FLORIDA INTERNATIONAL UNIVERSITY

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THE HIDDEN HAMMERHEAD SHARKS: UNRAVELING THE PHYLOGEOGRAPHY, TAXONOMY, AND CONSERVATION OF THE BONNETHEAD SHARK COMPLEX IN THE AMERICAN CONTINENT

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Cindy Gonzalez Rodriguez

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To: Dean Michael R. Heithaus
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This dissertation, written by Cindy Gonzalez Rodriguez, and entitled The Hidden Hammerhead Sharks: Unraveling the Phylogeography, Taxonomy, and Conservation of the Bonnethead Shark Complex in the American Continent, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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DEDICATION

Para mi Mami y mi Papi, Edelmira y Eduardo por todo su amor infinito e incondicional, creer en mí, apoyarme en mi carrera al conocimiento del Océano y el mundo, por brindarme la mejor educación no convencional donde siempre estuve rodeada de naturaleza y buenas gentes, ya contando con la fortuna de haber nacido y crecido en Colombia. Mamá eres el Buddha de mi vida, rompiste las barreras de una mujer convencional para ser una profesional y una guerrera, nada mejor que tus palabras, tu sabiduría, tu amor que arropa y tus plantas. Papá, el mundo tan diverso y precioso como me lo has mostrado a través de los viajes me han hecho una gran persona, gracias por mostrarme el valor de los indígenas y de las otras culturas. A los dos gracias por cultivar en mi la espiritualidad que caracteriza la comprensión de un universo y no solo de un planeta. A mi tío Guillermo por ser el mejor padre, amigo, y cómplice que pudimos tener. A mi tía Nerita por ser mi mejor amiga toda la vida y mostrarme que ser feliz es muy fácil. A mis hermanos Felipe y Javier por su apoyo incondicional. Para mis abuelos maternos porque creo que ser amante de la Naturaleza viene en la sangre, para mis abuelos paternos por brindarnos miras a otros mundos, en especial a mi abuela Manuela por su tenacidad para mostrarnos que no hay fronteras, imposibles, ni tiempo que lo defina, pero por sobre todo por su amor a las plantas y los animales. Estos sueños cumplidos son el fruto del esfuerzo de muchas generaciones.

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ABSTRACT OF THE DISSERTATION

THE HIDDEN HAMMERHEAD SHARKS: UNRAVELING THE PHYLOGEOGRAPHY, TAXONOMY, AND CONSERVATION OF THE BONNETHEAD SHARK COMPLEX IN THE AMERICAN CONTINENT

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Due to continued overexploitation and anthropogenic change, hammerhead sharks (Carcharhiniformes: Sphyrnidae) have experienced drastic declines over most of their geographic range. To date, there are ten described species of hammerhead sharks distributed in two genera (*Sphyrna* and *Eusphyra*). It has been assumed that the hammerheads represent a monophyletic group, and that the head shape evolved gradually, starting from carcharhinid ancestor. Interestingly there are four species of small hammerhead sharks (<150cm TL) that are only distributed in the American continent and have been poorly studied and assessed in fisheries (the smalleye hammerhead *S. tudes*, the scoophead shark *S. media*, and the scalloped bonnethead shark - *S. corona*, and the bonnethead shark - *S. tiburo*). These small sharks are rare and face significant threats from gillnetting, overfishing, and misidentification in fisheries.

The bonnethead shark, *Sphyrna tiburo*, is a small coastal shark distributed in the Western Atlantic from North Carolina (U.S) to southern Brazil, and in the eastern Pacific Ocean from California (U.S) to southern Brazil. Previous genetic studies based on mitochondrial markers revealed that bonnethead sharks comprise a species complex with at least two lineages in the Northwestern Atlantic and the Caribbean (*S. tiburo* and *S.* aff. *tiburo*, respectively), and one in the eastern Pacific (*S. tiburo vespertina*). Despite several efforts to provide a taxonomical placement their phylogeny and key aspects of their biology remain unresolved. Bonnethead sharks are assessed as Endangered by the International Union for Conservation of Nature (IUCN) and the family Sphyrnidae was listed in appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) to regulate their international trade as there is constant misidentification of the species in fisheries as they “look-alike”.

Chapter II focuses on resolving the phylogeography and population genetics of the two lineages identified in the Atlantic (*S. tiburo* from the Western Atlantic, and *S.* aff. *tiburo* from the Caribbean and the Southwestern Atlantic). Based on two mitochondrial markers [control region (mtCR) and cytochrome oxidase I (COI)] and landscape genetics, the findings confirmed that *S.* aff. *tiburo* and *S. tiburo* have been on independent evolutionary paths, we detected significant population differentiation within and between the two lineages, identified an isolation by distance (IBD) pattern, and updated the distribution range for each lineage.

Following these results, in Chapter III, I analyzed 67 morphological traits, two meristic characters (prevertebral counts and teeth counts), two mitochondrial markers (COI and mtCR), and 12 microsatellite loci (nuclear markers) to describe *S.* aff. *tiburo* as a new species (aka *Sphyrna alleni* sp. nov., Shovelbill shark; in press).

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ABBREVIATIONS AND ACRONYMS

ABH	Atlantic Bonnethead Shark
CBH	Caribbean Bonnethead Shark
CB	Caribbean
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
COI	Cytochrome Oxidase One
EP	Eastern Pacific
GOM	Gulf of Mexico
IBD	Isolation by Distance
IOP	Isthmus of Panama
IUCN	International Union for Conservation of Nature
NGS	Next Generation Sequencing
mtCR	Mitochondrial Control Region
mtDNA	Mitochondrial DNA
NDNA	Nuclear DNA
SNP’s	Single Nucleotide Polymorphisms
TEP	Tropical Eastern Pacific
WA	Western Atlantic

PREFACE

The following chapters have been published and have been formatted for those publications:

CHAPTER II

Gonzalez, C., Postaire, B., Domingues, R., Feldheim, K.A., Caballero, S., & Chapman, D. (2021). "Phylogeography and population genetics of the cryptic bonnethead shark *Sphyrna* aff. *tiburo* in Brazil and the Caribbean inferred from mtDNA markers". *Journal of Fish Biology*, 99(6), 1899–1911. https://doi.org/10.1111/jfb.14896

CHAPTER III

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The following chapter is in advanced preparation to be submitted for review in *Marine Biodiversity* and has been formatted for that publication.

CHAPTER IV

GONZALEZ C., POSTAIRE B., DRIGGERS W., CABALLERO S., CHAPMAN D. 2024. "RESURRECTING THE PACIFIC BONNETHEAD SHARK *SPHYRNA VESPERTINA* SPRINGER, 1940 (CARCHARHINIFORMES, SPHYRNIDAE)". (Manuscript under revision)

CHAPTER I

GENERAL INTRODUCTION

Carcharhiniform sharks comprise about 55% of the approximately 350 living shark species described to date. The Carcharhiniformes are divided into eight families, including the family Sphyrnidae that comprise all the hammerhead sharks (Naylor 1992). Hammerhead sharks are a monophyletic group that emerged in the Miocene around 20 Mya, characterized by their laterally expanded and dorsal–ventrally compressed head, structure better known as “cephalofoil” (Compagno, 1988). There are around 10 species of hammerhead sharks classified in two genera: *Sphyrna* (nine species) and *Eusphyra* (one species). Hammerheads present high variation in size and head shape depending on the species. Large-bodied species such as the great hammerhead (*S. mokarran*), the scalloped hammerhead (*S. lewini*), and the smooth hammerhead (*S. zygaena*) range in size from 300 to 600 cm of total length (TL), have large cephalofoils, and have a cosmopolitan distribution in the tropical and subtropical waters of the Atlantic, Pacific, and Indic Oceans (Gilbert 1967).

Contrary, there are around five species of small-bodied hammerheads that range in size from 120 cm to <150cm TL. From those, all but one of the species, have small cephalofoils and are restricted to one or both coasts of the American continent (*S. media*, *S. corona*, *S. tudes*, and *S. tiburo*). The winghead shark (*E. blochii)*, is the only small hammerhead species contained in the genus *Eusphyra* and is restricted to the waters of the Indo-West Pacific. It is assumed that these two lineages of small sharks diverged independently from the main lineage at two separate times during history (Martin 1993).

Other main differences between large-bodied and small hammerhead sharks are the age at maturation and reproductive cycles. While large hammerheads need around 12-14 years to reach their sexual maturity, only reproduce every two years, and have large gestation times (nine months), small hammerheads achieve sexual maturity at early ages between 2-4 years, have short gestation times (five months), and have small cephalofoils, characteristics that have given advantage to these small species to overcount for high mortality and predation rates (Martin 1993).

All hammerhead species are listed in threatened categories by the International Union for the Conservation of Nature (IUCN) after they have declined because of overfishing (UICN, 2020). Small hammerhead species and juveniles of the large species are mostly captured in gillnets in coastal environments (coral reefs, estuaries, muddy and sandy bottoms, and mangroves), being one of the main components of the catches in artisanal fisheries in many Latin American countries, where the fins are sold to different retailers, and their meat is consumed regionally (Gonzalez *et al.* 2021; Quinlan *et al.* 2021; Reis-Filho *et al.* 2014). On the other hand, the large-bodied species are within the top 3 components of the international shark fin trade and their fins are sold at high prices as a "exotic" seafood product (Cardeñosa *et al.* 2017, 2018a; b, 2020). Additionally, the whole family Sphyrnidae was listed on Appendix II of the Convention on International Trade of Endangered Species (CITES) in 2022 to regulate the trading of their products and subproducts as species identification can be challenging.

Traditionally, the taxonomical classification of species has relied on visual observations, morphological traits, and meristic features of the specimens (Winston 2000). Hammerhead sharks were originally classified based on their distribution ranges and morphology (being the head shape and size the most common used traits to differentiate between species) (Bigelow & Schoroeder 1948; Gilbert 1967; Linné Carl von & Salvius Lars 1758; Springer 1940; Springer & Garrick 1964). In most recent years, molecular methods have shown the potential on providing valuable information about the population structure, genetic diversity, current status of the populations, and with phylogenetic reconstructions is possible to estimate the temporal geological separation of cryptic species that are closely related and are morphologically similar but genetically different (Ebert *et al.* 2013; Gonzalez *et al.* 2021; Jörger & Schrödl 2013). The possibility of performing genetic comparations of individuals from different populations that were defined as one morphologically defined species, may aid to undercover one or more undescribed taxa (Baldwin *et al.* 2011).

A previous revision of the hammerhead sharks carried by Gilbert in 1967 based on systematic arrangements of the family Sphyrnidae, suggested that all species can be distinguished by the head type varying from evenly rounded and spadelike shape (*S. tiburo*) to an extremely big and wide "hammer" shape (*S. mokarran*). On his classification he used the presence or absence of a narial groove and the relative length of the latter, the size of the animal, the specimens available for each species, and the distribution ranges of the collected specimens, to identify and update the names of nine species of hammerhead sharks. Moreover, relying on morphological similarities of the head shape he reclassified the previous named species of bonnethead sharks *S. tiburo* and *S. vespertina* as subspecies (*S. tiburo tiburo*, and *S. tiburo vespertina*, respectively).

Some of the most important conclusions from Gilbert's study was that *S. tudes*, *S. corona*, and *S. media*, have numerous identical morphological characters, some of which are unique to these small species, therefore are the most closely related members of the genus *Sphyrna*. Additionally, *S. tiburo*, with many morphological characters like those in *S. tudes*, *S. corona*, and *S. media*, appears to occupy an intermediate phylogenetic position between these three species and the other *Sphyrna* large bodied species (e.g. *S. zygaena*, *S. mokarran*, and *S. lewini*) (Gilbert 1967).

Different hypothesis about the phylogeny of hammerhead sharks have been proposed based on the investigation of taxonomical and genetic information (serum proteins and mitochondrial markers), however the phylogenetic placement of the small species is still ambiguous. Lim et al. (2010) inferred the most recent phylogeny of eight hammerhead species (including the small species) based on the analysis of mitochondrial and nuclear genes. However, in recent years other cryptic species of hammerhead sharks have been identified as it is the case of the Carolina hammerhead shark (*S. gilberti*) (Quattro *et al.* 2013), and the discovery of two different lineages of bonnethead sharks in the Atlantic (*S. tiburo* and *S.* cf. *tiburo*) (Naylor *et al.* 2012), making necessary to produce an updated phylogeny with extreme priority for conservation and resource management on the small species and juveniles of hammerhead sharks.

Several phylogenetic studies have explored the role that biogeography and barriers to dispersal have played in the marine speciation processes and the divergence of lineages (Bowen *et al.* 2016; Bowler & Benton 2005; Lessios 2008; Pinheiro *et al.* 2018). Moreover, the clarification of species complexes in elasmobranchs has led to the resurrection of multiple synonymized taxa (Daly-Engel *et al.* 2019; Ebert *et al.* 2010, 2013; Long *et al.* 2011; Weigmann *et al.* 2023) and to the identification of new species with high degree of morphological similarity within the same genus (Aroca *et al.* 2022; Gonzalez *et al.* 2021; Quattro *et al.* 2013). These findings are essential for conservation and fisheries management as they provide both recent and historical information about key species (Caballero *et al.* 2013; Cardeñosa *et al.* 2020; Daly-Engel *et al.* 2012; Gonzalez *et al.* 2019; Pinhal *et al.* 2012; Quintanilla *et al.* 2015).

There is an interesting geographical distribution pattern of small hammerhead species, as they are restricted to the American continent. *Sphyrna corona* is restricted to the eastern Pacific Ocean, and *S. tudes* is restricted to the western Atlantic Ocean. *Sphyrna media* is found in both

Oceans, and for *S. tiburo*, there are two subspecies (*S. tiburo vespertina* from the Eastern Pacific, and *S. tiburo tiburo* from the Atlantic Oceans, respectively). Geographical barriers such as the rise and closure of the Isthmus of Panama (IOP) are important to consider as a driver of speciation and diversification of many marine species, as its uplifting changed the dynamics and conditions of the currents, salinity, depth, and biological properties of many ecosystems (Cowman & Bellwood 2013). This barrier isolated the tropical Western Atlantic and the Tropical Eastern Pacific Oceans promoting multiple vicariant events that have been a base of reference to understand phylogeographical processes and the biology of many marine species in the world, including sharks (Coates & Stallard 2013; Collins *et al.* 1996; O'Dea *et al.* 2016).

Despite there is no consensus about the uplifting and final closure of the IOP (Coates & Stallard 2013; Collins *et al.* 1996; Jaramillo *et al.* 2017; Molnar 2017; Montes *et al.* 2015; O'Dea *et al.* 2016), it is well known that the Pacific Ocean and the Caribbean Sea were still connected by shallow waters between 4.2 to 3.5 Mya while the formation and uplifting of the Isthmus was a gradual process of at least 12Mya (Jaramillo *et al.* 2017). Molecular clocks and fossil records calculated in other geminate species from the IOP have been used to estimate the divergence time between closely related lineages (Marko 2002).

As mentioned before the bonnethead shark *S. tiburo* might represent the missing link between small and large bodied hammerhead sharks, making it an excellent species of study. This is a small shark, reaching a maximum size of <150 cm (LT) (Compagno 1984). This species inhabits the estuaries and shallow coastal waters (coral reefs, sandy and muddy bottoms, and mangroves). Taxonomically, *S. tiburo* was assumed to be a single species distributed in the western Atlantic Ocean from North Carolina to southern Brazil, including the Gulf of Mexico (GOM) and the Caribbean Sea; and in the Eastern Pacific Ocean from California to Ecuador (Gilbert 1967).

Studies of the biology and behavior of *S. tiburo* in the GOM have shown clear patterns of philopatry and strong site fidelity (Driggers *et al.* 2014; Heupel *et al.* 2006), which could result in closed populations that are genetically different, while morphologically identical. Previously, Naylor et al. (2012) found large genetic divergence by analyzing a fragment of the mitochondrial ND2 gene from twelve bonnethead sharks from the GOM, and two individuals from Trinidad. It was suggested that the animals from Trinidad belonged to a different lineage (the Trinidad specimens were then designated as *S.* cf. *tiburo*). Additionally, Escatel-Luna et al. (2015) found significant population structure between 251 bonnethead sharks (*S. tiburo*) from neighboring estuaries in the U.S. Western North Atlantic and the Gulf of Mexico, suggesting that there are multiple populations within this well-studied region. Subsequently, Fields et al. (2016) provided strong evidence of significant population structure and cryptic speciation in bonnethead sharks from the Western Atlantic and Belize based on mitochondrial markers (COI and mtCR). Lastly Gonzalez et al. (2019) provided strong mitochondrial evidence and complemented the previous results with additional data from Panama in the Caribbean to determine that *S.* cf. *tiburo* is a differentiated lineage than *S. tiburo*, updating the taxonomical level to *S.* aff. *tiburo* following the rules of the formal scientific naming of the International Code of Zoological Nomenclature.

An improved understanding of the genetic population structure and the clarification of taxonomical differences between populations of *S. tiburo*, will add important phylogeographical resolution, since bonnethead sharks are commercially important in the U.S., Mexico, Brazil, Belize, Panama, and the Caribbean (Harper *et al.* 2014; Motta *et al.* 2005; Quinlan *et al.* 2021; White *et al.* 2008). This is essential for the elucidation of potential cryptic species, and to ensure that the different stocks (population units) can be harvested sustainably.

This dissertation first focuses on solving the phylogeographic and taxonomic relationships of the bonnethead complex with the aim to contribute to management and conservation frameworks for these rare hammerhead sharks. The research presented in chapter II sheds light on two major topics 1) provide genetic evidence to corroborate that *S.* aff. *tiburo* is a different lineage than *S. tiburo* in the Atlantic and 2) clarify and update the phylogeography of these two lineages.

Chapter III investigates further into the genetic diversity and taxonomy of *S.* aff. *tiburo* and *S. tiburo*. This study is based on integrative taxonomy and the analysis of two mitochondrial markers (COI and mtCR) and twelve microsatellite loci (nuclear markers). Here, I present meristic, morphological, and molecular evidence to describe *Sphyrna alleni* sp. nov., as a new species of Shovelbill shark. This new species is distributed in the Caribbean Sea from Belize to the southwestern Atlantic in Brazil. Focus was placed on combining the power of genetic markers and classic taxonomy to show the existence of two different species of sharks that once were classified as a single species and clarify their distribution ranges.

Lastly, chapter IV builds from both previous chapters to resurrect *Sphyrna vespertina* (Springer, 1940) as a species. I used the findings of my previous studies on *S. tiburo* and *S. alleni* sp. nov., complementing it with additional taxonomical and molecular analyses to test the hypothesis that the Pacific bonnethead shark, currently classified as a subspecies (*S. tiburo vespertina*), is in fact a third species for the bonnethead complex. In this chapter, I studied the historical ambiguity of taxonomical placement for this shark and present updated molecular, meristic, and morphological evidence to prove that *S. vespertina* is a different lineage and should not be considered as a subspecies. I also discuss the findings in light to understanding the role of the closure of the Isthmus of Panama in shaping independent evolutionary trajectories for these three lineages.

This is the first study to resolve the phylogeography and taxonomy of three cryptic species of bonnethead sharks in the American continent (*S. tiburo*, *S. alleni* sp. nov., and *S. vespertina* (survive)), that were previously assessed and classified as a single species. By combining genetic, taxonomical data, and evaluating geological, ecological, and biological aspects of these three species, I provide firm evidence to show the existence of at least three species of bonnethead sharks in the Atlantic and the Pacific Oceans. This comprehensive study serves as a baseline to delineate management and conservation policies for these underrepresented sharks in Latin America. Moreover, we urgently make a call for a reassessment of the population status by the IUCN, and proper implementation on the identification of small hammerhead sharks based on field characters and genetic profiling is imperative as they were also recently included in the Appendix II of CITES. This dissertation also provides novel information that will help inform policies for managing shark populations in Latin America. By synthesizing data from diverse fields including genetics, classic taxonomy, ecology, geology, and conservation biology, I create novel insights into the potential of species delimitation, taxonomic placement, and fisheries management.

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CHAPTER II

PHYLOGEOGRAPHY AND POPULATION GENETICS OF THE CRYPTIC BONNETHEAD SHARK *SPHYRNA* AFF. *TIBURO* IN BRAZIL AND THE CARIBBEAN INFERRED FROM MITOCHONDRIAL DNA MARKERS

Cindy Gonzalez, Bautisse Postaire, Rodrigo Domingues, Kevin Feldheim, Susana Caballero, Demian Chapman. (2021). "Phylogeography and population genetics of the cryptic bonnethead shark *Sphyrna* aff. *tiburo* in Brazil and the Caribbean inferred from mtDNA markers". *Journal of Fish Biology*, 99(6), 1899-1911. https://doi.org/10.1111/jfb.14896.

Abstract

Resolving the identity, phylogeny, and distribution of cryptic species within species complexes is an essential precursor to management. The bonnethead shark, *Sphyrna tiburo,* is a small coastal shark distributed in the Western Atlantic from North Carolina (U.S.A) to southern Brazil. Genetic analyses based on mitochondrial markers revealed that bonnethead sharks comprise a species complex with at least two lineages in the Northwestern Atlantic and the Caribbean (*Sphyrna tiburo,* and *Sphyrna* aff. *tiburo,* respectively). The phylogeographic and phylogenetic analysis of two mitochondrial markers (control region [mtCR] and cytochrome oxidase I [COI]) showed that bonnethead sharks from southeastern Brazil correspond to *S.* aff. *tiburo,* extending the distribution of this cryptic species > 5,000 km. Bonnethead shark populations are only managed in the U.S.A, and in the 2000s were considered to be regionally extinct or collapsed in southeast Brazil. Our results indicate that there is significant genetic differentiation between *S.* aff. *tiburo* from Brazil and other populations from the Caribbean ($\Phi_{ST} = 0.9053$, p <0.000), which means that collapsed populations in the former are unlikely to be replenished from Caribbean immigration. The species identity of bonnethead sharks in the Southwest Atlantic and their relationship to North Atlantic and Caribbean populations remains unresolved. Taxonomic revision and further sampling are required to reevaluate the status of the bonnethead shark complex through its distribution range.

Introduction

Sharks are mobile species that are widely distributed across large geographic areas and yet, recent studies provide evidence of strong population structure on finer spatial scales than expected based on movement potential (Ashe *et al.* 2015; Chapman *et al.* 2015; Petean *et al.* 2020; Portnoy *et al.* 2015). Some widely distributed elasmobranchs have proven to be a species complex with each of the cryptic species having a much narrower distribution, which can expose them to different management jurisdictions and potential threats (Fields *et al.* 2016; Lim *et al.* 2010; Naylor *et al.* 2012; Portnoy *et al.* 2010; Quattro *et al.* 2006, 2013). Elucidating the taxonomy and evolutionary relationships of species making up these complexes and determining their geographic distribution, is a necessary precursor to assessing their status and developing an informed management plan (Fields *et al.* 2016; Pinhal *et al.* 2012; Quattro *et al.* 2006).

The bonnethead shark *Sphyrna tiburo* (Linnaeus,1758) is one of the five small-bodied *Sphyrna* (hammerhead sharks) species, with a total length of <150 cm at maturity (Compagno 2002; Jawad 2013). Bonnethead sharks inhabit coastal areas of insular and continental shelves along the American continent having a preference for shallow waters, coral reefs, and estuaries (Compagno 2002). *Sphyrna tiburo* is distributed in the eastern Pacific from California (U.S.A) to Ecuador and was thought to be distributed throughout the Western Atlantic from North Carolina (U.S.A) to southern Brazil, including the Gulf of Mexico and the Caribbean (Compagno 2002; Jawad 2013). This species has the shortest known gestation period in sharks of about 4-5 months, and an early maturation time (2-3 years), therefore is considered a highly productive species when compared to other sharks (Driggers et al., 2014; Frazier et al., 2014; Parsons, 1993). Consequently, bonnetheads were assessed as Least Concern from 2006 until 2020 by the International Union for Conservation of Nature (IUCN) (Cortes 2016; Hayes *et al.* 2009). On 2019 a new IUCN evaluation on the current status of the bonnethead shark populations found that despite its high productivity, there is a decreasing population trend throughout its range (population declines 50 -80%), and therefore *S. tiburo* was reassessed as Endangered (Pollom 2020).

Bonnethead sharks are an important component of the fisheries in Latin-American countries like Mexico, Belize, Guatemala, Costa Rica, and Panama (Díaz-Jaimes *et al.* 2021; Harper *et al.* 2014; Quinlan *et al.* 2021). In Brazil, bonnethead populations have reportedly collapsed due to overexploitation and habitat destruction (Bressan *et al.* 2009; Cortes 2016; Reis-Filho *et al.* 2014). However, outside of the U.S.A bonnethead populations are not directly assessed and their fisheries are not actively managed for sustainability (Díaz-Jaimes *et al.* 2021; Quinlan *et al.* 2021; Simpfendorfer & Dulvy 2017), which means that the species status is underestimated throughout most of its range.

Previous genetic studies based on mitochondrial markers provide strong evidence that Western Atlantic bonnethead sharks are actually a species complex comprised of at least two lineages (Naylor et al. 2012; Fields et al., 2016, Gonzalez et al. 2019). The term lineage has been used to describe a group of organisms forming a single line of direct ancestry and descent (Queiroz 1999). For this study, we will use the term mitochondrial lineage defined as two or more species that are reciprocally monophyletic as a result of historically isolated populations who have had an independent evolutionary trajectory over a long period of time (Avise 2000; Kizirian & Donnelly 2004). These preceding studies have adopted the taxonomical name of the cryptic species designated by Naylor et al. (2012) since they were the first ones on finding significant genetic divergence between bonnetheads from the Gulf of Mexico (U.S.A.), and two individuals from Trinidad and Tobago by analyzing the mitochondrial *ND2* gene, suggesting that at least one cryptic bonnethead shark species exist in the region. They designated the Trinidad and Tobago specimens as *Sphyrna* cf. *tiburo* (Caribbean bonnethead shark - CBH), and the Gulf of Mexico specimens remaining as *S. tiburo* (Atlantic bonnethead shark - ABH). For the present study and hereafter, we have adopted the taxonomical name *Sphyrna* aff. *tiburo* instead of *S.* cf. *tiburo,* since the distinction between the two lineages is certain (Sigovini *et al.* 2016).

Both lineages (*S.* aff. *tiburo* and *S. tiburo*) exhibit significant population differentiation over relatively small spatial scales (*i.e.,* the western Florida coast, the south-eastern US Atlantic coast, and the southern Gulf of Mexico) (Díaz-Jaimes *et al.* 2021), which is consistent with evidence that both species present philopatric behavior (Driggers *et al.* 2014; Escatel-Luna *et al.* 2015; Feldheim *et al.* 2014; Fields *et al.* 2016; Gonzalez *et al.* 2019; Heupel *et al.* 2006; Portnoy *et al.* 2015).

In this study, we first used a combination of population genetics and phylogenetic methods to investigate the species identity of bonnethead sharks from northeastern Brazil, as their identity remains unknown in the Southwest Atlantic. Second, we explored the spatial genetic structure of the bonnethead complex to provide information on connectivity between locations of the Northwestern Atlantic (U.S.A and Mexico), the Caribbean Sea (Belize and Panama), and the Southwestern Atlantic (Brazil), to test if there could be a possible effect of isolation by distance between populations. This information will be useful to evaluate the current distribution range, the conservation status of the bonnethead shark complex, and to delineate fisheries management policies at a local level.

Methods

Fin clip tissue samples from twelve bonnethead sharks from Maranhão state, Brazil were collected in 2016 as part of local fisheries catches discards. Tissue samples were stored in 95% ethanol for genetic analyses, and total DNA extraction was performed using the Qiagen DNeasy Blood and Tissue Kit following the manufacturer´s protocol (Qiagen, Valencia, CA). A 558 base pair (bp) fragment of the mitochondrial cytochrome oxidase I (COI) was amplified for all individuals (n=12), using the primers FishCoxI F (5´TCWACCAACCACAAAGAYATYGGCAC3´), and FishCoxI R (TAR-ACTTCWGGGTGRCCRAAGAATCA), modified from Ward et al. (2005). Polymerase chain reaction (PCR) was carried out in 25 µl volumes and consisted of 12.5 µL of Master Mix 'Applied 2X' (Applied Biosystems), 10 µL of DNase/RNase-free water (Fisher Scientific), 0.75 µL of each primer (10 µM), and 1 µL of genomic DNA (15 ng/µL). PCR cycling protocol was as follows: initial denaturation at 94ºC for 2 minutes, followed by 35 cycles at 94ºC for 30 seconds, 55ºC for 45 seconds, and 72ºC for 40 seconds, and a final extension step of 72°C for 10 min. A 960 bp fragment of the mitochondrial control region (mtCR) was amplified for all samples (n=12), using the primers Pro-L (5`AGGGRAAGGAGGGTCAAACT3´), and 282H (5´AAGGCTAGGACCAAACCT3´) (Keeney *et al.* 2003). PCR was carried out in 25 µl volumes and consisted of 12.5 µL of Master Mix 'Applied 2X' (Applied Biosystems), 9 µL of DNase/RNase-free water (Fisher Scientific), 1 µL of each primer (10 µM), and 1.5 µL of genomic DNA (15 ng/µL). PCR was performed as follows: initial denaturation at 94ºC for 3 minutes, followed by 35 cycles at 95ºC for 30 seconds, 48ºC for 30 seconds, and 72ºC for 90 seconds, and a final extension step of 72°C for 10 min, modified from Quintanilla et al. (2015). Successfully amplified PCR products from the mitochondrial region were purified using Exo-SAP (Thermo Scientific) and sequenced with both amplifying primers on an ABI 3730 DNA analyzer.

