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Genetic impacts of a commercial aquaculture lease on adjacent oyster populations

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ABSTRACT

Commercial aquaculture leases typically support dense aggregations of shellfish that may contribute to wild populations through their reproductive efforts. Aggregations are thought to facilitate successful spawning and fertilization, potentially producing large numbers of larvae that may recruit to wild populations. We investigated the reproductive contribution of cultured oysters (*Crassostrea virginica*) to wild populations around a commercial aquaculture lease in Stump Sound, North Carolina. Through the late 1990s, the lease was stocked with oysters that originated from remotely set larvae that were produced in a hatchery in Louisiana using oysters from the Gulf of Mexico, which are genetically distinguishable from North Carolina oysters. We sequenced 359-bp of the mitochondrial 16S ribosomal (16S) gene to identify oysters exhibiting mitochondrial DNA haplotypes characteristic of Gulf Coast-derived oysters. An initial evaluation in 2001 of oysters collected from seven natural beds in and around the lease site showed a significantly elevated frequency of oysters exhibiting the 16S Gulf Coast (GC) haplotype than typically observed in NC oyster populations. When the same sites around the lease were resampled in 2015, the 16S GC haplotype was not detected, suggesting the elevated frequency of this haplotype in 2001 was transient. A variant of the 16S GC haplotype, not observed in the aquaculture stock, was observed at higher frequency in the second sampling period relative to the first. The detection of a mitochondrial haplotype with Gulf Coast ancestry not associated with recent aquaculture activities suggests that the genetic impacts on wild oyster populations may vary with the fitness of cultured oysters to local environmental conditions.

1. Introduction

Aquaculture of marine molluscs is an important industry globally, accounting for an estimated > 21% of world aquaculture production in 2014 (FAO, 2016). Contributing significantly to this industry, global aquaculture production of oysters exceeded 5 million tons in 2014 (FAO, 2016). Along the east coast of North America from Canada to the Gulf of Mexico, it is the eastern oyster, *Crassostrea virginica* that is the primary oyster species supporting the rapidly growing culture industry. Coincident with this expansion of oyster aquaculture, there is increasing concern over the potential impacts of aquaculture in the near shore environments where it occurs. Potential impacts that have drawn concern include habitat modification, introduction of and/or spread of pathogens, alterations in nutrient cycles, depletion of suspended particulate matter, and alteration of genetic diversity of wild populations (Naylor et al., 2000; McKindsey et al., 2006; Bert, 2007; Forrest et al., 2009). With the possible exception of introductions and the spread of pathogens, these potential impacts may result in either positive or negative effects on surrounding environments. Evaluation of these potential impacts have focused primarily on the environmental impacts (e.g. Castel et al., 1989; Mallet et al., 2006; Forrest et al., 2009; Comeau et al., 2014; Testa et al., 2015), with little attention being focused on directly assessing the genetic impacts. This lack is partially attributable to the inability to distinguish the cultured oysters from their wild conspecifics efficiently and economically. The increasing dependence of oyster growers on hatchery-produced seed and the increasing use of selectively-bred broodstock in the production of that seed, raises the possibility that the genetic signature of the cultured oysters may be distinct from that of the wild oysters on surrounding reefs (FOC, 2006; McKindsey et al., 2006; Dumbauld et al., 2009; Forrest et al., 2009; NRC, 2010). This distinction, if it exists, sets the stage for evaluating the possibility that through reproduction of the cultured oysters, aquaculture could have some genetic impact on wild populations (Gaffney, 2006).

One such opportunity occurred in the 1990's when an oyster aquaculture operation stocked seed derived from Louisiana oysters into a floating culture system in Stump Sound, North Carolina (Fig. 1). Floating systems concentrate potentially reproductive animals that

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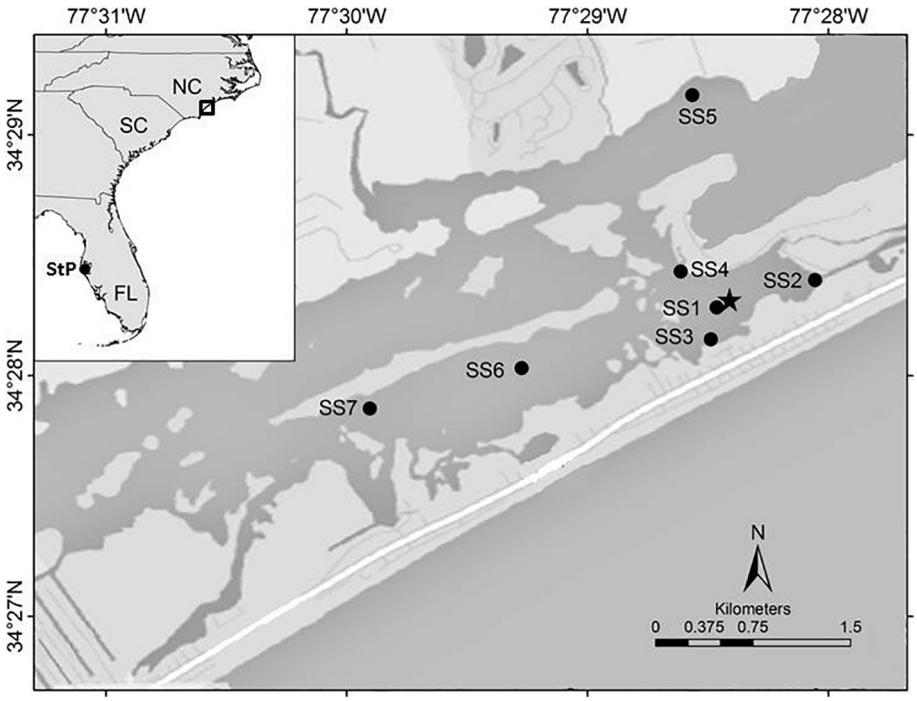

Fig. 1. Map of sample locations. Location of Stump Sound, North Carolina indicated by black square on map inset. Stump Sound sample sites 1 through 7 indicated by black circles and SS1-SS7, respectively. Louisiana lease site represented by black star. St. Petersburg, Florida sample site indicated by black circle and StP in map inset.

could produce large numbers of larvae that, in turn could recruit to the wild population. As cultured oysters are generally older than18 months of age when harvested, it is highly probable that they spawn before harvest. Anecdotal information provided by shellfish growers has suggested that there is increased larval settlement in and around aquaculture leases following the initiation of culture activities. This enhanced recruitment is commonly attributed to the spawning of the culture stock, although direct evidence of the magnitude or source of the "enhancement" is lacking. It is possible that the presence of high densities of cultured oysters modifies the local environment such that it is more conducive to oyster settlement, and thus the observation of higher recruitment near commercial operations is due to elevated recruitment of wild oysters, but again, direct evidence of this is lacking.

