Journal of Shellfish Research, Vol. 28, No. 4, 855–864, 2009.

POPULATION GENETICS OF THE EASTERN OYSTER *CRASSOSTREA VIRGINICA* (GMELIN, 1791) IN THE GULF OF MEXICO

ROBIN L. VARNEY,[1] CLARA E. GALINDO-SÁNCHEZ,[2,3] PEDRO CRUZ[3] AND PATRICK M. GAFFNEY[1]*

[1]*College of Earth, Ocean, and Environment, University of Delaware, Lewes, DE 19958;*
[2]*IFREMER, UMR M100 PE2M, Centre de Brest B.P. 70, 29280 Plouzané FRANCE;*
[3]*Laboratorio de Genética Acuícola, Centro de Investigaciones Biológicas del Noroeste (CIBNOR), A.P. 128 La Paz, Baja California Sur 23000 México*

ABSTRACT Genetic variation in eastern oysters (*Crassostrea virginica*) collected from 13 sites in the Gulf of Mexico was examined using a combination of mitochondrial DNA (mtDNA) sequencing, mtDNA restriction fragment length polymorphism analysis, and nuclear single nucleotide polymorphism analysis. Both mitochondrial and nuclear markers showed significant differentiation among samples. Combined with previous allozyme and microsatellite data, these results indicate considerable population subdivision throughout the Gulf of Mexico, despite the potentially homogenizing effect of larval dispersal.

KEY WORDS: *Crassostrea virginica*, oyster, genetics, single nucleotide polymorphism, mitochondrial DNA, Gulf of Mexico

INTRODUCTION

The eastern oyster *Crassostrea virginica* (Gmelin, 1791) occupies coastal and estuarine habitats from maritime Canada to subtropical Mexico, spanning a wide range of physical and biotic conditions. For more than half a century, biologists have asked whether locally or regionally adapted subpopulations might exist despite the possibility of extensive gene flow mediated by planktonic larval dispersal (Stauber 1950). Early surveys of allozyme polymorphisms suggested minimal genetic differentiation across the range of the species, with the exception of oysters from Nova Scotia and the Laguna Madre of southern Texas (Buroker 1983, Gaffney 1996). Reanalysis of Buroker's data by Cunningham & Collins (1994) revealed a split between populations from the Atlantic and the Gulf of Mexico, with the genetic break in northwest Florida. The Gulf–Atlantic split was underscored by restriction fragment length polymorphism (RFLP) analysis of mitochondrial DNA (mtDNA), which showed a striking divide between reciprocally monophyletic Gulf and Atlantic haplotype assemblages, although the genetic break occurred on the east coast of Florida rather than in the Gulf of Mexico (Reeb & Avise 1990).

Although evidence has been collected for population subdivision in the Atlantic (Wakefield & Gaffney 1996, Hoover & Gaffney 2005, Varney & Gaffney 2008), less attention has been given to oyster populations in the Gulf of Mexico. Here we report the genetic analysis of eastern oyster populations from throughout the region, using a combination of mitochondrial and nuclear DNA markers.

MATERIALS AND METHODS

Sample Collection and Preparation

Oysters were collected from 12 sites in the Gulf of Mexico (Table 1). Tissue snips were stored in 70–90% ethanol at room temperature prior to DNA extraction. DNA was extracted from adductor muscle or gill tissue using a Qiagen DNeasy extraction kit (Qiagen, Inc., Valencia, CA), following the extraction protocol for animal tissues. mtDNA extracts from an additional collection (Brownsville, TX) were provided by Dr. John Avise and were used only for mtDNA analyses.

*Corresponding author. E-mail: pgaffney@udel.edu

Mitochondrial DNA

Sequence variation was examined by direct sequencing or RFLP analysis of 5 mitochondrial fragments (Table 2). Polymerase chain reaction (PCR) conditions consisted of an initial denaturation of 2 min at 94°C, followed by 35 cycles of denaturing at 94°C for 45 sec, annealing at 55°C for 60 sec, extension at 72°C for 90 sec, and a final extension of 5 min at 72°C for all reactions, except that an annealing temperature of 60°C was used to generate the ND2-ND4 amplicon. For the 3 smaller amplicons, direct sequencing was performed on a SpectruMedix SCE2410 capillary sequencer (Transgenomic, Omaha, NE) using standard dye-terminator chemistry (ABI Big Dye 3.1) and the original PCR primers as sequencing primers. Sequence variation in the 2 larger amplicons was evaluated by RFLP analysis using restriction enzymes previously found to provide readily scored polymorphisms. Digests were run on 2–3% agarose gels and stained with ethidium bromide for visualization of DNA bands. Haplotypes were inferred from the pattern of DNA bands resulting from enzyme digestion.

Nuclear DNA

Primers were designed to amplify 10 nuclear regions from genomic DNA: activinlike type 1 receptor (ALR), arginine kinase (AK), chitinase (CH), cofilin (COF), elongation factor 1α (EF1α), filamin (FIL), γ-aminobutyric acid (GABA) receptor-associated protein (GABA), Ran protein (RAN), ribosomal protein L27 (RP), and thymosin beta (Thyβ; Table 2). All were based on reference sequences available in GenBank, except for CH (derived from a random *C. virginica* genomic clone; Gaffney, unpublished) and FIL (based on *C. gigas* EST contig FP000131.P.CG.5 obtained from SIGENAE AquaFirst (http://public-contigbrowser.sigenae.org:9090/Crassostrea_gigas/index.html).

To identify candidate single nucleotide polymorphisms (SNPs) for genotyping, we sequenced 17–20 individuals for

TABLE 1.

***Crassostrea virginica* collection sites in the Gulf of Mexico.**

Locality	CODE	Latitude	Longitude	*n*
1. Cedar Key, FL	CK	29.14	–83.04	15
2. Apalachicola, FL	AP	29.75	–85.03	24
3. Grand Isle, LA	LA	29.23	–89.99	14
4. Galveston Bay, TX	GV	29.29	–94.84	40
5. Port Aransas, TX	PA	27.82	–97.07	19
6. Brownsville, TX	BT	26.11	–97.17	8
7. Pueblo Viejo, Mexico	PV	22.18	–97.83	49
8. Laguna de Tamiahua, Mexico	TA	21.59	–97.55	45
9. Laguna Grande, Mexico	LG	20.03	–96.61	54
10. La Mancha, Mexico	LM	19.37	–96.23	47
11. Alvarado, Mexico	AL	18.77	–95.78	12
12. El Ostion, Mexico	LO	18.22	–94.60	47
13. Tabasco, Mexico	TB	18.32	–93.74	23

each target region. Aligned amplicon sequences were used to construct synthetic composite reference sequences containing all SNP and indel sites observed in the sequence sets (available from us upon request). Locations of SNPs chosen for genotyping (Table 3) were numbered according to the composite reference sequences. Samples were genotyped for 12 SNPs in the 10 nuclear loci. Three methods of moderate-throughput SNP genotyping were used in this study: RFLP, Amplifluor SNPs Genotyping System (Chemicon International, Inc., Billerica, MA), and single base extension (SBE). To provide templates for SNP genotyping, samples were amplified by PCR for each nuclear locus using the primers listed in Table 2.

Five SNPs in four nuclear loci—ALR (2 SNPs), COF, FIL, and GABA—were genotyped by RFLP analysis. PCRs consisted of an initial denaturation of 2 min at 94°C, followed by 35 cycles of denaturing at 94°C for 45 sec, annealing at 55°C for 60 sec, extension at 72°C for 90 sec, with a final extension of 5 min at 72°C. PCR products were digested with the appropriate restriction enzyme according to manufacturer protocol to target the SNP site of interest (Table 3). Digests were examined on 2–3% agarose gels stained with ethidium bromide for visualization of DNA bands. Genotypes were inferred from the pattern of DNA bands resulting from enzyme digestion.

