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Contemporary and historical influences on the genetic structure of the estuarine-dependent Gulf killifish *Fundulus grandis*

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ABSTRACT: In comparison to species living in open marine environments, estuarine-dependent species are expected to exhibit stronger genetic population structure due to dispersal limitations. Estuarine habitats are relatively transitory on geological time scales; thus, populations may not be at migration–drift equilibrium, which could confound estimates of current day gene flow or selection. We used 8 nuclear microsatellite loci to investigate the genetic structure of the estuarine Gulf killifish *Fundulus grandis* across 10 populations along the northwestern and northeastern Gulf of Mexico. Patterns of isolation by distance, spatial autocorrelation, and assignment tests indicate that dispersal is limited and occurs primarily between neighboring sites. Principal component analysis and Bayesian clustering revealed evidence for genetic discontinuities located in Mobile Bay and western Florida which are near hypothesized biogeographical boundaries. There was also a significant negative relationship between genetic diversity and latitude, a pattern consistent with the presence of hypothesized refugia in the southern Gulf regions during the Pleistocene that later recolonized the northern Gulf. Results suggest that populations may be at or near migration–drift equilibrium at a regional scale (e.g the western Gulf), but that dispersal barriers and potential historical signatures on population structure will need to be taken into consideration at larger spatial scales.

KEY WORDS: Genetic structure · Isolation by distance · Equilibrium · Estuary · Microsatellites · Gulf of Mexico

INTRODUCTION

Marine fish inhabiting the open ocean often exhibit low genetic population differentiation due to their large effective population sizes and high dispersal potential (Ward et al. 1994, Waples 1998, Grosberg & Cunningham 2001). In contrast, species that utilize near-shore habitats such as estuaries and lagoons are expected to exhibit more genetic structure than strictly marine species due to the discontinuous nature of these habitats and their relative isolation from ocean currents (Bilton et al. 2002, Watts & Johnson 2004). Decreased levels of genetic exchange combined with variable environmental conditions such as salinity and temperature should enhance the genetic divergence and local adaptation of populations in estuarine areas (e.g. Lemaire et al. 2000, Beheregaray & Sunnucks 2001).

At migration–drift equilibrium, dispersal between these near-shore habitats should approximate a model in which individuals are most likely to disperse to neighboring populations along a single dimension, thereby producing a genetic pattern of isolation by distance (IBD) (Kimura & Weiss 1964, Crow & Aoki 1984, Gold & Turner 2002, Burridge et al. 2004). The transitory distribution of these estuarine habitats on geological time scales, however, may prevent these fish populations from attaining migration–drift equilibrium

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and prevent the formation of IBD genetic structure (Chenoweth & Hughes 2003, Pampoulie et al. 2004, Durand et al. 2005). Determining the scale over which migration–drift equilibrium may exist in these systems is important since equilibrium is assumed when estimating migration rates using estimators of population subdivision such as Wright's fixation index (F_{ST}), or when inferring a signal of natural selection relative to neutral expectations (e.g. Whitehead & Crawford 2006, Duvernell et al. 2008).

The Gulf of Mexico's coastline and its connections to the Atlantic have undergone many changes. During the Pliocene (>1.8 million years before present [mybp]), sea levels were much higher, much of the Florida landmass was underwater, and the connection between the Atlantic Ocean and Gulf of Mexico was more expansive. During the Pleistocene (1 800 000 to 11 550 ybp), glaciation captured and stored much water and sea level dropped up to 150 m (McIntyre et al. 1976, Bloom 1983, Imbrie et al. 1983). With lower sea levels, Florida's shoreline extended 100 miles (ca. 161 km) west of its current position, which effectively isolated the Gulf of Mexico's marine populations from Atlantic populations, resulting in a strong genetic discontinuity for many taxa between the Gulf and the Atlantic (Reeb & Avise 1990, Avise 2000, Soltis et al. 2006). Additionally, Florida's landmass was also much more arid during the glacial maxima (~18 000 ybp), which may have resulted in fewer and more isolated estuarine habitats along the Gulf shore (Avise 1996). During interglacial periods in the Pleistocene, sea levels rose, Florida was a series of islands, and the Gulf and Atlantic Ocean were once again highly connected. Some genetic discontinuities have been described in the northern Gulf (e.g. Felder & Staton 1994, Gurgel et al. 2004, Bilodeau et al. 2005). During the glacial maxima, temperatures along the northern continental margins of the Gulf coast were 4 to 5°C lower than they are today (Brunner 1982). January temperatures were as much as 17°C lower than at present in the southeastern United States at this time, suggesting that shallow coastal environments would also have been considerably colder (Stanley 1986). These low temperatures are hypothesized to have forced some near-shore taxa into southern refugia in Mexico and south Florida (Barnwell & Thurman 1984, Felder & Staton 1994). With the rise of temperature and sea levels during the interglacial periods, these populations would have expanded back into the northern Gulf. These changes in the Gulf's shoreline, temperature, and connections to the Atlantic should have affected the distribution and population demographics of species inhabiting estuaries.