FIGURE 1 Map of sampling locations and geographical distribution of *Sphyrna tiburo* (ABH) and *S.* aff. *tiburo* (CBH). **ABH** (blue line): distributed from North Carolina (U.S.A) to Belize. **CBH** (red line): distributed from Belize to Southern Brazil. **COI sequences:** WA: Alabama (AL=1), Florida (FL=16), South Carolina (SC=4), Texas (TX=3); Bagdad Mexico (BM=4), Caribbean: Belize (BZ=16), Bocas del Toro (BDT=15). Southwest Atlantic: Brazil (BR=12). **CR sequences:** WA: Florida Bay, Florida (FB=25), Tampa Bay, Florida (TB=27), Panama City, Florida (PC=23), and North Carolina (NC= 23). Caribbean: Belize (BZ=54), Bocas del Toro, Panama (BDT=15). Southwest Atlantic: Brazil (BR=12).

All sequences (COI and mtCR) were aligned, edited, and checked manually using Geneious v.2020.2.2 (http://www.geneious.com) software, which was also used to identify haplotypes (Maddison & Maddison 2000). The Brazilian COI sequences (BR=12) were then compared to 59 bonnethead COI sequences obtained from GenBank (Table S1), from northern populations in the Western Atlantic (WA) and the Caribbean Sea, that included samples from the U.S.A: Alabama (AL=1), South Carolina (SC=4), Florida (FL=16), Texas (TX=3), Mexico: Bagdad Beach (BM=4), Belize: Placencia (BZ=16) (Wong *et al.* 2009), and Panama: Bocas del Toro (BDT=15), (Gonzalez *et al.* 2019) as depicted in Figure 1. Similarly, Brazilian mtCR sequences (BR=12) were compared to 169 CR sequences of bonnethead sharks obtained from GenBank (Table S2) from the WA (Figure 1), U.S.A: North Carolina (NC=23), Tampa Bay (TB=27), Florida Bay (FB=25), and Panama City (PC=25) (Portnoy *et al.* 2015), and from the

Caribbean Sea: Belize (BZ=54) (Fields *et al.* 2016), and Panama (BDT=15) (Gonzalez *et al.* 2019) (see S2 Table for GenBank accession numbers).

Haplotype diversity (*h*), and nucleotide diversity (π) of the COI and mtCR fragments were calculated in Arlequin 3.5.1.2 (Excoffier & Lischer, 2010) for the sampled localities. A statistical parsimony network for both mitochondrial markers was constructed using the software TCS v. 1.21 (Clement *et al.* 2000), providing a 95% plausible set for all haplotype linkages. The program jModelTest v.2.3.1 was used to obtain the best model for DNA substitution for both COI and mtCR (Posada 2008). After selecting the best model (TrN+I), PAUP (Excoffier & Heckel 2006) was used to build a COI maximum likelihood tree, including the outgroups *Sphyrna tudes* (GenBank: FJ519424.1), *Sphyrna lewini* (GenBank: FJ519636.1), and *Carcharhinus leucas* (GenBank: FJ519612.1). For the mtCR, the best model (TrN+I+G) was selected by jModelTest v.2.3.1, and was subsequently used to create a maximum likelihood tree in PAUP (Excoffier & Heckel 2006) including the outgroup *S. lewini* (GenBank: KY315830.1). We assessed population genetic differentiation at the COI and mtCR via pairwise comparisons of F_{ST} and Φ_{ST} using Arlequin v.3.5.1.2, with 10,000 permutations (Excoffier & Lischer 2010). An analysis of molecular variance (AMOVA) was used to quantify the genetic differences for the COI sequences of the *S.* aff. *tiburo* individuals (BDT, BZ, and BR), and for the mtCR sequences from *S. tiburo* and *S.* aff. *tiburo* (WA, BDT, BZ, BR), as implemented in Arlequin, using 10,000 permutations (Excoffier & Lischer 2010). A Bonferroni correction to adjust p-values was also performed, taking into account the number of comparisons of the localities.

Interspecific and intraspecific pairwise genetic distances were calculated for both COI (558 bp) and mtCR (960 bp) sequences to estimate the genetic divergence among and within ABH (*S. tiburo)* and CBH (*S.* aff. *tiburo)* individuals using the p- distance and the Kimura's two-parameter

(K2P) models, as implemented in MEGA 10.2.6 (Kumar *et al.* 2018; Stecher *et al.* 2020). The interspecific genetic distance estimated between the two bonnethead shark lineages was compared with the distance between *S. lewini* and the recently described sister species *S. gilberti*.

In order to evaluate the relationship between geographic distance and genetic divergence among populations from the WA, Brazil, Belize, and Panama a Mantel test was performed on the mtCR data by using the software Arlequin 3.5 (Excoffier & Lischer, 2010). For this instance, the genetic distance [F_{ST} / (1- F_{ST})] vs. the Euclidian geographical distance (ln km) across populations was used to estimate the level of isolation by distance with 10,000 permutations. The F_{ST} values were used since F_{ST} is an unbiased estimator of the proportion of genetic variability or divergence among and within pairs of populations under an island model, in which all populations diverged at the same time and are linked by similar migration rates (Diniz-Filho *et al.* 2013).

Finally, the software GENELAND version 4.0.9 is a Bayesian analysis that implements a Markov chain Monte Carlo (MCMC) clustering algorithm that identifies genetic discontinuities while taking into account the spatial distribution of the individuals sampled, classifying them into *K* clusters (Guillot *et al.* 2005). Two types of models can be performed, the spatial model (which includes both geographical coordinates and genotype data), and the non-spatial model (which only uses genetic information). For this analysis, we only included 81 *S.* aff. *tiburo* mtCR sequences represented in 31 haplotypes (H44- H75) since we wanted to test for population subdivision between Belize, Panama, and Brazil, and this marker is polymorphic, presenting higher genetic variation between individuals from the same localities. First, a non-spatial correlated model to estimate the *K* clusters a priori was run ten independent times with 100,000 MCMC of which every 100th one was saved. Initially, the tested number of genetic clusters (*K*) was set to vary between 1 and 10 (Guillot *et al.* 2005). Posteriorly, the *K* inferred from the model was set as *K*= 3, and was used to run a spatial correlated model, using the geographical coordinates (latitude and longitude) and the genetic information obtained for each individual (Guillot *et al.* 2005). The following parameters: K from 1 to 3, 100,000 MCMC iterations, and a thinning interval of 100 were set for ten independent runs. Lastly, to refine the geographical maps of the genetic clusters, a burn-in period of 100 iterations over the posterior probability of population membership was computed (Table 4).

TABLE 1 Pairwise F_{ST} (below diagonal) and Φ_{ST} (above diagonal) values for the COI gene from the Caribbean populations of *Sphyrna* aff. *tiburo:* Bocas del Toro (BDT), Belize (BZ), and Brazil (BR). Nucleotide % (π), and haplotype (h) diversity ± standard deviation (SD) diversity values are shown in the diagonal of each population unit.

Φ_{ST} F_{ST}	**BDT (n=15)**	**BZ (n=16)**	**BR (n=12)**
BDT	h = 0.0000 ± 0.0000 π = 0.00% ± 0.00	-0.00418 (0.9999 ± 0.0000)	0.9318 **(0.000 ± 0.000)**
BZ	-0.0042 (0.9909 ± 0.0000)	h = 0.7194 ± 0.0773 π = 12.5% ± 0.1064	0.6330 **(0.000 ± 0.000)**
BR	0.9260 **(0.000 ± 0.000)**	0.8570 **(0.000 ± 0.000)**	h = 0.1667 ± 0.1343 π = 0.03% ± 0.05

Probability values based on 10,000 permutations. Significant values in bold ($p < 0.05$). Numbers of samples of each locality are shown in parentheses. Values were taken after a Bonferroni correction.

Results

The species identity of the Brazilian bonnetheads was corroborated to be *S.* aff. *tiburo*. A fragment of 558 bp of the COI gene was obtained from 12 bonnetheads sampled in Brazil and haplotypes "ST04" and "ST05" were assigned following the nomenclature established in earlier studies, as they were not present in previously sampled bonnetheads from the Caribbean, the Gulf of Mexico, or the Northwest Atlantic. The maximum parsimony network for COI sequences consisted of two well-differentiated groups hereafter referred to as Atlantic bonnethead shark (ABH) or *S. tiburo,* and Caribbean bonnethead shark (CBH) or *S.* aff. *tiburo*. The CBH group included all Belize (except one sequence that grouped with the ABH), Panama, and Brazil sequences which were a minimum of 7 mutational steps away from haplotypes sampled in the Gulf of Mexico, Northwest Atlantic, and Bahamas (Figure 3). Consistently, the COI maximum likelihood tree revealed the same two reciprocally monophyletic clades, with strong bootstrap support as shown in Figure 2.

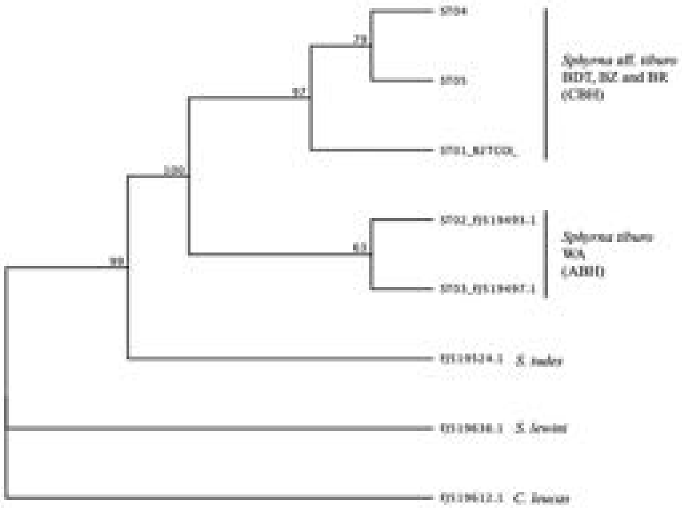

FIGURE 2 Maximum Likelihood distance tree of COI haplotype sequences from *Sphyrna tiburo* and outgroups: *S. tudes*, *S. lewini* and *Carcharhinus leucas*. CBH: Caribbean Bonnethead Shark, *S.* aff. *tiburo*. ABH: Atlantic Bonnethead Shark, *S. tiburo*.

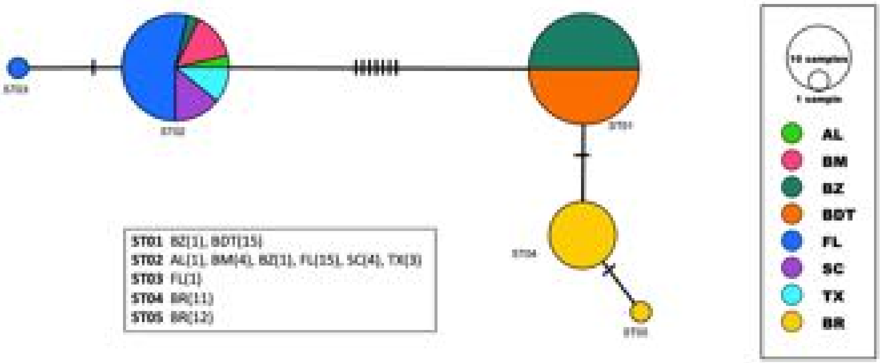

FIGURE 3 Maximum parsimony network of the cytochrome oxidase I (COI) for *S. tiburo* and *S.* aff. *tiburo*. Mutational steps are symbolized by dashes, and the diameter of the circles is proportional to the number of individuals that belong to each haplotype. Numbers of sequences per haplotype are given in parentheses. Sample sizes are as follow: Alabama (AL=1), Bagdad, Beach Mexico (BM=4), Belize (BZ=16), Bocas del Toro (BDT=15), Florida (FL=16), South Carolina (SC=4), Texas (TX=3).

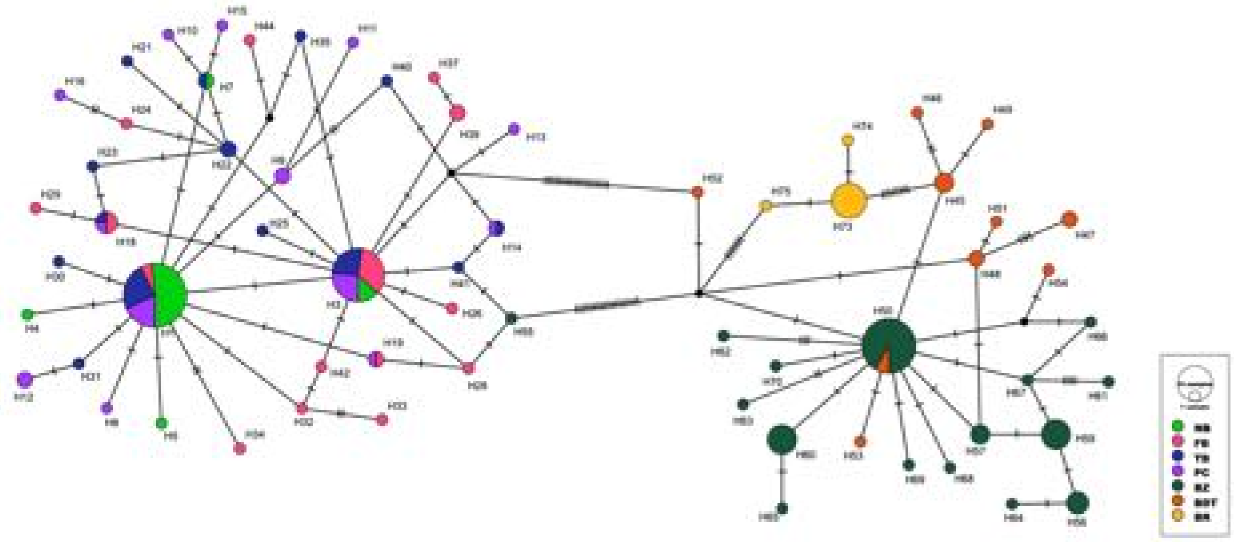

FIGURE 4 Maximum parsimony network of the control region (CR) for *S. tiburo* and *S.* aff. *tiburo.* Mutational steps are symbolized by dashes, and the diameter of the circles is proportional to the number of individuals that belong to each haplotype. Sample sizes are as follow: Western Atlantic haplotypes (H1- H44) that included North Carolina (NB=23), Florida Bay (FB=25), Tampa Bay (TB=27), and Panama City (PC= 25). Caribbean haplotypes from Bocas del Toro (BDT=15 - H45-H54), Belize (BZ=54 - H55- H72), and Brazil (BR=12 - H73- H75).

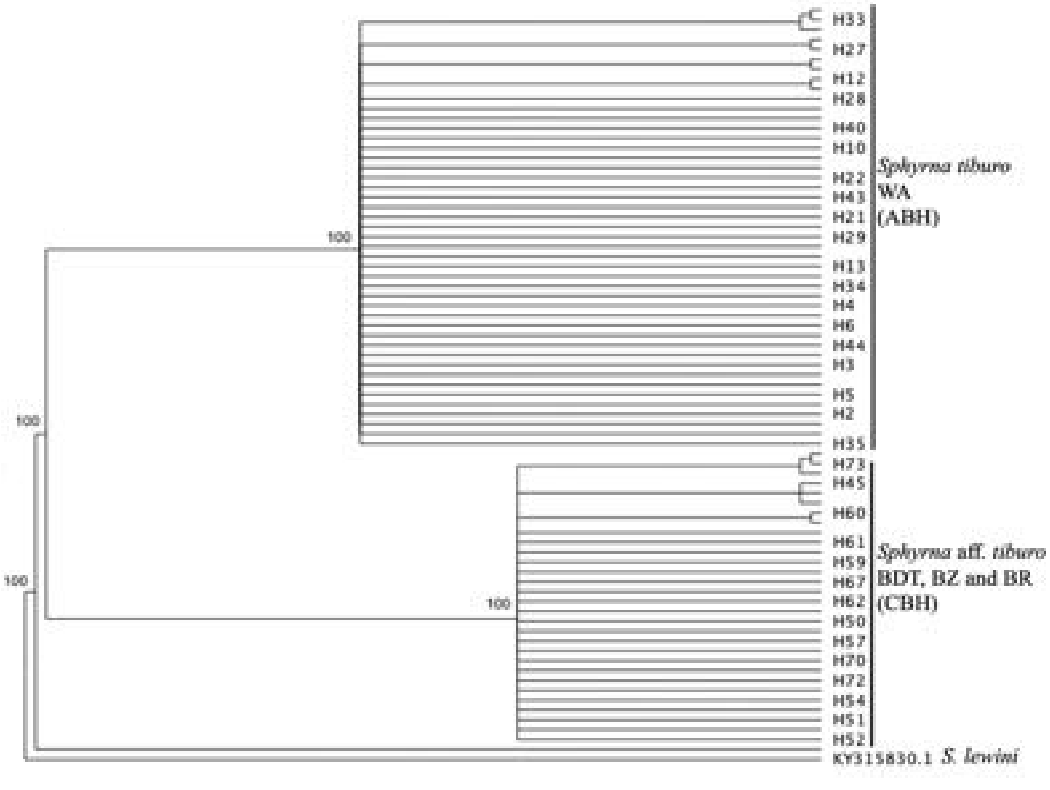

FIGURE 5 Maximum Likelihood distance tree of CR haplotype sequences from *Sphyrna tiburo*, and outgroup: *S. lewini.* ABH haplotypes from the Western Atlantic (WA= H1 - H44), and CBH from the Caribbean: Panama and Belize (BDT- BZ= H45 - H72), and Brazil (BR=H73 - H75).

TABLE 2 AMOVA analysis using pairwise genetic distances and conventional F_{ST} estimates for the COI gene. Significant values in bold ($p < 0.05$).

F_{ST}	Variance	%Total	F_{ST}	P value
Among groups G1 (BDT & BZ) G2 (BR)	0.4443	90.75	0.9074	0.3335 ± 0.0057
Among populations within groups	-0.0010	-0.21	-0.0229	1.000 ± 0.000
Among populations	0.0046	9.47	0.9053	**0.0000 ± 0.000**

The mtCR sequences obtained (960 bp) from the Brazilian bonnetheads consisted of three new haplotypes (H73- H75) and were assigned new names following the nomenclature established in previous studies. The mtCR haplotype network once again separated populations into the ABH and CBH lineages, differentiated by a minimum of 18 mutational steps (Figure 4). One CR haplotype (H55) sampled in one shark from Belize clustered within the ABH lineage. The maximum likelihood tree revealed reciprocal monophyly between the ABH and the CBH, with strong bootstrap support as shown in Figure 5**.** Pairwise comparisons of the F_{ST} and the Φ_{ST} were only analyzed within the CBH since the bonnetheads from Brazil clearly fall within this lineage (Table 1). Based on the COI, the Brazil samples were significantly differentiated from Panama ($F_{ST} = 0.9260$, $p < 0.000$), and Belize ($F_{ST} = 0.8570$, $p < 0.000$). The AMOVA comparison also indicated significant differentiation between populations ($\Phi_{ST} = 0.9053$, $p < 0.000$, Table 2). Likewise using the mtCR, the Brazil samples were genetically distant from Panama ($F_{ST} = 0.3527$, $p < 0.000$, $\Phi_{ST} = 0.8049$, $p < 0.000$), and Belize populations ($F_{ST} = 0.3544$, $p < 0.000$, $\Phi_{ST} = 0.8014$, $p < 0.000$) (Table 3). Overall haplotype diversity values ranged between 0.9467 ± 0.0289 (PC-Florida), and 0.3182 ± 0.1637 (Maranhão, Brazil), whereas nucleotide diversity values ranged between 0.36% ± 0.22 (BDT-Panama), and 0.03% ± 0.04 (Maranhão, Brazil) (Table 3). These values indicate that Brazil has the lowest nucleotide and haplotype diversity from all locations analyzed.

TABLE 3 Pairwise F_{ST} (below diagonal) and Φ_{ST} (above diagonal) values for the mitochondrial CR from the WA (*Sphyrna tiburo*), and Caribbean populations (*S.* aff. *tiburo*). WA: North Carolina (NC), Florida bay (FB), Tampa Bay (TB), Panama City (PC); Caribbean: Belize (BZ), Bocas del Toro (BDT), and Brazil (BR). Diagonal values: nucleotide % (π), and haplotype (h) diversity for each location.

Φ_{ST} F_{ST}	NC (n=23)	FB (n=25)	TB (n=27)	PC (n=25)	BZ (n= 54)	BDT (n=15)	BR (n=12)
NC	h = 0.7194 ± 0.0773 π = 0.05% ± 0.05	**0.2121*****	**0.1592*****	0.0626	**0.9194*****	**0.9322*****	**0.9847*****
FB	**0.1235*****	h = 0.9433 ± 0.0366 π = 0.22% ± 0.14	0.01766	0.0453	**0.9000*****	**0.8946*****	**0.9466*****
TB	**0.0427***	0.0190	h = 0.9402 ± 0.0314 π = 0.17% ± 0.12	-0.0020	**0.9038*****	**0.9038*****	**0.9556*****
PC	**0.0780****	-0.0062	0.0019	h = 0.9467 ± 0.0289 π = 0.23% ± 0.15	**0.8972*****	**0.8900*****	**0.9441*****
BZ	**0.2183 *****	**0.1160*****	**0.1171*****	**0.1144*****	h = 0.8316 ± 0.0413 π = 0.27% ± 0.16	**0.1117*****	**0.8014*****
BDT	**0.1754 *****	**0.0569*****	**0.0058*****	**0.0055*****	**0.1190*****	h = 0.9429 ± 0.0403 π = 0.36 %± 0.22	**0.8049*****
BR	**0.4467 *****	**0.3223*****	**0.3203*****	**0.3206*****	**0.3544*****	**0.3527*****	h = 0.3182 ± 0.1637 π = 0.03% ± 0.04

Significant P values in bold at <0.005*, 0.002**, 0.001***. Probability values based on 10,000 permutations.

TABLE 4 AMOVA results using pairwise genetic distances and conventional F_{ST} estimates for the mitochondrial CR. Significant values in bold ($p < 0.05$).

F_{ST}	**Variance**	**%Total**	**F_{ST}**	**P- value**
Among groups G1 (WA), G2 (BDT & BZ), G3 (BR)	0.0625	12.20	0.0645	0.01119 ± 0.001
Among populations within groups	-0.0290	5.66	0.1220	**0.0000 ± 0.000**
Among populations	0.4207	82.13	0.1787	**0.0000 ± 0.000**

TABLE 5 Estimates of genetic distances for COI and mtCR among and within *S. tiburo* (ABH) and *S.* aff. *tiburo* (CBH). Values for interspecific estimates in bold.

mtDNA	Species and sample size		Mean % divergence		Reference
			p - distance	K2P	
COI (558 bp)	S. tiburo (*n* = 28)	Intraspecific	0.1792	0.1795	This study
	S. aff. tiburo (*n* = 43)		0.2389	0.2396	
	S. tiburo (*n*=28) vs. *S.* aff. *tiburo* (*n* = 43)	**Interspecific**	**1.5233**	**1.5473**	
CR (960 bp)	S. tiburo (*n*= 100)	Intraspecific	0.3056	0.3065	
	S. aff. tiburo (*n* = 81)		0.5042	0.5073	
	S. tiburo (*n*=100) vs. *S.* aff. *tiburo* (*n* = 81)	**Interspecific**	**2.6204**	**2.6729**	
Other Studies					
COI	*S. tiburo* (*n*= 181) vs. *S.* cf. *tiburo* (*n*= 58)	**Interspecific**	**1.11**	-	Fields et al. (2016)
CR	*S. tiburo* vs. *S.* cf. *tiburo*		**3.25**	-	
CR (405 bp)	S. tiburo (*n*= 2)	Intraspecific	4.025	-	Pinhal et al. (2012)
	S. tiburo (n= 2) vs. Sphyrnidae (n= 51)	**Interspecific**	12.3474	-	

TABLE 6 Multiple runs for inferring the number of populations with GENELAND. Bold indicates the highest average posterior probability.

Run	Modal Number	Mean probability density	% of modal number
1	3	425.8839	99.6
2	3	848.9376	100
3	3	713.5293	99.9
4	3	1329.79	100
5	3	678.1013	100
6	3	2018.6467	99.9
7	3	1030.1222	99.9
8	**3**	**81.5211**	**99.9**
9	3	755.2413	100
10	3	241.6027	99.8

The pairwise genetic distance between ABH and CBH lineages was 1.5% for COI and 2.6% for the mtCR. The intraspecific genetic distances for both COI and mtCR are considerably smaller than the interspecific distances (Table 5). If we only analyze the most variable domain of the mtCR of 339 bp, the distance between the ABH and the CBH was 5.8%, which is similar to the estimates calculated by Fields et al. (2016) (COI: 1.11%, mtCR: 3.25%, and 6.2% for the variable domain). This estimate is also congruent with the genetic divergence calculated between

S. lewini and the new described cryptic species *S. gilberti* (5.6% - 5.8%) (Pinhal *et al.* 2012; Quattro *et al.* 2006).

The Mantel test based on the mtCR sequences detected a significant correlation between genetic distance and geographical distance (R^2=0.8450, p <0.0047) indicating an isolation-by-distance pattern among bonnethead populations from the WA, Belize, Panama, and Brazil (Figure 6). Lastly, the analysis of the 81 mtCR sequences from Belize, Panama, and Brazil performed in GENELAND, identified three differentiated clusters (*K*=3, Table 6) designating each one of these localities as a different population which is concordant with the Mantel test and the AMOVA results (Figure 7).

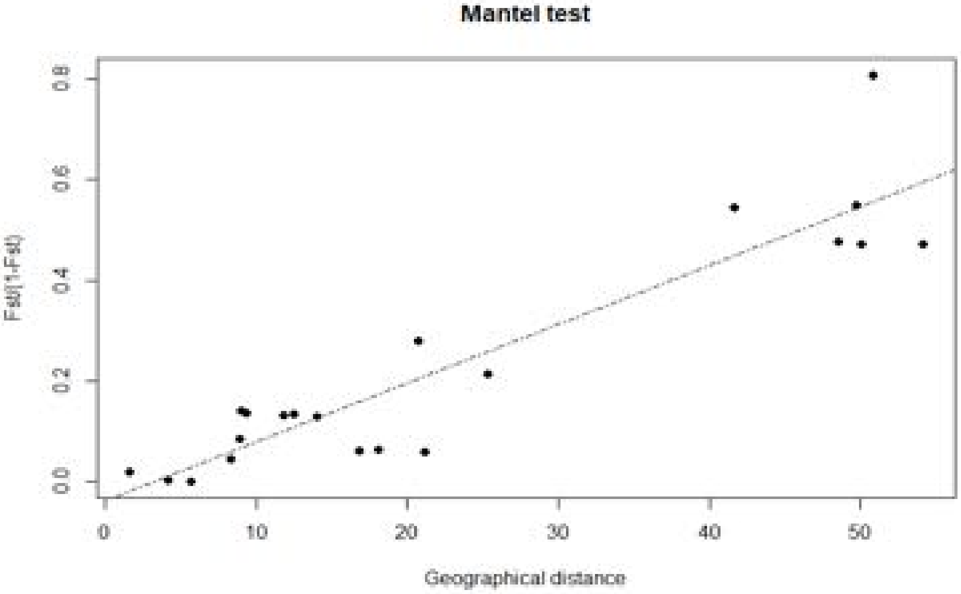

FIGURE 6 Mantel test of the relationships between genetic divergence (FST/(1-FST)) and geographic distance (ln km) among *Sphyrna tiburo* (ABH) and *S.* aff. *tiburo* (CBH) haplotypes. ABH haplotypes from the Western Atlantic (WA= H1- H44), and CBH from the Caribbean: Panama and Belize (BDT- BZ= H45- H72), and Brazil (BR= H73- H75).

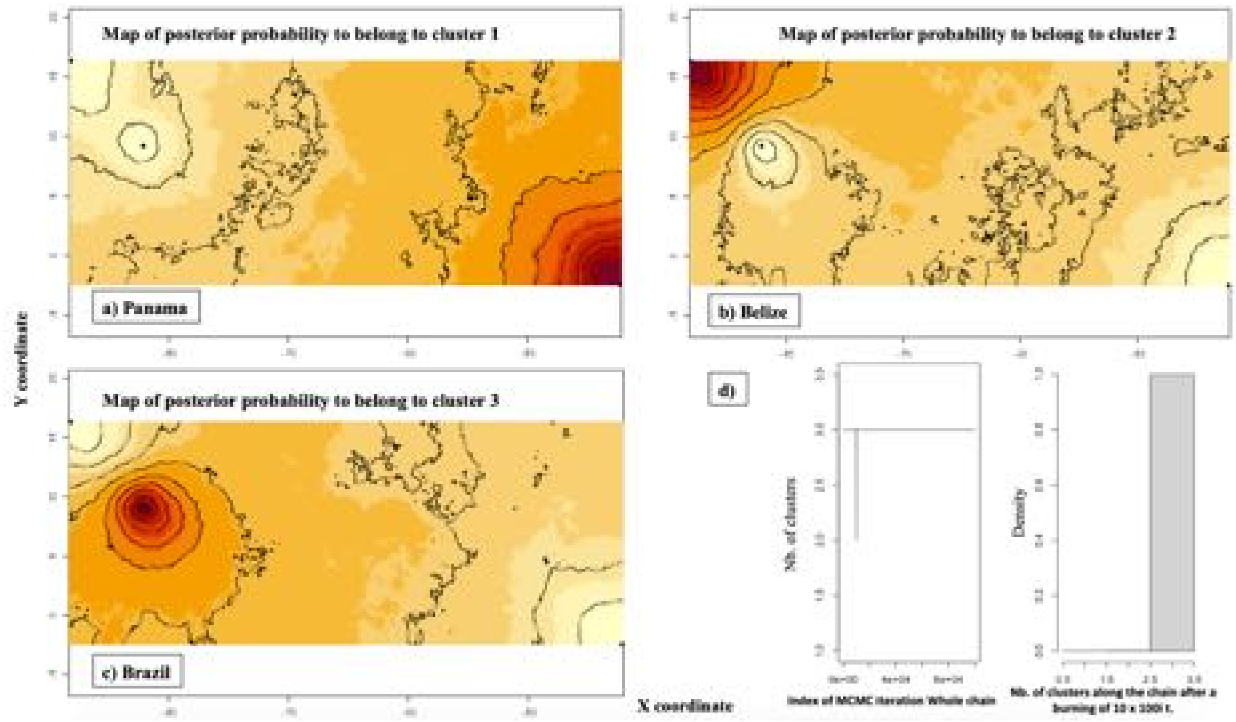

FIGURE 7 Maps of the posterior probabilities of population membership of *Sphyrna* aff. *tiburo* inferred by GENELAND with 100,000 permutations obtained from the mtCR. Spatial distribution of each group defined by *K*= 3. (a) Belize, (b) Bocas del Toro, and (c) Brazil; (d) number of clusters obtained. Contour lines indicate the spatial position of the genetic discontinuities. Darker-coloured areas indicates higher probabilities of population membership.

