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Resurrection of the sixgill shark *Hexanchus vitulus* Springer & Waller, 1969 (Hexanchiformes, Hexanchidae), with comments on its distribution in the northwest Atlantic Ocean

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Abstract

The sixgill sharks of the genus *Hexanchus* (Hexanchiformes, Hexanchidae) are large, rarely encountered deep-sea sharks, thought to comprise just two species: the bluntnose sixgill *Hexanchus griseus* (Bonaterre, 1788) and the bigeye sixgill *Hexanchus nakamurai* (Teng, 1962). Their distribution is putatively worldwide in tropical and temperate waters, but many verified records for these species are lacking, and misidentification is common. Taxonomic uncertainty has long surrounded *H. nakamurai* in particular, with debate as to whether individuals from the Atlantic constitute a separate species. Using 1,310 base pairs of two mitochondrial genes, *COI* and *ND2*, we confirm that bigeye sixgill sharks from the Atlantic Ocean (Belize, Gulf of Mexico, and Bahamas) diverge from those in the Pacific and Indian Oceans (Japan, La Reunion, and Madagascar) with 7.037% sequence divergence. This difference is similar to the genetic distance between both Atlantic and Indo-Pacific bigeye sixgill sharks and the bluntnose sixgill shark (7.965% and 8.200%, respectively), and between the entire genus *Hexanchus* and its sister genus *Heptranchias* (8.308%). Such variation far exceeds previous measures of species-level genetic divergence in elasmobranchs, even among slowly-evolving deep-water taxa. Given the high degree of morphological similarity within *Hexanchus*, and the fact that cryptic diversity is common even among frequently observed shark species, we conclude that these results support the resurrection of the name *Hexanchus vitulus* Springer and Waller, 1969 for bigeye sixgill sharks in the northwest Atlantic Ocean. We propose the common name "Atlantic sixgill shark" for *H. vitulus*, and provide new locality records from Belize, as well as comments on its overall distribution.

Keywords Systematics · Mitochondrial DNA · Phylogenetics · Speciation · Elasmobranchs

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Introduction

Of extant chondrichthyan species (sharks, skates, rays, and chimaeras), approximately 48% inhabit the deep ocean, and of these, fewer than 5% have life histories that are known to science (Cotton and Grubbs 2015). Among the largest deep-sea elasmobranchs are the sixgill sharks of the genus *Hexanchus* (Hexanchiformes, Hexanchidae), which has its origin in the early Jurassic (~200 mya; Musick et al. 2004), making it one of the oldest evolutionary lineages of vertebrates on the planet. Within the family Hexanchidae, the cowsharks, only four species are currently recognized, two of these in *Hexanchus* (Castro 2010). Typified by their large size, unique saw-like lower teeth, and unmistakable six or

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seven gill slits, cowsharks are nonetheless poorly understood by science and difficult to both observe and identify.

Sixgill sharks typically inhabit mesopelagic and bathypelagic waters (>200 m) in tropical and temperate oceans throughout the world (Castro 2010). Sixgills are elusive despite their size, and study has been hindered by their relatively inaccessible habitat. As a result, specimens are rare and difficult to obtain, precluding the type of in-depth examination of a range of individuals comprising different sexes, life stages, and locations that is ideal for taxonomy. Similar to other deep-water species, sixgill sharks likely display slower growth rates and other life history traits relative to shallow-water taxa, due to both biotic and abiotic factors in their environment, including low overall productivity and temperature. Such conditions may lead to lower metabolisms among deep-water organisms, which in turn induces generally slower rates of molecular evolution, speciation, and morphological divergence (Brown et al. 1979; Daly-Engel et al. 2010; Martin et al. 1992; Sorenson et al. 2014). Within the genus *Hexanchus*, highly conserved morphology coupled with the dearth of specimens available for study has produced decades of difficulty in describing alpha taxonomy, distribution, and detailed life-history characters in most areas where these animals occur.

Currently, two valid species are recognized in the genus *Hexanchus*. The bigeye sixgill shark, *Hexanchus nakamurai* (Teng, 1962), is a little-studied deep-water cowshark largely defined by its patchy distribution and still-debated taxonomi designation (Ebert et al. 2013; Springer and Waller 1969; Teng 1962). Often misidentified as small specimens of its larger congener the bluntnose sixgill shark, *Hexanchus griseus* (Bonaterre, 1788), the distribution of the bigeye sixgill shark is poorly known, but thought to be circumglobal throughout tropical and warm deep seas (Ebert et al. 2016). In the northwest Atlantic Ocean, the bigeye sixgill shark is known to inhabit the waters of the southern Mexican Yucatan and Caribbean, Florida, the Bahamas, Cuba, Nicaragua, Costa Rica, Venezuela, and the Guyanas (Castro 2010; Clark and Kristof 1990; Compagno 1984; Ebert 1990; Ebert et al. 2013; McLaughlin and Morrissey 2004), where it is reported to occur at depths between 90 and 701.5 m (Brooks et al. 2015; Ebert et al. 2016). The bigeye sixgill shark has been reported in bottom longline catches in the northern and eastern Gulf of Mexico since 2007 (Gulak et al. 2013; Hale et al. 2007; Morgan et al. 2009; Scott-Denton et al. 2011), but its full distribution and life history is uncertain, and its taxonomy remains unresolved.