Three SNPs in 3 nuclear loci—CH, EF1α, and RAN—were genotyped by Amplifluor technology. We used the Amplifluor AssayArchitect software (www.assayarchitect.com) to design the 2-tailed allele-specific primers and a reverse primer for each SNP. Although genomic DNA can be used in Amplifluor reactions, we found it necessary for best results to perform preamplification on our samples. PCRs consisted of an initial denaturation of 2 min at 94°C, followed by 45 cycles of

TABLE 2.

Primers for PCR amplification of mitochondrial and nuclear targets in *Crassostrea virginica*.

Target Region	Primers (5′ → 3′)	Size (bp)	Source
Mitochondrial			
ND2-trnR-trnH-ND4	AAATAGGTTAGGGGGACTCAGC GGAACCAGAAAAATCTCGACC	793	NC_007175:11251..12040; Hare and Avise (1996)
cox1	AGCACGTGAAAGAACTGTTATGTC AACTTCAGGATGGCCAAAAAATCA	718	NC_007175:84..801
ATP6-ND2	CTAGAGAAGGAACCGGATGAGTGT TGAAATTAGTAAAGCGCCATAATG	1,594	NC_007175:9702..11295
cob-cox2-trnS1-trnL1	TAATGCGGGATGCCAATTATGGAT CACTTCTCTGCCAGCATAGCTTAT	1,916	NC_007175:3855..5770
cox3	ATTTAGTTGATCCTAGGCCTTGACC CCCACAAAACAACAGCCCGCAAGT	635	NC_007175:2667..3301
Nuclear			
Activinlike type 1 receptor (ALR)	GGGCGTTATGGATCGGTGT GCGCTTGGTTCCCACCTTGTTGTT	492	AJ309316
Arginine kinase (AK)	GCGCCGCCTGGGTCTGAGTGAAAT TCCGGGTTTGCGTTTGGTTCTGGT	129	CD646841
Chitinase (CH)	CGGCAGAGTACTGGCACCAGAAGG CGTTATTGCTCCCGGAAACTG	272	Gaffney (unpublished)
ADF/cofilin (COF)	GGGGATCCACACAGAGATTCAAT CATTTCGTTAGCATTTTGATACGTCT	194	CV088058
Elongation factor 1α (EF1α)	TTCCACTGGCCATCTCATTTACAA GAAACGGCTCTCACTGTATGGTG	678	CD648970
Filamin (FIL)	CCGCAGAAAAACCACTCGAGCGTG TCACAAATGTACAACCCGCACCT	544	AquaFirst (unpublished)
GABA receptor-associated protein (GABA)	CCTGTCATCGTAGAAAAAGCACC CACTTTCATCACTGTAGGCAATG	801	BG624388
Ran protein (RAN)	AAATGTTCCCAACTGGCATAGAGA CTCCCACCAATTTCCTAGCTAACC	215	CD647917
RP L27A (RP)	GAAGCACCCTGGTGGTCGTGGTAA CCCGGGTTTTCTCTGAGAC	571	CD646506
Thymosin beta (Thyβ)	GCCTGAGAGCAGCTTTGTGTGT CCCAAGTTGTGCTTTATTCA	271	CD646676

TABLE 3.

Nuclear SNPs and genotyping methods.

Locus	SNP position	Method	Primers/Restriction Enzymes
ALR	294 (A/G) 333 (C/T)	RFLP	*Pst* I (CTGCA′G) *Nco* I (C′CATGG)
AK	34 (C/T)	SBE	TGGGTCTGAGTGAAATCGAGGCTAT
CH	162 (G/T)	SBE	TGCAATACCTCGTAATAGGACARGA
		Amplifluor	F: *GGCCCTATACAAGGGAGAAAGGTT
			F: #GCCCTATACAAGGGAGAAAGGTG
			R: TGCAATACCTCGTAATAGGACA
COF	149 (A/C)	RFLP	Hpy188I (TCN′GA)
EF1α	612 (A/G)	Amplifluor	F: *ACACCAATGATGAGCTGCTTT
			F: #ACACCAATGATGAGCTGCTTC
			R: CTGCTGGTACTGGAGAGTTTGAA
FIL	209 (C/T)	RFLP	SspI (AAT′ATT)
GABA	180 (A/C)	RFLP	BseLI (CCNNNNN′NNGG)
RAN	100 (A/G)	SBE	CAAAGTCGACATCAAGGATCGCAAAGTTAA
		Amplifluor	F: *CGGTGAAACACGATGGCTTTAGCT
			F: #GGTGAAACACGATGGCTTTAGCC
			R: GTGTGAAAACATCCCCATTGTGT
RP	443 (C/T)	SBE	AACTGACTAAACTAGAAAATAACCTGGATGGCTAAATGAA
Thyβ	95 (A/G) 130 (G/T)	SBE SBE	ATTTAATTTTGTAAAATGAATTCCA
			TTGTAACAAACATATGCTCT

SNPs are numbered according to composite reference sequence for each amplicon (see text). * SR tail, #FAM tail. Target SNPs in Amplifluor primers are underlined.

denaturing at 94°C for 45 sec, annealing at 55°C for 60 sec, extension at 72°C for 90 sec, with a final extension of 5 min at 72°C. PCR products were diluted 1:50 with water for use in the Amplifluor reaction. The Amplifluor reaction was performed in a 10-μL reaction containing 0.80 μL 2.5 mM dNTP (deoxynucleotide triphosphate) mix, 0.48 μL 25 mM $MgCl_2$ (Sigma, St. Louis, MO), 1.00 μL 10× Amplifluor Reaction Buffer S (Chemicon International, Billerica, MA), 0.50 μL 20× Amplifluor SNP FAM Primer (Chemicon), 0.50 μL 20× Amplifluor SNP SR Primer (Chemicon), 0.50 μL 20× SNP Specific Primer Mix, 0.06 μL Titanium *Taq* Polymerase (Clontech, Mountain View, CA), and 2 μL diluted PCR products. Amplifluor technology has an initial allele-specific amplification step using 2-tailed primers and a common reverse primer, and a second amplification step during which the Amplifluor primers bind to the template and further amplify the PCR products, incorporating the Amplifluor sequences to generate a fluorescent signal. Amplifluor reactions were performed on a Stratagene (La Jolla, CA) RoboCycler 96 Gradient Cycler, consisting of an initial denaturation of 4 min at 96°C on a preheated block, followed by 20 cycles of denaturing at 96°C for 15 sec, annealing at 51–55°C for 5 sec, extension at 72°C for 10 sec, then 24 cycles of denaturing at 96°C for 15 sec, annealing at 55°C for 20 sec, extension at 72°C for 40 sec, with a final extension of 3 min at 72°C. Products from the Amplifluor reaction were analyzed with a POLARstar OPTIMA fluorescence plate reader (BMG Labtech, Cary NC) and evaluated using the AssayAuditor Spreadsheet (www.chemicon.com) to assign the appropriate genotype to each sample.