Discussion

The present study confirms the presence of the cryptic bonnethead shark species *S.* aff. *tiburo* in the Southwest Atlantic of Brazil. Although we were only able to procure samples from twelve individuals from Brazil, it is important to reiterate the critical status of bonnetheads in this jurisdiction, which makes the collection of additional samples an uncertain prospect in the near term. All trees and networks from both COI and mtCR certainly placed the Brazilian specimens within the CBH lineage, albeit with unique haplotypes. Since we included samples from both extremes of the distribution in the WA and the Caribbean (North Carolina - U.S.A, and Maranhão - Brazil), we conclude that the ABH (*S. tiburo*) occurs in U.S.A., Mexico, and the Bahamas (Naylor et al. 2012; Escatel-Luna et al. 2015; Fields et al. 2016), while the CBH (*S.* aff. *tiburo*) occurs from

Belize to Brazil, including Panama, and Trinidad and Tobago (Naylor et al., 2012; Fields et al. 2016; Gonzalez et al. 2019; this study). These results change our understanding of the current distribution of *S. tiburo* and opens new insights for *S.* aff. *tiburo*, extending the distribution of *S.* aff. *tiburo* more than 5,000 kilometers south. Moreover, since CBH populations have been poorly studied, their life history, biological, and ecological processes remain unknown which could be masking negative effects from local fisheries.

Since Brazil is the southernmost margin of the distribution of this species complex, it also suggests that it is limited to two species in the Western Atlantic, although surveys in the Greater and Lesser Antilles islands of the Caribbean and other Latin-American countries (Colombia, Venezuela, Guyana) are needed to cover the known range of this complex both to the east and west. It is necessary to assess the current status of both lineages considering that their management is very different. *S. tiburo* (ABH) is only assessed and managed in the U.S.A., and the Bahamas (http://sedarweb.org/docs/sar/S34_Bonnethead_SAR.pdf; (Ward-Paige 2017)), and despite bonnethead sharks an important component of the fisheries in Mexico have not been assessed or protected in this country (Díaz-Jaimes *et al.* 2021; Escatel-Luna *et al.* 2015). *S.* aff. *tiburo* (CBH) is also fished from Belize to Brazil without active management for sustainability (Simpfendorfer & Dulvy 2017), and previous studies provide evidence of collapsing populations in the southern portion of its distribution (Bressan *et al.* 2009; Cortes 2016; Motta *et al.* 2005; Reis-Filho *et al.* 2014).

It is of notable interest that a possible secondary contact zone may exist between the Yucatan Peninsula (Mexico) and Belize since one individual sampled in Belize was grouped within the ABH lineage. This suggests that an important barrier is playing a key role in shaping bonnethead populations, pattern that has been identified in other species such as the blacktip shark (*Carcharhinus limbatus*) (Keeney *et al.* 2005), and the Nassau grouper (*Epinephelus striatus*) (Jackson *et al.* 2014). Other oceanographic studies have also detected a certain degree of genetic isolation in the Mesoamerican reef that could be associated with currents and cyclonic gyres moving northward along the Central American coastline (Callum 1997; Cowen *et al.* 2006; Richardson 2005; Shulman & Bermingham 1995). This may be sufficient to explain the genetic distinctness of many marine species and reef fish spawning aggregations in the Belizean Mesoamerican reef (e.g., black grouper (*Mycteroperca bonaci*), yellowfin grouper (*M. venenosa*), and tiger grouper (*M. tigris*), among others) (Heyman *et al.* 2008), which could also act as a potential barrier for both lineages of bonnethead sharks.

Both the divergence time estimated by Fields et al. (2016) between *S. tiburo* and *S.* aff. *tiburo* from 3.61 to 5.62 million years before present, and the genetic distances obtained in this and his study, reveal a clear historical partition between the two lineages. The absence of gene flow would prevent the occurrence of shared haplotypes, which is evident in both COI and the mtCR, and genetic drift could cause a complete divergence between populations of different regions, leading to reciprocally monophyletic lineages and speciation processes (Avise 2000).

Major geological changes took place in Belize during the late Pliocene to the Holocene including sea-level fluctuations and changes in the carbonate platform (Mazzullo 2006). These events could have separated bonnetheads in this region and enabled speciation to occur. Indeed, vicariant events caused by sea-level changes (maximum - 130m) during this geological time have been recognized as responsible for speciation in several plant and animal lineages (Briggs & Bowen 2013), including sharks (Domingues *et al.* 2018, 2019). Nonetheless, there is no contemporary physical barrier to dispersal preventing contact between the two lineages, so more sampling between Belize and Mexico (Yucatan Peninsula) could reveal the extent of sympatry and possibly hybridization between these species, as has been seen in other shark species complexes (Barker *et al.* 2019; Morgan *et al.* 2012).

All genetic studies of the bonnethead lineages so far show that populations are genetically differentiated on relatively small geographic scales (locations separated by as little as ~ 10^2- 10^3 km) (Díaz-Jaimes *et al.* 2021; Escatel-Luna *et al.* 2015; Fields *et al.* 2016), which makes them more susceptible to local threats than more mobile species that can be locally replenished through immigration (Portnoy *et al.* 2015). We detected significant population differentiation between *S.* aff. *tiburo* populations from Brazil and the Caribbean locations for both mitochondrial markers. Brazil also showed the lowest nucleotide and haplotype diversity from all locations. Since ancestral populations usually possess higher genetic diversity than derived populations (Maisano Delser *et al.* 2019), one could hypothesize that the Brazil bonnetheads were originated from an ancestral *S.* aff. *tiburo* population and got established by a founder effect, but these findings could be also attributed to the small number of samples analyzed. Brazil not only showed unique haplotypes but in the mtCR network was placed seven mutational steps from Belize and Panama, indicating that is a very differentiated population. This genetic differentiation of Brazilian *S.* aff. *tiburo* from populations from Panama and Belize may be a simple function of isolation by distance, but it is also possible that the Amazon river outflow that extends thousands of kilometers in the Atlantic Ocean acts as a soft barrier to dispersal (Gouveia *et al.* 2019; Pinheiro *et al.* 2018). Since bonnethead sharks are stenohaline species, has been documented that salinity plays a role in their distribution and movement (Ubeda *et al.* 2009), so unsuitable habitats might act as geographical barriers restricting species distribution (Alvarado-Serrano & Knowles 2014), as has been documented for other megafauna including stingrays (*Hypanus* spp.) (Petean *et al.* 2020), dolphins (*Sotalia* spp., *Stenella frontalis*) (Caballero *et al.* 2013, 2018), and the sawfish (*Pristis pristis*) (Faria *et al.* 2013). As a consequence of the lack of gene flow with adjacent populations the Brazilian *S.* aff. *tiburo* could be undergoing an independent evolutionary path.

Conclusions

Whether isolated by distance or geographic barriers, there is a need to consider Brazil and the Caribbean Sea *S.* aff. *tiburo* populations as a minimum of two management units. These results are concerning for *S.* aff. *tiburo* in Brazil as it implies that there may be a limited possibility of replenishment through immigration from the northern hemisphere populations. A limitation of our analysis is that the small sample of Brazilian bonnetheads we analyzed may not be spatially representative of the larger population distribution. We recommend further investigating this possible population differentiation using an increased sample size for Brazil, sampling other Latin-American countries (Venezuela, Guyana, Colombia, Costa Rica) and the Caribbean Islands, as well as using additional molecular markers (*e.g.,* microsatellites or SNPs), in order to clarify the degree of current and historical connectivity between what is believed to be different CBH stocks. If this stock differentiation along the Atlantic Coast of South America is confirmed, it would imply that management and conservation programs would need to manage these stocks as separate units.

This study highlights the particular urgency of reassessing the bonnethead species complex in the former region, given that the status and conservation of populations throughout their distribution range are either critical or unknown (Bressan *et al.* 2009; Díaz-Jaimes *et al.* 2021; Gonzalez *et al.* 2019; Lessa 1986; Reis-Filho *et al.* 2014). Furthermore, a formal taxonomic revision of the bonnethead shark species complex is required to identify morphological differences between the two lineages.

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INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC)

The methodology for sample collection and animal research was approved by the Wildlife Research group - Florida International University under the IACUC (Lab Animal Research - Record ID 30291259) and the Smithsonian Institute IACUC (Animal Care and Use - Record ID 20676904).

Supplemental material

All relevant data are within the manuscript and its Supporting Information files. Data from this study are also available in GenBank NCBI (https://www.ncbi.nlm. nih.gov/genbank/) through the following accession numbers: **Table S1** GenBank accession numbers, localities, and haplotypes for the mitochondrial Cytochrome Oxidase I (COI) sequences. **Table S2** GenBank accession numbers, localities, frequencies, and haplotypes numbers for the mitochondrial Control Region (CR) sequences. Samples from the Western Atlantic (WA): North Carolina (NC) and three locations along the Gulf Coast of Florida: Florida Bay (FB), Tampa Bay (TB), and Panama City (PC). Samples from the Caribbean: Bocas del Toro, Panama (BDT), and Belize (BZ). Samples from Southern Atlantic: Brazil (BR).

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Supplementary Material

Sample	Haplotype	Locality	Accession Number (GenBank)
OC-191	**ST01**	Placencia, Belize	FJ519523.1
OC-217	**ST01**	Placencia, Belize	FJ519521.1
OC-218	**ST01**	Placencia, Belize	FJ519520.1
OC-219	**ST01**	Placencia, Belize	FJ519519.1
OC-220	**ST01**	Placencia, Belize	FJ519518.1
OC-221	**ST01**	Placencia, Belize	FJ519517.1
OC-222	**ST01**	Placencia, Belize	FJ519516.1
OC-223	**ST01**	Placencia, Belize	FJ519515.1
OC-227	**ST01**	Placencia, Belize	FJ519513.1
OC-229	**ST01**	Placencia, Belize	FJ519514.1
OC-231	**ST01**	Placencia, Belize	FJ519511.1
OC-74	**ST01**	Placencia, Belize	FJ519510.1
OC-75	**ST01**	Placencia, Belize	FJ519509.1
OC-76	**ST01**	Placencia, Belize	FJ519508.1
B1TCOI - B15TCOI	**ST01**	Bocas del Toro, Panama	MH603121
FDA100	**ST02**	Alabama, U.S.A	KF461242.1
OC-69	**ST02**	Bagdad Beach, Mexico	FJ519286.1
OC-72	**ST02**	Bagdad Beach, Mexico	FJ519284.1
OC-198	**ST02**	Placencia, Belize	FJ519522.1
OC-31	**ST02**	Florida, U.S.A	FJ519504.1
OC-32	**ST02**	Florida, U.S.A	FJ519503.1

OC-65	**ST02**	Florida, U.S.A	FJ519502.1
OC-255	**ST02**	Florida, U.S.A	FJ519501.1
OC-287	**ST02**	Florida, U.S.A	FJ519499.1
OC-259	**ST02**	Florida, U.S.A	FJ519498.1
OC-239	**ST02**	Florida, U.S.A	FJ519495.1
OC-240	**ST02**	Florida, U.S.A	FJ519494.1
OC-245	**ST02**	Florida, U.S.A	FJ519493.1
OC-246	**ST02**	Florida, U.S.A	FJ519492.1
Stib018b(021)	**ST02**	Florida Cape Canaveral, U.S.A	FJ519643.1
Stib024b(114)	**ST02**	Florida Cape Canaveral, U.S.A	FJ519642.1
Stib016b(021)	**ST02**	Florida Crooked Island Sound, U.S.A	FJ519644.1
OC-173	**ST02**	South Carolina, U.S.A	FJ519491.1
OC-174	**ST02**	South Carolina, U.S.A	FJ519490.1
OC-181	**ST02**	South Carolina, U.S.A	FJ519489.1
Stib015n(020)	**ST02**	South Carolina Cape Romaine, U.S.A	FJ519645.1
ID44	**ST02**	Texas -Gulf of Mexico, U.S.A	FJ519505.1
OC-262	**ST02**	Florida, U.S.A	FJ519496.1
ID42	**ST02**	Texas -Gulf of Mexico, U.S.A	FJ519506.1
OC-230	**ST02**	Placencia, Belize	FJ519512.1
ID31	**ST02**	Texas -Gulf of Mexico, U.S.A	FJ519507.1
OC-283	**ST02**	Florida, U.S.A	FJ519500.1
OC-68	**ST02**	Bagdad Beach, Mexico	FJ519287.1
OC-71	**ST02**	Bagdad Beach, Mexico	FJ519285.1
OC-260	**ST03**	Florida, U.S.A	FJ519497.1
STBRA 01 - 06 STBRA 09 - 13	**ST04**	Maranhão, Brazil	MT881569
STBRA 08	**ST05**	Maranhão, Brazil	MT881570

TABLE S1 GenBank accession numbers, localities, and haplotypes for the mitochondrial Cytochrome Oxidase I (COI) sequences of *Sphyrna tiburo* and *S.* aff. *tiburo*.

GenBank Accession Number	Haplotype	NC	FB	TB	PC	BDT	BZ	BR	Total
KT031755.1	**H1**	6	1	2	2	0	0	0	11
KT031756.1	**H2**	11	1	6	3	0	0	0	21
KT031757.1	**H3**	1	0	0	0	0	0	0	1
KT031758.1	**H4**	1	0	0	0	0	0	0	1
KT031759.1	**H5**	1	0	0	0	0	0	0	1
KT031760.1	**H6**	2	6	2	5	0	0	0	15
KT031761.1	**H7**	1	0	0	0	0	0	0	1
KT031762.1	**H8**	0	0	0	1	0	0	0	1
KT031763.1	**H9**	0	0	0	2	0	0	0	2

KT031764.1	**H10**	0	0	0	1	0	0	0	1
KT031765.1	**H11**	0	0	0	1	0	0	0	1
KT031766.1	**H12**	0	0	0	2	0	0	0	2
KT031767.1	**H13**	0	0	0	1	0	0	0	1
KT031768.1	**H14**	0	0	1	1	0	0	0	2
KT031769.1	**H15**	0	0	0	1	0	0	0	1
KT031770.1	**H16**	0	0	0	1	0	0	0	1
KT031771.1	**H17**	0	2	3	1	0	0	0	6
KT031772.1	**H18**	0	1	1	1	0	0	0	3
KT031773.1	**H19**	0	0	0	1	0	0	0	1
KT031774.1	**H20**	0	0	0	1	0	0	0	1
KT031775.1	**H21**	0	0	1	0	0	0	0	1
KT031776.1	**H22**	0	0	2	0	0	0	0	2
KT031777.1	**H23**	0	0	1	0	0	0	0	1
KT031778.1	**H24**	0	1	0	0	0	0	0	1
KT031779.1	**H25**	0	0	1	0	0	0	0	1
KT031780.1	**H26**	0	1	0	0	0	0	0	1
KT031781.1	**H27**	0	1	0	0	0	0	0	1
KT031782.1	**H28**	0	1	0	0	0	0	0	1
KT031783.1	**H29**	0	1	0	0	0	0	0	1
KT031784.1	**H30**	0	0	1	0	0	0	0	1
KT031785.1	**H31**	0	0	1	0	0	0	0	1
KT031786.1	**H32**	0	1	0	0	0	0	0	1
KT031787.1	**H33**	0	1	0	0	0	0	0	1
KT031788.1	**H34**	0	1	0	0	0	0	0	1
KT031789.1	**H35**	0	0	1	0	0	0	0	1
KT031790.1	**H36**	0	0	1	0	0	0	0	1
KT031791.1	**H37**	0	1	0	0	0	0	0	1
KT031792.1	**H38**	0	1	0	0	0	0	0	1
KT031793.1	**H39**	0	2	0	0	0	0	0	2
KT031794.1	**H40**	0	0	1	0	0	0	0	1
KT031795.1	**H41**	0	0	1	0	0	0	0	1
KT031796.1	**H42**	0	1	0	0	0	0	0	1
KT031797.1	**H43**	0	0	1	0	0	0	0	1
KT031798.1	**H44**	0	1	0	0	0	0	0	1
MH603122.1	**H45**	0	0	0	0	3	0	0	3
MH603123.1	**H46**	0	0	0	0	1	0	0	1
MH603124.1	**H47**	0	0	0	0	2	0	0	2
MH603125.1	**H48**	0	0	0	0	2	0	0	2
MH603126.1	**H49**	0	0	0	0	1	0	0	1
MH603127.1	**H50**	0	0	0	0	2	0	0	2
MH603128.1	**H51**	0	0	0	0	1	0	0	1
MH603129.1	**H52**	0	0	0	0	1	0	0	1

MH603130.1	**H53**	0	0	0	0	1	0	0	1
MH603131.1	**H54**	0	0	0	0	1	0	0	1
Not Available	**H55**	0	0	0	0	0	1	0	1
Not Available	**H56**	0	0	0	0	0	4	0	4
Not Available	**H57**	0	0	0	0	0	3	0	3
Not Available	**H58**	0	0	0	0	0	20	0	20
Not Available	**H59**	0	0	0	0	0	7	0	7
Not Available	**H60**	0	0	0	0	0	7	0	7
Not Available	**H61**	0	0	0	0	0	1	0	1
Not Available	**H62**	0	0	0	0	0	1	0	1
Not Available	**H63**	0	0	0	0	0	1	0	1
Not Available	**H64**	0	0	0	0	0	1	0	1
Not Available	**H65**	0	0	0	0	0	1	0	1
Not Available	**H66**	0	0	0	0	0	1	0	1
Not Available	**H67**	0	0	0	0	0	1	0	1
Not Available	**H68**	0	0	0	0	0	1	0	1
Not Available	**H69**	0	0	0	0	0	1	0	1
Not Available	**H70**	0	0	0	0	0	1	0	1
Not Available	**H71**	0	0	0	0	0	1	0	1
Not Available	**H72**	0	0	0	0	0	1	0	1
MT913778	**H73**	0	0	0	0	0	0	10	10
MT913779	**H74**	0	0	0	0	0	0	1	1
MT913780	**H75**	0	0	0	0	0	0	1	1
	Total	**23**	**25**	**27**	**25**	**15**	**54**	**12**	**181**

TABLE S2 GenBank accession numbers, localities, frequencies, and haplotypes numbers for the mitochondrial Control Region (mtCR) sequences of *Sphyrna tiburo* and *S.* aff. *tiburo*. Samples from the Western Atlantic (WA): North Carolina (NC) and three locations along the Gulf Coast of Florida: Florida Bay (FB), Tampa Bay (TB) and Panama City (PC). Samples from the Caribbean: Bocas del Toro (BDT) and Belize (BZ). Samples from Southern Atlantic: Brazil (BR).

CHAPTER III

SPHYRNA ALLENI SP. NOV., A NEW HAMMERHEAD SHARK (CARCHARHINIFORMES, SPHYRNIDAE) FROM THE CARIBBEAN AND THE SOUTHWEST ATLANTIC.

Cindy Gonzalez, Bautisse Postaire, William Driggers, Susana Caballero, Demian Chapman. 2024. *Sphyrna alleni* sp. nov., a new hammerhead shark (Carcharhiniformes, Sphyrnidae) from the Caribbean and the Southwest Atlantic. *Zootaxa.* https://doi.org/10.11646/zootaxa.5512.4.2

http://zoobank.org/urn:lsid:zoobank.org:pub:E58F4A25-AAD5-4336-910B-30182CDD5903

Abstract

Hammerhead sharks (Family Sphyrnidae) comprise a monophyletic Miocene radiation of carcharhiniform sharks characterized by their laterally expanded and dorsoventrally compressed head ('cephalofoil'). The bonnethead shark (*Sphyrna tiburo*) is currently described as a single amphi-American hammerhead species composed of the subspecies *Sphyrna tiburo tiburo* in the Western Atlantic Ocean (WA) and *S. tiburo vespertina* in the Eastern Pacific Ocean (EP). Variation in mitochondrial DNA and cephalofoil shape suggest a species complex, with *S. tiburo* occurring in the U.S., Mexico, and Bahamas; *S.* aff. *tiburo* occurring from Belize to Brazil; and *S. vespertina* occurring in the EP. Morphometric, meristic, and genetic variation was used to resolve the bonnethead shark complex in the Western Atlantic. Twenty-three specimens (12 *S.* aff. *tiburo* from Belize and 11 *S. tiburo* from U.S.) were subject to sixty-one morphometric measurements and three meristic characters (counts of the number of precaudal vertebrae, lower and upper rows of functional teeth). An allometric formula was used to standardize any effect caused by differences in size of the individuals and data were analyzed with univariate and multivariate statistics. *Sphyrna* aff. *tiburo* and *S. tiburo* have non-overlapping vertebral counts (80-83 and 71-74 respectively) but no morphometric differences were detected. Although not captured in morphometric analysis, the cephalofoil of *S.* aff. *tiburo* has a more pointed anterior margin than *S. tiburo* that together with lobule shaped posterior margins gives the cephalofoil a distinctive shovel-shaped appearance. Concatenated mitochondrial sequences and 12 nuclear microsatellite markers clearly separated *S.* aff. *tiburo* and *S. tiburo.* We conclude that this complex comprises two species in the Western Atlantic, *S. tiburo* and *S. alleni* sp. nov., and we provide a description of the latter, which is distinguished by precaudal vertebral counts (80-83), a shovel-shaped cephalofoil with rounded posterior margins, and robust differences in mitochondrial and nuclear genetic markers.

We suggest nuclear genetic and meristic examination of EP bonnetheads is needed to update the taxonomical status and redescribe *S. vespertina*.

Introduction

Hammerhead sharks (Family Sphyrnidae) are a monophyletic lineage of carcharhiniform sharks first appearing in the Miocene (Gilbert 1967; Lim *et al.* 2010; Naylor 1992). They are characterized by their laterally expanded, dorsoventrally compressed head or 'cephalofoil' and currently comprise 10 named species (Gilbert 1967; Lim *et al.* 2010; Quattro *et al.* 2013). Hammerhead sharks are one of the most threatened shark families mainly due to overexploitation, with all species but one (*Sphyrna gilberti*) being Globally listed as 'Endangered' or 'Critically Endangered' by the International Union for the Conservation of Nature (IUCN. 2021). There are four species of small-bodied hammerheads (< 1.5 m total length at first maturity) that are endemic to the Americas (*Sphyrna tiburo*, *Sphyrna tudes*, *Sphyrna corona*, and *Sphyrna media*), with one species occurring only in the Eastern Pacific ("EP"; *S. corona*), one species occurring only in the Western Atlantic ("WA"; *S. tudes*) (Compagno 1984b; Gilbert 1967; Springer 1940), and two species occur in both oceanic basins, including the scoophead shark (*S. media*) and the bonnethead shark (*S. tiburo*).

The bonnethead shark (*S. tiburo*; Linnaeus, 1758) is unique among the sharks of the genus *Sphyrna* because it has only a slightly expanded cephalofoil. This species is distributed in the EP from California (U.S.) to Ecuador, and in the WA from North Carolina (U.S.) to southern Brazil, including the Gulf of Mexico and the Caribbean (Compagno 1984b; Jawad 2013). It was originally named and described as *Squalus tiburo* by Linnaeus in 1758 based on a dried specimen collected in an unknown location in 'America'. There are no type specimens. Almost two centuries later

Springer (1940) described the bonnetheads in the EP as a different species, separating them from *S. tiburo* based on cephalofoil shape, but no further description of the species was made. While *S. vespertina* has a cephalofoil that comes to a point on the anterior margin, *S. tiburo* has a slightly narrower and more evenly rounded cephalofoil (Springer 1940). The holotype for *S. vespertina* is CAS-SU 11584 with paratypes CM 5675 and CAS-SU 11881 (Springer 1940). Gilbert (1967) revised the hammerhead family and synonymized *S. vespertina* and *S. tiburo* following Bigelow and Schroeder (1948) on the grounds that cephalofoil shape variation within WA *S. tiburo* collected from a wide geographic area included pointed forms that resembled *S. vespertina*. Years later, Gilbert (1967) recognized the subspecies *S. tiburo tiburo* for the WA (Linnaeus, 1758), and *S. tiburo vespertina* for the EP (Springer 1940). However, a recent study comparing bonnethead shark specimens from the WA and EP support the species status of *S. vespertina* based on diagnostic differences in cephalofoil shape, electroreceptor densities, and mitochondrial genetic markers separating it from all WA bonnethead sharks (Aroca *et al.* 2022).

Genetic studies conducted on WA bonnethead sharks using up to three mitochondrial markers: cytochrome oxidase I (COI), control region (mtCR), and NADH dehydrogenase subunit 2- (NADH2), and one nuclear marker: internal transcribed spacer (ITS-2) indicate that a species complex comprised of at least two divergent lineages occurs in the region, hereafter referred as *S. tiburo* and *S.* aff. *tiburo* (Fields *et al.* 2016; Gonzalez *et al.* 2019; Naylor *et al.* 2012). *S. tiburo* occurs in the U.S., Mexico, and The Bahamas, while *S.* aff. *tiburo* has been found in Belize, Panama, Colombia, Trinidad & Tobago, and Brazil (Aroca *et al.* 2022; Fields *et al.* 2016; Gonzalez *et al.* 2019, 2021; Naylor *et al.* 2012). Morphometric analyses revealed distinct cephalofoil shapes for *S. tiburo*, *S.* aff. *tiburo*, and *S. vespertina* (Aroca *et al.* 2022). The cephalofoil of *S.* aff. *tiburo* has a more pointed anterior margin than *S. tiburo* and resembles but is still quantitively distinct from that of *S. vespertina,* usually because the latter has relatively straight rather than rounded posterior cephalofoil margins (Aroca *et al.* 2022). The probable inclusion of *S. tiburo* and *S.* aff. *tiburo* in bonnethead shark specimens examined by Bigelow and Schroeder (1948) explains why cephalofoil shape within WA *S. tiburo* appeared to be sufficiently variable to synonymize it with *S. vespertina.* No other morphological or meristic comparisons have been made between these WA cryptic species.

The present study combines classic taxonomy with genetics to describe *S.* aff. *tiburo* as a new species: "*Sphyrna alleni* sp. nov.". We performed 61 morphometric measurements and 3 meristic counts to evaluate potential differences in the morphology of these cryptic species. We also expand genetic analyses using two mitochondrial markers, cytochrome oxidase I (COI) and the control region (mtCR), and 17 nuclear microsatellite markers specific for *S. tiburo* (Price *et al.* 2014). We discuss the status of the broader bonnethead complex, suggesting that similar meristic and nuclear genetic analyses are needed in the EP to formally resurrect and redescribe *S. vespertina*. As the small hammerhead sharks are a common component of the local fisheries in many Latin-American countries and have been reported as collapsed, extirpated, or data deficient (Cardeñosa *et al.* 2020; Gonzalez *et al.* 2021; Harper *et al.* 2014; Reis-Filho *et al.* 2014) it is essential to resolve their taxonomy and phylogeography to support conservation.

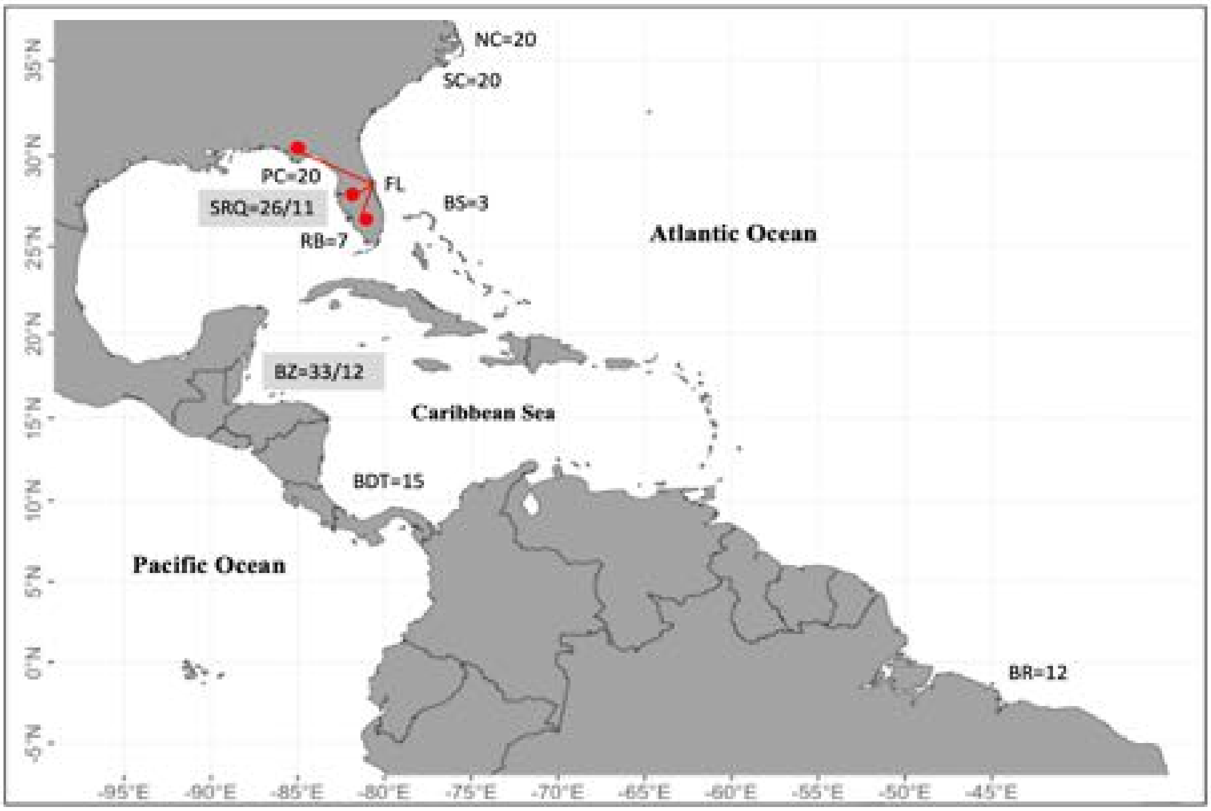

FIGURE 1 Sampling localities. Western Atlantic (WA): U.S. North Carolina (NC), South Carolina (SC), Florida (FL): Panama City (PC), Sarasota (SRQ), Rookery Bay (RB), Bahamas (BS); Caribbean: Belize (BZ), Bocas del Toro, Panama (BDT); Southwestern Atlantic: Brazil (BR). Grey boxes represent the two localities where specimens were also collected for morphometric, meristic, and genetic analyses (*S.* aff. *tiburo* N=12; *S. tiburo* N= 11). The information outside of boxes depict localities and number of samples used in the analysis of microsatellite DNA markers (*S.* aff. *tiburo* N=60; *S. tiburo* N=96).