The Gulf killifish *Fundulus grandis* is a common inhabitant of salt marshes along the coast from the St. Johns River in northeastern Florida (30° 23' 55.93' N, 81° 23' 53.47' W) to Laguna de Tamiahua, Veracruz, Mexico (21° 35' 36.51' N, 97° 33' 07.73' W) (Lee et al. 1980). We used nuclear microsatellite loci to investigate the population genetic structure of *F. grandis* throughout much of its range in the Gulf of Mexico. *F. grandis* deposits its eggs in dense mats of marsh vegetation that are usually only flooded during biweekly high tides every 2 wk (Greeley & MacGregor 1983). After an incubation period of about 2 wk, the returning tide induces hatching and the larvae then complete their development in the intertidal marsh. This egg stranding reproductive strategy is predicted to promote the retention of offspring in the natal estuary and contribute to significant genetic structure between estuaries. Direct estimates of adult dispersal distance are not known for *F. grandis*. Mark-recapture dispersal estimates for the closely related *F. heteroclitus*, which inhabits salt marshes along the Atlantic coast of North America, ranged from 200 m to 3.6 km within a single year (Sweeney et al. 1998, Skinner et al. 2005). If the genetic structure of *F. grandis* is predominantly shaped by current-day dispersal and distributional patterns, we would expect to find a pattern of IBD across the Gulf with significant genetic differentiation among populations due to low levels of dispersal. Alternatively, if past climatic events have had a significant impact on the genetic structure of *F. grandis* in the Gulf, then we might expect to find a lack of IBD across the Gulf, evidence for secondary contact in the northern Gulf between previously separated populations, and evidence of a negative relationship between latitude and genetic diversity.

MATERIALS AND METHODS

Sampling. *Fundulus grandis* were collected from 10 locations across the Gulf of Mexico from August 2005 to October 2006 either using baited minnow traps and seine nets, or they were purchased from local bait shops (Fig. 1). We attempted to capture this species at several sites along the southwestern coast of Florida (near Naples) and on the eastern coast near Miami; however, we were not successful in capturing any individuals in these areas. Four samples (100% of Kiln County, 100% of Lake Charles, 30% of Dauphin Island, and 50% of Sarasota individuals; see Table 1) were obtained at bait shops. The owners could verify the capture site for all of these populations. A total of 440 individuals were analyzed, with 40 ± 5.47 (mean ± SE) individuals collected from each location (see Table 1).

DNA was extracted from dried fin clips. The tissue was placed in 300 µl lysis buffer (75 mM NaCl, 25 mM

