosystems (Sandin et al. 2008). From this perspective, gray reef sharks in these pristine ecosystems may also provide a useful comparative context for genetic diversity assessment of this species relative to anthropogenically impacted ecosystems where they are known to have declined from overfishing and habitat degradation.

With gray reef shark populations declining in at least some portions of their distribution and concerns about the impact of apex predator removal on coral reefs, more information about gray reef shark population dynamics is necessary to aid in the formulation of effective conservation measures. In this study, we investigate the genetic population structure and comparative genetic diversity of the gray reef shark across much of its Indo-Pacific range.

Materials and Methods

Tissue samples (muscle or fin clips) were collected from a total of 305 gray reef sharks, from 2000 to 2008, encompassing most of the shark's known longitudinal geographic range (Figure 1). Samples were stored in 95% ethanol at room temperature until used for genetic analysis.

Laboratory Procedures

Genomic DNA for all genetic analyses was extracted from roughly 25mg of tissue using the QIAGEN Dneasy extraction kit (QIAGEN Inc, Valencia, California).

Mitochondrial genome control region locus sequencing

Polymerase chain reaction (PCR) amplification of the entire mitochondrial control region (mtCR) was performed using the primers CRF6 (5'AAGCGTCGACCTTGTAAGTC) (C. Testerman, unpublished) and DASR2 (5'GCTGAAACTTGCATGTGTAA) (V. Richards, unpublished). Reactions of 50µl consisted of 40µM dNTP's, 10x PCR buffer, 10pmol/µl of each primer, 10-25ng extracted DNA, and 1 unit of HotStart Taq DNA polymerase (QIAGEN Inc.). The PCR thermal profile included a denaturation step of 15min at 95°C, followed by amplification using 35 cycles at 94°C for 1 min, 50°C for 1min, 72°C for 2 min, and a final 5 min extension at 72°C in an iCycler thermocycler (Biorad, Hercules, California). All reactions were run with a negative control (master mix with no DNA) and the amplicons visualized on a 1.2% agarose gel. Amplicon purification was performed with the QIAquick PCR purification kit following manufacture's protocol (QIAGEN Inc.). Two gray reef shark internal sequencing primers were designed to achieve complete bi-directional coverage of the mtCR locus (GrRf531F – 5'CAAGAATGCCAGTCCTCTAGTT; GrRf862R – 5'TGCACTGTACACGCACTAT). Cycle sequencing was performed following standard ABI procedure using BigDye Terminator v3.1 (Applied Biosystems, Inc., Foster City, California). Cycle sequencing products were purified using DyeEx 2.0 Spin kits (QIAGEN Inc.). All sequencing was performed in-house on an ABI 3130 genetic analyzer (Applied Biosystems, Inc.).

Nuclear microsatellite genotyping

Fifteen nuclear genome microsatellite loci were used to assess population structure and genetic diversity. These microsatellite loci were developed by other

researchers for different carcharhinid shark species and optimized by us for use with gray reef sharks. The microsatellite loci used were from: Feldheim et al. (2001) [lemon shark; Ls11], Keeney and Heist (2003) [blacktip shark; Cli102, Cli103, Cli106], Portnoy et al. (2006) [sandbar shark; Cpl53, Cpl90, Cpl169], Ovenden et al. (2006) [spottail shark; Cs3, Cs8, Cs10, blacktip reef shark; Ct5, Ct6] and P. Prodöhl (unpublished) [blue shark; Pg2, Pg11, Pg13]. Forward primers were labeled with an M13 primer sequence (5'TGTAAAACGACGGCCAGT) attached to the 5' end and all microsatellite reactions included a matching, labeled M13 primer in 4 fluorescent dye colors (FAM, VIC, NED, PET) (Applied Biosystems, Inc.), with the exception of Pg2. Amplification was performed in 25µl reactions consisting of 40µM dNTP's, 10x PCR buffer, 25mM $MgCl_2$, 10pmol/µl forward, reverse and M13 primer, 10-25ng extracted DNA, and 1 unit of HotStart Taq DNA polymerase (QIAGEN Inc.). PCR thermal profiles consisted of an initial heating step of 95°C for 15 min, followed by 35 cycles at 94°C for 1 min, 54°C (Ct5), 56°C (Cli106), 58°C (Cs3, Cs8, Cs10, Ct6, Ls11, Pg11, and Pg13), or 60°C (Cli102, Cli103, Cpl53, Cpl90, Cpl169, and Pg2) for 1min, 72°C for 2 min and a final 5 min extension at 72°C in an iCycler thermocycler (Biorad). Microsatellite PCR products with different fluorescent dyes were pooled and genotyped with GENESCAN LIZ500 or LIZ600 (Pg2, Cs8, and Ct5) size standard (Applied Biosystems, Inc.). All microsatellites were genotyped on an ABI 3130 (Applied Biosystems, Inc.) and allele sizes scored using GENEMAPPER 3.0 (Applied Biosystems, Inc.).

Data Analysis

Mitochondrial DNA

Sequences were aligned using MacClade (Maddison and Maddison 1992) and edited manually. The number of unique haplotypes, haplotype diversity (*h*), nucleotide diversity (π), GC (%) content, and the ratio of transitions to transversions were calculated using DNASP v4.20.2 (Rozas et al. 2003) and MEGA v3.1 (Kumar et al. 2004). Within and among geographic sampling location diversity were calculated using analysis of molecular variance (AMOVA) performed in ARLEQUIN 2.0 (Schneider et al. 2000). Pairwise population ϕ_{ST} tests were implemented in ARLEQUIN 2.0 (Schneider et al. 2000) using the pairwise differences model of genetic distance, with significance determined by 10000 data permutations. To determine the influence of geographic distance on structuring of genetic populations, genetic isolation by distance between all sample sites was tested using the program IBDWS for both mitochondrial and nuclear data (Jensen et al. 2005). Geographic distances between sampling locations were calculated as the shortest distance around islands and landmasses.

Evolutionary relationships at the 95% confidence level among the gray reef shark mitochondrial sequence haplotypes were determined using statistical parsimony implemented in the program TCS v1.21 (Clement et al. 2000). To better visualize the evolutionary relationships, ambiguous loops were resolved using the criteria based on coalescent theory (Crandall and Templeton 1993). The criteria to resolve alternate statistical parsimony connections is given by Pfenninger and Posada (2002) and summarized as : 1) frequency criterion: haplotypes are more likely to be connected to haplotypes with a higher frequency than to singletons; 2) topological criterion: haplotypes are more likely to be connected to interior haplotypes than to tip haplotypes;

and 3) geographical criterion: haplotypes are more likely to be connected to haplotypes from the same population or a region than to haplotypes occurring in distant populations.

Microsatellite DNA

The Microsoft Excel toolkit add-in MS TOOLS (Park 2001) was used to estimate microsatellite summary statistics: unbiased heterozygosity (Nei 1987), observed heterozygosity, allele frequencies, and number of alleles per locus. The statistical power of the loci to detect population differentiation across all sample locations was assessed using the program POWSIM v4 (Ryman and Palm 2006). Simulations were run with a N_e=2000 and the number of generations (t) was varied for each run. MICRO-CHECKER v2.2.3 (Van Oosterhout et al. 2004) was used to check all geneotypes for null alleles, large allele dropout and scoring errors.

Deviations from Hardy-Weinberg equilibrium and population F-statistics (Weir and Cockerham 1984) for population differentiation both within loci and estimated between all loci was calculated in GENEPOP 3.4 (Raymond and Rousset 1995) and a sequential Bonferroni correction was applied to both to correct for multiple nominal tests (α=0.05, k=10, P<0.005) (Rice 1989). Overall F_{ST} estimate and its standard deviation and allelic richness were generated using FSTAT (Goudet 1995). Genetic differentiation for each population pair was assessed with 1000 dememorization steps, 100 batches and 1000 iterations, tested in GENEPOP 3.4 (Raymond and Rousset 1995).

As a complementary way to determine the most likely number of genetic populations (K) based on the microsatellite markers, we used the Bayesian model-based individual assignment program STRUCTURE v2.2 (Pritchard et al. 2000) which clusters

groups of individuals based on their multilocus genotypes and without using *a priori* defined putative populations. We used the program's default values (correlated allele frequencies and population admixture) with an initial burn-in of 100000 steps, 100000 Markov chain Monte Carlo steps and 10 iterations of each potential K (K =1-11) to assess convergence . Population structure was inferred by comparing the resulting log-likelihood values and the variance for each potential K.

Since sex-biased habitat segregation and female philopatry has been documented in some shark species (Hueter et al. 2005; Mucientes et al. 2008; Jorgenson et al. 2009) and even possibly in gray reef sharks (Wetherbee et al. 1997), we tested for sex-bias dispersal in gray reef sharks using the FSTAT program's biased dispersal option (Goudet 1995) where F_{ST} and F_{IS} indices were calculated. Given observations of comparatively low genetic diversity in the gray reef shark samples from the eastern Indian Ocean (western Australia) populations, we tested for signals of a potential genetic bottleneck using the program BOTTLENECK v1.2.02, incorporating the infinite allele model (I.A.M.) and the step-wise mutation model (S.M.M.) (Cornuet and Luikart 1997). All three default statistical tests were performed (sign test, standardized differences test, and Wilcoxon sign rank test) and the allele frequency distribution was assessed for an L-shaped distribution.

