Delaware Bay and Chesapeake Bay Populations of the Horseshoe Crab *Limulus polyphemus* are Genetically Distinct

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ABSTRACT: The Delaware Bay contains the world's largest population of horseshoe crabs, which constitute an ecologically significant component of this estuarine ecosystem. The North Atlantic species *Limulus polyphemus* has an extensive geographical distribution, ranging from New England to the Gulf of Mexico. Recent assessments of the Delaware Bay population based on beach spawning and trawling data have suggested a considerable decrease in the number of adult animals since 1990. Considerable debate has centered on the accuracy of these estimates and their impact on marine fisheries management planning. Compounding this problem is the lack of information concerning the genetic structure of Atlantic horseshoe crab populations. This study assessed patterns of genetic variation within and between the horseshoe crab populations of Delaware Bay and Chesapeake Bay, using both Random Amplification of Polymorphic DNA (RAPD) and DNA sequence analysis of the mitochondrial cytochrome oxidase I gene (COI). We examined 41 animals from Delaware Bay and 14 animals from the eastern shore of Chesapeake Bay. To provide high quality, uncontaminated genomic DNA for RAPD analysis, DNA was isolated from hemocytes by direct cardiac puncture, purified by spin column chromatography, and quantified by agarose gel electrophoresis. RAPD fingerprints revealed a relative paucity of polymorphic fragments, with generally homogeneous banding patterns both within and between populations. DNA sequence analysis of 515 bases of the 5′ portion of the mitochondrial COI gene showed haplotype diversity in the Chesapeake Bay sample to be significantly higher than in the Delaware Bay sample, despite the larger size of the latter. Haplotype analysis indicates minimal contemporary gene flow between Delaware Bay and Chesapeake Bay crab populations, and further suggests that the Delaware Bay population is recovering from a recent population decline.

Introduction

The horseshoe crab *Limulus polyphemus* is an ancient marine arthropod that inhabits waters along the Atlantic Coast of North America from Maine to Mexico. *Limulus* and its three Asian cousins (*Tachypleus tridentatus, T. gigas,* and *Carcinoscorpius rotundicauda*) are the only extant members of the class Merostomata, providing scientists with an opportunity to study a marine arthropod that was once a dominant predator in the ancient marine biosphere and that has shown very little morphological change over a relatively long evolutionary period (Selander et al. 1970). The largest concentration of horseshoe crabs in the world is presently found in Delaware Bay. These animals have an important role in both marine and estuarine ecosystems, most notably in their provision of food (eggs) for migrating shorebirds during the horseshoe crab spring spawning period (Botton et al. 1994; Penn and Brockmann 1994).

The spawning period for *Limulus* occurs each spring with the highest reproductive activity during May and June. Peak spawning activity generally occurs on or around the new and full moon high tides, although there is significant temporal variability seen depending on geographic location and estuarine environment (Shuster 1982). Paired adult female and male horseshoe crabs in amplexus migrate from deeper waters and prefer low energy, well-oxygenated sandy beaches for egg deposition and external fertilization. Satellite males often surround the nesting couples and release sperm in an attempt to fertilize deposited eggs (Brockmann 1996). Larvae hatch approximately four weeks after fertilization. Juvenile crabs are thought to spend their life stages at or near their breeding beaches. After the final molt, adult ani-

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mals are thought to migrate out to deeper bay waters and the continental shelf. There is no direct evidence that adult horseshoe crabs return to their natal beaches for spawning, although some studies support the possibility of local populations (Shuster 1982; Widener and Barlow 1999).

The abundance of horseshoe crabs worldwide appears to be on the decline, as a result of various factors including the loss of suitable spawning locations and over-harvesting (Widener and Barlow 1999). *L. polyphemus* was historically harvested for fertilizer and livestock feed and is now used primarily as bait for the eel and conch fisheries, as well as for biomedical purposes (Shuster and Botton 1995). Commercial harvests were typically well over one million crabs annually from the 1850s through the 1940s, dropping dramatically during the period 1950–1960 (Shuster 1996). Although the population subsequently recovered, fishery catch and effort have increased sharply in recent years, prompting renewed concern from a number of groups and agencies (Anonymous 1999; Widener and Barlow 1999). As a result, management plans have recently been developed to address these concerns (Anonymous 1998).

