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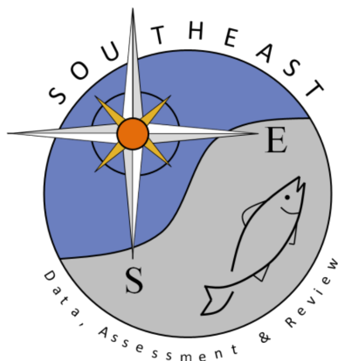

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RESEARCH ARTICLE

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Abstract Surveys of genetic variation within cosmopolitan marine species often uncover deep divergences, indicating historical separation and potentially cryptic speciation. Based on broad geographic (coastal eastern North America, Gulf of Mexico, western Africa, Australia, and Hawaii) and temporal sampling (1991–2003), mitochondrial (control region [CR] and cytochrome oxidase I [COI]) and nuclear gene (lactate dehydrogenase A intron 6 [LDHA6]) variation among 76 individuals was used to test for cryptic speciation in the scalloped hammerhead, *Sphyrna lewini* (Griffith and Smith). CR and COI gene trees confirmed previous evidence of divergence between Atlantic and Indo-Pacific scalloped hammerhead populations; populations were reciprocally monophyletic. However, the between-basin divergence recorded in the mtDNA genome was not reflected in nuclear gene phylogenies; alleles for LDHA6 were shared between ocean basins, and Atlantic and Indo-Pacific populations were not reciprocally monophyletic. Unexpectedly, CR, COI, and LDHA6 gene trees recovered a deep phylogenetic partition within the Atlantic samples. For mtDNA haplotypes, which segregated by basin, average genetic distances were higher among Atlantic haplotypes (CR: $D_{HKY}=0.036$, COI: $D_{GTR}=0.016$) than among Indo-Pacific haplotypes (CR: $D_{HKY}=0.010$, COI: $D_{GTR}=0.006$) and approximated divergences between basins for CR ($D_{HKY}=0.036$ within Atlantic; $D_{HKY}=0.042$ between basins). Vertebral counts for eight specimens representing divergent lineages from the western north Atlantic were consistent with the genetic data. Coexistence of discrete lineages in the Atlantic, complete disequilibrium between nuclear and mitochondrial alleles within lineages and concordant partitions in genetic and morphological characters indicates reproductive isolation and thus the occurrence of a cryptic species of scalloped hammerhead in the western north Atlantic. Effective management of large coastal shark species should incorporate this important discovery and the inference from sampling that the cryptic scalloped hammerhead is less abundant than *S. lewini*, making it potentially more susceptible to fishery pressure.

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Introduction

Application of molecular techniques to assay genetic variation within cosmopolitan marine species has revealed evidence of deep genetic partitions that suggests cryptic speciation within many taxa, including foraminiferans (de Vargas et al. 1999), cnidarians (Dawson and Jacobs 2001), crustaceans (Williams et al. 2001), copepods (Schizas et al. 1999; Lee 2000), gastropods (Etter et al. 1999; Quattro et al. 2001a), bony fishes (Colborn et al. 2001; Borsa 2002), birds (Friesen et al.1996), and mammals (Garcia-Rodrigues et al. 1998; Dalebout et al. 2002). Recent studies extend this trend to elasmobranchs, a relatively unstudied component of marine ecosystems. Newly discovered species of hound shark (*Mustelus*; Last and Stevens 1994; Heemstra 1997; Gardner and Ward 2002) and thresher shark (*Alopias*; Eitner 1995) were first recognized from studies of genetic variation. Unfortunately, too few comprehensive population genetic surveys have been completed to determine

the extent of cryptic speciation among cosmopolitan elasmobranch species.

We used broad geographic and genetic sampling to investigate the possibility of cryptic evolutionary lineages among hammerhead sharks in the genus *Sphyrna* Rafinesque, a group of eight widely distributed species (Gilbert 1967; Compagno 1984). The scalloped hammerhead (*S. lewini* Griffith and Smith) was the focal species because of morphological and mtDNA indications of a partition between Atlantic and Indo-Pacific populations. Springer (1941) detected sufficient morphological divergence between basins to recognize Atlantic (*S. diplana*) and Indo-Pacific (*S. lewini*) scalloped hammerhead species. However, these two forms were synonymized after broader geographic representation and larger samples sizes indicated that diagnostic characters were distributed across basins (Fraser-Brunner 1950; Gilbert 1967). In contrast, the first application of molecular techniques to evaluate genetic variation among hammerhead sharks, Martin's (1992) RFLP analysis of the mtDNA control region, indicated deep inter-basin divergence within *S. lewini*. Although based on two individuals per basin, subsequent analyses of sequence variation in the mitochondrial cytochrome *b* (Cyt*b*) and cytochrome oxidase (COI) genes (Martin 1993) supported the partition.

Additional incentives to survey scalloped hammerheads for cryptic species included commercial and recreational fishing pressure and use of *Sphyrna lewini* as 'utilized bycatch' (i.e., 'finning'; Bonfil 1997; Kotas 2002). *Sphyrna lewini* is an abundant coastal shark and, consequently, an important element of commercial fisheries worldwide. Also, scalloped hammerhead nursery grounds in shallow coastal bays and inlets are subject to high levels of commercial and recreational fisheries activity, and neonates and juveniles constitute a significant proportion of shark landings (e.g., Bonfil 1997; Kotas 2002). With a low intrinsic rate of increase (Smith et al. 1998) and increasing fishing pressure, prudent management of this fishery is warranted, especially if cryptic species are included.

To test for cryptic speciation in *S. lewini*, variation within two evolutionarily independent genomes (mitochondrial and nuclear loci) was assayed to reduce potential effects of bias in individual data sets. Specifically, taxonomic interpretations of mtDNA variation and gene trees are generally concordant for deeply divergent taxa (Weins and Penkrot 2002), such as bonefish (Colborn et al. 2001) and pygmy sunfishes (Quattro et al. 2001b), but can be misleading taxonomically at lower divergence levels, particularly for allopatric populations (e.g., Weins and Penkrot 2002). For example, mtDNA variation in *Carcharodon carcharius* is significantly structured between South Africa and Australia/New Zealand and could be interpreted as evidence for allopatric speciation. However, nuclear markers (microsatellite loci) did not support this fundamental genetic break, and the mtDNA data were interpreted as reflecting male-biased dispersal and female philopatry (Pardini et al. 2001). This dichotomy in mtDNA and nuclear inferences for *C. carcharius* emphasizes the importance of using independently evolving markers to test phylogeographic hypotheses (Slade et al. 1994; Quattro et al. 2001b; Weins and Penkrot 2002). Concordance among independent markers is strong support for species hypotheses, and a two-pronged strategy of mitochondrial and nuclear gene assessments is a valuable tool for detecting cryptic species (Avise and Ball 1990; Sites and Crandall 1997; Grady and Quattro 1999; Weins and Penkrot 2002).