Four SNPs in 3 nuclear loci—AK, RP, and Thyβ (2 SNPs)—were genotyped by SBE. For each SNP, a primer was designed with its 3′ terminus immediately upstream of the target SNP where a fluorophore-labeled ddNTP (dideoxynucleotide triphosphate) was incorporated (Li et al. 2002). SBE primers were designed with different lengths to facilitate multiplexing. PCRs consisted of an initial denaturation of 2 min at 94°C, followed by 40 cycles of denaturing at 94°C for 45 sec, annealing for 60 sec, extension at 72°C for 90 sec, with a final extension of 5 min at 72°C. To remove excess dNTPs and primers, 15 μL PCR product was treated with 5 U Antarctic Phosphatase (New England Biolabs, Ipswich, MA), 1 U 10× Antarctic Phosphatase buffer (New England Biolabs), and 2 U exonuclease I (*Exo*I) (New England Biolabs) diluted in the Exo buffer provided (New England Biolabs) and incubated at 37°C for 1 h followed by inactivation of enzymes at 65°C for 5 min. The SBE reaction was performed in a 10-μL reaction containing 1 μL 10× Therminator *Taq* Buffer (New England Biolabs), 1.5 μL 0.06 μM each SBE primer, 0.02 μL 0.01 mM each unlabeled ddNTP (New England Biolabs), 0.01 μL 1.0 pmol each labeled ddNTP (Fluorescein-12-ddATP, Rox-ddCTP, Tamra-ddGTP, R6G-ddUTP; Perkin Elmer, Waltham, MA), 0.5 μL Therminator *Taq* (New England Biolabs), and 6 μL treated PCR products. The SBE reaction was carried out on a PTC-100 programmable thermocycler (MJ Research, Inc., Waltham, MA) under the following conditions: 60 cycles of 95°C for 10 sec, 50°C for 10 sec, and 60°C for 30 sec. After the SBE reaction, 1 U Antarctic Phosphatase (New England Biolabs) diluted with 1 × X Antarctic Phosphatase buffer (New England Biolabs) was added to each sample to remove unincorporated ddNTPs and was incubated at 37°C for 1 h, followed by incubation at 65°C for 5 min. SNP genotypes were analyzed on a SpectruMedix SCE2410 capillary sequencer, and the data were viewed with the DNA fragment analysis software GenoSpectrum 2.08 (Transgenomic).

Statistical Analysis

Inferred genealogical relationships among mitochondrial haplotypes were depicted by a median joining network (Bandelt et al. 1999) constructed with NETWORK 4.5.1.0 (www.fluxus-engineering.com). DnaSP 5.0 (Librado & Rozas 2009) was used

to estimate mitochondrial haplotype and nucleotide diversity, and to examine mismatch distributions; these measures are useful for inferring the demographic history of populations. Fstat 2.9.3.2 (Goudet 1995) was used to estimate genotypic disequilibrium among SNP loci within and across populations. SPAGeDi 1.2 (Hardy & Vekemans 2002) was used to estimate the mean genetic differentiation (F_{ST}) (Weir & Cockerham 1984) among populations globally and regionally. Population pairwise F_{ST} values were estimated with Fstat, whereas Arlequin 3.11 (Excoffier et al. 2005) was used to estimate observed (H_o) and expected (H_e) heterozygosities. Deviations of heterozygote frequencies from Hardy-Weinberg equilibrium (HWE) (F_{IS}) (Weir & Cockerham 1984) and allelic frequencies for each population at each locus were estimated with GenePop 4.0 (Rousset 2008). Analysis of molecular variance (AMOVA) was conducted using Arlequin 3.11 (Excoffier et al. 2005) to investigate regional population differentiation. Exact RxC tests were implemented in StatXact 4.0.1 (Cytel Software, Cambridge, MA) to determine homogeneity of genotypic frequencies over all populations and among and within regions for each locus. Critical values were corrected for multiple tests using the modified false discovery rate method (B-Y) of Benjamini and Yekutieli (2001). This is a more powerful method of analyzing multiple tests than the Bonferroni correction (Narum 2006).

Six of 12 nuclear SNPs showed significant deviations from HWE in at least 1 population. MicroChecker (Van Oosterhout et al. 2004) was used to examine the data for possible genotyping errors and the presence of null alleles. Heterozygote deficiencies were consistently attributed to null alleles by the MicroChecker program. FreeNA (Chapuis & Estoup 2007) was used to estimate null allelic frequencies and to recalculate F_{ST} values after inclusion of null alleles using the excluding null alleles method.

For 2 allozyme data sets (Buroker 1983, Rosa-Vélez 1986), patterns of genetic relatedness of samples were visualized using nonmetric multidimensional scaling (MDS). Pairwise estimates of Nei's (1978) unbiased genetic similarity among samples were obtained using NTSYSpc 2.11x (Rohlf 2005), and was used for MDS as implemented in SYSTAT 11 (Wilkinson 1990). For nuclear SNP data, multilocus genotype data of each individual were used to perform 3-dimensional factorial correspondence analysis (FCA) of individuals and populations using Genetix 4.05 (Belkhir et al. 2004). FCA utilizes genotypic data to identify structural relationships among individuals and populations, making no *a priori* assumptions regarding the nature of the relationships. FCA coordinates were imported into SigmaPlot 9.0 (SYSTAT Software, Inc.) to display a 3-dimensional plot of genetic correspondence among oyster populations.

RESULTS

Mitochondrial DNA

Cox1

Alignment of a 599-bp portion of the 718-bp cox1 amplicon from 155 individuals showed 64 segregating sites and 61 haplotypes, with estimated haplotype diversity of 0.909 and nucleotide diversity (π) of 0.0081. The majority of individuals belonged to 2 haplogroups differing by 2 nucleotide substitutions, with a third group comprising several more distantly related haplotypes (Fig. 1).

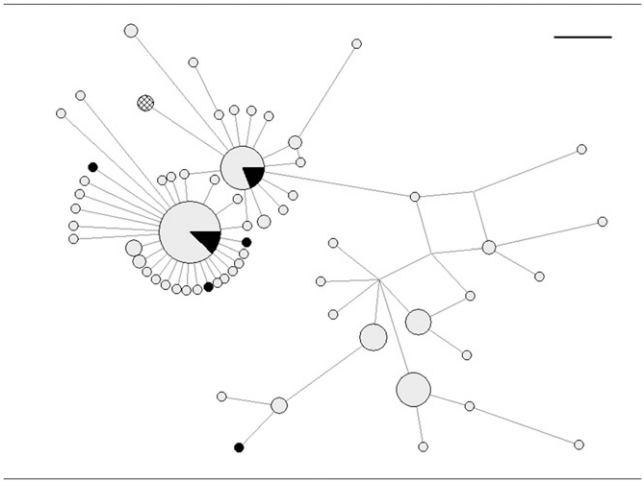

Figure 1. Median-joining network of cox1 haplotypes. Area of symbol is scaled to sample size. Shading indicates geographical source of haplotypes: Cedar Key (crosshatched), Apalachicola Bay to Port Aransas (black), Mexico (gray). Total sample size, $n = 159$. Scale bar in upper right = 1 mutational step.

Sample sizes from 6 collections from Mexico (total $n = 142$) and one from Florida ($n = 6$) were large enough to allow AMOVA. When the collections were divided into southern Gulf (Mexico) and northern Gulf (Florida) groups, AMOVA showed that 16.4% of the variation fell among groups ($F_{CT} = 0.164$, $P < 0.000005$), whereas 6 samples from Mexico were homogeneous ($F_{ST} = 0$, $P = 0.86$). Haplotype distributions within the 6 Mexican collections departed consistently from neutral expectations, with significant negative values of Fu's F_S and Fu and Li's D* and F* statistics, indicative of either historical population expansion or a selective sweep. In addition, each collection displayed a bimodal mismatch distribution, consistent with the admixture of 2 divergent lineages.

Cox 3

Amplicons from individuals ($n = 11$) from various sites in the Gulf were sequenced. Alignment of a 529-bp section of the 635-bp amplicon showed 13 segregating sites and 8 haplotypes, with estimated haplotype diversity of 0.927 and nucleotide diversity (π) of 0.0074. A median-joining network diagram of the haplotypes (Fig. 2) suggests the existence of 2 clades differing in their geographical distribution (northern vs. southern Gulf of Mexico). Digestion of cox3 amplicons from 46 individuals with 3 restriction enzymes (*Csp*6 I, *Afl* III, *Bsa*H I) resulted in 5 composite haplotypes, which varied significantly in frequency among sample sites (exact RxC test, $P < 0.00005$; Fig. 3).

ND2-ND4

Alignment of a 689-bp portion of the 793-bp ND2-ND4 amplicon from 76 individuals showed 52 segregating sites (including 1 single-base indel) and 38 haplotypes, with an estimated haplotype diversity of 0.856 and a nucleotide diversity (π) of 0.0053. The majority of individuals belonged to a widespread haplogroup containing numerous, closely related haplotypes, with a second highly divergent and less diverse haplogroup restricted to Mexico and southern Texas (Fig. 4).