One of the recommendations of the Atlantic States Marine Fisheries Commission management plan is to determine whether genetically differentiated geographic subpopulations of *L. polyphemus* exist (Anonymous 1999). Previous genetic studies showed modest but significant differences in allozyme frequencies between samples from the Gulf of Mexico and the Atlantic coast (Selander et al. 1970; Mitton 1994), while a Random Fragment Length Polymorphism (RFLP) analysis of mitochondrial DNA (mtDNA) showed marked differentiation between populations from these regions (Saunders et al. 1986). In the latter study, the Gulf-Atlantic break in mitochondrial haplotypes was localized to east-central Florida, a well-established zoogeographic boundary (Avise 1992). Shuster (1979) argued on the basis of morphometric data that discrete populations existed throughout the species' range, a view supported by the existence of significant among-locality morphometric size and shape variation (Riska 1981). The mtDNA RFLP analysis of Saunders et al. (1986) did not detect any population subdivision within the Atlantic region, although the study had limited resolution.

With the advent of molecular biotechnology, information concerning molecular sequences is readily generated and provides unambiguous data concerning the genotype of an organism (Ferraris and Palumbi 1996). In this study, two approaches were used: random amplification of polymorphic DNA (RAPD), and DNA sequence analysis of the mitochondrial cytochrome oxidase subunit I (COI) gene. A RAPD DNA fingerprint provides a "global" overview of the amount of DNA sequence variation in a population, although interpretation of the results is limited by the anonymous nature of the genetic polymorphisms recorded (Hadrys et al. 1992). Direct DNA sequence analysis is the most accurate tool for genotyping, but is more expensive and labor-intensive than other techniques. Mitochondrial DNA provides an excellent target for DNA sequence analysis in population genetic studies due to its haploid chromosome status, maternal inheritance, and relatively high mutation rate compared to nuclear DNA (Simon et al. 1994). The COI gene is highly conserved at the amino acid level and is a well-established target for phylogenetic studies (Palumbi 1996). Avise et. al. (1994) used the COI gene as a target for the determination of the phylogenetic relationships for all extant horseshoe crabs.

In order to examine population genetic structure on a smaller scale, we analyzed horseshoe crab samples obtained from two central mid-Atlantic sites, Chesapeake Bay and Delaware Bay. Both genomic RAPD analysis and mtDNA sequencing were performed to gauge the extent and patterns of genetic variation. While RAPD profiles exhibited substantial homogeneity within and between populations, mtDNA sequence variation revealed significant differences between Chesapeake and Delaware Bay populations.

Materials and Methods

Specimen Collections

Adult horseshoe crabs were collected from one site in Delaware Bay and one site in Chesapeake Bay (Fig. 1). Animals from Delaware Bay were collected during the spawning period (May to June) over the course of four years (1994–1997), at Highs Beach on the New Jersey side of Delaware Bay. This site, historically called King Crab Landing, has been a premier site for *Limulus* spawning and may have at one time been the site of a horseshoe crab fertilizer facility (Shuster personal communication). The Chesapeake Bay horseshoe crabs were collected in June 1997 at a spawning beach site on the eastern shore of the Bay immediately northeast of Kent Point. After bleeding and DNA isolation, all animals were returned to their respective collection sites.

Isolation of *Limulus* DNA

Initially, isolation of high quality genomic DNA from *Limulus* hemocytes was found to be surprisingly difficult. A "nuclease-like" digestion of high molecular weight DNA was observed after standard (protease K) genomic DNA isolation procedures
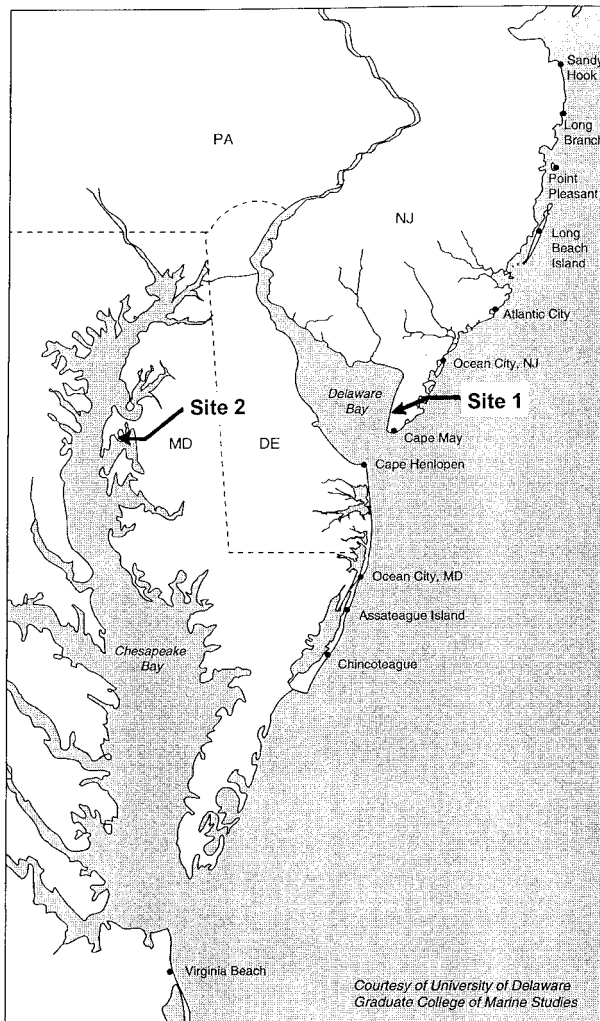
Fig. 1. Map of sampling sites. Site 1 is located at Highs Beach on the New Jersey side of the Delaware Bay (39°04′45″N, 74°54′32″W). Site 2 is located on the eastern shore of the Chesapeake Bay at Kent Point (38°54′09″N, 76°17′57″W). All animals were collected on the shoreline during the spring spawning period.