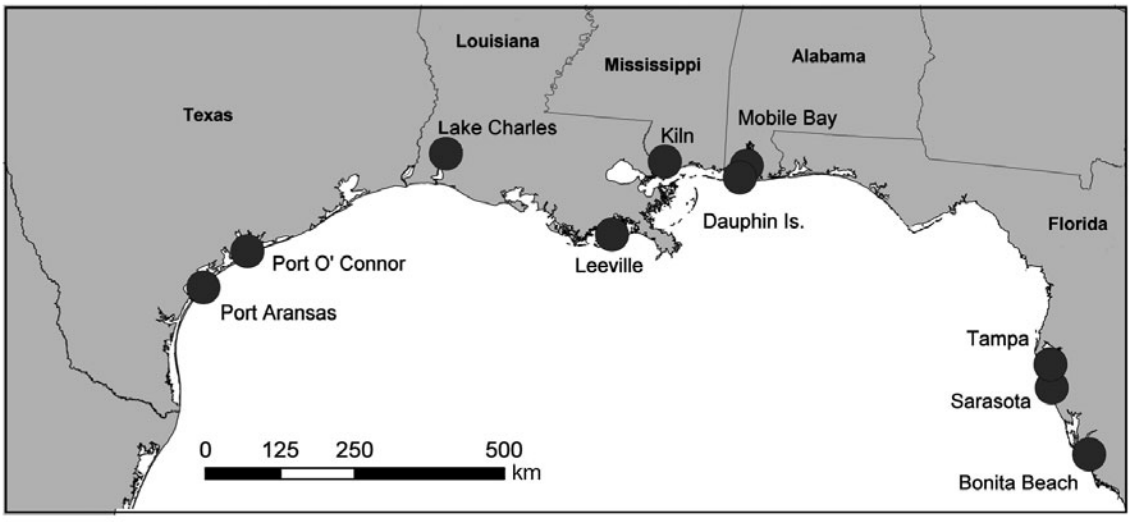

Fig. 1. Sampling localities of *Fundulus grandis* in the Gulf of Mexico

EDTA, 1% sodium dodecyl sulphate [SDS]) and incubated with 0.1 mg Proteinase K at 55°C for 2 h. Proteins were precipitated by adding a half volume of 7.5 M ammonium acetate followed by centrifugation for 10 min. DNA was precipitated from the supernatant by adding 0.7 volume of isopropanol followed by centrifugation for 15 min. The DNA pellet was washed with 70% ethanol, allowed to air dry for 30 min, and then resuspended in 50 µl 10 mM Tris-HCl pH 8.5.

DNA amplification and genotyping. We optimized 8 microsatellite loci previously characterized for *Fundulus heteroclitus* (Adams et al. 2005) for *F. grandis* in 3 fluorescently labeled multiplex primer groups containing the following final concentrations: A (0.05 µM *ATG-B101*, 0.05 µM *ATG-25*, 0.075 µM *ATG-20*), B (0.075 µM *CA-1*, 0.05 µM *ATG-12*), and C (0.06 µM *ATG-18*, 0.06 µM *ATG-B4*, 0.06 µM *ATG-B103*). Each 10 µl reaction contained 2.5 mM $MgCl_2$, 1X PCR buffer, 0.2 mM dNTPs, 0.4 U Taq DNA polymerase, 70 ng DNA, and one of 3 primer multiplexes (see above for concentrations). The PCR thermal cycling profile consisted of 94°C for 2 min, followed by 31 cycles of 94°C for 15 s, 55°C for 15 s, and 72°C for 30 s, ending with a 5 min extension step at 72°C. Following PCR amplification, the products were electrophoresed on an ABI 3730XL Genetic Analyzer (Applied Biosystems). All loci that did not amplify were reamplified in single locus reactions at least 2 times. Individuals that did not produce PCR products for more than half of the loci were discarded from the analyses.

We used GENEMAPPER version 4.0 (Applied Biosystems) to score the genotypes. All genotypes were checked by 2 individuals (D. A. Williams and S. D. Brown). To estimate our genotyping error rate, we extracted DNA from a duplicate set of fin tissue taken from a random subset of individuals (n = 53) across most populations and genotyped them at all loci. All of the test genotypes were identical to previously determined genotypes, indicating that our error rate was very low (<2%).

Statistical analysis. We tested the loci for deviation from Hardy-Weinberg equilibrium and linkage disequilibrium using GENEPOP version 3.3 (Raymond & Rousset 1995). The number of alleles (N_A), observed heterozygosity (H_O), and expected heterozygosity (H_E) were calculated using GenAlEx6 (Peakall & Smouse 2006). Allelic richness (A_R) for each locality was calculated using FSTAT version 2.9.3 (Goudet 2001) with a sample size adjustment of n = 17 individuals (the smallest sample size).

We used the program MICRO-CHECKER (Van Oosterhout et al. 2004) to infer whether heterozygote deficits within a population may have been due to technical difficulties such as null alleles, scoring difficulties due to stutter, or large allele dropout. Alternatively, biological processes such as population substructuring (i.e. Wahlund effect), inbreeding, and genetic bottlenecks can also cause heterozygote deficits in populations. These processes should generally produce consistent heterozygote deficits across all or most loci, whereas technical difficulties usually only affect one or a few loci. When null alleles were inferred at a locus, we estimated their frequency using Brookfield's equation, which assumes that non-amplifications are a result of null allele homozygotes (Brookfield 1996). We then corrected all allele frequencies for that locus before estimating levels of genetic diversity and calculating levels of differentiation.

The program BOTTLENECK (Cornuet & Luikart 1996, Piry et al. 1999) tested for the genetic signature of a recent reduction in the effective population size (N_e) in all samples. Populations that have experienced

a reduction in N_e are expected to have excess H_E relative to that expected under mutation–drift equilibrium. This occurs because allelic richness is lost at a significantly faster rate than heterozygosity after a population reduction. We evaluated all 3 mutation models (infinite allele model [IAM], 2 phase mutation model [TPM], and stepwise mutation model [SMM]). A Wilcoxon sign-rank test was then used to determine if a significant number of loci exhibited excess heterozygosity (Cornuet & Luikart 1996). We also used a graphical method to look for evidence of a bottleneck by plotting the number of alleles in 7 allele frequency categories (Luikart et al. 1998). A population that has not experienced a bottleneck is expected to show an L-shaped distribution (many low frequency alleles and few high frequency alleles), whereas bottlenecked populations will exhibit a mode shift.