Results

Mitochondrial DNA

The gray reef shark complete mtCR sequence was 1065-1068 base pairs (bp) in length and had a GC content of 32.9%, comparable to other members of the family Carcharhinidae (Keeney et al. 2005). We identified 37 polymorphic sites and 58

haplotypes among the 305 gray reef shark mtCR sequences, with a transition to tranversion ratio of 4.18. Summary statistics for each population, including number of samples, number of haplotypes, haplotype diversity (*h*) and nucleotide diversity (π) are listed in Table 1. Overall, haplotype diversity was relatively high compared to other Carcharhinidae sharks (Duncan et al. 2006; Keeney and Heist. 2006; Schultz et al. 2008) at 0.94500±0.00003 and nucleotide diversity was 0.00699±0.00183. The overall ϕ_{ST} (i.e., over all sampling locations) was 0.27906 (P<0.000) with the majority of genetic variation occurring within populations (72.09%) as opposed to among populations (27.91%) (Table 5). However, all population pairwise ϕ_{ST} values were significantly different ($p<0.05$), except for the following four pairwise comparisons: Hawaii and Fanning Atoll (p=0.22156), Fanning Atoll and Palymra Atoll (p=0.81091), Fanning Atoll and the Cocos (Keeling) Islands (p=0.07217) and the north GBR and central GBR (p=0.21889) (Table 4). There was no genetic isolation by distance signal detected for the mtCR sequence data (P=0.2610, $r^2 = 0.0127$), (Figure 3).

Sample locations from the Indian Ocean, including the Andaman Sea, the Cocos (Keeling) Islands, Madagascar and western Australia (Scott Reef and Rowley Shoals), were distinct in that they exhibited unique haplotypes. With the exception of Rowley Shoals, these populations had the smallest collection effort and unique haplotypes could be an artifact of incomplete sampling. However, only one individual sampled in the Pacific shared a haplotype with the 84 total samples from the Indian Ocean, a reasonable sampling effort for the Indian Ocean collectively (Figure 2). The Cocos (Keeling) Islands possessed a single, unique haplotype and exhibited the highest overall average pairwise ϕ_{ST} values compared to other sampling locations (average $\phi_{ST} = 0.5054$,

P<0.0000) (Table 4). Madagascar and the Andaman Sea had four and 12 haplotypes respectively, that were not shared with any other location and were the most divergent in the TCS network (Figure 2). Scott Reef and Rowley Shoals populations also had the lowest overall haplotype diversity (*h*=0.385±0.01745 and *h*=0.440±0.00686, respectively), possibly indicative of a genetic bottleneck or founder effect. A sample from Rowley Shoals was the only Indian Ocean sample site to share a haplotype with the Pacific, specifically the north GBR, Australia, (Figure 2) that could represent a remnant of past gene flow.

In contrast, the Pacific Ocean sample location's haplotypes were generally shared between other Pacific Ocean populations. The ancestral haplotypes in the network (based on outgroup weighting from the program TCS) includes those sites from the Pacific Ocean and include locations from both the central and western Pacific. Fanning Atoll and Palmyra Atoll were not differentiated from each other based on a negative ϕ_{ST} value and a non-significant *p*-value. Fanning Atoll was also not differentiated from Hawaii and the Cocos (Keeling) Islands based on non-significant ϕ_{ST} values. Waples and Gaggiotti (2006) suggested a chain of non-significant values is equivalent to one continuous population, which is a possibility of what is occurring in the central Pacific between Hawaii, Fanning Atoll and Palmyra Atoll. However, it could also be an artifact of relatively low sample numbers in Hawaii (n=23) because the nuclear microsatellite data (discussed below) in contrast show Hawaii to be a significantly differentiated population from both Fanning and Palmyra Atoll. The non-significant ϕ_{ST} value between the Cocos (Keeling) Islands and Fanning Atoll is most likely an artifact of the very small sample

sizes available for the Cocos (Keeling) Islands. The non-significant ϕ_{ST} values between north and central GBR also indicated one genetic population along the GBR.

Microsatellite markers

The number of samples, number of alleles, allele size range, allelic richness, observed heterozygosity, expected heterozygosity, and deviation from Hardy-Weinberg equilibrium are shown in Table 2 and 3. The total number of alleles per locus ranged from 5 to 46 (mean = 20.2) and the overall F_{ST} value was 0.071±0.02. The power analysis revealed significant power to detect structure at an F_{ST} = 0.0025 with a 98.5% probability and an alpha (α) error at 6.2%. The power analysis was run again excluding the collection sites with low sample numbers (Madagascar, n=8 and the Cocos (Keeling) Islands, n=6) to ensure that populations with low sample numbers were not significantly decreasing the statistical power. Under the same run parameters, the analysis showed a similar result that an F_{ST} = 0.0025 had a probability of 99.4% (α error = 6.7%) for detecting that level of differentiation, only slightly higher than when including sample locations with low sample numbers. All pairwise F_{ST} values observed in our analyses were greater than 0.003, indicating that the collective microsatellite markers has sufficient power to detect population differentiation in our samples.

Some populations had individual loci out of Hardy-Weinberg equilibrium after sequential Bonferroni correction (initial P = 0.05), suggestive of null alleles (Table 2). However, loci and populations found to be out of Hardy-Weinberg were retained for the final population differentiation analysis because 1) no single locus or population fell consistently out of Hardy-Weinberg equilibrium, and 2) the population differentiation

results did not change whether these non-equilibrium loci and/or populations were included or excluded (results not shown).

All microsatellite pairwise F_{ST} values were significant after sequential Bonferroni correction expect those between the central GBR, Australia and Scott Reef (F_{ST} =0.0079, P=0.03749) and between Palmyra Atoll and Fanning Atoll (F_{ST} =0.0031, P=0.00183) (Table 4). The non-significant F_{ST} value between the central GBR and the Scott Reef sample location could be due to the small sample size from Scott Reef (n=14). Gene flow between the central GBR to western Australia is unlikely and biologically difficult without mixing with the northern GBR population.

Individual-based analysis of the multilocus genotypes by STRUCTURE identified five populations of gray reef sharks compared to eight populations based on the population-level F_{ST} analysis. The five populations identified by STRUCTURE were: 1) Hawaii, 2) Palmyra Atoll, Fanning Atoll, central GBR, northern GBR, Scott Reef, and the Cocos (Keeling) Islands, 3) Madagascar, 4) the Andaman Sea, and 5) Rowley Shoals. Latch et al. (2006) indicated that STRUCTURE may not accurately estimate the true value of K when F_{ST} values are below 0.03. Over 15% of the gray reef shark pairwise F_{ST} values were below this 0.03 threshold, which could indicate why STRUCTURE failed to concordantly identify every population found to be significantly differentiated based on the F_{ST} values.

In contrast to the mitochondrial sequence analysis, there was a significant signal of genetic isolation by distance in the microsatellite data (P=0.0160, r^2 = 0.106) (Figure 3), suggesting geographic distance could contribute to the observed genetic structure.

There was no significant signal of sex - biased dispersal based on FSAT's biased dispersal test (Goudet 1995). All p-values were greater than 0.05 for both the F_{ST} and F_{IS} tests. The program BOTTLENECK v1.2.02 (Cornuet and Luikart 1997) did not detect a genetic bottleneck with the microsatellite data. All populations had a normal L-shaped distribution and *p*-values were non-significant.

Discussion

The nuclear microsatellite genotypes and mitochondrial control region sequences show highly significant population structure in gray reef sharks in the Indo-Pacific. In most cases, there was a high degree of concordance in the results obtained from these bi-organelle markers, strongly supporting the delineation of animals sampled from many of the ten geographic locations as individual genetic populations (stocks), with low to no gene flow among them. However, there were also some absences of concordance between the two marker types for some putative populations. Possible demographic and behavioral factors resulting in such high levels of overall population structure in gray reef sharks, reasons for the discordance between nuclear and mitochondrial markers in a few cases, caveats associated with some of the results and management and conservation implications of these findings are discussed below.

The absence of a genetic isolation by distance signal in the mtCR data and only a weak (although significant) correlation in the microsatellite data points to geographic distance between populations not playing an appreciable role in the strong overall population structuring observed in gray reef sharks. For the mtCR data, populations that differed substantially in geographic distance between them had relatively equivalent Φ_{ST}

values. For example, despite huge differences in the distances between Fanning Atoll and Madagascar (16,558 km) and between Madagascar and the Andaman Sea (5,229 km) populations, the pairwise Φ_{ST} values were similar (0.38391 and 0.34473, respectively). The absence of a mtCR isolation by distance signal is consistent with the emerging evidence suggesting that female gray reef sharks have relatively small home ranges (see below). It is unclear what to make of the small, but statistically significant signal of genetic isolation by distance in the nuclear microsatellite data. It is possible that this finding reflects dispersal behavior differences in female versus male gray reef sharks. Although both sexes do not migrate long distances (see below), males may disperse longer distances than females, resulting in sufficient male-mediated gene flow among relatively close reefs to provide a significant isolation by distance signal. Given the small correlation between genetic and geographic distance, however, this inference is necessarily tentative. Resolution of this issue will best be achieved by comparative analysis of gray reef shark populations along a long but more linearly defined gradient of coral reef habitats.