(data not shown). To generate high-quality genomic DNA preparations essential to RAPD analyses (Hadrys et al. 1992), a two-step isolation procedure, using a standard genomic DNA lysis procedure and a Qiagen Tissue DNA Kit protocol, was employed. Total genomic DNA was isolated from adult horseshoe crabs by direct cardiac puncture with an 18-gauge sterile needle after washing the crab and cleaning the dorsal cardiac membrane with an alcohol wipe. Two ml of hemolymph was aspirated into a 3-ml syringe containing 0.2 ml of bleeding buffer (0.5 M NaCl, 0.01 M N-ethyl maleimide, and 1% Tween 20 v/v) and then transferred to a sterile 1.5-ml microcentrifuge tube. The sample was then centrifuged at 1,000 rpm for 2

min at room temperature. The pellet was resuspended in 250 μl of lysis buffer (50 mm Tris HCl, pH 8.0, 0.1 M NaCl, 0.15 M EDTA, 1% SDS v/v, and 1 mg ml^{-1} proteinase K) and incubated at 50°C for 1 h. The gelatinous cellular lysate was sheared by gentle aspiration (five rounds) using an 18-gauge needle and 3-ml syringe. The lysate was transferred to a new 1.5-ml microcentrifuge tube and total DNA was purified using a Qiagen QIAamp Tissue spin-column chromatography system as directed by the manufacturer with a minor modification of the recommended elution conditions. DNA was eluted from the column using two consecutive 0.1-ml elutions of elution buffer (Qiagen AE buffer) preheated to 70°C for a total volume of 200 μl. Purified DNA samples were stored at −20°C. Samples of *Limulus* DNA were denoted according to the site and year collected; e.g., DB 97–02 refers to the second sample collected from the Delaware Bay in 1997.

NORMALIZATION OF DNA CONCENTRATIONS

Following DNA purification, 10 μl of each sample were analyzed by standard agarose gel electrophoresis and ethidium bromide staining (Sambrook et al. 1989). Samples were visually compared to DNA standards (molecular weight markers and genomic DNA control); samples that were visibly more concentrated than the controls were diluted to approximately 5 ng μl^{-1} with an appropriate amount of TE buffer (0.01 M Tris HCl, pH 8.0, 0.001 M EDTA). Each DNA sample was analyzed a second time by gel electrophoresis as described above, and a second series of dilutions was performed when required. This technique allowed for normalization of DNA concentrations between samples with very low possibility of contamination. Most of the DNA samples contained high molecular weight DNA with a minimal shearing (data not shown). Some samples showed significant lower molecular weight DNA, but this did not detract from the overall quality of the RAPD DNA profiles.

RANDOM AMPLIFICATION OF POLYMORPHIC DNA (RAPD) ANALYSIS

RAPD DNA profiles were generated by polymerase chain reaction (PCR) amplification from *Limulus* DNA extracts using random 10-base oligonucleotides as primers (Williams et al. 1990). Forty different 10-mer oligonucleotides (kits A and Z from Operon Technologies, Inc.) were used to screen a subset of the Delaware Bay DNA samples for polymorphisms. RAPD PCR reactions consisted of 1 μl of normalized DNA (approximately 5 ng), 10 mm Tris HCl, pH 8.3, 50 mm KCl, 0.001% gelatin, 2.5 mm MgCl$_2$, 1.0 μM 10-mer oligonucleotide, and 0.5 units Taq DNA polymerase, in a total

volume of 20 μl. Thermocycler conditions were 95°C for 1 min, 37°C for 1 min, 72°C for 2 min for 35 cycles, and a final cycle of 72°C for 7 min. Amplification products results were analyzed on a 1.3% agarose gel stained with ethidium bromide. Four of the oligonucleotide primers (A-2, A-4, A-19, and Z-10) that yielded well-resolved RAPD profiles were used to screen the entire set of samples. A subset of 10 individuals representing all COI haplotypes observed in this study was screened with the RAPD primers (Fig. 2).