First described by Teng (1962) from Taiwan as a subspecies of the bluntnose sixgill shark, *Hexanchus griseus nakamurai*, the bigeye sixgill shark was then separately described from the Bahamas as *H. vitulus* (Springer and Waller, 1969) based on observed morphological differences from *H. griseus*. Thought to be isolated to the northwest Atlantic Ocean, *H. vitulus* was for years differentiated from the Indo-Pacific *H. nakamurai* based on distribution alone, but two separate studies in the early 1990s compared the morphology of bigeye sixgill sharks from the Atlantic and the Pacific and found no discernible differences (Ebert 1990; Taniuchi and Tachikawa 1991). Subsequently Taniuchi and Tachikawa (1991) elevated the subspecies *Hexanchus griseus nakamurai* to the species *H. nakamurai* and synonymized it with *H. vitulus*; thus, *H. nakamurai* became the senior synonym and most widely accepted (Ebert 1990; Ebert et al. 2013; Taniuchi and Tachikawa 1991; Teng 1962). Currently, *H. nakamurai* is the only valid species nomenclature for the bigeye sixgill shark (Ebert et al. 2013, 2016; Taniuchi and Tachikawa 1991), though morphology between these species is highly conserved, and Ebert et al. (2013) suggested, based on genetic evidence from Naylor et al. (2012), that *H. nakamurai* and *H. vitulus* may comprise separate species in the Indo-Pacific and northwest Atlantic Oceans, respectively.

Due to its presumed low abundance and limited fishing effort in deep-sea habitats, *H.* cf. *nakamurai* is not captured frequently, and therefore its full distribution and conservation status are unknown. However, *H.* cf. *nakamurai* was the third most commonly captured shark species in deep-sea longline surveys in Exuma Sound in the Bahamas (Brooks et al. 2015), and was reported as common in the northern Gulf of Mexico by Castro (2010). It is likely that *H.* cf. *nakamurai* is more abundant than current data show, because there has been very little exploration of the deep tropical Atlantic Ocean to date. As commercial fishing moves increasingly into the deep ocean, however, the uncertain taxonomy of sixgill sharks and other deep-sea elasmobranchs may confound management efforts (Cotton and Grubbs 2015; Pfleger et al. 2018). Management issues are further exacerbated by a lack of perceived economic importance and representation in protective strategies or protocols at the local and international levels, including conventions such as the Convention on the Conservation of Migratory Species of Wild Animals (CMS) and the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES).

To help resolve taxonomic uncertainty and aid in conservation efforts, we undertook a detailed ecological and molecular examination of *H.* cf. *nakamurai* in the Atlantic Ocean. Using 1,310 base pairs (bp) of mitochondrial DNA and detailed catch records, we compared specimens of *H.* cf. *nakamurai* with *H. griseus* and the sevengill shark *Heptranchias perlo* (Hexanchiformes, Hexanchidae) in the Atlantic, as well as with *H. nakamurai* from the Indian and Pacific Oceans. A discussion of the distribution and biodiversity of *Hexanchus* in the Atlantic is also included.

Materials and methods

Captures

In Belize, a fishery-independent vertical longline survey was conducted from 2015 to 2016 in waters from 150 to 400 m (Fig. 1). Vertical longlines were hand deployed, and consisted of either a monofilament line terminating in 5–10 stainless steel 10/0 and 13/0 circle hooks, or a monofilament line terminating in a stainless-steel leader and 4, 16/0 circle hooks. A LAT1400 temperature depth recorder (TDR; Lotek Wireless, Newmarket, ON,Canada) was positioned just above the top hook, which measured temperature and pressure (dbar) every 15 s. Captured bigeye sixgill sharks were measured for fork length (FL) and total length (TL) (in centimeters) in a curved line, sexed, sampled (0.5 cm fin clip) for genetic analysis, and released (Table 1).

In the U.S. Gulf of Mexico (GoM), a fishery observer program administered by the National Oceanic and Atmospheric Administration (NOAA) Fisheries Service has monitored a portion of the shark and reef fish bottom longline fishery in the northwest Atlantic Ocean and US GoM since 2005 (Enzenauer et al. 2015). The survey deploys certified fisheries observers on commercial fishing vessels to witness all fishing activity; data collected include gear characteristics, set and haulback information, environmental conditions, catch composition, and specimen morphometrics. Observers also collect biological samples during their deployments at sea (Fig. 1; Enzenauer et al. 2015). Captured sixgill sharks were opportunistically sampled; observers recorded sex and FL (cm) measured in a straight line, then sampled a 0.5-cm fin clip for genetic analysis, and released the sharks alive when possible (Table 1). One whole *H.* cf. *nakamurai* specimen was obtained opportunistically from a NOAA Fisheries Cooperative Research Project (CRP) in the northeastern GoM (Fig. 1), which sampled for biological data from tilefishes and deep-water groupers (NA08NMF4540392) in 2009.