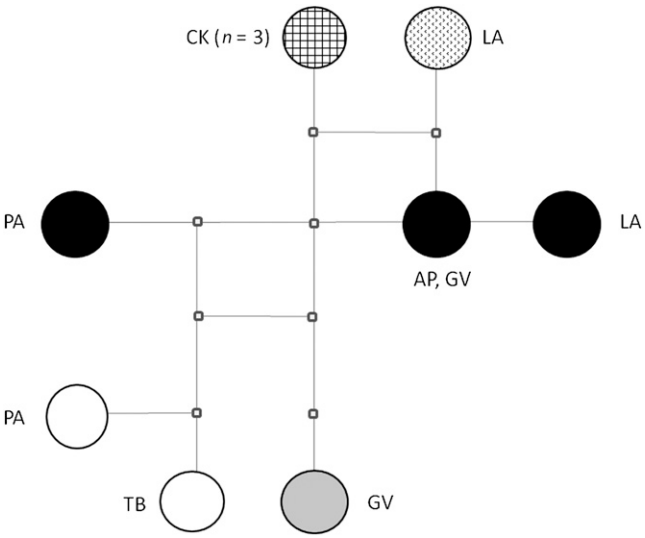

Figure 2. Median-joining network of cox3 haplotypes. Sources of sequenced amplicons are shown in parentheses; squares indicate unobserved haplotypes. Haplotypes are shaded to indicate RFLP haplogroups indicated in Figure 3. Total sample size, $n = 11$. AP, Apalachicola, FL; CK, Cedar Key, FL; GV, Galveston Bay, TX: LA, Grand Isle, LA; PA, Port Aransas, TX; TB, Tabasco, Mexico.

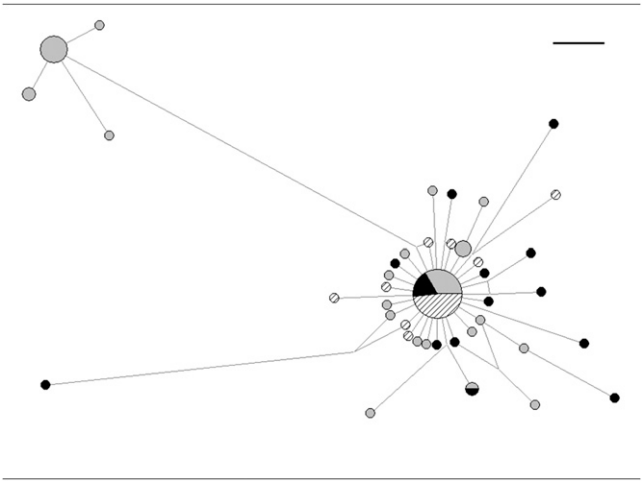

Figure 4. Median-joining network of ND2-ND4 haplotypes. Shading indicates geographical source of haplotypes: Cedar Key (crosshatched), Apalachicola Bay to Port Aransas (black), Mexico (gray). Area of haplotype symbol is scaled to sample size. A minor haplogroup (gray shading) was found only in the southern Gulf of Mexico. Total sample size, $n = 77$. Scale bar in upper right = 1 mutational step.

Frequencies of the 2 haplogroups varied significantly among sample sites (exact RxC test, $P < 0.00005$), with all 6 individuals from Tabasco belonging to the minor haplogroup (Fig. 5).

cob-trnL1

The 1,916-bp cob-trnL1 amplicon from 46 individuals was digested with 4 restriction enzymes (*Ase* I, *Bse*N I, *Bsa*H I, *Bsi*HKA I). Three haplotypes were observed after *Bsa*H I digestion: a common haplotype seen in all samples, and 2 uncommon haplotypes restricted to southern Texas and Mexico (data not shown). Frequencies of the 3 haplotypes varied significantly among sample sites (exact RxC test, $P = 0.040$).

ATP6-ND2

The 1,594-bp ATP6-ND2 amplicon from 47 individuals was digested with 2 restriction enzymes (*Apa*L I, *Ase* I). Two haplotypes were observed after *Apa*L I digestion: a common haplotype seen in all samples and a less common haplotype not found in the Florida samples (data not shown). Frequencies of the 2 haplotypes were homogeneous among sample sites (exact RxC test, $P = 0.177$).

Nuclear DNA

Numbers of individuals successfully SNP genotyped (N), minor allele frequency (q), observed (H_O) and expected (H_E) heterozygosities, and deviations from HWE (F_{IS}) for each marker in each population are given in Table 4. Exact tests

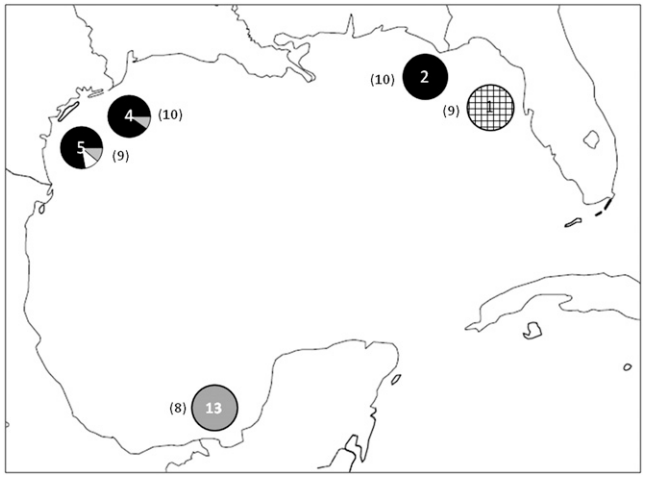

Figure 3. Distribution of cox3 RFLP haplotypes. Shading in pie charts corresponds to Figure 2; sample sizes are given in parentheses. Location numbers from Table 1 are superimposed on pie charts.

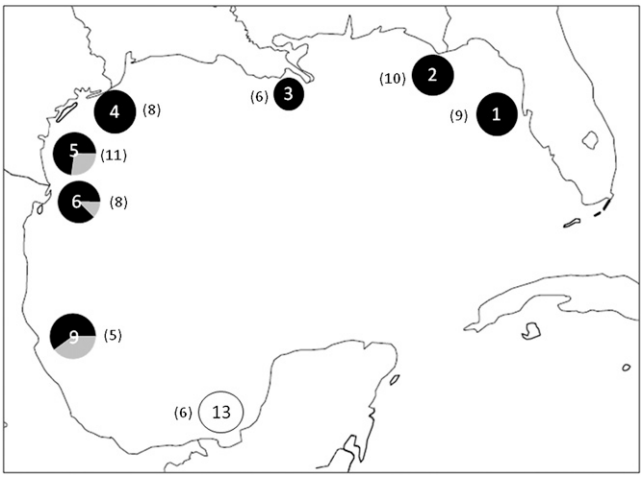

Figure 5. Distribution of major ND2-ND4 haplogroups. Size of pie chart is scaled to sample size in parentheses. Location numbers from Table 1 are superimposed on pie charts.

TABLE 4.

Twelve *C. virginica* nuclear SNP markers evaluated in 12 *C. virginica* Gulf populations.