PCR Amplification of the Mitochondrial Cytochrome Oxidase I Gene

Oligonucleotide primers based on GenBank Accession U09391 were designed to amplify a 582 base pair fragment of the COI gene (Fig. 3). Primer sequences were (Lim 01) 5′-GGGCATCCTGA-AGTCTAC-3′ and (Lim 582) 5′-ACCTAG-GAAATGTTGAGGG-3′. PCR reactions were prepared by adding 1 μl of purified/normalized genomic DNA (approximately 5 ng) to a reaction cocktail as described above for the RAPD PCR with the following thermocycler conditions: 95°C for 1 min, 45°C for 1 min, and 72°C for 1.5 min, for a total of 30 cycles. PCR samples were analyzed on a 1.3% agarose gel stained with ethidium bromide.

DNA Sequencing of Cytochrome Oxidase I PCR Products

DNA sequencing reactions were performed using Perkin Elmer Applied Biosystems FS Taq dye terminator kits and analyzed on an ABI Prism 310 Capillary Genetic Analyzer. Sequencing primers used were (Lim 172) 5′-ACCTAGGAAATGTT-GAGGG-3′ and (Lim 481) 5′-ATGGAAATCAAT-GAGTGACC-3′. PCR primers (Lim 01 and Lim 582) were also used as sequencing primers to help elucidate ambiguous base calling in some sequences. One-half to two μl of PCR product were added to a DNA sequencing reaction containing 6 μl of FS Taq sequencing mix and 0.2 μM sequencing primer in a total of 20 μl final volume reaction. Sequencing thermocycler conditions were 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min, for 25 cycles. DNA termination products were precipitated by adding the entire reaction volume to 75 μl of 70% ethanol and 0.5 mm $MgCl_2$, incubated at room temperature for 20 min, and centrifuged at maximum speed for 15 min. The supernatant was discarded and the pellet dried at 37°C for 2–3 min. The sample was resuspended in Template Suppression Reagent (PE Applied Biosystems) and heated for 3 min at 95°C. Samples were analyzed on a 61-cm capillary using standard ABI 310 conditions.

Sequence Analyses

Electropherograms were generated for each DNA primer/template combination and sequence alignments were made using the MACDNASIS software program (Hitachi). Sequences were aligned against the published *Limulus* mitochondrial cytochrome oxidase I haplotypes A and B (Avise et al. 1994). Each COI sequence alignment was manually edited and compared to the original electropherogram for accuracy. Statistical analyses of sequence data were conducted with Arlequin 1.1 (Schneider et al. 1997) and DnaSP 3.14 (Rozas and Rozas 1999).

Results

Random Amplification of Polymorphic DNA Fingerprints

RAPD profiles were generally similar for all individuals tested, with few clear polymorphisms (Fig. 2). In the four selected RAPDs (A-2, A-4, A-19, and Z-10), no major DNA fragment pattern differences were seen between the Delaware and Chesapeake samples. Most of the variation present was in the form of PCR fragment intensity rather than the presence or absence of bands. For example, RAPD A2, lane 10 (Chesapeake sample CB 97-12) shows a very weak signal for the middle band at approximately 1 kb. RAPD A19 shows a 650 bp band with variable intensity seen most dramatically with a strong band in lanes 1 and 3 (DB 94-01 and DB 96-07) and fainter bands in lanes 2, 4, 5, and 7 (DB 95-04, DB 97-06, DB 97-09, and CB 97-03). Other observed RAPD pattern variations include apparent size polymorphisms. RAPD A4 shows a size difference for the middle approximately 1 kb band (lane 3, sample DB 96-07). Lanes 9 and 10 (samples CB 97-07 and CB 97-12) are putative heterozygotes for this size polymorphism. The pattern for RAPD Z10 exhibits an apparent size polymorphism for the lower band at about 400 bp for sample DB 94-01 (lane 1). There is no correlation between mitochondrial COI sequence haplotype and RAPD fingerprint pattern from total DNA either within or between the Delaware and Chesapeake populations.