Materials and methods

Specimen Collection and Preservation. Twelve mature bonnethead sharks (six males and six females) diagnosable in the field as *S.* aff. *tiburo* based on capture location and having cephalofoils with pointed anterior margins were provided by fishermen from Robinson's Point and Riversdale Village, Belize (Table 1, Figure 1). Another 11 mature bonnethead sharks (six males and five females) identified as *S. tiburo* based on capture location and having a semi-circular cephalofoil were collected in Panama City and Sarasota, Florida, U.S (Table 1, Figure 1). For all the specimens, a set of samples (fin, muscle, liver) was collected and preserved (95% ethanol, RNAlater, and frozen). The whole sharks were kept in ice baths to avoid decay. Sixty-one morphometric measurements were performed on each shark and meristic data were collected. Specimens were then injected with formalin 10% and fixed for three months in a container with formalin 10%. The final step of the preservation was made at the Florida Museum of Natural History and consisted of preserving the specimens in a 95% ethanol pool for another three months. Details of all specimens can be found in the 'Materials Examined" section. Fin and muscle samples were subsequent used for genetic analyses (see mitochondrial and nuclear markers sections).

Morphometric data. Measurements of precaudal (PCL), fork (FL), total (TL) and stretch total (STL) lengths were taken on a straight line along the axis of the body for 12 *S.* aff. *tiburo* and 11 *S. tiburo*. Subsequently, 61 morphometric features (Table 2) were measured following the methods from (Compagno 1984a).

Meristic data. Three meristic traits of all the specimens were counted: the total number of teeth on the upper and lower functional rows of the jaws, and the number of precaudal vertebrae (Springer & Garrick 1964). A dissection pin was inserted into the anterior margin of the precaudal pit and perpendicular to the body axis. Precaudal vertebrae counts (PVC) were then made in one of two ways. All the soft tissue was removed from 12 sharks (six *S.* aff. *tiburo* and six *S. tiburo*) from the tail to the brainstem, cleaning all the spine and counting the PCV by hand. The second approach consisted of taking X-rays of six well-preserved specimens (three *S.* aff. *tiburo* and three *S. tiburo*). Radiographs were taken of each specimen's vertebral column at the Veterinary Hospital of the University of Florida. Precaudal vertebrae counts were then performed by hand from the X-ray images obtained (Table 1).

TABLE 1 Summary of *S.* aff. *tiburo* and *S. tiburo* specimens used in this study. PVC= precaudal vertebrae count.

Species	Sex	TL (cm)	PVC	Specimen	UF-Museum Catalog #	Type	Collection site	Sample ID	Collector
S. aff. *tiburo*	F	103	83	Preserved Formalin 10%	UF245705;	Holotype	Stann Creek, Riversdale, Belize	001BZN	Cindy Gonzalez
S. aff. *tiburo*	M	60.5	82		UF245723;	Paratype		002BZN	
S. aff. *tiburo*	F	83	80		UF245724;			003BZN	
S. aff. *tiburo*	F	71.5	81		UF245725;			004BZN	
S. aff. *tiburo*	M	63	83		UF245726;			005BZN	
S. aff. *tiburo*	M	64.5	82		UF245727;			006BZN	
S. aff. *tiburo*	F	85	81	Dissected	Collected 2016	Not Preserved		017BZO	Demian Chapman
S. aff. *tiburo*	F	77.5	80					008BZO	
S. aff. *tiburo*	F	74.8	82					001BZO	
S. aff. *tiburo*	M	81.6	81					019BZO	
S. aff. *tiburo*	M	90.5	80					005BZO	
S. aff. *tiburo*	M	88.7	80					009BZO	
S. tiburo	F	86	73	Preserved Formalin 10%	UF 247324;	Comparative material	Terra Ceia Bay, Sarasota, Panama City FL, U. S	6FLN	Jayne Gardiner, Tonya Wiley, John Carlson
S. tiburo	F	74.5	72		UF 247324;			12FLN	
S. tiburo	F	82	72		UF 247324;			10FLN	
S. tiburo	M	80.5	74		UF 247325;			7FLN	
S. tiburo	M	73.5	73		UF 247325;			8FLN	
S. tiburo	M	59.5	72		UF 247326;			11FLN	
S. tiburo	F	71.6	72	Dissected	Collected 2015			023FLO	
S. tiburo	F	82	72					008FLO	
S. tiburo	M	82.3	73					017FLO	
S. tiburo	M	76	74					006FLO	
S. tiburo	M	83.3	73					004FLO	

Morphometric and Meristic Data Analysis. To remove any size effect on the 61 morphological traits, the data were size standardized by implementing an allometric formula, and later analyzed by using univariate and multivariate statistics following the methods by Elliot et al., (1995) and Quattro et al., (2013). Stretched total length (STL) was used as the measure of body length. All individual character measurements were standardized according to the formula (Elliott *et al.* 1995):

$M_{(Adj)} = M_o\ (L_s / L_o)^b$; where $M_{(Adj)}$ = *size-adjusted* measurement, Mo = original morphometric measurement, Ls= mean STL of each bonnethead from all samples for each variable, Lo= STL of specimen. and b was estimated by the allometric growth equation $M = aL''$. Parameter b was estimated as the slope of the regression of log M_o, on log L_o, for each character from the observed data.

Principal Component Analysis (PCA) was used to independently examine the morphometric (continuous variables), and the meristic traits (discontinuous variables). The morphological variation to define groups among *S. tiburo* and *S.* aff. *tiburo* was assessed by examining principal components with eigenvalues greater than one and assessing the proportion of variation explained by those components, character weightings on components, and plots of component scores. A Discriminant Function Analysis (DFA) was used to evaluate the meristic and adjusted morphometric characters to identify the variables that explain differentiation between groups. Finally, the original data (Mo) were standardized as percent of STL and used to test for interspecific differences between means of individual morphometrics by using an ANOVA. When data were not normally distributed a Kruskal-Wallis was used to test whether samples originated from the same distribution. All statistical tests were considered significant at $\alpha = 0.05$.

Genetic Data and Analysis

Mitochondrial markers

Fin clip tissue samples from the *S. tiburo* and *S.* aff. *tiburo* specimens described earlier from Sarasota, Florida U.S (n=11) and Placencia, Belize (n=12) respectively, were stored in 95% ethanol and total DNA extraction was performed with the Qiagen DNeasy Blood and Tissue Kit following the manufacturer´s protocol (Qiagen, Valencia, CA). A 563 base pair (bp) fragment of the mitochondrial cytochrome oxidase I (COI) was amplified for all the samples using the primers FishCoxI F (5´TCWACCAACCACAAAGAYATYGGCAC3´), and FishCoxI R (5´TAR-ACTTCWGGGTGRCCRAAGAATCA3´), modified from Ward *et al.* (2005) and following their PCR conditions and thermal cycling profiles. A 579 bp fragment of the mitochondrial control region (mtCR) was amplified for all samples using the primers Pro-L (5`AGGGRAAGGAGGGTCAAACT3´), and 282H (5´AAGGCTAGGACCAAACCT3´) and the reaction and thermal cycling conditions described in Keeney *et al.* (2003). PCR products were purified using Exo-SAP (Thermo Scientific) and sequenced with both amplifying primers on an ABI 3730 DNA analyzer.

All sequences (COI and mtCR) were aligned, edited, and checked manually using Geneious v.2020.2.2 (http://www.geneious.com) software, which was also used to identify haplotypes (Maddison & Maddison 2000). Haplotype diversity (*h*), and nucleotide diversity (π) of the COI and mtCR fragments were calculated in Arlequin 3.5.1.2 (Excoffier & Lischer 2010) for the sampled localities. Ten sequences (five of each cryptic species) were selected and a concatenated alignment of both the COI and the CR was built. The program jModelTest v.2.3.1 was used to obtain the best model for DNA substitution (Posada 2008). After selecting the best model (TrN+I), PAUP (Excoffier & Heckel 2006) was used to build a concatenated neighbor joining tree, using *Sphyrna lewini* as the outgroup.

Nuclear Markers

PCR amplification and microsatellite genotyping. Fin clip tissue samples from *S. tiburo* (n=96) and *S.* aff. *tiburo* (n= 60) collected from a wide geographic area (Figure 1) were stored in

95% ethanol for genetic analyses, and genomic DNA extraction was performed using the Qiagen DNeasy Blood and Tissue Kit following the manufacturer´s protocol (Qiagen, Valencia, CA). They were genotyped for 17 polymorphic microsatellite markers (Price *et al.* 2014). Microsatellite primer and multiplexing details are provided in Table S1. PCR for microsatellite amplification were conducted using the Type-it Kit Qiagen Multiplex PCR Master Mix®, 25–70 ng of DNA and 0.4 µM of each primer in 5 µL of final reaction volume. Loci were organized into four multiplex sets per PCR reaction and were performed with sets of *S. tiburo* or *S.* aff. *tiburo*. The PCR protocol consisted on an initial step of 5 min at 95°C, followed by 29 cycles of 30 s at 95°C for denaturation, 90 s at 60°C for annealing, and 30 s at 72°C for extension, with an additional step for final extension of 30 min at 60°C PCR (Díaz-Jaimes *et al.* 2021). The PCR products for microsatellite loci were visualized using capillary electrophoresis and sized with Alexa725™ to score with the Geneious v.2020.2.2 software (http://www.geneious.com). The software Micro-Checker 2.2 (Van Oosterhout *et al.* 2004) was used to identify possible genotyping errors.

All loci successfully amplified were tested for presence of null alleles using the software Micro-Checker 2.2 (Van Oosterhout *et al.* 2004) and confirmed using GenePop (Rousset 2008). Nuclear genetic diversity was estimated as the number of alleles observed (N_a), and the average number of alleles per locus. Deviations from the Hardy–Weinberg equilibrium (HW) and linkage disequilibrium between all pairs of loci overall and within each cryptic species and sampling site were assessed by the exact test implemented in ARLEQUIN v. 3.5.2.2 (Excoffier & Lischer 2010), that was also used to calculate the observed heterozygosity (*HO*), expected heterozygosity (*HE*), and estimated inbreeding coefficient (F_{IS}) for each sampling site (burn-in period: 10,000 iterations, MCMC 100,000). Genetic differentiation between *S. tiburo* and *S.* aff. *tiburo* was tested using pairwise F_{ST} values calculated in ARLEQUIN v. 3.5.2.2, and *Jost's D* values (Jost *et al.* 2018) calculated using the software GENODIVE (Meirmans & Van Tienderen 2004). Average genetic diversity (AGD) defined as the gene diversity over all loci in each population, was calculated in ARLEQUIN v. 3.5.2.2. Loci or samples with more than 15% of missing data were removed from the analyses. All p-values were Bonferroni corrected.

We tested the hypothesis of two separate gene pools for *S. tiburo* and *S.* aff. *tiburo* by implementing the admixture model with correlated allele frequencies to estimate the most likely number of 'populations' (*K* clusters) in the data by using the software STRUCTURE (Pritchard *et al.* 2000). The Evanno method implemented in STRUCTURE HARVESTER v.1.0 (Earl & vonHoldt 2012) was used to estimate the best number of *K* clusters. Three independent runs were conducted (length burn-in period: 50,000; MCMC 500,000). The results were summarized in CLUMPP v.1.0 (Jakobsson & Rosenberg 2007) and were formatted and visualized in DISTRUCT (Rosenberg 2004).

Results

Morphometric analysis. The possible effects of the size on the 61 morphometric variables analyzed were eliminated. None of the correlation coefficients were significant, indicating that body size has no effect on any of the variables. PCA analysis extracted 14 components with eigenvalues greater than 1.0, accounting for 94.33% of the variability. The proportion of variation for the individual components and among components indicated that these continuous morphological characters cannot resolve *S. tiburo* and *S.* aff. *tiburo* (Figure 2).

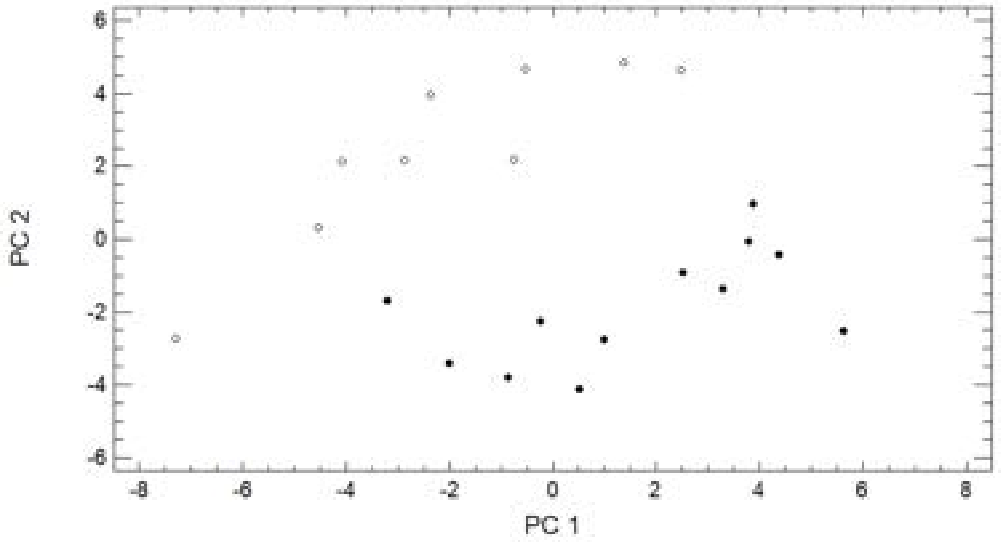

FIGURE 2 Plot of the first two principal components for 61 size-adjusted morphometric variables analyzed. Black circles indicate *S.* aff. *tiburo* specimens from Placencia, Belize and white circles indicate *S. tiburo* specimens from Florida, U. S.

However, the anterior margin of all examined *S.* aff. *tiburo* cephalofoils have a noticeable triangular apex, with an additional bulge present in that of adult males (Figure 3). They also had noticeable lobule shaped head posterior margins, making the entire cephalofoil appear shovel shaped. In comparison, all *S. tiburo* specimens had a more evenly rounded, semi-circular cephalofoil, with mature males having a bulge on the anterior margin (Figure 3). When comparing *S.* aff. *tiburo* and *S. vespertina* they also have triangular/ pointed cephalofoils, but the posterior margins of the head are straight, and the lobules are not present (Figure 3). These observations are all in line with the geometric morphometric analyses reported by Aroca et al. (2022).

FIGURE 3 Cephalofoil comparison (dorsal and ventral views of each specimen are provided). Bar scale: 5cm. TL: Total length of specimen. A) *S. alleni* sp. nov.; B) *S. tiburo*; C) and *S. vespertina*. Photos: Cindy Gonzalez.

Meristic Analysis. PCA analysis was run with three variables (PVC number of precaudal vertebrae, UT- upper functional teeth row, and LT- lower functional teeth row). The purpose of the analysis is to obtain a small number of linear combinations of the three variables, which account for most of the variability in the data. In this case, one component was extracted since only one component had an eigenvalue greater than or equal to 1.0. It accounted for 41.1213% of the variability in the original data.

The DFA of the meristic characters was also run with the three variables PVC, UT, and LT and was used to classify the meristic characters into two groups corresponding to *S. tiburo* and *S.* aff. *tiburo*. The rows of the UT and the LT were 25 in average for both species therefore this character was not useful to discriminate between species. The size adjusted data DFA identified the number of precaudal vertebrae as the only discriminator of *S. tiburo* and *S.* aff. *tiburo* (Figure 4), with 100% of specimens classified correctly (Table 2).

Species	**Size (n)**	**Species assigned**	
		S.* aff. *tiburo	***S. tiburo***
S.* aff. *tiburo	12	12	0
		100.00%	0.00%
S. tiburo	11	0	11
		0.00%	**100.00%**

TABLE 2 Discriminant Function Analysis (DFA) of the percent of cases/specimens correctly classified: 100.00%.

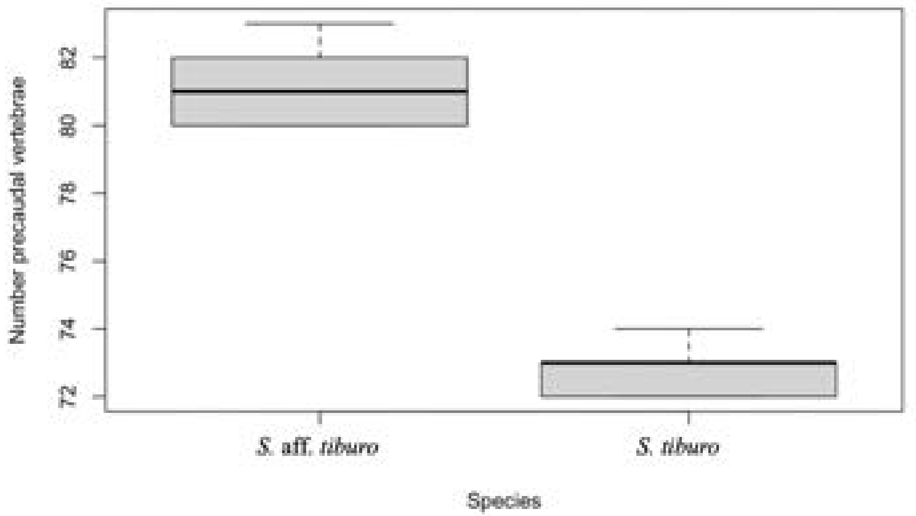

FIGURE 4 Boxplot of the number of precaudal vertebrae for *S.* aff. *tiburo*. (Belize, N = 12) and *S. tiburo* (Florida, U.S, N = 11) specimens.

Mitochondrial DNA. The neighbor joining tree based on the concatenated 1,142 bp sequence (COI + mtCR) revealed *S. tiburo* and *S.* aff. *tiburo* to be reciprocally monophyletic with 89% bootstrap support (Figure 5).

Microsatellites. Twelve microsatellite loci were successfully amplified for *S. tiburo* (n= 96) and *S.* aff. *tiburo* (n= 60). After excluding individuals with >15% missing genotypes, 117 samples were included in our analyses: S. tiburo (n= 63), and S. aff. tiburo (n= 54). No evidence of null alleles was detected. The number of alleles ranged from 2 (locus Spti4) to 16 (locus Spti41) for S. tiburo, and between two (locus Spti44) and 16 (locus Spti48) for S. aff. tiburo. Genetic diversity values including the observed HO and expected heterozygosities HE, were obtained for all loci and by locality across all loci (Table 3), along with deviations from HW equilibrium (Table 4). Allele frequencies only peripherally overlapped, and most alleles were unique to putative species (Figure 6).

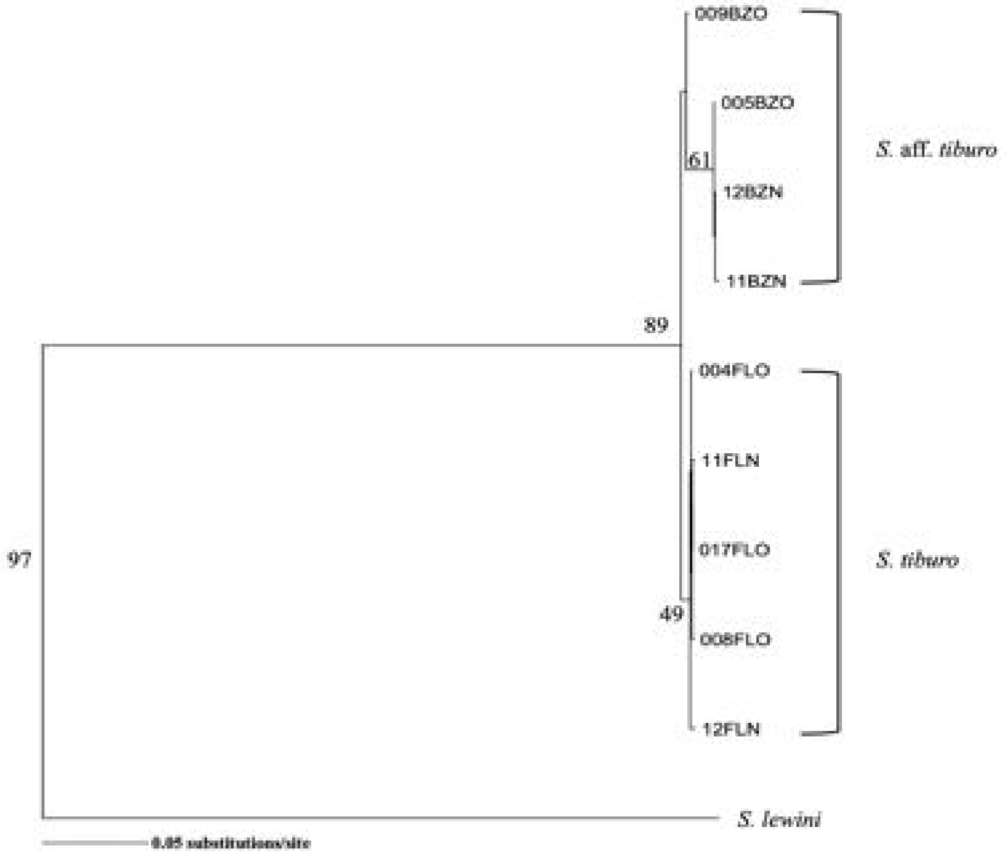

FIGURE 5 Neighbor joining concatenated tree (COI + mtCR) for *S.* aff. *tiburo* and *S. tiburo*. Branch lengths and scale represent the proportion of polymorphic sites between haplotypes, and bootstrap values given in percentage (%).

The K values calculated by STRUCTURE using the Evanno method identified *K*= 2 as the most likely number of clusters in the data, clearly separating the *S. tiburo* and *S.* aff. *tiburo* as distinct gene pools (Figure 7). Pairwise F_{ST} values and *Jost's D* values were calculated and significant population differentiation was detected in all cases except between NC and FL (F_{ST}) and BZ and BDT (*Jost's D*) (Table 5).

TABLE 3 Genetic diversity per population across all loci. N= total sample size, H_O = observed and H_E expected heterozygosities, F_{IS}= inbreeding coefficient.

Species	Locality	N	H_O	H_E	F_{IS}
S. tiburo	FL	38	0.594	0.765	**0.177*****
	NC	13	0.652	0.766	**0.085***
	SC	12	0.613	0.760	**0.157****
S.* aff. *tiburo	BDT	15	0.658	0.718	0.058
	BR	7	0.597	0.747	**0.167****
	BZ	32	0.688	0.755	0.005

H_O, H_E, and F_{IS} were calculated keeping one representative per MLG and per sampling site. With the F_{IS} is indicated the test significance for deviation to Hardy–Weinberg equilibrium: $p < 0.05$*; $p < 0.01$**; and $p < 0.001$***. Localities: Florida, U.S (FL), North Carolina, U.S (NC), South Carolina, U.S (SC), Bocas del Toro, Panama (BDT), Brazil (BR), Belize (BZ).

TABLE 4. Genetic diversity for 12 microsatellite loci for *S. tiburo* and *S.* aff. *tiburo*.

		S. tiburo (n=63)									*S.* aff. *tiburo* (n=54)								
		FL (n=38)			**NC (n=13)**			**SC (n=12)**			**BDT (n=15)**			**BR (n=7)**			**BZ (n=31)**		
Locus	***A***	***nA***	H_O	H_E	***nA***	H_O	H_E	***nA***	H_O	H_E	***nA***	H_O	H_E	***nA***	H_O	H_E	***nA***	H_O	H_E
Spti2	10	10	0.71	0.87	9	0.92	0.89	6	0.80	0.80	8	**0.73**	**0.88**	8	**0.57**	**0.90**	10	0.87	0.88
Spti4	4	3	0.11	0.13	2	0.08	0.08	1	0	0	5	**0.85**	**0.73**	3	**0.20**	**0.64**	6	**0.60**	**0.77**
Spti9	6	5	0.63	0.70	5	0.77	0.79	5	0.83	0.81	3	0.13	0.13	1	0	0	3	0.22	0.23
Spti3	7	7	0.79	0.77	5	0.77	0.82	5	0.67	0.67	7	0.71	0.72	7	0.86	0.90	8	0.74	0.84
Spti10	14	13	0.84	0.87	12	1.00	0.92	7	0.75	0.74	5	**0.80**	**0.78**	5	0.80	0.87	8	**0.64**	**0.85**
Spti34	10	8	**0.55**	**0.84**	8	**0.45**	**0.90**	4	0.75	0.74	4	0.53	0.61	3	0.43	0.38	5	0.79	0.63
Spti44	7	7	**0.36**	**0.78**	5	**0.50**	**0.80**	4	**0.08**	**0.58**	2	0.50	0.49	2	0.00	0.26	3	0.45	0.52
Spti48	17	15	**0.69**	**0.92**	12	**0.67**	**0.95**	9	**0.67**	**0.90**	10	**0.57**	**0.89**	10	0.86	0.95	16	**0.58**	**0.94**
Spti42	11	11	0.89	0.89	7	0.91	0.88	7	0.88	0.82	9	0.80	0.82	7	0.86	0.89	10	0.87	0.87
Spti46	5	5	**0.35**	**0.75**	3	0.46	0.55	4	**0.08**	**0.75**	7	0.93	0.84	5	0.71	0.82	7	0.81	0.81
Spti26	8	8	0.65	0.77	5	0.69	0.73	6	0.64	0.69	6	**0.47**	**0.84**	4	0.57	0.70	8	0.69	0.81
Spti41	16	16	**0.56**	**0.90**	9	**0.60**	**0.89**	8	**0.60**	**0.88**	9	0.87	0.88	8	0.71	0.89	13	1.00	0.90

U.S locations: FL: Florida; NC: North Carolina; SC: South Carolina; Caribbean: BDT: Bocas del Toro, Panama; BR: Maranhao, Brazil; BZ: Placencia, Belize. *n*: samples per location; *A*: number of alleles across all populations; *n*A: number of alleles at each locus; H_O and H_E observed and expected heterozygosity. Loci out of Hardy Weinberg equilibrium shown in bold before Bonferroni correction (P<0.05), and in bold highlighted in grey for loci out of HW equilibrium after Bonferroni correction (P<0.001) for *S. tiburo* and (P<0.001) for *S.* aff. *tiburo.*

TABLE 5 Pairwise F_{ST} values (above diagonal) and *Jost's D* values (below diagonal) for the microsatellites analyzed. Western Atlantic, U.S: Florida (FL), North Carolina (NC), South Carolina (SC). Caribbean and Southwestern Atlantic: Bocas del Toro, Panama (BDT), Brazil (BR), and Belize (BZ).

F_{ST} *Jost's D*	**FL (n=38)**	**NC (n=13)**	**SC (n=12)**	**BDT (n=15)**	**BR (n=7)**	**BZ (n=31)**
FL	AGD= 0.6884 +/- 0.3575	0.0116	**0.0344*****	**0.1572*****	**0.1553*****	**0.1366*****
NC	0.0204	AGD= 0.6943 +/-0.3794	**0.0322****	**0.1701*****	**0.1579*****	**0.1547*****
SC	**0.0997*****	0.0708	AGD= 0.6393 +/- 0.3535	**0.2176*****	**0.20389****	**0.1931*****
BDT	**0.5287*****	**0.5689*****	**0.6546*****	AGD= 0.6709 +/- 0.3566	**0.0724*****	0.0055
BR	**0.4805*****	**0.4709*****	**0.5727*****	**0.1922*****	AGD= 0.6703 +/- 0.3777	**0.0621*****
BZ	**0.4886*****	**0.5363*****	**0.6089*****	0.0117	**0.1695****	AGD= 0.6405 +/- 0.3378

Significant P values at <0.005*, <0.002** and < 0.001***. Probability values based on 10,000 permutations. Significant P values (P <0.002 after Bonferroni correction) in bold. Average genetic diversity values (AGD) are shown in the diagonal for each locality. Numbers of samples of each locality in parentheses.

