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Microsatellite and mitochondrial DNA analyses of the genetic structure of blacktip shark (*Carcharhinus limbatus*) nurseries in the northwestern Atlantic, Gulf of Mexico, and Caribbean Sea

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Abstract

We investigated the genetic structure of blacktip shark (*Carcharhinus limbatus*) continental nurseries in the northwestern Atlantic Ocean, Gulf of Mexico, and Caribbean Sea using mitochondrial DNA control region sequences and eight nuclear microsatellite loci scored in neonate and young-of-the-year sharks. Significant structure was detected with both markers among nine nurseries (mitochondrial $\Phi_{ST} = 0.350$, $P < 0.001$; nuclear $\Phi_{ST} = 0.007$, $P < 0.001$) and sharks from the northwestern Atlantic, eastern Gulf of Mexico, western Gulf of Mexico, northern Yucatan, and Belize possessed significantly different mitochondrial DNA haplotype frequencies. Microsatellite differentiation was limited to comparisons involving northern Yucatan and Belize sharks with nuclear genetic homogeneity throughout the eastern Gulf of Mexico, western Gulf of Mexico, and northwestern Atlantic. Differences in the magnitude of maternal vs. biparental genetic differentiation support female philopatry to northwestern Atlantic, Gulf of Mexico, and Caribbean Sea natal nursery regions with higher levels of male-mediated gene flow. Philopatry has produced multiple reproductive stocks of this commercially important shark species throughout the range of this study.

Keywords: blacktip shark, *Carcharhinus limbatus*, control region, microsatellites, philopatry, population structure

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Introduction

Reproductive behaviour and genetic population structure have been investigated for few shark species inhabiting the coastal waters of North and Central America, despite the commercial importance of many species and concern regarding population sustainability and conservation (Musick *et al*. 2000). Genetic homogeneity was observed between western Atlantic and Gulf of Mexico (Gulf) populations of sandbar (*Carcharhinus plumbeus*) and Atlantic sharpnose sharks (*Rhizoprionodon terraenovae*) (Heist *et al*. 1995, 1996; Heist & Gold 1999) and was attributed to the dispersal ability of sharks throughout the study areas. However, the low intraspecific diversity of the genetic markers employed may have led to underestimates of population structure for these species (Heist *et al*. 1995, 1996; Heist & Gold 1999). Low, but statistically significant, levels of microsatellite heterogeneity were detected among lemon shark (*Negaprion brevirostris*) populations within the western Atlantic (Feldheim *et al*. 2001). Allozyme differentiation was detected between northern and southern populations of the Channel Island Pacific angelshark (*Squatina californica*) (Gaida 1997).

The blacktip shark inhabits coastal tropical and subtropical waters around the world and is common in the US Atlantic from South Carolina to the Florida Keys, the Gulf, and the Caribbean Sea (Castro 1996). It is an important commercial and recreational species in the southeastern

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US large coastal shark fishery (NMFS 2001) and is targeted by shark fisheries throughout the southern Gulf and eastern Caribbean Sea (Bonfil 1997; Castillo-Géniz *et al*. 1998). Life history traits, such as moderately slow growth and low fecundity, limit recruitment rates making blacktip shark populations susceptible to collapses from overfishing. Blacktip sharks in the northwestern Atlantic and Gulf are highly mobile, with movements documented between the US Atlantic and eastern Gulf, eastern and western Gulf, and the Texas Gulf coast and Mexican Gulf coast (Kohler *et al*. 1998).

Blacktip sharks use shallow coastal bays as nurseries where young remain up to several months after parturition. These highly productive areas presumably provide juveniles with protection from predators and abundant food (Simpfendorfer & Milward 1993; Heupel & Hueter 2002; Heupel & Simpfendorfer 2002). Female blacktip sharks along US Atlantic and Florida Gulf coasts migrate to nursery areas beginning in May, where juveniles are born and remain until surface water temperatures decline below approximately 20 °C and then migrate to wintering grounds near southern Florida and the Florida Keys (Castro 1993, 1996; Hueter 1999; Heupel & Hueter 2001). Seasonal migrations of female blacktip sharks from nurseries throughout the remainder of the Gulf are poorly known (Bonfil 1997). Data from blacktip sharks tagged within Laguna Yalahau in the northern Yucatan Peninsula suggest that juvenile blacktip sharks spend 1 or 2 years along the Yucatan coast (Hueter *et al*. in press). Movements of blacktip sharks in the Caribbean Sea have not been characterized.

The lack of geographical barriers to movement and the documented mobility of blacktip sharks suggest that sufficient dispersal may occur to prevent the development of genetic population structure throughout the northwestern Atlantic, Gulf, and Caribbean Sea. Despite the species' dispersal potential, a previous mitochondrial DNA (mtDNA) analysis detected significant haplotype frequency differences between neonates from nurseries on the Gulf coast of Florida and the Atlantic coast of South Carolina (Keeney *et al*. 2003). Results were interpreted as supporting female philopatry to natal nursery areas. The reproductive structure of blacktip shark nurseries throughout the remainder of the Gulf and Caribbean Sea is unknown. The characterization of genetic heterogeneity and subsequent use of this information by fishery managers is critical to the long-term success of conservation and fishery management programs (Shaklee & Bentzen 1998). Deviations from panmixia throughout a species' range necessitate management of regional populations as separate stocks. Failure to detect existing reproductive structure can allow the removal of too many individuals from small regional populations, resulting in localized extirpations and reduced overall recruitment.

In this study, we used mtDNA control region sequences and eight nuclear microsatellite loci to examine the genetic structure of blacktip shark nurseries within the northwestern Atlantic Ocean, Gulf, and Caribbean Sea. Our goals were to determine and compare the extent of mtDNA and nuclear differentiation throughout the study area. Heterogeneity in both markers would support either limited dispersal or reproductive philopatry by both sexes. Nuclear homogeneity and mtDNA heterogeneity would indicate mating of females with males from different nursery areas, with females returning to natal nursery regions for parturition. This study increases current knowledge of blacktip shark biology by characterizing adult philopatry as well as providing important management information by determining whether multiple blacktip shark stocks exist throughout the study region.