The large degree of genetic structure in gray reef sharks could result from their ecological and behavioral patterns as highly coral reef-associated species. Stomach contents analysis of gray reef sharks in Hawaii suggests their diet is comprised mostly of teleosts that are reef dwelling and found in shallow water (Papastamatiou et al. 2006). McKibben and Nelson (1986) showed that gray reef sharks do not appear to leave the reef or atoll that they inhabit and have a small overall activity space. The observed distance traveled for gray reef sharks at Enewetak, Marshall Islands was 0.20km – 16km (McKibben and Nelson 1986). This distance is much smaller compared to the home

range of a closely related and similar-sized, non-coral reef associated species such as the blacktip shark (*Carcharhinus limbatus*), that has been observed to migrate up to 1,865km (Kohler et al. 1998), and exhibits a lesser degree of genetic population structuring (Keeney et al. 2005).

Another behavioral pattern that could contribute to the high genetic structuring in gray reef sharks is their apparent tendency to form predictable and large aggregations in the central Pacific starting in March and occurring through June on a biennial pattern (Economakis and Lobel 1998). Some aggregations observed were as large as 160 individuals and were around for the daylight hours. Economakis and Lobel (1998) suggested that water temperature coincided with the maximum number of sharks observed each day. Some reasons given for this aggregation behavior include a pre-pupping ritual in which females use the warmth to aid in embryo development or accelerate female growth rate, or as a refuge from males during the reproductive season (Economakis and Lobel 1998). Most importantly, the same individuals returned to the aggregation on multiple days, suggesting a high degree of behavioral site fidelity by gray reef sharks.

All these behaviors point to a small home territory range of the gray reef shark (McKibben and Nelson 1986). The behavior and biology of gray reef sharks suggesting high site fidelity, shallow water preference and strong coral reef association suggest that gray reef shark movements are typically limited to short distances only. The strong genetic structuring we observed in both mtCR and microsatellite data is consistent with low gene flow among populations resulting from limited physical movements of individuals.

It has been shown in other sharks, like the lemon and blacktip sharks (Feldheim et al. 2001; Keeney et al. 2005), that females exhibit site fidelity and utilize shallower nursery grounds for parturition, whereas males disperse over longer distances. In the gray reef shark, evidence of sexual habitat segregation is indicated by males typically occupying deeper water, while females more often utilize inshore habitat (Wetherbee et al. 1997). The relative differences in population differentiation seen in our mitochondrial and microsatellite results could superficially be used to infer sexual segregation and female philopatry as the microsatellite data define fewer genetically significant populations than the mtCR data and provide a positive signal of isolation by distance for male-mediated gene flow. A statistical test for sex-biased dispersal, however, did not indicate any support for this hypothesis. It should be noted, however, that sex-biased dispersal would have to be intense in order for detection by statistical means (Goudet et al. 2002). Also, we did not have complete sex data for all sampled individuals and some populations were devoid of any sex information entirely based on method of collection in certain locations (i.e. Fanning Atoll samples came from a local market in which only the shark fins are sold). Lacking sufficient samples with sex data could give an incomplete picture of dispersal and skew the results. Since gray reef sharks are highly structured also for microsatellite markers, males also likely have low dispersal distances, making it difficult to statistically detect sex-biased dispersal even though it may nominally exist. Similar to the gray reef shark, the sand tiger shark (*Carcharias taurus*) demonstrated limited gene flow with both mitochondrial and nuclear microsatellite data, with disjunct distributions separated by large expanses of deep, open-ocean suggested as the main factors limiting male and female gene flow (Ahonen et al. 2009). Lack of concordance

between mitochondrial and nuclear data sets can also occur due to the differences in the mode of inheritance between mitochondrial and nuclear genes. Mitochondrial genes have an effective population size that is four times smaller then the effective population size of nuclear genes, causing populations to demonstrate more subdivision at mitochondrial genes (Birky et al. 1989).

Population genetic diversity comparisons

Gray reef sharks from western Australia (Scott Reef and Rowley Shoals) exhibited the lowest overall mitochondrial haplotype diversity values, even with a reasonable sample size examined (total N=56 animals). This comparatively low mitochondrial genetic diversity can be indicative of a genetic bottleneck event (Bouzat et al. 1998) possibly caused by past overfishing or a relatively recent founder effect. Tests for a genetic bottleneck using the microsatellite data from these animals, however, did not show any statistical evidence for this phenomenon. In fact, microsatellite based genetic diversity did not appear correspondingly low in sharks from this region (Table 2) Keeney et al. (2005) suggested that because microsatellites have a high mutation rate male dispersal after a bottleneck can replenish nuclear diversity faster than mitochondrial diversity.

The TCS network suggested ancestral haplotypes comprising samples mostly from the central Pacific (Hawaii and Fanning Atoll). This ancestral position in the network and the comparatively high genetic diversity observed in the central and western Pacific animals is suggestive of the Pacific as the evolutionary origin for gray reef sharks. Radiation from the central Pacific into the western Pacific and Indian Ocean likely

occurred via the geographically proximal islands and atolls in oceania and the continental shorelines suggesting a stepping stone model of dispersal (Kimura and Weiss 1964). Interestingly, the TCS analysis suggests that the haplotypes from the Andaman Sea, Madagascar, western Australia and the Cocos (Keeling) Islands are more closely related to Pacific Ocean haplotypes then they are to each other, despite all of them being sampled in Indian Ocean locations. One possible explanation for these evolutionary relationships is that the Indian Ocean might have been colonized by independent waves of gray reef sharks entering and utilizing different migratory pathways, either via the Indonesian archipelago or along northern Australia.

Management and conservation implications

Knowledge of the degree of genetic structure in any exploited marine species is a necessary prerequisite for informed management and conservation measures, including the establishment of protective marine reserves, no-take zones or allowed fishing zones. The strong genetic structuring observed in gray reef sharks with almost every one of the ten sampling locations being significantly genetically differentiated from each other supports recognition and implementation of independent management measures on local scales. Eight of the ten sampling locations for gray reef sharks were their own significant population based on the mitochondrial DNA analysis. At the minimum, based on results of the microsatellite, individual-based STRUCTURE analysis, the ten sampling locations should be considered to comprise of at least five genetically distinct populations, each deserving of independent management focus. Overall, the low to no gene flow scenario

between populations suggests that gray reef shark replenishment of overfished reefs is not likely to occur from adjoining reefs as there is little connectivity between populations.

Although the imminent decline of the gray reef shark throughout its distribution is unlikely considering it is one of the most abundant sharks on Indo-Pacific reefs, accumulating new information indicating that this species is being overfished in some regions opens the possibility that it is also being overfished in many other parts of its range. Furthermore, well documented declines in coral reef habitats in the Indo-Pacific (Bruno and Selig 2007) due to habitat degradation from human development and now climate change impacts may also be negatively impacting the population status of gray reef sharks. We suggest that preservation of genetic diversity in gray reef sharks and their adaptability (and therefore resiliency) to ongoing climate change will require that the high degree of genetic structuring documented here be incorporated into management measures, including potential implementation of fishing prohibitions in regions showing a high degree of genetic isolation and ongoing population declines.

Finally, we note that we only examined gray reef shark populations from scattered parts of the species' overall distribution. However, the very high degree of population genetic structuring observed is strongly suggestive that unsampled populations of this species will likely display similarly high degree of population isolation. A comprehensive picture of population structure in this charismatic and ecologically important coral reef apex predator will require further sampling and investigation. The genetic markers and first assessment provided here provides a useful foundation for such future studies

Table 1. Gray Reef mtCR summary statistics: N, sample size; *n*, number of haplotypes; *h*, haplotype diversity; SD, standard deviation; π, nucleotide diversity

Geographical Location	Population	N	*n*	*h* ± SD	π ± SD
Central Pacific Ocean	Fanning Atoll	76	24	0.938 ± 0.01100	0.00371 ± 0.00023
	Hawaii	23	5	0.692 ± 0.00495	0.00168 ± 0.00017
	Palmyra Atoll	47	15	0.871 ± 0.03600	0.00350 ± 0.00031
Southwestern Pacific Ocean	Central Great Barrier Reef	30	8	0.651 ± 0.08600	0.00241 ± 0.00027
	North Great Barrier Reef	29	13	0.865 ± 0.05200	0.00309 ± 0.00039
Eastern Indian Ocean	Andaman Sea, Indonesia	21	12	0.933 ± 0.03100	0.00420 ± 0.00036
	Cocos (Keeling) Islands	6	1	0.000 ± 0.00000	0.00000 ± 0.00000
	Scott Reef	13	2	0.385 ± 0.01745	0.00216 ± 0.0000006
	Rowley Shoals	36	4	0.440 ± 0.00686	0.0065 ± 0.0000001
Western Indian Ocean	Madagascar	8	4	0.536 ± 0.12300	0.00453 ± 0.00104
	Overall	289	58	0.942 ± 0.00003	0.00447 ± 0.00014

Table 2. Gray reef shark microsatellite summary statistics: N, Number of samples; N_A, Number of alleles; A_{SR}, Allele size range (in base pairs); A_R, Allelic Richness; H_E, Expected heterozygosity; H_{obs}, Observed heterozygosity; HW, Hardy-Weinberg P-values, *indicates value is not in Hardy-Weinberg equilibrium after sequential Bonferroni correction (P<0.05).