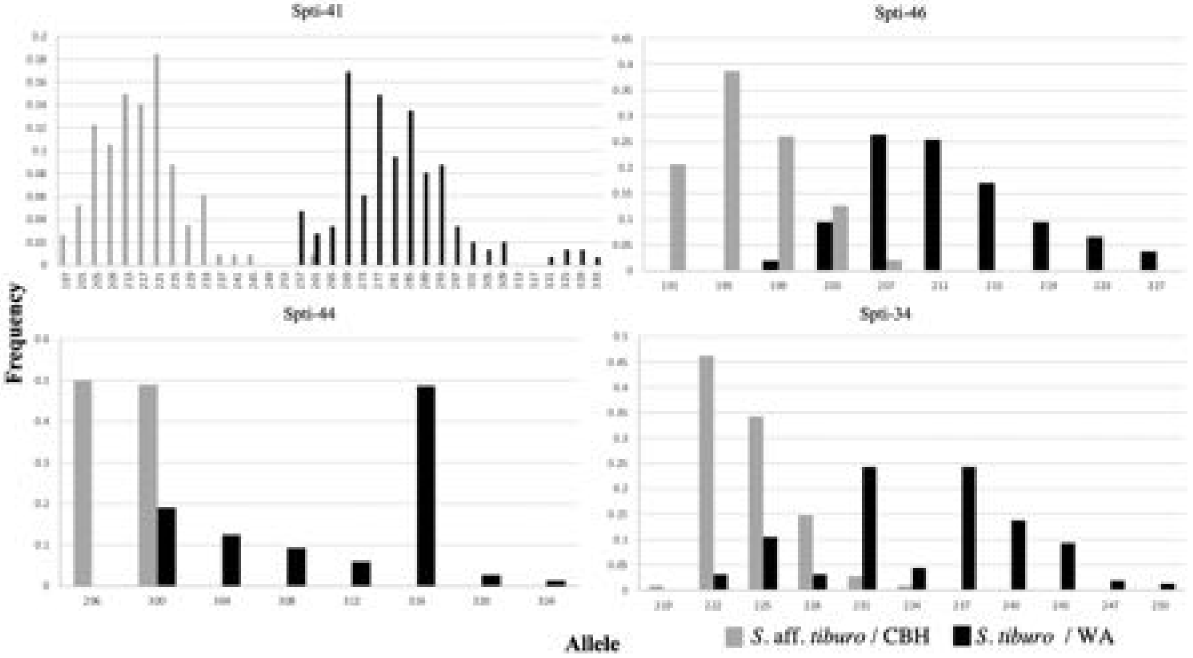

FIGURE 6 Allele frequencies for four microsatellite loci: Spti 41, Spti 44, Spti 46, and Spti 34. Grey bars represent *S.* aff. *tiburo* and black bars represent *S. tiburo* specimens analyzed (See supplementary materials: S2 for the other eight loci analyzed).

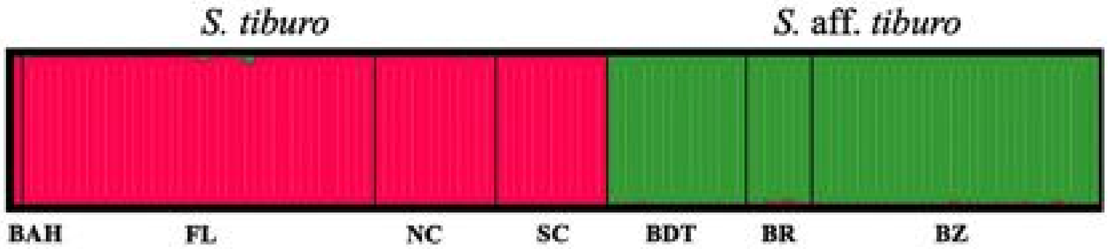

FIGURE 7. STRUCTURE bar plot showing the assignment probabilities (*K*=2) of each genotyped individual of *S. tiburo* and *S.* aff. *tiburo* from seven different localities. BAH: Bahamas; FL: Florida, U.S; NC: North Carolina, U.S; SC: South Carolina, U.S; BDT: Bocas del Toro, Panama; BR: Maranhão State, Brazil; BZ: Placencia, Belize. *K*-groupings correspond to: Red: Western Atlantic (Bahamas and U.S locations: North Carolina South Carolina, U.S); and Green: Southwestern Atlantic and the Caribbean (Bocas del Toro, Panama; Maranhão State, Brazil; and Placencia, Belize).

***Sphyrna alleni* sp. nov.**

Family: Sphyrnidae, Carcharhiniformes; Genus *Sphyrna,* Gilbert 1967.

Sphyrna tiburo Bigelow & Schroeder, 1948; Gilbert, 1967; Compagno, 1973, 1979, 1988.

Sphyrna cf. *tiburo* Naylor, 2012; Fields, 2016; Gonzalez, 2019; Gonzalez, 2021.

Sphyrna aff. *tiburo* Gonzalez, 2021; Aroca, 2022.

Proposed common name. Shovelbill Shark (EN), Caraïbe Bec-selle Requin-marteau (FR), Tiburón Cabeza de Pala del Caribe (SP) (Figure 8).

Materials examined. *Sphyrna alleni* sp. nov. specimens were collected at Robinson Point and Riversdale Village, Belize, and were provided by local fishermen in 2016 and 2019. *S. tiburo* specimens were collected in Panama City, Terra Ceia Bay, and Sarasota Bay, FL between 2015 - 2021 and provided by Dr. J. Carlson from NOAA and Dr. J Gardiner from New College of Florida. Type specimens were placed in the collection of the Florida Museum of Natural History (see Table 1 for details).

Holotype. UF 245705, female, 103 cm TL. Placencia, Riversdale, Belize; Collected: February 2019 by C. Gonzalez (Figure 9).

Paratypes. All from Placencia, Riversdale, Belize., and collected by C. Gonzalez in February 2019. UF 245723, male, TL 60.5 cm; UF 245724, female, 83 cm; UF245725, female, TL 71.5 cm; UF245726, male, TL 63 cm, UF245727, male, 64.5 cm.

Non-Types. Six *S. alleni* sp. nov., sharks collected by D. Chapman and local fishermen in 2016 at Robinson Point, Riversdale, Belize, decayed after fixing them with 10% formalin, probably because the meat was decomposed after shipping delays. After taking the measurements and correspondent genetic samples, the vertebrae and the jaws were kept frozen at the Mote Marine Laboratory & Aquarium, Sarasota, FL.

Diagnosis. Small hammerhead shark (<150 cm at maturity) with a flat, shovel shaped head that lacks indentations on its anterior edge. Cephalofoil anterior margin is pointed (like a tringle) in both sexes and the posterior margins are lobule shaped. The anterior margin of males exhibits a pronounced bulge. Enlarged, molariform posterior teeth, first dorsal rear tip in front of pelvic origins, and shallowly concave posterior anal margin. It is distinct from *S. tiburo* because in this species the anterior margin of the head is more rounded and the lobules on the posterior margin are not present. Precaudal vertebral counts for *S. alleni* sp. nov., are between 80-83 (~10 more vertebrae than *S. tiburo*).

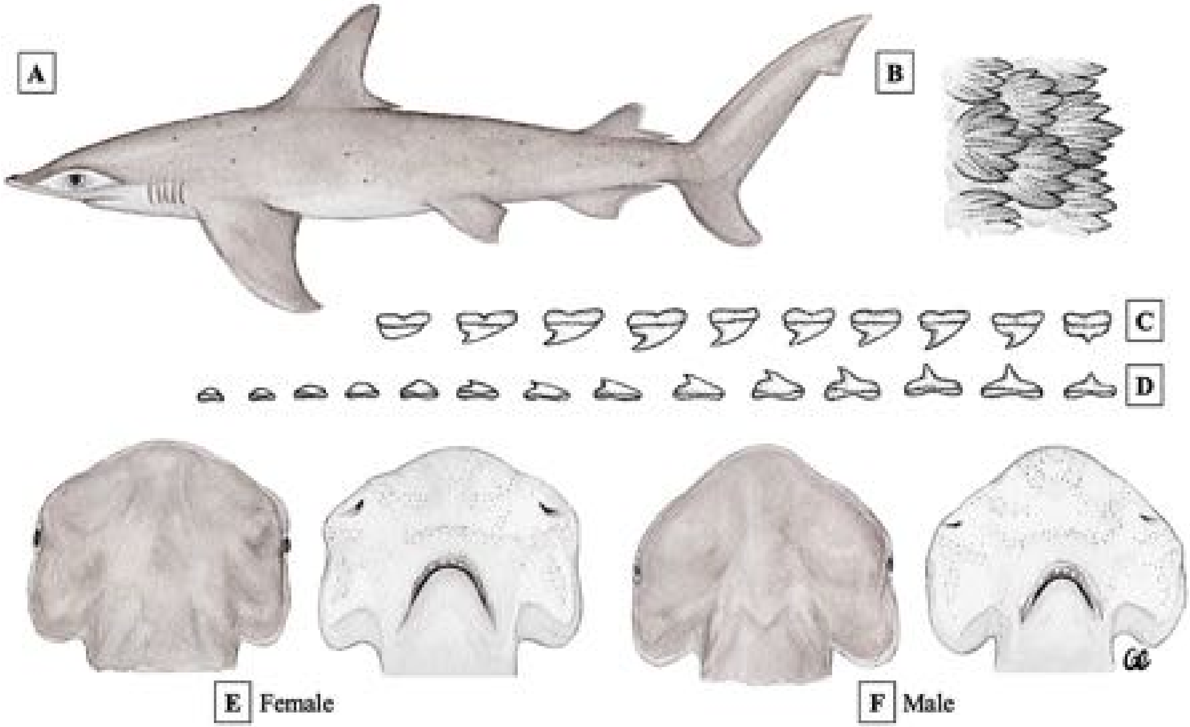

FIGURE 8 *Sphyrna alleni* sp. nov. A) Horizontal view of the shark; B) dermal denticles; C) upper and D) lower teeth; Dorsal and ventral representation of the head shape of E) Female and F) Male. Illustrations: Gina Clementi.

TABLE 6 Proportional measurements for *S. alleni*. sp. nov., and *S. tiburo*. ANOVAs values are followed by p-values in parentheses. Values not reported when the data was non normal distributed. When the data was non normally distributed a Kruskal-Wallis (K-W) test was performed and p-values are reported in parentheses.

	Morphological Character		***n***	**Mean (cm)**	**(S.D)**	**Range**	**ANOVA**	**K-W test**
1	**Head length**	*S. alleni*	12	16.48	2.13	13.4 - 19.71	0.488 (0.49)	
		S. tiburo	11	16.15	1.53	12.5 - 18		
2	**Pre-first dorsal length**	*S. alleni*	12	24.81	4.31	19.5 - 35	3.673 (0.07)	
		S. tiburo	11	23.75	2.41	18.4 - 27.3		
3	**Pre-second dorsal length**	*S. alleni*	12	50.02	8.92	38 - 68.5	3.717 (0.07)	
		S. tiburo	11	48.4	5.19	36 - 54.5		
4	**Pre-pectoral length**	*S. alleni*	12	16.31	2.24	13.5 - 21	0.805 (0.38)	
		S. tiburo	11	15.9	1.49	12.2 - 17		
5	**Pre-pelvic length**	*S. alleni*	12	36.58	6.65	28 - 51	3.289 (0.08)	
		S. tiburo	11	35.12	3.69	26.7 - 40.9		
6	**Preanal length**	*S. alleni*	12	47.25	8.51	36 - 65	0.359 (0.55)	
		S. tiburo	11	46.59	4.99	35.3 - 54.2		
7	**Snout-vent length**	*S. alleni*	12	38.85	7.51	28.8 - 54.5		6.367 (0.012)*
		S. tiburo	11	36.33	4.69	27.5 - 45.2		
8	**Inter-dorsal space**	*S. alleni*	12	19.08	3.53	14.2 - 26.2	0.877 (0.36)	
		S. tiburo	11	18.40	2.46	12.5 - 21.5		
9	**Second dorsal-caudal space**	*S. alleni*	12	6.06	1.16	4.5 - 8.2	24.73 (6.39e-05) ***	
		S. tiburo	11	6.55	0.66	5.1 - 7.7		
10	**Pectoral-pelvic space**	*S. alleni*	12	17.18	3.53	12 - 23.6		7.670 (0.005)
		S. tiburo	11	15.82	2.06	12 - 18		
11	**Pelvic-anal space**	*S. alleni*	12	7.02	1.69	5 - 10.1	0.635 (0.43)	
		S. tiburo	11	7.30	1.40	5 - 9.5		
12	**Anal-caudal space**	*S. alleni*	12	4.60	0.86	3.5 - 6	31.54 (1.42e-05) ***	
		S. tiburo	11	5.63	0.66	3.9 - 6.36		

13	**Pelvic-caudal space**	*S. alleni*	12	16.80	2.68	13.2 - 21.2	8.093 (0.009) **	
		S. tiburo	11	17.75	1.99	13.8 - 20.4		
14	**Vent-caudal length**	*S. alleni*	12	40.91	6.17	32.1 - 53		6.368 (0.012)*
		S. tiburo	11	35.54	6.00	24.7 - 43.8		
15	**Head width**	*S. alleni*	12	13.17	1.44	10.97 - 15.3	14.9 (0.000) ***	
		S. tiburo	11	12.31	1.01	10.45 - 14.1		
16	**Snout length**	*S. alleni*	12	5.63	0.75	4.68 - 7.2	5.188 (0.033) *	
		S. tiburo	11	5.31	0.44	4.42 - 6		
17	**Eye length**	*S. alleni*	12	1.40	0.21	1.1 - 1.74		0.640 (0.428)
		S. tiburo	11	1.38	0.08	1.25 - 1.55		
18	**Eye height**	*S. alleni*	12	1.01	0.14	0.85 - 1.35	0.066 (0.8)	
		S. tiburo	11	1.02	0.13	0.7 - 1.15		
19	**Internarial space**	*S. alleni*	12	9.05	1.13	7.61 - 11.5	46.17 (1.02e-06) ***	
		S. tiburo	11	8.32	0.65	6.8 - 9.3		
20	**Nostril length**	*S. alleni*	12	1.28	0.72	0.78 - 3.53	19.64 (0.000) ***	
		S. tiburo	11	1.05	0.06	1 - 1.2		
21	**Mouth width**	*S. alleni*	12	5.26	1.09	3.79 - 7.68	0.002 (0.962)	
		S. tiburo	11	5.13	0.54	4.1 - 6		
22	**Mouth length**	*S. alleni*	12	3.33	0.55	2.55 - 4.32	0.493 (0.49)	
		S. tiburo	11	3.20	0.34	2.7 - 3.75		
23	**Intergill length**	*S. alleni*	12	3.99	0.93	2.85 - 6.55		0.0038 (0.951)
		S. tiburo	11	3.82	0.50	3.2 - 4.6		
24	**First gill slit height**	*S. alleni*	12	2.24	0.44	1.54 - 2.96	2.968 (0.09)	
		S. tiburo	11	2.05	0.28	1.5 - 2.67		
25	**Second gill slit length**	*S. alleni*	12	2.45	0.41	1.78 - 3.12		3.640 (0.06)
		S. tiburo	11	2.27	0.29	1.8 - 2.9		
26	**Third gill slit length**	*S. alleni*	12	2.53	0.42	1.97 - 3.57	0.053 (0.82)	
		S. tiburo	11	2.48	0.23	2.13 - 3		
27	**Forth gill slit length**	*S. alleni*	12	2.54	0.48	1.85 - 3.34	0.221 (0.64)	

		S. tiburo	11	2.55	0.27	2.23 - 3.15		
28	**Fifth gill slit length**	*S. alleni*	12	2.27	0.49	1.6 - 3.22	0.078 (0.78)	
		S. tiburo	11	2.19	0.33	1.85 - 2.82		
29	**Pectoral anterior margin**	*S. alleni*	12	11.55	2.06	8.6 - 15.3	4.563 (0.04) *	
		S. tiburo	11	11.67	0.98	9.62 - 12.7		
30	**Pectoral base**	*S. alleni*	12	4.35	0.97	2.95 - 6.3		1.670 (0.19)
		S. tiburo	11	4.32	0.40	3.5 - 4.9		
31	**Pectoral inner margin**	*S. alleni*	12	4.20	0.73	3.05 - 5.35	0.073 (0.79)	
		S. tiburo	11	4.15	0.53	3 - 4.8		
32	**Pectoral posterior margin**	*S. alleni*	12	9.80	2.08	7.07 - 13.44	4.071 (0.05)	
		S. tiburo	11	9.02	0.90	7.67 - 10.4		
33	**Pectoral height**	*S. alleni*	12	10.44	2.08	7.45 - 14.69		3.409 (0.06)
		S. tiburo	11	9.74	0.97	8 - 11.2		
34	**First dorsal anterior margin**	*S. alleni*	12	12.93	2.12	10.12 - 17.2	7.188 (0.01) *	
		S. tiburo	11	12.19	1.07	9.8 - 14		
35	**First dorsal base**	*S. alleni*	12	6.52	1.25	4.83 - 9.15	0.001 (0.97)	
		S. tiburo	11	6.38	0.85	4.8 - 7.56		
36	**First dorsal inner margin**	*S. alleni*	12	4.65	2.69	2.65 - 12.45	0.192 (0.66)	
		S. tiburo	11	4.02	0.87	2.83 - 5.55		
37	**First dorsal posterior margin**	*S. alleni*	12	9.12	1.74	6.5 - 12.2	0.058 (0.81)	
		S. tiburo	11	9.01	1.01	6.7 - 10.3		
38	**First dorsal height**	*S. alleni*	12	9.84	2.17	6.32 - 12.33	0.254 (0.62)	
		S. tiburo	11	9.38	1.08	7.1 - 10.71		
39	**Second dorsal anterior margin**	*S. alleni*	12	4.89	1.40	3 - 8.75		0.969 (0.325)
		S. tiburo	11	4.87	0.55	3.87 - 5.83		
40	**Second dorsal base**	*S. alleni*	12	2.80	0.44	1.95 - 3.44	2.337 (0.14)	
		S. tiburo	11	3.03	0.58	2 - 4		
41	**Second dorsal inner margin**	*S. alleni*	12	4.18	0.69	3.03 - 5.35	4.316 (0.050)	
		S. tiburo	11	4.37	0.47	3.35 - 4.95		

42	**Second dorsal posterior margin**	*S. alleni*	12	4.61	0.81	3.35 - 6.2	2.514 (0.13)	
		S. tiburo	11	4.81	0.72	3.6 - 5.8		
43	**Second dorsal height**	*S. alleni*	12	3.45	0.77	2.25 - 4.44	0.238 (0.63)	
		S. tiburo	11	3.47	0.55	2.4 - 4.2		
44	**Pelvic anterior margin**	*S. alleni*	12	5.52	0.93	4 - 7	0.219 (0.64)	
		S. tiburo	11	5.51	0.74	4.3 - 6.48		
45	**Pelvic base**	*S. alleni*	12	4.44	0.91	2.66 - 6.13	0.058 (0.81)	
		S. tiburo	11	4.39	0.67	3.43 - 5.52		
46	**Pelvic inner margin**	*S. alleni*	12	2.99	0.47	2.13 - 4	2.41 (0.14)	
		S. tiburo	11	3.21	0.42	2.45 - 3.8		
47	**Pelvic posterior margin**	*S. alleni*	12	4.93	0.94	3.49 - 6.88	1.737 (0.20)	
		S. tiburo	11	4.60	0.48	3.56 - 5.14		
48	**Pelvic height**	*S. alleni*	12	4.43	1.13	2.52 - 6	0.957 (0.34)	
		S. tiburo	11	4.57	0.73	3.5 - 5.66		
49	**Anal anterior margin**	*S. alleni*	12	3.41	0.72	2.38 - 5.05	2.993 (0.09)	
		S. tiburo	11	3.61	0.43	2.86 - 4.3		
50	**Anal base**	*S. alleni*	12	5.38	0.95	3.85 - 7.07	1.947 (0.18)	
		S. tiburo	11	5.06	0.64	4 - 5.8		
51	**Anal inner margin**	*S. alleni*	12	3.05	0.48	2.23 - 3.9		2.004 (0.157)
		S. tiburo	11	2.81	0.56	1.6 - 3.42		
52	**Anal posterior margin**	*S. alleni*	12	5.96	1.04	4.32 - 7.75	3.894 (0.062)	
		S. tiburo	11	5.46	0.79	4.22 - 6.57		
53	**Anal height**	*S. alleni*	12	2.32	0.67	1.41 - 3.5	0.112 (0.742)	
		S. tiburo	11	2.34	0.70	1.46 - 3.85		
54	**Dorsal caudal margin**	*S. alleni*	12	20.73	2.73	17.2 - 26.3	3.787 (0.06)	
		S. tiburo	11	19.94	1.62	16.3 - 21.5		
55	**Pre-ventral caudal margin**	*S. alleni*	12	8.28	1.20	6.57 - 10.54	9.828 (0.005) **	
		S. tiburo	11	7.59	0.91	6.05 - 9.33		
56	**Upper post-ventral caudal margin**	*S. alleni*	12	12.72	1.88	10.27 - 16.3	24.87 (6.18e-05) ***	

		S. tiburo	11	11.18	1.27	9 - 12.5		
57	**Lower post-ventral caudal margin**	*S. alleni*	12	3.78	0.75	3 - 5.55	0.732 (0.40)	
		S. tiburo	11	3.57	0.58	2.7 - 4.9		
58	**Caudal fork length**	*S. alleni*	12	5.83	0.72	4.72 - 6.66	4.737 (0.04) *	
		S. tiburo	11	5.50	0.58	4.3 - 6.4		
59	**Caudal fork width**	*S. alleni*	12	5.48	0.83	4.35 - 7.25	0.136 (0.72)	
		S. tiburo	11	5.47	0.50	4.7 - 6.5		
60	**Subterminal caudal margin**	*S. alleni*	12	2.42	0.42	1.97 - 3.4		0.136 (0.712)
		S. tiburo	11	2.15	0.36	1.35 - 2.52		
61	**Terminal caudal margin**	*S. alleni*	12	4.11	0.79	2.15 - 5.22		0.852 (0.356)
		S. tiburo	11	4.23	0.62	3.1 - 4.85		

Description. Proportional measurements expressed as a percentage of the total length (TL) are given for the specimens analyzed (Table 6).

Body slender; expanded and narrow pre-branchial head that is shovel-shaped, no indentation on the anterior margin, tip of the head with a triangular contour shape (Figure 8). Head dorsoventrally flattened and narrow but longitudinally elongated, measuring from 19 to 22% of total length (TL) in the specimens analyzed. Distance from tip of snout to mouth about a 34-36% of the head length; posterior margins of head short, forming a lobule shape with a pronounced angular termination; pre-narial grooves not present; mucous pores on the ventral side and on the top of the head distributed in a diffuse pattern, pores highly concentrated in the tip of snout, and surrounding the eyes. There is sexual dimorphism in the individuals, so males have a more pronounced triangular termination of the head than females that have a slightly more rounded head (Figure 3). Eyes oval, small, located at the lateral sides of the head with a diameter of about 1.2 cm (1.2- 1.5% of TL). Nostrils small, apertures transversely oval to tear shaped; internarial space about 66% of the total head width (11% of TL). Prominent rostral cartilage on the top of the head. Mouth about a 40% of the head width, broadly arched; symphysis of mouth; 25 teeth in average in the upper and lower rows of functional teeth; anterior teeth not serrated with short, smooth-edged cusps; posterior molar teeth with a broader basis, flattened, cusp-less, keeled, expanded, and rounded; lower teeth shorter than uppers. Labial furrow around corner of mouth on the lower jaw, but none on the upper jaw (Figure 3).

First dorsal moderately hooked or curved like a sickle on its anterior margin, its origin over the end of the inner margins of pectoral fins, vertical height of the dorsal fin about 60% and base about a 40% of the length of head; posterior margin slightly curved with very small serrations on the end, its free rear tip usually somewhat anterior to pelvic origins. Interdorsal space about 25%

of the total length. Second dorsal fin small, moderately high, height is about 35% of the dorsal fin height, one centimeter less than anal fin, concave posterior margin like an “L” shape; inner margin moderately long, longer than fin height. Five gill openings extended after the posterior head lobules, evenly spaced; each slit increases a little bit after the first one, each slit is about 2.2-2.5 cm in length. Pectoral fins originate just after the gill openings, about 74% of the head length and proportional to 14% of the total length. Pelvic fins slightly curved at the corners, with posterior margins almost straight to slightly curved. Anal fins larger and longer than second dorsal fin; base of anal fin is 6.8 to 7.8% of the total length, its origin well in front of second dorsal origin; posterior margin shallowly concave. Caudal fin long about 25% - 28% of the total length; well defined precaudal pit; lower lobule of the fork about 1/3 (36 - 40%) of the upper lobule; upper margin and subterminal caudal margin straight, fork width about ¼ (26%) of total caudal fin length. Precaudal vertebrae counts between 80 to 83, ten or nine vertebrae more than *S. tiburo* (72-74).

Teeth counts of the functional rows for the upper and lower jaw were 25 in average for each row. When compared to *S. tiburo* no significant differences in shape or structure of the jaws were found. The *S. alleni* sp. nov., sharks have anterior teeth that are pointier, narrower, and more sharpened than *S. tiburo,* that have wider teeth. Also, *S. alleni* sp. nov., the anterior and posterior teeth seem to be bigger than *S. tiburo*. Lower jaw: *S. alleni* sp. nov., have fewer posterior teeth without cusps with a flatter crown foot, fewer rows of teeth exposed (3-2 rows), and are more spaced than *S. tiburo*. Contrary, the arrangement of posterior teeth in *S. tiburo* is a clumped pattern and more rows of teeth are exposed (5-3 rows). Upper jaw: *S. alleni* sp. nov., have only one column of posterior teeth without cusps and flattened crown foots. The second column of teeth have a small cusp developed, that starts to get more prominent until the anterior teeth with well-defined cusp ends. In comparison, the upper jaws of *S. tiburo* have 3 columns of posterior teeth that are cusp-less and flattened. Only in the 4th column of posterior teeth the cusps start to develop, but they are not as pointed and sharp as the new species. Dermal denticles with 5 ridges imbricated or loosely spaced in the body (Figure 8).

Coloration. Color after preservation varies from light brown to dark gray; the ventral side tends to become "yellower" with formalin and keeps its original color if preserved in ethanol (Figure 9). In life color varies between gray or gray-brown on the dorsal side; white, or light beige on the ventral side, often with small dark spots on lateral sides of the body (Figure 10).

FIGURE 9 Holotype of *Sphyrna alleni* sp. nov. collected in Riversdale, Belize. UF 245705, Female, 103 cm TL. Photo: Zachary Randall, Digital Imaging Division, Florida Museum.

Distribution range. *S. alleni* sp. nov. is distributed in coastal waters, estuaries, coral reefs, seagrass beds, and sand bottoms from Belize to Brazil. Presence has been confirmed in the Caribbean in Belize, Panama, Colombia, Trinidad, and Tobago, and in the southwestern Atlantic in Brazil. *S. tiburo* is distributed from North Carolina, U.S to Belize where a potential contact zone for the two species could occur between Mexico and Belize (Yucatan Peninsula).

Etymology. *S. alleni* sp. nov., is named after Paul G. Allen (1953-2018) who was an investor, co-founder of Microsoft, and philanthropist who, through the Paul G. Allen Family Foundation, has generously supported shark research and conservation.

Comparative material. Eleven *S. tiburo* specimens from Sarasota Bay, FL, U.S were used. From those, six specimens were fixed and kept at the Florida Museum of Natural History. UF 247324 (n=3): female, TL 86; female, TL 74.5; female, TL 82, Collected 07/20/20; UF 247325 (n=2): male, TL 80.5; male, TL 73.5, Collected 10/13/20; UF 247326 (n=1): male (Figure 10), TL 59.5 Collected 08/18/20, all specimens collected by J. Gardiner. The other five specimens were measured, genetic samples were taken, and the vertebrae and teeth were cleaned and counted by hand. The vertebrae and jaws were kept frozen at the Predator Ecology and Conservation Lab at Florida International University. They correspond to the following individuals: FLO-017, male, TL 82.3 cm; FLO-023, female, TL 71.6; FLO-004, male TL 83.3; FLO- 008, female TL 82cm; FLO-006, male, TL 76 cm, collected by J. Carlson from NOAA in 2015 in Panama City, FL.

FIGURE 10 Paratype. External morphology and coloration of a fresh specimen of *Sphyrna alleni* sp. nov., collected in Placencia, Belize. UF: 247326, Male, 64.5 cm TL. Photos: Cindy Gonzalez.

Discussion

Here, we present multiple lines of evidence confirming that *S. tiburo* and *S. alleni* sp. nov., are distinct species. We reconfirm that they are reciprocally monophyletic when examining mitochondrial DNA, and they also separate into two gene pools when examining 12 nuclear microsatellites, with numerous private alleles observed (i.e., alleles unique to one species or the other). Although none of the morphometric measurements were able to separate them, visual inspection reveals a more pointed anterior margin of the cephalofoil in *S. alleni* sp. nov., which is aligned with previous geometric morphometric analysis (Aroca *et al.* 2022). This species is also separated from *S. tiburo* by non-overlapping precaudal vertebral counts, which is a common meristic trait used to classify closely related and cryptic shark species (Ebert & Compagno 2009; Quattro *et al.* 2013; Springer & Garrick 1964). We propose the new species: *Sphyrna alleni* sp. nov., with the common name 'Shovelbill', to acknowledge the name it is referred to by the people of Belize where the holotype was collected.