Materials and methods

Sample collection and DNA extraction

Neonate (open umbilical scar) and young-of-the-year (YOY) (closed umbilical scar) blacktip sharks were sampled within one South Carolina Atlantic coast nursery (Bulls Bay, Charleston County) and three Florida Gulf coast nurseries (Pine Island Sound, Lee County; Terra Ceia Bay, Manatee County; and Withlacoochee Bay near Yankeetown, Levy County) as described in Keeney *et al*. (2003), one Texas Gulf coast nursery (Nueces County), and one nursery in Quintana Roo, Mexico on the northern Yucatan Peninsula (Laguna Yalahau) in 2000 and 2001. Neonate and YOY blacktip sharks were also collected along the Atlantic coast of Georgia (Glynn County) in 2002 and from two local fish markets on the Caribbean Sea coast of Belize (Belize City in 2001 and Dangriga in 2002). Sample sizes for each site are listed in Table 1 and sample locations are shown in Fig. 1.

Sharks were collected within the same nursery for two consecutive years to investigate temporal genetic heterogeneity within nurseries and to decrease full-sibling sampling bias [females do not reproduce in consecutive years (Castro 1996)]. Neonate and YOY blacktip sharks were sampled within coastal nurseries (with the exception of Belize) to measure genetic heterogeneity among nurseries rather than among potential nonresident adults and juveniles moving through an area. If adult sharks return to specific regions for reproduction, genetic structure may be present among animals that mix at other times of the year. Neonates were collected using gillnets or rod and reel. The tip of the dorsal fin (approximately 1 cm long) was removed from each shark and stored in 20% DMSO saturated with NaCl. Immediately following tissue collection, the live sharks were tagged and released. Any dead animal was retained for other studies.

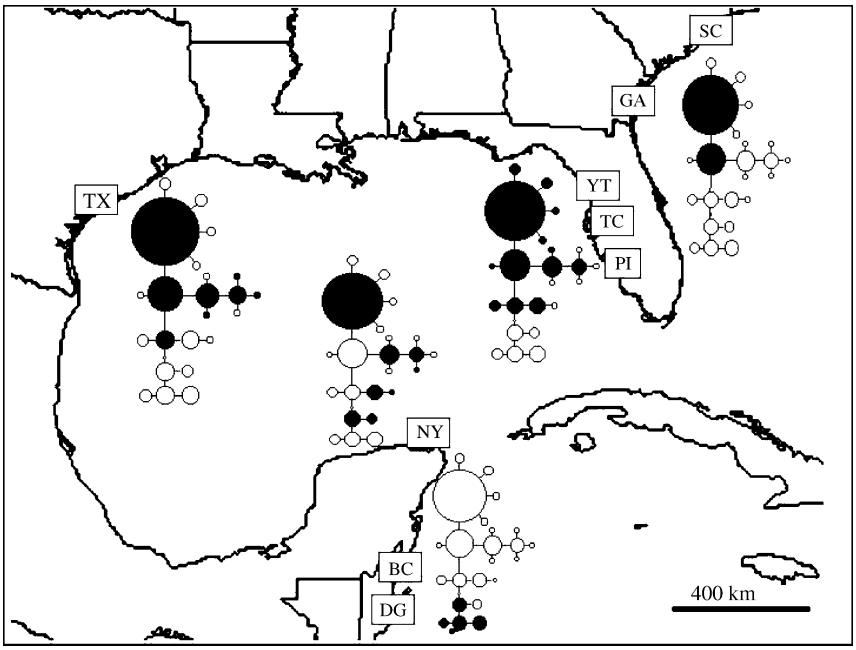

Fig. 1 Sample sites with haplotype parsimony networks for five regions. SC, Bulls Bay South Carolina; GA, Georgia; PI, Pine Island Sound Florida; TC, Terra Ceia Bay Florida; YT, Yankeetown Florida; TX, Padre Island Texas; NY, Laguna Yalahau northern Yucatan; BC, Belize City; and DG, Dangriga. Parsimony networks are provided for northwestern Atlantic (SC and GA), eastern Gulf of Mexico (YT, TC, and PI), western Gulf of Mexico (TX), Northern Yucatan (NY), and Belize (BC and DG). Haplotypes are represented by circles with diameters proportional to haplotype frequencies. Haplotypes recovered in each region are darkened. All haplotypes are connected to nearest haplotypes by one mutation except the five haplotypes at the bottom of each network which were found only in northern Yucatan and Belize and are separated from the rest of the network by two mutations. Alternate connections are not shown.

Table 1 Sample sizes for control region and microsatellite analyses for each site during each year of sampling

Sample Site	Year	mtDNA	msat
Bulls Bay, South Carolina	2000	8	8
	2001	26	26
Georgia	2002	13	13
Pine Island Sound, Florida	2000	30	35
	2001	15	37
Terra Ceia Bay, Florida	2000	30	37
	2001	15	34
Yankeetown, Florida	2000	30	35
	2001	15	31
Padre Island, Texas	2000	18	18
	2001	31	32
Laguna Yalahau, Mexico	2000	30	32
	2001	30	52
Belize City, Belize	2001	13	10
Dangriga, Belize	2002	19	19
Total		323	419

mtDNA refers to the number of sharks for which the control region was sequenced and msat refers to the number of sharks for which microsatellite genotypes were determined.

Laboratory procedures

Total genomic DNA from each shark was isolated from approximately 25 mg of fin tissue using the QIAGEN DNeasy® Tissue kit. An initial polymerase chain reaction (PCR) product containing the entire mtDNA control region was amplified using primers within the proline tRNA (light strand primer Pro-L: 5′-AGGGRAAGGAGGGTCAAACT-3′) and 12S rRNA (heavy strand primer 282: 5′-AAGGCTAGGACCAAACCT) genes. Control region PCR amplification, product purification, and sequencing were performed as described in Keeney *et al*. (2003). The entire control region from a minimum of 15 neonate and YOY blacktip sharks sampled in each of 2000 and 2001 ($n \geq 30$ per nursery) was sequenced for sharks from each nursery in the northwestern Atlantic and Gulf (with the exception of South Carolina in 2000 and Georgia in 2002, where eight and 13 neonates were analysed, respectively). The control regions of all 13 Belize City and 19 Dangriga sharks were sequenced (Table 1).

The genotypes for eight microsatellite loci (*Cli-2*, *Cli-7*, *Cli-12*, *Cli-13*, *Cli-55*, *Cli-100*, *Cli-107*, and *Cli-108*) of at least 50 neonate and YOY blacktip sharks from each Gulf nursery were determined as described in Keeney & Heist (2003), except loci *Cli-12* and *Cli-108*. These two loci were multiplexed in the same reaction by adding one radiolabelled and one nonradiolabelled primer for each locus with an annealing temperature of 56 °C. The genotypes for the eight loci of all northwestern Atlantic and Caribbean Sea sharks were determined (the majority of loci in three Belize City sharks could not be amplified and these sharks were not included in microsatellite analyses) (Table 1).