Locus	Locations	Fanning Atoll	Hawaii	Palmyra Atoll	Central GBR	North GBR	Andaman Sea	Cocos Islands	Scott Reef	Rowley Shoals	Madagascar	Total
Cs3	N	67	22	43	23	32	21	6	6	36	8	264
	N_A	4	3	3	4	4	5	3	1	3	1	
	A_{SR}	390-400	390-400	390-400	390-400	390-398	386-402	390-398	396	386-390	396	
	A_R	1.358	1.648	1.579	1.418	1.457	2.501	1.667	1.000	1.513	1.000	
	H_{exp}	0.1801	0.3203	0.2881	0.2048	0.2287	0.6818	0.3182	-	0.2664	-	
	H_{obs}	0.1791	0.3636	0.2791	0.1304	0.1250	0.0952	0.3333	-	0.1389	-	
	HW	0.1654	1.0000	0.5113	0.2118	0.0244	0.0000*	1.0000	-	0.0086	-	
Cs8	N	67	23	47	25	35	21	6	14	36	7	281
	N_A	22	9	23	21	21	17	10	18	25	6	
	A_{SR}	259-307	273-299	259-305	255-299	255-303	265-327	265-301	263-305	253-307	269-313	
	A_R	3.705	2.947	3.702	3.713	3.719	3.652	3.818	3.769	3.755	2.633	
	H_{exp}	0.9491	0.7874	0.9483	0.9502	0.9516	0.9384	0.9697	0.9603	0.9577	0.6813	
	H_{obs}	0.8657	0.7826	0.9362	0.8400	0.9429	1.0000	0.8333	0.9286	0.9444	0.5714	
	HW	0.0068	0.8681	0.8091	0.0091	0.8255	1.0000	0.1666	0.6154	0.986	0.2838	
Cs10	N	72	22	47	17	35	20	6	14	35	7	275
	N_A	23	16	18	19	22	20	9	14	19	10	
	A_{SR}	366-487	366-440	370-496	364-444	364-487	366-458	370-444	365-433	365-478	406-432	
	A_R	3.620	3.519	3.522	3.770	3.647	3.681	3.655	3.651	3.485	3.626	
	H_{exp}	0.9331	0.9112	0.9151	0.9608	0.9383	0.9436	0.9394	0.9392	0.906418	0.9341	
	H_{obs}	0.7361	0.7273	0.7021	0.8236	0.6571	1.0000	1.0000	0.7857	0.8857	0.7143	
	HW	0.0000*	0.0026*	0.0032*	0.0432	0.0000*	0.4083	1.0000	0.0038*	0.6863	0.0655	
Ct5	N	57	21	44	30	31	21	6	14	35	7	266
	N_A	18	6	15	16	18	13	5	16	19	9	

	A_{SR}	231-287	241-265	241-289	225-277	225-307	225-271	247-269	225-287	235-277	241-257	
	A_R	3.273	2.248	3.442	3.422	3.453	3.394	2.990	3.709	3.563	3.615	
	H_{exp}	0.8443	0.5842	0.8981	0.8927	0.8995	0.8897	0.8030	0.9497	0.9222	0.9341	
	H_{obs}	0.6842	0.5238	0.7045	0.7000	0.7742	0.9048	0.8333	0.9286	0.8571	1.0000	
	HW	0.0000*	0.1318	0.0006*	0.0000*	0.0080	0.9980	1.0000	0.344	0.1818	1.0000	
Ct6	N	73	22	47	30	32	21	3	14	35	7	284
	N_A	6	5	6	4	7	4	3	4	5	3	
	A_{SR}	300-328	290-328	300-330	322-328	300-328	320-326	322-326	322-328	320-328	302-326	
	A_R	1.934	1.868	1.734	1.718	2.142	2.099	2.333	1.977	2.268	1.667	
	H_{exp}	0.4547	0.4186	0.3523	0.3486	0.5233	0.5424	0.6000	0.4683	0.5797	0.2727	
	H_{obs}	0.4384	0.5000	0.3404	0.4000	0.4063	0.2857	0.6667	0.5714	0.5143	0.2857	
	HW	0.2401	1.0000	0.7347	1.0000	0.0209	0.007	1.0000	1.0000	0.1999	1.0000	
Pg2	N	75	22	47	33	35	21	6	14	36	6	295
	N_A	7	3	7	6	6	5	4	6	7	4	
	A_{SR}	132-150	138-144	132-150	132-150	132-150	132-150	135-144	126-144	132-150	132-141	
	A_R	2.834	2.220	2.709	2.627	2.645	2.470	2.475	2.671	2.676	2.982	
	H_{exp}	0.7644	0.5909	0.7280	0.7110	0.7155	0.6585	0.6515	0.7249	0.7136	0.8182	
	H_{obs}	0.6133	0.6818	0.7021	0.5152	0.7143	0.6190	0.6667	0.5714	0.5278	0.8333	
	HW	0.0385	1.0000	0.4807	0.0318	0.5887	0.2739	0.5177	0.3129	0.0026*	0.8764	
Pg11	N	75	22	47	28	34	21	6	14	36	8	291
	N_A	12	6	9	10	11	7	4	8	9	2	
	A_{SR}	142-164	150-162	144-168	144-168	144-168	130-160	140-160	144-160	144-160	140-148	
	A_R	3.016	2.747	3.017	3.169	3.266	2.366	2.925	3.188	3.211	1.727	
	H_{exp}	0.8043	0.7505	0.8014	0.8390	0.8635	0.6098	0.8030	0.8492	0.8533	0.4000	
	H_{obs}	0.8133	0.5455	0.7872	0.9286	0.7941	0.7143	0.8333	0.9286	0.7778	0.2500	
	HW	0.3142	0.0509	0.4936	0.6197	0.4183	0.4161	0.7466	0.9925	0.6545	0.3853	

Pg13	N	76	23	47	23	35	21	6	14	36	8	289
	N_A	28	14	29	19	23	19	7	18	25	11	
	A_{SR}	192-254	202-252	192-256	196-248	192-254	212-264	204-250	196-242	196-262	216-256	
	A_R	3.739	3.286	3.768	3.730	3.745	3.737	3.400	3.784	3.782	3.628	
	H_{exp}	0.9549	0.8638	0.9600	0.9536	0.9561	0.9547	0.8939	0.9630	0.9628	0.9333	
	H_{obs}	0.9342	0.8697	0.9574	0.8696	0.8857	1.0000	1.0000	0.8571	0.9444	1.0000	
	HW	0.9138	0.184	0.2016	0.0209	0.0679	0.8678	0.7099	0.1605	0.2612	1.0000	
Cpl53	N	72	21	47	27	34	20	6	14	35	6	282
	N_A	36	15	24	23	26	21	4	23	24	7	
	A_{SR}	191-275	207-259	191-265	155-269	155-261	197-269	155-239	155-275	155-258	235-261	
	A_R	3.758	3.613	3.735	3.752	3.760	3.735	2.925	3.906	3.715	3.473	
	H_{exp}	0.9582	0.9326	0.9545	0.9574	0.9587	0.9534	0.8030	0.9841	0.9507	0.9091	
	H_{obs}	0.9444	0.8095	0.8085	0.9260	0.7941	0.8500	0.5000	0.9841	0.8571	0.8333	
	HW	0.0966	0.4024	0.0000*	0.3039	0.0209	0.2706	0.4234	1.0000	0.3852	0.561	
Cpl90	N	76	22	47	31	32	20	3	14	36	6	287
	N_A	8	6	8	8	8	7	2	8	9	3	
	A_{SR}	233-263	233-263	233-263	233-263	233-263	235-247	239-241	235-263	239-267	237-241	
	A_R	3.084	2.788	3.108	2.967	3.056	2.972	2.000	3.298	3.171	2.329	
	H_{exp}	0.8251	0.7558	0.8302	0.7970	0.8466	0.7987	0.6000	0.8730	0.8443	0.5185	
	H_{obs}	0.7763	0.7273	0.7234	0.7097	0.8750	0.6000	0.3333	0.9286	0.7222	0.5000	
	HW	0.899	0.5223	0.0687	0.2055	0.2708	0.0558	1.0000	0.9861	0.0000*	1.0000	
Cpl169	N	75	22	47	30	35	21	6	14	36	8	294
	N_A	29	10	26	24	24	13	6	16	25	4	
	A_{SR}	122-192	126-184	122-186	126-200	126-184	122-184	128-178	122-182	120-194	132-176	
	A_R	3.601	3.307	3.710	3.791	3.664	2.310	3.180	3.780	3.737	1.949	
	H_{exp}	0.9273	0.8721	0.9497	0.9644	0.9408	0.5587	0.8485	0.9630	0.9542	0.4417	
	H_{obs}	0.8267	0.7273	0.9362	0.9000	0.8000	0.5714	0.3333	0.9286	0.9444	0.5000	