Mitochondrial Cytochrome Oxidase I Sequence Analysis

The 5′ end of the COI gene was sequenced to determine the extent of genetic variation in Delaware Bay and Chesapeake Bay horseshoe crab samples (Fig. 3a). Nucleotides 1 to 515 in our sequence alignment correspond to positions 37 to 551 in GenBank Accession U09391 (Fig. 3b). The sequence alignment shows the sequence for the A
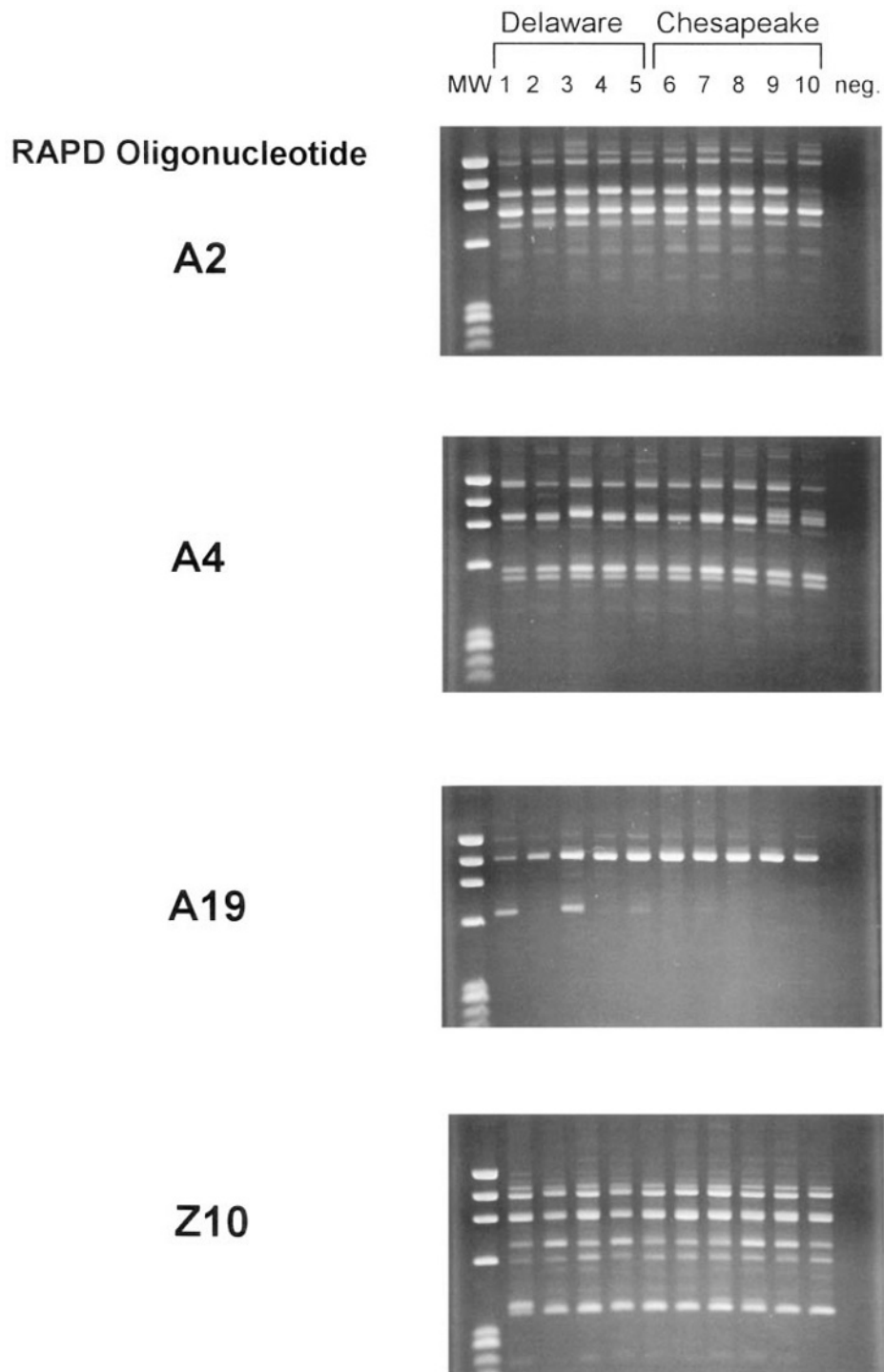
Fig. 2. Random Amplified Polymorphic DNA (RAPD) screen of representative Delaware Bay and Chesapeake Bay individual DNA samples. RAPD 10-base oligonucleotides used were A2 (5′ TGCCGAGCTG), A4 (5′ AATCGGGCTG), A19 (5′ CAAACGTCGG), and Z10 (5′ CCGACAAACC). Molecular weight marker (MW) was φX174 DNA digested with *Hae* III (fragment size from top to bottom; 1353, 1078, 872, 603, 310, 281/271, 234, and 194 base pairs). The last lane of each agarose gel is a negative control, which contains all of the RAPD PCR reaction mix minus genomic template DNA. *Limulus polyphemus* DNA samples used in this screen are: DB 94-01, 95-04, 96-07, 97-06, 97-09 and CB 97-01, 97-03, 97-04, 97-07, 97-12.