Samples of *S. lewini* and appropriate outgroups (Table 1) were first characterized for mitochondrial CR variation. To test the CR interpretation of scalloped hammerhead evolution within and across basins and to interpret mtDNA variation in a broader phylogenetic and taxonomic context, select samples were screened for variation in the COI subunit I locus of the mitochondrial genome. To expand taxonomic representation and eliminate the possibility of misidentified individuals, COI data for *S. lewini* were combined with Martin's (1993) COI data set (available at http://spot.colorado.edu/~am/Cyb.COI.Data.1), which included sequences for all members of the genus *Sphyrna*, except *S. zygaena*, and several outgroups (*Eusphyra blochii*, *Negaprion brevirostris*, and *Prionace glauca*). The data set was expanded to include *S. zygaena* and a broader geographic representation of *S. lewini*. To test phylogenetic patterns in mtDNA, samples were then screened for variation in a nuclear gene, the sixth intron of the muscle-type lactate dehydrogenase-A locus (Stoner et al. 2003).

Finally, as a comparison to the genetic data, a small subset of the *S. lewini* samples was evaluated for variation in total vertebrae. The impetus for this portion of the study was Gilbert's (1967) analysis of morphological variation in *S. lewini* in which he reported wide variation in vertebral counts for nine scalloped hammerhead specimens. Total vertebrae ranged from 174 to 204, but variation was considerably narrower across eight of the nine specimens, with counts ranging from 192 to 204. As noted by Gilbert (1967), the broader range was due to substantially fewer total vertebrae (174) in one specimen from the western north Atlantic, notably coastal South Carolina.

Materials and methods

Specimens, DNA extraction, PCR amplification, sequencing

Blood, fin clip, muscle, or liver tissue was obtained from specimens taken during 1991–2003 collections and identified in the field as scalloped hammerhead sharks, *S. lewini* (Griffith and Smith). Collections were made primarily by the Marine Resources Division, South Carolina Department of Natural Resources Marine Forensics Branch, Center for Coastal Ecosystem Health and Biomolecular Research, National Ocean Service,

Charleston, South Carolina, the National Marine Fisheries Service, North Carolina Division of Marine Fisheries, and local fishermen. Sample distribution included the western north Atlantic (coastal North Carolina to Florida), the Gulf of Mexico (western Florida to Louisiana), southeastern Atlantic (coastal Africa), central Pacific (Hawaii), western Pacific (eastern Australia), and eastern Indian Ocean (northwestern Australia) (Table 1). Tissues were also obtained from specimens of *S. zygaena* taken in the southern Atlantic (coastal Africa). Tissues were stored frozen or in 70% ethanol until total nucleic acids were extracted with QiAmp tissue extraction columns, following the manufacturer's (Qiagen) protocol. Total nucleic acids were isolated from blood through standard phenol–chloroform extraction (Hillis et al. 1996). Tissue samples from all individuals sequenced and specimens used for vertebral counts were stored at the University of South Carolina (available through JMQ).

The complete mitochondrial control region (~1100 bp) was amplified from genomic DNA extracts, using the primers ElasmoCR15642F (5′ - TTG GCT CCC AAA GCC AAR ATT CTG - 3') and ElasmoCR16638R (5′ - CCC TCG TTT TWG GGG TTT TTC GAG - 3′) designed by Stoner et al. (2003). Amplifications were conducted in 50-µl volumes, which included ~10 ng of total DNA, 10 mM Tris (pH 8.3), 2.5 mM $MgCl_2$, 50 mM KCl, 0.01% Triton X-100, 10 pmol of each primer, 200 µM each dNTP, and 2 U of *Taq* DNA polymerase. PCR conditions were: 4 min at 94°C; 40 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 2 min, and a final extension of 7 min at 72°C. A 750-bp portion of the- COI subunit I gene was amplified with the universal primers CO1e and CO1f (Palumbi 1996) and reaction ingredients and conditions described above for the CR. The sixth intron (~200 bp) of the LDHA locus (LDHA6) was amplified by hemi-nested PCR described by Stoner et al. (2003). The first round of PCR used primers ElasmoLDHA6F1 (5′ - GCT TAT GAR GTG ATW AAA CTG AA - 3′) and ElasmoLDHAR1 (5′ - GAA RAC CTC RTT YTY WAT RCC ATA - 3′) and the reaction mixture described above, with an annealing temperature of 52°C. The second PCR included a 2-µl aliquot of the first PCR product, primers ElasmoLDHA6F2 (5′ - GGG WTG TCT GTG GCA GAC CTC GC - 3′) and ElasmoLDHAR1, and the reaction mixture and cycling parameters described for the first PCR (Stoner et al. 2003).

Amplification products were sequenced on an ABI 377 automated sequencer. Approximately 460 and 400 bases, respectively, of the COI and CR amplicons were characterized in the forward direction. For LDHA6 products, heterozygotes were diagnosed as individuals with two equally intense peaks at single base positions on chromatograms. Suspected heterozygotes were rare; however, when encountered, both strands were sequenced to confirm peak intensity at individual positions and subsequently compared to homozygotes to infer the phase of mutations.

Sequence and phylogenetic analyses

Chromatograms were edited and aligned in Sequencher (version 4.1; Gene Codes Corp., Inc.) or BioEdit (version 5.09; T. Hall, North Carolina State University). Sequences for each gene were sufficiently homologous for ingroup and outgroup taxa to be aligned by eye. Aligned COI sequences were checked for correct reading frame by translation to amino acid sequence in Sequencher and BioEdit. McClade (Maddison and Maddison 1992) was used to determine the number of alleles/haplotypes and to identify and remove repeated occurrences of each in the COI, CR, and LDHA6 sequence datasets, each consisting of sequences for 76 individuals. Genetic distance calculations and phylogenetic analyses were conducted in PAUP* (version 4.0b10, D. L. Swofford, Florida State University). Data files are available from the corresponding author.

Parsimony trees were reconstructed using the exhaustive search (CR and LDHA6) or branch-and-bound (COI) algorithm. For parsimony analyses, COI, CR, and LDHA6 characters were weighted equally. Additional COI parsimony analyses weighted characters according to an empirically derived 10:1 transition to transversion (ti:tv) ratio. Bootstrap resampling (1,000 pseudoreplicates) was used to test support for hypothesized relationships (Felsenstein 1985).