Differentiation among populations was calculated in FSTAT using the F_{ST} analog theta (Weir & Cockerham 1984). We tested for pairwise differences between populations using the log likelihood statistic *G* (Goudet et al. 1996) (not assuming Hardy-Weinberg equilibrium) in FSTAT. To illustrate the relationships among populations we used principal component analysis (PCA) of allele frequencies using PCAGEN (www2.unil.ch/popgen/softwares/pcagen.htm). The significance of the axes was calculated based on 10 000 permutations. We tested for IBD by correlating pairwise $F_{ST}/(1 - F_{ST})$ between populations with geographic distance using 1000 permutations in a Mantel test in GenAlEx6. We also tested for genetic spatial autocorrelation using GenAlEx6 (Peakall & Smouse 2006). The autocorrelation coefficient, r, is a measure of the genetic similarity between populations that fall within a defined distance class. The significance of r is determined by random permutation of all populations among distance classes and recalculating r 1000 times to set the upper and lower 95% confidence limits around this value. If the r value fell above or below these limits, then significant spatial structure was inferred. We also calculated 95% CI around each r value by bootstrapping r values within each distance class 1000 times. If these 95% CI did not include r = 0, then significant spatial structure was inferred. We also used a simple assignment test to ask whether more individuals would be assigned to their population of capture or to neighboring populations than to populations further away as expected by a stepping stone model of dispersal. Individuals were assigned to populations in which they had the highest log likelihood score using the Bayesian based assignment method of Rannala & Mountain (1997) in GeneClass2 (Cornuet et al. 1999, Piry et al. 2004).

A Bayesian clustering method implemented in the program STRUCTURE version 2.1 was used to estimate the number of populations (K) in the Gulf of Mexico and to assign individuals to these populations (Pritchard et al. 2000, Pritchard & Wen 2003). The membership of each individual in a population was estimated using the ancestry coefficient *q*, which varies on a scale from 0 to 1.0, with 1.0 indicating full membership. We ran the Monte Carlo Markov Chain (MCMC) for 10^6 iterations following a burn-in period of 10^5 iterations for K = 1 to 10 using the correlated allele frequencies model and assuming admixture (the default values) 10 times. The most likely K was then estimated using the method of Evanno et al. (2005).

RESULTS

All loci were highly polymorphic, with an average of 22.1 ± 3.4 alleles per locus (range = 13 to 35 alleles) for all populations combined (Table 1). None of the pairwise comparisons between loci within populations exhibited significant genotypic linkage disequilibrium after Bonferroni correction ($p < 0.0001$). This suggests that the loci we used provide independent measures of genetic diversity and genetic structure. We found no significant heterozygote excess in any sample. However, there was a significant heterozygote deficit at locus *ATG-25* in 2 populations and at loci *ATG-B101*, *ATG-B4*, and *CA-1* in the Bonita Beach population (Table 1). The lack of a clear pattern across loci or populations suggests that genetic bottlenecks or population admixture were unlikely explanations for the observed heterozygote deficits. The MICROCHEKER analysis indicated taht the most likely technical cause of these deficits was null alleles rather than problems associated with scoring stutter peaks or large allele drop out.

None of the tests for a genetic bottleneck was significant. There was not a significant excess of heterozygosity relative to equilibrium expectations under any mutation model nor was there evidence of a mode shift in the frequency distribution of alleles (data not presented).

Average allelic richness across the 8 loci decreased with increasing latitude ($y = -0.36x + 20.5$, $r^2 = 0.54$, $p = 0.01$) (Fig. 2a). The pattern of allelic richness with longitude revealed that the samples from southern Texas and Florida have the highest allelic richness and that allelic richness decreases in populations further north (Fig. 2b). This resulted in a U-shaped distribution of allelic richness with longitude in the Gulf and is best explained with a second order polynomial relationship ($y = 0.03x^2 - 4.5x + 207.4$, $r^2 = 0.87$, $p = 0.001$) (Fig. 2b).