Location	ALR294	ALR333	AK	CH	COF	EF1α	FIL	GABA	RAN	RP	Thyβ95	Thyβ130	Mean
CK													
n	15	14	15	15	15	15	15	14	15	15	15	15	
q	0.300	0.000	0.433	0.000	0.267	0.233	0.000	0.000	0.200	0.433	0.400	0.267	
H_O	0.467	—	0.600	—	0.267	0.467	—	—	0.267	0.467	0.400	0.533	0.433
H_E	0.480	—	0.508	—	0.469	0.421	—	—	0.384	0.508	0.536	0.405	0.464
F_{IS}	–0.077	—	–0.189	—	0.349	–0.273	—	—	0.200	0.084	0.200	–0.333	–0.005
AP													
n	17	17	23	21	23	21	21	18	24	21	23	23	
q	0.235	0.000	0.261	0.095	0.457	0.190	0.048	0.000	0.313	0.405	0.348	0.130	
H_O	0.235	—	0.261	0.190	0.304	0.381	0.000	—	0.292	0.238	0.435	0.174	0.251
H_E	0.371	—	0.394	0.177	0.507	0.316	0.093	—	0.439	0.494	0.464	0.232	0.349
F_{IS}	0.373	—	0.343	–0.081	0.405	–0.212	1.000	—	0.340	0.524	0.064	0.254	0.301
LA													
n	10	10	14	14	14	14	14	13	14	14	14	14	
q	0.250	0.000	0.179	0.143	0.250	0.286	0.036	0.000	0.321	0.143	0.286	0.143	
H_O	0.100	—	0.357	0.286	0.357	0.429	0.071	—	0.357	0.143	0.429	0.286	0.281
H_E	0.395	—	0.304	0.254	0.389	0.423	0.071	—	0.452	0.254	0.423	0.254	0.322
F_{IS}	0.757	—	–0.182	–0.130	0.085	–0.013	0.000	—	0.217	0.447	–0.013	–0.130	0.104
GV													
n	21	21	40	40	40	40	39	39	40	39	40	40	
q	0.286	0.048	0.388	0.038	0.425	0.263	0.244	0.013	0.263	0.192	0.400	0.138	
H_O	0.095	0.000	0.475	0.075	0.500	0.375	0.282	0.026	0.425	0.282	0.500	0.275	0.276
H_E	0.418	0.093	0.481	0.073	0.495	0.392	0.373	0.026	0.392	0.315	0.486	0.240	0.315
F_{IS}	**0.777**	1.000	0.012	–0.026	–0.010	0.044	0.247	0.000	–0.085	0.105	–0.029	–0.147	0.157
PA													
n	9	10	19	19	19	19	4	15	19	19	19	19	
q	0.333	0.200	0.211	0.105	0.237	0.079	0.375	0.000	0.184	0.105	0.026	0.000	
H_O	0.000	0.000	0.421	0.105	0.053	0.158	0.250	—	0.368	0.105	0.053	—	0.174
H_E	0.471	0.337	0.341	0.193	0.371	0.149	0.536	—	0.309	0.193	0.053	—	0.277
F_{IS}	**1.000**	**1.000**	–0.241	0.463	**0.862**	–0.059	0.571	—	–0.200	0.463	0.000	—	0.386
PV													
n	37	37	49	49	49	48	47	46	49	49	49	49	
q	0.216	0.041	0.143	0.010	0.459	0.240	0.319	0.022	0.337	0.143	0.490	0.082	
H_O	0.054	0.027	0.245	0.020	0.429	0.438	0.340	0.043	0.265	0.163	0.531	0.122	0.223
H_E	0.344	0.079	0.247	0.020	0.502	0.368	0.439	0.043	0.451	0.247	0.505	0.151	0.283
F_{IS}	**0.845**	0.660	0.010	0.000	0.147	–0.191	0.227	–0.011	**0.415**	0.343	–0.051	0.193	0.216
TA													
n	25	25	45	45	45	39	45	36	45	45	45	45	
q	0.280	0.060	0.267	0.000	0.389	0.141	0.333	0.000	0.478	0.078	0.411	0.100	
H_O	0.080	0.120	0.356	—	0.422	0.282	0.444	—	0.556	0.111	0.511	0.111	0.299
H_E	0.411	0.115	0.396	—	0.481	0.245	0.449	—	0.505	0.145	0.490	0.182	0.342
F_{IS}	**0.809**	–0.044	0.102	—	0.123	–0.152	0.011	—	–0.102	0.236	–0.044	0.392	0.133
LG													
n	34	34	54	54	54	52	54	53	54	53	54	54	
q	0.368	0.029	0.278	0.102	0.491	0.317	0.167	0.019	0.269	0.151	0.389	0.093	
H_O	0.088	0.000	0.041	0.204	0.352	0.596	0.296	0.038	0.426	0.113	0.333	0.148	0.220
H_E	0.490	0.087	0.405	0.201	0.514	0.437	0.296	0.056	0.410	0.259	0.480	0.170	0.317
F_{IS}	**0.815**	1.000	–0.006	–0.104	0.305	–0.368	–0.057	–0.010	–0.075	**0.565**	0.565	0.128	0.230
LM													
n	34	34	47	46	47	45	47	34	47	46	47	47	
q	0.147	0.059	0.287	0.054	0.394	0.444	0.330	0.000	0.255	0.130	0.266	0.053	
H_O	0.059	0.000	0.447	0.109	0.234	0.222	0.489	—	0.340	0.174	0.404	0.064	0.231
H_E	0.255	0.112	0.414	0.104	0.483	0.499	0.447	—	0.384	0.229	0.395	0.102	0.311
F_{IS}	**0.772**	**1.000**	–0.081	–0.047	**0.518**	**0.558**	–0.096	—	0.115	0.244	–0.025	0.376	0.303
AL													
n	9	9	12	12	12	12	12	9	12	12	12	12	
q	0.111	0.000	0.375	0.083	0.458	0.208	0.083	0.056	0.333	0.167	0.458	0.000	
H_O	0.222	—	0.583	0.167	0.583	0.250	0.167	0.111	0.667	0.167	0.583	—	0.350
HE	0.209	—	0.489	0.159	0.518	0.344	0.159	0.111	0.464	0.290	0.518	—	0.326

continued on next page

TABLE 4.

continued

Location	ALR294	ALR333	AK	CH	COF	EF1α	FIL	GABA	RAN	RP	Thyβ95	Thyβ130	Mean
F_{IS}	–0.067	—	–0.203	–0.048	–0.132	0.283	–0.048	0.000	–0.467	0.436	–0.132	—	–0.038
LO													
n	36	36	47	46	47	40	43	28	47	46	47	44	
q	0.375	0.014	0.298	0.022	0.436	0.488	0.221	0.000	0.319	0.065	0.340	0.068	
H_O	0.361	0.028	0.468	0.043	0.532	0.275	0.256	—	0.383	0.087	0.426	0.136	0.272
H_E	0.475	0.028	0.423	0.043	0.497	0.506	0.348	—	0.439	0.123	0.454	0.129	0.315
F_{IS}	0.243	0.000	–0.108	–0.011	–0.071	**0.460**	0.268	—	0.129	0.297	0.063	–0.062	0.110
TB													
n	22	22	23	23	23	23	23	23	23	23	23	23	
q	0.250	0.045	0.174	0.022	0.478	0.261	0.261	0.000	0.109	0.152	0.413	0.109	
H_O	0.227	0.000	0.348	0.043	0.261	0.435	0.261	—	0.130	0.217	0.478	0.217	0.238
H_E	0.384	0.089	0.294	0.043	0.510	0.394	0.394	—	0.198	0.264	0.496	0.198	0.297
F_{IS}	0.413	1.000	–0.189	0.000	0.494	–0.106	0.343	—	0.347	0.179	0.036	–0.100	0.220
F_{ST}	0.000	0.003	0.019	**0.027**	**0.034**	**0.138**	**0.050**	0.000	**0.024**	**0.052**	**0.044**	0.017	**0.043**

Numbers of individuals scored (*n*), minor allele frequency (q), observed (H_O) and expected (H_E) heterozygosities, and F_{IS} are listed for each marker in each population. Bold F_{IS} and F_{ST} values are significant at a tablewide $\alpha' = 0.05$ with the modified false discovery rate method of Benjamini and Yekutieli (2001).

for homogeneity of genotype frequencies over all populations indicated significant heterogeneity among populations (exact RxC test, $P < 0.05$) for all loci except AK. Within populations, mean observed heterozygosity (H_O) ranged from 0.174 in PA to 0.433 in CK. We found significant (after B-Y correction) deficiencies of heterozygotes relative to Hardy-Weinberg proportions in 6 of 12 of nuclear SNPs; none showed significant deficiencies of heterozygotes in all populations. AK, CH, FIL, GABA, Thyβ95, and Thyβ130 did not exhibit significant deviations from HWE after B-Y correction. Seven of the 12 populations we sampled showed significant heterozygote deficiencies at one or more loci when corrected for multiple tests by the B-Y method. Analysis with MicroChecker suggested the presence of null alleles in at least 1 population in 10 SNP loci; null alleles were not detected at AK and GABA in any population. Eight of the 12 populations were fixed for the GABA C allele.