Likelihood ratio tests (Goldman 1993), as implemented in MODELTEST (version 3.0; Posada and Crandall 1998), were used in conjunction with PAUP* to select sequence evolution models appropriate to each sequence data set. Maximum likelihood trees were reconstructed using the heuristic search routine, including likelihood bootstrapping (100 pseudoreplicates).

A partition homogeneity test (Farris et al. 1994, 1995) was conducted in PAUP* to assess congruence of phylogenetic signal across a combined CR/LDHA6 data set. Invariant positions and gaps were excluded for these analyses. Tree lengths for 1,000 random partitions of the combined data set were not significantly different from trees generated from the CR and LDHA6 components individually ($P=0.28$), and the data were pooled for subsequent analyses. Unweighted parsimony analyses on the pooled data set used the exhaustive search strategy implemented in PAUP*. Maximum likelihood reconstructions used a sequence evolution model obtained from MODELTEST (Posada and Crandall 1998). Bootstrapping (Felsenstein 1985) was used to estimate the reliability of parsimony and likelihood reconstructions (1,000 and 100 pseudoreplicates, respectively).

Vertebral counts

Eight juvenile scalloped hammerheads, three from Bulls Bay and five from St. Helena Sound, SC (Table 1), were characterized for pre-caudal and total vertebrae by X-radiography using a Summit generator (62 mA at a

Table 1 *Sphyrna lewini*. Sampling locations, collection date, sex, fork length (FL), and haplotypes of specimens

Region Location	Haplotype (Allele)						
	Date	Sex	FL	CR	COI	LDHA6	Clade
Western Atlantic (**WA**)							
Oregon Inlet, North Carolina	May 1990	♀	120	1		2	Atlantic
	May 1990	♀	129	1	6	2	Atlantic
	June 1990	♂	169	1	7	2	Atlantic
	June 1990	♂	153	1	6	3	Atlantic
	June 1990	♀	198	1	6	2	Atlantic
	June 1990	♂	169	1	6	2	Atlantic
	June 1990	♂	170	1		2	Atlantic
	June 1990	♀	136	1	6	2	Atlantic
Folly River, North Carolina	30 Aug 2003	N/A	70	1		2	Atlantic
	30 Aug 2003	N/A	36	5		6	Cryptic
Cape Romaine, South Carolina	12 July 1994	♂	33.5	5		6	Cryptic
Bulls Bay, South Carolina	14 May 2002	♀	34.8	5		6	Atlantic (189)
	11 July 2002	N/A	N/A	5		6	Cryptic (173)
	19 July 2001	N/A	N/A	5		6	Cryptic (179)
	19 July 2001	N/A	N/A	5		6	Cryptic
Coastal South Carolina	27 July 1994	♂	37	5		6	Cryptic
	27 July 1994	♀	35	5	8	6	Cryptic
	27 July 1994	♀	35	5	8	6	Cryptic
	14 Aug 1995	♂	103.8	5	8	6	Cryptic
	29 Sept 1999	♂	54.3	5	8	6	Cryptic
	29 Sept 1999	♀	54	6	8	6	Cryptic
	29 Sept 1999	♂	43	5		6	Cryptic
St. Helena Sound, South Carolina	26 Aug 2002	♂	55.8	5		6	Cryptic
	30 Aug 2002	♂	50.3	5		6	Cryptic (162)
	30 Aug 2002	♂	41.8	1		1	Atlantic
	30 Aug 2002	♂	52.6	1		1	Atlantic (195)
	30 Aug 2002	♂	46.0	1		2	Atlantic
	30 Aug 2002	♂	52.0	1		2	Atlantic (199)
	30 Aug 2002	♂	54.0	1		2	Atlantic (191)
	3 Sep 2002	♀	43.8	5		6	Cryptic
	3 Sep 2002	♀	42.7	5		6	Cryptic (171)
	3 Sep 2002	♀	48.9	1		1	Atlantic
St. Augustine, Florida	12 July 1995	N/A	N/A	1		1	Atlantic
	27 Feb 1995	♀	42	1		2	Atlantic
Cocoa Beach, Florida	2 May 1995	♀	62	1		1	Atlantic
	2 May 1995	N/A	N/A	1	6	2	Atlantic
Fort Lauderdale, Florida	N/A	N/A	N/A	5		6	Cryptic
Gulf of Mexico (**GM**)							
Panama City, Florida	12 June 2003	♂	39	1		1	Atlantic
	12 June 2003	♀	36	1		2	Atlantic
	12 June 2003	♀	37	1		2	Atlantic
	12 June 2003	♀	38	1		2	Atlantic
	12 June 2003	♂	79	1		1	Atlantic
	10 June 2003	♀	39	1		2	Atlantic
	10 June 2003	♂	46	1		2	Atlantic
	11 Sept 2003	♂	48	1		1	Atlantic
	11 Sep 2003	♀	49	1		2	Atlantic
	7 Oct 2003	♀	53	1		2	Atlantic
	23 Oct 2003	♂	53	1		2	Atlantic
	30 Oct 2003	♀	31	1		2	Atlantic
S of New Orleans, Louisiana	5 Aug 1995	♀	187	1	6	2	Atlantic
	8 Aug 2001	N/A	N/A	1		4	Atlantic
	9 Aug 2001	♀	81	1	6	2	Atlantic
	12 Aug 2001	♀	87	1	6	1	Atlantic
	13 Aug 2001	♂	70	1		2	Atlantic
Southeastern Atlantic (**AF**)							
Abidjam, Ivory Coast	14 Oct 1999	N/A	169	1	6	1	Atlantic
	14 Oct 1999	N/A	163	1	6	1	Atlantic
	14 Oct 1999	N/A	182	1	6	1	Atlantic
	14 Oct 1999	N/A	172	1		2	Atlantic
	14 Oct 1999	N/A	157	1	6	2	Atlantic
	14 Oct 1999	N/A	195	1	6	1	Atlantic

Table 1 (Contd.)

