Chapter 4
Conservation Genetics of the American Horseshoe Crab (*Limulus polyphemus*): Allelic Diversity, Zones of Genetic Discontinuity, and Regional Differentiation

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Abstract Extensive studies of genetic variation of Atlantic horseshoe crab *Limulus polyphemus* populations have revealed the presence of considerable allelic diversity and population structuring that appear to reflect the actions of various evolutionary processes. We have expanded on our previous efforts to gain a more refined understanding of *L. polyphemus* population structure by surveying 792 additional animals distributed among 12 additional spawning aggregations. Here we report on variation at 13 microsatellite DNA markers for 1,684 horseshoe crabs sampled from 33 spawning assemblages from northern Maine to the Yucatan Peninsula, Mexico. Average unbiased heterozygosity (uH_E) was high (0.74 ± 0.01), the number of private alleles was low (0.06 ± 0.04), effective population size (N_e) ranged from 22 to 187, inbreeding (F) ranged from −0.07 to 0.07, and tests for genic differentiation among populations indicated shallow but statistically significant differentiation within regions and highly significant differences among regions ($P < 0.005$). Current findings are consistent with previous research by this group in suggesting a series of genetic discontinuities across the species' range that could indicate regional adap-

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tive significance or reflect vicariant geographic events. Additional collections allowed improved delineation of structuring (as reflected by two new zones of genetic discontinuity) along the southeast Atlantic coast as well as identification of previously undetected shallow but significant structuring along the Florida Gulf coast. Regional groupings may warrant management unit recognition based on the patterns observed among multiple genetic metrics. The integration of this information with previously identified genetic variation and ecological data is essential to developing an ecologically and evolutionarily sound conservation management strategy.

Keywords *Limulus polyphemus* • American horseshoe crab • Microsatellite DNA • Genetic diversity • Differentiation • Genetic discontinuity • Management units

4.1 Introduction

The American horseshoe crab, *Limulus polyphemus*, is a resource of economic and biological significance along North America's Atlantic coast, particularly in the mid-Atlantic region of the United States (Shuster et al. 2003). *Limulus polyphemus* eggs are a primary food source for migratory shorebirds such as the western Atlantic red knot, *Calidris canutus rufa* (Baker et al. 2004), and other life stages serve as a food source for many species of finfish and the protected Atlantic loggerhead sea turtle (*Caretta caretta*). Horseshoe crabs are commercially harvested in large numbers as bait for American eel (*Anguilla rostrata*) and whelk (*Busycon* spp.) fisheries (Walls et al. 2002). In addition, *L. polyphemus* are harvested for non-destructive use in the production of *Limulus* amoebocyte lysate (LAL, Levin and Bang 1968), the standard reagent used to detect bacterial endotoxins in patients, injectable drugs, intravenous devices, and other medical applications.

As a direct result of perceived declines in the abundance of *L. polyphemus*, the Atlantic States Marine Fisheries Commission (ASMFC 1998) adopted a fishery management plan (FMP) for the species. The FMP (as amended) makes stock identification a principal research need in regional management of the species. Heritable genetic information offers an objective means of defining management units and provides an evolutionary framework from which to develop and evaluate conservation priorities (Ryder 1986; Moritz 1994). Molecular genetics occupies an important place in contemporary conservation biology as a robust tool for identifying fine-scale population structure, determining the degree of reproductive isolation among populations, and identifying the presence and extent of metapopulation structure (Hallerman 2003).

A range of molecular genetic techniques has been utilized in attempts to assess population structure in *L. polyphemus*. A survey of allozyme variation among four collections suggested that Atlantic Ocean and Gulf of Mexico populations of *L. polyphemus* were genetically differentiated (Selander et al. 1970). A subsequent study of

mitochondrial DNA (mtDNA) variation identified a major genetic discontinuity distinguishing northern from southern populations, with the phylogeographic break occurring around Cape Canaveral, Florida (Saunders et al. 1986). Pierce et al. (2000) reported little evidence of gene flow between Delaware and Chesapeake Bay *L. polyphemus* populations as reflected by sequence variation in the mtDNA COI region, although variation at randomly amplified polymorphic DNA (RAPD) markers was uniform, implying that gene flow may be sex-biased. Simple-sequence repeat loci, often termed microsatellites, have become the standard markers for identification of population structure due to their hypervariability and codominant expression (Ellegren 2004). These markers have provided previously unrealized utility at the individual level, including genetic tagging, assignment (or allocation) to population or collection of origin (Cornuet et al. 1999), assessment of relatedness (Brockmann et al. 1994, 2000), and demonstrations of sex-biased dispersal (Goudet et al. 2002). Most recently, King et al. (2005) surveyed selectively neutral genetic variation at 14 microsatellite DNA markers of 892 *L. polyphemus* sampled at 21 locations from northern Maine to the Yucatan Peninsula, Mexico. This extensive intraspecific examination of the nuclear genome revealed the presence of considerable allelic diversity and population structuring that appeared to reflect various evolutionary processes. Their findings suggested the presence of similar levels of genetic diversity and variation among the collections, punctuated by a series of genetic discontinuities of varying "depth" across the species' range that could indicate demographic independence, regional adaptation, and/or reflect vicariant geographic events. Gender-specific estimates of population differentiation (F'_{ST}) and assignment to collection of origin suggested the presence of male-biased dispersal throughout the study area by virtue of observing higher differentiation among females across populations than for males. Moreover, patterns of population relatedness were consistent with the observations that populations at both ends of the species' range are more differentiated from proximal populations than those in the middle, the zone of greatest abundance. Faurby et al. (2010) applied Bayesian coalescent-based methods to these microsatellite data to infer the historic demography of *L. polyphemus* populations. Their results showed strong declines in population sizes throughout the species' distribution except in the geographically isolated southernmost Mexico population, where a strong increase in population size was observed. Analyses suggested that demographic changes in the core of the distribution occurred within the last 150 years and thus were likely caused by anthropogenic effects including past overharvest of the species for fertilizer, and current use of the animals as bait and for biomedical testing (i.e., for production of *Limulus* amebocyte lysate or LAL). Declines of the peripheral northern and southern populations that occurred during the "Little Ice Age" are suggested to most likely have been climatically driven (Faurby et al. 2010).

A key short-term goal for horseshoe crab management is to sustain populations demographically, whereas the long-term goal is to conserve adaptively important genetic variation to maintain the species' evolutionary potential. The management process must begin with a definition of the biologically appropriate units of conservation. Against this background, we here expand on the previous surveys of microsatellite DNA variation among spawning aggregations (predictable gatherings of

adults with the specific purpose of reproducing) of *L. polyphemus* (King et al. 2005; Faurby et al. 2010) to identify previously undetected population, phylogeographic, and evolutionary relationships. We report on the screening of 13 polymorphic markers from 1,684 animals collected throughout the species' range from 33 spawning aggregations to estimate allelic diversity, characterize allelic patterns within and among collections, identify phylogeographic structure, and assess the demographic status of each collection. This research, which includes collections from 12 previously untested populations distributed from Connecticut to Florida's Gulf Coast (Table 4.1; Fig. 4.1), provides a refined view of the nature and extent of neutral (assumed) genetic variation in the southeastern portion of the *L. polyphemus* range. The integration of this information with previously identified allozyme (Selander et al. 1970), mitochondrial DNA (Saunders et al. 1986; Pierce et al. 2000), microsatellite DNA (King et al. 2005; Faurby et al. 2010), and ecological data is essential to developing an ecologically and evolutionarily sound conservation management strategy.

4.2 Methods

4.2.1 Sample Collections, DNA Extraction, and Microsatellite Genotyping

Limulus polyphemus were sampled in 32 spawning aggregations along the United States Atlantic coast from Franklin, Maine to St. Joseph Bay, Florida (Table 4.1; Fig. 4.1). A collection also was obtained from the north coast of the Yucatan Peninsula in the Ria Lagartos estuary, Mexico. Crabs were hand collected from shallow waters. Non-lethal somatic tissue samples were obtained by either clipping a 5 mm section from the terminus of a locomotive appendage or collecting a 100 µL sample of hemolymph via syringe. Solid samples were preserved in 95 % ethanol and stored at 4 °C; hemolymph was spotted onto two quadrants of an FTA card (Whatman, GE Health Care Life Sciences) and allowed to air-dry. After documenting sex and measuring prosomal width, each specimen was returned live to the location from which it was collected. Genomic DNA from somatic tissue was extracted with one of two extraction methods: the Puregene DNA extraction kit (Gentra Systems, Inc., Minneapolis, MN, used according to manufacturer's guidelines), or using glass milk according to the method outlined by Höss and Pääbo (1993). DNA isolated with the Puregene kit was resuspended in 100 µl of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA before use in PCR. The supernatant resulting from the glass milk extraction was not diluted and used directly in PCR. For hemolymph samples preserved on FTA cards, DNA was extracted from the card following the manufacturer's instructions.

Thirteen polymorphic microsatellite loci (King et al. 2005) were used for the present analysis. The PCR reaction mixtures consisted of 100–200 ng of genomic

Table 4.1 Abbreviation, general location, and sample size for 33 spawning collections of Atlantic horseshoe crabs *Limulus polyphemus* genotyped at 13 microsatellite DNA loci

Abbreviation	Spawning collection site	Sample size
MEH	Hog Bay, Franklin, Maine	47
MET	Thomas Point Beach, Maine	45
MEM	Middle Bay, Brunswick, Maine	48
NHS	Chadman's Landing, Squamscott River, New Hampshire	48
MAP	Pleasant Bay, Massachusetts	48
RIN	Green Island, Narragansett Bay, Rhode Island	48
CTH	***Housatonic River*, *Milford Point*, *Connecticut***	***48***
NYP	Great Peconic Bay, Long Island, New York	48
NJF	Fortescue Beach, New Jersey	48
NJR	Reeds Beach, New Jersey	48
NJH	Highs Beach, New Jersey	49
DKH	Kitts Hummock Beach, Delaware	36
DBS	Big Stone Beach, Delaware	31
DFB	Fowler Beach, Delaware	47
MDT	Turkey Point, Chesapeake Bay, Maryland	30
MDF	Flag Pond State Park, Chesapeake Bay, Maryland	29
MD5	***Ocean City*, *Maryland – 2005***	***48***
MD6	***Ocean City*, *Maryland – 2006***	***48***
VAC	***Chincoteague*, *Virginia***	***48***
NCS	Shackleford Banks, North Carolina	55
SBB	Bulls Bay, South Carolina	53
SBE	***Beaufort*, *South Carolina***	***48***
GSA	***Savannah*, *Georgia***	***48***
GSI	***Sapelo Island*, *Georgia***	***32***
FIR	Indian River, Florida (Atlantic coast)	46
FBB	***Biscayne Bay***	***20***
FMI	***Tiger Tail Beach*, *Marco Island*, *Florida* (*Gulf coast*)**	***81***
FCH	***Charlotte Harbor*, *Florida***	***51***
FTB	***Tampa Bay*, *Florida***	***141***
FCK	Seahorse Key, Cedar Keys NWR, Florida	132
FAP	***Alligator Point*, *Apalachicola Bay*, *Florida***	***92***
FSJ	St. Joseph Bay, Florida	23
MXY	Ria Lagartos and San Felipe, Yucatan, Republic of Mexico	20
	Total	1,684

This presentation expands on the previous surveys of microsatellite DNA variation among populations of *L. polyphemus* (King et al. 2005; Faurby et al. 2010) by including collections from 12 previously untested spawning aggregates identified here by ***bolded*** and *italicized* text

DNA, 1× PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 2 mM $MgCl_2$, 0.15 % Tween 20, 0.25 mM dNTPs, 0.5 μM forward and reverse primer, and 0.1 U *Taq* DNA polymerase (Promega, Madison, WI, USA) in a total volume of 10–20 μl.

