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ORIGINAL PAPER

Deep mitochondrial lineage divergence among populations of the southern stingray (*Hypanus americanus* (Hildebrand & Schroeder, 1928)) throughout the Southeastern United States and Caribbean

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Abstract
Although over half of all known elasmobranchs are batoids, with many species exploited and several of conservation concern, little is known of their population genetic structure and micro-evolutionary history. Here, we used sequence variation in 648 bp of the mitochondrial control region to study the phylogeography of the southern stingray (*Hypanus americanus* (Hildebrand & Schroeder, 1928)) (previously *Dasyatis americana*) throughout the Carolinas, Florida, and the Caribbean. Out of 267 individuals sampled from eight locations, 67 haplotypes were identified and analysis of molecular variance revealed a high level of genetic partitioning ($\Phi_{ST} = 0.49$; $P < 0.00001$) that was delineated into three geographic regions: (i) the USA and Belize, (ii) the Bahamas and the West Indies, and (iii) Grand Cayman Islands. Phylogenetic and statistical parsimony analyses identified three divergent lineages that were largely concordant with the population structure. However, the geographic distribution of haplotypes described a complex phylogeographic pattern with numerous haplotypes from the divergent lineages co-occurring at the same sampling site. The strong genetic partitioning detected for the Grand Cayman population suggests that this small and isolated population might warrant individualized conservation management.

Keywords Batoid · Elasmobranch · Population structure · Conservation · Control region

Introduction

Investigation into the spatial and temporal distribution of intraspecific gene lineages provides valuable insight into the processes that have affected gene flow and helped shape present-day patterns of population structure (Avise 2000; Templeton 2006). By attempting to unravel the evolution of a species, we can also hope to gain a better understanding of its migratory and demographic history. In combination, these data have the potential to provide us with the predictive power to make effective conservation and management decisions (Palumbi 2003; Hellberg 2007).

Despite their ubiquity in many marine and freshwater ecosystems, stingrays (Myliobatoidei) are a relatively understudied group and data on their population genetics are limited. Many batoids are viviparous and lack a larval phase, so dispersal is restricted to juvenile and adult movements, leading to the expectation that genetic population structure will be closely linked to life history and behavior patterns. For example, demersal species, which inhabit shallow coastal waters, are likely to show the highest levels of genetic partitioning (Plank et al. 2010; Sandoval-Castillo and Rocha-Olivares 2011; Borsa et al. 2012; Le Port and Lavery 2012; Arlyza

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et al. 2013). Although populations of larger, more mobile species are expected to be highly connected, Phillips et al. (2011) found significant subdivision among populations of three species of *Pristis* sawfish, which can reach up to 7 m, in Australia.

A possible explanation for genetic partitioning detected among elasmobranch populations when mitochondrial markers are used is female philopatry (Portnoy et al. 2010; Mourier and Planes 2013). However, other factors such as temperature (Richards et al. 2009), salinity (Chevolot et al. 2007), currents (Walker et al. 1997), historical and/or seasonal migration patterns (Chevolot et al. 2006a, b), deep or open expanses of water (Le Port et al. 2008), and historical changes in sea level (Arlyza et al. 2013) likely also contribute to genetic discontinuities among batoid populations.

Many batoid phylogeography studies have focused on species in the Indo-Pacific and Australia (Schluessel et al. 2010; Phillips et al. 2011; Borsa et al. 2012; Arlyza et al. 2013; Li et al. 2013; Puckridge et al. 2013), the north Atlantic (Newby et al. 2014; Sellas et al. 2015; Griffiths et al. 2010, 2011), and Baja California (Smith et al. 2009; Sandoval-Castillo and Rocha-Olivares 2011; Castillo-Páez et al. 2014) or have taken a global view (Richards et al. 2009; Kashiwagi et al. 2012; Le Port and Lavery 2012; Le Port et al. 2013). Here, we add to the larger picture of batoid phylogeography by presenting the first data for the southern stingray, *Hypanus americanus* (Hildebrand & Schroeder, 1928) (Hildebrand and Schroeder 1928) (previously *Dasyatis americana*), in the Southeastern United States and Caribbean. Recently, Last et al. (2016) proposed a revision of the Dasyatidae subdividing the genus into eight genera (*Hemigtrygon*, *Telatrygon*, *Bathytoshia*, *Pteroplatytrygon*, *Dasyatis*, *Taeniurops*, *Hypanus*, and *Megatrygon*). This revision included the renaming of *H. americanus* from *D. americana*. *H. americanus* is a large demersal batoid that ranges from New Jersey, USA, and northern Gulf of Mexico to southern Brazil (Stehmann et al. 1978). It is common throughout its range and the growing practice of hand feeding in numerous Caribbean locations has made it an important commodity to the tourism industry (Corcoran et al. 2013; Vaudo et al. 2018). A 2-year tracking study at Grand Cayman Island in the Caribbean found that the longest movement was just 4.3 km (Corcoran et al. 2013), suggesting limited dispersal over short (ecological) time scales for this species.