Further, another fishery-independent bottom longline survey was conducted in the northeastern GoM and Bahamas from 2011 to 2016 by scientists from Florida State University. A standard longline/trap set consisted of 550 m of mainline anchored and deployed along the bottom with 50 baited gangions of five hook sizes (10/0, 11/0, 12/0, 14/0 and 18/0) spaced 10 m apart and set on the bottom at depths between 160 m and 2645 m deep. Each set terminated with a LAT1400 TDR (Lotek Wireless, Inc.) that measured temperature and depth every 10 s. Captured sixgill sharks were measured [precaudal length (PCL), FL, TL], sexed, and sampled (0.5 cm fin clip) for genetic analysis (Table 1). Sampling map

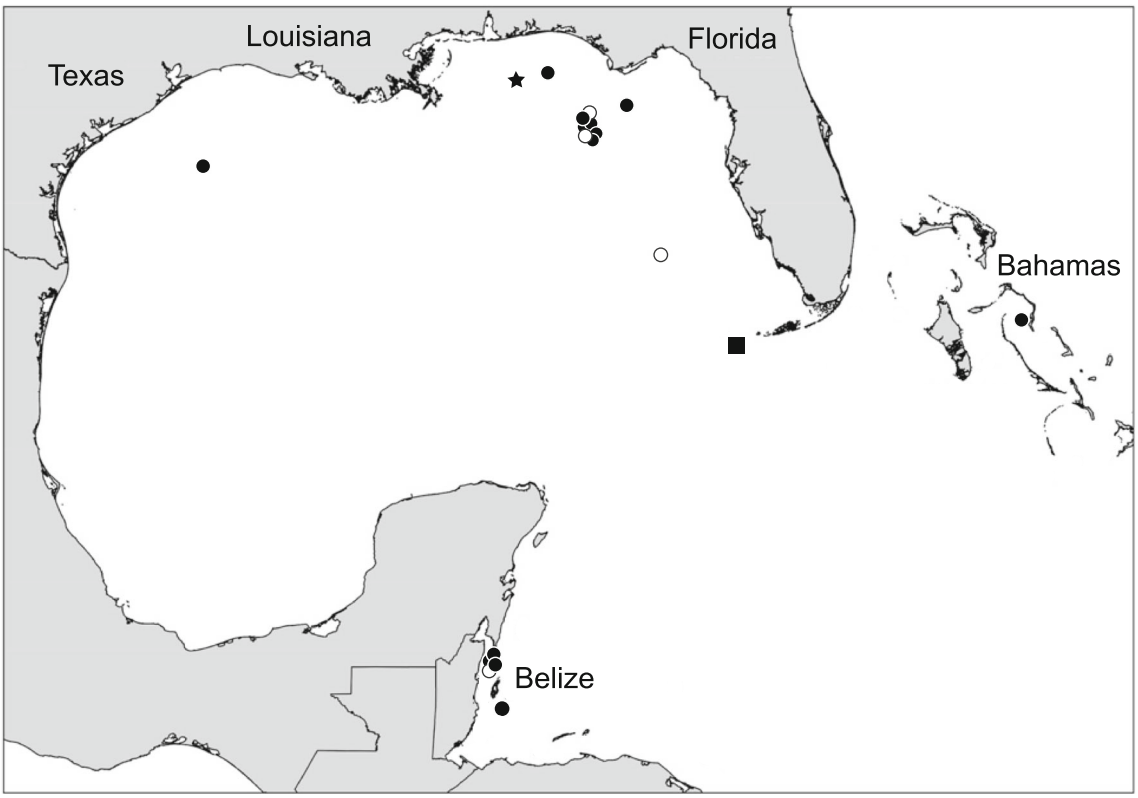

Fig. 1 Capture locations of sixgill sharks in Belize, the northern Gulf of Mexico, and the Bahamas from 2007 to 2016. *Circles* represent *Hexanchus vitulus*, and the *square* represents *H. griseus*. *Closed circles* were IDs confirmed by genetic sequencing, and the *star* represents the capture location of the neonate *H. vitulus*

Table 1 Capture dates, locations, sizes and sexes of *Hexanchus vitulus* captured in the northern Gulf of Mexico (*GoM*), Bahamas, and Belize from 2007 to 2016