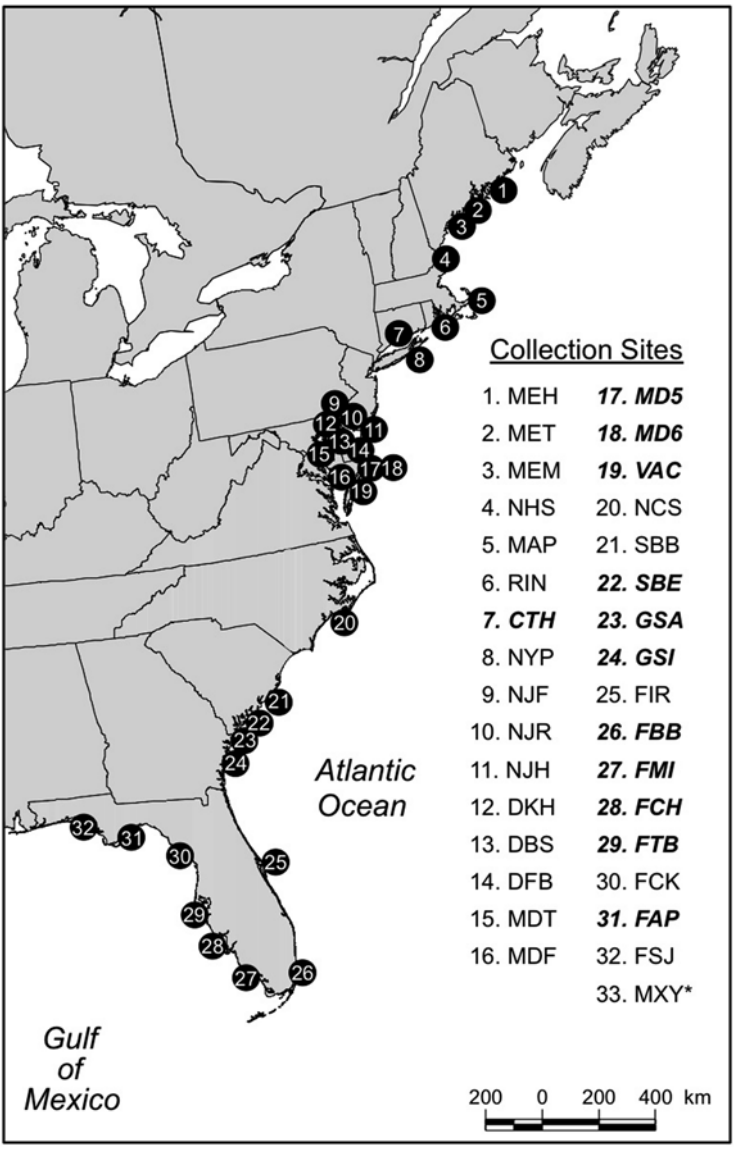

Fig. 4.1 Map showing general locations for 32 of 33 collection sites of *Limulus polyphemus* along the Atlantic and Gulf coasts of the United States. Collection names are provided in Table 4.1. The 12 new collections are identified by *bolded* and *italicized* text. The collection from a site on Mexico's Yucatan Peninsula is not depicted

Amplifications were carried out on either a PTC-200 or PTC-225 Thermal Cycler (MJ Research) using the following procedure: initial denaturing at 94 °C for 2 min; 35 cycles of 94 °C for 40 s, 58 °C for 40 s, 72 °C for 1 min; and a final extension at

72 °C for 5 min. Fragment electrophoresis and scoring were performed according to protocols described by King et al. (2005).

4.2.2 Statistical Analyses

4.2.2.1 Basic Population Genetic Analyses

Genetic diversity of all collections was quantified using GenAlEx (Peakall and Smouse 2006, 2012) to calculate allelic frequencies, number of alleles per locus (N_A), effective number of alleles (A_E), observed heterozygosity (H_O), unbiased expected heterozygosity (uH_E), and the average (across loci) inbreeding coefficient (F_{IS}). Observed genotype frequencies were tested for consistency with Hardy-Weinberg and linkage equilibrium expectations using randomization tests implemented by GENEPOP 4.3 (Raymond and Rousset 1995). The Hardy-Weinberg test used the Markov chain randomization test of Guo and Thompson (1992) to estimate exact two-tailed *p*-values for each locus in each sample. Global tests combined these results over loci and sampling locations using Fisher's method (Sokal and Rohlf 1994). Linkage disequilibrium tests used the randomization method of Raymond and Rousset (1995) for all pairs of loci. Sequential Bonferroni adjustments (Rice 1989) were used to determine statistical significance for these and all other multiple tests.

4.2.2.2 Family Structure Analysis

Unidentified family structure can be problematic for detection of hidden population structure using Bayesian clustering programs like STRUCTURE, as collections dominated by one or a few families can lead to the false interpretation of genetic differentiation or an entire population being out of Hardy-Weinberg equilibrium (Ramilo and Wang 2012). Therefore, to determine whether our collections consisted of a small number of families, we analyzed each collection for the presence of full-sibling families using the program COLONY v2.0 (Wang and Santure 2009). Settings for COLONY analyses included the assumption of male and female polygamy, no genotyping error information, no inbreeding, long run length with the full likelihood analysis method, high likelihood precision, no allele frequency updates, and no sibship prior for Bayesian analysis. Samples were analyzed as offspring without separation into candidate male and female genotypes and progeny, as these data were not available. Although the inference of family relationships is weakened in this situation with no sex, age, relationship information, and the assumption of polygamy for both sexes, COLONY is considered to be more accurate than pairwise estimates of relationships (Wang and Santure 2009). As a representation of the genetic effects of breeding structure, family size, and previous inbreeding, an estimate of the effective number of breeders, also known as the effective population size, N_e,

was estimated by sibship assignment using the COLONY full likelihood method assuming random mating.

4.2.2.3 Estimates of Population Differentiation (F'_{ST})

We calculated F'_{ST} in GenAlEx using the scaling approach of Meirmans (2006), which provides a true measure of allelic differentiation between populations where $F'_{ST}=1$ when populations share no alleles. Significance of all pairwise F'_{ST} comparisons was assessed through 9,999 permutations. We estimated the effective number of migrants (N_em) among collections using the private alleles method of Barton and Slatkin (1986) implemented in GENEPOP, instead of the F_{ST} -based estimate. These estimates of N_em are intended to approximate the relative magnitude of historical gene flow among collections.

4.2.2.4 Analysis of Molecular Variance

To describe differentiation among various regional assemblages of populations, we used a hierarchical AMOVA (Excoffier et al. 1992) implemented in GenAlEx to partition the genetic variance among collections relative to the total variance (F_{RT}), as well as among collections within embayments (F'_{ST}). Within regional groupings, the collections were further divided into various groups of collections to investigate whether there was significant partitioning of genetic variance among spawning assemblages. Significance of all hierarchical AMOVA analyses was assessed through 9,999 permutations.

4.2.2.5 Evolutionary Relationships Among Populations

Evolutionary relationships among the *L. polyphemus* collections were visualized by analysis of the pair-wise genetic distance matrices calculated using the Cavalli-Sforza and Edwards (1967) chord distance in BIOSYS (Swofford and Selander 1981). Principal coordinate analyses were used to graphically compare the pairwise chord distances without imposing the appearance of a bifurcating evolutionary history (ordinated with PAlaeontological STatistics ver. 2.17c, PAST; Hammer et al. 2001).

The Bayesian clustering program STRUCTURE ver 2.3.1 (Pritchard et al. 2000) was utilized to determine the number of populations (or clusters, K) present among the collections sampled. Unlike F'_{ST} estimates of population structure, STRUCTURE does not rely on a priori grouping of populations for inference. Because a single STRUCTURE analysis on a set of populations may only reveal the uppermost level of population structure (Evanno et al. 2005), we performed a hierarchical STRUCTURE analysis similar to that employed by King et al. (2006). In the initial phase, $K=1$ to $K=33$ clusters were considered for all collections pooled together

using 100,000 iterations discarded as burn-in followed by 200,000 iterations, and ten independent runs for each K using the admixture model and un-correlated allele frequencies. Subsequent analysis of each cluster tested $K=1$ to $K=C+3$ (the number of collections [C] included in the subset plus three) using the admixture and correlated allele frequencies model with the same number of burn-in and iterations. Sample location was incorporated as prior information, as populations separated by extreme distances presumably do not exchange migrants. The number of clusters for each analysis was determined using the ΔK method of Evanno et al. (2005) performed in the program Structure Harvester (Earl and vonHoldt 2011).

Isolation-by-distance as a mechanism to explain phylogeographic structuring was examined by correlating the matrices of genetic (D_C) and geographic distances (kilometers) between each pair-wise comparison. Geographic distance was estimated as the shortest ocean distance between collection sites. The statistical significance of the correlation between genetic and geographic distance matrices was assessed with a Mantel randomization test performed by the MXCOMP routine in NTSYS-PC 2.10 (Rohlf 2000).

4.2.2.6 Assignment Testing

Maximum likelihood assignment tests (after Paetkau et al. 1995) were used to determine the likelihood of each individual's multilocus genotype being found in the collection from which it was sampled (without replacement) using the program GeneClass II (Piry et al. 2004). In the event of null frequencies, a constant likelihood of 0.01 was assumed. Based on the results of population structure analyses (see below), we also assessed assignment success to various region of origin scenarios.

4.3 Results

4.3.1 Basic Population Genetic Parameters

Genotype data were collected at 13 microsatellite DNA loci for 1,684 *L. polyphemus* sampled from 33 locations encompassing the range of the species. A high degree of genetic diversity was detected; 251 alleles were observed across the 13 loci ranging from 11 at *Lpo*A37 and LpoA315 to 65 at *Lpo*D6. The mean number of alleles per locus was lowest at the extremes of the species' range (3.0, MEH and 5.5, MXY) and greatest in the collections from the Gulf coast of the U.S. (e.g., 16.0, FTB). Heterozygosity was lowest in animals collected at the extremes of the range (Table 4.2) and relatively uniform (average 76 %) for the remainder of the collections. Estimates of individual pair-wise genetic distances, using the proportion of shared alleles, indicated that levels of genetic diversity observed among the 13

Table 4.2 Summary genetic data for 33 Atlantic horseshoe crabs *Limulus polyphemus* collections from throughout their native range in the coastal USA and Mexico

Collection	N	N_a	A_E	uH_E	H_O	HWE	F_{IS}	Private alleles	SE private alleles	N_e	Lower CI_{95}	Upper CI_{95}	NFSF
MEH	47	3.00	1.93	0.42	0.40	0.05	0.06	0.00	0.00	22	14	42	3
MET	45	6.85	4.01	0.72	0.72	0.00	0.01	0.00	0.00	56	36	86	0
MEM	48	8.23	3.71	0.71	0.70	0.50	0.01	0.00	0.00	62	42	96	1
NHS	48	7.92	4.11	0.73	0.75	0.83	–0.04	0.00	0.00	50	33	79	0
MAP	48	8.92	4.77	0.77	0.75	***<0.001***	0.03	0.08	0.08	60	41	92	0
RIN	48	9.38	4.82	0.76	0.76	0.72	0.02	0.00	0.00	65	44	99	0
CTH	48	9.23	4.51	0.75	0.71	***<0.001***	0.05	0.15	0.10	56	38	87	0
NYP	48	9.31	4.90	0.76	0.72	0.55	0.04	0.00	0.00	64	43	101	1
NJF	48	9.15	4.94	0.77	0.73	0.24	0.05	0.00	0.00	69	46	105	0
NJR	48	9.23	4.70	0.76	0.74	0.17	0.03	0.00	0.00	62	41	96	0
NJH	49	9.15	4.53	0.75	0.72	0.35	0.04	0.00	0.00	71	49	107	1
DKH	36	8.46	4.40	0.75	0.72	0.05	0.04	0.00	0.00	68	42	121	1
DBS	31	8.46	4.67	0.76	0.74	0.22	0.03	0.08	0.08	53	33	90	0
DFB	47	9.31	4.82	0.75	0.71	0.08	0.05	0.00	0.00	66	45	102	2
MDT	30	7.77	4.24	0.72	0.70	0.15	0.01	0.00	0.00	40	24	70	1
MDF	29	8.31	4.66	0.75	0.76	0.06	0.00	0.00	0.00	54	34	94	0
MD5	48	9.46	4.97	0.76	0.73	0.20	0.04	0.00	0.00	65	44	101	0
MD6	48	8.62	4.78	0.76	0.74	0.01	0.06	0.00	0.00	65	44	100	2
VAC	48	9.15	4.94	0.77	0.74	0.01	0.05	0.00	0.00	54	37	87	0
NCS	55	10.00	5.16	0.78	0.80	0.86	-0.03	0.00	0.00	78	55	116	0
SBB	53	10.31	5.15	0.75	0.72	***<0.001***	0.07	0.08	0.08	82	56	124	1
SBE	48	9.62	5.13	0.75	0.73	0.17	–0.01	0.00	0.00	55	37	87	0