	HW	0.0125	0.0161	0.1035	0.2003	0.0026*	0.4889	0.0026*	0.5622	0.1053	1.0000	
Cli102	N	73	22	45	25	27	21	2	14	36	4	269
	N_A	4	3	5	4	4	5	2	4	5	1	
	A_{SR}	134-142	134-142	130-142	134-142	134-142	130-142	138-140	134-142	130-142	140	
	A_R	1.968	1.569	2.098	1.807	2.138	2.238	2.000	2.209	2.609	1.000	
	H_{exp}	0.4576	0.2844	0.5036	0.3829	0.5374	0.5610	0.5000	0.5582	0.6999	-	
	H_{obs}	0.3151	0.3182	0.3556	0.2800	0.2963	0.2857	0.5000	0.5000	0.7778	-	
	HW	0.0000*	1.0000	0.0001*	0.0176	0.0000*	0.0036*	-	0.4708	0.5621	-	
Cli103	N	75	22	45	24	27	20	2	14	36	4	269
	N_A	4	3	5	4	3	4	2	4	5	2	
	A_{SR}	113-131	113-131	113-133	113-133	113-131	121-131	129-131	113-131	113-135	129-131	
	A_R	2.205	2.043	2.201	1.859	2.206	2.032	2.000	2.257	2.348	1.500	
	H_{exp}	0.5984	0.5539	0.5950	0.4441	0.6010	0.5013	0.5000	0.6164	0.6405	0.2500	
	H_{obs}	0.5333	0.5909	0.6222	0.4583	0.7037	0.5000	0.5000	0.5714	0.4722	0.2500	
	HW	0.0879	1.0000	0.3225	0.6011	0.7217	0.1442	-	1.0000	0.1593	-	
Cli106	N	76	19	44	30	29	21	3	14	36	5	277
	N_A	7	3	5	7	7	7	2	6	7	2	
	A_{SR}	195-209	197-203	197-211	197-209	193-211	197-209	197-203	197-209	197-209	197-203	
	A_R	2.724	2.440	2.738	2.573	2.645	2.679	1.933	2.522	2.709	1.952	
	H_{exp}	0.7376	0.6785	0.7474	0.6847	0.7181	0.7247	0.5333	0.6534	0.7387	0.5556	
	H_{obs}	0.5789	0.6316	0.7727	0.6333	0.7586	0.7619	0.6667	0.5714	0.7778	0.6000	
	HW	0.0094	0.4269	0.5122	0.1548	0.3402	0.0921	1.0000	0.1687	0.8106	1.0000	
Ls11	N	75	22	47	33	35	21	6	14	36	7	296
	N_A	7	4	6	6	6	5	4	4	7	3	
	A_{SR}	242-254	242-252	242-254	242-252	242-252	248-256	244-252	244-252	246-258	248-252	
	A_R	2.874	2.426	2.873	2.665	2.800	2.537	2.754	2.560	2.965	1.791	

	H_{exp}	0.7777	0.6617	0.7758	0.7156	0.7594	0.6725	0.7576	0.6958	0.8013	0.3846	
	H_{obs}	0.8000	0.5909	0.6596	0.6061	0.7429	0.4762	0.6667	0.5714	0.8333	0.4286	
	HW	0.2277	0.5843	0.3168	0.4446	0.5589	0.0064	0.1936	0.2092	0.7565	1.0000	

Table 3. Gray reef shark microsatellite summary statistics for all loci combined: N_A, Number of alleles; A_R, Average allelic richness; H_{exp}, Expected heterozygosity; H_{obs}, Observed heterozygosity

	Fanning Atoll	Hawaii	Palmyra Atoll	Central GBR	North GBR	Andaman Sea	Cocos Islands	Scott Reef	Rowley Shoals	Madagascar
N_A	215	106	189	175	190	152	67	150	194	68
A_R	2.9129	2.5779	2.9291	2.8654	2.9562	2.8269	2.6703	2.9521	3.0333	2.3248
H_{exp}	0.7445	0.6644	0.7498	0.7205	0.7626	0.7326	0.7014	0.7999	0.7861	0.6179
H_{obs}	0.6693	0.6260	0.6858	0.6481	0.6847	0.6443	0.6444	0.7591	0.7317	0.5974

Table 4. Microsatellite F_{st} values (above diagonal) and mtCR Φ_{st} values (below diagonal). Non-significant values are in bold (F_{st} values: P>0.005 after sequential Bonefferoni correction; Φ_{st} values: P>0.05).

	Fanning Atoll	Hawaii	Palmyra Atoll	Central GBR	North GBR	Andaman Sea	Cocos Islands	Scott Reef	Rowley Shoals	Madagascar
Fanning Atoll		0.0483	**0.0031**	0.0129	0.0094	0.1112	0.0676	0.0084	0.1239	0.1571
Hawaii	**0.0304**		0.0410	0.0502	0.0479	0.1408	0.1327	0.0702	0.1629	0.1935
Palmyra Atoll	**-0.0066**	0.1275		0.0120	0.0034	0.0997	0.0587	0.0110	0.1248	0.1520
Central GBR	0.0638	0.2200	0.2011		0.0173	0.1207	0.0933	**0.0079**	0.1331	0.1749
North GBR	0.0377	0.1774	0.1305	**0.0120**		0.0963	0.0404	0.0071	0.1138	0.1401
Andaman Sea	0.1888	0.5246	0.3807	0.4948	0.4405		0.1145	0.1002	0.1104	0.1019
Cocos Islands	**0.1364**	0.7045	0.3740	0.6527	0.5340	0.4837		0.0694	0.1228	0.2013
Scott Reef	0.1496	0.5815	0.3555	0.5655	0.4486	0.5537	0.5743		0.1163	0.1740
Rowley Shoals	0.2775	0.7600	0.5361	0.7328	0.6525	0.7133	0.7542	0.1229		0.2056
Madagascar	0.3839	0.5393	0.5393	0.5390	0.5031	0.3447	0.2751	0.4125	0.6359	

Table 5. AMOVA results

Source of Variation	Degrees of Freedom	Sum of Squares	Variance Components	Percentage of Variation
Among Populations	9	292.698	1.08228 Va	27.91
Within Populations	280	782.906	2.79609 Vb	72.09
Total	289	1075.603	3.87837	

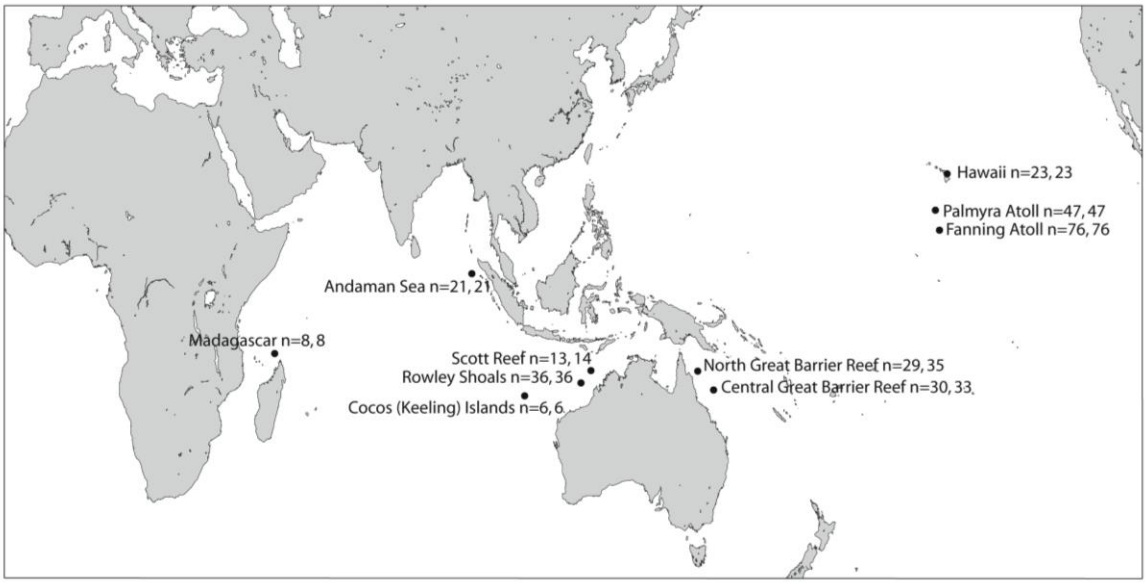

Figure 1. Map displaying sample locations and sample numbers of gray reef sharks. The number of individuals sequenced (mt DNA) and genotyped (microsatellites) are indicated respectively by the two n values at each location.