Spyrna tiburo is distributed from North Carolina, U.S to Belize, while *S. alleni* sp. nov*.,* is distributed from Belize to Brazil (Gonzalez *et al.* 2021). Major geological changes took place in Belize during the late Pliocene to the Holocene including sea-level fluctuations and changes in the carbonate platform (Mazzullo 2006). These events could have separated bonnetheads in this region and enabled speciation to occur. Indeed, vicariant events caused by sea-level changes (maximum 130 m) during this geological time have been recognized as responsible for speciation in several plant and animal lineages in the Mesoamerican reef (Briggs & Bowen 2013), including sharks (Domingues *et al.* 2018, 2019). There is no contemporary geophysical barrier preventing contact between the two lineages and more sampling between Belize and Mexico (Yucatan Peninsula) could reveal the extent of sympatry and possibly hybridization between these species, as has been seen in other shark species complexes (Barker *et al.* 2019; Morgan *et al.* 2012). However, according to a relaxed molecular clock calculated by Fields et al., (2016) these species diverged between 3·61 and 5·62 Mya., which means they could have diverged prior to the uplifting of the Isthmus of Panama (Montes *et al.* 2015; O'Dea *et al.* 2016). Given some similarity in cephalofoil shape in *S. alleni* sp. nov. and *S. vespertina* it is possible that they are sister lineages and *S. tiburo* diverged from them as it expanded into the subtropical and temperate Atlantic, with a later separation of *S. vespertina* and the incipient *S. alleni* sp. nov. by the Isthmus closure.

The phylogenetic and head morphology reconstruction from Aroca *et al.* (2022) also indicates that *S. tiburo* is a species complex composed of at least three distinguishable entities: two in the Western Atlantic: a southern central lineage (Caribbean), and a north- western one (i.e., Gulf of Mexico, Florida, South and North Carolina), and one in the eastern Pacific (Aroca *et al.* 2022). Detailed phylogenetic analysis of this complex is required to better understand their evolutionary history. We also highlight the need for further meristic and genetic investigation into *S. vespertina* to resurrect its species status and provide a fuller description.

Conclusion

Bonnetheads are currently assessed as Globally 'Endangered' by the International Union for the Conservation of Nature (IUCN) but they have been assessed as one amphi-American species. The assessment highlights that the species is well managed in higher latitude parts of its Northern Hemisphere Atlantic range (U.S., Bahamas) but heavily fished and poorly managed elsewhere, with evidence of population collapse in Brazil and throughout much of the Tropical Eastern Pacific (Cardeñosa *et al.* 2020; Harper *et al.* 2014; Pérez-Jiménez 2014; Reis-Filho *et al.* 2014). Reevaluating this assessment considering the geographic distribution of *S. tiburo* and *S. alleni* sp. nov. is now warranted. Given how fishing and management is distributed it is likely that the IUCN status of *S. tiburo* would improve and *S. alleni sp. nov.* would warrant a highly threatened status. Greater management attention is necessary to rebuild populations of *S. alleni* sp. nov., which could take the form of restrictions on gillnets and trawls as these gear types are responsible for most catches of this coastal species (Pollom *et al.*, 2021).

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Conflict of Interest

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Institutional Animal Care and Use Committee (IACUC)

All the samples were collected under the Belize fisheries department marine scientific research permit (Most recent permit for this work #0065-19). The methodology for sample collection and animal research was approved by the Wildlife Research group - Florida International University under the IACUC (Lab Animal Research -Record ID 30291259) and the Smithsonian Institute IACUC (Animal Care and Use - Record ID 20676904).

Author Contributions

Study concept and design: Cindy Gonzalez and Demian Chapman. Analysis and interpretation of data: Cindy Gonzalez, Bautisse Postaire, Susana Caballero, William Driggers, and Demian Chapman. Statistical Analysis: Cindy Gonzalez, Bautisse Postaire, William Driggers. Critical revision of the manuscript for important intellectual content: Demian Chapman, Susana Caballero. Study supervision: Demian Chapman, Susana Caballero.

Data Availability Statement

All relevant data are within the manuscript and its Supporting Information files.

Supporting Information

All relevant data are within the manuscript and its Supporting Information files. Data from this study are also available in GenBank NCBI (https://www.ncbi.nlm. nih.gov/genbank/).

Table S1 Microsatellite primer and multiplexing details. **Table S2** Allele frequencies for eight microsatellite loci: Spti 2, Spti 3, Spti 4, Spti 9, Spti 10, Spti 48, Spti 26, Spti 42.

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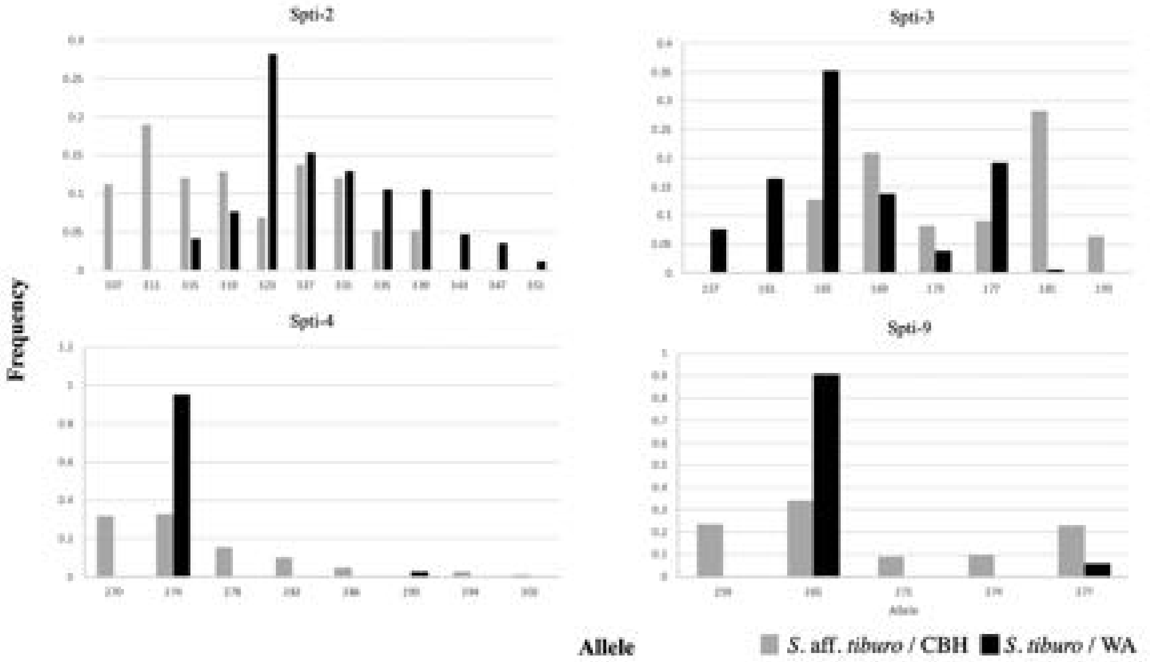
Supplementary material

Multiplex	Locus Name	Primer	Reference
1	**Spti2**	F: ATGGATGTGTTGACGCATGG R: ACCTTGTGCTCCTACATTTATCTATCC	Price et al., 2014
	Spti3	F: TCAATGGCTGTGAAACAGTGG R: ACTAGTACTTGCCAAGGTGTGGG	
	Spti4	F: ATCAGTCCAGCCATACACCC R: AGAGAGCAGCTAGACACAAAGCC	
	Spti9	F: AGTCTGTTGTTTAACTTCCAGCC R: ATGGAGAAGTTCCACTTCAGG	
2	**Spti10**	F: GAAGTGAGAGAAATGCGGGC R: ATGTGTGGATGGCAGGTGG	
	Spti23	F: AAACCAGACTTTGTCCCATCG R: CTGTCCATGTTGCTCTTGCC	
	Spti25	F: AAAGCTCTAGTCGAAATGGTTGG R: GCCCAAGCCTCAAAGAAGG	
	Spti34	F: ACAAGAGGGAGATCCGAGGC R: TATCTCCTGGTGTCGGGTCC	
3	**Spti26**	F: AAGAATGATATGCTTTGCCTGC R: GCTGTCAGATCTTGACTTCCTAGC	
	Spti42	F: CCAACATGTTTAGGTCAAGCG R: TGATTGACAACGAGATTTACTGG	
	Spti44	F: GGGTGTCCGAGTGCTTGC R: TGGTTTAAGCACGAGCAATCC	
	Spti46	F: GCCGAAGGCCTGTAATGC R: TTAGTGCAGAGTGACACCCG	
	Spti48	F: TGTTTGTCGATTTCTTTGAGTGG R: CAAGTGCAGGGAGTCTTTGG	
4	**Spti36**	F: CAAGTGCAGGGAGTCTTTGG	

	R: TCCCTGTCTATCCATTCACCC
Spti38	F: AAGAAGAGAGAGACAGGCAGGC R: TCTCTCAAACTCCCTCTTTCAGC
Spti40	F: TCTCTCTGCTGTAGCTGCCC R: TCTGGTATCTGGCACCATGC
Spti41	F: ACATCTGCCATGGCTAACCC R: TGACAATCAGCGGTTGAAGC

Table S1 Microsatellites: Multiplex and primer details.



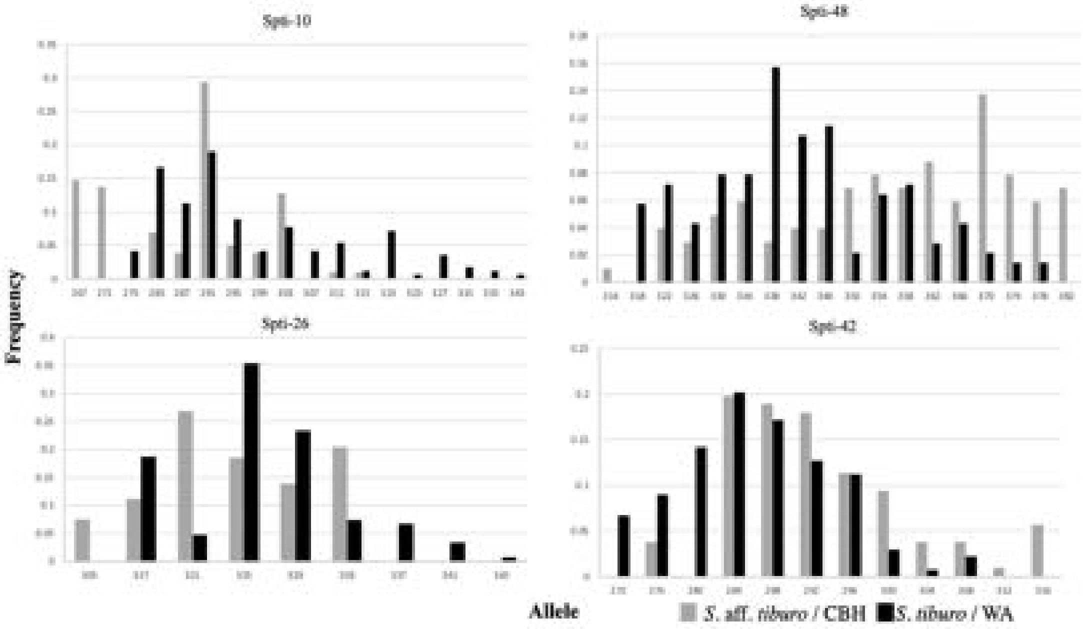


Figure S1 Allele frequencies for eight microsatellite loci: Spti 2, Spti 3, Spti 4, Spti 9, Spti 10, Spti 48, Spti 26, and Spti 42. Grey bars represent *S.* aff. *tiburo* and black bars represent *S. tiburo* specimens analyzed.

CHAPTER VI

RESURRECTING THE PACIFIC BONNETHEAD SHARK *SPHYRNA VESPERTINA* SPRINGER, 1940 (CARCHARHINIFORMES, SPHYRNIDAE).

Cindy Gonzalez, Bautisse Postaire, Jose Tavera, Susana Caballero, William Driggers, Kevin Feldheim, Arturo Acero, Demian Chapman. 2024. Resurrecting the Pacific bonnethead shark *Sphyrna vespertina* Springer, 1940 (Carcharhiniformes, Sphyrnidae). (Under Review)

Abstract

The Pacific bonnethead *Sphyrna tiburo vespertina* is a small subspecies of coastal hammerhead shark distributed in the tropical waters of the Eastern Pacific from California to Ecuador. It was first described as a species in 1940 (*Sphyrna vespertina*, Springer 1940), and then reclassified as a subspecies by Gilbert in 1967, so the Pacific bonnethead shark has a prolonged history of taxonomic ambiguity. Recent studies based on genetic and morphological evidence showed that bonnethead sharks are in fact a species complex, comprised at least by three different lineages distributed in the Western Atlantic, the Caribbean Sea and the Southwestern Atlantic, and the Eastern Pacific, respectively. The present study uses integrative taxonomy and molecular approaches (mitochondrial and nuclear markers) to provide evidence to resurrect the species *Sphyrna vespertina* (Springer 1940). Morphometric, meristic, and molecular analyses were performed along with a comparison of cephalofoil shape between the three lineages to provide useful field characters for species identification. The total number of precaudal vertebrae and the head shape were the main morphological discriminators between the three lineages and complementing these findings the molecular data showed clear genetic divergence to consider *S. vespertina* as a species entity. We urge for a reassessment of the small hammerhead species through their distribution range and to effectively develop local management strategies to protect these underrepresented sharks.

Introduction

Hammerhead sharks (Carcharhiniformes: Sphyrnidae) have experienced drastic declines over most of their geographic range due to continued overexploitation and anthropogenic pressures over the oceans (Cardeñosa *et al.* 2020; Chapman *et al.* 2009; Cortes & Neer 2005; Pérez-Jiménez 2014). Around 10 species of hammerhead sharks have been described in two genera: *Sphyrna* (9) and *Eusphyra* (1) (Compagno 1984b; Gilbert 1967). Four of them (*S. tiburo*, *S. tudes*, *S. media*, *S. corona*) are small coastal sharks restricted to the tropical waters of the American continent each species with restricted distribution ranges. From these species, the bonnethead shark *S. tiburo* is characterized by having the least laterally expanded cephalofoil with a shovel/ spade-head shape, that has been the most useful morphological character to identify it as a different species. However, after many studies the taxonomy of these sharks remains unresolved (Fields *et al.* 2016; Gonzalez *et al.* 2019, 2021; Naylor *et al.* 2012).

Linnaeus first described the bonnethead shark as *Squalus tiburo* (Linné Carl von & Salvius Lars 1758). This description is very short and based on a specimen "from America", but there is no type material or information about the location of capture (Linné Carl von & Salvius Lars 1758). In 1810 the genus name was changed to *Sphyrna* by Rafinesque, updating the name to *Sphyrna tiburo* (Rafinesque C. S. 1810). Springer (1940) described *Sphyrna vespertina* as a new species of bonnethead shark from the eastern Pacific Ocean. He examined one holotype (79 cm TL, Female, Stanford No. 11584) collected in Panama City, Panama, and two paratypes (93.8 cm TL, male, Carnegie Museum No. 5675) collected in Guayaquil, Ecuador; and (52 cm TL, Female, Stanford No. 1188) collected in Panama City, Panama (Springer 1940). Springer's description defined the morphological characters to distinguish *S. vespertina* from *S. tiburo*. The most noticeable difference is the head shape that in the frontal margin former is more rounded, while *S. vespertina* has a broader shorter head that resembles a shovel shape. When comparing the two species he noticed that the internarial distance is greater in *S. vespertina* than in *S. tiburo*, suggesting that there is a higher level of body compression (Springer 1940). Gilbert (1967) in his "Revision of the hammerhead sharks (Family Sphyrnidae)" placed *S. tiburo* and *S. vespertina* as subspecies, determining the Atlantic bonnethead sharks as *S. tiburo tiburo*, and *S. tiburo vespertina* for the Pacific Bonnetheads (Gilbert 1967).

More recent studies based on molecular markers and taxonomy have resulted in clear evidence of a bonnethead shark species complex, with at least three lineages: *S. tiburo* distributed in the western Atlantic from North Carolina to the Yucatan Peninsula; the recently described *S. alleni* sp. nov. distributed in the Caribbean Sea from Belize to Brazil in the southwestern Atlantic (Fields *et al.* 2016; Gonzalez *et al.* 2021; Naylor *et al.* 2012, Gonzalez *et al. 2024*); and *S. tiburo vespertina* distributed in the Tropical Eastern Pacific (TEP) from California to Ecuador (Aroca *et al.* 2022; Gilbert 1967; Springer 1940). Despite resolution of the taxonomy and phylogeography of *S. tiburo* and *S. alleni* sp. nov., in the Atlantic, to date there is no taxonomical consensus about the status of the Pacific bonnethead currently classified as a subspecies (*S. tiburo vespertina*). Robertson and Allen (2015) recognized a split at the subspecies level, while the Eschmeyer's Catalog of Fishes recognize them as different species (Fricke *et al.* 2024).

The most recent study conducted by Aroca et al., (2022) is the only one in which the general shape of the cephalofoil and the density of the ampullae of Lorenzini of the three lineages were evaluated with morphological analyses and complemented with genetic markers. Both morphometric and molecular analysis supported the existence of three different evolutionarily significant units that deserve specific taxonomic status (Aroca *et al.* 2022). Based on these previous studies and evidence, for clarity purposes, from here we will treat *S. vespertina* as a species entity.

To finally resolve the taxonomy of this lineage we investigated the morphology and genetic diversity of *S. vespertina* in the eastern Pacific Ocean. We used 61 morphometric measurements, three meristic counts; two mitochondrial markers (COI and mtCR), and 12 nuclear microsatellite loci and compared our findings with the specimens of *S. tiburo* and *S. alleni* in Gonzalez et al., 2024. Here we tested the hypothesis of three separate species in the bonnethead complex and provide robust evidence of the species status of *S. vespertina*. We urge for a reassessment of this complex by the International Union for the Conservation of Nature because two of these three species are heavily fished in Latin American countries where fisheries governance is generally in its infancy.

Materials and Methods

A total of seven specimens from the Tropical Eastern Pacific (TEP) in Panama and thus identified as *S. vespertina* were used for morphometrics analyses. From those, four specimens were used for meristic counts. Another eleven tissue samples from *S. vespertina* from Colombia were provided by Jose Tavera from La Universidad del Valle in Colombia, so a total of 15 individuals were included in the genetic analyses (Panama n=4; Colombia n=11). These data were compared with the study from Gonzalez et al., (2024).

Specimen Collection and Preservation

Seven mature Pacific bonnethead sharks (five females and two males) diagnosable in the field as *S. vespertina* were provided by fishermen from the Gulf of Chiriquí, in the eastern Pacific of Panama (Table 1, Figure 1). They were caught in artisanal fisheries with gillnets. For all the specimens, a set of samples (fin, muscle, liver) were collected and preserved in three ways (95%

ethanol, RNAlater, and frozen). The whole sharks were kept in ice baths to avoid decay. Sixty-one morphometric measurements were performed on each shark and meristic data were collected. Two specimens (one male and one female) were then injected and fixed with formalin 10% to be deposited as museum specimens. Fin and muscle samples were subsequent used for genetic analyses (see mitochondrial and nuclear markers sections).

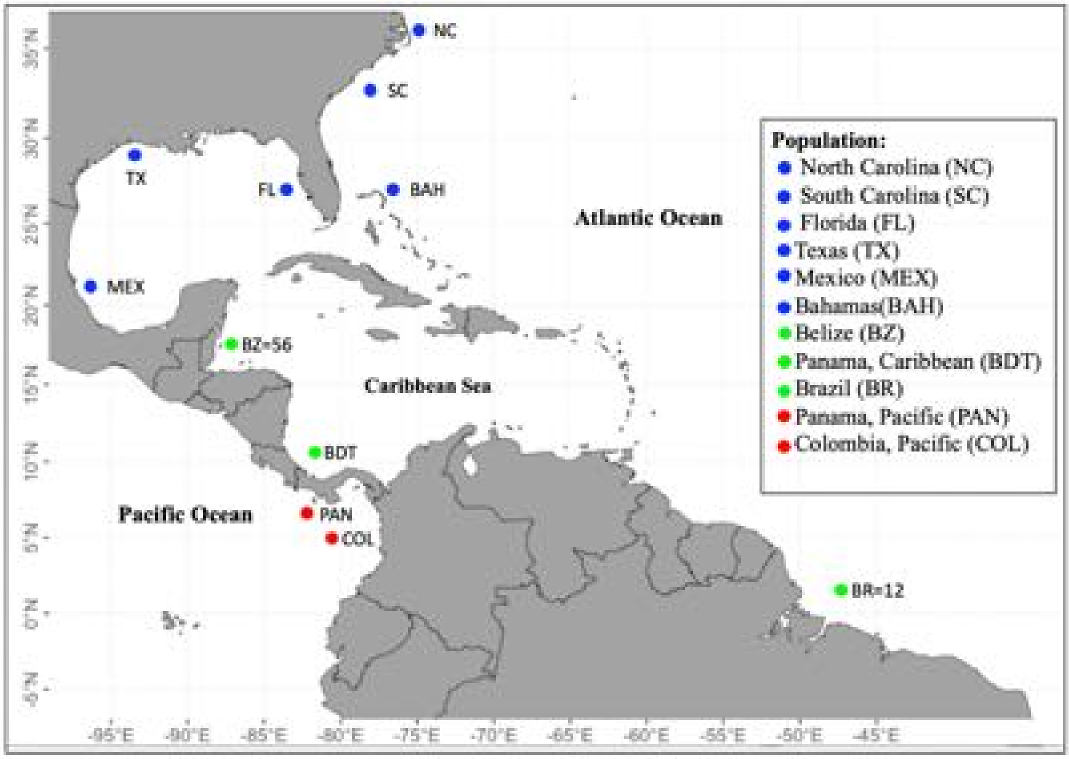


FIGURE 1 Sampling localities. Red: *S. vespertina* from the eastern Pacific (Gulf of Chiriquí, Panama and Gulf of Tribugá, Colombia); Green: *S. alleni* sp. nov., from the Caribbean Sea (Belize and Panama), and the southwestern Atlantic (Brazil); Blue: *S. tiburo* from the western Atlantic (U.S.: North Carolina, South Carolina, Florida; Mexico; and Bahamas). Number of *S. vespertina* specimens collected for morphometric analyses (Panama, Pacific n=7), meristic analyses (Panama Pacific, n=4), and tissue samples for genetic analyses (Panama, Pacific n= 4; Colombia, Pacific n=11).

Morphometric data

Measurements of precaudal (PCL), fork (FL), total (TL), and stretch total (STL) lengths were taken on a straight line along the axis of the body of seven *S. vespertina* specimens. For each shark 61 morphometric features (Table 2) were measured following the methods from (Compagno

1984a). We compared our specimens with the morphometric data from eleven *S. tiburo* and twelve *S. alleni* sp. nov., from Gonzalez et al., (2024).

Meristic data

Four specimens (three females and one male) were dissected and three meristic characters were enumerated: the precaudal vertebrae count (PVC), the total number of teeth on the upper (UT) and the lower (LT) functional rows of the jaws (Springer & Garrick 1964). All the soft tissue from these *S. vespertina* specimens was removed from the tail to the brainstem, until each vertebra was exposed. Posteriorly, a dissection pin was inserted into the anterior margin of the precaudal pit, and the total number of vertebrae were counted by hand for each shark (Table 1).

Table 1 Summary of *S. vespertina, S. alleni.* sp. nov. and *S. tiburo* specimens used for the morphological and meristic analyses. PVC: Number of Precaudal Vertebrae

Species	Sex	TL (cm)	PVC	Specimen	Museum Catalog number	Type	Collection site	Sample ID	Collector
S. vespertina	F	86	89	Dissected	STRI-354, Collected 09/27/22	No type material, not preserved	Gulf of Chiriquí, Panama Pacific	354	Cindy Gonzalez
S. vespertina	F	92	90		STRI-368, Collected 09/27/22			368	
S. vespertina	M	50.3	91		STRI-374, Collected 01/15/24			374	
S. vespertina	F	57	86		STRI-375, Collected 01/15/24			375	
S. vespertina	F	106	N/A	Preserved Formalin 10%	STRI-360, Collected 09/27/22	Holotype		360	
S. vespertina	M	99	N/A		STRI-364, Collected 09/27/22	Paratype		364	
S. vespertina	F	73	N/A	Frozen	STRI_ETC0301, Collected 03/05/23	Comparative material		ETC0301	Helio Quintero
S. alleni	F	103	83	Preserved Formalin 10%	UF245705; Collected 02/21/19	Comparative material	Staan Creek, Riversdale, Belize	001BZN	Cindy Gonzalez
S. alleni	M	60.5	82		UF245723; Collected 02/21/19			002BZN	
S. alleni	F	83	80		UF245724; Collected 02/21/19			003BZN	
S. alleni	F	71.5	81		UF245725; Collected 02/21/19			004BZN	
S. alleni	M	63	83		UF245726; Collected 02/21/19			005BZN	
S. alleni	M	64.5	82		UF245727; Collected 02/21/19			006BZN	

S. alleni	F	85	81	Dissected	Collected 2016	Not Preserved		017BZO	Demian Chapman
S. alleni	F	77.5	80					008BZO	
S. alleni	F	74.8	82					001BZO	
S. alleni	M	81.6	81					019BZO	
S. alleni	M	90.5	80					005BZO	
S. alleni	M	88.7	80					009BZO	
S. tiburo	F	86	73	Preserved Formalin 10%	UF 247324; Collected 07/20/20	Comparative material	Terra Ceia Bay, Sarasota, FL, U. S	6FLN	Jayne Gardiner, Tonya Wiley
S. tiburo	F	74.5	72		UF 247324; Collected 07/20/21			12FLN	
S. tiburo	F	82	72		UF 247324; Collected 07/20/22			10FLN	
S. tiburo	M	80.5	74		UF 247325; Collected 10/13/20			7FLN	
S. tiburo	M	73.5	73		UF 247325; Collected 10/13/21			8FLN	
S. tiburo	M	59.5	72		UF 247326; Collected 08/18/20			11FLN	
S. tiburo	F	71.6	72	Dissected	Collected 2016			023FLO	
S. tiburo	F	82	72					008FLO	
S. tiburo	M	82.3	73					017FLO	
S. tiburo	M	76	74					006FLO	
S. tiburo	M	83.3	73					004FLO	

Morphometric and Meristic Data Analysis

The data was size standardized to remove any body size effect on the 61 morphological traits by implementing Elliot's et al., (1995) allometric formula: $M_{(Adj)} = M_o (L_s / L_o)^b$; where $M_{(Adj)}$= size-adjusted measurement, M_o = original morphometric measurement, Ls = mean STL of each bonnethead from all samples for each variable, L_o = STL of specimen. and b was estimated by the allometric growth equation $M = aL''$. Parameter b was estimated as the slope of the regression of log M_o, on log L_o, for each character from the observed data. Stretched total length (STL) was used as the measure of body length. After the data standardization, it was analyzed by using univariate and multivariate statistics following the methods by Elliot et al., (1995), Quattro et al., (2013), and Gonzalez et al., (2024).

Principal Component Analysis (PCA) were run independently to examine the morphometric (continuous variables), and meristic traits (discontinuous variables). The morphological variation to define groups among *S. vespertina, S. tiburo,* and *S. alleni* sp. nov., was assessed by examining principal components with eigenvalues greater than one and evaluating the proportion of variation explained by those components, character weightings on components, and plots of component scores. A Discriminant Function Analysis (DFA) was used to evaluate the meristic and adjusted morphometric characters to identify the variables that explain differentiation between groups. Finally, the original data (M_o) were standardized as percent of STL and used to test for interspecific differences between means of individual morphometrics by using an ANOVA. When data were not normally distributed a Kruskal-Wallis was used to test whether samples originated from the same distribution. All statistical tests were considered significant at $\alpha = 0.05$.

Mitochondrial markers

Fin clip tissue samples from *S. vespertina* from the Pacific of Panama (Gulf of Chiriquí, n= 4) and the Pacific of Colombia (Gulf of Tribugá, n= 11), were stored in 95% ethanol and total DNA extraction was performed using the Qiagen DNeasy Blood and Tissue Kit following the manufacturer´s protocol (Qiagen, Valencia, CA). A 571 base pair (bp) fragment of the mitochondrial cytochrome oxidase I (COI) was amplified for all the samples using the primers FishCoxI F (5´TCWACCAACCACAAAGAYATYGGCAC3´), and FishCoxI R (5´TAR-ACTTCWGGGTGRCCRAAGAATCA3´), modified from Ward et al. (2005) and following their PCR conditions and thermal cycling profiles. A 408 bp fragment of the mitochondrial control region (mtCR) was amplified for all samples using the primers Pro-L (5`AGGGRAAGGAGGGTCAAACT3´), and 282H (5´AAGGCTAGGACCAAACCT3´) and the reaction and thermal cycling conditions described in (Keeney *et al.* 2003). PCR products were purified using Exo-SAP (Thermo Scientific) and sequenced with both amplifying primers on an ABI 3730 DNA analyzer.

All sequences (COI and mtCR) were aligned, edited, and checked manually using Geneious v.2020.2.2 (http://www.geneious.com), which was also used to identify haplotypes (Maddison & Maddison 2000). Fourteen sequences (five *S. vespertina*, five *S. tiburo,* and four *S. alleni* sp. nov. sequences) were selected and a concatenated alignment of the mtCR and COI sequences was built. The program jModelTest v.2.3.1 was used to obtain the best model for DNA substitution (Posada 2008). After selecting the best model (TIM2+I), PAUP (Excoffier & Heckel 2006) was used to build a concatenated neighbor joining tree, using *Sphyrna lewini* as the outgroup.

Nuclear Markers

PCR amplification and microsatellite genotyping

Fifteen fin clip tissue samples from *S. vespertina* (n= 15), *S. tiburo* (n= 96), and *S. alleni* sp. nov. (n= 60), collected from a wide geographic area (Figure 1) were stored in 95% ethanol for genetic analyses, and genomic DNA extraction was performed using the Qiagen DNeasy Blood and Tissue Kit following the manufacturer´s protocol (Qiagen, Valencia, CA). Samples were genotyped for 12 polymorphic microsatellite markers (Price *et al.* 2014). Microsatellite primer and multiplexing details are provided in Table S1. PCR for microsatellite amplification were conducted using the Type-it Kit Qiagen Multiplex PCR Master Mix®, 25–70 ng of DNA and 0.4 µM of each primer in 5 µl of final reaction volume. Loci were organized into four multiplex sets per PCR reaction. The PCR protocol consisted on an initial step of 5 min at 95°C, followed by 29 cycles of 30 s at 95°C for denaturation, 90 s at 60°C for annealing, and 30 s at 72°C for extension, with an additional step for final extension of 30 min at 60°C PCR (Díaz-Jaimes *et al.* 2021). The PCR products for microsatellite loci were visualized using capillary electrophoresis and sized with Alexa725™ (Maddox & Feldheim 2014) to score with the Geneious v.2020.2.2 software (http://www.geneious.com). The software Micro-Checker 2.2 (Van Oosterhout *et al.* 2004) was used to identify possible genotyping errors.