Oyster populations along the US Atlantic Coast and the Gulf of Mexico exhibit distinct differences in their mitochondrial genome, which can be detected using standard molecular genetic techniques (Reeb and Avise, 1990). Previous studies based on genetic analysis of the mitochondrial large (16S) ribosomal subunit gene have revealed subtle but distinct differences among regional populations of *C. virginica* (Ó Foighil et al., 1995; Wakefield, 1997). Major regional haplotypes can be identified by variation at two positions (sites 288 and 296, as reported in Ó Foighil et al., 1995). Populations north of the Chesapeake Bay are dominated by one haplotype ("North Atlantic") described by the following sequence for sites 288–296: 5′-TAAATTCTA-3′. South Atlantic populations ("South Atlantic") are dominated by oysters having 5′-GAAATTCTA-3′ and Gulf Coast populations ("Gulf") are dominated by oysters having 5′-GAAATTCTG-3′ (Ó Foighil et al., 1995; Wakefield, 1997). Maternal inheritance of mitochondrial DNA in *C. virginica* limits the detection of gametic contributions of males by mtDNA markers; however, *C. virginica* is a protandrous hermaphrodite that generally functions as male when they first mature after which a portion of the population transforms to reproduce as females with the proportion of females increasing in each size class as they grow (Coe, 1934; Galtsoff, 1964). All progeny of oysters reproducing as females (increasingly likely in subsequent years) will possess a regionally diagnostic mitochondrial haplotype.

The objective of this study was to determine the proportion of oysters collected from intertidal reefs near the aquaculture lease site that exhibited a Gulf mitochondrial haplotype. The presence of this distinctive genetic signature in oysters on the adjacent reefs would suggest that not only are oysters on the aquaculture leases spawning, but would also provide evidence of local recruitment. The proportion of oysters collected from nearby reefs exhibiting the Gulf mitochondrial haplotype was assessed three years and again 17 years after larvae originating from the Gulf of Mexico were remotely set in NC and stocked on the aquaculture lease. Sampling wild populations shortly after the aquaculture leases were initially stocked with Gulf oysters and then again 14 years later provides insight into short and long-term genetic impacts of oyster aquaculture on wild populations.

2. Materials and methods

2.1. Sampling

Throughout the late 1990s, J&B AquaFood grew out oysters resulting from seed or larvae obtained from a hatchery in Louisiana on a 1–1.5 acre plot in Stump Sound, North Carolina amid relayed oysters from Virginia Creek, NC. In 2001, 70–100 oysters were collected from seven reef sites in and around the commercial lease and sequenced for the mitochondrial 16S ribosomal subunit gene (Fig. 1). Additionally, approximately 50 oysters were sampled from the culture stock being held on the lease in fall of 2000 and analyzed to confirm the presence of the diagnostic 16S mtDNA Gulf haplotype. A sample of oysters was also obtained from the Gulf Coast (St. Petersburg, Florida) to further substantiate the robustness of our regional genetic identifications. A subset of five of the original seven sample sites in Stump Sound were revisited in 2015, and 50 oysters were collected from each site for genetic analysis. Two of the original sites (SS1 and SS5) were omitted from the sampling in 2015 due to lack of access (on private property). The oysters sampled from the natural reefs were measured (shell height), and the adductor muscle was removed and frozen at −80 °C until genetic analysis.

2.2. DNA extraction and sequencing

Total genomic DNA was extracted using the PureGene DNA extraction protocol (Qiagen, Inc.) with slight modification for use with small tissue weights. Resuspended DNA extracts were stored at −20 °C

Table 1
Distribution of 16S mtDNA haplotypes observed in oyster samples from Stump Sound, North Carolina, Louisiana lease, and St. Petersburg, Florida.

Region	Haplotype	Variable nucleotide site															
		6	7	10	11	12	15	16	20	27	42	46	54	143	154	155	159
South	Hap1	A	G	T	A	T	G	A	T	T	A	T	C	G	A	T	T
	Hap4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap24	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.
	Hap40	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap60	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.
	Hap54	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap55	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap70	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap2	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.
	Hap18	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.
	Hap43	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.
	Hap51	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap52	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.
	Hap53	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap56	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap57	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
	Hap58	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap59	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.
	Hap61	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.
	Hap35	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap62	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap63	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap64	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap14	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap79	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.
	Hap65	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.
	Hap66	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C
	Hap26	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.
	Hap67*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap68	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap69	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap81	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.
	Hap82	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap83	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap84	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap85	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
	Hap21	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap22	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap31	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap47	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap126	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap162	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Gulf	Hap13	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap29	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap50	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.
	Hap77	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap78	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap80	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
North	Hap7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Total																

Region	Haplotype	Variable nucleotide site															
		160	162	163	176	187	191	197	201	203	217	233	249	265	284	288	300
South	Hap1	T	T	T	T	A	T	G	G	T	C	A	T	G	T	A	T
	Hap4	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.
	Hap5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C
	Hap24	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap40	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.
	Hap60	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap54	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap55	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap70	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap18	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

(continued on next page)

Table 1 (*continued*)