GSA	48	9.77	5.06	0.75	0.73	0.46	0.02	0.00	0.00	68	46	105	0
GSI	32	9.23	5.07	0.75	0.74	0.02	0.02	0.08	0.08	57	36	97	0
FIR	46	8.46	4.51	0.74	0.71	0.49	0.04	0.08	0.08	70	47	107	0
FBB	20	9.31	6.34	0.81	0.80	0.75	0.02	0.08	0.08	63	37	138	0
FMI	81	13.54	6.87	0.79	0.78	0.52	0.02	0.00	0.00	106	77	146	1
FCH	51	13.15	7.42	0.83	0.82	0.25	0.00	0.08	0.08	82	56	124	0
FTB	141	16.00	7.78	0.82	0.78	0.00	0.05	1.00	0.59	187	126	232	4
FCK	132	14.62	7.32	0.80	0.78	0.04	0.04	0.23	0.12	145	110	189	0
FAP	92	13.46	7.15	0.81	0.79	***<0.001***	0.01	0.08	0.08	112	82	152	0
FSJ	23	10.00	6.18	0.80	0.78	0.13	0.00	0.00	0.00	92	51	254	0
MXY	20	5.46	3.01	0.47	0.50	1.00	–0.07	0.08	0.08	38	21	83	0

Data include the sample size (N), mean number of alleles observed (*Na*), effective number of alleles (A_E), unbiased expected heterozygosity (uH_E), observed heterozygosity (H_O), the multi-locus *P*-value of conformance to Hardy-Weinberg equilibrium (HWE) determined using Fisher's method output by GENEPOP, the inbreeding coefficient (F_{IS}) output by GENEPOP averaged over loci, the mean and standard error of private alleles per population, the effective population size (with lower and upper 95 % confidence intervals) determined by COLONY, and the number of full-sibling families (NFSF) identified by COLONY. Values for the diversity measures are averaged over the 13 microsatellite DNA loci. Bold italicized values are those that remained significant after sequential Bonferroni correction

microsatellite loci were sufficient to produce unique multilocus genotypes (i.e., genetic distances > zero) for all animals surveyed.

Randomization tests showed that genotypes for most collections and most loci surveyed in this study were consistent with Hardy-Weinberg expectations. When *p*-values were combined over loci and analyzed for significance using Fisher's method, four collections deviated from HWE expectations (MAP, CTH, FAP, and SBB). Minimal linkage disequilibrium was observed as only 11 of 2,808 (0.4 %) comparisons among loci by population were found to be significant after correction for multiple tests (overall $\alpha = 0.00002$, $p \leq 0.0004$). In each instance, disequilibria involved different pairs of loci from different collections suggesting that the disequilibrium was likely a result of sampling error, null alleles, inbreeding, year-class mixing, population mixing, or a combination of the three rather than physical linkage among loci.

4.3.2 Demographics and Family Structure

American geneticist Sewall Wright (1931) defined effective population size (N_e) as "the number of breeding individuals in an idealized population that would show the same amount of dispersion of allele frequencies under random genetic drift or the same amount of inbreeding as the population under consideration." The effective population size is usually less than the census population size (N) as not every individual within a population spawns with equal success. In general, the genetic variability levels in this study paralleled the estimates of effective population size, N_e, which ranged from lows of 22 (MEH) and 38 (MXY), to a high of 187 (FTB). The average N_e over all collections was 71. COLONY analyses indicated that no single collection was dominated by a small number of large families, with the largest number of inferred full-sib relationships occurring in the MEH and FTB collections ($n = 3$ and $n = 4$ full-sibling families respectively). Estimates of the inbreeding coefficient F_{IS} also were low, ranging from −0.07 to 0.07.

4.3.3 Levels of Population Differentiation

The fixation index, F'_{ST}, is a measure of differentiation between populations. Pair-wise estimates of the fixation index, F'_{ST}, (Table 4.3; above diagonal) ranged from <0.01 (multiple comparisons within the Mid-Atlantic region from MAP to NCS) to 0.91 between the two collections from the extremes of the range (MEH and MXY). Of the 528 tests of significance in pair-wise F'_{ST} values, 431 (82 %) were significantly greater than zero ($p < 0.0005$; data not shown) indicating the presence of considerable population structure throughout the species' range. Inter-regional comparisons between collections comprising the five groups identified previously by King et al. (2005) were all statistically significant (Table 4.4).

Table 4.3 Pairwise matrix of F'_{ST} values (above diagonal) for collections of Atlantic horseshoe crabs, *Limulus polyphemus* (negatives converted to zero). Pairwise chord distances (Cavalli-Sforza and Edwards 1967) are provided below the diagonal

	MEH	MET	MEM	NHS	MAP	RIN	CTH	NYP	NJF	NJR	NJH	DKH	DBS	DFB	MDT	MDF	MD5	MD6
MEH	0.000	0.479	0.511	0.520	0.494	0.516	0.545	0.508	0.484	0.499	0.503	0.491	0.500	0.549	0.583	0.606	0.528	0.533
MET	0.506	0.000	0.021	0.113	0.079	0.076	0.126	0.086	0.077	0.091	0.084	0.062	0.086	0.148	0.146	0.164	0.097	0.123
MEM	0.512	0.184	0.000	0.090	0.109	0.132	0.176	0.155	0.136	0.163	0.150	0.108	0.122	0.216	0.189	0.219	0.156	0.182
NHS	0.521	0.227	0.221	0.000	0.087	0.096	0.132	0.133	0.113	0.130	0.088	0.117	0.075	0.180	0.161	0.146	0.133	0.134
MAP	0.516	0.243	0.251	0.259	0.000	0.005	0.013	0.016	0.000	0.013	0.017	0.015	0.003	0.087	0.052	0.037	0.026	0.028
RIN	0.527	0.251	0.268	0.253	0.196	0.000	0.009	0.011	0.002	0.013	0.018	0.020	0.003	0.081	0.059	0.043	0.000	0.017
CTH	0.543	0.266	0.281	0.270	0.192	0.172	0.000	0.012	0.005	0.017	0.014	0.033	0.010	0.096	0.026	0.016	0.018	0.016
NYP	0.533	0.263	0.277	0.269	0.204	0.193	0.189	0.000	0.000	0.000	0.000	0.000	0.002	0.056	0.036	0.010	0.007	0.018
NJF	0.519	0.246	0.260	0.255	0.170	0.168	0.166	0.194	0.000	0.000	0.000	0.000	0.000	0.062	0.045	0.016	0.008	0.018
NJR	0.520	0.241	0.266	0.266	0.178	0.182	0.182	0.168	0.164	0.000	0.000	0.000	0.003	0.070	0.045	0.018	0.011	0.025
NJH	0.520	0.228	0.259	0.236	0.173	0.173	0.178	0.181	0.170	0.173	0.000	0.009	0.000	0.068	0.038	0.012	0.017	0.031
DKH	0.516	0.243	0.248	0.247	0.204	0.203	0.197	0.188	0.184	0.191	0.193	0.000	0.011	0.091	0.042	0.044	0.011	0.029
DBS	0.527	0.273	0.287	0.273	0.214	0.205	0.212	0.218	0.198	0.207	0.206	0.217	0.000	0.060	0.032	0.013	0.012	0.026
DFB	0.546	0.275	0.308	0.291	0.217	0.210	0.227	0.213	0.217	0.215	0.200	0.242	0.227	0.000	0.149	0.092	0.069	0.095
MDT	0.555	0.290	0.312	0.307	0.226	0.243	0.232	0.236	0.233	0.224	0.227	0.246	0.249	0.283	0.000	0.000	0.047	0.069
MDF	0.567	0.314	0.326	0.306	0.233	0.221	0.217	0.219	0.206	0.212	0.222	0.241	0.236	0.247	0.207	0.000	0.042	0.048
MD5	0.525	0.257	0.285	0.273	0.190	0.171	0.179	0.194	0.178	0.174	0.170	0.209	0.217	0.207	0.225	0.225	0.000	0.010
MD6	0.524	0.245	0.265	0.253	0.180	0.176	0.184	0.177	0.190	0.167	0.174	0.183	0.210	0.202	0.238	0.236	0.181	0.000
VAC	0.545	0.245	0.260	0.261	0.185	0.203	0.201	0.224	0.190	0.196	0.189	0.210	0.216	0.236	0.253	0.247	0.207	0.208
NCS	0.544	0.259	0.271	0.263	0.173	0.190	0.189	0.174	0.175	0.184	0.175	0.202	0.214	0.213	0.232	0.236	0.183	0.169
SBB	0.560	0.310	0.319	0.304	0.235	0.224	0.228	0.226	0.224	0.235	0.218	0.237	0.261	0.266	0.267	0.246	0.218	0.239
SBE	0.578	0.302	0.318	0.299	0.251	0.233	0.227	0.229	0.232	0.243	0.223	0.249	0.262	0.267	0.272	0.248	0.231	0.234
GSA	0.574	0.303	0.323	0.310	0.247	0.238	0.232	0.237	0.224	0.239	0.232	0.253	0.268	0.263	0.280	0.252	0.231	0.244
GSI	0.554	0.314	0.324	0.309	0.256	0.256	0.251	0.243	0.246	0.257	0.253	0.257	0.263	0.285	0.280	0.264	0.255	0.255
FIR	0.600	0.479	0.467	0.477	0.423	0.393	0.413	0.408	0.412	0.411	0.419	0.422	0.413	0.428	0.450	0.417	0.400	0.412
FBB	0.631	0.486	0.459	0.461	0.431	0.416	0.430	0.435	0.437	0.437	0.438	0.433	0.448	0.445	0.476	0.450	0.425	0.431
FMI	0.636	0.484	0.461	0.459	0.432	0.411	0.421	0.422	0.425	0.432	0.430	0.437	0.440	0.432	0.475	0.449	0.412	0.431
FCH	0.645	0.490	0.468	0.465	0.437	0.425	0.427	0.431	0.436	0.435	0.434	0.438	0.446	0.436	0.474	0.451	0.425	0.435
FTB	0.638	0.481	0.455	0.451	0.431	0.415	0.421	0.420	0.425	0.426	0.426	0.432	0.436	0.433	0.469	0.440	0.417	0.430
FCK	0.659	0.489	0.464	0.463	0.427	0.417	0.424	0.421	0.427	0.431	0.428	0.440	0.439	0.434	0.471	0.443	0.422	0.435
FAP	0.666	0.493	0.469	0.464	0.437	0.426	0.432	0.430	0.437	0.441	0.435	0.448	0.444	0.442	0.479	0.453	0.434	0.446
FSJ	0.675	0.504	0.484	0.477	0.434	0.420	0.419	0.432	0.427	0.438	0.432	0.446	0.442	0.437	0.479	0.447	0.434	0.441
MXY	0.791	0.679	0.683	0.676	0.664	0.656	0.667	0.684	0.666	0.676	0.665	0.677	0.662	0.647	0.689	0.673	0.656	0.676