Data analyses

Control region sequences were read manually and aligned using the CLUSTALW program of MACVECTOR™ 6.5 (1998).

The number of unique haplotypes, haplotype frequencies, number of polymorphic sites, numbers of transitions and transversions, nucleotide composition, haplotype diversity (h), and nucleotide sequence diversity (π) were calculated using ARLEQUIN version 2.000 (Schneider *et al*. 2000). The chi-squared (χ^2) Monte Carlo resampling procedure of Roff & Bentzen (1989) was used to test for temporal haplotype heterogeneity between year 2000 and year 2001 cohorts within northwestern Atlantic and Gulf nurseries and spatial haplotype heterogeneity among nurseries.

Microsatellite genotypes were determined manually. Sharks missing data at more than three loci were omitted from the analyses ($n = 3$). Numbers of alleles per locus, expected and observed heterozygosities, deviations from Hardy–Weinberg expectations for each locus within each nursery, and tests of linkage disequilibrium between all pairs of loci over all nurseries and within each nursery were calculated using GENEPOP version 3.3 (Raymond & Rousset 1995). Significance of deviations from Hardy–Weinberg expectations was determined using the Markov chain exact probability test of Guo & Thompson (1992) and significance of linkage disequilibrium values was determined with Fisher exact test as implemented in GENEPOP 3.3.

Analysis of molecular variance (AMOVA) (Excoffier *et al*. 1992) was used to examine temporal genetic heterogeneity within nurseries and spatial genetic heterogeneity among nurseries for both control region haplotypes and microsatellite genotypes with ARLEQUIN 2.000. AMOVA calculates analogs of Wright's (1951, 1965) hierarchical *F*-statistics, designated as Φ-statistics, which incorporate allele frequencies and evolutionary distances among haplotypes. A two-nucleotide insertion-deletion (indel) at nucleotide positions 1045 and 1046 was treated as one event by omitting the second nucleotide from the control region haplotype distance matrix. Significance of Φ-statistics was determined via nonparametric permutation (Excoffier *et al*. 1992) with 1000 data permutations. AMOVA was performed among all nine nurseries and between each pair of nurseries. Genetically similar and geographically proximate nurseries were grouped into northwestern Atlantic (South Carolina and Georgia) and eastern Gulf (Pine Island Sound, Terra Ceia Bay, and Yankeetown) regions and AMOVA was performed to assess genetic heterogeneity within and among regions and between pairs of regions. For all pairwise comparisons, the initial $\alpha = 0.05$ significance level was adjusted for simultaneous pairwise comparisons using the sequential Bonferroni approach to maintain a type I error rate < 0.05 (Rice 1989).

Isolation by distance among populations based on control region and microsatellite data was assessed using Mantel tests implemented by the ISOLDE program of GENEPOP 3.3. Pairwise population values of $\Phi_{ST}/(1 - \Phi_{ST})$ were permuted against coastal geographical distances 10 000 times.

Pairwise genetic divergence between regions was estimated for control region haplotypes with Nei's corrected average genetic divergence (Nei 1987; p. 276) incorporating Tamura & Nei's (1993) model of sequence evolution with ARLEQUIN 2.000. For microsatellites, Cavalli-Sforza & Edwards's (1967) chord distance was calculated using the GENDIST program of PHYLIP version 3.6b (Felsenstein 2004). Neighbour-joining (Saitou & Nei 1987) trees were produced from distance matrices with MEGA version 2.0 (Kumar *et al*. 2001).

Wilcoxon signed rank tests for microsatellite heterozygote excess and the allele frequency distribution test of BOTTLENECK version 1.2.02 (Cornuet & Luikart 1996) were used to test for evidence of a nuclear genetic bottleneck in northwestern Atlantic blacktip sharks. Genetic bottlenecks reduce allelic diversity faster than heterozygosity (Nei *et al*. 1975), causing populations to display an excess of heterozygosity at a greater number of microsatellite loci than predicted by chance until mutation–drift equilibrium is established (Cornuet & Luikart 1996). A loss of low-frequency alleles in populations following recent bottlenecks can also cause a mode-shift from the expected L-shaped distribution frequency of microsatellite alleles (Luikart *et al*. 1998). Heterozygote excess was tested using the infinite alleles model (IAM), stepwise-mutation model (SMM), and two-phase microsatellite evolution model with 30% IAM and 70% SMM (TPM).

Results

Summary statistics

The control region from 323 blacktip sharks [including 169 sequences previously reported in Keeney *et al*. (2003)] was 1067–1070 nucleotides long and 13% guanine, 32% adenine, 35% thymine, and 20% cytosine (33% GC content). Fifteen polymorphisms (11 transitions, two transversions, and two indels) resulted in 23 unique haplotypes (Table 2). Twenty sharks (1 from Pine Island Sound, 2 from Yankeetown, 6 from Texas, and 11 from Laguna Yalahau) were heteroplasmic for a 12th adenine at nucleotide 933 as described in Keeney *et al*. (2003) and were treated as possessing 11 adenines for all analyses. Sample size, number of haplotypes, π, and h for each sample site are listed in Table 3. Overall h and π were 0.805 ± 0.018 and 0.00214 ± 0.00130, respectively.