Region	Haplotype	Variable nucleotide site															
		160	162	163	176	187	191	197	201	203	217	233	249	265	284	288	300
	Hap43	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap51	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
	Hap52	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap53	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.
	Hap56	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap57	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap58	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.
	Hap59	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap61	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
	Hap35	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap62	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap63	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.
	Hap64	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.
	Hap8	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.
	Hap14	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap79	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap65	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.
	Hap66	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap26	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap67*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap68	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap69	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap81	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.
	Hap82	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap83	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap84	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	C
	Hap85	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap21	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap22	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap31	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap47	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap126	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.
	Hap162	.	.	.	C	.	.	A	.	.	.	.	.	.	.	.	.
Gulf	Hap13	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap29	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap50	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hap77	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.
	Hap78	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.
	Hap80	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
North	Hap7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Total																

Region	Haplotype	Variable nucleotide site												Numbers of observed haplotypes			
														Stump Sound		Louisana Lease	St. Petersburg, FL
		304	305	306	308	310	311	312	318	325	327	334	336	2001	2015	2000	2001
South	Hap1	A	T	T	C	G	A	A	A	T	T	A	T	469	222		3
	Hap4	.	.	.	.	.	.	.	.	.	.	.	.	28	8		
	Hap5	.	.	.	.	.	.	.	.	.	.	.	.	2	1		
	Hap24	.	.	.	.	.	.	.	.	.	.	.	.	4	3		
	Hap40	.	.	.	.	.	.	.	.	.	.	.	.	1	1		
	Hap60	.	.	.	.	.	.	.	.	.	.	.		1	1		
	Hap54	.	.	.	.	.	G	.	.	.	.	.		1			
	Hap55	.	.	.	.	.	.	.	.	C	.	.	.	1			
	Hap70	.	.	.	.	.	.	G	.	.	.	.	.	1			
	Hap2	.	.	.	.	.	.	.	.	.	.	.	.	3			
	Hap18	.	.	.	.	.	.	.	.	.	.	.	.	7			
	Hap43	.	.	.	.	.	.	.	.	.	.	.	.	1			
	Hap51	.	.	.	.	.	.	.	.	.	.	.	.	1			
	Hap52	.	.	.	.	.	.	.	.	.	.	.	.	1			
	Hap53	.	.	.	.	.	.	.	.	.	.	.	.	1			
	Hap56	G	.	.	.	.	.	.	.	.	.	.	.	2			
	Hap57	.	.	.	.	.	.	.	.	.	.	.	.	1			
	Hap58	.	.	.	.	.	.	.	.	.	C	.	.	1			
	Hap59	.	.	.	.	.	.	.	.	.	.	.	.	1			
	Hap61	.	.	.	.	.	.	.	.	.	.	.	.	1			
	Hap35	.	.	.	.	.	.	.	.	.	.	.	.	1			

(continued on next page)

Table 1 (*continued*)

Region	Haplotype	Variable nucleotide site												Numbers of observed haplotypes			
														Stump Sound		Louisana Lease	St. Petersburg, FL
		304	305	306	308	310	311	312	318	325	327	334	336	2001	2015	2000	2001
	Hap62	.	C	.	.	.	.	.	.	.	.	.	.	1			
	Hap63	.	.	.	.	.	.	.	.	.	.	.	.	1			
	Hap64	.	.	.	.	.	.	.	.	.	.	.	.	2			
	Hap8	.	.	.	.	.	.	.	.	.	.	.	.	1			
	Hap14	.	.	.	.	.	.	.	.	.	.	.	.	1			
	Hap79	.	.	.	.	.	.	.	.	.	.	.	.	1			
	Hap65	.	.	.	.	.	.	.	.	.	.	.	.	1			
	Hap66	.	.	.	.	.	.	.	.	.	.	.	.	1			
	Hap26	.	.	.	.	.	.	.	.	.	.	.	.	1			
	Hap67*	.	.	.	.	A	.	.	.	.	.	.	.	1			
	Hap68	.	.	.	.	.	.	.	.	.	.	G	.	1			
	Hap69	.	.	.	.	.	.	.	.	.	.	.	.	1			
	Hap81	.	.	.	.	.	.	.	.	.	.	.	.	2			
	Hap82	.	.	C	.	.	.	.	.	.	.	.	.	1			
	Hap83	.	.	.	.	.	.	.	.	.	.	.	.	1			
	Hap84	.	.	.	.	.	.	.	.	.	.	.	.	1			
	Hap85	.	.	.	.	.	.	.	.	.	.	.	.	1			
	Hap21	.	.	.	.	.	.	.	.	.	.	.	.		1		
	Hap22	.	.	.	T	.	.	.	.	.	.	.	.		1		
	Hap31	.	.	.	.	.	.	.	.	.	.	.	.		1		
	Hap47	.	.	.	.	.	.	.	.	.	.	.	G		1		
	Hap126	.	.	.	.	.	.	.	.	.	.	.	.		1		
	Hap162	.	.	.	.	.	.	.	.	.	.	.	.		1		
Gulf	Hap13	.	.	.	.	.	G	.	G	.	.	.	.	6	6		
	Hap29	.	.	.	.	.	.	.	G	.	.	.	.	4		40	32
	Hap50	.	.	.	.	.	.	.	G	.	.	.	.			1	
	Hap77	.	.	.	.	.	.	.	G	.	.	.	.				1
	Hap78	.	.	.	.	.	.	.	G	.	.	.	.				1
	Hap80	.	.	C	.	.	.	.	G	.	.	.	.				1
North	Hap7	.	.	.	.	T	.	.	.	.	.	.	.		1		
	Total													558	249	41	38

until polymerase chain reaction (PCR) was performed. Amplification of a 400 base pair fragment of the mitochondrial large (16S) ribosomal subunit was performed with PCR using a 16S ribosomal subunit gene primer (16SAR) (Palumbi et al., 1991) and an oyster specific primer (16SOBR) (Wakefield, 1997). Reactions of 25 μL total volume, containing 1 μL of DNA template, 5 μL 5× PCR Buffer, 0.4 μM of each primer, 0.2 mM dNTPs, 1.5 mM MgCl2, and 0.04 U/μL GoTaq® Flexi DNA Polymerase (Promega), were conducted on either a MJ Research PTC-100 or a Techne 3Prime Thermal Cycler. Cycling parameters consisted of an initial denaturing step of 5 min at 94 °C, followed by 40 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min, and a final extension of 72 °C for 5 min. Polymerase chain reaction products were run on 1% agarose gels and stained with ethidium bromide. Successful amplification products were used in a cycle sequencing protocol using BigDye™ Dye Terminator chemistry v3.1 (Applied Biosystems, Inc.). The products were purified with Sephadex® columns and sequenced on an Applied Biosystems 3130XL capillary DNA sequencer. Sequences were visually scored for accuracy and polymorphisms using Sequencher v5.3 (Gene Codes, Inc.).