	VAC	NCS	SBB	SBE	GSA	GSI	FIR	FBB	FMI	FCH	FTB	FCK	FAP	FSJ	MXY
MEH	0.582	0.533	0.542	0.595	0.566	0.527	0.617	0.676	0.682	0.710	0.683	0.708	0.710	0.744	0.913
MET	0.150	0.109	0.197	0.190	0.172	0.140	0.389	0.349	0.405	0.405	0.385	0.404	0.386	0.412	0.746
MEM	0.195	0.156	0.246	0.239	0.240	0.185	0.406	0.344	0.406	0.405	0.391	0.395	0.375	0.414	0.741
NHS	0.183	0.107	0.188	0.187	0.191	0.133	0.409	0.364	0.435	0.421	0.412	0.410	0.393	0.403	0.758
MAP	0.067	0.002	0.084	0.085	0.070	0.044	0.321	0.294	0.354	0.357	0.338	0.339	0.331	0.315	0.763
RIN	0.054	0.012	0.080	0.057	0.073	0.052	0.297	0.268	0.326	0.333	0.307	0.316	0.305	0.308	0.739
CTH	0.065	0.017	0.075	0.048	0.062	0.065	0.331	0.342	0.378	0.377	0.357	0.368	0.352	0.326	0.771
NYP	0.092	0.000	0.058	0.062	0.050	0.049	0.304	0.318	0.343	0.352	0.322	0.334	0.330	0.316	0.776
NJF	0.076	0.000	0.070	0.079	0.059	0.047	0.319	0.301	0.349	0.355	0.335	0.346	0.342	0.317	0.765
NJR	0.095	0.001	0.069	0.087	0.065	0.069	0.331	0.315	0.358	0.357	0.327	0.338	0.341	0.318	0.770
NJH	0.095	0.004	0.063	0.061	0.054	0.062	0.327	0.327	0.378	0.369	0.349	0.361	0.348	0.332	0.773
DKH	0.092	0.019	0.088	0.107	0.099	0.093	0.337	0.310	0.362	0.359	0.343	0.361	0.351	0.349	0.783
DBS	0.077	0.004	0.079	0.081	0.075	0.053	0.306	0.300	0.358	0.359	0.347	0.356	0.336	0.343	0.755
DFB	0.147	0.077	0.150	0.138	0.128	0.127	0.366	0.351	0.377	0.370	0.371	0.369	0.367	0.345	0.714
MDT	0.112	0.043	0.108	0.092	0.097	0.122	0.365	0.397	0.446	0.437	0.425	0.430	0.412	0.403	0.783
MDF	0.109	0.019	0.060	0.060	0.050	0.065	0.336	0.364	0.414	0.413	0.395	0.396	0.392	0.365	0.771
MD5	0.033	0.016	0.081	0.058	0.070	0.070	0.306	0.278	0.307	0.298	0.293	0.311	0.301	0.319	0.753
MD6	0.037	0.025	0.110	0.083	0.097	0.090	0.314	0.323	0.370	0.378	0.354	0.368	0.365	0.352	0.784
VAC	0.000	0.075	0.148	0.100	0.123	0.134	0.363	0.325	0.352	0.345	0.339	0.356	0.337	0.358	0.776
NCS	0.193	0.000	0.045	0.057	0.038	0.034	0.311	0.282	0.339	0.332	0.318	0.315	0.311	0.273	0.751
SBB	0.245	0.204	0.000	0.019	0.003	0.006	0.313	0.329	0.389	0.392	0.380	0.372	0.366	0.317	0.764
SBE	0.257	0.207	0.158	0.000	0.018	0.038	0.310	0.329	0.366	0.363	0.351	0.346	0.329	0.291	0.726
GSA	0.248	0.202	0.180	0.170	0.000	0.008	0.341	0.332	0.388	0.393	0.378	0.373	0.366	0.315	0.770
GSI	0.280	0.232	0.206	0.213	0.229	0.000	0.298	0.304	0.368	0.378	0.357	0.347	0.344	0.303	0.740
FIR	0.425	0.411	0.388	0.391	0.401	0.409	0.000	0.223	0.339	0.361	0.351	0.349	0.360	0.332	0.687
FBB	0.422	0.420	0.419	0.421	0.417	0.439	0.381	0.000	0.065	0.083	0.080	0.104	0.098	0.162	0.663
FMI	0.424	0.409	0.421	0.416	0.415	0.439	0.426	0.276	0.000	0.009	0.011	0.045	0.049	0.152	0.691
FCH	0.427	0.415	0.421	0.425	0.420	0.443	0.433	0.290	0.166	0.000	0.018	0.053	0.037	0.169	0.692
FTB	0.420	0.407	0.417	0.414	0.417	0.434	0.428	0.280	0.151	0.161	0.000	0.030	0.040	0.142	0.695
FCK	0.418	0.409	0.416	0.408	0.412	0.432	0.426	0.309	0.197	0.203	0.183	0.000	0.015	0.059	0.649
FAP	0.427	0.415	0.420	0.412	0.420	0.439	0.444	0.305	0.205	0.204	0.184	0.154	0.000	0.092	0.663
FSJ	0.426	0.410	0.415	0.397	0.410	0.432	0.432	0.369	0.323	0.330	0.314	0.258	0.272	0.000	0.630
MXY	0.655	0.651	0.656	0.647	0.661	0.658	0.643	0.633	0.621	0.619	0.625	0.593	0.612	0.578	0.000

Table 4.4 Pairwise matrix of F_{ST} values (below diagonal) for collections of Atlantic horseshoe crabs, *Limulus polyphemus* (negative values were converted to zero)

	MEH	MET	MEM	NHS	MAP	RIN	CTH	NYP	NJF	NJR	NJH	DKH	DBS	DFB	MDT	MDF	MD5	MD6
MEH	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
MET	0.206	0.000	0.022	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
MEM	0.222	0.006	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
NHS	0.220	0.031	0.025	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
MAP	0.198	0.020	0.028	0.021	0.000	0.269	0.094	0.066	0.461	0.089	0.064	0.095	0.371	0.001	0.001	0.013	0.013	0.007
RIN	0.208	0.019	0.034	0.024	0.001	0.000	0.163	0.116	0.357	0.089	0.048	0.049	0.368	0.001	0.001	0.011	0.464	0.074
CTH	0.222	0.032	0.046	0.033	0.003	0.002	0.000	0.111	0.267	0.052	0.064	0.007	0.206	0.001	0.024	0.107	0.038	0.063
NYP	0.208	0.022	0.041	0.034	0.004	0.003	0.003	0.000	0.454	0.455	0.454	0.451	0.414	0.001	0.011	0.193	0.181	0.045
NJF	0.196	0.020	0.035	0.028	0.000	0.001	0.001	0.000	0.000	0.469	0.435	0.435	0.474	0.001	0.001	0.091	0.199	0.050
NJR	0.204	0.023	0.043	0.033	0.003	0.003	0.004	0.000	0.000	0.000	0.459	0.435	0.371	0.001	0.003	0.083	0.138	0.010
NJH	0.207	0.022	0.040	0.023	0.004	0.004	0.003	0.000	0.000	0.000	0.000	0.180	0.460	0.001	0.006	0.140	0.053	0.006
DKH	0.206	0.016	0.029	0.030	0.003	0.005	0.008	0.000	0.000	0.000	0.002	0.000	0.182	0.001	0.008	0.005	0.163	0.019
DBS	0.211	0.022	0.032	0.019	0.001	0.001	0.002	0.000	0.000	0.001	0.000	0.003	0.000	0.002	0.039	0.166	0.145	0.038
DFB	0.224	0.038	0.057	0.045	0.020	0.019	0.023	0.013	0.015	0.017	0.016	0.022	0.014	0.000	0.001	0.001	0.001	0.001
MDT	0.256	0.040	0.053	0.043	0.013	0.015	0.006	0.009	0.011	0.011	0.010	0.011	0.008	0.037	0.000	0.474	0.004	0.001
MDF	0.262	0.044	0.060	0.038	0.009	0.010	0.004	0.002	0.004	0.004	0.003	0.011	0.003	0.022	0.000	0.000	0.006	0.006
MD5	0.209	0.024	0.039	0.032	0.006	0.000	0.004	0.002	0.002	0.002	0.004	0.003	0.003	0.015	0.011	0.010	0.000	0.132
MD6	0.214	0.031	0.047	0.033	0.006	0.004	0.004	0.004	0.004	0.006	0.007	0.007	0.006	0.022	0.017	0.011	0.002	0.000
VAC	0.225	0.036	0.047	0.043	0.014	0.012	0.014	0.020	0.016	0.021	0.021	0.020	0.016	0.032	0.026	0.024	0.007	0.008
NCS	0.210	0.027	0.040	0.026	0.000	0.003	0.004	0.000	0.000	0.000	0.001	0.004	0.001	0.018	0.010	0.005	0.004	0.006
SBB	0.223	0.053	0.067	0.049	0.020	0.019	0.018	0.014	0.017	0.017	0.016	0.022	0.019	0.037	0.028	0.015	0.019	0.026
SBE	0.243	0.049	0.063	0.048	0.020	0.013	0.011	0.015	0.018	0.021	0.015	0.026	0.019	0.033	0.023	0.015	0.013	0.019
GSA	0.233	0.045	0.064	0.049	0.016	0.017	0.015	0.012	0.014	0.016	0.013	0.024	0.018	0.031	0.025	0.012	0.016	0.023
GSI	0.225	0.037	0.050	0.035	0.010	0.012	0.016	0.012	0.011	0.017	0.015	0.023	0.013	0.031	0.032	0.016	0.016	0.021
FIR	0.258	0.104	0.111	0.108	0.077	0.072	0.081	0.076	0.078	0.082	0.082	0.084	0.076	0.090	0.096	0.086	0.072	0.075
FBB	0.282	0.083	0.084	0.084	0.061	0.056	0.073	0.069	0.063	0.068	0.072	0.067	0.064	0.075	0.091	0.080	0.056	0.067
FMI	0.247	0.093	0.095	0.097	0.072	0.067	0.079	0.072	0.072	0.075	0.081	0.076	0.074	0.079	0.099	0.089	0.061	0.075
FCH	0.259	0.088	0.090	0.090	0.068	0.064	0.074	0.070	0.069	0.071	0.075	0.071	0.070	0.073	0.092	0.083	0.055	0.072
FTB	0.234	0.086	0.089	0.090	0.067	0.062	0.073	0.067	0.068	0.067	0.073	0.071	0.070	0.076	0.092	0.083	0.057	0.071
FCK	0.246	0.091	0.091	0.091	0.068	0.064	0.076	0.070	0.071	0.070	0.076	0.075	0.073	0.076	0.095	0.084	0.061	0.074
FAP	0.252	0.087	0.086	0.087	0.066	0.062	0.072	0.069	0.069	0.071	0.073	0.073	0.069	0.075	0.090	0.083	0.059	0.073
FSJ	0.311	0.100	0.103	0.096	0.067	0.066	0.071	0.070	0.068	0.070	0.075	0.077	0.075	0.076	0.095	0.083	0.066	0.075
MXY	0.511	0.287	0.288	0.286	0.270	0.263	0.277	0.281	0.272	0.278	0.282	0.289	0.279	0.257	0.304	0.294	0.262	0.277