Genotypes of 418–419 sharks were determined for eight microsatellite loci. Total number of alleles and observed heterozygosities for each locus ranged from 4 to 42 (mean = 14) and 0.096 to 0.962 (mean = 0.500), respectively (Table 3). Number of individuals analysed, number of alleles, observed heterozygosity (H_O), expected

Table 2 Polymorphic nucleotide positions for 23 blacktip shark haplotypes

	Nucleotide Position															
Haplo	25	53	88	139	144	208	280	324	378	550	576	644	911	1044	1053
1	C	T	T	T	C	C	A	G	T	A	T	T	A	–	A
2	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.
3	.	.	.	.	.	.	.	.	.	.	.	C	.	.	G
4	.	C	.	.	.	.	.	.	.	.	.	C	.	.	.
5	.	.	.	.	.	.	.	.	.	.	.	C	–	.	.
6	.	.	.	.	T	.	.	.	.	.	.	C	–	.	.
7	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.
8	.	.	C	.	.	.	.	.	.	.	.	C	–	.	.
9	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.
10	.	.	.	.	.	.	.	.	.	G	.	C	.	.	G
11	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.
12	.	.	.	.	.	.	.	.	.	.	C	C	.	.	G
13	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G
14	.	.	.	.	T	.	.	.	.	.	.	C	.	.	.
15	.	.	.	.	.	.	.	.	A	.	.	C	.	AT	G
16	.	.	.	.	.	T	.	.	A	.	.	C	.	AT	G
17	.	.	.	.	T	.	G	.	.	.	.	C	–	.	.
18	.	.	.	.	.	.	.	.	.	G	.	.	.	.	G
19	.	.	.	.	T	.	.	.	.	G	.	C	–	.	.
20	T	.	.	.	.	.	.	.	.	.	.	C	–	.	.
21	.	.	.	.	.	.	.	.	A	.	.	.	.	AT	G
22	.	.	.	.	.	T	.	.	A	.	.	.	.	AT	G
23	.	.	.	.	.	.	.	.	A	G	.	.	.	AT	G

Haplotype numbers (Haplo) are listed in the left column and the positions of polymorphic base pairs are listed across the top row. The nucleotide at each position is given for haplotype 1. Only nucleotides different from haplotype 1 are given for all other haplotypes. Nucleotides identical to haplotype 1 are indicated with periods (.) and deletions are indicated with dashes (–). Complete haplotype sequences are deposited in GenBank (accession nos: AY208861–AY208873 for haplotypes 1–13, accession nos: AY766123–AY766132 for haplotypes 14–23).

heterozygosity (H_E), and deviation from Hardy–Weinberg expectations (F_{IS}) for each locus within each sample site are provided in Table 3. Seven out of 72 tests of Hardy–Weinberg equilibrium within sample sites were nominally significant ($P < 0.05$) and three were significant (*Cli-7* heterozygote excess in Terra Ceia Bay and Belize City and *Cli-107* heterozygote deficiency in Belize City) after sequential Bonferroni adjustment of alpha (initial $\alpha = 0.006$) (Table 3). Eighteen out of 252 tests of linkage disequilibrium within sample sites were nominally significant, 15 of which involved Belize sharks, and one test (*Cli-7*/*Cli-13* in Belize City) was significant after sequential Bonferroni adjustment of alpha (initial $\alpha = 0.002$). No loci were in disequilibrium globally, supporting the independent assortment of alleles at different loci.

Table 3 Summary of microsatellite and mtDNA control region (CR) data for each nursery and entire data set

		Microsatellite loci								
Population	Parameter	*Cli-2*	*Cli-7*	*Cli-12*	*Cli-13*	*Cli-55*	*Cli-100*	*Cli-107*	*Cli-108*	All Loci	mtDNA CR
SC	alleles/haplotypes	1	24	2	18	7	4	6	7	69 (n = 34)	2 (n = 34)
	H_O	0.000	0.912	0.059	0.912	0.382	0.147	0.765	0.677	0.482	
	H_E/h	0.000	0.955	0.058	0.894	0.381	0.142	0.734	0.748	0.489	0.371±0.079
	F_{IS}/π	0.000	0.046	−0.015	−0.020	−0.002	−0.038	−0.042	0.096	0.015	0.00035±0.00038
GA	alleles/haplotypes	2	18	3	10	5	1	4	8	51 (n = 13)	2 (n = 13)
	H_O	0.077	1	0.385	0.692	0.308	0.000	0.923	0.846	0.529	
	H_E/h	0.077	0.972	0.335	0.905	0.289	0.000	0.677	0.779	0.504	0.462±0.110
	F_{IS}/π	0.000	−0.030	−0.154	0.242	−0.067	0.000	−0.385	−0.091	−0.051	0.00043±0.00046
PI	alleles/haplotypes	4	34	3	21	5	4	4	8	83 (n = 72)	10 (n = 45)
	H_O	0.083	0.972	0.097	0.875	0.250	0.167	0.653	0.681	0.472	
	H_E/h	0.082	0.956	0.094	0.915	0.252	0.157	0.677	0.721	0.482	0.785±0.041
	F_{IS}/π	−0.023	−0.018	−0.031	0.044*	0.009	−0.064	0.036	0.056	0.020	0.00120±0.00085
TC	alleles/haplotypes	4	31	3	21	5	4	5	8	81 (n = 71)	8 (n = 45)
	H_O	0.056	0.958	0.042	0.944	0.239	0.197	0.714	0.704	0.482	
	H_E/h	0.083	0.952	0.042	0.920	0.242	0.184	0.692	0.738	0.482	0.720±0.049
	F_{IS}/π	0.320	−0.007**	−0.010	−0.026	0.011	−0.071	−0.033	0.046	−0.001	0.00106±0.00078