Region Location	Haplotype (Allele)						
	Date	Sex	FL	CR	COI	LDHA6	Clade
	14 Oct 1999	N/A	157	1		2	Atlantic
14 Oct 1999	N/A	163	1		2	Atlantic	
14 Oct 1999	N/A	166	1	6	2	Atlantic	
Hawaii (**HW**)							
Kaneohe Bay, Hawaii	20 Feb 200	♀	55.5	4		1	Indo-Pacific
	20 Feb 200	♂	57.1	4	2	2	Indo-Pacific
	20 Feb 200	♂	59.9	4	2	1	Indo-Pacific
	20 Feb 200	♀	68.5	4		2	Indo-Pacific
	29 Mar 2000	♂	51.7	4		2	Indo-Pacific
	29 Mar 2000	♂	47.8	4		2	Indo-Pacific
	29 Mar 2000	♂	49.1	4		2	Indo-Pacific
	26 June 2000	N/A	N/A	4	2	2	Indo-Pacific
	26 June 2000	♂	N/A	4	4	2	Indo-Pacific
Australia (**AU**)							
NW Australia	5 Sept 2001	♂	129	2	2	1	Indo-Pacific
	11 Sept 2001	♂	125	3	2	1	Indo-Pacific
	13 Sept 2001	♂	121	2	3	5	Indo-Pacific
	13 Sept 2001	♀	161	2	3	5	Indo-Pacific

Acronyms for regions are used in other tables, figures, and text. Two CO1 hapotypes (1 and 5) were obtained from Martin (1993). For clarity and consistency with the text, specimens are identified to one of three phylogenetically divergent clades (*Cryptic*, *Atlantic*, and *Indo-Pacific*) recovered in the CR and COI trees (Fig. 1). Numbers in parentheses after 'Clade' designation are total vertebral counts for select individuals; see text for detail

N/A-data not available

voltage of 2.5 kV). Specimens were positioned so that the left lateral surface of the body was perpendicular to the tube. The object-film distance was increased to facilitate magnification of the image and aid accuracy of vertebral counts. Prior to exposure a dissection pin was placed through the precaudal pit of each specimen to ensure a consistent point of reference for all counts. To be consistent with Gilbert (1967), only counts for total vertebrae are reported. Total number of vertebrae was counted on each radiograph twice, once each by independent researchers; if a difference between the two counts was observed, a third count was conducted.

Table 2 *Sphyrna lewini*. Mitochondrial and nuclear gene variation in *Sphyrna lewini*, including specimens of the *Cryptic* lineage. See Table 1 and text for explanation

Category	Locus	Length (bp)	% A+T	Ti/Tv	Variable sites (proportion)	Phylogenetically informative
Mitochondrial	Control Region	408	71.2	0.99	94 (23%)	60 (14.7%)
	Cytochrome Oxidase - I	461	61.9	9.63	111 (24.1%)	85 (18.4%)
Nuclear Intron	LDHA6	171	61.5	1.07	9 (5.3%)	4 (2.3%)

Table 3 *Sphyrna lewini*. Characterization, geographic distribution, and abundance (numbers in table) of mtDNA control region (CR) haplotypes. Only variable sites (28) and haplotypes for *S. lewini* (haplotypes 1–4) and the *Cryptic* lineage (haplotypes 5 and 6) are shown

Distribution						
Haplotype	Variable site	Atlantic			Indo-Pacific	
		WA	GM	AF	AU	HW

	`   1111111222222222222333333`
	`4455234447822466666888155669`
	`8912260145218002568348408167`					

1	`TATAAATAATTTCGCA-CCCTACAACGA`	19	17	9		
2	`.....C..TA.A.AT.T.T.C.T....G`				3	
3	`.....C..TA.ATATGT..TC.......`				1	
4	`.....C..TA.A.ATGT..TC.......`					9
5	`ATGTCCATCACATAT.CT...T.TGTA.`	17				
6	`ATGTCCATCACA.AT.CT...T.TGTA.`	1				

Geographic codes are as follows: *WA* western Atlantic, *GM* Gulf of Mexico, *AF* western Africa, *AU* Australia, and *HW* Hawaii

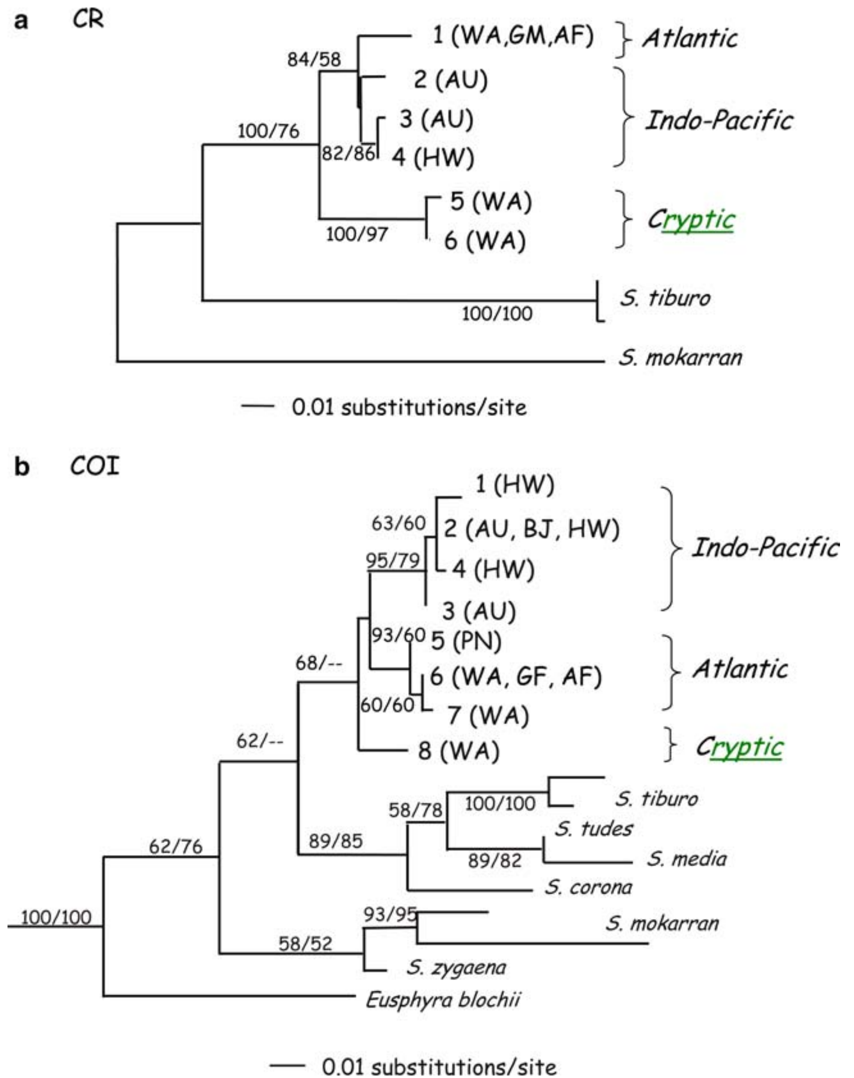

Fig. 1 *Sphyrna lewini*. **a** Phylogenetic relationships among mitochondrial control region and **b** cytochrome oxidase I haplotypes recovered from within *S. lewini* and among species of hammerhead sharks (*Sphyrna*). Parsimony reconstructions are depicted. Numbers near nodes are maximum parsimony/maximum likelihood bootstrap values; only nodes supported by >50% shown. Population codes for *S. lewini* haplotypes (in parentheses after terminal taxa) follow Table 1. CR lineages within *S. lewini* are designated *Cryptic*, *Atlantic*, and *Indo-Pacific* and noted in the COI tree. Haplotype numbers (Tables 3, 4) within *S. lewini* are indicated to the right of terminal nodes