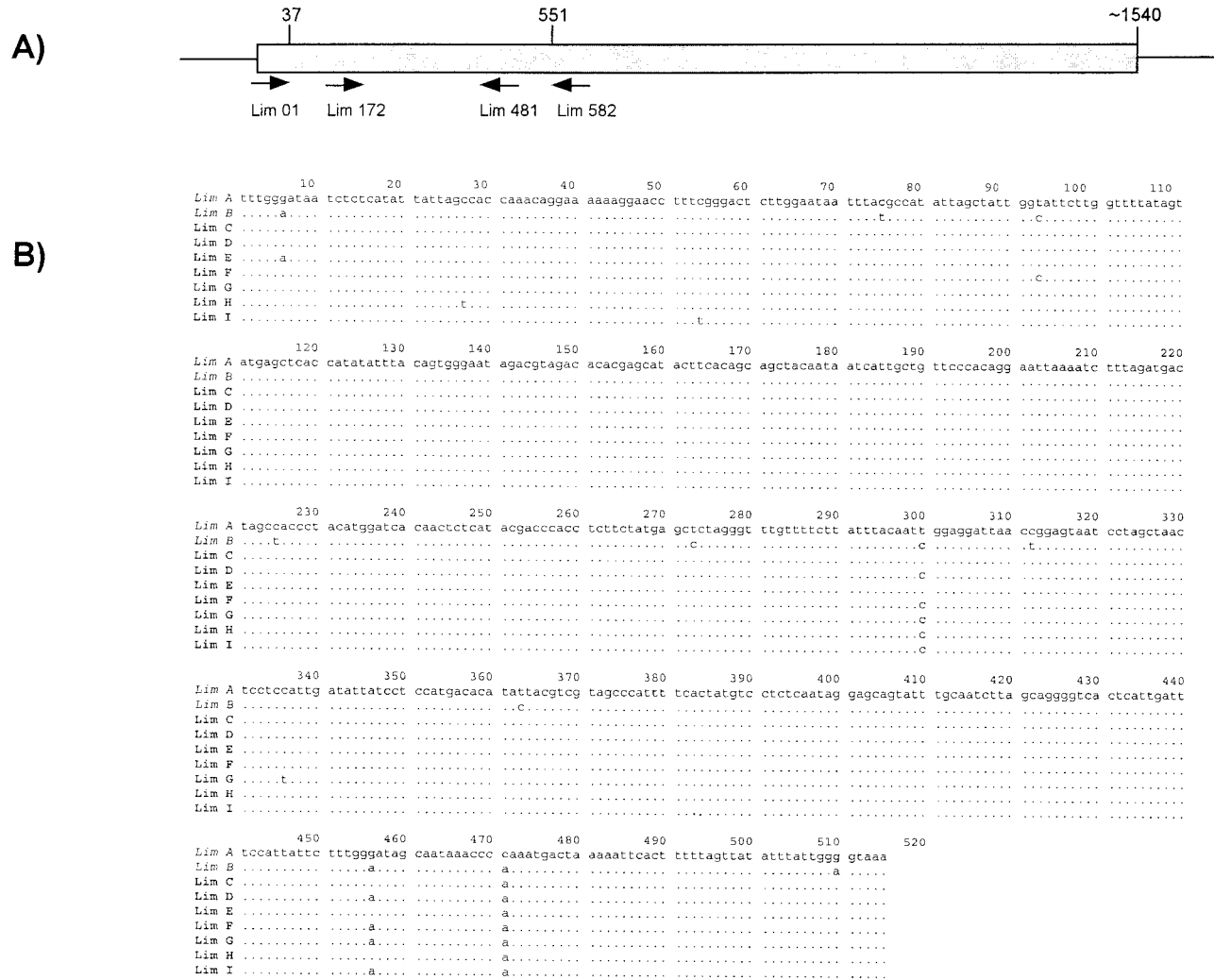
Fig. 3. Mitochondrial cytochrome c oxidase I gene sequence. (A) Diagram of the ~1,500 base pair *Limulus polyphemus* mitochondrial COI gene used in the sequence analysis. Sequence positions 37 to 551 are used in this molecular analysis and correspond to sequence positions 1 to 515 used in the sequence comparison. Oligonucleotide primers used in the PCR and in DNA sequencing are shown below the COI gene diagram. Primer numbers refer to the position of the 5′ nucleotide of the oligonucleotide. (B) Sequence alignment of all haplotypes (C to I) observed in this study. Haplotypes A and B are the sequence haplotypes reported by Avise et al. (1994), and represent the genetically distinct "northern" and "southern" types respectively. Nucleotide substitutions are shown below the haplotype A sequence. Dots indicate nucleotides identical to the type A COI sequence.

haplotype, originally described by Avise et al. (1994) for the northern (Atlantic) type *Limulus*.