Sample ID	Location	Date of capture	Sex	FL (cm)	Straight or curved	Mean depth (m)	Temp (°C)	Source
Hna115	GoM	13-Jan-07	F	69.0	S	299.0	NM	BLLOP
Hna150	GoM	14-Jan-07	M	55.0	S	294.5	NM	BLLOP
Hna151	GoM	14-Jan-07	F	49.0	S	294.5	NM	BLLOP
Hna152	GoM	14-Jan-07	M	54.0	S	294.5	NM	BLLOP
Hna126	GoM	10-Apr-10	F	55.0	S	238.0	NM	BLLOP
Hna120	GoM	13-Apr-10	F	40.0	S	270.0	NM	BLLOP
Hna101	GoM	16-Apr-10	F	59.0	S	253.0	NM	BLLOP
Hna014	GoM	30-Nov-10	F	62.5	S	146.0	NM	BLLOP
Hna016	GoM	22-Apr-11	F	62.5	S	250.0	NM	BLLOP
Hna017	GoM	22-Apr-11	F	64.0	S	250.0	NM	BLLOP
Hna110	GoM	25-Oct-11	F	59.0	S	247.5	NM	BLLOP
Hna142	GoM	26-Oct-11	M	61.0	S	248.5	NM	BLLOP
Hna107	GoM	31-Oct-11	M	57.0	S	232.5	NM	BLLOP
Hna122	GoM	21-Jan-12	F	70.0	S	114.5	NM	BLLOP
NBZ3001	Belize	11-Feb-16	F	100.0	C	242.0	NM	MA
GLO026	Belize	24-Aug-16	M	116.0	C	269.0	16.0	MA
NBZ3005	Belize	19-Sep-16	F	104.0	C	333.0	13.0	MA
NBZ2503	Belize	20-Sep-16	F	104.0	C	285.0	15.1	MA
–	Belize	20-Sep-16	F	72.0	C	285.0	15.1	MA
–	Belize	21-Oct-16	M	101.0	C	288.0	14.0	MA
–	Belize	21-Oct-16	M	86.0	C	265.0	14.0	MA
–	Belize	22-Oct-16	M	74.0	C	277.0	14.2	MA
–	Belize	22-Oct-16	F	100.0	C	277.0	14.2	MA
–	Belize	14-Nov-16	M	99.0	C	250.0	16.6	MA
–	Belize	15-Nov-16	M	93.0	C	313.0	13.9	MA
–	GoM	13-Jan-07	F	55.0	S	289.5	NM	BLLOP
–	GoM	14-Jan-07	F	54.0	S	294.5	NM	BLLOP
–	GoM	22-Jul-07	F	240.0	S	141.0	NM	BLLOP
–	GoM	29-Apr-08	F	80.0	S	184.0	NM	BLLOP
–	GoM	11-Apr-10	M	70.0	S	231.5	NM	BLLOP
–	GoM	13-Apr-10	F	58.0	S	279.0	NM	BLLOP
–	GoM	16-Apr-10	F	66.0	S	253.0	NM	BLLOP
–	GoM	16-Apr-10	F	43.0	S	253.0	NM	BLLOP
–	GoM	24-Oct-11	F	50.0	S	244.0	NM	BLLOP
–	GoM	3-May-14	F	45.0	S	288.0	12.5	FSU
–	GoM	3-May-14	F	38.5	S	288.0	12.5	FSU
–	GoM	3-May-14	M	47.0	S	288.0	12.5	FSU
–	Belize	20-Sep-16	F	72.0	C	285.0	15.1	MA
–	Belize	21-Oct-16	M	101.0	C	288.0	14.0	MA
–	Belize	21-Oct-16	M	86.0	C	265.0	14.0	MA
–	Belize	22-Oct-16	M	74.0	C	277.0	14.2	MA
–	Belize	22-Oct-16	F	100.0	C	277.0	14.2	MA
–	Belize	14-Nov-16	M	99.0	C	250.0	16.6	MA
–	Belize	15-Nov-16	M	93.0	C	313.0	13.9	MA
CEI074	Bahamas	28-Oct-10	M	117.0	S	NM	NM	FSU
CEI090	Bahamas	13-Nov-10	F	87.0	S	NM	NM	FSU
CEI094	Bahamas	25-Nov-10	M	123.0	S	NM	NM	FSU
CEI103	Bahamas	1-Dec-10	M	124.0	S	NM	NM	FSU
CEI104	Bahamas	1-Dec-10	M	113.5	S	NM	NM	FSU

Sample ID refers to the DNA sample; if no ID is listed that individual was not genetically sequenced. *FL* fork length, *S* straight measurement, *C* curved measurement, *NM* not measured, *BLLOP* Bottom Longline Observer Program, *MA* MarAlliance, *FSU* Florida State University Deep-C Consortium

was generated with QGIS and modified in Adobe Illustrator (Fig. 1).

Identification

Deep-sea fishers in Belize were interviewed in 2015–2016 about the species of sharks captured in waters >150 m. Of those that reported catching sixgill sharks, further inquiries were made to clarify the size, shape and size of the eye, and location of the capture of each animal. Detailed photos and a guide (Ebert et al. 2016) were used to distinguish between *H.* cf. *nakamurai* and *H. griseus;* only recent observations from fishers who demonstrated the interest and ability to differentiate between the species were recorded. Additionally, fishers were interviewed in 2004–2007, and jaws of landed specimens were examined.

Identification of species in the field was made using tooth plate counts when possible (Ebert et al. 2016), with animals possessing five lower tooth plates on each side of the jaw being identified as *H.* cf. *nakamurai* and those with six lower tooth plates being *H. griseus*. In addition, the greater dorsal-caudal space resulting from the placement of the dorsal fin well anterior to the anal fin was used to distinguish *H.* cf. *nakamurai* from *H. griseus*. Genetic species identification of a subset of 22 individuals was accomplished by comparing tissues from the current study with congeners using 1,310 bp of concatenated mitochondrial genes, NADH dehydrogenase 2 (*ND2*) and cytochrome oxidase I (*COI*). Samples of *H.* cf. *nakamurai* from the Bahamas, GoM, and Belize, *H. griseus* from the northeast GoM, *H. nakamurai* from the Indian Ocean (La Reunion), and *Heptranchias perlo* from the Bahamas were obtained through direct sampling; sequences from the Bahamas and four *H. griseus* were obtained from previous studies (Brooks et al. 2015). An additional three mtDNA genomic sequences were downloaded from Genbank for *H. nakamurai* from the Indo-Pacific: two from Japan (Accession nos. NC022733 and AB560491.1) and one from Madagascar (*ND2* only, Accession no. JQ518726.1).