Results

Sequence characteristics and variation

Sequence variation in mitochondrial and nuclear genes was assayed in 76 *S. lewini*, representing samples from all major ocean basins (Table 1). Both mitochondrial genes were considerably more variable (proportion of variable and phylogenetically informative sites) than the nuclear gene (Table 2). Variation in a 408-bp fragment of the CR defined six haplotypes in *S. lewini* (Table 3; GenBank accessions DQ168917–DQ168922), which were segregated by ocean basin (three each from the Atlantic and Indo-Pacific), two for *S. tiburo* (GenBank accession DQ168923–DQ168924), and one for *S. mokarran* (GenBank accession DQ168925). The COI data set encompassed 461 bp (Table 2) for the species included in Martin (1993) plus 29 additional *S. lewini* and three *S. zygaena*. The 11 taxa included in the COI dataset were represented by 19 haplotypes, including eight from the additional *S. lewini* samples (Table 4 and GeneBank accessions DQ68934–DQ168941) and one for *S. zygaena* (GenBank accession DQ168942). Variation among COI haplotypes was distributed across codon positions but was most common at third positions (10 first position changes, 1 second position, and 100 third positions). Like the CR variants, COI haplotypes recovered from scalloped hammerhead samples were segregated by ocean basin, with four haplotypes unique to each basin (Table 4).

Taxa characterized for mitochondrial genes were subsequently examined for variation in a 171-bp portion of LDHA6. Six alleles (GenBank accessions DQ168926–DQ168931) were recovered from samples of *S. lewini*; three were common and distributed across ocean basins (Atlantic–Indo-Pacific), two were singletons from the Atlantic Ocean, and one was restricted to samples from the western Atlantic (Table 5). One LDHA6 allele was recovered for the two outgroup species, *S. tiburo* (GenBank accession DQ168933) and *S. mokarran* (GenBank accession DQ168932).

Table 4 *Sphyrna lewini*. Characterization, geographic distribution, and abundance (numbers in table) of COI haplotypes. Only variable sites (26) and haplotypes for *S. lewini* (haplotypes 1–7) and the cryptic lineage (haplotype 8) are illustrated. Geographic codes follow Table 3, with the addition of PN (Panama) and BJ (Baja California), which were included in Martin's (1993) data set

Distribution								
Haplotype	Position	Atlantic				Indo-Pacific		
		WA	GM	PN	AF	AU	BJ	HW
	11111222222333333333444							
	35559456770244682235699016							
	50392657393158616926208731							
1	CCCACTCGTATTTTTCTTGAATTCTA							1
2	.................A.T.C...A					2	1	3
3	.................AAT.C...A					2		
4	.................A.T.C...T							1
5	TTT.TCTAC.C..CC.CAAT.C...A			1				
6	TTT.TCTAC.C..CC.CAAT.CC..A	6	3		6			
7	TTT.TCTAC.C..CCTCAAT.CC..A	1						
8	.T.GTC.A.GCCC.C.CAATTC.T.A			5				

Table 5 *Sphyrna lewini*. Characterization, geographic distribution, and abundance (numbers in table) of alleles for the nuclear LDHA6 locus. Only variable sites (6) and alleles for *S. lewini* (allele 1–5) and the cryptic lineage (allele 6) are shown. Geographic codes follow Table 1

Distribution						
Allele	Position	Atlantic			Indo-Pacific	
		WA	GM	AF	AU	HW
	1					
	456671					
	980260					
1	TGAGAA	5	4	4	2	2
2	....C.	13	12	5	7	
3	..T.C.	1				
4	.C.T..		1			
5	.....-				2	
6	C.....	18				

Table 6 *Sphyrna lewini*. Genetic distances within (diagonal elements) and between (off-diagonal elements) scalloped hammerhead lineages calculated as HKY corrected distances for CR, GTR corrected distances for COI, and F81 corrected distances for LDHA6. Lineage designations correspond to those in Table 1 and Fig. 1. *Atlantic* and *Indo-Pacific* locations were pooled for LDHA6 since only two well-supported lineages were recovered at this locus

Locus	Lineage	Genetic Distance		
		Cryptic	*Atlantic*	*Indo-Pacific*
CR	*Cryptic*	0.003		
	Atlantic	0.053	0.000	
	Indo-Pacific	0.051	0.025	0.010
COI	*Cryptic*	0.000		
	Atlantic	0.030	0.003	
	Indo-Pacific	0.036	0.032	0.005
LDHA6	*Cryptic*	0.000		
	Atlantic/Indo-Pacific	0.018	0.011	

Comparisons of genetic distance estimates within and between basins revealed trends that were consistent across mitochondrial and nuclear genes (Table 6). Average sequence divergence was lowest among Indo-Pacific haplotypes and highest between basins (Atlantic–Indo-Pacific). However, divergence among Atlantic haplotypes was substantially higher than within the Indo-Pacific, and comparable to between basin estimates for CR. This pattern reflected two suites of Atlantic haplotypes for each gene. One group was widely distributed within the basin, and a second was restricted to the northwestern Atlantic (*Cryptic* in Table 6), notably from coastal North Carolina, South Carolina, and Florida. Haplotypes within these groups differed minimally, but divergence between groups was substantial.

Haplotype divergence within the Atlantic was consistent across genes and across individuals. Specimens with the divergent northwestern Atlantic haplotype for CR also had the northwestern Atlantic COI and LDHA6 haplotypes.