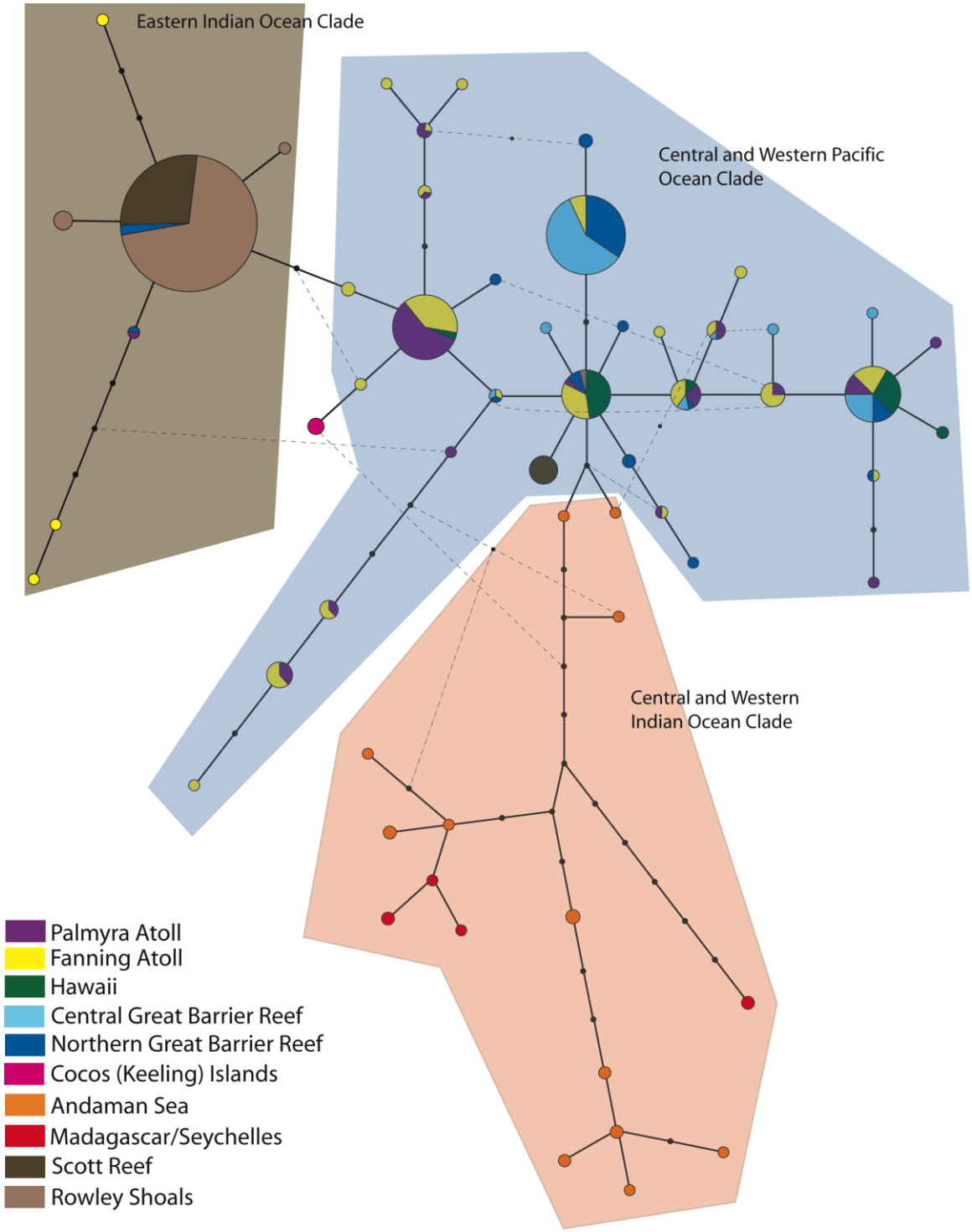

Figure 2. TCS network (95%) of gray reef shark haplotypes. Black dots indicate theoretical un-sampled haplotypes. The size of each haplotype is equivalent to the frequency of that haplotype in the population. Dashed lines are alternative connections.

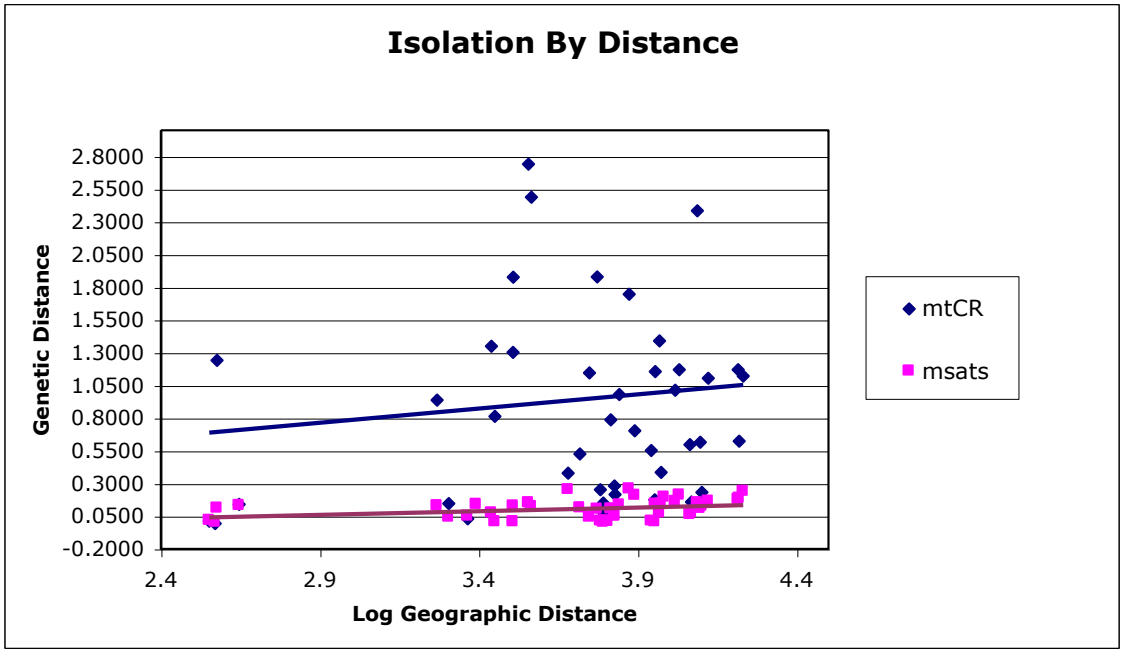

Figure 3. Isolation by distance plot for mtCR (P=0.2610, r^2=0.0127) and microsatellite data (P=0.0160, r^2=0.106).

Appendix C. Alignment of entire CR haplotype sequences. Dots indicate identical sequence to top sequence. OC is the identifying number for each haplotype.

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                        20                  40                  60
OC01  : ATTGCTATTATAGCGAATAAATGCTGAATTATAAAAATTCAGTTTTTGTACGCCAGAGTGACATATTAAT :   70
OC03  : ..........................................................................:   70
OC10  : ..........................................................................:   70
OC13  : ..........................................................................:   70
OC19  : ..........................................................................:   70
OC23  : ..........................................................................:   70
OC32  : ..........................................................................:   70
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OC64  : ..........................................................................:   70
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OC66  : ..........................................................................:   70
OC67  : ..........................................................T...............:   70
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OC250 : ..........................................................................:   70
OC273 : ..........................................................................:   70
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OC293 : ..........................................................................:   70
OC294 : ..........................................................................:   70
OC295 : ..........................................................................:   70
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OC301 : ..........................................................................:   70
OC302 : ..........................................................................:   70
OC303 : ..........................................................................:   70
OC304 : ..........................................................................:   70
OC305 : ..........................................................................:   70
OC306 : ..........................................................................:   70
OC325 : ..........................................................................:   70
OC352 : ..........................................................................:   70
OC353 : ..........................................................................:   70
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Appendix C continued

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OC360 : ..............................................................  :   70


                80                  100                 120                 140
OC01  : GATATAGTCCACATACCTTAATATACCACATAATACCCTCATTCCATAATAGCTATAACAGATATTATAC :  140
OC03  : ..............................................................  :  140
OC10  : ..............................................................  :  140
OC13  : ..............................................................  :  140
OC19  : ..............................................................  :  140
OC23  : ..............................................................  :  140
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OC215 : .......C......................................................  :  140
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OC306 : ..............................................................  :  140
OC325 : ..............................................................  :  140
OC352 : ..............................................................  :  140
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Appendix C continued

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OC353 : ..................................................................... :  140
OC360 : ..................................................................... :  140


                      160                 180                 200
OC01  : TACCTCATTACATATACTATGCTTAATCCTCATTAGTCGATATTCCACTATATCATTACATACTATGCTT :  210
OC03  : ..................................................................... :  210
OC10  : ..................................................................... :  210
OC13  : ..................................................................... :  210
OC19  : ..................................................................... :  210
OC23  : ...T.......................C..................................... :  210
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OC67  : ...T.......................C..................................... :  210
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OC303 : ............................C.....................................T.. :  210
OC304 : ............................C.....................................T.. :  210
OC305 : ...T.................................................................. :  210
OC306 : ...T.................................................................. :  210
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Appendix C continued

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OC325 : ...T.......................................................... :  210
OC352 : .............................................................. :  210
OC353 : ...T.......................C.................................. :  210
OC360 : ...T.......................C.................................. :  210


              220                 240                 260                 280
OC01  : AATCCACATTAGTCTACTGTCAGCTATTTCATTTCATTAAATTATTTAACCCTCATTAATCTATAATCAA :  280
OC03  : .............................................................. :  280
OC10  : .............................................................. :  280
OC13  : .............................................................G :  280
OC19  : .............................................................. :  280
OC23  : ...........A.................................................. :  280
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OC78  : .............................................................G :  280
OC79  : ...........A.................................................G :  280
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OC304 : ...........A.................................................. :  280
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Appendix C continued

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OC305 : ..........A.......................................................... :  280
OC306 : ..............................................................G :  280
OC325 : ..............................................................G :  280
OC352 : ..................................................................... :  280
OC353 : ..........A.......................................................... :  280
OC360 : ..........A.......................................................... :  280


                        300                 320                 340
OC01  : TAATTTCATAGCATAATATTTTTCACTTAACCCTACTTTACATGGTATTATTTAATGCCGTTGGTAAGAA :  350
OC03  : ..................................................................... :  350
OC10  : ..................................................................... :  350
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OC19  : ..................................................................... :  350
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OC295 : ..........................................A.......................... :  350
OC300 : .....C................................................................ :  350
OC301 : ..........................................A.......................... :  350
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Appendix C continued

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OC303 : .....C.....................................................................  :  350
OC304 : .....C.....................................................................  :  350
OC305 : ..........................................A..................................  :  350
OC306 : .....................................................................         :  350
OC325 : .....................................................................         :  350
OC352 : ..........................................A..................................  :  350
OC353 : ..........................................A..................................  :  350
OC360 : ..........................................A..................................  :  350


              360                 380                 400                 420
OC01  : ACCCCCATTAACCTAATAAATGAAAAAAATTGTACGGTTTGTGGTACATTACTGTTTTATCCCCTACTAT :  420
OC03  : .T.....................................................................  :  420
OC10  : .T.....................................................................  :  420
OC13  : .T.....................................................................  :  420
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OC295 : .T.....................................................................T...  :  420
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OC305 : .T.....................................................................  :  420
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OC325 : .T.....................................................................  :  420
OC352 : .T.....................................................................  :  420
OC353 : .T.....................................................................  :  420
OC360 : .T.....................................................................  :  420


                        440                 460                 480
OC01  : TGATCAAAACTGACATTTGATTATGGTTGAACTTCATATAATCCTTGATCGTATCAAGAATGCCAGTCCT  :  490
OC03  : .......................................................................  :  490
OC10  : .......................................................................  :  490
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OC352 : ..........................................................................  :  490
OC353 : ............................G.............................................  :  490
OC360 : ..........................................................................  :  490


             500                 520                 540                 560
OC01  : CTAGTTCCCTTTAATGGCATATTTATCCTTGATCGTCTCAAGATTTATCTTCCGCCCTGTTTTTTTAGTT  :  560
OC03  : ..........................................................................  :  560
OC10  : ..........................................................................  :  560
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OC295 : ..................................................................... :  560
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OC360 : .................. : 1068
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Acknowledgements

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References

Abercrombie DL, Clarke SC, Shivji MS (2005) Global-scale genetic identification of hammerhead sharks: Application to assessment of the international fin trade and law enforcement. *Conservation Genetics*, **6**, 775-788.