There was significant genetic structure across all 10 populations (F_{ST} = 0.041 ± 0.02 SE; 95% CI = 0.02–0.08). The average pairwise F_{ST} between populations was 0.05 ± 0.004 (n = 45, range = 0.003 to 0.11)

Table 1. *Fundulus grandis*. Genetic diversity measures at microsatellite loci within 10 populations of *F. grandis*. N = number of individuals genotyped, N_A = number of alleles at a locus, H_O = observed heterozygosity (values in bold represent significant heterozygote deficits), H_E = expected heterozygosity, F_{IS} = inbreeding coefficient (calculated after correction for possible null alleles, see 'Materials and methods'), A_R = allelic richness corrected for sample size using 17 individuals

Sample (abbreviation)	Statistic	*ATG-12*	*ATG-18*	*ATG-20*	*ATG-25*	*ATG-B101*	*ATG-B103*	*ATG-B4*	*CA-1*	Mean
Pt. Aransas (AR)	N	34	34	34	34	34	34	34	34	34.0
	N_A	12	8	10	13	9	20	20	22	14.3
	H_O	1.00	0.79	0.68	0.82	0.88	0.91	0.91	0.91	0.86
	H_E	0.89	0.82	0.65	0.86	0.80	0.92	0.92	0.94	0.85
	F_{IS}	−0.104	0.074	−0.030	0.060	−0.036	0.027	0.018	0.045	0.007
	A_R	11.0	7.1	7.6	10.6	7.0	15.3	15.6	17.7	11.5
Pt. O'Connor (OC)	N	43	43	43	43	43	43	43	43	43.0
	N_A	14	7	9	11	10	20	18	19	13.5
	H_O	0.86	0.77	0.67	0.91	0.81	0.88	0.95	0.91	0.85
	H_E	0.87	0.81	0.62	0.87	0.78	0.93	0.92	0.91	0.84
	F_{IS}	0.013	0.035	−0.076	−0.036	−0.080	0.063	−0.020	0.015	−0.011
	A_R	11.1	6.3	7.3	9.0	7.6	15.5	14.4	14.5	10.7
Lake Charles (LC)	N	36	36	36	36	36	36	36	36	36.0
	N_A	10	9	9	10	8	17	18	16	12.1
	H_O	0.81	0.72	0.44	0.92	0.75	0.92	0.94	0.86	0.80
	H_E	0.79	0.80	0.49	0.84	0.80	0.93	0.90	0.88	0.80
	F_{IS}	−0.002	0.109	0.121	−0.073	0.037	0.027	−0.036	0.003	0.023
	A_R	8.5	7.1	7.0	8.8	6.8	14.8	13.4	12.8	9.9
Leeville (LV)	N	59	59	59	59	59	59	59	59	59.0
	N_A	10	7	8	10	8	21	19	18	12.6
	H_O	0.81	0.83	0.46	**0.75**	0.73	0.97	0.88	0.83	0.78
	H_E	0.81	0.77	0.49	0.85	0.79	0.94	0.93	0.84	0.80
	F_{IS}	0.033	−0.080	0.072	0.076	0.088	−0.026	0.066	0.035	0.033
	A_R	8.2	6.1	5.8	8.2	6.7	15.8	15.0	11.3	9.6
Kiln County (KC)	N	124	123	124	124	124	124	124	123	123.8
	N_A	11	13	9	9	9	27	28	20	15.8
	H_O	0.85	0.77	0.59	0.87	0.81	0.93	0.94	0.85	0.83
	H_E	0.81	0.80	0.59	0.83	0.81	0.94	0.92	0.83	0.82
	F_{IS}	−0.038	0.044	0.008	−0.046	−0.001	0.015	−0.02	−0.027	−0.01
	A_R	8.29	7.26	5.43	7.74	6.64	16.00	14.73	10.22	9.54
Dauphin Island (DI)	N	23	23	23	22	22	23	23	23	22.8
	N_A	6	8	7	8	8	17	16	11	10.1
	H_O	0.87	0.83	0.78	0.86	0.64	1.00	1.00	0.65	0.83
	H_E	0.69	0.80	0.76	0.81	0.62	0.92	0.91	0.68	0.77
	F_{IS}	−0.243	−0.006	−0.008	−0.030	−0.038	−0.065	−0.078	0.017	−0.056
	A_R	5.5	7.6	6.2	7.7	7.4	15.0	14.7	9.0	9.1
Mobile Bay (MB)	N	55	55	55	55	55	55	55	55	55.0
	N_A	10	8	8	9	8	20	17	19	12.4
	H_O	0.82	0.69	0.86	0.78	0.73	0.93	0.95	0.84	0.82
	H_E	0.77	0.75	0.81	0.81	0.74	0.93	0.91	0.83	0.82
	F_{IS}	−0.056	0.073	−0.051	0.038	0.053	0.017	−0.027	−0.018	0.004
	A_R	7.2	7.2	6.7	7.5	6.4	15.3	13.1	11.5	9.4
Tampa Bay (TB)	N	17	17	17	17	17	17	17	17	17.0
	N_A	12	6	4	8	11	12	15	18	10.8
	H_O	0.94	0.88	0.29	0.94	0.88	0.77	0.94	0.94	0.82
	H_E	0.89	0.78	0.27	0.78	0.84	0.87	0.88	0.93	0.78
	F_{IS}	−0.034	−0.093	−0.110	−0.106	−0.028	0.135	−0.056	0.077	−0.027
	A_R	12	6	4	8	11	12	15	18	10.8
Sarasota (SS)	N	31	31	31	31	31	31	31	31	31.0
	N_A	15	7	4	8	13	14	12	17	11.3
	H_O	0.94	0.81	0.68	**0.61**	0.97	0.77	0.71	0.94	0.80
	H_E	0.89	0.75	0.61	0.84	0.89	0.87	0.77	0.92	0.82
	F_{IS}	−0.039	−0.061	−0.081	0.117	−0.073	0.141	0.113	−0.032	0.011
	A_R	12.4	6.2	4.0	8.5	11.2	12.2	10.1	14.3	9.9
Bonita Beach (BB)	N	17	18	18	18	18	18	18	18	17.9
	N_A	12	6	5	10	8	15	12	18	10.8
	H_O	0.78	0.79	0.79	0.94	0.94	**0.67**	**0.68**	**0.79**	0.80
	H_E	0.90	0.69	0.67	0.86	0.84	0.90	0.86	0.94	0.83
	F_{IS}	0.168	−0.107	−0.144	−0.074	−0.101	0.174	0.246	0.192	0.044
	A_R	12.0	5.9	4.9	9.9	7.9	14.0	11.6	17.6	10.5