Gene trees

Control region

Parsimony analysis of partial CR sequences recovered one shortest tree (L: 116, CI: 0.91, RI: 0.89; Fig. 1a) that recognized three lineages within a monophyletic *S. lewini*. One lineage included the divergent northwestern Atlantic haplotypes (referred to as the *Cryptic* lineage), the *Indo-Pacific* lineage encompassed haplotypes that are widely distributed but restricted to the Indo-Pacific basin, and the *Atlantic* lineage included CR haplotypes that were widely distributed in the Atlantic basin. CR sequences and bootstrap analysis supported early divergence of the C*ryptic* lineage, with a subsequent *Atlantic–Indo-Pacific* split. Based on the HKY85 model of sequence evolution (Hasegawa et al. 1985), empirically derived base frequencies (A: 0.372, C: 0.197, G: 0.072, T: 0.359), and a gamma distribution with a shape of α = 0.248, the best maximum likelihood reconstruction ($-\ln l$ = 1059.134) recovered relationships supported by parsimony analyses. Likelihood bootstrapping indicated support for basal isolation of the *Cryptic* lineage but did not distinguish the *Atlantic–Indo-Pacific* partition due to ambiguous placement of one Australian haplotype (Fig. 1a).

Cytochrome oxidase-I

Unweighted and weighted parsimony analyses of partial COI sequences recovered one tree (L: 229, CI: 0.528, RI: 0.666; Fig. 1b) that differed from Martin's (1993) combined COI and Cytochrome *b* phylogeny only in the placement of *S. lewini*. In the COI parsimony tree, *S. lewini* diverges deeper relative to Martin's (1993) tree and is sister to a lineage consisting of *S. corona* ([*S. tudes*, *S. media*] *S. tiburo*). The COI parsimony analyses recognized three haplotype lineages within a monophyletic *S. lewini* that corresponded to the *Cryptic*, *Atlantic*, and *Indo-Pacific* lineages in the CR trees. However,

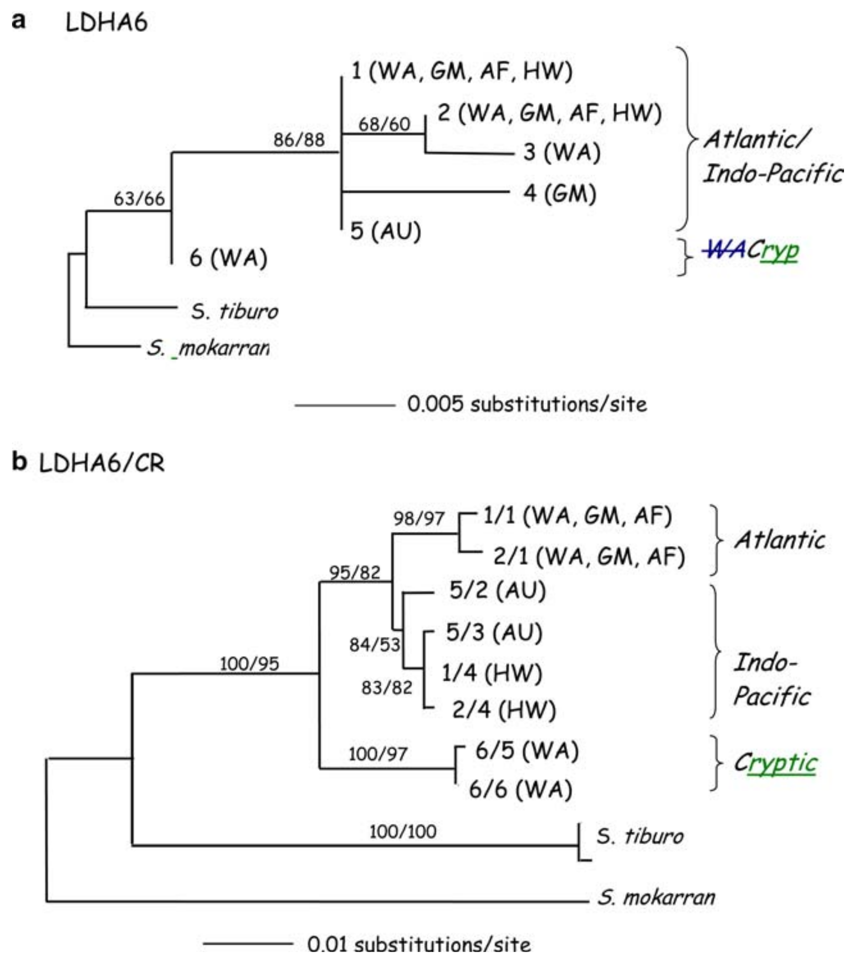

Fig. 2 *Sphyrna lewini*. **a** Phylogenetic relationships among alleles of Lactate Dehydrogenase-A Intron Six (LDHA6) and **b** as recovered from a combined analysis of mitochondrial CR and nuclear LDHA6 sequence data (2B). Parsimony reconstructions are depicted. CR and LDHA6 sequences were combined by individual. Numbers near nodes are maximum parsimony/ maximum likelihood bootstrap values; only nodes supported by > 50% shown. Population codes for *S. lewini* haplotypes (in *parentheses* after certain terminal taxa) follow Table 1. For illustration, CR lineages, *Cryptic*, *Atlantic*, *Indo-Pacific*, are applied to the trees. Allele/ haplotype numbers indicated to the right of the terminal nodes

relationships among these clades were not well resolved. Using a general time reversible model with rate heterogeneity (GTP + G; MODELTEST), empirically derived base frequencies (A: 0.273, C: 0.205, G: 0.170, T: 0.353), and a gamma distribution with a shape of α = 0.156, likelihood analyses recovered a single shortest tree (−lnl = 1,653.063) that recognized the *Cryptic, Atlantic*, and *Indo-Pacific* lineages. However, likelihood analyses did not recover a monophyletic *S. lewini*. The *Cryptic* and *Indo-Pacific* lineages were placed in a clade with four other hammerhead species, but relationships among these clades were unresolved (tree not shown). The *Atlantic* lineage was basal to other clades within *S. lewini*.

Lactate dehydrogenase-A intron six

The shortest tree (L: 9, CI: 1.000, RI: 1.000) recovered in parsimony analyses of the LDHA6 sequences recognized two clades within a monophyletic *S. lewini* (Fig. 2a). One corresponded to the *Cryptic* lineage in mitochondrial trees and included a single allele in 18 individuals. A second clade encompassed five alleles that were distributed across the Atlantic and Indo-Pacific basins. The best likelihood tree (−lnl = 293.430), reconstructed under the F81 (Felsenstein 1981) model with empirically derived nucleotide frequencies (A: 0.283, C: 0.151, G: 0.234, T: 0.332), repeated relationships portrayed in the parsimony tree (Fig. 2a). Likelihood and parsimony bootstrap analyses indicated moderate support for the *Cryptic–Atlantic/Indo-Pacific* partition, but relationships among haplotypes in the *Atlantic/Indo-Pacific* clade generally were not supported.