DNA sequence analysis of a more variable region of the *C. virginica* mitochondrial genome, ND2 (partial)-tRNA^{Arg}-tRNA^{His}-ND4 (partial) (ND4), has revealed separation of North Atlantic, South Atlantic, and Gulf Coast haplogroups by an insertion/deletion polymorphism occurring in a noncoding region between tRNA^{Arg} and tRNA^{His} at nucleotides 11,411–11,412 in GenBank Accession AY905542 (Gaffney, 2006). The three major haplogroups are distinguished by variation among nucleotides 11405-11412 in AY905542: North Atlantic – 5′-GGGTAAA—C-3′, South Atlantic – 5′-GGGTAAA–AC-3′, Gulf Coast – 5′-GGGCAAAACAC-3′ (Gaffney, 2006). This additional genetic analysis was used to strengthen our identification of oysters as the potential offspring of the Louisiana lease stock. All oysters exhibiting a 16S mtDNA Gulf haplotype were amplified with PCR primers for ND4 as described in Milbury (2007) and sequenced as described above. From the 790 bp ND4 product, 750 base pairs were aligned with nucleotides 11273-12019 in AY905542 to determine the correct haplogroup assignment for each sample.

2.3. Data analysis

Mitochondrial 16S ribosomal subunit gene sequences were edited (primer sequence removed), 359 base pairs were aligned using BioEdit software (Hall, 1999), and individuals exhibiting identical haplotypes were identified using Arlequin v3.5.2.2 (Excoffier and Lischer, 2010). In these alignments, the diagnostic positions were at 310 and 318 of the sequenced fragment. Standard genetic indices, including haplotype (h) and nucleotide diversity (π), and assessment of geographic and temporal genetic structure via analysis of molecular variance (AMOVA) (Excoffier et al., 1992) were also calculated in Arlequin. Tajima's D test (Tajima, 1989) was implemented in Arlequin to test the null hypothesis of neutral evolution of the 16S mtDNA gene. Values of D differing significantly from zero can indicate selection, population expansion, or bottlenecks (Tajima, 1989).

Potential spatial and temporal population subdivision for 16S mtDNA was assessed using Φ_{ST}, which takes into account the relationships between haplotypes based on molecular distance (Excoffier et al., 1992). Using Arlequin, hierarchical AMOVAs were performed to estimate the amount of genetic variability within and among four groups – 2001 Stump Sound, 2015 Stump Sound, Louisiana lease, and St. Petersburg. Differences in pairwise Φ_{ST} and haplotype frequency values between spatial and temporal groups were tested via

permutation (10,000 permutations) and exact tests (100,000 steps in a Markov chain with 10,000 step dememorization) implemented in Arlequin. For multiple comparisons of pairwise Φ_{ST} and exact tests, significant differences were determined using the modified false discovery rate (FDR) adjusted P-values based on (Benjamini and Yekutieli, 2001).

Resampling statistics (Resampling Stats, Arlington, VA) were used to assess whether the frequencies of Gulf Coast haplotypes in Stump Sound were elevated relative to the frequencies observed in a statewide (12 locations from Wanchese in northern Pamlico Sound to the Cape Fear River) survey (Sackett, 2002). To test this, 7 collections of 80 oysters from this study was simulated 500 times using the data from the statewide survey as the base population.

3. Results

3.1. 16S mtDNA polymorphism

In 2001, 558 oysters from seven natural beds in Stump Sound, NC were genetically typed for a fragment of the mitochondrial 16S ribosomal subunit gene. Forty distinct 16S mtDNA haplotypes were observed (Table 1). The vast majority (84%, n = 469) of the oysters exhibited a single haplotype (Hap1, Table 1), which was identical to the South Atlantic (SA) haplotype identified in previous studies (Ó Foighil et al., 1995; Wakefield, 1997). An additional 14.2% (n = 79) were variants of the SA haplotype, exhibiting the characteristic substitution, as well as one or two additional substitutions elsewhere in the fragment (Table 1). A small percentage (0.72%, n = 4) exhibited the characteristic haplotype of the Gulf Coast (GC) (Hap29, Table 1). Among the seven natural beds sampled, 2 individuals were found to have the GC haplotype in SS2 and one individual each in SS6 and SS7. This GC haplotype was the dominant haplotype found in the Louisiana lease stock (98%, Table 1), as well as the sample collected from a natural reef in St. Petersburg, Florida (84%, Table 1). The remaining 1.1% (n = 6) of the samples collected in Stump Sound in 2001 exhibited a variant of the GC haplotype (Hap13, Table 1) that had the diagnostic substitution at position 318 and an additional substitution at position 311. This 16S GC-variant haplotype was not observed in either the Louisiana lease or St. Petersburg samples.

Five of the seven previously sampled natural beds in Stump Sound were revisited in 2015, and 249 oysters were collected and genotyped for the 16S mtDNA fragment. Fourteen distinct 16S mtDNA haplotypes were observed among these samples, seven of which were not observed in 2001 (Table 1). Similar to 2001, the majority of the oysters (89.2%, n = 222) exhibited the common SA haplotype (Hap1), with an additional 8% (n = 20) of the oysters exhibiting variants of the SA haplotype (Table 1). One oyster (0.4%) from Site 6 exhibited the North Atlantic haplotype (Hap7) identified by Wakefield (1997). The remaining 2.4% (n = 6) of samples collected from Stump Sound in 2015 exhibited the GC-variant haplotype (Hap13, Table 1). None of the samples collected in 2015 exhibited the 16S GC haplotype found in the Louisiana lease stock. Comparison of the 2015 data to 2001 data shows a disappearance of the 16S GC haplotype (Hap29) but an overall increase (1.3%, p = 0.07) in the frequency of the 16S GC-variant haplotype (Hap13).