Table 3 *Continued*

Population	Parameter	Microsatellite loci									
		Cli-2	*Cli-7*	*Cli-12*	*Cli-13*	*Cli-55*	*Cli-100*	*Cli-107*	*Cli-108*	All Loci	mtDNA CR
YT	alleles/haplotypes	3	33	3	21	5	4	5	7	81 (n = 66)	9 (n = 45)
	H_O	0.167	0.985	0.106	0.955	0.258	0.136	0.727	0.727	0.508	
	H_E/h	0.157	0.956	0.102	0.912	0.233	0.158	0.696	0.710	0.490	0.796±0.041
	F_{IS}/π	−0.064	−0.030	−0.036	−0.048	−0.108	0.135	−0.045	−0.024	−0.035	0.00134±0.00093
TX	alleles/haplotypes	3	29	3	17	7	4	5	7	75 (n = 50)	8 (n = 49)
	H_O	0.120	0.920	0.120	0.960	0.280	0.120	0.660	0.760	0.493	
	H_E/h	0.116	0.946	0.116	0.912	0.273	0.153	0.677	0.740	0.492	0.813±0.027
	F_{IS}/π	−0.039	0.028	−0.037	−0.053	−0.025	0.216*	0.026	−0.027	−0.002	0.00147±0.00099
NY	alleles/haplotypes	4	31	3	25	7	4	6	8	88 (n = 84)	8 (n = 60)
	H_O	0.179	0.964	0.095	0.917	0.417	0.214	0.607	0.810	0.525	
	H_E/h	0.167	0.950	0.092	0.921	0.360	0.219	0.627	0.764	0.513	0.790±0.035
	F_{IS}/π	−0.067	−0.015	−0.031	0.005*	−0.159	0.023	0.032	–0.061	−0.025	0.00282±0.00165
BC	alleles/haplotypes	3	10	1	9	2	3	4	3	35 (n = 10)	4 (n = 13)
	H_O	0.500	1	0.000	1	0.400	0.400	0.700	0.500	0.563	
	H_E/h	0.426	0.932	0.000	0.905	0.526	0.358	0.742	0.484	0.547	0.680±0.112
	F_{IS}/π	−0.184	−0.078**	0.000	−0.111*	0.250	–0.125	0.060**	−0.035	−0.031	0.00077±0.00066
DG	alleles/haplotypes	3	20	3	17	4	4	5	5	61 (n = 19)	2 (n = 19)
	H_O	0.167	1	0.105	0.947	0.316	0.474	0.632	0.632	0.534	
	H_E/h	0.256	0.952	0.104	0.939	0.474	0.479	0.667	0.590	0.558	0.526±0.040
	F_{IS}/π	0.354	−0.052	−0.014	−0.009	0.339	0.012	0.055	−0.072	0.043	0.00049±0.00048
Total	Total # sharks	418	419	419	419	419	419	418	419	418–419	312
	Alleles/haplotypes	5	42	4	26	14	5	7	10	113	23
	Mean H_O	0.122	0.962	0.096	0.921	0.306	0.184	0.684	0.726	0.500	
	Mean H_E/h	0.126	0.955	0.092	0.917	0.308	0.189	0.678	0.731	0.500	0.805±0.018
	F_{IS}/π	0.028	−0.007	−0.036	−0.005	0.009	0.026	−0.010	0.007	−0.001	0.00214±0.00130

Parameters are as follows: alleles, number of microsatellite alleles; haplotypes, number of CR haplotypes; n, number of sharks analysed for each marker; H_O, observed microsatellite heterozygosity; H_E, expected microsatellite heterozygosity; h, CR haplotype diversity; F_{IS}, microsatellite inbreeding coefficient [negative values = heterozygote excess and positive values = heterozygote deficiency; values marked with * and **deviate from Hardy–Weinberg expectations nominally ($P < 0.05$) and after sequential Bonferroni adjustment of alpha, respectively], π, CR nucleotide diversity.

Table 4 Geographic distribution of blacktip shark control region haplotypes

	Sample Site									
Haplotype	SC	GA	PI	TC	YT	TX	NY	BC	DG	Total
1	26	9	16	20	17	14	23	–	–	125
2	8	4	13	13	8	6	—	—	—	52
3	—	—	3	4	8	6	—	—	—	21
4	—	—	1	—	—	—	—	—	—	1
5	—	—	5	2	4	13	5	—	—	29
6	—	—	2	1	1	7	6	—	—	17
7	—	—	—	2	1	—	—	—	—	3
8	—	—	—	—	1	—	—	—	—	1
9	—	—	1	—	3	—	—	—	—	4
10	—	—	—	—	2	—	11	—	—	13
11	—	—	2	—	—	—	—	—	—	2
12	—	—	1	2	—	—	—	—	—	3
13	—	—	1	1	—	—	—	—	—	2
14	—	—	—	—	—	—	1	—	—	1
15	—	—	—	—	—	—	8	1	—	9
16	—	—	—	—	—	—	5	—	—	5
17	—	—	—	—	—	1	—	—	—	1
18	—	—	—	—	—	—	1	—	—	1
19	—	—	—	—	—	1	—	—	—	1
20	—	—	—	—	—	1	—	—	—	1
21	—	—	—	—	—	—	—	7	9	16
22	—	—	—	—	—	—	—	2	10	12
23	—	—	—	—	—	—	—	3	—	3
Total	34	13	45	45	45	49	60	13	19	323

The number of sharks possessing each haplotype is listed under each nursery. Data from nurseries sampled within consecutive years have been pooled. Nursery abbreviations are as listed for Fig. 1 and haplotype numbers refer to haplotypes listed in Table 2.

Within- and among-nursery genetic heterogeneity

Two control region haplotypes (1 and 2) were found in 55% of the blacktip sharks analysed and accounted for all 47 northwestern Atlantic sharks. Identical haplotypes were not recovered from all sampling localities and Belize sites possessed only unique haplotypes, except one haplotype (15) from Belize City that was common in the northern Yucatan (Table 4). Chi-squared tests and AMOVA did not detect differences in control region or microsatellite allele frequencies between 2000 and 2001 cohorts within nurseries ($P > 0.220$), except for Texas control region χ^2 ($P = 0.043$), so sharks collected in 2000 and 2001 were pooled within nurseries for spatial analyses. Significant heterogeneity was detected among the nine nurseries with mtDNA ($\chi^2 = 633.45$, $P < 0.001$; $\Phi_{ST} = 0.350$, $P < 0.001$) and microsatellites ($\Phi_{ST} = 0.007$, $P < 0.001$). Population structure was not detected between Georgia and South Carolina nurseries with mtDNA ($\chi^2 = 0.26$, $P = 0.472$; $\Phi_{ST} = -0.042$, $P = 0.719$) or microsatellites ($\Phi_{ST} = -0.008$, $P = 0.892$) or between any Florida nurseries with mtDNA (Keeney *et al*. 2003) or microsatellites (overall $\Phi_{ST} = -0.002$, $P = 0.876$). Significant structure was detected between sharks collected from Belize City in 2001 and Dangriga in 2002 with mtDNA ($\chi^2 = 8.77$, $P = 0.015$; $\Phi_{ST} = 0.195$, $P = 0.010$), but not with microsatellites ($\Phi_{ST} = 0.007$, $P = 0.246$). Lower sample sizes between years resulted in reduced power to detect temporal compared to spatial genetic differences. Nevertheless the among-year differences were much smaller than those among widespread nurseries indicating that sampling of siblings did not significantly affect haplotype and allele frequency differences among nurseries.