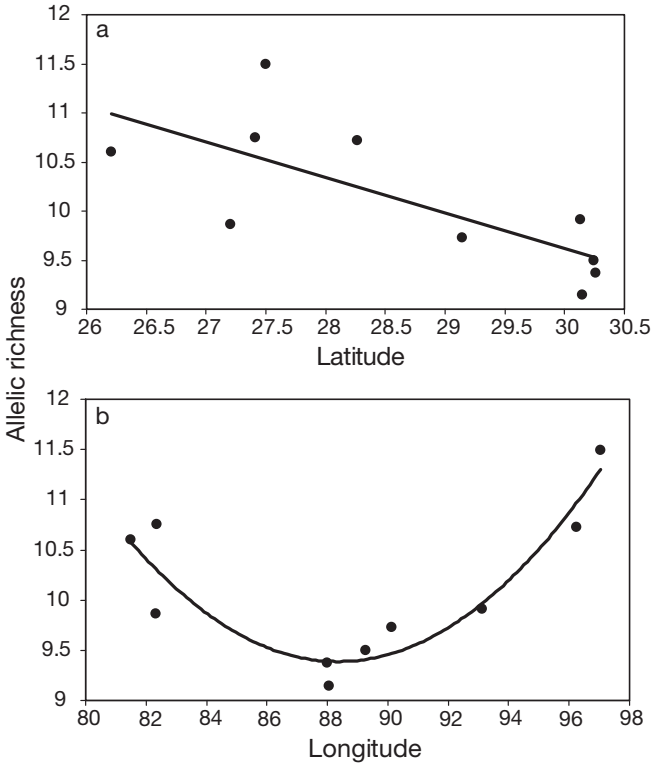

Fig. 2. *Fundulus grandis*. Relationship of average allelic richness within a population (n = 8 microsatellite loci) and (a) latitude, (b) longitude

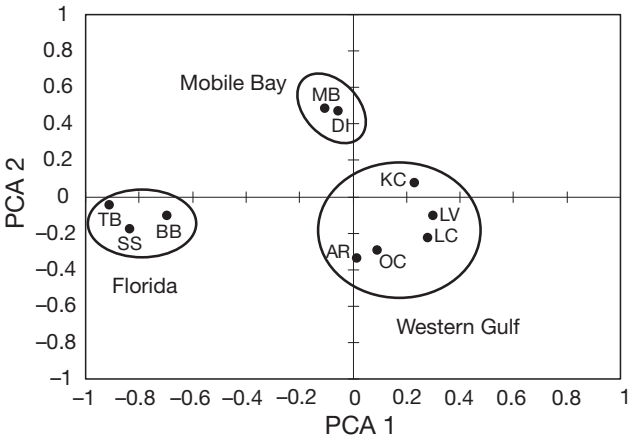

Fig. 3. *Fundulus grandis*. Principal component analysis (PCA) of allele frequencies in populations of *F. grandis*. Circles encompass clusters of genetically similar populations. The first axis explains 42% of the variation (p = 0.0001) and the second axis explains 20% of the variation (p = 0.0001) in allele frequency differences between populations. Abbreviations as in Table 1