Mitochondrial 16S mtDNA intrapopulation diversity indices are shown in Table 2. The numbers of haplotypes and haplotype frequencies varied among years and populations sampled (Table 2). Haplotype and nucleotide diversities also varied among years and populations, with considerably lower values found in the Louisiana lease than in Stump Sound and St. Petersburg populations (Table 2). The results of Tajima's *D*-test for Stump Sound and St. Petersburg populations showed significant ($P < 0.05$) negative values (Table 2), suggesting recent population expansion. The negative Tajima's *D* value for the Louisiana lease was not significantly different from zero (Table 2).

The outcome of the 500 simulated studies (7 samplings of 80 oysters in 2001) is shown in Fig. 2. The probability of sampling four individuals (out of 560 oysters) with the 16S GC haplotype, as was seen in the 2001analysis, was low (P = 0.014).

3.2. Genetic differentiation

A hierarchical AMOVA of 16S mtDNA sequence data revealed significant genetic variation among samples from Stump Sound 2001, Stump Sound 2015, Louisiana lease, and St. Petersburg ($\Phi_{CT} = 0.4694$, $P < 0.0001$). No significant population differentiation was detected between samples collected from Stump Sound in 2001 and 2015 ($\Phi_{CT} = -0.00004$, P = 0.4781). Comparison of Louisiana lease and St. Petersburg oysters revealed significant genetic differences between the two populations ($\Phi_{ST} = 0.0255$, $P < 0.05$). No significant spatial genetic structure was detected throughout the Stump Sound sites sampled in 2001 ($\Phi_{ST} = 0.0018$, P = 0.2070) or in 2015 ($\Phi_{ST} = 0.0006$, P = 0.4100). Significant temporal genetic variation between samples collected in 2001 and 2015 from Stump Sound populations was only detected at Site 7 (Table 2).

Pairwise Φ_{ST} tests of genetic differentiation and exact tests of haplotype frequency of 16S mtDNA sequence data revealed significant differences between Stump Sound populations and the Louisiana lease oysters, and Stump Sound populations and St. Petersburg oysters (Tables 3, 4). After FDR correction of the P-value, no significant genetic differences were detected among Stump Sound populations or between Louisiana lease and St. Petersburg oysters via pairwise Φ_{ST} or exact tests (Tables 3, 4).

3.3. ND4 mtDNA haplotype analysis

The ND4 mtDNA sequence analysis of the 16 oysters collected from Stump Sound in 2001 and 2015 exhibiting a 16S GC or GC-variant haplotype showed all samples exhibited the insertion expected of Gulf Coast oysters. All of the Louisiana lease and St. Petersburg oysters exhibiting 16S GC haplotypes also exhibited the insertion expected of Gulf Coast oysters. Of the four oysters collected in 2001 exhibiting the 16S GC haplotype (Hap29), three oysters exhibited ND4 haplotypes identical to the most common ND4 haplotype found in the Louisiana lease and St. Petersburg oysters, with the remaining sample having one additional nucleotide substitution elsewhere in the fragment. Eleven of the 12 oysters collected from Stump Sound in 2001 and 2015 exhibiting the 16S GC-variant haplotype (Hap13) exhibited identical ND4 haplotypes that had several substitutions different from the oysters exhibiting the 16S GC haplotype and the Louisiana lease and St. Petersburg oysters, with the remaining sample having one additional substitution.

4. Discussion

Few studies have evaluated the effects of bivalve aquaculture on the genetics of wild populations. Previous studies examining the effects of clam aquaculture on the genetics of wild clam populations in South Carolina and Florida showed varying results from negligible to substantial depending on the source of the cultured seed and the timing of sampling in regard to aquaculture activities (Metzner-Roop, 1994; Arnold et al., 2004; Arnold et al., 2009). The present study investigated the reproductive contribution of cultured oysters (*Crassostrea virginica*) sourced from a hatchery in Louisiana to wild populations around a commercial aquaculture lease in Stump Sound, North Carolina at two different times – during aquaculture activities and then again 14 years later. The results show a potential enhancement of a Gulf Coast mitochondrial haplotype in oyster reefs in Stump Sound in 2001 that could have been attributed to spawning activity of the Louisiana stock maintained on J&B AquaFood leases in the late 1990s; however, reassessment of 16S mtDNA haplotypes in wild populations 14 years later suggests that this enhancement was transient. A variant of the 16S Gulf Coast haplotype not observed in the culture population was observed in increasing frequency in wild populations over the sampling period.

Table 2
Haplotype frequencies, number of haplotypes, haplotype diversity, nucleotide diversity, Tajima's *D* values and corresponding P-values, Φ_{ST} values and corresponding P-values from temporal AMOVAs for samples collected from Stump Sound, North Carolina sample sites 1 through 7 (SS1-SS7) in 2001 and 2015, Louisiana lease in 2000, and St. Petersburg, Florida in 2001.