Significant genetic heterogeneity was detected among the six regions with mtDNA ($\Phi_{CT} = 0.394$, $P < 0.001$) and microsatellites ($\Phi_{CT} = 0.011$, $P = 0.003$), but not within regions (mtDNA $\Phi_{SC} = -0.013$, $P = 0.638$; microsatellite $\Phi_{SC} = 0.002$, $P = 0.942$). Six out of eight individual microsatellite loci (*Cli-2*, *Cli-7*, *Cli-12*, *Cli-55*, *Cli-100*, and *Cli-108*) possessed nominally significant Φ_{ST} values ($P < 0.05$) and four (*Cli-2*, *Cli-7*, *Cli-55*, and *Ci-108*) were significant after sequential Bonferroni adjustment of alpha (initial $\alpha = 0.006$) with six regional groups. All pairwise comparisons were significant between regions with control region sequences and significant microsatellite differentiation was detected with all comparisons involving Belize sites (except Dangriga vs. Belize City) and the northern Yucatan. All significant microsatellite Φ_{ST} values were approximately an order of magnitude

Table 5 Pairwise regional AMOVA estimations for control region sequences and microsatellites

	Western Atlantic	Eastern Gulf	Western Gulf	Northern Yucatan	Belize City	Dangriga
Western Atlantic	—	0.089/<0.001*	0.317/<0.001*	0.212/<0.001*	0.866/<0.001*	0.886/<0.001*
Eastern Gulf	0.002/0.091	—	0.110/<0.001*	0.099/<0.001*	0.702/<0.001*	0.731/<0.001*
Western Gulf	0.002/0.222	–0.002/0.949	—	0.114/<0.001*	0.707/<0.001*	0.745/<0.001*
Northern Yucatan	0.007/0.004*	0.007/<0.001*	0.004/0.026*	—	0.431/<0.001*	0.496/<0.001*
Belize City	0.063/<0.001*	0.067/<0.001*	0.065/<0.001*	0.049/<0.001*	—	0.195/0.010*
Dangriga	0.022/<0.001*	0.021/<0.001*	0.022/0.003*	0.011/0.012*	0.007/0.246	—

Values above diagonal are for mtDNA control region sequences and values below diagonal are for microsatellite loci. Values are Φ_{ST}/P value. AMOVA P values > 0.001 are estimated within ±0.012. Probabilities marked with * are significant after sequential Bonferroni adjustment of α.

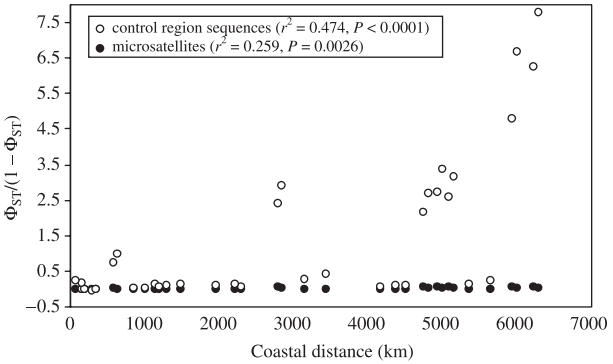

Fig. 2 Pairwise $\Phi_{ST}/(1-\Phi_{ST})$ values between sample sites plotted against coastal distances for control region sequences and microsatellites.

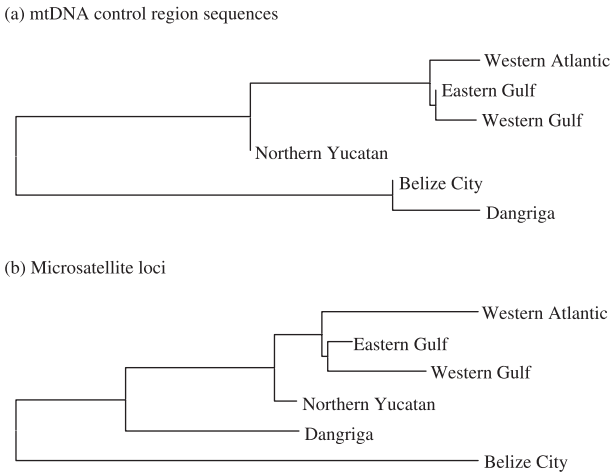

Fig. 3 Neighbour-joining trees from genetic distances for six sample regions using (a) Nei's genetic distance corrected for within-population pairwise differences [incorporating Tamura and Nei's model of sequence evolution with gamma distribution shape parameter (α) = 0.7117] for mtDNA control region sequences and (b) Cavalli-Sforza and Edwards chord distance for eight microsatellite loci.

lower than mtDNA Φ_{ST} values between the same regions (Table 5). The greatest magnitude of genetic differentiation for both markers occurred between Belize sites and all other regions (Table 5). Significant genetic heterogeneity was detected among the four Gulf and northwestern Atlantic regions with mtDNA (Φ_{CT} = 0.138, P < 0.001) and microsatellites (Φ_{CT} = 0.005, P = 0.039) when Belize samples were removed from analyses.

Isolation-by-distance, neighbour-joining, and bottleneck analyses

Mantel tests detected significant associations between genetic divergence and geographical distance with control region sequences (r^2 = 0.474, P < 0.0001) and microsatellites (r^2 = 0.259, P = 0.0026) (Fig. 2), indicating an isolation-by-distance pattern for both markers. Neighbour-joining trees reflected the genetic structure throughout the northwestern Atlantic, Gulf, and Caribbean Sea by clustering populations into successively more widespread regional groups (Fig. 3). Belize sites were the most genetically divergent and were most similar to the northern Yucatan. Topologies were identical for control region and microsatellite trees, except that Dangriga was most similar to Belize City with the control region and most similar to the northern Yucatan with microsatellites. Significant heterozygote excess was not detected in the northwestern Atlantic with the IAM (P = 0.809), SMM (P = 0.996), or TPM (P = 0.973), and the microsatellite allele frequency distribution was L-shaped, suggesting the population is at mutation–drift equilibrium for these microsatellite loci.

Discussion

Blacktip shark philopatry

Genetic structure was detected among blacktip shark nurseries with mtDNA and microsatellites. Mitochondrial DNA displayed a high overall level of genetic structure and genetic differentiation in all regional comparisons. Considering the documented long-distance movements of

blacktip sharks (Kohler *et al*. 1998), the magnitude and geographical scale of genetic differentiation indicate a strong tendency for female blacktip sharks to utilize their natal nursery regions for parturition. Sampling neonate animals facilitated detecting reproductive genetic structure. Genetic homogeneity between year 2000 and year 2001 nursery cohorts indicates that spatial genetic heterogeneity reflects geographical differences in the genetic structure of nurseries and is not heavily biased by temporal heterogeneity within nurseries due to sampling siblings (Waples 1998). Results from Texas may include a slight sibling bias as genetic differences were detected for mtDNA between years.