All loci successfully amplified were tested for presence of null alleles using the software Micro-Checker 2.2 (Van Oosterhout *et al.* 2004) and confirmed using GenePop (Rousset 2008). Nuclear genetic diversity was estimated as the number of alleles observed (N_a), and the average number of alleles per locus. Deviations from the Hardy– Weinberg equilibrium (HWE) and linkage disequilibrium between all pairs of loci overall and within each cryptic species and sampling site were assessed by the exact test implemented in ARLEQUIN v. 3.5.2.2 (Excoffier & Lischer 2010), that was also used to calculate the observed heterozygosity (*HO*), and the expected heterozygosity (*HE*) (burn-in period: 10,000 iterations, MCMC 100,000). Genetic differentiation between the three lineages was tested using pairwise F_{ST} values calculated in ARLEQUIN v. 3.5.2.2, and *Jost's D* values (Jost *et al.* 2018) were calculated using the software GENODIVE (Meirmans & Van Tienderen 2004). Average genetic diversity (AGD) defined as the gene diversity over all loci in each population, was calculated in ARLEQUIN v. 3.5.2.2. Loci or samples with more than 15% of missing data were removed from the analyses. All p-values were Bonferroni corrected.

We tested the hypothesis of three separate gene pools for *S. vespertina, S. alleni* sp. nov., and *S. tiburo* by implementing the admixture model with correlated allele frequencies to estimate the most likely number of 'populations' (*K* clusters) in the data by using the software STRUCTURE (Pritchard *et al.* 2000). The Evanno method implemented in STRUCTURE HARVESTER v.1.0 (Earl & vonHoldt 2012) was used to estimate the best number of *K* clusters. Three independent runs were conducted (length burn-in period: 50,000; MCMC 500,000). The results were summarized in CLUMPP v.1.0 (Jakobsson & Rosenberg 2007) and were formatted and visualized in DISTRUCT (Rosenberg 2004).

Results

Morphometric analysis

Elliot's equation was used to standardize the data eliminating the possible effects of the body size of the specimens on the 61 morphometric variables analyzed (Table 2). PCA identified 16 components with eigen values greater than 1.0 that accounted for 90.20 % of the variability in the data. However, the morphological characters evaluated cannot resolve *S. vespertina* from S. *tiburo* and *S. alleni* sp. nov., as the proportion of variation for the individual components and among components was not significant indicating high phenotypic similarity between the three lineages.

Although the morphological analyses did not reflect any differences, it is noticeable that *S. vespertina* sharks have distinctive pointed and triangular cephalofoils. In contrast, *S. tiburo* specimens have a more evenly rounded, semi-circular cephalofoil. When comparing *S. vespertina* and *S. alleni* sp. nov., both have triangular head shapes, but in *S. vespertina* the "V" termination in the anterior margin is more pronounced, has a visible white edge, and the posterior margin has a straight end (Figure 2). For *S. alleni* sp. nov., the anterior margin of all examined cephalofoils came to a noticeable point, with an additional bulge present in that of adult males, and the posterior margin of the head has noticeable lobule shaped ends, making the entire cephalofoil appear shovel-shaped (Figure 2). These observations are supported by the geometric morphometric analyses of the cephalofoils reported by Aroca et al. (2022).

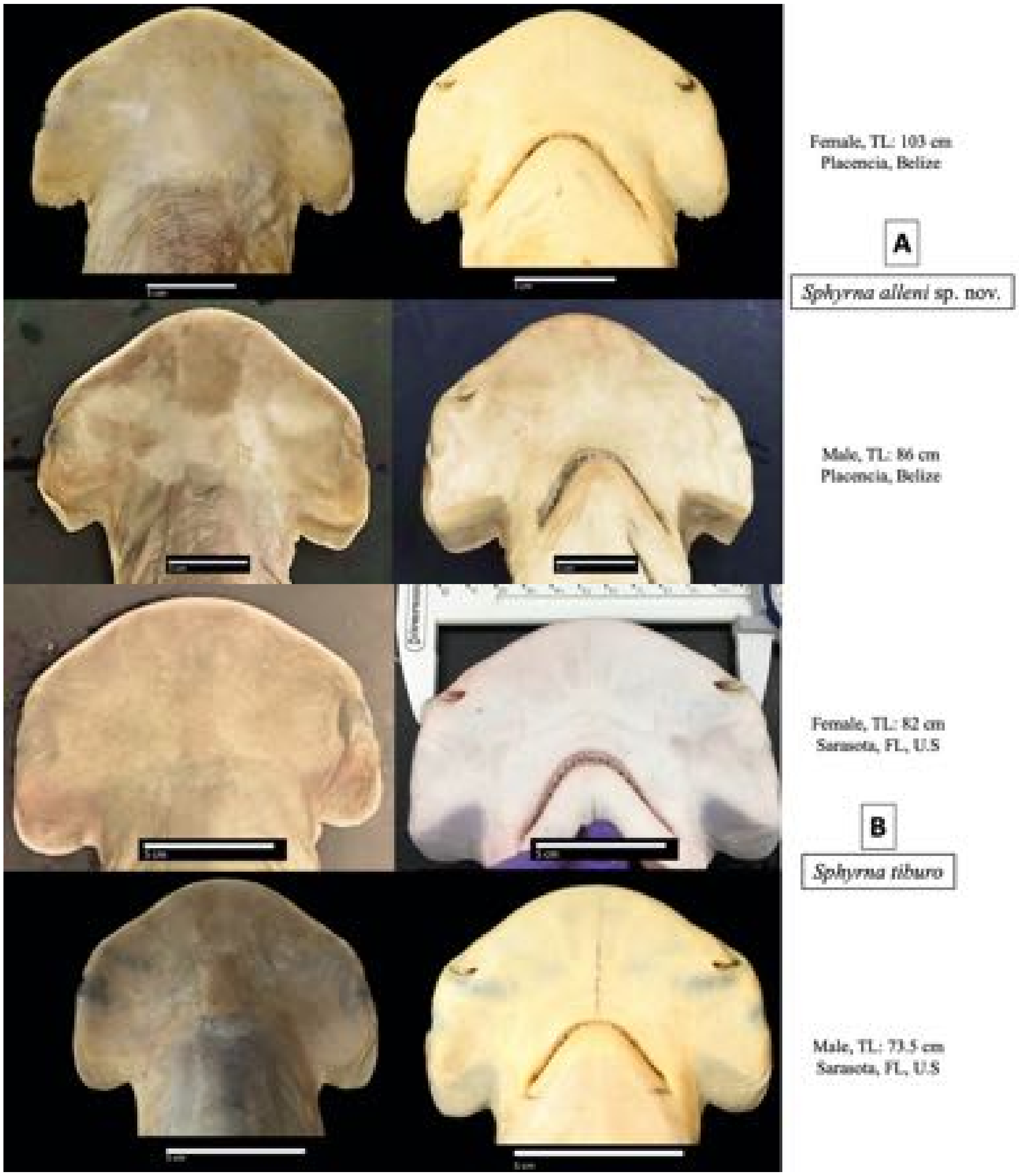


FIGURE 2 Cephalofoil comparison (dorsal and ventral views of each specimen are provided). Bar scale: 5cm. TL: Total length of specimen. A) *S. alleni* sp. nov.; B) *S. tiburo*; C) and *S. vespertina*. Photos: Cindy Gonzalez.

Meristic Analysis

Three variables (number of precaudal vertebrae - PVC, number of upper functional teeth row- UT, and number of lower functional teeth row- LT) were used to run the meristics PCA analysis. In this case, only the PVC component was extracted with an eigenvalue greater than 1.0, accounting for the 63.241% of the variability in the data.

A discriminant function analysis (DFA) was also run with the three variables PVC, UT, and LT. It was implemented to randomly classify the meristic characters into three groups corresponding to *S. vespertina*, *S. tiburo,* and *S. alleni* sp. nov. This analysis identified the number of precaudal vertebrae (PVC) as the only discriminator between *S. vespertina, S. tiburo*, and *S. alleni* sp. nov., (Figure 3), with 100% of specimens classified correctly (Table 3).

Table 2 Proportional measurements for *S. vespertina*, S. *alleni*. sp. nov., and *S. tiburo*. ANOVAs values are followed by p-values in parentheses. Values not reported when the data was non normal distributed. When the data was non normally distributed a Kruskal-Wallis (K-W) test was performed and p-values are reported in parentheses.

	Morphological Character		*n*	Mean (cm)	(S.D)	Range	ANOVA	K-W test
1	**Head Length**	*S. vespertina*	7	17.71	4.32	11.7 - 22.5		
		S. alleni	12	16.48	2.13	13.4 - 19.71	1.418 (0.244)	
		S. tiburo	11	16.15	1.53	12.5 - 18		
2	**Pre-first dorsal length**	*S. vespertina*	7	26.41	6.75	16.4 - 34.5		
		S. alleni	12	24.81	4.31	19.5 - 35	1.563 (0.222)	
		S. tiburo	11	23.75	2.41	18.4 - 27.3		
3	**Pre-second dorsal length**	*S. vespertina*	7	49.86	13.71	30.2 - 66		
		S. alleni	12	50.02	8.92	38 - 68.5	0.141 (0.71)	
		S. tiburo	11	48.4	5.19	36 - 54.5		
4	**Prepectoral length**	*S. vespertina*	7	17.78	4.46	11.59 - 23.5		
		S. alleni	12	16.31	2.24	13.5 - 21	1.961 (0.172)	
		S. tiburo	11	15.9	1.49	12.2 - 17		
5	**Prepelvic length**	*S. vespertina*	7	37.67	10.16	23 - 49.5		
		S. alleni	12	36.58	6.65	28 - 51	0.659 (0.424)	
		S. tiburo	11	35.12	3.69	26.7 - 40.9		
6	**Preanal length**	*S. vespertina*	7	47.33	12.73	30 - 62		
		S. alleni	12	47.25	8.51	36 - 65	0.38 (0.847)	
		S. tiburo	11	46.59	4.99	35.3 - 54.2		
7	**Snout-vent length**	*S. vespertina*	7	39.37	10.72	24 - 53		
		S. alleni	12	38.85	7.51	28.8 - 54.5	0.828 (0.371)	
		S. tiburo	11	36.33	4.69	27.5 - 45.2		
8	**Interdorsal space**	*S. vespertina*	7	18.27	5.76	10.7 - 26		
		S. alleni	12	19.08	3.53	14.2 - 26.2	0 (0.997)	
		S. tiburo	11	18.40	2.46	12.5 - 21.5		
9	**Second dorsal-caudal space**	*S. vespertina*	7	6.12	1.56	3.86 - 8		
		S. alleni	12	6.06	1.16	4.5 - 8.2	0.805 (0.377)	
		S. tiburo	11	6.55	0.66	5.1 - 7.7		

10	**Pectoral-pelvic space**	*S. vespertina*	7	16.17	4.66	9.67 - 22.8		
		S. alleni	12	17.18	3.53	12 - 23.6	0.123 (0.729)	
		S. tiburo	11	15.82	2.06	12 - 18		
11	**Pelvic-anal space**	*S. vespertina*	7	7.46	1.56	5.73 - 9.6		
		S. alleni	12	7.02	1.69	5 - 10.1	0.16 (0.9)	
		S. tiburo	11	7.30	1.40	5 - 9.5		
12	**Anal-caudal space**	*S. vespertina*	7	4.58	1.22	2.9 - 6		
		S. alleni	12	4.60	0.86	3.5 - 6		5.9656 (0.051)
		S. tiburo	11	5.63	0.66	3.9 - 6.36		
13	**Pelvic-caudal space**	*S. vespertina*	7	17.17	4.45	11.22 - 23		
		S. alleni	12	16.80	2.68	13.2 - 21.2	0.243 (0.626)	
		S. tiburo	11	17.75	1.99	13.8 - 20.4		
14	**Vent-caudal length**	*S. vespertina*	7	42.07	11.55	26.5 - 57		
		S. alleni	12	40.91	6.17	32.1 - 53	3.637 (0.067)	
		S. tiburo	11	35.54	6.00	24.7 - 43.8		
15	**Head width**	*S. vespertina*	7	11.78	1.57	13.9 - 10.34		
		S. alleni	12	13.17	1.44	10.97 - 15.3		4.5651 (0.102)
		S. tiburo	11	12.31	1.01	10.45 - 14.1		
16	**Snout length**	*S. vespertina*	7	5.85	1.15	4.2 - 7		
		S. alleni	12	5.63	0.75	4.68 - 7.2	2.245 (0.145)	
		S. tiburo	11	5.31	0.44	4.42 - 6		
17	**Eye length**	*S. vespertina*	7	1.28	0.17	1 - 1.45		
		S. alleni	12	1.40	0.21	1.1 - 1.74	1.443 (0.24)	
		S. tiburo	11	1.38	0.08	1.25 - 1.55		
18	**Eye height**	*S. vespertina*	7	1.06	0.18	0.82 - 1.35		
		S. alleni	12	1.01	0.14	0.85 - 1.35	0.154 (0.698)	
		S. tiburo	11	1.02	0.13	0.7 - 1.15		
19	**Internarial space**	*S. vespertina*	7	9.89	1.95	7.08 - 12.5		
		S. alleni	12	9.05	1.13	7.61 - 11.5	7.276 (0.012)**	
		S. tiburo	11	8.32	0.65	6.8 - 9.3		
20	**Nostril length**	*S. vespertina*	7	1.32	0.19	1.5 - 1.04		
		S. alleni	12	1.28	0.72	0.78 - 3.53		9.0604 (0.011)*

		S. tiburo	11	1.05	0.06	1 - 1.2		
21	**Mouth Width**	*S. vespertina*	7	5.80	1.51	3.72 - 8		
		S. alleni	12	5.26	1.09	3.79 - 7.68	1.671 (0.207)	
		S. tiburo	11	5.13	0.54	4.1 - 6		
22	**Mouth length**	*S. vespertina*	7	3.45	0.85	2.16 - 4.6		
		S. alleni	12	3.33	0.55	2.55 - 4.32	0.874 (0.358)	
		S. tiburo	11	3.20	0.34	2.7 - 3.75		
23	**Intergill length**	*S. vespertina*	7	4.32	1.39	2.55 - 6.25		
		S. alleni	12	3.99	0.93	2.85 - 6.55		0.8947 (0.6393)
		S. tiburo	11	3.82	0.50	3.2 - 4.6		
24	**First gill slit height**	*S. vespertina*	7	2.54	0.77	1.46 - 3.55		
		S. alleni	12	2.24	0.44	1.54 - 2.96	4.321 (0.0471)*	
		S. tiburo	11	2.05	0.28	1.5 - 2.67		
25	**Second gill slit length**	*S. vespertina*	7	2.79	0.88	1.67 - 4.26		
		S. alleni	12	2.45	0.41	1.78 - 3.12		2.4667 (0.2913)
		S. tiburo	11	2.27	0.29	1.8 - 2.9		
26	**Third gill slit length**	*S. vespertina*	7	2.98	0.82	1.74 - 4.15		
		S. alleni	12	2.53	0.42	1.97 - 3.57		2.8337 (0.2425)
		S. tiburo	11	2.48	0.23	2.13 - 3		
27	**Forth gill slit length**	*S. vespertina*	7	2.83	0.80	1.72 - 4.05		
		S. alleni	12	2.54	0.48	1.85 - 3.34	1.094 (0.305)	
		S. tiburo	11	2.55	0.27	2.23 - 3.15		
28	**Fifth gill slit length**	*S. vespertina*	7	2.64	0.78	1.54 - 3.6		
		S. alleni	12	2.27	0.49	1.6 - 3.22	3.009 (0.0934)	
		S. tiburo	11	2.19	0.33	1.85 - 2.82		
29	**Pectoral anterior margin**	*S. vespertina*	7	11.17	3.59	6.4 - 15.3		
		S. alleni	12	11.55	2.06	8.6 - 15.3	0.209 (0.651)	
		S. tiburo	11	11.67	0.98	9.62 - 12.7		
30	**Pectoral base**	*S. vespertina*	7	4.33	1.20	2.56 - 5.8		
.		*S. alleni*	12	4.35	0.97	2.95 - 6.3	0.002 (0.961)	
		S. tiburo	11	4.32	0.40	3.5 - 4.9		
31	**Pectoral inner margin**	*S. vespertina*	7	4.80	1.25	3.12 - 6.12		

		S. alleni	12	4.20	0.73	3.05 - 5.35	2.302 (1.14)	
		S. tiburo	11	4.15	0.53	3 - 4.8		
32	**Pectoral posterior margin**	*S. vespertina*	7	10.30	3.63	5.74 - 14.65		
		S. alleni	12	9.80	2.08	7.07 - 13.44	1.526 (0.227)	
		S. tiburo	11	9.02	0.90	7.67 - 10.4		
33	**Pectoral height**	*S. vespertina*	7	10.44	3.26	6.13 - 14.25		
		S. alleni	12	10.44	2.08	7.45 - 14.69	0.587 (0.45)	
		S. tiburo	11	9.74	0.97	8 - 11.2		
34	**First dorsal anterior margin**	*S. vespertina*	7	13.48	3.88	8.15 - 18.8		
		S. alleni	12	12.93	2.12	10.12 - 17.2	1.385 (0.249)	
		S. tiburo	11	12.19	1.07	9.8 - 14		
35	**First dorsal base**	*S. vespertina*	7	6.50	1.56	4 - 8.15		
		S. alleni	12	6.52	1.25	4.83 - 9.15	0.05 (0.824)	
		S. tiburo	11	6.38	0.85	4.8 - 7.56		
36	**First dorsal inner margin**	*S. vespertina*	7	3.41	0.93	2.29 - 4.9		
		S. alleni	12	4.65	2.69	2.65 - 12.45		1.5449 (0.4573)
		S. tiburo	11	4.02	0.87	2.83 - 5.55		
37	**First dorsal posterior margin**	*S. vespertina*	7	8.66	2.05	5.86 - 12.1		
		S. alleni	12	9.12	1.74	6.5 - 12.2	0.164 (0.689)	
		S. tiburo	11	9.01	1.01	6.7 - 10.3		
38	**First dorsal height**	*S. vespertina*	7	9.74	3.28	5.56 - 13.7		
		S. alleni	12	9.84	2.17	6.32 - 12.33	0.158 (0.694)	
		S. tiburo	11	9.38	1.08	7.1 - 10.71		
39	**Second dorsal anterior margin**	*S. vespertina*	7	4.73	1.30	3 - 6.54		
		S. alleni	12	4.89	1.40	3 - 8.75		0.4422 (0.8016)
		S. tiburo	11	4.87	0.55	3.87 - 5.83		
40	**Second dorsal base**	*S. vespertina*	7	3.10	0.86	2 - 4.1		
		S. alleni	12	2.80	0.44	1.95 - 3.44	0.006 (0.941)	

		S. tiburo	11	3.03	0.58	2 - 4		
41	**Second dorsal inner margin**	*S. vespertina*	7	3.83	0.93	2.5 - 5.45		
		S. alleni	12	4.18	0.69	3.03 - 5.35	2.667 (0.114)	
		S. tiburo	11	4.37	0.47	3.35 - 4.95		
42	**Second dorsal posterior margin**	*S. vespertina*	7	4.51	1.13	2.7 - 5.94		
		S. alleni	12	4.61	0.81	3.35 - 6.2		1.5649 (0.4573)
		S. tiburo	11	4.81	0.72	3.6 - 5.8		
43	**Second dorsal height**	*S. vespertina*	7	3.00	0.91	1.8 - 4.28		
		S. alleni	12	3.45	0.77	2.25 - 4.44	1.588 (0.218)	
		S. tiburo	11	3.47	0.55	2.4 - 4.2		
44	**Pelvic anterior margin**	*S. vespertina*	7	5.61	1.68	3.5 - 7.3		
		S. alleni	12	5.52	0.93	4 - 7	0.035 (0.852)	
		S. tiburo	11	5.51	0.74	4.3 - 6.48		
45	**Pelvic base**	*S. vespertina*	7	4.31	1.37	2.43 - 6.5		
		S. alleni	12	4.44	0.91	2.66 - 6.13	0.023 (0.88)	
		S. tiburo	11	4.39	0.67	3.43 - 5.52		
46	**Pelvic inner margin**	*S. vespertina*	7	3.70	2.02	1.92 - 7.1		
		S. alleni	12	2.99	0.47	2.13 - 4		1.3547 (0.508)
		S. tiburo	11	3.21	0.42	2.45 - 3.8		
47	**Pelvic posterior margin**	*S. vespertina*	7	5.44	1.53	3.3 - 7.75		
		S. alleni	12	4.93	0.94	3.49 - 6.88	3.212 (0.084)	
		S. tiburo	11	4.60	0.48	3.56 - 5.14		
48	**Pelvic height**	*S. vespertina*	7	5.13	1.81	2.58 - 7.5		
		S. alleni	12	4.43	1.13	2.52 - 6	0.697 (0.411)	
		S. tiburo	11	4.57	0.73	3.5 - 5.66		
49	**Anal anterior margin**	*S. vespertina*	7	4.82	2.36	2.24 - 8.58		
		S. alleni	12	3.41	0.72	2.38 - 5.05		2.642 (0.267)
		S. tiburo	11	3.61	0.43	2.86 - 4.3		
50	**Anal base**	*S. vespertina*	7	5.90	1.47	3.71 - 7.54		
		S. alleni	12	5.38	0.95	3.85 - 7.07	3.026 (0.093)	

		S. tiburo	11	5.06	0.64	4 - 5.8		
51	**Anal inner margin**	*S. vespertina*	7	3.00	0.73	1.93 - 3.82		
		S. alleni	12	3.05	0.48	2.23 - 3.9	0.643 (0.429)	
		S. tiburo	11	2.81	0.56	1.6 - 3.42		
52	**Anal posterior margin**	*S. vespertina*	7	5.45	1.69	3.66 - 8.15		
		S. alleni	12	5.96	1.04	4.32 - 7.75	0.021 (0.886)	
		S. tiburo	11	5.46	0.79	4.22 - 6.57		
53	**Anal height**	*S. vespertina*	7	6.77	10.72	1.6 - 31		
		S. alleni	12	2.32	0.67	1.41 - 3.5	2.693 (0.112)	
		S. tiburo	11	2.34	0.70	1.46 - 3.85		
54	**Dorsal caudal margin**	*S. vespertina*	7	23.05	5.79	14.96 - 31		
		S. alleni	12	20.73	2.73	17.2 - 26.3	3.453 (0.0737)	
		S. tiburo	11	19.94	1.62	16.3 - 21.5		
55	**Preventral caudal margin**	*S. vespertina*	7	9.91	6.92	4.82 - 25		
		S. alleni	12	8.28	1.20	6.57 - 10.54		1.6104 (0.447)
		S. tiburo	11	7.59	0.91	6.05 - 9.33		
56	**Upper postventral caudal margin**	*S. vespertina*	7	14.31	4.19	8.45 - 21		
		S. alleni	12	12.72	1.88	10.27 - 16.3		4.6094 (0.0998)
		S. tiburo	11	11.18	1.27	9 - 12.5		
57	**Lower postventral caudal margin**	*S. vespertina*	7	3.77	1.22	2.26 - 5.44		
		S. alleni	12	3.78	0.75	3 - 5.55	0.315 (0.579)	
		S. tiburo	11	3.57	0.58	2.7 - 4.9		
58	**Caudal fork length**	*S. vespertina*	7	5.57	1.32	3.74 - 7.5		
		S. alleni	12	5.83	0.72	4.72 - 6.66	0.091 (0.765)	
		S. tiburo	11	5.50	0.58	4.3 - 6.4		
59	**Caudal fork width**	*S. vespertina*	7	6.26	1.65	3.92 - 8.3		
		S. alleni	12	5.48	0.83	4.35 - 7.25	2.365 (0.135)	
		S. tiburo	11	5.47	0.50	4.7 - 6.5		

60	**Subterminal caudal margin**	*S. vespertina*	7	2.71	4.49	1.67 - 4.25		
		S. alleni	12	2.42	0.42	1.97 - 3.4		2.4875 (0.2883)
		S. tiburo	11	2.15	0.36	1.35 - 2.52		
61	**Terminal caudal margin**	*S. vespertina*	7	4.87	1.41	3.04 - 7.25		
		S. alleni	12	4.11	0.79	2.15 - 5.22	1.552 (0.223)	
		S. tiburo	11	4.23	0.62	3.1 - 4.85		

Species	**Size (N)**	**Species assigned**	
		***S. tiburo* and *S.* alleni sp. nov.**	***S. vespertina***
***S. tiburo* and *S. alleni* sp. nov.**	23	23	0
		100.00%	0.00%
S. vespertina	4	0	4
		0.00%	**100.00%**

TABLE 3 Discriminant Function Analysis (DFA) of the percent of cases/ specimens correctly classified: 100.00%.

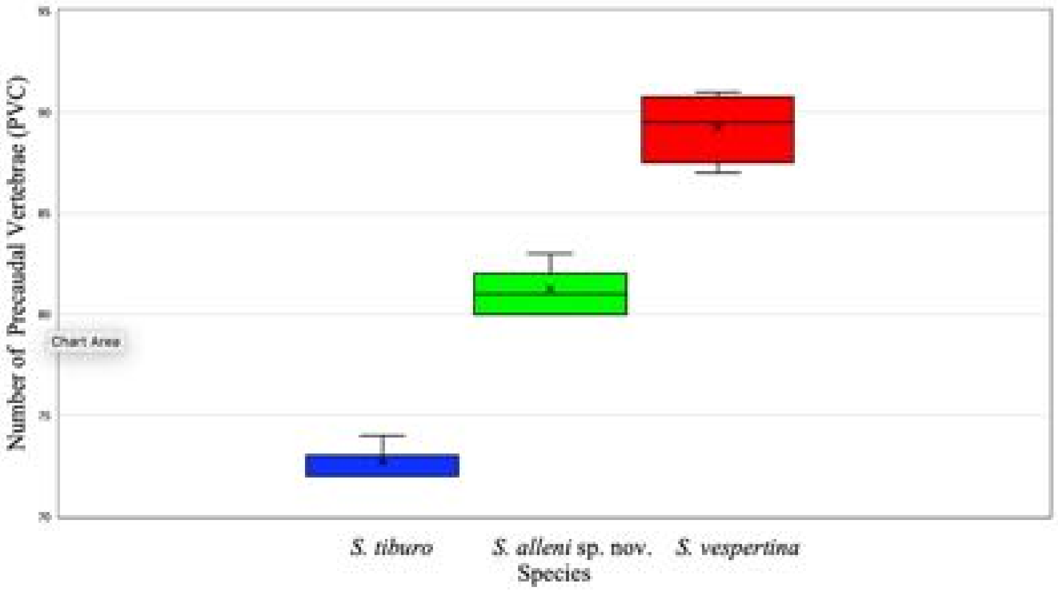


FIGURE 3 Boxplot of the number of precaudal vertebrae for *S. tiburo* (Florida, U.S, N = 11), *S. alleni* sp. nov., (Belize, N = 12), and *S. vespertina* (Panama - Pacific, N=4) specimens.

DNA Sequence data

Mitochondrial DNA

A total of 971 bp of the mitochondrial genome (COI + mtCR) was amplified and sequenced from 15 *S. vespertina* tissue samples from the TEP from Panama and Colombia. Eight additional samples from *S. alleni* sp. nov., (n= 3) and *S. tiburo* (n= 5) from different locations were also sequenced and included in the analyses. The concatenated neighbor joining tree revealed a polytomy of three lineages with 100% bootstrap support (Figure 4). The low substitution rates were in average 0.14%, therefore was not possible to resolve the phylogeny of these shark complex based on these two mitochondrial markers.

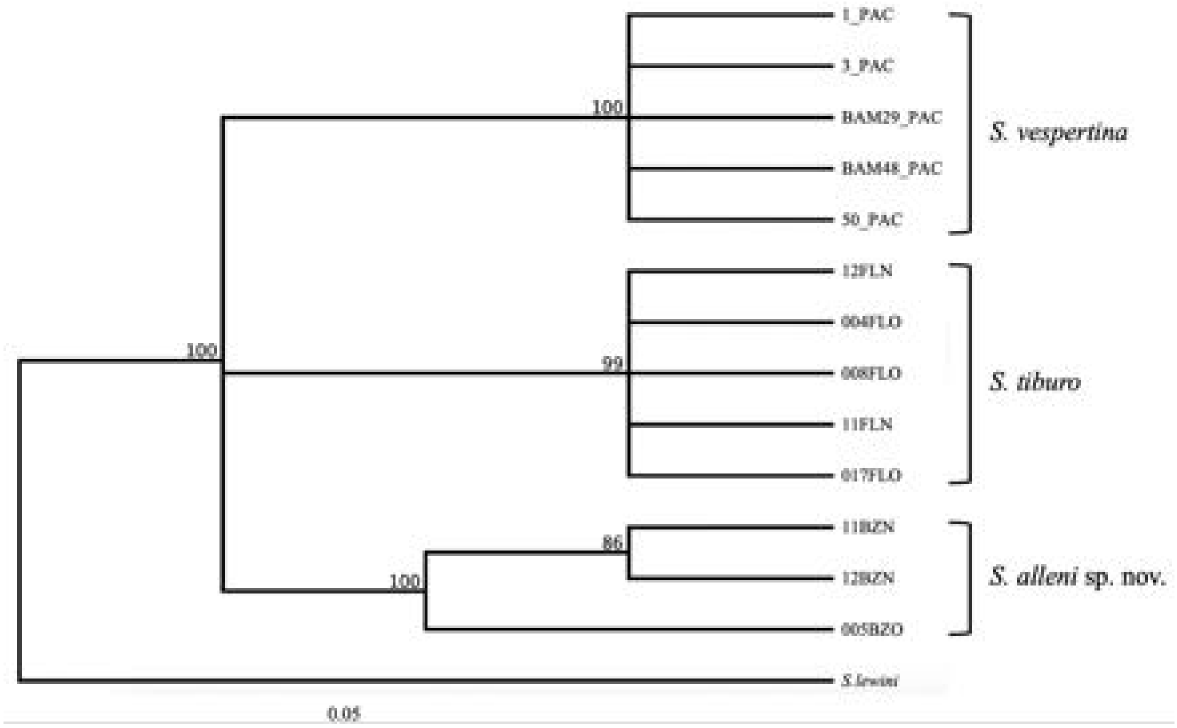


FIGURE 4 Neighbor joining concatenated tree (COI + mtCR) for *S. vespertina*, *S. tiburo*, and *S. alleni* sp. nov. Branch lengths and scale represent the proportion of polymorphic sites between haplotypes, and bootstrap values given in percentage (%).