(Table 2). Virtually all pairwise comparisons between populations exhibited significant genotypic frequency differences after a Bonferroni correction for multiple tests ($p < 0.001$) (Table 2). The PCA, which is based on the correlation matrix of allele frequencies between populations, indicated some clear subdivisions among the populations (Fig. 3). The first axis of the PCA (Fig. 3) accounted for 42% of the variation and clearly discriminated between the Florida populations (Bonita Beach, Sarasota, Tampa Bay) and the rest of the Gulf populations (p = 0.0001). This can also be seen in the pairwise F_{ST} values (Table 2), where the average F_{ST} among Florida populations is 0.027, yet the mean F_{ST} between the next geographically close populations in the Mobile Bay area (Mobile Bay and Dauphin Island) and the Florida populations is 0.075, similar to the mean F_{ST} of 0.080 between Florida populations and all other populations. The second axis accounted for 20% of the variation and separates the Mobile Bay and Dauphin Island populations from the other Gulf populations and also reveals a pattern of IBD across the western Gulf (p = 0.0001). This separation can also be seen in the pairwise F_{ST} values (Table 2), where among the populations west of the Mobile Bay area the average F_{ST} is 0.014, while between Mobile Bay populations and the populations west of them the average F_{ST} is 0.042.

Table 2. *Fundulus grandis*. Pairwise estimates of F_{ST} between populations of *F. grandis*. Values in bold indicate pairwise comparisons that do not exhibit significant differentiation in genotype frequencies using the log likelihood statistic *G* after Bonferroni correction. Samples are ordered west to east. Abbreviations as in Table 1

	AR	OC	LC	LV	KC	DI	MB	TB	SS	BB
AR	–	**0.003**	0.014	0.022	0.025	0.055	0.044	0.075	0.061	0.049
OC		–	0.013	0.014	0.019	0.046	0.042	0.079	0.065	0.062
LC			–	0.004	0.014	0.051	0.045	0.106	0.087	0.078
LV				–	0.010	0.036	0.039	0.103	0.086	0.078
KC					–	0.035	0.025	0.092	0.084	0.077
DI						–	**0.013**	0.086	0.075	0.073
MB							–	0.069	0.072	0.054
TB								–	**0.012**	0.035
SS									–	0.028
BB										–

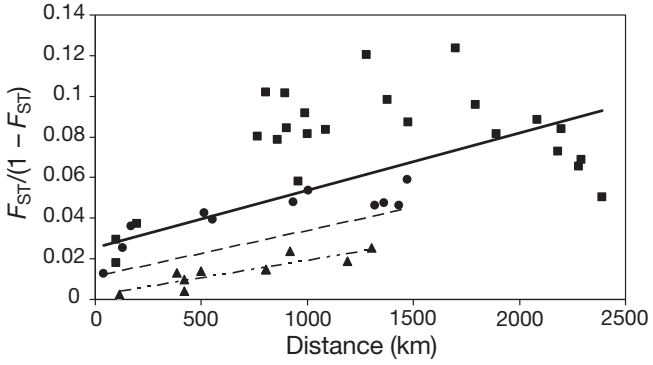

Fig. 4. *Fundulus grandis*. Relationship between pairwise genetic and geographic distance for 10 populations of *F. grandis*. Solid line is regression line for all 10 populations (■, ●, ▲, $y = 3 \times 10^{-5}x + 0.025$); dashed line is 7 populations excluding the Florida populations (●, ▲, $y = 2 \times 10^{-5}x + 0.011$); dot-dashed line is 5 populations west of Mobile Bay (▲, $y = 2 \times 10^{-5}x + 0.002$). ■: pairwise comparisons between Florida and all other populations; ●: comparisons excluding the Florida populations; ▲: comparisons excluding Mobile Bay and Florida populations. F_{ST}: Wright's fixation index

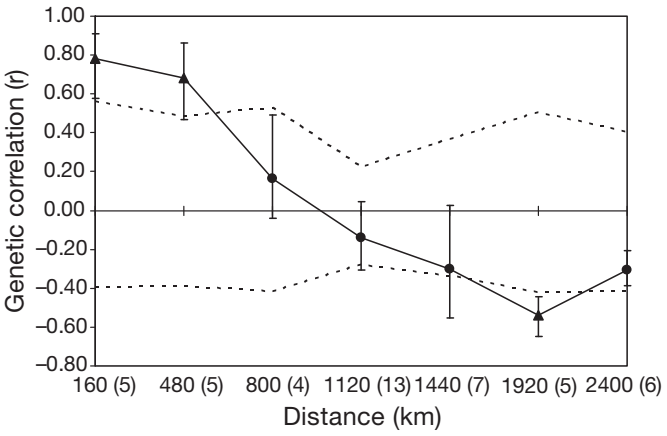

Fig. 5. *Fundulus grandis*. Genetic spatial autocorrelation of pairwise genetic (F_{ST}) and geographic distance for 10 populations of *F. grandis*. Dashed lines are 95% CI around r = 0; vertical lines are 95% bootstrapped CI around each calculated r value. ▲: significantly different from r = 0 ($p \leq 0.005$). Numbers in parentheses next to the distance classes are the number of pairwise comparisons in that class