Significant genotypic linkage disequilibrium was found within and among loci (Table 5). SNPs located in the same amplicon (ALR294/ALR333 and Thyβ95/Thyβ130) had significant genotypic linkage disequilibrium across populations. Three pairs of independent nuclear loci had significant genotypic linkage disequilibrium across populations after B-Y correction: CH–RP, EF1α–Thyβ95, and FIL–Thyβ95. Significant genotypic disequilibrium was not detected between the 2 ALR SNPs in any individual population, but genotypic disequilibrium was found between the 2 Thyβ SNPs in 3 populations (GV, LG, and LM). Significant genotypic disequilibrium was found between 5 pairs of independent nuclear loci in 4 different populations—AK–RAN in LM, CH–RP in PA, EF1α–FIL in LG, EF1α–Thyβ95 in LM and LO, and RAN–Thyβ95 in LO.

Significant genetic differentiation among samples was detected ($F_{ST} = 0.043$, $P < 0.001$), with F_{ST} values among individual loci from 0–0.138. Population pairwise F_{ST} estimates ranged from 0–0.272 (Table 6). Because null alleles may lead to incorrect estimates of population differentiation, null allele frequencies were estimated with FreeNA (Chapuis & Estoup 2007) and used to recalculate F_{ST} values (without *P* values). When corrected for null alleles, the global multilocus estimate for F_{ST} was 0.047. Population pairwise F_{ST} values differed slightly from initial values when the data were reanalyzed to account for null alleles, ranging from 0.001–0.283 (Table 6).

Genetic similarity depicted by 3-dimensional factorial correspondence analysis (Fig. 6) showed separation of samples into 3 groups: northeastern Gulf (CK to LA), Texas to Mexico, and Port Aransas. Examination of individual SNPs that displayed significant F_{ST} values (CH, COF, EF1α, FIL, RAN, RP, Thyβ95) showed diverse patterns of allelic frequencies, often with extreme values for the PA sample and separation of northeastern Gulf samples from the remainder (Varney 2009).

DISCUSSION

This study is the first geographically comprehensive study of population structure of the eastern oyster in the Gulf of Mexico. Previous surveys have largely been limited to U.S. waters (Buroker 1983, Grady et al. 1989) or have had a limited sampling range (Hedgecock & Okazaki 1984, Rosa-Vélez 1986, King et al. 1994, Galindo-Sánchez et al. 2008). Buroker (1983) noted that Gulf of Mexico populations from Florida to Texas appeared genetically similar (pairwise Nei's unbiased I values, 0.927–0.996), with the exception of oysters inhabiting the lower Laguna Madre of Texas (pairwise Nei's unbiased I values, 0.853–0.868 with other Gulf of Mexico populations), and that allozyme heterozygosity and allelic frequencies at some loci varied clinally along the coastline. Hedgecock and Okazaki (1984) reported that oysters from the Bay of Campeche were considerably different from a sample collected near Apalachicola Bay, FL (Nei's I = 0.910). Reanalysis of Buroker's (1983) data for 22 allozyme loci shows a pattern of genetic similarity among populations mirroring their geographical locations (Fig. 7). In a complementary allozyme study of *C. virginica* collected from coastal lagoons in Mexico, Rosa-Vélez (1986) found substantial differentiation among populations (pairwise Nei's

TABLE 5.

***P* values for estimates of pairwise genotypic linkage disequilibrium.**

	ALR294	ALR333	AK	CH	COF	EF1	FIL	GABA	RAN	RPL27	Thyβ95
ALR333	**<0.001**										
AK	0.534	0.782									
CH	0.873	0.967	0.486								
COF	0.385	0.299	0.657	0.220							
EF1	0.758	0.713	0.405	0.128	0.012						
FIL	0.812	0.631	0.654	0.338	0.907	0.059					
GABA	0.880	0.829	0.894	0.027	0.504	0.898	0.381				
RAN	0.985	0.972	0.224	0.642	0.977	0.213	0.516	0.251			
RPL27	0.297	0.692	0.111	**<0.001**	0.651	0.899	0.387	0.575	0.784		
Thyβ95	0.130	0.025	0.839	0.308	0.599	**<0.005**	**<0.010**	0.357	0.054	0.432	
Thyβ130	0.904	0.941	0.776	0.486	0.431	0.027	0.396	0.280	0.054	0.623	**<0.001**

Boldface values are significant after B-Y correction (tablewide $\alpha' = 0.05$).

I values, 0.908–0.994), with clustering of 3 southernmost samples and distinct separation of a sample from the hypersaline Laguna Madre of Mexico (Fig. 8). Collectively, allozyme survey data suggest a pattern of isolation by distance among Gulf of Mexico populations, except for highly divergent populations inhabiting the hypersaline Laguna Madre region.

A similar picture is obtained when genetic distances based on nuclear SNPs are visualized by multidimensional scaling. Northern Gulf samples cluster separately from Texas and Mexico samples, with the Port Aransas sample as an outlier (Fig. 6). Allozyme surveys showed the latter locality to occupy a transition zone between the distinctive lower Laguna Madre population and populations to the north, which exhibit the common Gulf Coast profile (Groue & Lester 1982, King et al. 1994).

The 6 samples collected along the Veracruz coast (PV, TA, LG, LM, AL, LO) were also surveyed for 5 microsatellite loci (Galindo-Sánchez et al. 2008), allowing a comparison of patterns of genetic heterogeneity derived from 2 nuclear marker classes (microsatellites and SNPs) and 1 mtDNA region (cox1). Both types of nuclear markers showed significant heterogeneity in allelic frequencies among samples (11 of 15 pairwise F_{ST} values for microsatellites, 5 of 15 pairwise F_{ST} values for SNPs), but cox1 haplotype frequencies were homogeneous among samples (Monte Carlo RxC test, $P = 0.489$). Pairwise F_{ST} values estimated for microsatellites and SNPs were not correlated (Mantel test, $P = 0.18$), nor were they correlated with geographical distance between sample collection sites. Local heterogeneity in allelic frequencies, termed ''chaotic genetic patchiness'' (Johnson & Black 1982), has frequently been observed in sedentary marine invertebrates with planktonic dispersal stages, and is generally attributed to spatial and temporal variation in the genetic composition of recruits (Arnaud-Haond et al. 2008). The admixture of cohorts of genetically differentiated recruits may also be the source of the linkage disequilibrium observed here among nuclear SNPs.

Mitochondrial sequence profiles likewise reveal a significant population structure in Gulf of Mexico oysters that is not entirely concordant with the patterns shown by nuclear markers. RFLP haplotype frequencies vary clinally along the coastline for 2 of 3 mtDNA regions surveyed (Figs. 3 and 5), similar to the patterns shown by nuclear markers; but in contrast, samples from the Laguna Madre region are not distinct from neighboring samples. The greater differentiation of nuclear markers (allozymes and SNPs) compared with mtDNA for Laguna Madre oysters is unexpected from the

TABLE 6.

Pairwise F_{ST} estimated without excluding null alleles method (ENA; above diagonal) and with ENA (below diagonal).