Ahonen H, Harcourt RG, Stow AJ (2009) Nuclear and mitochondrial DNA reveals isolation of imperiled grey nurse shark populations (*Carcharias taurus*). *Molecular Ecology*, **18**, 4409-4421.

Baum JK, Myers RA, Kehler DG, Worm B, Harley SJ, Doherty PA (2003) Collapse and conservation of shark populations in the Northwest Atlantic. *Science*, **299**, 389-392.

Birky CW, Fuerst P, Maruyama T (1989) Organelle gene diversity under migration, mutation, and drift: Equilibrium expectations, approach to equilibrium, effects of heteroplasmic cells, and comparison to nuclear genes. *Genetics*, **121**, 613-627.

Bouzat JL, Cheng HH, Lewin HA, Westemeier RL, Brawn JD, Paige DN (1998) Genetic evaluation of a demographic bottleneck in the greater prairie chicken. *Conservation Biology*, **12,** 836-843.

Bruno JF, Selig ER (2007) Regional decline of coral cover in the Indo-Pacific: Timing, extent, and subregional comparisons. *Plos One*, 2.

Castro JI, Woodley CM, Brudeck RL (1999) A preliminary evaluation of the status of shark species. In: FAO Fisheries Technical Paper No. 380. Rome: FAO.

Castro ALF, Stewart BS, Wilson SG, Hueter RE, Meekan MG, Motta PJ, Bowen BW, Karl SA (2007) Population genetic structure of the Earth's largest fish, the whale shark (*Rhincodon typus*). *Molecular Ecology*, **16,** 5183-5192.

Cavalcanti MJ (2007) A phylogenetic supertree of the hammerhead sharks (Carcharhiniformes: Sphyrnidae). *Zoological Studies*, **46**, 6-11.

Chapman DD, Abercrombie DL, Douady CJ, Pikitch EK, Stanhope MJ, Shivji MS (2003) A streamlined, bi-organelle, multiplex PCR approach to species identification: Application to global conservation and trade monitoring of the great white shark, Carcharodon carcharias. *Conservation Genetics*, **4**, 415-425.

Chapman DD, Pinhal D, Shivji MS (2009) Tracking the fin trade: genetic stock identificatioin in western Atlantic scalloped hammerhead sharks (*Sphyrna lewini*). *Endangered Species Research*, in press.

Clement M, Posada D, Crandall KA (2000) TCS: A computer program to estimate gene genealogies. *Molecular Ecology*, **9,** 1657-1660.

Clarke SC, Magnussen JE, Abercrombie DL, Mcallister MK, Shivji MS (2006) Identification of shark species composition and proportion in the Hong Kong shark fin market based on molecular genetics and trade records. *Conservation Biology*, **20**, 201-211.

Compagno LJV (1984) FAO species catalogue Vol. 4, Part 2 Sharks of the World. An annotated and illustrated catalogue of shark species known to date. FAO Fisheries Synopsis, 125, 251-655.

Compagno LJV, Dando M, Fowler S (2005) Sharks of the World. Collins Field Guide, pp368. HarperCollins Publishers Ltd., London.

Cornuet JM, Luikart G (1997) Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. *Genetics*, **144**, 2001-2014.

Crandall KA, Templeton AR (1993) Empirical tests of some predictions from coalescent theory with applications to intraspecific phylogeny reconstruction. *Genetics*, **134,** 959-969.

Dasmahapatra KK, Mallet J (2006) DNA barcodes: recent successes and future prospects. *Heredity*, **97**, 254-255.

DeMartini E, Friedlander A, Sandin S, Sala E (2008) Differences in fish-assemblage structure between fished and unfished atolls in the northern Line Islands, central Pacific. *Marine Ecology Progress Series*, **365**, 199-215.

Duncan KM, Martin AP, Bowen BW, DeCouet GG (2006) Global phylogeography of the scalloped hammerhead shark (*Sphyrna lewini*). *Molecular Ecology*, **15,** 2239-2251.

Dulvy NK, Freckleton RP, Polunin NVC (2004) Coral reef cascades and the indirect effects of predator removal by exploitation. *Ecology Letters*, **7,** 410–416.

Dulvy NK, Rogers SI, Jennings S, Stetzenmiller V, Dye SR, Skjoldal HR (2008) Climate change and deepening of the North Sea fish assemblage: a biotic indicator of warming seas. *Journal of Applied Ecology*, **45**, 1029-1039.

Economakis AE, Lobel PS (1998) Aggregation behavior of the grey reef shark, Carcharhinus amblyrhynchos, at Johnston Atoll, Central Pacific Ocean. *Environmental Biology of Fishes*, **51** 129-139.

Feldheim KA, Gruber SH, Ashley MV (2001) Population genetic structure of the lemon shark (*Negaprion brevirostris*) in the western Atlantic: DNA microsatellite variation. *Molecular Ecology*, **10,** 295-303.

Ferretti F, Myers RA, Serena F, Lotze HK (2008) Loss of large predatory sharks from the Mediterranean Sea. *Conservation Biology*, **22**: 952-964.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, **3**, 294-299.

Friedlander AM, DeMartini EE (2002) Contrasts in density, size, and biomass of reef fishes between the northwestern and the main Hawaiian islands: the effects of fishing down apex predators. *Marine Ecology Progress Series*, **230,** 253-264.

Goudet J (1995) Fstat version 1.2: A computer program to calculate Fstatistics. *Journal of Heredity*, **86,** 485-486.

Hauser L, Carvalho GR (2008) Paradigm shifts in marine fisheries genetics: ugly hypothese slain by beautiful facts. *Fish and Fisheries*, **9,** 333-362.

Hayes CG, Jiao Y, Cortes E (2009) Stock assessment of scalloped hammerheads sharks in the western North Atlantic Ocean and Gulf of Mexico. *North American Journal of Fisheries Management*, **29**, 1406-1417.

Hebert PDN, Cywinska A, Ball SL, deWaard JR (2003) Biological identification through DNA barcodes. *Proceedings of the Royal Society of London*, B 270, 313-321.

Heist EJ, Gold JR (1999) Microsatellite DNA variation in sandbar sharks (*Carcharhinus plumbeus*) from the Gulf of Mexico and Mid-Atlantic Bight. *Copeia*, **1,** 182-186.

Hillis DM, Dixon MT (1991) Ribosomal DNA: Molecular evolution and phylogenetic inference. *The Quarterly Review of Biology*, **66**, 411-446.

Hueter RE, Heupel MR, Heist EJ, Keeney DB (2005) Evidence of Philopatry in Sharks and Implications for the Management of Shark Fisheries. *Journal of Northwest Atlantic Fisheries Science*, **35,** 239-247.

Ivanova NV, Zemlak TS, Hanner RH, Hebert PDN (2007) Universal primer cocktails for fish DNA barcoding. *Molecular Ecology Notes*, **7,** 544-548.

Jensen LJ, Bohonak AJ, Kelley ST (2005) Isolation by distance, web service. BMC *Genetics*, **6,** 13, v3.15 http://ibdws.sdsu.edu/.

Jorgensen SJ, Reeb CA, Chapple TK, Anderson S, Perle C, Van Sommeran SR, Fritz-Cope C, Brown AC, Klimley AP, Block BA (2009) Philopatry and migration of Pacific white sharks. Proceedings of the Royal Society B, doi: 10.1098/rspb.2009.1155.

Keeney DB, Heist EJ (2003) Characterization of microsatellite loci isolated from the blacktip shark and their utility in requiem and hammerhead sharks. *Molecular Ecology Notes*, **3,** 501-504.

Keeney DB, Heupel MR, Hueter RE, Heist EJ (2005) Microsatellite and mitochondrial DNA analyses of the genetic structure of the blacktip shark (*Carcharhinus limbatus*) nurseries in the northwestern Atlantic, Gulf of Mexico, and Caribbean Sea. *Molecular Ecology*, **14,** 1911-1923.

Keeney DB, Heist EJ (2006) Worldwide phylogeography of the blacktip shark (*Carcharhinus limbatus*) inferred from mitochondrial DNA reveals isolation of western Atlantic populations coupled with recent Pacific dispersal. *Molecular Ecology*, **15,** 3669-3679.

Kimura M, Weiss GH (1964) The stepping stone model of population structure and the decrease of genetic correlation with distance. *Genetics*, **49,** 561-576.