	SS1	SS2		SS3		SS4		SS5	SS6		SS7		Louisiana lease	St. Petersburg
Haplotype	2001	2001	2015	2001	2015	2001	2015	2001	2001	2015	2001	2015	2000	2001
Hap1	0.8904	0.8000	0.8600	0.9079	0.8600	0.8667	0.9200	0.8293	0.8810	0.8980	0.7347	0.9200		0.0789
Hap4	0.0548	0.0571	0.0600	0.0526	0.0400	0.0133	0.0400	0.0244	0.0476	0.0204	0.0918			
Hap5			0.0200			0.0133			0.0119					
Hap24	0.0137	0.0143			0.0200	0.0133	0.0200	0.0122				0.0200		
Hap40				0.0132			0.0200							
Hap60						0.0133				0.0204				
Hap54	0.0137													
Hap55	0.0137													
Hap70	0.0137													
Hap2		0.0143				0.0133					0.0102			
Hap18		0.0143				0.0267		0.0244			0.0204			
Hap43		0.0143												
Hap51		0.0143												
Hap52		0.0143												
Hap53		0.0143												
Hap56				0.0132				0.0122						
Hap57				0.0132										
Hap58						0.0133								
Hap59						0.0133								
Hap61						0.0133								
Hap35								0.0122						
Hap62								0.0122						
Hap63								0.0122						
Hap64								0.0122			0.0102			
Hap8								0.0122						
Hap14								0.0122						
Hap79								0.0122						
Hap65									0.0119					
Hap66									0.0119					
Hap26											0.0102			
Hap67*											0.0102			
Hap68											0.0102			
Hap69											0.0102			
Hap81											0.0204			
Hap82											0.0102			
Hap83											0.0102			
Hap84											0.0102			
Hap85											0.0102			
Hap21												0.0200		
Hap22												0.0200		
Hap31			0.0200											
Hap47					0.0200									
Hap126												0.0200		
Hap162										0.0204				
Hap13		0.0143	0.0400		0.0600			0.0122	0.0238	0.0204	0.0204			
Hap29		0.0286							0.0119		0.0102		0.9756	0.8421
Hap50													0.0244	
Hap77														0.0263
Hap78														0.0263
Hap80														0.0263
Hap7										0.0204				
Total	73	70	50	76	50	75	50	82	84	49	98	50	41	38
Number of haplotypes	6	11	5	5	5	10	4	13	7	6	16	5	2	5
Haplotype diversity (*h*)	0.2062	0.3594	0.2596	0.1747	0.2596	0.2501	0.1543	0.3135	0.2232	0.1956	0.4540	0.1551	0.0488	0.2902
Nucleotide diversity (π)	0.0006	0.0012	0.0010	0.0005	0.0011	0.0009	0.0004	0.0010	0.0008	0.0008	0.0016	0.0004	0.0001	0.0009
Tajima's D	−1.780	−2.152	−1.660	−1.624	−1.580	−2.160	−1.578	−2.386	−1.887	−2.017	−2.216	−1.862	−1.122	−1.637
P-value	0.0065	0.0007	0.0214	0.0146	0.0238	0.0008	0.0200	0.0001	0.0045	0.0014	0.0006	0.0040	0.1285	0.0192
Temporal Φ_{ST}		−0.0098		0.0162		−0.0037			−0.0091		0.0246			
P-value		0.9535		0.1042		0.6816			0.9182		0.0108			

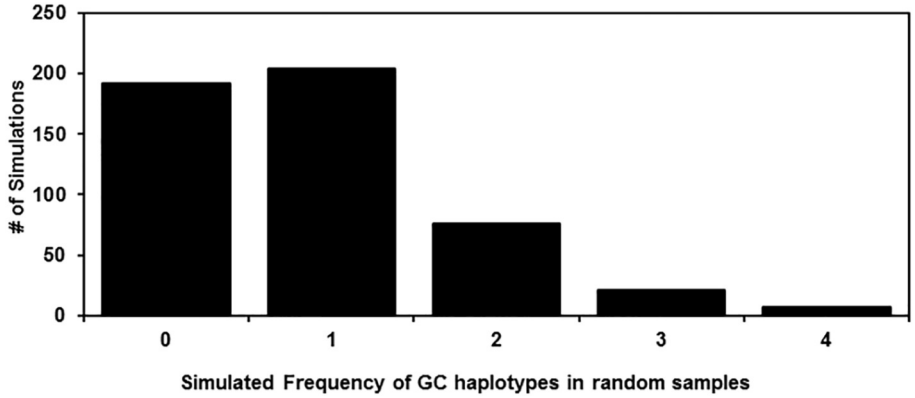

Fig. 2. Number of individuals exhibiting 16S GC haplotype in simulated samplings (7 independent samples of 80 individuals repeated 500 times) of a population exhibiting the observed statewide frequency of 0.18%.

Table 3
Pairwise Φ_{ST} values (below the diagonal) and P-values (above the diagonal). Significant values ($P < 0.05$) after FDR adjustment are indicated in bold. Population abbreviations: 01SS1-01SS7 = 2001 Stump Sound sample sites 1 through 7, 15SS2-15SS7 = 2015 Stump Sound sample sites 2, 3, 4, 6, 7, Louisiana lease = samples collected from J&B AquaFood, StP = St. Petersburg, Florida.

	01SS1	01SS2	01SS3	01SS4	01SS5	01SS6	01SS7	15SS2	15SS3	15SS4	15SS6	15SS7	Louisiana lease	StP
01SS1	–	0.5758	0.8569	0.3114	0.6144	0.5915	0.1490	0.5195	0.2034	0.9650	0.7998	0.1544	**< 0.0001**	**< 0.0001**
01SS2	−0.0031	–	0.3945	0.3790	0.6182	0.9519	0.5514	0.9531	0.7445	0.6839	0.9020	0.1050	**< 0.0001**	**< 0.0001**
01SS3	−0.0070	−0.0002	–	0.2723	0.4708	0.4462	0.1105	0.3607	0.1080	0.9687	0.5828	0.0619	**< 0.0001**	**< 0.0001**
01SS4	0.0016	0.0007	0.0030	–	0.9316	0.1620	0.0299	0.2079	0.0625	0.6824	0.5619	0.3585	**< 0.0001**	**< 0.0001**
01SS5	−0.0019	−0.0022	−0.0010	−0.0046	–	0.4750	0.0406	0.4761	0.3186	0.9545	0.9555	0.7221	**< 0.0001**	**< 0.0001**
01SS6	−0.0031	−0.0069	−0.0005	0.0047	−0.0009	–	0.2960	0.9707	0.6639	0.5793	0.9133	0.1177	**< 0.0001**	**< 0.0001**
01SS7	0.0047	−0.0022	0.0072	0.0129	0.0111	0.0018	–	0.6799	0.2700	0.1566	0.3561	0.0106	**< 0.0001**	**< 0.0001**
15SS2	−0.0032	−0.0098	0.0019	0.0057	−0.0007	−0.0120	−0.0051	–	0.9999	0.6820	0.9340	0.1247	**< 0.0001**	**< 0.0001**
15SS3	0.0069	−0.0066	0.0162	0.0135	0.0028	−0.0061	0.0032	−0.0126	–	0.3328	0.6131	0.1847	**< 0.0001**	**< 0.0001**
15SS4	−0.0106	−0.0044	−0.0112	−0.0037	−0.0064	−0.0029	0.0067	0.0004	0.0092	–	0.7063	0.7469	**< 0.0001**	**< 0.0001**
15SS6	−0.0069	−0.0079	−0.0044	−0.0018	−0.0071	−0.0091	0.0010	−0.0103	−0.0048	−0.0072	–	0.1383	**< 0.0001**	**< 0.0001**
15SS7	0.0081	0.0076	0.0117	0.0015	−0.0024	0.0090	0.0246	0.0157	0.0176	0.0001	0.0039	–	**< 0.0001**	**< 0.0001**
Louisiana lease	**0.8657**	**0.7629**	**0.8817**	**0.8177**	**0.7892**	**0.8081**	**0.6838**	**0.8107**	**0.7896**	**0.9017**	**0.8442**	**0.9009**	–	0.0269
StP	**0.7748**	**0.6680**	**0.7922**	**0.7295**	**0.7036**	**0.7192**	**0.6039**	**0.6993**	**0.6764**	**0.7917**	**0.7330**	**0.7913**	0.0255	–