Tagging studies have demonstrated philopatry in juvenile blacktip sharks (Hueter 1998; Hueter *et al*. 2004). Genetic studies (Pardini *et al*. 2001; Feldheim *et al*. 2002; Schrey & Heist 2003) and tagging data (Pratt & Carrier 2001; Sims *et al*. 2001; Feldheim *et al*. 2002) support philopatry in other shark species (Hueter *et al*. 2004). The observed philopatry of juvenile blacktip sharks, repeated use of seasonal nurseries by adult female blacktip sharks, and mtDNA genetic differentiation among northwestern Atlantic, Gulf of Mexico, and Belize nurseries provide strong evidence for nursery area philopatry in adult female blacktip sharks throughout the study area. These results expand the previous detection of female blacktip shark philopatry by Keeney *et al*. (2003) between South Carolina and Gulf coast of Florida nurseries. Natural selection can lead to adult female philopatry to natal sites if parturition in natal areas increases juvenile shark survival. Philopatry to nurseries that enhance neonate survival would be an advantageous reproductive strategy for blacktip sharks because each female produces a small number of young that have been heavily invested in, similar to marine mammal species that display genetic structure characteristic of nursery philopatry (Baker *et al*. 1990). The high blacktip shark natural mortality that can occur during the neonate stage (Heupel & Simpfendorfer 2002) would aid in the development of philopatry to nurseries if these areas facilitate neonate survival.

Genetic differentiation of microsatellites was also detected among nursery regions. Locus-by-locus analysis indicated that heterogeneity was not the result of only one or two loci displaying strong differentiation. Pairwise genetic differentiation was limited to comparisons involving northern Yucatan and Belize sites, with microsatellite homogeneity throughout the eastern Gulf, western Gulf, and northwestern Atlantic. The control region's higher level of genetic differentiation may indicate stronger female philopatry with increased male straying among Gulf and northwestern Atlantic nursery areas. Female philopatry coupled with higher levels of male gene flow has been detected in many marine species, including humpback whales (*Megaptera novaengliae*) (Palumbi & Baker 1994), beluga whales (*Delphinapterus leucas*) (Brown Gladden *et al*. 1999), green sea turtles (*Chelonia mydas*) (Karl *et al*. 1992), white sharks (*Carcharodon carcharias*) (Pardini *et al*. 2001), and possibly the shortfin mako (*Isurus oxyrinchus*) (Schrey & Heist 2003). The mating behaviours and breeding areas of blacktip sharks are poorly known. Therefore, the selective pressure on males and females to return to specific breeding areas is unknown and males may display lower levels of fidelity to breeding sites than pregnant females do for nurseries, resulting in lower levels of genetic differentiation with microsatellites. Our data suggest that female blacktip sharks in the Gulf and northwestern Atlantic may mate with males that originated in other regions, then return to their natal areas for parturition.

The genetic differentiation detected with both types of markers in comparisons involving northern Yucatan and Belize suggests stock structure involving both sexes. Males and females do not appear to be mating randomly throughout these areas and gene flow is limited between the northwestern Atlantic/eastern Gulf/western Gulf and northern Yucatan/Belize regions, as well as between northern Yucatan and Belize (conclusions drawn from Belize microsatellite data are tentative in that small sample sizes may not accurately reflect microsatellite allele frequencies). Movements of blacktip sharks along the Yucatan Peninsula and Belize coasts are poorly known. It is unclear whether migratory male and female blacktip sharks return to these areas for mating or if less migration occurs within these waters than within the more northern Gulf and northwestern Atlantic regions. Blacktip sharks may be less migratory along the Yucatan Peninsula and within the Caribbean Sea, because equatorial currents keep sea surface temperatures high throughout the tropical zone. Southward migrations along the northwestern Atlantic and Florida Gulf coasts coincide with declines in sea surface temperatures below approximately 20 °C (Castro 1996; Heupel & Hueter 2001).

Although our results are consistent with higher levels of male- vs. female-mediated gene flow, the different effective population sizes of the genetic markers employed and population demography should be considered when inferring patterns of population structure (Buonaccorsi *et al*. 2001). Because mtDNA is maternally inherited and haploid, its effective population size is approximately one-fourth that of nuclear DNA, resulting in increased rates of genetic drift and a faster approach to equilibrium between drift and migration in populations that do not have highly skewed sex ratios or extreme male polygyny (Birky *et al*. 1983). Therefore, lower levels of nuclear genetic heterogeneity can result not only from increased male gene flow, but also from sampling nursery areas that have not been isolated long enough for differentiation of nuclear markers. Northern Yucatan and Belize populations may have been separated from each other and from all other populations

long enough to develop genetic differences with both mtDNA and nuclear markers. In contrast, populations in the eastern Gulf, western Gulf, and northwestern Atlantic may not have been separated long enough for genetic drift to produce nuclear differentiation. This nonequilibrium scenario could apply if eastern Gulf, western Gulf, and northwestern Atlantic populations were (re)established via successive range expansions from southern Gulf refugia as water temperatures increased and sea levels rose in the last 10 000–15 000 years, as has been proposed for king mackerel (*Scomberomorus cavalla*) in this region (Broughton *et al*. 2002). Range expansions from the southern Gulf into the northwestern and northeastern Gulf, and eventually into the northwestern Atlantic coupled with low contemporary gene flow, could produce the isolation-by-distance pattern observed in our data. The similarity of population relationships recovered for both markers with neighbour-joining trees suggests that sufficient time has elapsed for microsatellites to develop distinct genetic patterns.

In populations that have reached equilibrium, mtDNA's smaller effective population size results in higher Φ_{ST} values than those of nuclear markers when equal gene flow occurs with both sexes. In this study, the magnitude of the discrepancy in genetic differentiation between markers supports strong female philopatry with increased male gene flow throughout the Gulf and northwestern Atlantic. Also, microsatellite loci with high mutation rates can produce spuriously high gene flow estimates among isolated populations through homoplasy and high within-population variation (Hedrick 1999), but are not biasing our results because loci with a wide range of heterozygosities displayed differentiation among nurseries.