Combined control region and lactate dehydrogenase

A data set comprising mitochondrial and nuclear gene sequences was constructed by pairing observed control region and nuclear intron data for each individual. Unweighted parsimony analyses of the pooled data set recovered one tree (L: 126, CI: 0.910, RI: 0.910; Fig. 2b) that differentiated the three lineages recognized in all mitochondrial gene trees. Combining data sets produced trees that were better resolved and in which nodes received greater support than in single gene trees. Seven nodes in the combined tree were supported in 50% or more bootstrap replicates, compared to five and three nodes for the CR and LDHA6 gene trees, respectively. The *Cryptic*, *Atlantic*, and *Indo-Pacific* lineages in *Sphyrna lewini* also were strongly supported by bootstrapping. Likelihood analysis of the pooled data set recovered a single tree (-lnL = 1414.027) under the HKY + G model (Hasegawa et al. 1985); this tree supported monophyly of *S. lewini* and the *Cryptic* clade as basal to the *Atlantic–Indo-Pacific* partition.

Vertebral variation

Eight juvenile *S. lewini*, three from Bulls Bay and five from St. Helena Sound, South Carolina, were characterized for total vertebrae and CR and LDHA6 haplotypes. Sharks from St. Helena Sound represented individuals within the *Cryptic* and *Atlantic* lineages that were captured in late August and early September 2002. Counts for total vertebrae segregated according to mitochondrial and nuclear haplotype lineages. Control region and LDHA6 haplotypes identified four each of the *Atlantic* and *Cryptic* clades among the specimens sampled for vertebrae. Specimens of the *Cryptic* lineage had significantly (two-tailed *t* test, $P < 0.002$) fewer total vertebrae (mean/SD 171.3/7.0, range 162 to 179) than samples with *Atlantic* haplotypes (mean/SD 193.5/4.4, range 189 to 199).

Discussion and conclusions

Uplift of the Isthmus of Panama has partitioned variation in many marine, freshwater, and terrestrial species (reviewed by Bermingham et al. 1999), as first recognized by Jordan's (1908) 'law of geminate species'. The scalloped hammerhead, *S. lewini*, is circumtropical to subtropical; thus divergence between allopatric Atlantic and Indo-Pacific populations of *S. lewini* is predicted from geological history and was indicated in limited geographic sampling of the mtDNA genome (two specimens per basin; Martin1993). A primary concern was whether inter-basin divergence was sufficient to warrant taxonomic recognition, prompting our evaluation of evolutionarily independent markers. Mitochondrial and nuclear gene trees and allelic distributions support cryptic speciation among hammerhead sharks, but the event recorded in these genes was within the Atlantic basin rather than between basins as predicted by geological history and Martin's (1993) preliminary data.

Genetic sampling recovered three mtDNA lineages among *S. lewini* samples. Two lineages correspond to the predicted divergence between Atlantic and Indo-Pacific populations. However, a third, deeper mtDNA lineage was recovered and was restricted in our sampling to the western north Atlantic (coastal North Carolina to Florida). The second Atlantic lineage was first recorded in CR sequences and gene trees but was confirmed with broader taxonomic sampling of a second mitochondrial gene, COI. Inclusion of all recognized hammerhead species in the COI data set confirmed the occurrence of two divergent lineages in the western Atlantic.

Interestingly, sequence divergence estimates for the mitochondrial genes provide different impressions of population subdivision and scalloped hammerhead lineage diversification. COI sequence divergence (GTR corrected) is comparable among the *Atlantic*, *Cryptic*, and *Indo-Pacific* scalloped hammerhead lineages, suggesting coincident separation (Table 6). Whereas, CR sequences record earlier isolation of the *Cryptic* lineage,

based on 5.0% divergence (HKY corrected) relative to *Atlantic* and *Indo-Pacific* haplotypes, followed by a more recent separation of *Atlantic* and *Indo-Pacific* clades (2.5% divergence). Also, the lowest divergence estimate among lineages for COI is between the coexisting Atlantic haplotypes (*Cryptic* and *Atlantic*), which, curiously, record the highest CR sequence divergence. Potentially, these differences reflect variation in substitution patterns and saturation effects between coding (COI) and noncoding (CR regions) regions of the mtDNA genome.

Likelihood and parsimony reconstructions of mitochondrial gene trees generally recognize monophyly of the *Atlantic*, *Cryptic*, and *Indo-Pacific* lineages, but relationships among lineages vary across trees and bootstrap support for some nodes is weak. Assuming a roughly uniform mtDNA clock, disparity between divergence estimates for COI and CR haplotypes might reflect saturation effects. Variation among COI haplotypes in *S. lewini* was limited to third positions as anticipated of coding genes, whereas differences among CR haplotypes were distributed across the fragment. Similarly, base frequencies varied between genes (Table 2), with COI third positions having greater disparity in the proportion of purines. Divergent A and G frequencies might be contributing to saturation effects, even at low divergence levels (Kocher and Carleton 1999).

With all possible phylogenetic arrangements (three-taxon statements) portrayed in various mitochondrial gene trees, the origin and factors contributing to isolation and divergence of *Sphyrna lewini* lineages are unresolved. The (*Cryptic* (*Atlantic* + *Indo-Pacific*)) arrangement is more tenable than alternatives in terms of conventional biogeography and is consistent with a monophyletic *S. lewini*. Of course, any phylogenetic arrangement of scalloped hammerhead lineages requires sympatric divergence of the *Atlantic* and *Cryptic* lineages. More extensive sampling is required to evaluate alternative scenarios for speciation in *S. lewini*. Similarly, variable sequence divergence estimates for COI and CR haplotypes prevents application of a general mtDNA molecular clock to date separations and identify corresponding geological events and ecological factors.

Even with well-resolved mtDNA relationships, determining the taxonomic status of recently diverged mitochondrial lineages is problematic (Moritz 1994; Sites and Crandall 1997; Weins and Penkrot 2002). The *Atlantic* and *Indo-Pacific* clades of *S. lewini* have divergent COI and CR haplotypes, do not share haplotypes, and are exclusive (following Weins and Penkrot 2002). However, divergence is anticipated among allopatric populations. Conversely, the *Atlantic* and *Cryptic* hammerhead lineages are sympatric and divergent for mtDNA. While mtDNA divergence estimates and gene trees suggest speciation, co-occurrence of divergent haplotypes could reflect retention of ancestral polymorphisms (Campton et al. 2000).