Kohler NE, Casey JG, Turner PA (1998) NMFS cooperative shark tagging program, 1962-93 – An atlas of shark tag and recapture data. *Marine Fisheries Review*, **60,** 1-87.

Kumar S, Tamura K, Nei M (2004) MEGA 3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics*, **5,** 150-163.

Latch EK, Dharmarajan G, Glaubitz JC, Rhodes Jr OE (2006) Relative performace of Bayesian clustering software for inferring population structure and individual assignment at low levels of population differentiation. *Conservation Genetics*, **7,** 295-302.

Madison WP, Maddison DR (1992) MacClade: Analysis of phylogeny and character evolution. Sinauer, Sunderland, Mass.

Magnussen JE, Pikitch EK, Clarke SC, Nicholson C, Hoelzel AR, Shivji MS (2007) Genetic tracking of basking shark products in international trade. *Animal Conservation*, **10**, 199-207.

Martin AP, Naylor GJP, Palumbi SR (1992) Rates of mitochondrial DNA evolution in sharks are slow compared with mammals. *Nature*, **357**, 153-155.

McKibben JN, Nelson DR (1986) Patterns of movement and groups of gray reef sharks, *Carcharhinus amblyrhynchos*, at Enewetak, Marshall Islands. *Bulletin of Marine Science*, **38,** 89-110.

Messing J (1983) New M13 vectors for cloning. *Methods in Enzymology*, **101**, 20-78.

Moore WS (1995) Inferring phylogenies from mtDNA variation: Mitochondrial-gene tree versus nuclear-gene tree. *Evolution*, **49**, 718-726.

Mucientes GR, Queiroz N, Sousa LL, Tarroso P, Sims DW (2009) Sexual segregation of pelagic sharks and the potential threat from fisheries. *Biology Letters,* **5,** 156-159.

Musick JA, Burgess G, Cailliet G, Camhi M, Fordham S (2000) Management of sharks and their relatives (Elasmobranchii). *Fisheries*, **25**, 9-13.

Myers RA, Baum JK, Shepherd TD, Powers SP, Peterson CH (2007) Cascading effects of the loss of apex predatory shark from a coastal ocean. *Science*, **315,** 1846-1850.

Nei M (1987) Molecular Evolutionary Genetics. Columbia University Press, New York, NY, USA.

Ovenden JR, Street R, Broderick D (2006) New microsatellite loci for Carcharhinid sharks (*Carcharhinus tilstoni* and *C. sorrah*) and their cross-amplification in other shark species. *Molecular Ecology Notes*, **6,** 415-418.

Pank M, Stanhope M, Natanson L, Kohler N, Shivji MS (2001) Rapid and simultaneous identification of body parts from the morphologically similar sharks *Carcharhinus obscurus* and *Carcharhinus plumbeus* (Carcharhinidae) using multiplex PCR. *Marine Biotechnology*, **3,** 231-240.

Papastamatiou YP, Wetherbee BM, Lowe CG, Crow GL (2006) Distribution and diet of four species of carcharhinid shark in the Hawaiian Islands: evidence for resource partitioning and competitive exclusion. *Marine Ecology Progress Series*, **320,** 239-251.

Park SDE (2001) Trypanotolerance in West African cattle and the population genetic effects of selection. PhD Thesis. University of Dublin.

Pfenninger M, Posada D (2002) Phylogeographic history of the land snail *Candidula unifasciata* (Helicellinae, Stylommatophora): Fragmentation, corridor migration and secondary contact. *Evolution*, **56,** 1776-1788.

Polovina JJ, Abecassis M, Howell EA, Woodworth P (2009) Increases in the relative abundance of mid-trophic level fishes concurrent with declines in apex predators in the subtropical North Pacific, 1996-2006. *Fishery Bulletin*, **107**, 523-531.

Portnoy DS, McDowell JR, Thompson D, Musick JA, Graves JE (2006) Isolation and characterization of five dinucleotide microsatellite loci in the sandbar shark, *Carcharhinus plumbeus*. *Molecular Ecology Notes*, **6,** 431-433.

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

Quattro JM, Stoner DS, Driggers WB, Anderson CA, Priede KA, Hoppmann EC, Campbell NH, Duncan KM, Grady JM (2006) Evidence of cryptic speciation within hammerhead sharks (genus Sphyrna). *Marine Biology*, **148,** 1143-1155.

Raymond M, Rousset F (1995) GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity*, **86,** 248-249.

Rice WR (1989) Analyzing tables of statistical tests. *Evolution*, **43,** 223-225.

Robbins WD, Hisano M, Connolly SR, Choat JH (2006) Ongoing collapse of coral-reef shark populations. *Current Biology*, **16,** 2314-2319.

Rose DA (1996) An overview of world trade in sharks and other cartilaginous fishes. Cambridge, United Kingdoms: TRAFFIC International.

Rozas J, Sánchez-DelBarrio JC, Messeguer X, Rozas R (2003) DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics*, **19,** 2496-2497.

Rubinoff D (2006) Utility of Mitochondrial DNA Barcodes in Species Conservation. *Conservation Biology*, **20**, 1026-1033.

Ryman N, Palm S (2006) POWSIM: a computer program for assessing statistical power when testing for genetic differentiation. *Molecular Ecology*, **6,** 600-602.

Saccone C, DeGiorgi C, Gissi C, Pesole G, Reyes A (1999) Evolutionary genomics in Metazoa: the mitochondrial DNA as a model system. *Gene*, **238**, 195-209.

Sandin SA, Smith JE, DeMartini EE, Dinsdale EA, Donner SD, Friedlander AM, Konotchick T, Malay M, Maragos JE, Obura D, Pantos O, Paulay G, Richie M, Rohwer F, Schroeder RE, Walsh S, Jackson JBC, Knowlton N, Sala E (2008) Baselines and Degradation of Coral Reefs in the Northern Line Islands. *PLoS One*, **3**, e1548.

Scicluna SM, Tawari B, Clark CG (2006) DNA barcoding of Blastocystis. *Protist*, **157**, 77-85.

Schneider S, Roessli D, Excoffier L (2000) Arlequin: A software for population genetics data analysis. Ver 2.000. Genetics and Biometry Lab, Dept of Anthropology, University of Geneva.

Schrey AW, Heist EJ (2003) Microsatellite analysis of population structure in the shortfin mako (*Isurus oxyrinchus*). *Canadian Journal of Fisheries and Aquatic Sciences*, **60,** 670-675.

Schultz JK, Feldheim KA, Gruber SH, Ashley MV, McGovern TM, Bowen BW (2008) Global phylogeography and seascape genetics of the lemon sharks (genus *Negaprion*). *Molecular Ecology*, **17,** 5336-5348.

Shepherd TD, Myers RA (2005) Direct and indirect fishery effects on small coastal elasmobranchs in the northern Gulf of Mexico. *Ecology Letters*, **8**, 1095-1104.

Shivji M, Clarke S, Pank M, Natanson L, Kohler N, Stanhope M (2002) Genetic identification of pelagic shark body parts for conservation and trade monitoring. *Conservation Biology*, **16**, 1036-1047.

Shivji MS (2009) DNA Forensic Applications in Shark Management and Conservation. In: *Sharks and Their Relatives: Physiological Adaptations, Behavior, Ecology, Conservation, and Management*. Chapter 15. (Eds: J.C. Carrier, M.R. Heithaus, J.A. Musick). CRC Press. In Press.

Stevens JD, Bonfil R, Dulvy NK, Walker PA (2000) The effects of fishing on sharks, rays, and chimaeras (chondrichthyans), and the implications for marine ecosystems. *ICES Journal of Marine Science*, **57**, 476-494.

Stevenson C, Katz LS, Micheli F, Block B, Heiman KW, Perle C, Weng K, Dunbar R, Witting J (2007) High apex predator biomass on remote Pacific islands. *Coral Reefs*, **26,** 47-51.

Stoeckle M, Hebert PN (2008) Barcode of life. *Scientific American*, **299**, 82-88.

Swofford DL (2003) PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

VanOosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes*, **4**, 553-538.

Waples RS, Gaggiotti O (2006) What is a population? An empirical evaluation of some genetic methods for identifying the number of gene pools and their degree of connectivity. *Molecular Ecology*, **15**, 1419-1439.

Ward RD, Zemlak TS, Innes BH, Last PR, Hebert PDN (2005) DNA barcoding Australia's fish species. *Philosophical transactions of the Royal Society of London, B* **360**, 1847-1857.

Ward RD, Holmes BH, White WT, Last PR (2008) DNA barcoding Australasian chondrichthyans: results and potential uses in conservation. *Marine and Freshwater Research*, **59**, 57-71.

Waugh J (2007) DNA barcoding in animal species: progress, potential and pitfalls. *BioEssays*, **29**, 188-197.

Weir BS, Cockerham CC (1984) Estimating *F*-statistics for the analysis of population structure. *Evolution*, **38,** 1358-1370.

Wetherbee BM, Crow GL, Lowe CG (1997) Distribution, reproduction and diet of the gray reef shark *Carcharhinus amblyrhynchos* in Hawaii. *Marine Ecology Progress Series*, **151,** 181-189.

Yancy HF, Zemlak TS, Mason JA Washington JD, Tenge BJ, Nguyen NT, Barnett JD, Savary WE, Hill WE, Moore MM, Fry FS, Randolph SC, Rogers PL, Hebert PDN (2007) Potential use of DNA barcodes in regulatory science: applications of the Regulatory Fish Encyclopedia. *Journal of Food Protection*, **71**