	VAC	NCS	SBB	SBE	GSA	GSI	FIR	FBB	FMI	FCH	FTB	FCK	FAP	FSJ	MXY
MEH	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
MET	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
MEM	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
NHS	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
MAP	0.001	0.380	0.001	0.001	0.001	0.003	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
RIN	0.001	0.096	0.001	0.001	0.001	0.003	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
CTH	0.001	0.043	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
NYP	0.001	0.452	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
NJF	0.001	0.472	0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
NJR	0.001	0.386	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
NJH	0.001	0.276	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
DKH	0.001	0.036	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
DBS	0.001	0.329	0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
DFB	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
MDT	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
MDF	0.001	0.064	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
MD5	0.006	0.052	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
MD6	0.004	0.014	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
VAC	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
NCS	0.016	0.000	0.001	0.001	0.002	0.008	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
SBB	0.033	0.011	0.000	0.024	0.314	0.239	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
SBE	0.022	0.013	0.005	0.000	0.047	0.003	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
GSA	0.027	0.009	0.001	0.004	0.000	0.213	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
GSI	0.030	0.008	0.002	0.009	0.002	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
FIR	0.082	0.074	0.080	0.077	0.086	0.075	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
FBB	0.062	0.058	0.074	0.071	0.073	0.066	0.050	0.000	0.004	0.002	0.001	0.001	0.001	0.001	0.001
FMI	0.067	0.069	0.084	0.077	0.083	0.078	0.074	0.012	0.000	0.138	0.024	0.001	0.001	0.001	0.001
FCH	0.061	0.063	0.081	0.072	0.079	0.076	0.075	0.014	0.001	0.000	0.015	0.001	0.001	0.001	0.001
FTB	0.064	0.063	0.081	0.072	0.079	0.074	0.075	0.015	0.002	0.003	0.000	0.001	0.001	0.001	0.001
FCK	0.067	0.063	0.080	0.072	0.079	0.073	0.075	0.019	0.008	0.009	0.005	0.000	0.005	0.001	0.001
FAP	0.063	0.062	0.078	0.068	0.077	0.072	0.077	0.018	0.009	0.006	0.007	0.003	0.000	0.001	0.001
FSJ	0.071	0.058	0.073	0.064	0.071	0.068	0.076	0.031	0.029	0.030	0.027	0.011	0.017	0.000	0.001
MXY	0.262	0.262	0.281	0.262	0.281	0.278	0.255	0.237	0.222	0.219	0.216	0.203	0.209	0.227	0.000

Probability of value being greater than zero is provided above the diagonal

Most of the non-significant F'_{ST} values were observed among intra-regional comparisons. Of the 97 non-significant values, 72 were observed in comparisons among collections from the Mid-Atlantic region from Massachusetts to Maryland. Both the MEH and MXY collections from the extremes of the distribution were highly differentiated from all other collections with pair-wise F'_{ST} values averaging 0.57 and 0.74, respectively.

4.3.4 Evolutionary Relationships Among Populations

4.3.4.1 Genetic Distance

Pair-wise genetic distance (D_C) values between all pairs of collections (Table 4.3, below diagonal) were consistent with F'_{ST} estimates as the greatest distances were observed between the collections representing the extremes of the range (0.791 between MEH and MXY) and lowest among populations within regions (0.184 between MEM and MET; 0.164 between NJR and NJF; 0.158 between SBB and SBE; 0.151 between FMI and FTB). Initial principal coordinates analyses (PCoA) of genetic distances showed that the geographically extreme collections surveyed in this study (MEH and MXY) exhibited large genetic distances that distorted the overall structure, and these data were eliminated from further graphical comparisons. In a PCoA ordination of the 31 remaining collections, seven distinct clusters (regional groupings) of collections were apparent (Fig. 4.2): (1) MET, MEM, and NHS (hereafter referred to as Gulf of Maine); (2) MAP to NCS (Mid-Atlantic); (3) SBB to GSI (Southeast Atlantic); (4) FIR (Florida East); (5) FBB (Florida South); (6) FMI to FAP (Florida Gulf of Mexico); and (7) FSJ. At the broadest scale, clustering corresponded to the geographical distribution of the collections, with greater distances separating the southernmost regional collections from the northern collections; at this scale there appeared little correspondence to geographic distribution within the groupings. Upon closer inspection of the collections within each grouping by subsequent PCoA, additional zones of genetic discontinuity were visible along the coasts of the Atlantic Ocean (Fig. 4.3) and Gulf of Mexico (Fig. 4.4) coasts. Along the Atlantic coast (Fig. 4.3), a moderate to high degree of relatedness (or recent gene flow; Table 4.5) was inferred among collections within the Gulf of Maine, from Cape Cod, Massachusetts (MAP) to the outer banks of North Carolina (NCS), and among the collections from South Carolina and Georgia. Within the Mid-Atlantic grouping, a slightly higher degree of relatedness was observed among collections within Delaware Bay than among those with immediate access to the Atlantic coast. In contrast, the two collections from the Chesapeake Bay (MDT, MDF) were moderately differentiated from the other Mid-Atlantic collections. As with the range-wide PCoA, the greatest zones of genetic discontinuity within the study area occurred to the north (GSI) and south (FBB) of the Indian River, FL (FIR) collection (Figs. 4.3 and 4.4).

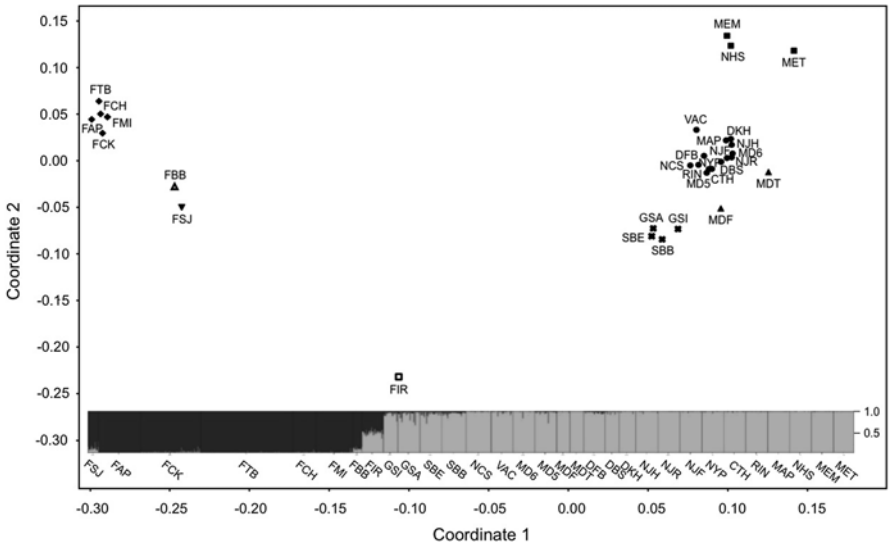

Fig. 4.2 Combined graphical representation of principal coordinates (scatter plot of pairwise chord distance, Cavalli-Sforza and Edwards 1967) and STRUCTURE (histogram) analyses among 1,617 *Limulus polyphemus* sampled from 31 locations along the Atlantic and Gulf coasts of the United States (collections from Hog Bay, Franklin, ME and Mexico's Yucatan Peninsula were eliminated as outliers) surveyed at 13 microsatellite DNA loci. For the STRUCTURE histograms, each individual is represented by a *single vertical bar*, broken into $K=2$ colored segments, the length of which is proportional to the membership fraction in each of the K clusters. *Black lines* partition the collections

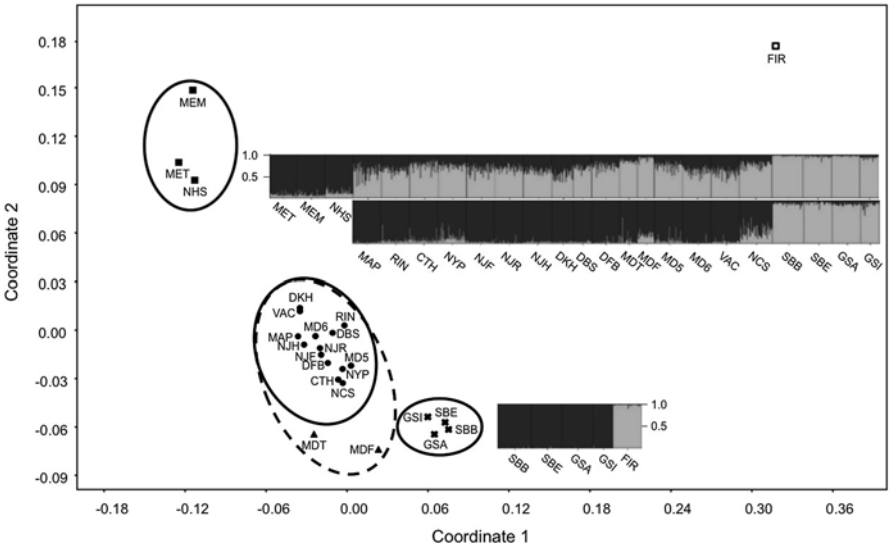

Fig. 4.3 Combined graphical representation of principal coordinates (scatter plot of pairwise chord distance, Cavalli-Sforza and Edwards 1967) and STRUCTURE (histogram) analyses among *Limulus polyphemus* sampled from 24 locations along the Atlantic coast of the United States (collections from Hog Bay, Franklin, ME and Biscayne Bay, FL eliminated) surveyed at 13 microsatellite DNA loci. Ellipses enclose groups of similar collections. For the STRUCTURE histograms, each individual is represented by a *single vertical bar*, broken into K colored segments, the length of which is proportional to the membership fraction in each of the K clusters. *Black lines* partition the collections

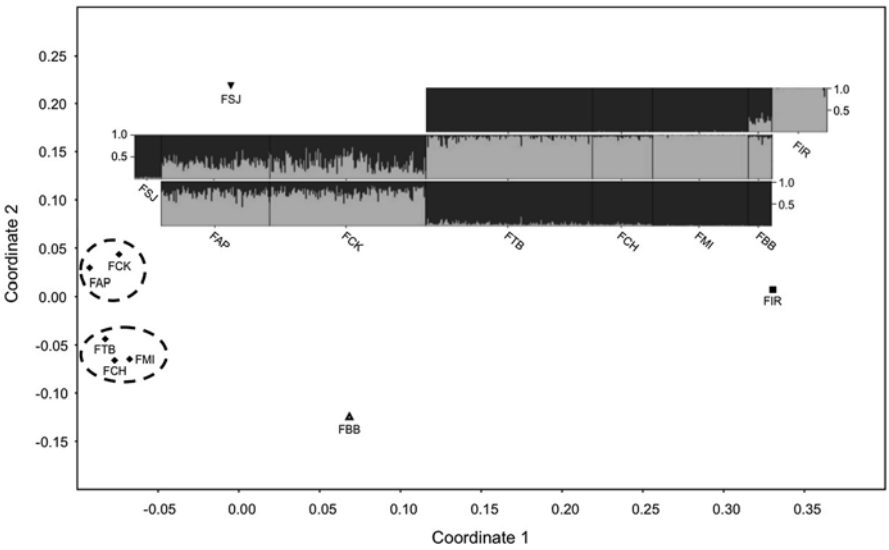

Fig. 4.4 Combined graphical representation of principal coordinates (scatter plot of pairwise chord distance, Cavalli-Sforza and Edwards 1967) and STRUCTURE (histogram) analyses among *Limulus polyphemus* sampled from eight locations along the Florida Atlantic and Gulf coasts of the United States surveyed at 13 microsatellite DNA loci. *Ellipses* enclose groups of similar collections and *boxes* highlight single collections. For the STRUCTURE histograms, each individual is represented by a *single vertical bar*, broken into *K* colored segments, the length of which is proportional to the membership fraction in each of the *K* clusters. *Black lines* partition the collections

In addition to the deep zone of discontinuity that exists to the north and south of Indian River, FL, a regional PCoA of the Florida coast identified shallow but significant differentiation along the central portion of the Gulf coast that was not detectable in the range-wide analysis (Fig. 4.4). This brings to four the number of genetically discontinuous zones detected within this state: (1) FIR (Florida East) and FBB (Florida South); (2) FBB and FMI (Florida Southwest; (3) Florida Southwest and FCK (Florida West); and 4) Florida West and the collection from St. Joseph Bay (FSJ).