We use mitochondrial control region sequence data to investigate the genetic population structure and microevolutionary history of *H. americanus* throughout the southeast coast of the USA and the Caribbean. Although phylogeographic and phylogenetic patterns are most robust when inferred from multiple, independent loci (Edwards 2009), preliminary surveys of mitochondrial data for many individuals are cost-effective and uncover important questions for further investigation with deeper sequencing efforts, particularly for non-model systems (Bowen et al. 2014).

Materials and methods

Sampling sites and collections

A total of 267 individuals were sampled from eight major locations throughout the Caribbean and along the Eastern USA coast (Fig. 1). Tissue samples from six species representing four genera were collected for outgroup analyses (*Hypanus say* and *Hypanus sabinus*: Tampa, FL, USA; *Bathytoshia centroura*: Virginia, USA; *Hypanus dipterurus*: Almejas Bay, Mexico; *Bathytoshia lata*: Hawaii, USA; and *Hemitrygon fluviorum*: Queensland, Australia). All samples were preserved in 95% ethanol at 4 °C. Genomic DNA was extracted from 25 mg of tissue using the DNeasy Tissue Kit (QIAGEN Inc.) and stored at − 20 °C until use.

Polymerase chain reaction and sequencing

The following primer pair was designed for initial polymerase chain reaction (PCR) amplification and sequencing of the mitochondrial control region: DamProF1 (5′-TCAG GAAAAAGGGGGCCAAACC-3′) and DsaCRR4 (5′-CCTCGTTTTTGGGGTTTTTCGAG-3′). DamProF1 starts at the first base of Proline and DsaCRR4 is approximately 1.5 kbp from the 5′ end of the control region. Three internal primers were subsequently designed: DamProF3 (5′-GCCA AACCCTTATCCTTGGCT-3′), DamCRR1 (5′-TGGA AATATTATGCCCGC TTAAGG-3′), and DamCRR3 (5′-TAGGTTAGTGCCAGGAGATGGTTG-3′). The combination of DamProF1 and DamCRR1 produced 782 bp of PCR product. The internal combination of DamProF3 (14 bp from the beginning of Proline) and DamCRR3 produced 648 bp of sequence data. DsaCRR4 lies within a 27-bp region that is highly conserved among elasmobranchs. This region corresponds to the conserved sequence block (CSB3) identified by Sbisà et al. (1997) for mammals.

The 50 μL PCR reactions contained 1 μL of DNA template, 5 μL 10× PCR buffer, 50 μM of each dNTP, 0.25 μM of each primer, and 0.75 units of HotStar Taq™ DNA Polymerase (QIAGEN Inc.). PCR was performed in a Mastercycler Gradient (Eppendorf Inc.) thermal cycler as follows: 95 °C initial heating for 15 min to activate the hot start DNA polymerase, followed by 35 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min, and a 5-min final extension step at 72 °C. Success of each reaction was assessed by gel electrophoresis and a negative control was included to check for contamination. PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN Inc.) prior to