Location	CK	AP	LA	GV	PA	PV	TA	LG	LM	AL	LO	TB
CK	—	0.018	**0.110**	**0.048**	**0.272**	**0.055**	**0.116**	**0.053**	**0.099**	**0.048**	**0.106**	**0.069**
AP	0.022	—	0.014	0.013	0.213	0.024	**0.052**	0.013	**0.049**	0.006	**0.059**	0.024
LA	0.106	0.022	—	0.011	0.213	0.036	0.031	0.004	0.051	0.020	0.024	0.020
GV	0.054	0.020	0.021	–	**0.160**	0.004	0.012	–0.004	0.021	–0.001	0.015	0.000
PA	0.283	0.220	0.225	0.190	—	**0.149**	**0.218**	0.139	0.077	0.245	0.100	0.164
PV	0.088	0.034	0.035	0.015	0.221	—	0.012	0.002	0.017	0.004	0.020	0.000
TA	0.111	0.048	0.037	0.016	0.260	0.009	—	0.024	**0.052**	0.022	0.041	0.032
LG	0.053	0.015	0.011	0.001	0.157	0.017	0.029	—	0.021	0.006	0.005	0.001
LM	0.098	0.049	0.058	0.023	0.096	0.035	0.054	0.022	—	0.037	0.019	0.016
AL	0.043	0.014	0.028	0.010	0.266	0.024	0.034	0.014	0.048	—	0.041	0.019
LO	0.104	0.057	0.030	0.019	0.129	0.035	0.041	0.015	0.018	0.040	—	0.026
TB	0.071	0.028	0.031	0.004	0.192	0.002	0.034	0.006	0.018	0.029	0.029	—

Boldface values are significant (tablewide $\alpha' = 0.05$) after B-Y correction. *P* values not available for F_{ST} using ENA.

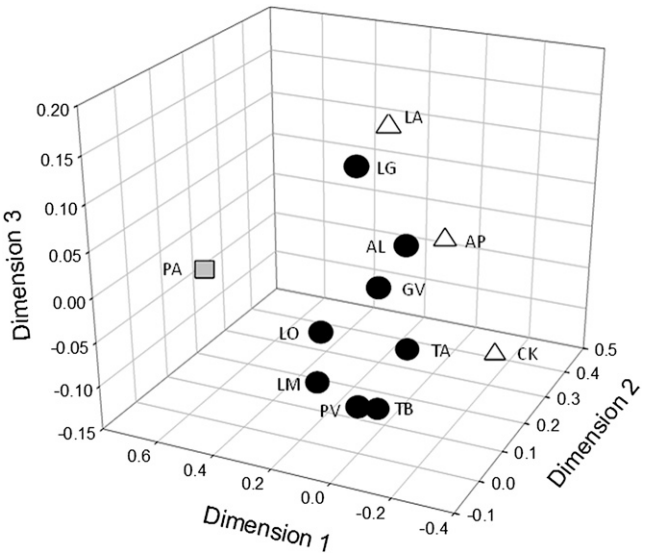

Figure 6. Three-dimensional FCA representation of genetic similarities among Gulf of Mexico *C. virginica* populations based on nuclear SNP data. Open triangles, northern Gulf samples; gray square: Port Aransas; black circles, Texas and Mexico samples. AL, Alvarado, Mexico; AP, Apalachicola, BT, Brownsville, TX; FL; CK, Cedar Key, FL; GV, Galveston Bay, TX: LA, Grand Isle, LA; LG, Laguna Grande, Mexico; LM, La Mancha, Mexico; LO, El Ostion, Mexico; PA, Port Aransas, TX; PV, Pueblo Viejo, Mexico; TA, Laguna de Tamiahua, Mexico; TB, Tabasco, Mexico.

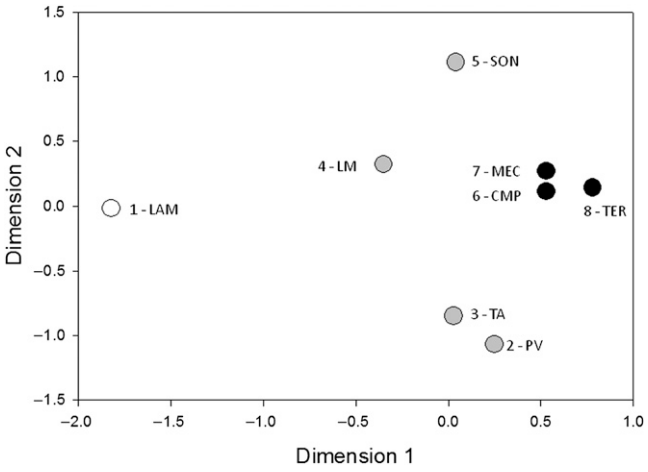

Figure 8. Nonmetric MDS representation of genetic similarities (Nei's unbiased I) among Mexican *C. virginica* populations, based on data from 16 allozyme loci presented by Rosa-Vélez (1986). Sites are numbered from northernmost (open circle, Laguna Madre of Mexico) to southernmost (black circles, sites in Tabasco and Campeche). Locality abbreviations not listed in Table 1: CMP,= Laguna Carmen y Machona, Tabasco; LAM, Laguna Madre, Tamaulipas; MEC, Laguna de Mecoacán, Tabasco; SON, Laguna Sontecomapan, Veracruz; TER, Laguna de Términos, Campeche.

conventional neutral model (Birky et al. 1989), and may reflect the action of natural selection in an environment with higher temperature and salinity than neighboring estuarine environments (King et al. 1994).

It is clear from both mitochondrial and nuclear markers that *C. virginica* inhabiting the Gulf coast do not belong to a single panmictic unit. Population subdivision within the Gulf of Mexico has been observed in a number of fish and invertebrate species, even those with high dispersal capability and apparently continuous ranges (Neigel 2009). The data presented here suggest multiple genetic breaks along the coastline from Florida to the Yucatan peninsula, which coincide approximately with the boundaries of 5 faunal zones suggested by Pulley (1952) on the basis of bivalve distributions: (1) southwest Florida (Cape Romano to Anclote Keys), (2) northeast Gulf (Anclote Keys to Mississippi River), (3) northwest Gulf (Mississippi River to Matagorda Island), (4) Texas transitional (Matagorda Island to Cabo Rojo, Veracruz), and (5) Cabo Rojo to Cabo Catoche, Yucatan. The evidence presented here points to genetic differentiation along the west coast of Florida (Tampa Bay vs. northwestern Florida, Fig. 7; Cedar Key vs. Apalachicola Bay, Fig. 3), in Laguna Madre, and along the southern coastline of the Gulf of Mexico (Figs. 3, 5, and 8). In addition, mitochondrial and nuclear gene profiles are not completely concordant, which may reflect both the stochastic nature of individual gene genealogies and the action of natural selection on individual loci. More comprehensive genetic surveys throughout the Gulf of Mexico will be needed to evaluate the relative importance of historical isolation, selective forces, and hydrographic barriers to dispersal in shaping current population structure in the region.

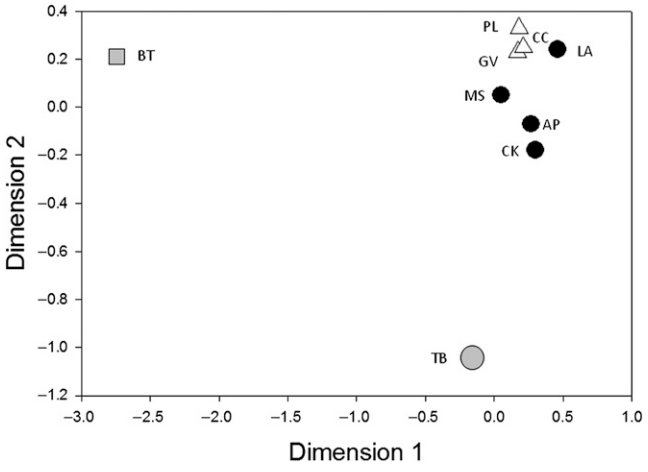

Figure 7. Nonmetric MDS representation of genetic similarities (Nei's unbiased I) among Gulf of Mexico *C. virginica* populations, based on 22 allozyme loci (Buroker 1983). Black circles, northern Gulf of Mexico; open triangles, Texas; gray square, Laguna Madre, TX; open circle, Tampa Bay. Abbreviations not listed in Table 1: CC, Corpus Christi; MS, Horn Island, MS, TX; PL, Port Lavaca, TX; TB, Tampa Bay, FL.