The initial assessment of recruitment from the aquaculture lease to wild populations in 2001 revealed a considerably higher percentage of the 16S GC haplotype within Stump Sound than among other North Carolina oyster populations sampled the same year. Although only four of the 558 (0.72%) oysters sampled in Stump Sound exhibited the 16S GC haplotype, a statewide survey of intertidal oyster reefs conducted by Sackett (2002) examining 564 oysters collected from 12 other sites along the NC coast from Wanchese to the Cape Fear River uncovered only a single oyster exhibiting the 16S GC haplotype (1 oyster collected from Ocracoke, 0.18%). Subsequent surveys of wild oyster populations in NC from 2002 to 2009 revealed varying, but low, frequencies of oysters exhibiting the 16S GC haplotype: 2002 – 1 of 285 (0.35%); 2003 – 1 of 447 (0.22%); 2004 – 0 of 335; 2005 – 0 of 203; 2006 – 1 of 338 (0.26%); 2009 – 3 of 869 (0.34%). In no case did the frequencies approach that observed in Stump Sound in 2001(0.72%) (Varney et al., 2016).

The observed contribution of the 16S GC haplotype in 2001, while modest, reflects a substantial contribution to the wild population in Stump Sound when compared to studies of restoration efforts utilizing the same molecular marker. Through analysis of 16S mtDNA haplotypes, Milbury et al. (2004) evaluated the success of a restoration effort in the Choptank River which is a tributary of the Chesapeake Bay in Maryland. Four million Louisiana oysters were deployed in 1997, and of the 3545 spat collected from 1999 to 2001, only 3 (0.08%) exhibited a Gulf Coast mtDNA haplotype (Milbury et al., 2004). The low overall impact of this restoration project was due in part to reduced numbers of planted oysters surviving during the initial 3 year period after deployment (Milbury et al., 2004).

Monitoring restoration efforts or aquaculture impacts on wild populations of *C. virginica* with maternally inherited mitochondrial markers shortly after initiation may result in an underestimate of the genetic contribution. Given the protandrous hermaphroditism exhibited in *C. virginica*, it is likely that many of the stocked oysters spawn as males during their first year or two and the gametic contribution from those oysters to the wild population is not detectable using the methods employed here. Additionally, as NC oysters typically reach harvest size in 18 to 24 months, cultured oysters may be harvested before reproducing as females limiting contributions that would be detected by methods used in this study. In 2001, however, there were ~70,000 oysters of Gulf origin on the lease, with similar numbers having been stocked for the previous 3 years. Histological examination (monthly from June to November 2001, N = 12) of those oysters revealed the Gulf oysters to be reproductively active and mostly female (60–80%).

To assess the persistence of this modest genetic impact on wild populations in Stump Sound after an extended period of time, five of the seven previously sampled wild reefs were revisited 14 years after initial sampling was conducted and subsequent changes in the frequency of the 16S GC haplotype were evaluated. When the wild oyster populations in Stump Sound were resampled in 2015, the 16S GC haplotype was not detected, suggesting the elevated frequency of this haplotype in 2001 was transient. This transience could be a result of Gulf oysters not being adapted to the local environmental conditions. Reciprocal transplant experiments have shown that oysters exhibit better fitness when in their home environment (Burford et al., 2014), but other studies have not observed such local adaptation (Hughes et al., 2017). If introduced genotypes are deleterious in the new

Table 4
P-values of exact tests. Significant values ($P < 0.05$) after FDR adjustment are indicated in bold. Population abbreviations: 01SS1-01SS7 = 2001 Stump Sound sample sites 1 through 7, 15SS2-15SS7 = 2015 Stump Sound sample sites 2, 3, 4, 6, 7, Louisiana lease = samples collected from J&B AquaFood, StP = St. Petersburg, Florida.

	01SS1	01SS2	01SS3	01SS4	01SS5	01SS6	01SS7	15SS2	15SS3	15SS4	15SS6	15SS7	Louisiana lease	StP
01SS1	–													
01SS2	0.4769	–												
01SS3	0.8506	0.2280	–											
01SS4	0.4462	0.4868	0.1425	–										
01SS5	0.6807	0.7449	0.6707	0.9810	–									
01SS6	0.6095	0.6083	0.6452	0.1855	0.4506	–								
01SS7	0.4551	0.9719	0.3628	0.1026	0.3445	0.6053	–							
15SS2	0.3969	0.8684	0.3086	0.3683	0.8218	0.8920	0.9294	–						
15SS3	0.2609	0.7743	0.1329	0.3011	0.8436	0.6624	0.8029	1.0000	–					
15SS4	0.9633	0.9526	0.9108	0.9110	0.9861	0.7795	0.8483	0.4797	0.4292	–				
15SS6	0.4103	0.7411	0.3075	0.8364	0.9293	0.6367	0.6327	0.7008	0.7157	0.7786	–			
15SS7	0.2267	0.3340	0.0732	0.8520	0.8678	0.1548	0.1196	0.0740	0.1434	0.7427	0.7416	–		
Louisiana lease	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	–	
StP	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	0.0252	–

environment, it is possible any introduction would be short-lived with no evidence of the introduction after the source of the genotypes was removed (Arnold et al., 2004). In cases where culture stocks are locally sourced, the enhancement effect that is caused by reproductive activity of the cultured stock may be longer lasting.