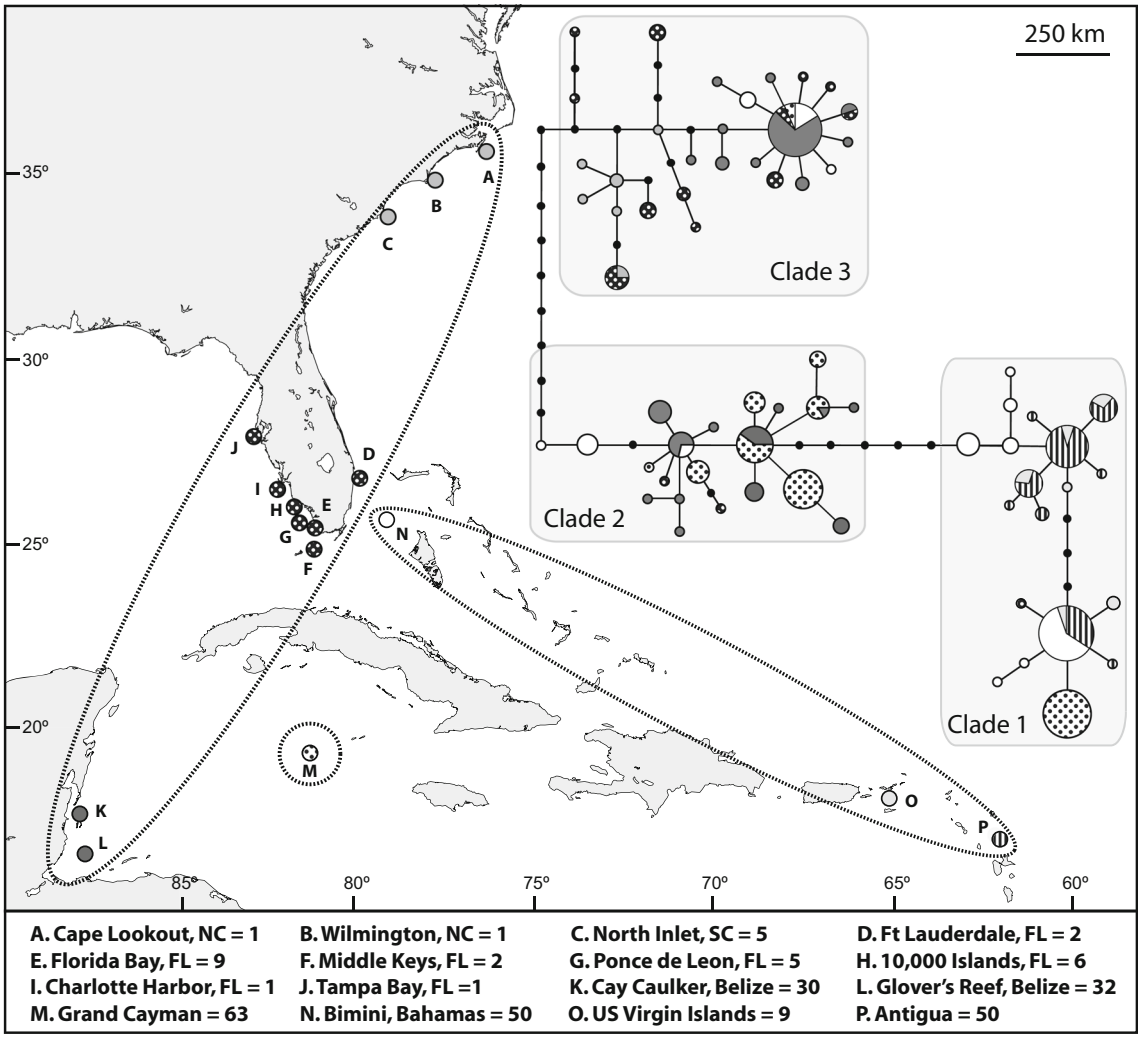

Fig. 1 Map showing individual sampling sites throughout the Carolinas, Florida, and the Caribbean. Dashed lines encircle the three differentiated populations delineated by the AMOVA. The statistical parsimony network depicts relationships among haplotypes where circle size is proportional to haplotype frequency, connecting lines represent single mutational steps, and small solid black circles represent hypothetical missing haplotypes not sampled. The three major clades are shaded in gray. Sample sizes for each location (labeled A through P) are listed below the map

sequencing. Samples were sequenced in both directions using standard protocols on ABI 377 and 3730xl genetic analyzers. Individual sequences are available from GenBank (accession numbers LN623712–LN623978).

Nuclear mitochondrial pseudogenes

Nuclear insertion of mitochondrial DNA can result in a nuclear mitochondrial pseudogene (numt) and numerous examples involving vertebrate taxa have been reported (Bensasson et al. 2001). To investigate the possibility that we were inadvertently amplifying a numt, we isolated mitochondria from a select individual prior to DNA extraction using differential centrifugation on homogenized fin tissue (Palva and Palva 1985). We confirmed the effectiveness of this mtDNA extraction protocol by checking for nuclear DNA contamination via PCR using nuclear internal transcribed spacer 2 (ITS2) primers. Comparison of the control region sequence obtained using this protocol to the sequence obtained from total genomic DNA showed them to be identical and confirmed that we were not amplifying a numt.