Seven variable nucleotide positions were observed among the 55 individual horseshoe crabs examined, resulting in seven COI haplotypes (C to I). Six of seven substitutions were transitions; all were synonymous (Fig. 4). The single transversion substitution at nucleotide position 471 was observed in all individuals, as well as in other Atlantic specimens sequenced (Gaffney unpublished data), suggesting a sequencing error in the GenBank sequence U09391.

Despite their geographic proximity, the Chesapeake Bay and Delaware Bay populations showed highly significant differences in haplotype frequencies (Fig. 5; exact test of Raymond and Rousset 1995; $p < 10^{-5}$). The 42 individuals from Delaware Bay possessed three haplotypes, predominantly C. The Chesapeake Bay sample contained six haplotypes in a sample of 14 individuals, exhibiting a haplotype diversity (0.846 ± 0.061 SE) and nucleotide diversity (0.0040 ± 0.00032 SE) much higher than observed in the larger Delaware Bay sample (haplotype diversity of 0.262 ± 0.083 and nucleotide diversity of 0.00095 ± 0.00031). These differences lead to an estimate of gene flow of only 1.07 migrants per generation, using the method of Hudson et al. (1992).

	Nucleotide Position and Sequence								# Individuals	
Haplotype	6	27	54	93	300	336	456	471	DB	CB
Lim A	g	c	c	t	t	c	g	c	0	0
Lim C								a	35	4
Lim D				c			a	a	5	1
Lim E	a							a	1	0
Lim F				c		c	a	a	0	4
Lim G					c	t	a	a	0	2
Lim H		t				c		a	0	2
Lim I			t			c	a	a	0	1

Fig. 4.   Summary of COI sequence diversity observed in the Delaware and Chesapeake Bay *Limulus polyphemus* samples. Sequence substitutions shown are compared to haplotype A. Nucleotide position refers to the numbering of the sequence alignment described previously. The number of individuals represent individual sample haplotypes observed from Delaware Bay (DB) and Chesapeake Bay (CB).
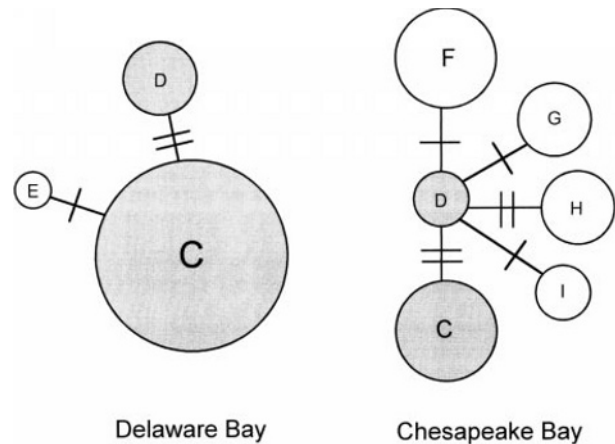

Delaware Bay   Chesapeake Bay

Fig. 5.   Parsimony network illustrating the relationships among *Limulus polyphemus* COI haplotypes observed in Chesapeake Bay and Delaware Bay. Size of the symbol is proportional to haplotype abundance (not to scale). The number of nucleotide substitutions separating two haplotypes is indicated by the number of hash marks on the line between them.

Discussion

Although the existence of a genetic division between the Gulf of Mexico and Atlantic populations of *Limulus polyphemus* reported by Saunders et al. (1986) reflects a biogeographical boundary that has been observed for a variety of species (Avise 1992), the discovery of substantial population subdivision on a smaller scale in *L. polyphemus* is surprising. Despite small sample sizes, the patterns of mtDNA variation in Chesapeake Bay and Delaware Bay samples show clearly that gene flow between populations inhabiting the sampled sites is restricted. The most plausible explanation is that horseshoe crabs in the upper Chesapeake Bay population are resident, separate from the larger shelf-inhabiting population found in Delaware Bay, as has been postulated for horseshoe crabs inhabiting New England estuaries (Botton and Ropes 1987). Whether the Chesapeake Bay and Delaware Bay populations are themselves genetically subdivided cannot be determined from the data presented here. Since the Chesapeake Bay sample represented a single spawning event at a single site, while the Delaware Bay sample was collected over the course of four years, spatial or temporal genetic heterogeneity within bays is unlikely to have contributed to the inter-bay differences.