Genetic analyses

Small (<1 cm^3) samples of fin or muscle tissue were taken at the time of capture and stored in 2-mL screw-top vials containing 1.5 mL 20% dimethylsulfoxide (DMSO) saturated salt (NaCl) buffer (Seutin et al. 1991) or >75% ethanol (EtOH). DNA was extracted from fin clips using a DNeasy Blood & Tissue Kit from Qiagen (Germantown, MD, USA) and amplified using primers obtained from Integrated DNA Technologies (Coralville, IN, USA). The *COI* gene was amplified using HCO/LCO primers (Folmer et al. 1994), and the *ND2* gene was amplified using primers tMetShkND2_F/tAlaShkND2_R from O'Brien et al. (2013). PCR reactions consisted of 14 uL BioMix Red from Bioline (London, UK) at the recommended concentration, 2 uL (3 ng) template DNA, and 2 uL (1.0 M) each primer (20 uL total PCR volume). PCR amplification on a C1000 Touch Thermal Cycler (Bio-Rad; Hercules, CA, USA) consisted of an initial denaturation at 95 °C for 4 min, followed by 36 cycles of 1 min at 95 °C, followed by 30s at 58 °C, and 30s at 72 °C with a final extension at 72 °C for 20 min. Products were cleaned with a standard exonuclease 1/thermosensitive alkaline phosphatase (FastAP) cleaning protocol using a 7:1 product: enzyme ratio, and sequenced on an Applied Biosystems 3730XL DNA Analyzer at the University of Arizona Genetics Core.

DNA sequences were trimmed and concatenated in Geneious v. 9.1.8 (Kearse et al. 2012), and aligned using the Mafft (Katoh et al. 2002) plugin for Geneious. A mutational model was calculated using jModeltest (Posada 2008) for each gene separately as well as for the concatenated sequences. MrBayes v. 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) was used to construct a Bayesian inference phylogenetic tree. First, analyses of Markov Chain Monte Carlo (MCMC) chains were run for 10,000,000 generations, while sampling one tree per 200 generations. Convergence between simultaneous runs was reached when the average standard deviation of split frequencies fell below 0.01 (Ronquist et al. 2005). Following a burn-in of 10,000 steps, posterior clade probabilities were calculated and the likelihood scores for all the topologies averaged. Genetic distance expressed as percent sequence divergence was calculated in MrBayes (Huelsenbeck and Ronquist 2001). A maximum likelihood analysis was run using the Geneious plugin for PhyML (Guindon et al. 2010), and a Bayesian phylogenetic tree was generated in Geneious and modified in FigTree v. 1.4.2 (Fig. 2).

Results

Captures

A total of 45 sixgill sharks were captured by the four fishery-independent and two fishery-dependent surveys in Belize, Bahamas, and the northern Gulf of Mexico (GoM) from 2007 to 2016 (Table 1, Fig. 1). Of these, fin clips from *Hexanchus* cf. *nakamurai* from Belize ($n = 4$), the GoM ($n = 14$), and the Bahamas ($n = 5$) were sequenced for genetic identification. These were genetically compared with a number of DNA sequences for individuals obtained for a separate study: *H. griseus* from the GoM ($n = 5$), and *H. nakamurai* from the Indo-Pacific ($n = 4$; Fig. 2). In Belize, 11 bigeye sixgill sharks (*H.* cf. *nakamurai*), six males and five females, were captured by fishery-independent surveys in 2016 (Table 1, Fig. 1). All males had non-calcified claspers and therefore were presumed juvenile, and the females were also presumed juvenile due to their sizes (<135 cm TL). In the GoM, *H.* cf. *nakamurai* were reported by fishery observers on 19 bottom longline sets from 2007 to 2016 in the northern GoM, with a total of 23 sixgill sharks sampled. Genetic sequencing confirmed the identification of 14 *H.* cf. *nakamurai* and one *H. griseus* from these captures (Table 1, Fig. 1). All bigeye sixgill sharks captured in the northern GoM were <80 cm FL and therefore classified as juveniles. In addition, three newborn *H.* cf. *nakamurai* (52–61 cm TL, possessing yolk scars) were captured in the fishery-independent surveys in the northern GoM in May 2014. It is notable that though nearly 500 longline sets were made in this survey and 31 *H. griseus* were captured, *H.* cf. *nakamurai* were only captured on a single set (Table 1, Fig. 1). During Fall 2010 and 2011, a total of 14 *H.* cf. *nakamurai* (11 males and 3 females) and one sevengill shark (*Heptranchias perlo* (Bonnaterre, 1788)) were captured