ACKNOWLEDGMENTS

We are grateful to Coren Milbury and Ami Wilbur for contributing to sequencing and RFLP analysis of mitochondrial amplicons, and to John Avise and Carlos Pérez-Rostro for providing tissue samples and DNA extracts. This work was supported in part by Delaware Sea Grant R-F/22 to PMG and SEP-COSNET 808.03.04 to Jorge De la Rosa-Vélez. CEG-S was supported by a graduate fellowship from CONACYT of Mexico.

LITERATURE CITED

Arnaud-Haond, S., V. Vonau, C. Rouxel, F. Bonhomme, J. Prou, E. Goyard & P. Boudry. 2008. Genetic structure at different spatial scales in the pearl oyster (*Pinctada margaritifera cumingii*) in French Polynesian lagoons: beware of sampling strategy and genetic patchiness. *Mar. Biol.* 155:147–157.

Bandelt, H.- J., P. Forster & A. Röhl. 1999. Median-joining networks for inferring intraspecific phylogenies. *Mol. Biol. Evol.* 16:37–48.

Belkhir, K., P. Borsa, L. Chikhi, M. Raufaste & F. Bonhomme. 2004. GENETIX 4.05, logiciel sous Windows TM pour la génétique des populations. Montpellier, France: Laboratoire Génome, Populations, Interactions, CNRS UMR 5171, Université de Montpellier II.

Benjamini, Y. & D. Yekutieli. 2001. The control of the false discovery rate in multiple testing under dependency. *Ann. Stat.* 29:1165–1188.

Birky, C. W., Jr., P. Fuerst & T. Maruyama. 1989. Organelle gene diversity under migration, mutation and drift: equilibrium expectations, approach to equilibrium, effects of heteroplasmic cells, and comparison to nuclear genes. *Genetics* 121:613–627.

Buroker, N. E. 1983. Population genetics of the American oyster *Crassostrea virginica* along the Atlantic coast and the Gulf of Mexico. *Mar. Biol.* 75:99–112.

Chapuis, M.- P. & A. Estoup. 2007. Microsatellite null alleles and estimation of population differentiation. *Mol. Biol. Evol.* 24:621–631.

Cunningham, C. W. & T. M. Collins. 1994. Developing model systems for molecular biogeography: vicariance and interchange in marine invertebrates. In: B. Schierwater, B. Streit, G. P. Wagner & R. DeSalle, editors. Molecular ecology and evolution: approaches and applications. Basel: Birkhäuser Verlag. pp. 405–433.

Excoffier, L., G. Laval & S. Schneider. 2005. Arlequin version 3.0: an integrated software package for population genetics data analysis. *J. Exp. Zool.* 1:47–50.

Gaffney, P. M. 1996. Biochemical and population genetics. In: V. S. Kennedy, R. I. E. Newell & A. F. Eble, editors. The eastern oyster *Crassostrea virginica*. College Park, MD: Maryland Sea Grant College. pp. 423–441.

Galindo-Sánchez, C. E., P. M. Gaffney, C. I. Pérez-Rostro, J. De la Rosa-Vélez, J. Candela & P. Cruz. 2008. Assessment of genetic diversity of the eastern oyster *Crassostrea virginica* in Veracruz, Mexico using microsatellite markers. *J. Shellfish Res.* 27:721–727.

Goudet, J. 1995. FSTAT (version 1.2): a computer program to calculate F-statistics. *J. Hered.* 86:485–486.

Grady, J. M., T. M. Soniat & J. S. Rogers. 1989. Genetic variability and gene flow in populations of *Crassostrea virginica* (Gmelin) from the northern Gulf of Mexico. *J. Shellfish Res.* 8:227–232.

Groue, K. J. & L. J. Lester. 1982. A morphological and genetic analysis of geographic variation among oysters in the Gulf of Mexico. *Veliger* 24:331–335.

Hardy, O. J. & X. Vekemans. 2002. SPAGeDi: a versatile computer program to analyse spatial genetic structure at the individual or population levels. *Mol. Ecol. Notes* 2:618–620.

Hare, M. P. & J. C. Avise. 1996. Molecular genetic analysis of a stepped multilocus cline in the American oyster (*Crassostrea virginica*). *Evolution* 50:2305–2315.

Hedgecock, D. & N. B. Okazaki. 1984. Genetic diversity within and between populations of American oysters (*Crassostrea*). *Malacologia* 25:535–549.

Hoover, C. A. & P. M. Gaffney. 2005. Geographic variation in nuclear genes of the eastern oyster, *Crassostrea virginica* Gmelin. *J. Shellfish Res.* 24:103–112.

Johnson, M. S. & R. Black. 1982. Chaotic genetic patchiness in an intertidal limpet, *Siphonaria sp. Mar. Biol.* 70:157–164.

King, T. L., R. Ward & E. G. Zimmerman. 1994. Population structure of eastern oysters (*Crassostrea virginica*) inhabiting the Laguna Madre, Texas, and adjacent bay systems. *Can. J. Fish. Aquat. Sci.* 51(Suppl. 1):215–222.

Li, Q., Z. Liu, H. Monroe & C. T. Culiat. 2002. Integrated platform for detection of DNA sequence variants using capillary array electrophoresis. *Electrophoresis* 23:1499–1511.

Librado, P. & J. Rozas. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 25:1451–1452.

Narum, S. R. 2006. Beyond Bonferroni: less conservative analyses for conservation genetics. *Conserv. Genet.* 7:783–787.

Nei, M. 1978. Estimation of average heterozygosity and genetic distances from a small number of individuals. *Genetics* 89:583–590.

Neigel, J. E. 2009. Population genetics and biogeography of the Gulf of Mexico. In: D. F. Felder & C. K. Camp, editors. Gulf of Mexico: its origins, waters, and biota. College Station, TX: Texas A&M University Press.

Pulley, T. E. 1952. Distribution of mollusks in the Gulf of Mexico. *Rep. Am. Malac. Union* 1952:2–3.

Reeb, C. A. & J. C. Avise. 1990. A genetic discontinuity in a continuously distributed species: mitochondrial DNA in the American oyster, *Crassostrea virginica*. *Genetics* 124:397–406.

Rohlf, F. J. 2005. NTSYSpc: numerical taxonomy system, version 2.11x. Setauket, NY: Exeter Publishing.

Rosa-Vélez, J. 1986. Variabilidad genetica poblacional en ostiones de la especie *Crassostrea virginica* del Golfo de Mexico. Ph.D. diss. Colegio de Ciencias y Humanidades, Mexico: Universidad Nacional Autónoma de México. 124 pp.

Rousset, F. 2008. GENEPOP'007: a complete re-implementation of the GENEPOP software for Windows and Linux. *Mol. Ecol. Res.* 8:103–106.

Stauber, L. A. 1950. The problem of physiological species with special reference to oysters and oyster drills. *Ecology* 31:109–118.

Van Oosterhout, C., W. F. Hutchinson, D. P. M. Wills & P. Shipley. 2004. MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Mol. Ecol. Notes* 4:535–538.

Varney, R. L. 2009. Assessment of nuclear DNA variation and population structure in the eastern oyster *Crassostrea virginica* through discovery and analysis of single nucleotide polymorphisms (SNPs). Ph.D. diss. College of Earth, Ocean, and Environment. Newark, DE: University of Delaware.

Varney, R. L. & P. M. Gaffney. 2008. Assessment of population structure in *Crassostrea virginica* throughout the species range using single nucleotide polymorphisms. *J. Shellfish Res.* 27:1061.

Wakefield, J. R. & P. M. Gaffney. 1996. DGGE reveals additional population structure in American oyster (*Crassostrea virginica*) populations. *J. Shellfish Res.* 15:513.

Weir, B. S. & C. C. Cockerham. 1984. Estimating F-statistics for the analysis of population structure. *Evolution* 38:1358–1370.

Wilkinson, L. 1990. SYSTAT 11Richmond, CA: SYSTAT Software, Inc.