Patterns of mtDNA sequence variation may also yield some insight into the demographic history of the two populations. Relationships among haplotypes may be summarized by the distribution of pairwise sequence differences, i.e., the number of nucleotides at which a given pair of sequences are different (Avise et al. 1988). The Chesapeake Bay sample yields a ragged distribution of pairwise differences as might be expected from a stable population at equilibrium (Fig. 6). The average number of pairwise differences observed in the Chesapeake Bay sample, 2.08, is consistent with an effective population size of females (N_f) of 5,000–10,000, if the usual estimate of mitochondrial mutation rate (2–4% per million years) is applied. A similar estimate of N_f is obtained from the observed nucleotide diversity (Wills 1990).

The Delaware Bay population exhibits an approximately Poisson distribution of pairwise differences (Fig. 6) with a mean pairwise difference of only 0.49 nucleotides. Such low sequence diversity is consistent with a four-fold smaller population at equilibrium, i.e., 1,250–2,500 females. This estimate is considerably smaller than census estimates;
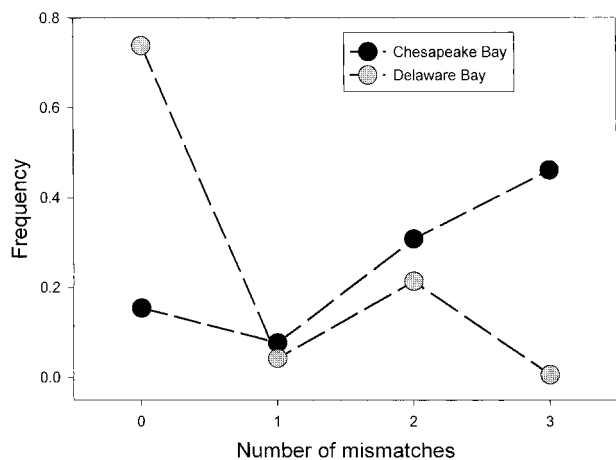

Fig. 6.   COI sequence mismatch distributions in Chesapeake Bay (CB) and Delaware Bay (DB) samples.

the commercial bait landings in New Jersey and Delaware during the years 1995–1997 averaged more than one million individuals (Anonymous 1998). Although the effective population size estimated using genetic markers is typically less than the census size (Nei and Graur 1984; Avise 1992), the disparity observed is usually less than an order of magnitude. The simplest interpretation is that the current Delaware Bay horseshoe crab population is not at mutation-drift equilibrium, but is exhibiting the effects of a recent population bottleneck.

One visible genetic consequence of a population bottleneck is the loss of haplotype diversity (Nei et al. 1975; Maruyama and Fuerst 1985). The Delaware Bay horseshoe crab population underwent a dramatic reduction in size earlier this century until the cessation of the commercial fishery in the 1960s, after which it rebounded (Shuster and Botton 1995). It is likely that the low haplotype diversity observed in the Delaware Bay population can be attributed to this recent bottleneck and the absence of significant gene flow from neighboring populations that did not experience population reductions. Conservation efforts directed at maintaining a large *Limulus* population in the Delaware Bay that will support migratory shorebirds during the spring spawning period should recognize the relative genetic homogeneity of this local population. The upper Chesapeake Bay with its significant habitat heterogeneity could provide a reservoir of horseshoe crab genetic variation for future marine management plans and this population should be especially conserved.

The primary conclusion of this study is that horseshoe crabs exhibit marked population subdivision even over a relatively small geographic range. Tagging records indicate that adults are relatively sedentary (Sokoloff 1978), and larvae are thought to settle in the shallow intertidal areas near their natal beaches (Rudloe 1981). The genetic data presented here indicate that gene flow between the upper Chesapeake Bay and Delaware Bay populations is extremely limited, suggesting the existence of substantial natal site fidelity. It should be noted, though, that the mitochondrial genome is typically maternally inherited, and as a result marks only the demographic history of females. If male horseshoe crabs are more vagile, gene flow among populations could be substantial yet remain undetected by mtDNA analyses (Palumbi and Baker 1994). Our RAPD fingerprinting analysis was not able to differentiate between the two subpopulations of horseshoe crabs. This result may reflect either the limitations of the RAPD technique or a general lack of polymorphic loci in the *Limulus* genome. Additional studies using both mitochondrial and nuclear DNA markers would be invaluable in delineating the subpopulation structure of Atlantic and Gulf Coast horseshoe crab populations.

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