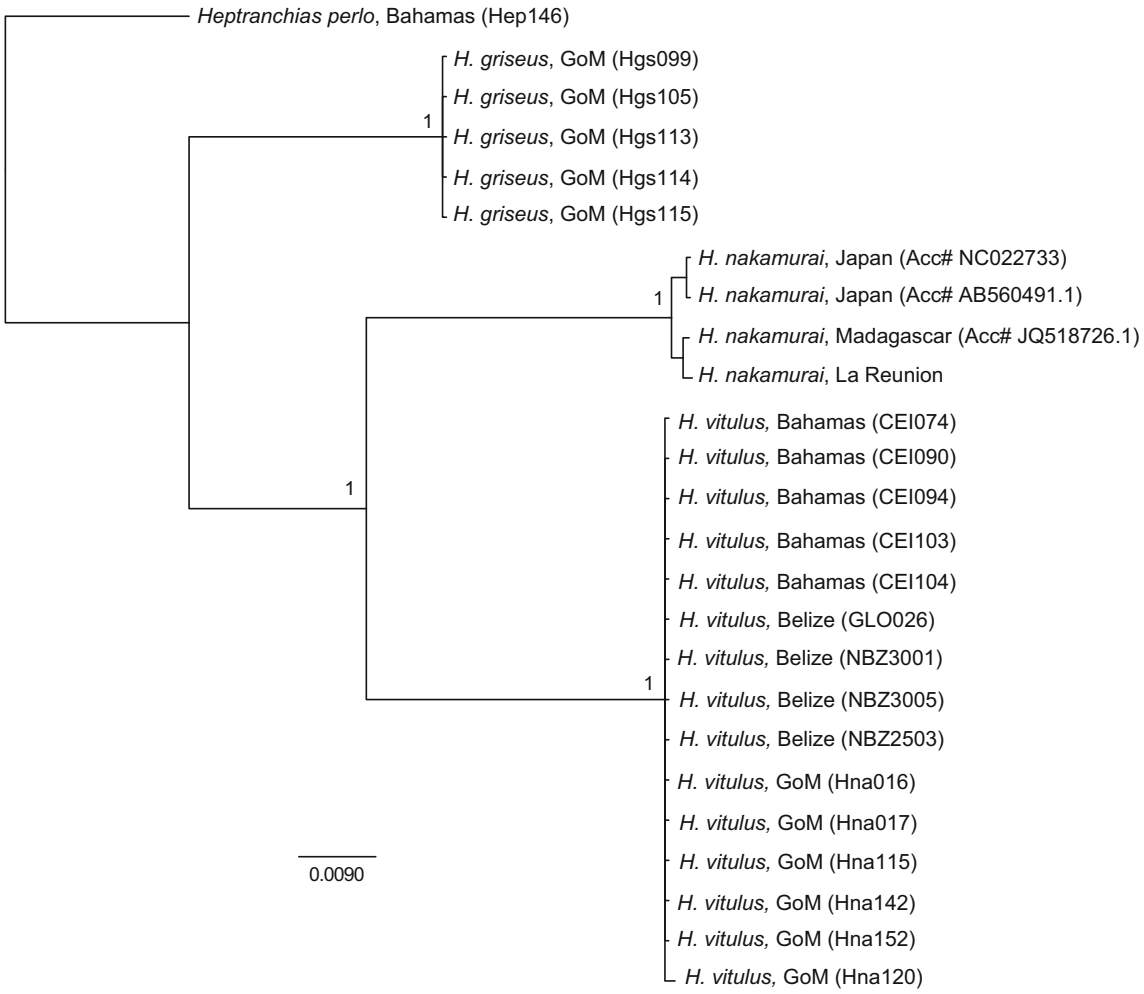

Fig. 2 Bayesian phylogenetic tree of concatenated mitochondrial *ND2* and *COI* gene sequences from *Hexanchus* species with a *Heptranchias* outgroup (1,310 bp total). *GoM* Gulf of Mexico. *Numbers at nodes* represent posterior probability, and *numbers in parentheses* refer to the genetic sample IDs for *H. vitulus* in Table 1

in Exuma Sound, Bahamas at depths ranging from 504 to 701 m and temperatures of 10.0–14.5 °C (Brooks et al. 2015). All except one male were mature. A subset of five *H.* cf. *nakamurai* was used for comparison with the GoM and Belize individuals, and the sevengill shark was used as a genetic outgroup.

Genetic analyses

Genetic sequencing for this study obtained 712 bp (bp) of *ND2* (Genbank Accession nos. MG589390-MG589411) and 598 bp of *COI* (Genbank Accession nos. MG573219-MG573240), for a total of 1,310 bp. For comparison's sake, a subset of six out of the 14 GoM *H.* cf. *nakamurai* were included in the phylogenetic analysis. The model of molecular evolution for separate and concatenated mitochondrial genes was estimated by jModeltest to be GTR + I (Aikake Information Criteria), and Bayesian analyses returned unrooted topologies identifying species groups with unambiguous 100% posterior support (Fig. 2). The maximum likelihood analysis returned an identical, well-supported topology, so only the results from the Bayesian tree are presented here. The 15 samples of *H.* cf. *nakamurai* from Belize The Bahamas, and the GoM all grouped together with little within-group variation (0.011), while *H. nakamurai* from the Indo-Pacific contained slightly more (0.340; Table 2). All five samples of *H. griseus* were found to be the same haplotype. Between-species genetic distances within the genus *Hexanchus*, expressed as percent sequence divergence, ranged from 7.037 to 8.200%, while within-species divergence was exponentially lower (<0.001 to 0.340; Table 2). *H. nakamurai* in the Indo-Pacific was separated from *H.* cf. *nakamurai* in the Atlantic by 7.037% sequence divergence, while *H. griseus* in the GoM was differentiated from both Atlantic and Indo-Pacific congeners by 7.965 and 8.200%, respectively. The two *H. nakamurai* from Japan belonged to the same haplotype, but the *H. nakamurai* from Madagascar and La Reunion were 0.142% different from one another. Genetic distance between Madagascar and Japan was 0.567%, while distance between