Data analysis

Individual sequences were aligned using MUSCLE as implemented in GENEIOUS PRO v4.5.4 (Kearse et al. 2012). Molecular diversity indices (number of haplotypes, haplotype diversity, and nucleotide diversity) were estimated using the program DNASP v4.0 (Librado and Rozas 2009). Genetic population structure was examined by an analysis of molecular variance (AMOVA) as implemented in ARLEQUIN v3.11 (Excoffier and Lischer 2010). Significance of Φ statistics was

Table 1 Pairwise Φ_{ST} values between individual sampling sites

	Carolinas	Florida	Cay Caulker	Glover's Reef	Cayman	Bimini	USVI	Antigua
Carolinas		1.000	0.004	1.000	0.000	0.000	0.000	0.000
Florida	0.032		0.006	1.000	0.000	0.000	0.000	0.000
Cay Caulker	*0.367*	*0.281*		0.069	0.000	0.000	0.000	0.000
Glover's Reef	*0.188*	0.058	*0.209*		0.000	0.000	0.000	0.000
Cayman	*0.707*	*0.633*	*0.286*	*0.599*		0.000	0.000	0.000
Bimini	*0.591*	*0.531*	*0.284*	*0.511*	*0.155*		0.466	0.000
USVI	*0.882*	*0.700*	*0.498*	*0.686*	*0.413*	0.140		1.000
Antigua	*0.879*	*0.781*	*0.612*	*0.763*	*0.464*	0.196	− 0.046	

Significant Φ_{ST} values after sequential FDR correction are shown in italics below the diagonal. Corrected *P* values are listed above the diagonal

USVI, US Virgin Islands

determined by permuting haplotypes among populations 10,000 times and we corrected for multiple comparisons using the FDR method (Benjamini and Hochberg 1995).

We estimated evolutionary relationships among *H. americanus* haplotypes using three methods. We constructed an unrooted statistical parsimony network in TCS v1.21 (Clement et al. 2000), a Bayesian phylogeny in BEAST2 v2.4.4 (Bouckaert et al. 2014), and a maximum likelihood (ML) phylogeny in RaxML v8.2.10 (Stamatakis 2014). The unrooted parsimony network included all *H. americanus* individuals we sampled and were conducted using the default settings, resulting in the most parsimonious connections among haplotypes at the 95% confidence level. We estimated rooted Bayesian and ML phylogenies two ways: using all individuals and using one representative of each of the 67 control region haplotypes. For the Bayesian analyses, we ran two independent MCMC analyses for 10 million steps, sampling every 1000 steps. Convergence was determined by viewing the log files in Tracer v1.6.0. All parameters had effective sample sizes (ESS) > 300. Treefiles were combined in LogCombiner v2.4.4 with a 10% burn-in, and the maximum clade credibility (MCC) tree for the combined file was calculated in TreeAnnotator v2.4.4. In RAxML, we estimated the ML tree under the GTR model with rate heterogeneity and the proportion of invariant sites estimated and performed 100 bootstrap replicates. The following six species were used as outgroups: *Hemitrygon fluviorum*, *Bathytoshia centroura*, *Bathytoshia lata*, *Hypanus dipterurus*, *Hypanus say*, and *Hypanus sabinus*. We tested for expansion in population size using Fu's Fs statistic (Fu 1997) in DnaSP. Significance of the test statistic was evaluated using a coalescent simulation (1000 replicates).

Table 2 Results of hierarchical analyses of molecular variance (AMOVA)

Source of variation	% variance	Φ statistic	*P* value
Among groups	42.08	$\Phi_{CT} = 0.421$	0.028
Among locations within group	11.98	$\Phi_{SC} = 0.207$	0.000
Within locations	45.94	$\Phi_{ST} = 0.541$	0.000

Groups: Belize-USA, Grand Cayman, Bimini-West Indies. Italic values are significant

Results

Diversity indices

The 648-bp fragment of the mtDNA control region contained no insertions or deletions and 49 polymorphic sites, which defined 67 distinct haplotypes. Overall haplotype and nucleotide diversity were 0.948 and 0.01803 respectively.