Despite the power of mtDNA to recover population divergences, the strongest evidence of speciation is concordant partitioning of evolutionarily independent characters (Avise and Ball 1990; Sites and Crandall 1997; Grady and Quattro 1999; Weins and Penkrot 2002). Allelic distributions and trees for the nuclear encoded LDHA6 gene confirm the evolutionary independence of two scalloped hammerhead lineages recognized in the mtDNA data and trees. Individuals with mtDNA haplotypes corresponding to the *Cryptic* lineage were fixed for an LDHA6 allele that was not recovered from Indo-Pacific or other Atlantic samples. Absence of heterozygotes for LDHA6 and disequilibrium across mitochondrial and nuclear loci indicates that the sympatric *Atlantic* and *Cryptic* clades do not share a gene pool.

LDHA6 trees also support evolutionary independence of the *Cryptic* lineage. Likelihood and parsimony reconstructions for LDHA6 consistently recover basal divergence of the *Cryptic* allele relative to an *Atlantic–Indo-Pacific* assemblage. Parsimony and likelihood analyses on the pooled LDHA6 and CR data (Fig. 2b) yielded well-supported relationships among lineages within *S. lewini*. As in the individual datasets, three distinct phylogenetic partitions are apparent. However, unlike single gene analyses, partitions and inter-relationships in the combined tree received strong bootstrap support, particularly a monophyletic *S. lewini* and a sister group relationship between the *Cryptic* and an *Atlantic* + *Indo-Pac*ific clade.

Like genetic data, variation in total vertebrae for western north Atlantic specimens of *S. lewini* is strongly partitioned, and the division is consistent with genetic lineages. Although the meristic data are preliminary due to small sample size and limited geographic representation, concordant morphological and genetic (mitochondrial and nuclear) variation strongly supports the occurrence of two scalloped hammerhead shark species in the Atlantic. Moreover, Gilbert's (1967) inability to discriminate morphologically between lineages indicates that acquisition of reproductive isolation (as supported by genetic data) preceded morphological differentiation, i.e., speciation was cryptic. The sampling bias of this study, i.e., small samples largely from the western north Atlantic and primarily juveniles, precludes confident assessment of the geographic distribution of the cryptic hammerhead species. Available samples indicate that the cryptic species occurs in the northwestern Atlantic, specifically in coastal areas from North Carolina to Florida. Within this range, the two Atlantic scalloped hammerhead lineages occurred sympatrically in North and South Carolina, where they were taken syntopically, but other samples of the cryptic species also were taken within the range of *S. lewini*. An independent, global survey of *S. lewini* based on ITS2 variation recorded three specimens of the cryptic species, all from eastern coastal Florida (Abercrombie et al. in press). A phylogeographic assessment of *S. lewini* is underway (KMD) and might illuminate the geographic distribu-

tion of the cryptic species, particularly whether it is limited to the Atlantic.

In addition to the intrinsic significance of cryptic speciation among cartilaginous fishes and among cosmopolitan species, thorough geographic and genetic surveys of broadly distributed shark species are fundamental to comprehensive management plans. The rapid expansion of commercial shark fisheries in conjunction with marine habitat degradation has prompted international concern for the sustainability of these fisheries and persistence of target species (Compagno and Cook 1995; Walker 1998; Castro et al. 1999; Stevens et al. 2000). The undetected presence of cryptic species within commercially exploited sharks, such as the hound shark (genus *Mustelus*; Last and Stevens 1994; Heemstra 1997; Gardner and Ward 2002), points to a growing conservation crisis.

The scalloped hammerhead is a common and abundant element of the large coastal shark fishery, which is currently considered over-fished (NMFS 2001). The tendency for scalloped hammerheads to aggregate makes this species vulnerable to increasing fishing efficiency. For example, reports of scalloped hammerhead catches include estimates of nearly 35 tons taken in individual purse seine hauls in the northwestern Atlantic (Bonfil 1997). Limited catch data for the scalloped hammerhead indicate decreasing CPUE in the western Atlantic (Brown 1998; Cramer 1998) and substantial declines in many areas of the northwestern Atlantic (Baum et al. 2002). Recognition of two sympatric scalloped hammerheads species in the western Atlantic should prompt careful re-evaluation of the current management plan. Intense coastal fishing pressure on scalloped hammerheads places at least two species at greater risk for over fishing.

Data from this study and related efforts suggest a lower abundance of the cryptic species relative to its sister species *S. lewini*. Sampling for this study was more intense in coastal South Carolina, where 16 of 22 specimens were of the cryptic species. However, only two of the other 54 specimens screened for genetic variation and taken from across the range of *S. lewini* were identified as the cryptic species. Similarly, an independent assessment of genetic variation in *S. lewini* sampled more extensively than this study, yet only three specimens of the cryptic species, also from the western north Atlantic, were detected among 140 samples (Abercrombie et al. in press).

The apparent high relative abundance of the cryptic species in coastal South Carolina could be an artifact of sampling but also might highlight a conservation focus. Most specimens screened in this study were neonates to juveniles, including those from coastal South Carolina. High relative abundance of juveniles of the cryptic species in South Carolina estuaries and its rarity in other coastal areas (i.e., Gulf of Mexico) suggests that South Carolina bays are among the more important nursery grounds for the cryptic species. Protecting prime nursery habitat is vital to the persistence of the cryptic species, since species with narrow geographic distributions, overall or during critical life history stages, inherently are at higher risk of extinction. Concentrated reproduction in South Carolina costal waters also could increase the risk of extinction of the cryptic species. Population declines due to intense coastal fisheries could be exacerbated by a gender-biased harvest of the cryptic species as female density increases during the reproductive season. If South Carolina coastal waters are the primary nursery grounds for the cryptic species and females aggregate during reproductive season, these areas are conservation priorities. Data on the geographic distribution and relative abundance of both scalloped hammerhead species is critical at this juncture and should be used to evaluate current management plans.

Mitochondrial haplotype and nuclear allele distributions and phylogenies support the existence of two sympatric scalloped hammerhead species in the northwestern Atlantic. The diagnosis of a cryptic species of hammerhead shark is based on sympatric occurrence of two deep genetic lineages and complete disequilibrium between mitochondrial haplotypes and nuclear alleles within lineages. Coexistence of these lineages in the north Atlantic and the lack of genetic exchange inferred from disequilibrium strongly suggest independent gene pools and reproductive isolation, two properties of species integrity in some species concepts (e.g., see Mayr 1940; Dobzhansky 1950). Preliminary morphological data, notably vertebral counts, support these genetic findings.

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