Only mitochondrial haplotypes 1 and 2 were recovered from sharks collected off Georgia in 2002, consistent with results from South Carolina in 2000 and 2001 that were interpreted as supporting a northwestern Atlantic bottleneck (Keeney *et al*. 2003). Also, haplotype and nucleotide diversities were lower in the northwestern Atlantic than in the Gulf, supporting a northwestern Atlantic genetic bottleneck (Avise 2000). Postglacial range expansion from the Gulf into the northwestern Atlantic could produce a northwestern Atlantic population with low genetic diversity and a subset of Gulf haplotypes (Grosberg & Cunningham 2001), consistent with our results. High microsatellite mutation rates and postbottleneck male dispersal may have replenished nuclear diversity faster than mtDNA diversity in the northwestern Atlantic, consistent with the observation that nuclear diversity in the northwestern Atlantic is similar to that in the Gulf.

Genetic differences were not detected among Florida nurseries or between northwestern Atlantic nurseries with either marker, indicating sufficient straying of both sexes to homogenize genotype and haplotype frequencies among these proximate nurseries. Low levels of straying can serve as a dispersal mechanism for marine species with strong regional philopatry to continental nurseries (Bowen *et al*. 1992) and allow these species to attain widespread distributions. Because there are no physical barriers to blacktip dispersal throughout the study region, the isolation-by-distance pattern is consistent with natal homing of migratory sharks and/or limited movements from natal regions throughout the study site, with straying most common to proximate nurseries. Because gene flow is inversely related to distance between natal regions for both sexes, long-distance dispersal is rare, movements are primarily along coastlines, and minimal gene flow occurs between northwestern Atlantic/Gulf and northwestern Caribbean blacktip shark populations. Better geographical sampling within the Caribbean is needed to ensure that our results are not artefacts of sampling only two sites in Belize.

Fisheries management and conservation

The economic importance of, and high commercial and recreational harvest experienced by, blacktip shark populations necessitates proper management of fisheries for the species to ensure sustainability. The magnitude of genetic structure detected in this study is consistent with the presence of multiple blacktip shark stocks within the southeastern US Atlantic, Gulf, and Caribbean Sea. Genetic stock structure was detected between populations in the northwestern Atlantic and Gulf, northwestern Atlantic and Belize, Gulf and Belize, and within the Gulf. Within the Gulf, our data support separate eastern and western stocks as well as a distinct northern Yucatan population that may be part of a larger southern Gulf stock. The low northwestern Atlantic mtDNA diversity reflects the region's smaller female effective population size and may indicate a greater susceptibility to collapse compared to Gulf populations. However, discrepancies in effective population sizes do not necessarily reflect parallel discrepancies in census sizes, especially if a population has experienced fluctuations in size (Wright 1938; Vucetich *et al*. 1997). Previous genetic bottlenecks may have produced the low mtDNA diversity and smaller female effective population size in the northwestern Atlantic, while nuclear diversity has been maintained or recovered via rapid microsatellite mutation and male migration.

The isolation-by-distance pattern indicates that we have not identified specific blacktip shark stocks with well-defined geographical boundaries, but have identified the presence of multiple blacktip shark stocks within the study area (Gold *et al*. 1999). The genetic differences observed among continental nurseries indicate that reproductive straying among distant regions is minimal and blacktip populations in the northwestern Atlantic, eastern Gulf, western Gulf, and Caribbean Sea are likely to respond to regional management practices incorporating local population

parameters. High neonate mortality coupled with natal philopatry signify that the conservation of nursery areas is critical to blacktip shark recruitment. Further sampling along the northwestern Atlantic, northern and southern Gulf, and Caribbean Sea is needed to determine whether distinct geographical boundaries exist for blacktip stocks. Even if such boundaries do not exist, regional management practices will still have larger effects on proximate nursery areas. The Florida peninsula may divide blacktip populations into eastern Gulf and northwestern Atlantic stocks, as is the case for many marine fishes (Bowen & Avise 1990; Gold & Richardson 1998). Moreover, there is a decline in the extent of blacktip nursery habitat along the southeastern tip of Florida (NMFS 2003). The magnitude of mtDNA differentiation between Gulf and Atlantic blacktip shark populations and the lack of haplotype discontinuity detected in several coastal, marine species (reviewed in Avise 2000) reflect low levels of reproductive straying between these regions as discussed in Keeney *et al*. (2003). Other breaks in nursery habitat occur along the northwest coast of Florida and the southwest coast of Louisiana (NMFS 2003), and genetic discontinuities may occur at these locations. The genetic structure detected in this study was based on sampling nurseries and identifies multiple reproductive stocks. These results do not rule out blacktip sharks mixing during nonbreeding times. The adult populations of blacktip sharks may therefore include sharks originating from multiple nurseries, which would indicate that harvesting blacktip sharks during nonbreeding periods affects multiple stocks.

Although our sampling was not exhaustive throughout the northwestern Atlantic and Gulf, our results are consistent with maternal genetic differences occurring across discontinuities in blacktip nursery habitat (NMFS 2003): nurseries within continuous nursery habitats were genetically similar, while nurseries on opposite sides of habitat breaks were genetically distinct. Because genetic heterogeneity also increased with geographical distance, attributing genetic differences to breaks in nursery habitat is tentative. Comparisons need to be made between proximate nurseries lacking suitable habitat between them, such as the middle Louisiana coast and eastern Texas coast. Nursery habitat disruptions are not likely barriers to gene flow in the classic habitat fragmentation sense with migratory sharks, but they may facilitate females returning to natal nursery regions by setting habitat boundaries. Genetic discontinuities along the Yucatan Peninsula need to be identified because the genetic structure of blacktip shark nurseries throughout Texas and the Yucatan could have important international management implications. Laguna Yalahau may represent the end of genetically 'Gulf' blacktip sharks because it is located at the junction of the Gulf and Caribbean Sea and is genetically distinct from Belize populations. Better geographical sampling of the Caribbean is required before a disjunction in blacktip shark populations can be identified with confidence.

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This study represents a major portion of Devon Keeney's Ph.D. research on the genetic structure of *Carcharahinus limbatus* populations and was a collaborative effort between Southern Illinois University Carbondale and Mote Marine Laboratory. Michelle Heupel is a staff scientist and program manager of the Elasmobranch Behavioral Ecology Program at Mote Marine Laboratory. Michelle's research focuses on the movements and behavior patterns of juvenile sharks to define use of nursery areas and responses to changes within those habitats. Robert Hueter is a senior scientist and director of the Center for Shark Research at Mote Marine Laboratory, where his research interests include aspects of the ecology, behavior, life history and population status of elasmobranchs. Ed Heist is associate professor in zoology and fisheries at Southern Illinois University Carbondale where he studies conservation and population genetics of marine and freshwater fishes.