Table 2 Genetic distance within and between species, expressed as percent sequence divergence across 1,310 bp of mtDNA

	Hexanchus vitulus	*Hexanchus nakamurai*	*Hexanchus griseus*	*Heptranchias perlo*
Hexanchus vitulus, $n = 15$	0.011[a]			
Hexanchus nakamurai, $n = 4$	7.037	0.340[a]		
Hexanchus griseus, $n = 5$	7.965	8.200	0.000[a]	
Heptranchias perlo, $n = 1$	8.752	9.125	7.046	–

[a] Average within-species variation

Japan and La Reunion was 0.382%. Despite belonging to different genera, genetic distances between *Heptranchias perlo* and all three sixgill species were comparable to those within *Hexanchus* (7.046–9.125%; Table 2).

Distribution of *Hexanchus* cf. *nakamurai*

Bigeye sixgill sharks *H.* cf. *nakamurai* were captured in the northern GoM during winter, spring, and fall (Table 1), indicating that they may be present year-round. Likewise, bigeye sixgill sharks in Belize were captured in February and August–November of 2016. Individuals captured in Belize were found at depths of 242–333 m (avg. 280 m) and temperatures of 13.0–16.6 °C (avg. 14.6 °C), while those in the northern GoM ranged between 114 and 299 m (avg. 240 m); the neonate sharks were captured on a single set at a depth of 288 m at 12.5 °C. Captures in the GoM extended from Texas to Florida, with a cluster of captures off the coast of northwest Florida (Fig. 1). Ten sharks were captured along the barrier reef in northern Belize and one on the northern tip of Glover's Reef Atoll (Fig. 1).

Fisher interviews confirmed that *H.* cf. *nakamurai* are present throughout the offshore atolls of Belize, with captures reported on the western slope of Glover's Reef, western and southern Turneffe Atoll, and western Lighthouse Reef Atoll (Fig. 1). Further confirmation of their distribution at Lighthouse Reef Atoll was made in 2004–2007 by examination of jaws from landed sharks (R. Graham, unpublished data). Several interviewed fishers indicated that seasonal aggregations exist at the offshore atolls.

Discussion

We found that mitochondrial DNA sequencing firmly supports the existence of two distinct species of bigeye sixgill sharks: *Hexanchus nakamurai* from the Indian and Pacific Oceans, and *H.* cf. *nakamurai*, hereafter referred to as *Hexanchus vitulus* (Springer and Waller 1969). *H. vitulus* is currently assumed to be restricted to the Atlantic, where its only congener is the circumglobally distributed *Hexanchus griseus*. Phylogenetic analysis showed high statistical support for separation among all three species in the genus (posterior probability = 1.00), with individual sequences clustering tightly together into species groups and well apart from others (Fig. 2). Between-species distances were exponentially larger than within-species variation: 7.037–8.200% sequence divergence compared with <0.001–0.340% (Table 2). Little average variation was seen among the 15 *H. vitulus* individuals sampled for this study (0.011%); of these, only one belonged to a different haplotype than the rest. Significantly more variation was seen among the four *H. nakamurai*, which represented a much wider distribution of sampling sites than *H. vitulus*. Within species, all samples that grouped together by sampling site were genetically similar: *H. nakamurai* specimens in the Indian Ocean were more closely genetically related to each other, for example, than to *H. nakamurai* in the Pacific. While isolation by distance is the most likely cause for such separation, these differences were still exponentially smaller than the observed between-species variation.

Genetic divergence between the two bigeye sixgill species (7.037%) was considerable, and equivalent to that seen between both bigeye sixgills and the well-described bluntnose sixgill, *H. griseus* (*H. vitulus–H griseus* = 7.965%; *H. nakamurai–H griseus* = 8.200%). Interestingly, divergence between all three species in the genus *Hexanchus* was similar in scale to the divergence observed between *Hexanchus* and the genus *Heptranchias*, as represented by the outgroup *Heptranchias perlo*, the sharpnose sevengill shark. There, only 7.046% divergence separated *H. perlo* from *H. griseus*, 8.752% separated *H. perlo* from *H. vitulus*, and 9.125% separated *H. perlo* from *H. nakamurai*. Fossil and molecular evidence indicate that the genera *Hexanchus* and *Heptranchias* split from one another no later than 50 mya (Musick et al. 2004; Sorenson et al. 2014); results from the current study and others indicate that *H. nakamurai* and *H. vitulus* may have evolved soon after (Naylor et al. 2012), perhaps in response to similar climatic events. Though further investigation is warranted before genus-level revision is undertaken, these results highlight our lack of knowledge about the evolution and taxonomy of the deep-water cowsharks.