Microsatellites

Twelve microsatellite loci were successfully amplified for 15 samples of *S. vespertina* and for another 96 samples from *S. tiburo* and 60 from *S. alleni* sp. nov., from the previous study from Gonzalez et al. (2024) were included in the analyses. Individuals with more than >15% of missing genotypes were excluded. Evidence of null alleles was detected. The number of alleles ranged from two (loci Spti44) to 15 (loci Spti48 and Spti41) for *S. vespertina,* from four (loci Spti4) to 17 (loci Spti41) for *S. tiburo,* and between three (loci Spti9 and Spti44) and 16 (loci Spti48) for *S.*

alleni sp. nov., (Table 4). Genetic diversity values including the observed H_O and expected heterozygosities H_E, were obtained for all loci, along with deviations from HW equilibrium (Table 4).

The K estimate values calculated by STRUCTURE using the Evanno method identified *K*= 3 as the most likely number of clusters in the data, clearly separating the three lineages: *S. vespertina*, *S. tiburo* and *S. alleni* sp. nov., (Figure 5). Pairwise F_{ST} values and *Jost's D* values also confirmed significant population differentiation in all cases (Table 5).

TABLE 4 Genetic diversity for 12 microsatellite loci for *S. tiburo, S. alleni* sp. nov., and *S. vespertina*.

		Species								
		S. tiburo (n= 63)			*S. alleni* (n= 53)			*S. vespertina* (n= 16)		
Locus	***A***	***nA***	***Ho***	***HE***	***nA***	***Ho***	***HE***	***nA***	***Ho***	***HE***
Spti2	14	10	0.77	0.85	9	**0.77**	**0.87**	14	0.90	0.35
Spti4	7	4	0.09	0.10	7	**0.64**	**0.76**	6	0.69	0.84
Spti9	6	6	0.71	0.75	3	0.18	0.19	5	0.85	0.78
Spti3	8	7	0.78	0.78	8	0.79	0.84	7	0.69	0.81
Spti10	14	14	0.86	0.89	11	**0.71**	**0.84**	10	**0.31**	**0.86**
Spti34	10	10	**0.53**	**0.84**	6	0.65	0.64	4	0.69	0.70
Spti44	7	7	**0.35**	**0.78**	3	0.40	0.51	2	**0.00**	**0.15**
Spti48	17	17	**0.67**	**0.93**	16	**0.61**	**0.93**	15	**0.69**	**0.95**
Spti42	11	11	0.87	0.87	11	0.85	0.87	7	**0.58**	**0.87**
Spti46	8	5	**0.35**	**0.73**	8	0.83	0.82	6	0.77	0.74
Spti26	8	8	0.65	0.76	8	**0.60**	**0.83**	5	**0.69**	**0.76**
Spti41	17	17	**0.57**	**0.89**	13	0.91	0.89	15	0.85	0.90

A: number of alleles across all populations; *nA*: number of alleles at each locus; *Ho* observed, and *HE* expected heterozygosity; n: number of samples analyzed per species. Loci out of Hardy Weinberg equilibrium are shown in bold.

TABLE 5 Pairwise F_{ST} values (above diagonal) and *Jost's D* values (below diagonal) for the 12 microsatellite loci analyzed.

F_{ST} **Jost's D**	***S. tiburo*** **(n= 63)**	***S. alleni*** **(n= 53)**	***S. vespertina*** **(n= 15)**
S. tiburo	AGD= 0.699 +/-0.361	**0.1461*****	**0.223*****
S. alleni	**0.512*****	AGD= 0.687 +/-0.355	**0.2353*****
S. vespertina	**0.892*****	**0.927*****	AGD= 0.763 +/-0.403

Probability values based on 10,000 permutations. Average Genetic Diversity (AGD) ± values are shown in the diagonal for each species; n= numbers of samples are shown in parentheses. Significant P values in bold at <0.005*, <0.002**, and < 0.001***.

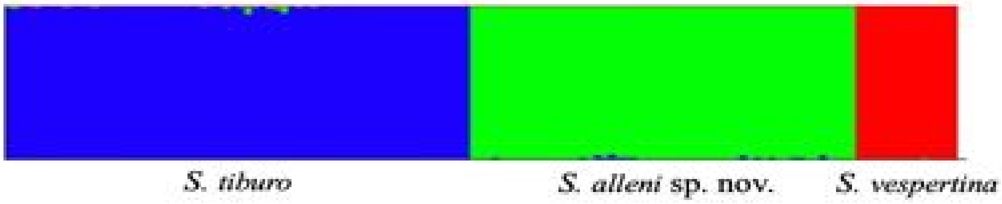


FIGURE 5 SRUCTURE bar plot showing the assignment probabilities (*K*=3) of each genotyped individual of *S. tiburo, S alleni* sp. nov., and *S. vespertina*. *K* groupings correspond to: Blue - Western Atlantic (Bahamas and U.S locations: North Carolina, and South Carolina, U.S); Green – Caribbean and the Southwestern Atlantic (Bocas del Toro, Panama; Placencia, Belize; and Maranhão State, Brazil); and Red - Tropical eastern Pacific (Gulf of Chiriquí, Panama; and Gulf of Tribugá, Colombia).

FIGURE 6 Fresh specimen of *S. vespertina* collected by local fishermen in the Gulf of Chiriqui, Panama (Eastern Pacific). Specimen #364/STRI- Naos Laboratories, Male, TL=99cm. Picture: Cindy Gonzalez.

Taxonomic Treatment

Sphyrna vespertina (Springer 1940) (survive)

Classification. Elasmobranchii, Carcharhiniformes, Sphyrnidae, *Sphyrna.*

Common name. Pacific bonnethead shark (Figure 6)

Synonyms, combinations, and misspellings. *Sphyrna tiburo vespertina, Platysqualus tiburo vespertina.*

Reference of the original description. Springer, S. (1940). Three new sharks of the genus *Sphyrna* from the Pacific coast of tropical America. Stanford Ichthyological Bulletin, 1(5): 161–172.

Types. Holotype: Female,769mm, Panama City, Panama, SU: 11584; Paratypes: Male, 938mm, Guayaquil, Ecuador, CM: 5675; and Female, 520 mm, Panama City, Panama SU:11881.

Neotype. SV360_STRI. Adult female 106 cm, Gulf of Chiriquí, Boca Chica, Panama. Collected in 2023 by Cindy Gonzalez. Specimen repository at Naos Marine Labs, Smithsonian Tropical Research Institute.

Description. Diagnosis after Springer, 1940 [2259]: "A small species (a 938-mm individual is apparently sexually mature), body moderately compressed. head flattened, spade-shaped, its greatest width about 1. 5 times the greatest length of the oculonarial expanse in full-grown individuals, the front margin not lobed, no indentation in the front margin before the nasal apertures, front and side margins of head not clearly confluent in half-grown and mature individuals; length of snout in internasal distance about 1.75 times for large specimens; mouth well arched, a line through its angles in advance of the posterior edges of the, oculonarial expanse; teeth in 12 2 12/12 1 12 to 14 1 14/13 1 13 rows, upper teeth low, the cusps angled toward the outer margins of the jaws, progressively shorter toward the angles of the jaws and nearly or entirely absent on the last row; teeth of the lower jaw entire with slender cusps, cusps low and progressively shorter toward the angles of the jaws, indistinct or absent on the last 4 or 5 rows; first dorsal high, its origin a little behind the end of the pectoral bases; second dorsal with the posterior lobe not greatly produced, length of lobe less than 1.5 times length of base of fin; eyes close to nasal apertures, separated from them by a distance equal to about 1.5 times the diameter of the eye. This species may be distinguished from others of the genus by characters given in the accompanying key. It is closely allied to *Sphyrna tiburo* of the Caribbean, Gulf of Mexico, and the Atlantic coast of the Americas. Direct comparison of specimens of vespertina and tiburo shown that the former has a broader and shorter head, greater internasal distance, higher first dorsal, shorter pectoral, greater body compression, and longer caudal fin. The area of the oculonarial expanse has an important relation to the swimming equilibrium of the sharks of this genus, and its development is accompanied by changes in the shape of the body and the relative sizes of the fins. Thus, the reduced pectorals, the relatively high first dorsal fin, the compressed body, and the large caudal fin which are characteristic of the genus, reflect the degree of development of the flattened head. Data from Englewood specimens of *S. tiburo* and *S. tudes* indicate that growth is accompanied by a proportionate increase in the width and decrease in the length of the head, by a reduction in the relative size of the pectoral fins, and by an increase in the relative height of the first dorsal fin. In adults of tiburo from Englewood there is a measurable difference in proportions of the head in the sexes, the females having slightly wider and shorter heads. It is probable that the maturity factor is important in the determination of any hammerhead" (Springer 1940).

Distribution. Endemic to the Eastern Pacific, from southern California to Ecuador (Ebert et al. 2013). Bonnetheads reside on continental and insular shelves over sandy or muddy bottoms, mangroves, coral reefs, estuaries, and shallow bays from depths <15m (50 ft.) (Compagno 1984a).

Diagnosis. Small and coastal hammerhead shark. Head flattened, triangularly shaped, no indentations in the front margin, straight anterior margin of the head -not lobed. Pacific bonnetheads reach an average size of 100-120 cm with a maximum length of approximately 150 cm (Frazier *et al.* 2014). Females are larger than males on average. Females mature at 84 cm, while males mature between 52-75 cm or less. Life span is estimated at 17.9 years for females and 16.0 years for males (Frazier et al., 2014).

Artificial Key

1a. Narrow and round-shovel shaped head with straight terminations on the anterior margins of the head; the lowest length of the internarial space (8.32 cm in adults); lowest length of snout (from front of mouth to middle of the front margin of the head) (5.31 cm in adults); smallest mouth (width 5.13 cm, length 3.20 cm). Number of precaudal vertebral counts (PVC= ~73). Distributed in the western Atlantic from North Carolina to the Yucatan Peninsula in Mexico..Bonnethead Shark, *Sphyrna tiburo* (Linnaeus, 1758).

2b. Wider and spade-like heart head shape with pronounced lobules on the anterior margins of the head; medium length of the internarial space (9.05 cm in adults); intermediate length of snout (from front of mouth to middle of the front margin of the head) (5.63 cm in adults); mouth (width 5.26 cm, length 3.33 cm). Number of precaudal vertebral counts (PVC= ~80). Distributed in the Caribbean Sea from Belize to the southwestern Atlantic in Brazil...Shovelbill Shark, *Sphyrna alleni* sp. nov., (Gonzalez et al. 2024).

3b. Triangular head broadly spade-shaped, white coloration margin all around the anterior and posterior margins of the head, and straight terminations on the anterior margins of the head; greatest length of the internarial space (9.89 cm in adults); greatest length of snout (from front of mouth to middle of the front margin of the head) (5.85 cm in adults); biggest mouth (width 5.80 cm, length 3.45 cm). Number of precaudal vertebral counts (PVC= ~90). Distributed in the tropical eastern Pacific from California to Ecuador.............................Pacific Bonnethead Shark, Sphyrna vespertina (Springer, 1940).

Discussion

We found that taxonomical, mitochondrial, and nuclear DNA analyses firmly support the existence of three distinct species of bonnethead sharks: *S. tiburo* distributed in the western Atlantic from North Carolina to the Yucatan Peninsula; *S. alleni* sp. nov. distributed in the Caribbean Sea from Belize to Brazil in the southwestern Atlantic; and *S. vespertina* distributed in the eastern Pacific from California to Ecuador (should not be considered a subspecies or synonym of *S. tiburo*).

Although most of the morphological characters evaluated overlap between these three species indicating a high degree of morphological similarity, meristic characters such as precaudal vertebral counts consistently revealed a distinct separation between these three forms. We found the higher number of precaudal vertebral counts in *S. vespertina* (mean= 89, range= 86–91), followed by *S. alleni* sp. nov., (mean = 82, range = 80–83), and we found slightly lower counts in *S. tiburo* (mean = 73, range = 72–74). Vertebral counts are important systematic characters in sharks and in this study PVC constitute the discriminant phenotypic character to classify these cryptic species (Springer & Garrick 1964).

Regarding the external morphology, we found that the highest percentage of variation between *S. vespertina*, *S. alleni* sp. nov., and *S. tiburo* is in the head shape. Our results showed that *S. vespertina* specimens have longer heads than the other two species, the front margin has a pronounced triangular termination shape with a white coloration margin and straight anterior endings on the head. In contrast *S. alleni* sp. nov., has the wider cephalofoil of the three species, has a triangular posterior margin without a white coloration border and a round-lobule termination in the anterior margin of the head. Finally, *S. tiburo* has the smaller and narrowest head with a round termination on the posterior margin, no coloration border, and straight ends on the anterior margin. Our findings are consistent with the results from Aroca et al. (2022) in which they evaluated the head shape with geometric morphometrics and found the same patterns. Despite the differences in the head shape are obvious, it is necessary to conduct more detailed studies to investigate the drivers of speciation, movement patterns, and identify which environmental factors caused these changes in the morphology of the cephalofoil.

The results of our mitochondrial DNA analyses (971 bp of COI + mtCR) also support the existence of three species. The concatenated neighbor joining tree revealed a polytomy of three lineages with 100% of bootstrap support. Despite a common ancestor was shared between *S. vespertina*, *S. alleni* sp. nov., and *S. tiburo*, the estimated substitution rates from the mitochondrial genes evaluated in this study were very low (0.13%-0.15%), therefore it was not possible to resolve the phylogeny of this species complex.

To complement our evidence, the 12 microsatellite loci analyzed showed unique alleles and size composition for each lineage. The existence of three independent genetic pools ($K=3$) was confirmed with the population analyses implemented in STRUCTURE. There is a clear evolutionary separation of the three species reflected in the few alleles shared and the significant population differentiation (both Jost's D and F_{ST} values in all cases). Specifically for *S. vespertina*, such genetic differentiation is a result of a gradual process of geographical isolation that happened during and after the closure of the Isthmus of Panama (IOP), barrier that interrupted the gene flow between the populations of bonnetheads from the Tropical Eastern Pacific and the Tropical Western Atlantic Oceans, promoting independent processes of speciation for these sharks (Aroca *et al.* 2022; Cowman & Bellwood 2013; Gonzalez *et al.* 2021; Lessios 2008; Marko 2002). The uplifting of the IOP triggered multiple vicariant events that have been a base of reference to understand phylogeographical processes and the speciation of many marine species in the world (Coates & Stallard 2013; Collins *et al.* 1996; O'Dea *et al.* 2016).

Despite there is no consensus about the geological time of uplifting and final closure of the IOP (Coates & Stallard 2013; Collins *et al.* 1996; Jaramillo *et al.* 2017; Molnar 2017; Montes *et* *al.* 2015; O'Dea *et al.* 2016), it is accepted that the Pacific Ocean and the Caribbean Sea were still connected by shallow waters between 4.2 to 3.5 Mya, and the formation of the Isthmus was a gradual process of at least 12Mya (Jaramillo *et al.* 2017). Fossil records and Isthmian calibrations (time of divergence between closely related species) assume that the morphologically similar species that splitted in the two coasts of the isthmus (better known as "geminate species'') were isolated geographically around 3.1 to 3.5 Mya (Knowlton & Weigt 1998; Marko 2002).

Fields et al. (2016) estimated the divergence time between *S. tiburo* and *S. alleni* sp. nov., in the Atlantic Ocean and they seem to have separated around 3.61–5.62 Mya. This indicates that vicariant events were also occurring in the Atlantic and the Caribbean region previous to the Isthmus closure (Lessios 2008; Marko 2002). In a most recent study, Aroca et al. (2022) based on a mitochondrial DNA (COI) calibrated tree, proposed that *S. vespertina* splitted from the Atlantic species around 6.7- 1.47 Mya. Reproductive isolation and cessation of the gene flow promoted the speciation process between *S. vespertina* and the other two lineages form the Atlantic, as it has occurred with other species of fishes, echinoderms, and crustaceans (Cowman & Bellwood 2013; Lessios 2008; Robertson & Allen 2015). As the rates of evolution are low and constant in COI and mtCR genes in sharks (Martin *et al.* 1992) we were not able to find which is the most ancestral lineage. Additional mitochondrial markers and genomic approaches such as Single Nucleotide Polymorphisms (SNP's) are needed to solve the phylogeny of this shark complex (Morin et al. 2004; Pazmiño et al. 2017; Vargas-Ramírez et al. 2020). At the same time, as significant population differentiation has been detected in bonnetheads distributed in closely geographical areas (e.g., *S. tiburo* from Texas, Florida, and Mexico), it is necessary to conduct additional genetic, dietary, and biological studies of *S. vespertina* in other locations of the Eastern Pacific to investigate the status of their populations and find if other cryptic species could be hidden (Aroca *et al.* 2022; López-Angarita *et al.* 2021; Smith *et al.* 1998).

Conclusions

As *S. vespertina*, *S. alleni* sp. nov., and *S. tiburo* are small coastal sharks (TL <150cm), these species are vulnerable to predation throughout their lifespan, they have countered by evolving in short generation times to outproduce their losses to predators and natural mortality (Smith *et al.* 1998). Despite these species have a fast rate of recovery to population declines, to date there is no management framework for *S. vespertina*, and *S. alleni* sp. nov., through their distribution ranges. The only protected and regulated species is *S. tiburo* that is managed in the U.S and the Bahamas but not in the rest of its distribution. Sharks of the bonnethead complex are heavily fished mostly in gillnets and are an important component of the local fisheries of many Latin-American countries. There are reported populations that have been collapsed, extirpated, or not seen in decades in California U.S, Mexico, Brazil, and Panama (Bonfil *et al.* 1990; Cardeñosa *et al.* 2020; Harper *et al.* 2014; Reis-Filho *et al.* 2014). It is imperative to call for a new reassessment of the conservation status by the IUCN for these small hammerhead sharks.

The clarification of species complexes needs the supportive combination of diagnostic morphological characters that allow for species recognition and genetic approaches, as an inability to recognize individuals in the field creates difficulties for future research, management for conservation, and fisheries purposes. Additional studies that encompass the full range of this species complex is needed. Uncovering these cryptic species is essential for the comprehension of their evolutionary processes, biogeography, ecology, and to conservation strategies, as distribution ranges need to be redefined and can be smaller than initially assumed meaning a higher risk of local extinction for these species (Baldwin *et al.* 2011; Bickford *et al.* 2007; Dulvy *et al.* 2014).

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CHAPTER V

GENERAL CONCLUSIONS

Hammerheads are one of the most threatened and exploited groups of sharks globally with most of the species with declining population trends (Cardeñosa *et al.* 2018; Chapman *et al.* 2009). There are ten described species distributed in two genus (*Sphyrna* and *Eusphyra*), with almost each species having a unique head shape (Compagno 1984; Gilbert 1967; Rafinesque C. S. 1810). Most of the species in the genus *Sphyrna* have been assessed as Vulnerable, Endangered or Critically Endangered on the IUCN Red List of Threatened Species (Cardeñosa *et al.* 2018; Fowler 2014; Miller *et al.* 2013).

Interestingly, from the *Sphyrna* species four of them are small coastal sharks (TL <150 cm) restricted to the tropical waters of the American continent (the smalleye hammerhead *S. tudes*, the scoophead shark *S. media*, the scalloped bonnethead shark - *S. corona*, and the bonnethead shark - *S. tiburo*). These small hammerhead sharks inhabit sandy or muddy bottoms, mangroves, coral reefs, estuaries, and seagrass beds until a depth of less than 100 m (Bigelow & Schoroeder 1948; Compagno 1984). These species are less susceptible to population declines than other congeneric shark species, as they have high population growth rates, early maturation times, and shorter gestation times (Driggers *et al.* 2014; Frazier *et al.* 2014; Parsons 1993). Notwithstanding, these coastal sharks are a prevalent component of the local fisheries in Mexico, Guatemala, Honduras, Belize, Panama, Venezuela, and Brazil (Pollom 2020), where meat is consumed or sold locally and the fins are traded internationally (Cardeñosa *et al.* 2020). Small hammerheads are mostly fished in gillnets as target and as bycatch (Pollom 2020), and previous studies have reported collapsed or extirpated populations in California, Mexico, and Brazil (Bressan *et al.* 2009; Domingues *et al.* 2019; Pérez-Jiménez 2014). Except for the U.S. and Bahamas, small hammerhead shark fisheries are not directly assessed or actively managed for sustainability (Simpfendorfer & Dulvy 2017).

Drastic population declines (50-80%) were identified by the UICN in 2019, reassessing *S. tiburo* as Endangered species, and *S. media, S. tudes,* and *S. corona* as Critically Endangered (IUCN, 2020). Later, in 2022 the family Sphyrnidae was listed in CITES appendix II to regulate their international trade and increase enforcement, as there is constant misidentification of the hammerhead species as they “look-alike”. Subsequently, a growing effort on research has been dedicated to direct effective conservation strategies and sustainable management, with a specific focus on hammerhead shark distributional patterns and the scale of population subdivision on both a regional (Aroca *et al.* 2022; Díaz-Jaimes *et al.* 2021; Escatel-Luna *et al.* 2015; Gonzalez *et al.* 2019; Quintanilla *et al.* 2015), and a global scale (Daly-Engel *et al.* 2012; Duncan *et al.* 2006; Grobler *et al.* 2023; Lim *et al.* 2010).

Hammerhead sharks represent a monophyletic group (including all the descendants of a common ancestor) (Cavalcanti 2007). Despite several taxonomical and some genetic studies, to date, there is no consensus about the phylogenetic placement of the small hammerhead shark species, but it is assumed that the bonnethead shark *S. tiburo* is the intermediate species between the large bodied and small hammerhead sharks (Lim *et al.* 2010). *Sphyrna tiburo* (Linnaeus,1758), is a small coastal shark (<150 cm at maturity), distributed in the EP from California (U.S.) to Ecuador, and in the western Atlantic Ocean (WA) from North Carolina (U.S.) to southern Brazil (Compagno 1984; Jawad 2013). Taxonomically, only *S. tiburo tiburo* for the WA (Linnaeus, 1758), and *S. tiburo vespertina* for the EP (Springer, 1940) are recognized as valid subspecies (Gilbert 1967). Nonetheless, previous studies based on mitochondrial markers evidenced that bonnethead sharks from the WA and the Caribbean are a species complex with two cryptic lineages (*S. tiburo,* and *S.* aff. *tiburo,* respectively) (Fields *et al.* 2016; Gonzalez *et al.* 2019; Naylor *et al.* 2012).

This dissertation provides genetic, morphological, and ecological evidence to detangle three cryptic species that were considered as a single species (*S. tiburo*). In the Chapter II, based on the analysis of mitochondrial markers (COI, and mtCR) and landscape genetics I corroborated that WA bonnethead sharks are a species complex with at least two cryptic lineages, complementing the findings from previous studies (aka *S. tiburo* and *S.* aff. *tiburo*) (Fields *et al.* 2016; Gonzalez *et al.* 2019; Naylor *et al.* 2012). Both lineages exhibit significant population differentiation over relatively small spatial scales (Díaz-Jaimes *et al.* 2021), which is consistent with evidence that small hammerhead shark species display a philopatric behavior and do not perform long migrations (Driggers *et al.* 2014; Escatel-Luna *et al.* 2015; Feldheim *et al.* 2014; Fields *et al.* 2016; Gonzalez *et al.* 2019; Heupel *et al.* 2006; Portnoy *et al.* 2015). Our findings suggest that *S. tiburo* is distributed only from North Carolina (U.S.) to Belize, while *S.* aff. *tiburo* is distributed from Belize to Brazil, extending the distribution range of this new lineage >5.000 km to the south. We also suggest that a potential contact zone for both lineages exists between the Yucatan Peninsula and Belize. These findings changed our understanding of the current distribution of *S. tiburo tiburo,* that was originally thought to be distributed from North Carolina, U.S all the way down to Brazil. To date, no geographical barriers in the Western Atlantic or the Caribbean Sea have been identified to be disrupting the genetic flow between the two lineages, but a series of geological changes in the sea level in the Mesoamerican reef could explain these distribution patterns (Heyman *et al.* 2008; Kramer *et al.* 2015). An important lesson we learned through all these years of research is that small hammerhead sharks are residential to their natal grounds, and therefore remain during their lifespan in their natal grounds (Escatel-Luna *et al.* 2015; Gonzalez *et al.* 2019; Heupel *et al.* 2006; Portnoy *et al.* 2015).

In chapter III I consolidated the necessary evidence to propose and describe *S.* aff. *tiburo* as a new species of hammerhead shark: *Sphyrna alleni* sp. nov., (Common name: Shovelbill shark). As vertebral counts constitute a meristic character for species delimitation in sharks (Springer & Garrick 1964), I found that *S. alleni* sp. nov., has higher precaudal vertebral counts than *S. tiburo* (~80 vs ~71 respectively). The bodies of the two species are very similar (cryptic species), but differences in the head shape and distribution of teeth constitute the morphological characters to differentiate this species visually. While *S. tiburo* has a shovel head shape with a rounded anterior margin, *S. alleni* sp. nov., has a triangular termination on the anterior margin and developed lobules in the posterior margin of the head. Respect the teeth *S. tiburo* has less cuspless rows of teeth distributed in the back part of the jaw, while *S. alleni* sp. nov., has more rows of cuspless teeth in a clumpy distribution pattern in the same part of the jaw. Finally, both the mitochondrial and nuclear analyses reflected a high degree of genetic differentiation with almost different allele sizes and very few alleles shared, suggesting that the two species have been on independent evolutionary paths for at least 3.5 Mya. In this chapter I presented an original description for the species following the rules of the International Code of Zoologial Nomenclature.

The identification of genetically distinct populations is essential for the comprehension and clarification of evolutionary processes such as the adaptative radiation that shaped the current distribution patterns and adaptations to the environment of these particular sharks (Gonzalez *et al.* 2021; Reis-Filho *et al.* 2014). Fields et al. (2016) estimated a shared common ancestor for the two lineages between 3.61-5.62 Mya, which indicates that they separated before or during the uplifting and final closure of the Isthmus of Panama.

In chapter IV I tested the hypothesis whether the Pacific bonnethead *S. tiburo vespertina* subspecies distributed in the Eastern Pacific constitutes a third cryptic species for the bonnethead complex. Building up from the findings and data from chapter III, I implemented the same methods to resurrect *S. vespertina* (Springer, 1940- survive). as a species. Precaudal vertebral counts represented the higher counts from the three species (~90 PVC). The shape head is unique with an anterior pointy and triangular margin, and straight posterior ends with no lobules developed. There is also a white coloration edge all around the head of the *S. vespertina* individuals examined. Regarding the genetic findings, the phylogenetic reconstruction showed a polytomy, so I could not resolve the phylogeny of the bonnethead complex only based in the two mitochondrial markers analyzed. Other powerful methods such as SNP's should be implemented to produce an updated phylogeny with enough resolution to correctly place the bonnethead complex and other species of small hammerhead sharks.

The 12 microsatellite loci analyzed clearly showed genetic differentiation between the three species (*S. tiburo*, *S. alleni* sp. nov., and *S. vespertina*), and the STRUCTURE results showed three groups with unique allele composition and non or very few allele sizes overlapping.

The closure and rising of the IOP modified the environment affecting the current flow, primary productivity, temperature, and salinity of the Caribbean Sea and the Pacific Ocean, triggering independent evolutionary trajectories for S. vespertina and the other two species from the Atlantic Ocean, as it has happened with many other species of fishes (Lessios 2008). Although different hypotheses of the formation of the Isthmus of Panama are still in debate (Coates & Stallard 2013; Collins *et al.* 1996; Jaramillo *et al.* 2017; Molnar 2017; Montes *et al.* 2015; O'Dea *et al.* 2016), all agree that between 4.2 to 3.5 Ma the Caribbean Sea and the Pacific Ocean were still connected by shallow waters (Jaramillo *et al.* 2017). This suggests that the diversification inside the bonnethead complex happened before or during the closure of the Isthmus, barrier that interrupted the genetic flow between *S. vespertina* from the TEP and *S. alleni* sp. nov., from the Caribbeans Sea.

Overall, this dissertation highlights the complexity of integrating taxonomical approaches with molecular methods for species delimitation and clarification. The data presented here fill important gaps in understanding the origins of the small hammerhead sharks in the American continent. Moreover, by studying cryptic species I was able to resolve the phylogeography and taxonomy of the bonnethead complex, with three different species (*S. tiburo* and *S. alleni* sp. nov., in the Atlantic, and *S. vespertina* in the eastern Pacific Ocean). The ultimate goal of this dissertation is to make a call for an urgent, updated IUCN red list assessment for this species complex throughout their distribution ranges, to provide a conservation framework and improve fisheries policies in Latin-American countries.

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