4.3.4.2 Population Structure

Population structure can be inferred from the results of the individual-based STRUCTURE analyses among 33 *L. polyphemus* collections, which were congruent with the PCoA (Figs. 4.2, 4.3, and 4.4). The number of inferred clusters (*K*) determined by STRUCTURE for the initial (uppermost hierarchical level) analysis was two, corresponding to the Atlantic coast collections north of Indian River, Florida (FIR) and collections south of FIR around to St. Joseph Bay, Florida the western most collection of the Gulf of Mexico included in the analysis (Fig. 4.2). Due to the

Table 4.5 Pair-wise estimates of the effective number of migrants per generation, N_em, among 31 collections of Atlantic horseshoe crabs *Limulus polyphemus* sampled from throughout their native range in the coastal USA and Mexico

	MEH	MET	MEM	NHS	MAP	RIN	CTH	NYP	NJF	NJR	NJH	DKH	DBS	DFB	MDT	MDF	MD5	MD6
MET	0.5																	
MEM	0.6	9.9																
NHS	0.5	6.6	6.5															
MAP	0.8	4.7	4.9	4.8														
RIN	0.8	4.3	3.5	3.5	6.1													
CTH	0.7	5.0	6.3	4.6	6.0	7.7												
NYP	0.8	5.8	6.4	4.7	6.7	7.2	7.5											
NJF	0.8	4.2	5.7	4.0	6.0	6.7	8.6	6.9										
NJR	0.8	7.3	7.0	4.6	8.5	7.9	7.5	7.8	9.5									
NJH	0.8	9.1	6.3	5.1	8.2	8.2	8.9	8.1	8.5	9.1								
DKH	0.7	4.8	7.2	5.6	6.9	8.5	9.7	8.2	8.2	7.5	10.7							
DBS	0.8	4.1	4.4	3.9	5.4	9.1	7.1	6.8	5.8	6.5	7.3	5.4						
DFB	0.8	3.2	2.4	2.6	8.4	4.2	7.0	7.7	3.5	7.2	8.3	7.9	4.7					
MDT	0.7	3.1	3.4	4.4	6.8	6.4	5.3	5.7	5.6	6.3	8.0	4.5	4.3	3.5				
MDF	0.6	2.5	3.2	3.1	4.2	5.6	6.0	6.1	5.9	5.8	5.7	4.6	4.4	3.0	4.0			
MD5	0.9	5.4	5.2	5.4	5.8	10.4	10.8	7.8	8.5	12.2	9.9	8.9	7.8	4.6	5.5	5.4		
MD6	0.8	4.7	3.9	2.9	8.0	8.1	7.4	7.4	6.1	9.3	8.6	8.6	8.1	7.6	3.8	3.8	6.9	
VAC	0.8	4.7	6.5	4.5	9.2	7.0	7.5	6.9	7.4	10.2	10.6	8.3	5.3	5.9	5.4	5.2	7.9	5.8
NCS	0.7	5.5	6.1	3.9	10.0	7.3	7.8	8.8	8.3	10.5	10.5	7.3	7.5	8.4	6.5	5.2	9.0	9.9
SBB	0.7	3.8	4.0	3.8	6.6	5.5	7.5	7.1	6.6	5.8	7.5	8.4	5.9	3.5	6.5	6.4	8.9	5.2
SBE	0.6	3.4	4.7	3.5	3.8	5.6	6.6	5.6	6.4	6.0	4.8	7.1	6.2	3.6	3.3	3.8	7.1	7.4
GSA	0.7	3.2	3.8	3.3	4.3	4.4	6.6	5.8	5.2	5.4	6.1	5.2	4.0	4.0	4.6	4.2	7.9	4.8
GSI	0.8	2.9	3.6	3.6	4.6	5.6	5.2	5.8	4.4	5.1	6.5	5.2	5.0	3.7	3.4	4.4	6.9	5.8
FIR	0.6	0.9	1.0	0.9	1.8	2.3	1.6	1.9	1.4	1.9	1.5	1.8	1.9	1.7	1.6	1.5	1.9	1.6
FBB	0.5	0.9	1.2	1.1	1.0	1.5	1.4	1.5	1.0	1.3	1.4	1.6	1.2	1.1	1.4	1.3	1.7	1.2
FMI	0.7	1.2	1.8	1.8	1.7	2.1	2.2	2.2	1.4	1.7	1.9	2.3	1.6	1.8	1.7	2.0	2.1	2.0
FCH	0.8	1.4	1.8	1.9	1.6	1.9	2.0	2.2	1.4	1.6	1.9	2.5	1.8	1.9	1.8	2.1	2.2	2.1
FTB	0.5	0.9	1.3	1.2	1.1	1.4	1.4	1.4	1.0	1.2	1.3	1.4	1.1	1.1	1.2	1.3	1.4	1.2
FCK	0.6	1.0	1.2	1.3	1.4	1.7	1.9	1.8	1.3	1.4	1.9	1.7	1.3	1.6	1.5	1.7	1.7	1.6
FAP	0.5	1.1	1.3	1.5	1.3	1.5	1.5	1.7	1.1	1.3	1.8	1.7	1.4	1.5	1.4	1.5	1.4	1.5
FSJ	0.4	0.8	1.1	1.1	1.3	1.5	1.5	1.5	1.1	1.5	1.7	1.7	1.3	1.4	1.5	1.5	1.3	1.5
MXY	0.1	0.2	0.3	0.3	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.5	0.5	0.4	0.4	0.4	0.3	0.3

	VAC	NCS	SBB	SBE	GSA	GSI	FIR	FBB	FMI	FCH	FTB	FCK	FAP	FSJ
MET														
MEM														
NHS														
MAP														
RIN														
CTH														
NYP														
NJF														
NJR														
NJH														
DKH														
DBS														
DFB														
MDT														
MDF														
MD5														
MD6														
VAC														
NCS	9.5													
SBB	6.0	7.0												
SBE	4.5	6.3	12.0											
GSA	6.0	6.8	9.9	9.3										
GSI	3.7	4.7	7.2	6.6	5.7									
FIR	1.6	1.7	2.1	1.6	1.4	2.0								
FBB	1.2	1.3	1.5	1.2	1.1	1.4	1.4							
FMI	1.7	2.1	1.9	2.1	2.0	1.9	1.8	5.3						
FCH	1.7	2.0	2.0	2.0	1.9	2.0	2.2	5.6	9.4					
FTB	1.2	1.3	1.6	1.4	1.2	1.3	1.5	3.9	13.0	11.1				
FCK	1.5	1.7	2.0	1.9	1.5	1.5	1.7	3.6	7.9	7.4	9.3			
FAP	1.3	1.5	1.8	1.6	1.5	1.4	1.5	3.1	8.6	6.2	11.1	8.4		
FSJ	1.2	1.8	2.4	1.9	1.9	1.7	2.4	2.4	4.3	3.8	3.4	8.6	4.1	
MXY	0.5	0.4	0.4	0.4	0.4	0.5	0.4	0.8	0.9	1.0	0.6	0.7	0.8	1.0

apparent complex migration patterns (zones of genetic discontinuity) existing among the two regions, a sequential method of inferring the number of clusters was employed to identify within-cluster structure. Sequential STRUCTURE analysis of the Atlantic coast collections (MEH to GSI) indicated $K=2$ delineating the Gulf of Maine collections from the Mid-Atlantic and Southeast Atlantic (SBB to GSI) collections (Fig. 4.3). Upon removal of the Gulf of Maine collections, analysis of the remaining collections again identified $K=2$ clusters discriminating between the Mid-Atlantic and Southeast Atlantic collections. Including the highly differentiated Florida East (FIR) collection this brings to four the number of distinct clusters of *L. polyphemus* populations identified along the Atlantic coast from Maine to central Florida (Figs. 4.3, 4.4, and 4.5). A relatively high degree of relatedness was observed among the *L. polyphemus* collections grouped within the identified clusters.

Multiple sequential STRUCTURE analyses of all eight Florida collections (FIR to FSJ) were consistent with the patterns observed for genetic distance in the PCoA ordination (Fig. 4.4). Three analyses were performed with each resulting in $K=2$ clusters. The Indian River (FIR) and St. Joseph Bay (FSJ) collections were identified as two distinct clusters highly differentiated from adjacent populations and from each other. These analyses resulted in a total of four clusters (excluding FIR) bringing the number of total clusters to eight. However, the level of differentiation among the Florida South (FBB), Florida Southwest (FMI to FTB), and Florida West (FCK to FAP) clusters did not appear as great as that observed between the St. Joseph Bay, FL (FSJ) collection and the other Gulf of Mexico collections or as seen among the Atlantic coast clusters.

4.3.4.3 Patterns of Genetic Variation: A Test for Isolation by Distance

A Mantel matrix regression test identified a statistically significant correlation ($r=0.74$; $t=10.3$; $p<0.0001$) between pair-wise matrices of ocean (km) and genetic (chord) distances among 31 *L. polyphemus* collections (excluding the MEH and MXY collections; Fig. 4.6). This graphical representation of the matrix comparison illustrates the deep genetic discontinuity that exists along the Florida Atlantic coast around the Indian River (FIR) collection. All pair-wise comparisons with the FIR collection (denoted by ■) exhibited large genetic distances (>0.35) relative to geographic distance. Most other pair-wise comparisons (denoted by ○) exhibited an isolation-by-distance pattern in which the comparisons with collections south and west of FIR were separated by a distributional gap that appears to correspond with a zone of deep genetic discontinuity.

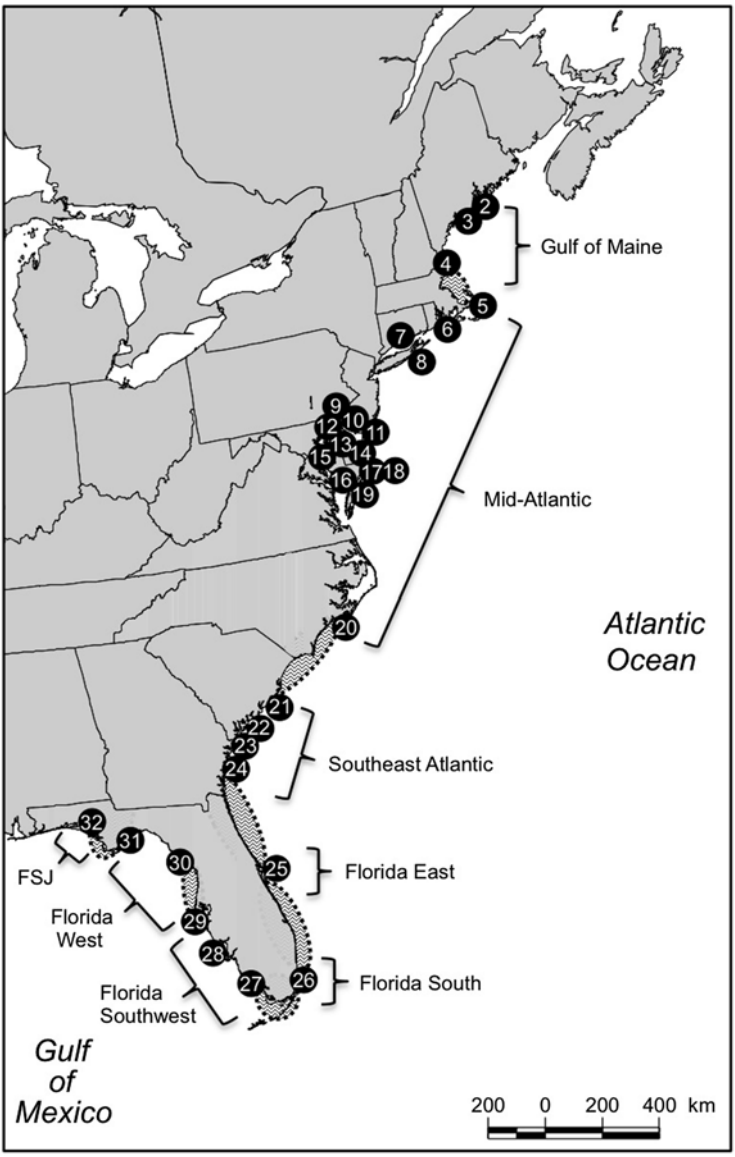

Fig. 4.5 Map showing locations of 32 of 33 collection sites of *Limulus polyphemus* along the Atlantic and Gulf coasts of the United States with *hashed* regions (▨) demarking zones of genetic discontinuity and areas in need of additional sampling. Collections within each delineated area could be considered management units. The collections representing the extremes of the species' range from Hog Bay, Maine and Mexico's Yucatan Peninsula (not depicted) are not shown