Population structure

AMOVA results among the eight sampling sites revealed a high degree of genetic partitioning, with an overall Φ_{ST} of 0.49 ($P = 0.0000$). Non-significant pairwise Φ_{ST} values suggested three isolated groups: (i) Glovers Reef, Cay Caulker, Florida, Carolinas; (ii) Bimini, US Virgin Islands, Antigua; and (iii) Grand Cayman Island (Table 1). A second AMOVA performed on the grouped locations showed them to be well differentiated (Belize-USA vs. Bimini-West Indies $\Phi_{ST} = 0.64$ ($P = 0.0000$), Belize-USA vs. Cayman $\Phi_{ST} = 0.48$ ($P = 0.0000$), and Bimini-West Indies vs. Cayman $\Phi_{ST} = 0.48$ ($P = 0.0000$)) and a hierarchical AMOVA showed significant variation was partitioned among these groups ($\Phi_{CT} = 0.421$, $P = 0.028$, Table 2).

Statistical parsimony and phylogenetic analysis

At the 95% probability level, TCS joined all haplotypes into a single 33-step network (Fig. 1). The Bayesian and ML phylogenetic analyses of the 67 distinct haplotypes and six outgroups produced well-supported phylogenies, which recovered the same three divergent clades as the statistical

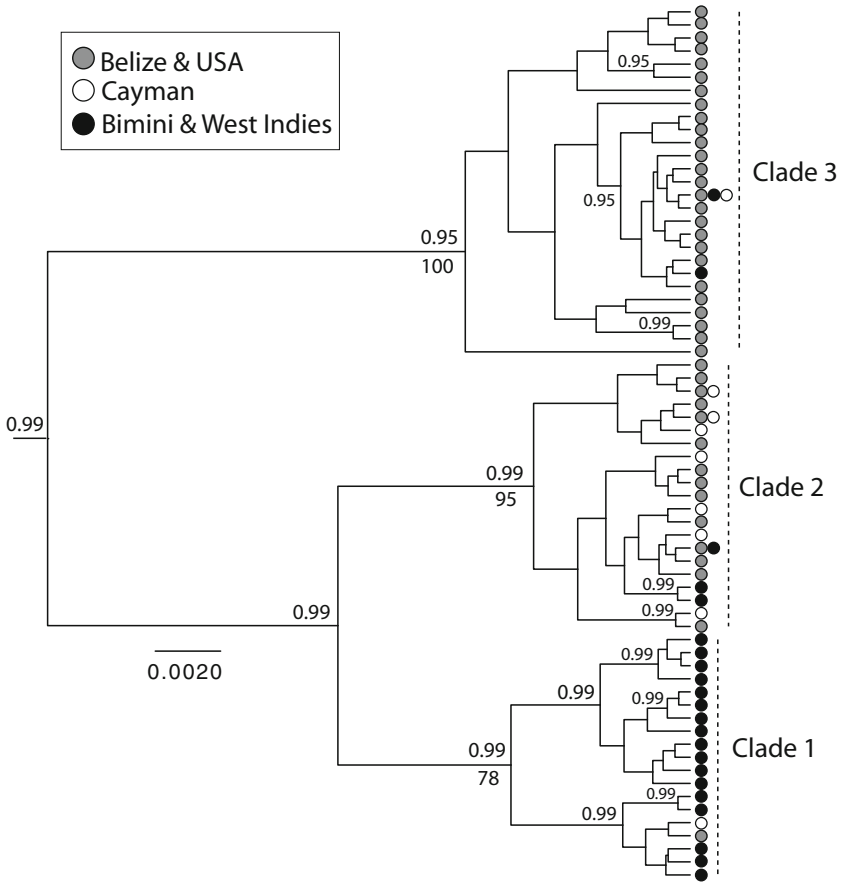

Fig. 2 Bayesian phylogeny of *H. americanus* haplotypes. Bayesian posterior probabilities are shown over branches and ML bootstrap values above 75 are shown under branches. Dotted lines indicate the three major mitochondrial clades. Shaded circles at the tips represent the locations where each haplotype occurs

parsimony analysis and showed clade 3 to be ancestral to clades 1 and 2 (Fig. 2).

Demographic history

Fu's Fs statistic was only significant for Belize-USA (Fs = − 11.62, $P = 0.012$). For Bimini-West Indies, Fs = − 1.55 ($P = 0.379$) and for Cayman, Fs = 4.42 ($P = 0.935$).