The between-species distances obtained here are equivalent to or greater than those observed in studies of the same genes

previously conducted on sixgills and other deep-water sharks (Naylor et al. 2012; Ward et al. 2007; Ward et al. 2005), the taxonomy of which is complicated by highly conserved morphology. Specifically, Ward et al. (2007) used DNA barcoding to discriminate between 15 species of deep-water dogfishes in the genus *Squalus* with 654 bp of the *COI* gene. In 127 individuals, the authors found an average within-species sequence divergence of 0.17 ± 0.05% and an average between-species divergence of 4.35 ± 0.23%. The use of the*ND2* gene in the current study, which has a relatively higher diversity and rate of molecular evolution, may account for the greater distances seen here. Divergence times between hexanchid lineages are much greater than those in dogfish sharks, however, with fossil evidence of *Hexanchus* first appearing in the lower Jurassic (200–50 mya), while the radiation of most *Squalus* species is thought to have occurred within the past 10 mya (Musick et al. 2004; Sorenson et al. 2014).

Museum records suggest *Hexanchus vitulus* may have a restricted distribution relative to what is currently known for both *H. nakamurai* and *H. griseus*. Records for *H. vitulus* exist in the northwest Atlantic from the northern Bahamas throughout the Gulf of Mexico (U.S., Mexico and Cuba) through Central America at least to Costa Rica. The catch records reported here confirm that *H. vitulus* are likely year-round residents of northern Gulf of Mexico (GoM) and Belize, and the relatively high capture rate in northern Belize indicates that this area may be a local hotspot for juvenile *H. vitulus*. The range of the species most likely includes the entire Meso-American Barrier Reef, including Guatemala and Honduras, where one *H. vitulus* was captured by fishers and landed in Quetzalito, Guatemala in 2016 (A. Hacohen, pers. comm.).

All sampled *H. vitulus* specimens from Belize and the northern GoM were juveniles captured at depths <350 m. Three of the individuals captured on a single longline in the northern GoM were neonates with visible yolk bellies measuring 52–61 cm TL, indicating that there may be a nursery in that region as suggested by Castro (2010), though no pregnant females were observed. The maximum recorded depth for the species is 701 m (Brooks et al. 2015); therefore, there is likely some stratification of the distribution by size, presumably with larger individuals present in deeper waters. Brooks et al. (2015) captured mostly adult *H. vitulus* in the Bahamas at an average depth of 639.5 m, indicating that mature *H. vitulus* may inhabit deeper waters than the juveniles. Alternatively, the lack of adults in Belize could indicate that these waters act as refuge for *H. vitulus*, and the larger animals' distribution does not overlap with the smaller juveniles. Resource and habitat partitioning between juvenile *H. vitulus* and much larger adult bluntnose sixgill sharks has been suggested as predation avoidance (Barnett et al. 2012), though Brooks et al. (2015) reported overlapping depth distributions for both *Hexanchus* species in the Bahamas. Larger sharks, including the night shark *Carcharhinus signatus* (Poey, 1868), have been captured by vertical longline and recorded on deep camera installments in the same areas as *H. vitulus* in Belize, though in much lower frequencies (Baremore, unpublished data).

Though morphology is the traditional technique used in alpha taxonomy, genetic tools are becoming increasingly common in studies describing new species, especially when morphological data are ambiguous, as is the case with *H. vitulus*. Though not ideal when used in isolation, estimates of neutral genetic diversity can provide a powerful indicator of cryptic speciation in elasmobranchs, especially when combined with other tools (Ebert et al. 2013; White et al. 2017). DNA samples are informative even when specimens are few, can be obtained non-lethally, and do not require frequency estimates of elements such as teeth and scales from a prescribed set of multiple individuals (i.e., one or more mature females). Finally, while environmental factors can result in convergence among a set of physical characters difficult for taxonomists to distinguish, genetic analyses can pinpoint long-standing reproductive isolation despite complete morphological homogeneity. Here, where multiple investigators have compared specimens of bigeye sixgills from both the Atlantic and the Pacific without finding any definitive characters to separate the two (Ebert 1990; Ebert et al. 2013; Taniuchi and Tachikawa 1991), molecular data represent a crucial resource for taxonomists.

Our results support the resurrection of *Hexanchus vitulus* as a valid species as described by Springer and Waller (1969). The original description was based on specimens and records from the Bahamas, Cuba, Nicaragua, and Costa Rica. The holotype (USNM No. 299674) and paratype (USNM No. 200675) were designated by Springer and Waller (1969) and reside in the Smithsonian Museum of Natural History. Because Springer and Waller (1969) did not propose a common name for this species, we designate the name Atlantic sixgill shark to distinguish *H. vitulus* from its congeners. As commercial fishing efforts move steadily further into the deep ocean, it is increasingly vital to use as many tools as at the disposal of science to clarify the evolutionary diversity of elasmobranchs in this region. Through a more thorough understanding of its biology and behavior, the Atlantic sixgill shark—a flagship species of the deep—can help reveal the biotic and abiotic drivers that specifically shape deep water shark evolution and distribution. As this species forms a keystone in top-down trophic control of the deep ocean (Barnett et al. 2012), such findings are key to molding fisheries gears and practices to reduce threats to population and ecosystem persistence.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors.

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