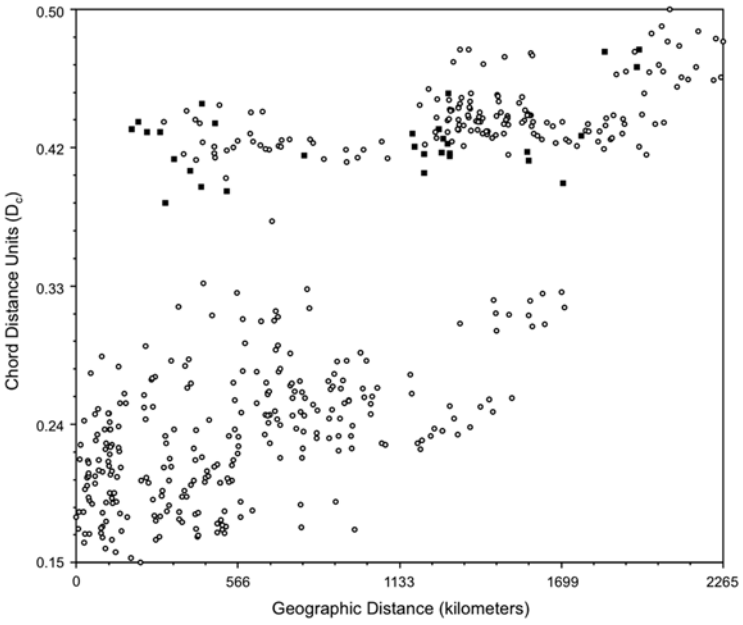

Fig. 4.6 Scatterplot illustrating the significant correlation between geographic distance (coastal distance measured in kilometers) and genetic distance (chord distance; Cavalli-Sforza and Edwards 1967) for 31 *Limulus polyphemus* collections (Hog Bay, Maine, and the Yucatan Peninsula were omitted; Mantel test $r = 0.74$; $t = 10.3$; $P < 0.0001$). Pairwise comparisons with the highly differentiated Indian River, Florida (FIR) collection are denoted by the filled square (■)

4.3.4.4 Assignment Testing

Maximum likelihood assignment tests also revealed structuring of genotypic frequencies in *L. polyphemus*. Individual animals were correctly assigned to collection of origin 25.1 % of the time, on average, across collections, and all assignments to collection of origin were higher than expected by chance ($p < 0.001$). When the *L. polyphemus* collections were pooled into eight regional groups including the MEH and MXY collections, 1,399/1,684 (83.1 %) of the specimens were correctly classified to region of origin (Table 4.6). Of the 285 misclassified animals, 263 (92.2 %) were assigned incorrectly to the adjacent regional grouping. The MEH and MXY collections exhibited 100 % correct assignment, and no animals were incorrectly assigned to either collection. Removal of the MEH and MXY collections from this analysis reduced the correct assignment rate slightly to 82.4 %. Attempts to take into consideration shallower levels of differentiation resulted in lower assignment success (Table 4.6). Splitting the Florida Gulf coast collections FMI to FSJ into FMI-FTB and FCK-FSJ groupings resulted in correct assignment of 75.2 % of *L. polyphemus*. At a finer level of resolution the FCK to FSJ collections were split into FCK-FAP and FSJ, which resulted in correct assignment of 73.2 %.

Table 4.6 Results of maximum likelihood assignment tests for all *Limulus polyphemus* collections using multilocus genotypes derived from 13 microsatellite DNA markers

Region	N	MEH	Gulf of Maine	Mid-Atlantic	SE	FL-east	FL-south	FL-gulf	MXY	% Correct assign
MEH	47	**47**	0	0	0	0	0	0	0	**100.0**
Gulf of Maine	141	0	**114**	23	4	0	0	0	0	**80.9**
Mid-Atlantic	709	0	66	**534**	103	4	1	1	0	**75.3**
Southeast	181	0	5	34	**140**	0	1	1	0	**77.4**
FL-east	46	0	0	1	0	**44**	0	1	0	**95.7**
FL-south	20	0	0	0	0	0	**9**	11	0	**45.0**
FL-gulf	520	0	0	0	3	0	26	**491**	0	**94.4**
MXY	20	0	0	0	0	0	0	0	**20**	**100.0**
Eight Regions: (MEH) (MET-NHS) (MAP-NCS) (SBB-GSI) (FIR) (FBB) (FMI-FSJ) (MXY) – 83.1 %										
Six Regions: (MET-NHS) (MAP-NCS) (SBB-GSI) (FIR) (FBB) (FMI-FSJ) – 82.4 %										
Seven Regions: (MET-NHS) (MAP-NCS) (SBB-GSI) (FIR) (FBB) (FMI-FTB) (FCK-FSJ) – 75.2 %										
Eight Regions: (MET-NHS) (MAP-NCS) (SBB-GSI) (FIR) (FBB) (FMI-FTB) (FCK-FAP) (FSJ) – 73.2 %										

Values along the diagonal are the number of correct assignments to that regional grouping. Regions consist of eight proposed groupings delineated by zones of genetic discontinuity observed in analyses of phylogeographic structuring: Hog Bay, Maine (MEH); Gulf of Maine (MET-NHS); Mid-Atlantic (MAP-NCS); Southeast (SBB-GSI); Florida-East (FIR); Florida-South (FBB); Florida-Gulf (FMI-FSJ); and Yucatan Peninsula, Mexico (MXY). Percent correct classification for six, seven, and eight (without extreme collections) region models are also provided

4.3.4.5 Partitioning of Molecular Variance

When *L. polyphemus* collections were grouped into regions according to ordination of genetic distances and clusters inferred from STRUCTURE analysis, quantitative estimates of hierarchical gene diversity indicated significant genetic population structure at every level, the greatest amount owing to variation within collections; a pattern commonly seen in intraspecific comparisons utilizing microsatellite DNA markers. Grouping all collections into eight regions (six as described above and two collections from the extremes of the species' range) revealed that 8 % ($p<0.0001$) of the genetic variation was distributed among regions, 1.0 % ($p<0.0001$) among collections within regions, and 91 % ($p<0.0001$) within collections (Table 4.7A). Elimination of the northernmost and southernmost collections from this analysis reduced the amount of variation among regions to 6 % ($p<0.001$) and increased that

Table 4.7 Analysis of molecular variance among 33 *Limulus polyphemus* collections from (A) Maine to the Yucatán Peninsula pooled into eight regional groupings (including the regional extreme populations MEH and MXY); (B) 31 collections excluding the two regional extremes (Hog Bay, Franklin, Maine and Ria Lagartos, Yucatan Peninsula, Mexico) pooled into six regional groupings; (C) the same collections with the Florida Gulf of Mexico collections split between Tampa Bay (FTB) and Cedar Key (FCK); and (D) the St. Josephs Bay (FSJ) collection split from the other northern Gulf of Mexico collections Each variance component and fixation index (F'_{ST}) was significantly greater than zero at $\alpha=(0.05)$ ($P<0.001$)

Source	*Df*	SS	Variance	% Variance	F'_{ST}
A. Eight Regions: (MEH) (MET-NHS) (MAP-NCS) (SBB-GSI) (FIR) (FBB) (FMI-FSJ) (MXY)					
Among regions	7	1074.6	0.43	8 %	0.33
Among pops. within regions	25	229.7	0.04	1 %	0.04
Within pops	3335	16592.8	4.98	91 %	
Total	3367	17897.1	5.44	100 %	
B. Six Regions: (MET-NHS) (MAP-NCS) (SBB-GSI) (FIR) (FBB) (FMI-FSJ)					
Among regions	5	748.7	0.32	6 %	0.27
Among pops. within regions	25	229.7	0.04	1 %	0.04
Within pops	3203	16219.1	5.06	93 %	
Total	3233	17197.4	5.42	100 %	
C. Seven Regions: (MET-NHS) (MAP-NCS) (SBB-GSI) (FIR) (FBB) (FMI-FTB) (FCK-FSJ)					
Among regions	6	772.9	0.30	6 %	0.26
Among pops. within regions	24	205.4	0.04	<1 %	0.03
Within pops	3203	16219.1	5.06	94 %	
Total	3233	17197.4	5.40	100 %	
D. Eight Regions: (MET-NHS) (MAP-NCS) (SBB-GSI) (FIR) (FBB) (FMI-FTB) (FCK-FAP) (FSJ)					
Among regions	7	784.1	0.30	6 %	0.26
Among pops. within regions	23	194.3	0.03	<1 %	0.03
Within pops	3203	16219.1	5.06	94 %	
Total	3233	17197.4	5.40	100 %	

within collections to 93 % ($p<0.001$) (Table 4.7B). Other groupings based on shallower levels of differentiation (Table 4.7C, D) failed to increase the amount of variation among regions. The low level of variation observed among collections within the regions (1.0 %) supports the appropriateness of these groupings in all models.

4.4 Discussion

The pattern of genetic variation observed in this study is consistent with that identified previously in surveys of morphological and genetic variation (Shuster 1979; Riska 1981; King et al. 2005), suggesting a series of discontinuities across the species' range that could reflect regional adaptive significance and/or vicariant geographic events (e.g., a change in sea level giving rise to the Florida peninsula). Regional groupings (Fig. 4.5) that were consistent across analyses warrant management unit status based on the presence of statistically significant allele frequency heterogeneity, allocation of genetic diversity (Table 4.6), and a high percentage of correct classification to region of origin (Table 4.7). There appears to be substantial gene flow between each population and its nearest neighbors, with some notable exceptions (discussed below). Moreover, genetic diversity was sufficiently high at these nDNA markers that each individual surveyed possessed a unique multilocus genotype. This allowed assessment of family structure and estimates of effective population sizes.

American horseshoe crabs, *L. polyphemus*, often are considered to be evolutionarily static due to their morphological similarity to mid-Mesozoic taxa and have been referred to as phylogenetic relics (Selander et al. 1970). However, the presence of considerable variability and geographic differentiation in both morphology (Shuster 1979; Riska 1981) and genetic diversity illustrate that this species is far from evolutionarily static. Genetic distances estimated in the present study suggest the presence of eight or more regional management units of *L. polyphemus* within the United States, at a minimum: Hog Bay, Maine, Gulf of Maine, Mid-Atlantic, Southeast Atlantic, Florida East, Florida South, Florida Southwest, Florida West, St. Joseph Bay, Florida. This regional genetic structuring conforms generally to patterns of geographic variation in morphometric characters reported by Shuster (1979) and the findings of Riska (1981). Furthermore, the molecular genetic characteristics lend support to the suggestion of Shuster (1979) that there may be physiological races within the species.

Genetic distance analyses indicate clustering at the regional level (Figs. 4.2, 4.3, and 4.4) that conforms to the estimates of gene flow between each population and its nearest neighbors within groupings. Notable exceptions are the Hog Bay, Maine (MEH), Indian River, Florida (FIR), St. Joseph Bay, Florida (FSJ), and Yucatan Peninsula Mexico (MXY) collections (Table 4.5). The MEH collection is both the northernmost sampling location and a body of water that is almost completely isolated from the Gulf of Maine by strong currents that form a retention zone, trapping *L. polyphemus* in the bay and effectively eliminating migration/gene flow in or out

of the bay. If it were to be shown that movement of *L. polyphemus* into Hog Bay from other bays does occur, then one would have to invoke some strong selective force to prevent successful reproduction of immigrants. A more plausible explanation is that geographic isolation combined with small population (and founder) size and inbreeding has afforded stochastic processes (such as random genetic drift) the opportunity to dramatically reduce the level of genetic diversity and heterozygosity in MEH.