Discussion

Levels of genetic differentiation among *H. americanus* sampling sites revealed contrasting patterns of population structure throughout the southeastern USA and Caribbean. For example, Belize-USA and Bimini-West Indies populations were significantly structured across only 85 km of the Florida channel, whereas sampling sites along approximately 1800 km of USA coastline (Cape Lookout, NC, to Tampa Bay, FL) showed no significant genetic partitioning. This result suggests that high levels of coastal migration facilitate gene flow in *H. americanus*. Similar findings of population connectivity along a coastline have been reported for other batoids. For example, Le Port and Lavery (2012) found *Dasyatis brevicaudata* (short-tail stingray) populations were less divergent along continuous coastal sites than between ocean basins while Chevolot et al. (2006a, b) found high gene flow along approximately 3000 km of European coastline for the thornback ray, *Raja clavata*. *Aetobatus narinari* eagle rays sampled from three locations around the Florida peninsula suggested a single genetic population (Newby et al. 2014). Complementing our finding that Belizean and Southeastern United States sites represent one genetic population, Sellas et al. (2015) found that the majority of *A. narinari* individuals sampled in Cuba originated from Florida or the Yucatan peninsula, suggesting Cuba is an important migration corridor between Central and North America for some batoid populations. Although a continuous coastline does not connect Bimini, the US Virgin Islands, and Antigua, our data suggest that the numerous small stretches of open water in this island chain are not significant impediments to gene flow for *H. americanus*. One possibility is that migration via island hopping along the windward coasts of Cuba, Haiti, Dominican Republic, and Puerto Rico contributed to the lack of genetic partitioning among our greater and lesser Antilles sampling sites.

Deep water over short distances does not appear to restrict gene flow in *H. americanus*. For example, sampling sites in the US Virgin Islands and Antigua, separated by approximately 340 km of water spanning the Anegada Passage and

reaching a sill depth of 1915 m, showed no significant partitioning. We also found lack of significant structure between Glovers Reef and Cay Caulker separated by approximately 110 km of water at a depth of 300–400 m. These significant depths would likely preclude dispersal along the bottom and suggest that despite *H. americanus*' demersal life history, open water migration is possible. Indeed, satellite-tagging data from *A. narinari* showed movements over water greater than 800 m, but a maximum dive depth of 24.5 m, suggesting a preference for the upper photic zone (Sellas et al. 2015). Our data do, however, also suggest there is a limit on *H. americanus*' ability to migrate through open water. Grand Cayman Island is significantly partitioned from all other sampling sites and its remote location in the middle of the Caribbean Sea has likely restricted genetic exchange between it and neighboring locations.

The last major phylogeographic break identified for *H. americanus* was across the Straits of Florida, which separates the Belize-USA and Bimini-West Indies populations and reaches a depth of approximately 800 m (Neumann and Ball 1970). This region has been shown to be an important break for a range of taxa including sponges, corals, mollusks, crustaceans, and fish (reviewed in DeBiasse et al. 2016). The distance across the Florida Channel (85 km) is less than the distance between Glovers Reef and Cay Caulker (~ 110 km) and between the US Virgin Islands and Antigua (~ 340 km), yet both of these later comparisons showed no significant partitioning and were separated by deep water. Temperature has been shown to be an important factor affecting migration patterns for *Hypanus* and *Bathytoshia* stingrays (Struhsaker 1969; Snelson et al. 1988), and differences in both temperature and salinity were thought to be responsible for a genetic discontinuity between thorny skate (*Amblyraja radiata*) populations in Europe (Chevolot et al. 2007). Consequently, it is possible that the dramatic changes in temperature and salinity across the Florida Channel due to geostrophic adjustments created by the high velocity and complex directional patterns of the Florida Current (Neumann and Ball 1970; Lynch-Stieglitz et al. 1999) may be factors contributing to the observed phylogenetic break in *H. americanus*.

Conclusion

Hypanus americanus is significantly differentiated into three populations throughout the Eastern USA and Caribbean. Two of these populations (Belize-USA and Bimini-West Indies) extend over wide geographic areas, whereas the third (Cayman) has a much smaller geographic range. This *H. americanus* population is a global tourist attraction that contributes significantly to this small island's economy and the restricted range and isolation detected render it vulnerable to environmental impact and consequently it should be regarded a priority for conservation management.

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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.

Sampling and field studies All necessary permits for sampling and observational field studies were obtained by the authors from the competent authorities and are mentioned in the acknowledgements, if applicable.

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