Although 16S mtDNA haplotype frequencies varied between 2001 and 2015 in Stump Sound, no significant spatial or temporal genetic variation was detected among wild oyster populations in this area. Temporal genetic stability of *C. virginica* populations within Stump Sound is consistent with the results of Varney et al. (2016) that showed little temporal genetic variation over a nine-year sampling period among 36 oyster populations along the NC Coast. The planktonic larval phase, high fecundity, and large and stable population sizes of *C. virginica*, as well as the close proximity of sampling sites in Stump Sound, may maintain the genetic stability of populations in this area (Toonen and Grosberg, 2011).

Significant genetic differences were observed in an AMOVA between the Louisiana lease and St. Petersburg populations sampled. Although significant genetic differentiation has been noted among *C. virginica* populations throughout the Gulf of Mexico using mitochondrial and nuclear DNA markers (Varney et al., 2009), the genetic differences between these samples may be the result of hatchery selection on the Louisiana lease oysters. Hatchery stocks of shellfish have been shown to exhibit reduced allelic diversity and lowered heterozygosity due to propagation of offspring from a small number of parents (Gaffney et al., 1992; Hedgecock et al., 1992; Gaffney, 2006). The cultured oysters originating from a hatchery in Louisiana analyzed in this study exhibited considerably lower haplotype and nucleotide diversities and a reduced number of haplotypes than the wild populations.

This study revealed the presence of a variant of the known 16S Gulf Coast haplotype in wild North Carolina oysters that was not detected in the Louisiana lease or St. Petersburg samples. This 16S GC-variant haplotype occurred at a notable frequency (1.1%) in Stump Sound in 2001, and increased in frequency (2.4%) in the 2015 samples. Although the subtle increase of 1.3% in the frequency of the 16S GC-variant between 2001 and 2015 is difficult to interpret, there are two likely sources for this variant. First, it may represent a contribution from earlier stockings that were genetically different from the oysters held on the lease at the time this study was conducted. Stocks cultured in other years may have exhibited different haplotypes and the 16S GC-variant observed in this study could reflect the reproductive contribution of these earlier efforts. Alternatively, this 16S GC-variant haplotype may occur at low frequency in southeastern North Carolina estuaries and thus its presence in Stump Sound may have little to do with the ongoing culture efforts. The 16S GC-variant haplotype has also been observed in other wild NC oyster populations at varying frequencies ranging from 1.1% to 4.2% (Varney et al., 2016). The apparent increase could be a result of temporal fluctuations in a native haplotype.

As oyster mitochondrial DNA is inherited maternally and not subject to crossing-over, progeny inherit it unaltered from the mother with variation introduced by new mutations. The phylogeographic pattern of haplotype variation in *C. virginica* 16S mtDNA has been confirmed in the more variable mitochondrial ND2-ND4 (ND4) region (Gaffney, 2006). Analysis of ND4 mtDNA sequence data confirms the presence of the ND4 Gulf regional signature in individuals exhibiting both the 16S GC and GC-variant haplotypes. Additional variable sites in ND4 sequences separate individuals exhibiting the 16S GC-variant haplotype from those exhibiting the 16S GC haplotype. All Stump Sound samples exhibiting the 16S GC haplotype had ND4 sequences identical to the most common ND4 haplotype found in Louisiana lease and St. Petersburg samples, with the exception of one Stump Sound individual with an additional unique single base change. Stump Sound samples exhibiting the 16S GC-variant haplotype had ND4 sequences that were identical to one another (with the exception of one individual with one additional unique single base change), but had several polymorphisms

differentiating them from the Louisiana lease and St. Petersburg samples.

Comparison of ND4 sequences exhibited in Stump Sound individuals with other *C. virginica* ND4 sequences available in GenBank revealed similarities between oysters collected in North Carolina and Texas. Stump Sound individuals exhibiting the 16S GC and GC-variant haplotypes had ND4 sequences almost identical to haplotypes exhibited in oysters collected along the Texas coast. Alignment of the Texas ND4 haplotypes shows separation into two groups based on several nucleotide variations (R.D. Overath, unpublished data). Most of the 112 Texas ND4 haplotypes available in GenBank align with the ND4 sequences exhibited by Stump Sound individuals exhibiting the 16S GC haplotype; however, Stump Sound individuals exhibiting the 16S GC-variant haplotype had ND4 sequences almost identical to five unique haplotypes exhibited in oysters collected from Galveston Bay, Matagorda Bay, and San Antonio Bay in the northwestern Gulf of Mexico (R.D. Overath, personal communication). Although 16S mtDNA sequence data are not available for the Texas individuals, the similarity of the ND4 mtDNA sequences with individuals exhibiting the16S GC-variant indicate that this variant may have originated in Gulf populations. The source of this haplotype may have been Gulf oysters stocked on this lease in previous years, with the increase in frequency observed in wild oyster populations possibly a result of successful reproduction of earlier introductions.

As bivalve aquaculture production becomes more prevalent along coasts, the genetic impacts on wild populations should be investigated. Depending on the source of the aquaculture seed, successful reproduction of culture stocks may introduce new genotypes into an area potentially changing the genetic composition of wild populations (Gaffney, 2006). The results of this study suggest that oysters on aquaculture leases can contribute to natural recruitment, effectively introducing new genotypes to wild populations. While most aquaculture operations are not designed to augment wild populations, the small but detectable presence of oysters bearing mitochondrial haplotypes consistent with a Gulf Coast ancestry on the wild reefs points to the potential for enhancement in and around aquaculture operations during aquaculture activities. The detection of a mitochondrial haplotype with Gulf Coast ancestry not associated with recent culture activities suggests that the longevity of genetic impacts on wild populations vary with fitness of cultured oysters to the environment. Additionally, it remains to be determined whether the contribution traceable to the culture oysters represents supplementation or displacement of natural recruitment.

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