Along the Florida coasts, both Indian River Lagoon (FIR) and St. Joseph Bay (SJB) are physically isolated populations that have recently undergone major population declines. Indian River Lagoon is a 156-mile estuary that lies behind barrier islands along the east coast of Florida. Exchange of lagoon water with the Atlantic Ocean is limited to one tidal inlet at the north end, and four tidal inlets in the southern part of the estuary act to geographically isolate FIR *L. polyphemus* populations to the north and south. In addition, the environment of the Indian River Lagoon is quite different from that of other coastal areas in the southeast: salinity is highly variable and depends on distance from the inlets and the magnitude of freshwater inputs (e.g. groundwater, rivers) (Ehlinger and Tankersley 2004), and most of the lagoon is microtidal (tidal fluctuations occur only near the inlets; Ehlinger and Tankersley 2009). American horseshoe crabs in the Indian River Lagoon are also thought to be in decline (Provancha 1997) due to disease (Scheidt and Lowers 2001), entrapment in power plant intakes (Ehlinger and Tankersley 2007) and shoreline development. Genetic differences between Indian River horseshoe crabs and populations to the north or south are likely due to their physical isolation, recent population declines and to the unique environment of the lagoon to which they are adapted.

St. Joseph Bay is a nearly enclosed, 24 km long by 10 km wide bay that is open to the Gulf of Mexico only at its north end. In 1999 bait harvesters removed nearly 100,000 adult horseshoe crabs from this bay (Gerhart 2007). Moreover, the FSJ collection consisted of the smallest sample size along the Florida Gulf coast. Therefore, the genetic differences between FSJ and other Gulf coast populations may be due to sampling error, geographic isolation and/or demographic stochasticity associated with bottleneck effects.

Interestingly, reduced genetic variation and high levels of differentiation also were observed in the collection from the southern extreme of the range (MXY; Yucatan Peninsula, Republic of Mexico). *Limulus polyphemus* has a disjunct distribution in the Gulf of Mexico. Horseshoe crabs are found in the Florida Keys and along the west coast of Florida to the Mississippi and Louisiana barrier islands (Fulford and Haehn 2012), but are not generally found elsewhere in the Gulf of Mexico until one reaches the west (Campeche) and northern (Yucatan) coasts of the Yucatan Peninsula (Shuster 1979; Gomez-Aguirre 1993; Zaldivar-Rae et al. 2009) where they nest on beaches that are not unlike those found in Florida. The MXY collection was from a large population that occupies an extensive estuarine and coastal area along the northern coast of the Yucatan. Geographic isolation from other *L. polyphemus* populations combined with the absence of larval migration between proximal bay systems (reported by Botton and Loveland 2003),

affords the possibility that founder effect, inbreeding, and genetic drift likely have played important roles in this population's genetic diversity. Indeed, the level of genetic differentiation between southeastern U.S. and Yucatan Peninsula *L. polyphemus* combined with the observations of discrete populations based on morphometric differentiation (Shuster 1979) may warrant further investigations regarding taxonomic revision.

Several shallow and moderate zones of genetic discontinuity are inferred from all analyses conducted, separating the: (1) Gulf of Maine (MET-NHS) from Mid-Atlantic (MAP-NCS); (2) Mid-Atlantic from Southeast Atlantic (SBB-GSI); (3) Southeast Atlantic from Florida East (FIR); (4) Florida East from Florida South (FBB); (5) Florida South from Florida Southwest (FMI-FTB); (6) Florida Southwest from Florida West (FCK-FAP); (7) Florida West from St. Joseph Bay, Florida (FSJ); and (8) St. Joseph Bay from Yucatan Peninsula, Mexico (MXY). Moderate (or narrower) zones of genetic discontinuity are evident between: (a) the Gulf of Maine and Mid-Atlantic collections; (b) Mid-Atlantic and Southeast collections; and (c) Atlantic and Gulf of Mexico Florida collections. This phylogeographic pattern implies that there are at least eight demographically distinct assemblages across the species' range that are relevant to conservation considerations. In addition to support for recognition of these zones of discontinuity, the observation of genetic differentiation suggested low levels of gene exchange between collections on either side of these genetic discontinuities. The presence of demographically distinct and evolutionary significant lineages delineated by zones of genetic discontinuity is consistent with the findings of researchers assessing behavioral patterns and large differences observed by Graham et al. (2009) in body size within spawning aggregates sampled along the Northeastern Atlantic coast. Integration of the information from the nuclear genome with previously identified variation in allozymes (Selander et al. 1970) and mitochondrial DNA (Saunders et al. 1986; Pierce et al. 2000), and with ecological data should prove essential to developing an ecologically and evolutionarily sound management strategy.

The correlation between genetic distance and geographic distance among collections supports isolation-by-distance as a mechanism underlying the population structure of *L. polyphemus* along the Atlantic coast of North America (Fig. 4.6). Even within the continuum of isolation-by-distance, and providing for gene flow between neighboring collections, there still are regions that are discrete with respect to each other, as indicated by clustering of specific populations using the PCoA and STRUCTURE results (Figs. 4.2, 4.3, and 4.4). For example, the NH and ME collections (minus MEH) form a discrete Gulf of Maine population unit, the southern New England collections (MAP, from the Atlantic side of Cape Cod, MA, and RIN, Rhode Island) group with the collections from CT, NY, NJ, DE, MD, and NC to form a large mid-Atlantic cluster, the SC and GA collections cluster, and all but one Florida collections (FIR) form a cluster. In addition, the collection from the north coast of Mexico's Yucatan Peninsula is more differentiated from the Florida collections than the Florida collections are from the more northerly populations. This

population is, however, still within the bounds of the isolation-by-distance model developed for the U.S. populations.

The percentage of genetic variance among populations (7 %) and/or regions (6–8 %) bodes well for using these molecular data for stock identification and mixed-stock analysis. Furthermore, the high probability (83.1 %) of correctly assigning individuals to the management unit from which they were collected indicated by multilocus assignment tests confirms that these data will be useful for identifying the source of *L. polyphemus* that are intercepted as by-catch in commercial fisheries. Of the 285 crabs mis-assigned to management units other than their collection of origin (16.9 % error rate), only 22 were mis-assigned to management units that were not adjacent to their true source (1.3 % of misclassifications). This finding adds further support to the isolation-by-distance model for explaining the observed patterns of genetic variation.

Pair-wise estimates of F'_{ST}, hierarchical gene diversity, and PCoA results suggest relative homogeneity among collections within each of the regions (proposed management units) defined above. Most pair-wise F'_{ST} values that were not significantly different from zero were between collections within the same proposed management unit. Hierarchical gene diversity analysis shows only 1 % of the total genetic variation being found among collections within proposed management units, as opposed to 6 % of genetic variation being found among these regional units (that figure increases to 8 % when the MEH and MXY collections are included; Table 4.4).

Given the large latitudinal range (19–45° North) encompassed by the extant distribution of *L. polyphemus*, it is instructive to investigate the extent to which Pleistocene ice ages and the associated palaeoclimatic oscillations may have influenced observed patterns of allelic diversity. The zone of discontinuity observed between *L. polyphemus* collections surveyed north and south of Cape Cod, MA (~41.7° north latitude) corresponds roughly to the median latitude of maximum Pleistocene glacial advance in North America and has been observed to be a breakpoint between nucleotide diversity and latitudinal distribution of freshwater and anadromous fish species (Bernatchez and Wilson 1998), with nucleotide diversity decreasing with increasing latitude. However, *L. polyphemus*, an estuarine species that exhibits anadromous tendencies (Shuster 1979), showed no general trend of reduced allelic diversity with increasing latitude in the present study. We do not assert that recent glacial and interglacial periods have not impacted *L. polyphemus* in this portion of the species' range, only that any effects appear to have been ameliorated by natural and anthropogenic gene flow, sufficient available habitat, and/or demographic robustness sufficient to have prevented the loss of allelic diversity through stochastic processes. Hence, the zone of discontinuity that exists in this area may simply reflect that Cape Cod is a zoogeographic barrier that influences oceanic and near-shore currents in such a manner that ultimately results in restricted gene flow among *L. polyphemus* populations.

Although a modicum of caution should be exercised when drawing phylogeographic inferences from microsatellite DNA variation (due to the potential for homoplasy in this class of marker), patterns observed in the present study are consistent with the well-documented intraspecific phylogeographic relationships

observed among a host of marine taxa in the southeastern U.S. zoogeographic province (Avise 1992), including previous findings for *L. polyphemus* (Selander et al. 1970; Saunders et al. 1986). Microsatellite DNA variation in *L. polyphemus* reveals clear genetic separation of Atlantic and Gulf of Mexico collections, suggesting that the Florida Peninsula has served as a barrier to gene exchange. However, as stated above, physical isolation of the FIR and FBB collections from other Florida populations cannot be excluded as a cause for the observed levels of differentiation in this area. Additional sampling along the Atlantic coastline to south Florida will be required to better understand the population structure of this region (Fig. 4.5).

4.5 Management Implications

Management units (MUs) are defined as populations that are demographically independent of one another (Allendorf and Luikart 2007), meaning that their population dynamics depend mostly on local birth and death rates, and not on genetically effective migration from other spawning assemblages. Identification of MUs – similar in concept to "stocks" widely referred to in fisheries management – is useful for short-term management, such as delineating fishing areas, setting harvest rates, and monitoring population status. Offering an operational definition, Moritz (1994) suggested that MUs are populations that have substantially divergent allele frequencies at many loci. One possible limitation of this approach, however, is that allele frequency differentiation cannot be interpreted directly as evidence for demographic independence (Allendorf and Luikart 2007). A related issue is the difficulty in determining whether migration from nearby spawning assemblages would be sufficient to reestablish an MU should it become overharvested or extinct. Palsbøll et al. (2007) proposed that the identification of MUs from population genetic data be based upon the amount of genetic divergence at which populations become demographically independent; wherein MU status would be assigned when the observed estimate of genetic divergence is significantly greater than a pre-defined threshold value. To illustrate the application of the MU concept, Ramstad et al. (2004) analyzed approximately 100 sockeye salmon from 11 spawning sites throughout the Lake Clark drainage of the Bristol Bay system in Alaska at 11 microsatellite DNA loci to determine whether the spawning assemblages were demographically isolated. The effective population size, N_e, for each of the Lake Clark spawning sites was ~1,000. Using the criterion of at least 10 % exchange (Hastings 1993), groups spawning at these sites would be demographically isolated if they exchanged fewer than about 100 adults, which corresponds to genetic differentiation (F_{ST}) of 0.0025 under a classical Wright-Fisher island model of migration-drift equilibrium. Therefore, one might conclude that these spawning sites constitute separate MUs if their genetic divergence, F_{ST}, exceeds 0.0025. The overall value of F'_{ST} among these sites excluding one outlier was ~0.007 (95 % CI of 0.004–0.010), which was greater than the threshold of 0.0025. The authors concluded that these 11

spawning aggregations were demographically isolated and should be considered separate MUs.

Considering the current data for American horseshoe crabs, it seems clear that there exist multiple MUs, corresponding to the proposed regional management areas. In addition to the demographically discrete lineages identified for American horseshoe crabs, a series of metapopulations and other individual collections delineated within each discrete lineage may be considered distinct management/recovery units for future management planning purposes. Furthermore, metapopulations may exist in the Northern Atlantic (Gulf of Maine and New Hampshire collections), the Mid-Atlantic region (with some substructure within), the upper Chesapeake Bay collections (MDT, MDF), the Southeast Atlantic (SBB, SBE, GSA, and GSI), southwest Florida Gulf of Mexico (FMI, FCH, FTB), and the northwest Florida Gulf of Mexico (FCK, FAP). Within areas bounded by zones of genetic discontinuity, there appears to be substantial gene flow between each population and its nearest neighbors; the presence of these metapopulations appears to bode well for the demographic viability of horseshoe crabs in some regions.

Given the restriction in female-mediated gene flow observed by King et al. (2005) and that of Pierce et al. (2000), and the absence of larval migration reported by Botton and Loveland (2003), the appreciable amount of gene flow we have documented along North America's Atlantic coast is most likely to be the result of effective migration by sub-adult or adult males. These findings suggest that management efforts may best be targeted at local populations rather than at a regional level, as an absence of an effective number of females may result in local functional extinctions. In the most extreme cases, it may become necessary to institute harvest limitations on female *L. polyphemus* for populations known to have low census sizes. The patterns of genetic differentiation observed in this study also suggest that relocations from adjacent embayments could serve as a source for future restoration efforts.

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