There was a significant positive relationship between genetic and geographic distance (r = 0.57, p = 0.01) (Fig. 4). Most of the Florida comparisons with the western Gulf, however, form a cluster of points above the regression line, suggesting that these populations are more differentiated from the rest of the Gulf than predicted by distance alone. At the furthest distances, F_{ST} drops below the predicted relationship which may indicate the presence of homoplasy. There was still a significant relationship between genetic and geographic distance after removing the Florida samples (r = 0.61, p = 0.02), although these comparisons revealed that the Mobile Bay samples cluster above the predicted relationship (Fig. 4). Comparing only the populations west of Mobile Bay resulted in a strong relationship between genetic and geographic distance (r = 0.89, p = 0.02) with an origin very close to zero as expected when populations with a stepping stone population structure are at migration–drift equilibrium (Fig. 4). The effect of genetic differentiation between the Mobile Bay and Florida populations with the western Gulf populations can be seen in Fig. 4 by an increase in the *y*-intercept of the regression line for genetic versus geographic distance and is consistent with the groupings revealed by the PCA.

There was significant positive genetic spatial autocorrelation for pairwise F_{ST} values across the entire Gulf only in the 160 km (p = 0.001) and 480 km (p = 0.003) distance classes (Fig. 5), which generally encompasses 2 to 3 of our sampling sites. There was a steep decline in autocorrelation values for populations separated by distance classes of 726 km or more, with the correlogram crossing the *y*-axis at ~977 km and becoming significantly negative at the 1920 km distance category, reflecting the distinct population clusters of Florida versus the western Gulf revealed by the PCA analysis and STRUCTURE (see below). Restricting the spatial autocorrelation only to the populations west of the Mobile Bay area revealed significant positive spatial autocorrelation in the first of 4 distance classes (160 km, r = 0.68, p = 0.02, data not presented).

One assumption of the individual-based assignment tests, such as the assignment method of Rannala & Mountain (1997) and the algorithm in STRUCTURE, is that the populations are in Hardy-Weinberg equilibrium. We therefore performed the analyses with and without locus *ATG-25*, which exhibited putative null alleles, since it is not possible to correct allele frequencies for null alleles and retain the correct individual genotypes. We also performed the analyses with and without the Bonita Beach population because it had heterozygote deficits at 3 loci. Excluding locus *ATG-25* or the Bonita Beach population had virtually no effect on the final results. The assignment test of Rannala & Mountain (1997) supported an IBD relationship. Most individuals were assigned to their population of capture (55.4%) or to an adjacent population (29.5%). STRUCTURE estimated 3 population clusters in the Gulf (Fig. 6). The Florida samples formed the strongest cluster, with an average proportion of ancestry (q) of 89.8% for Cluster A (Fig. 6). Although the other populations were a mix of the 3 clusters, the proportions of each cluster were strongly related to geography and exhibited a pattern consistent with IBD. Excluding the Florida populations (which were predominantly Cluster A), there was a strong positive relationship be-

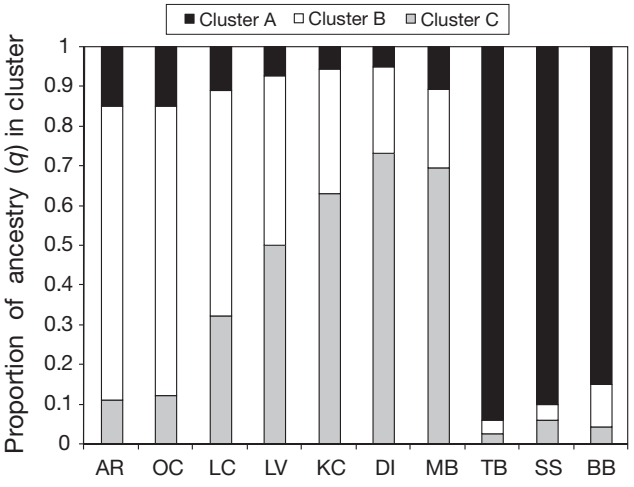

Fig. 6. *Fundulus grandis*. Proportion of ancestory (*q*) in each of the 3 clusters determined with STRUCTURE (see 'Material and methods') for each sampling locality of *F. grandis*. Abbreviations as in Table 1

tween longitude and the *q* that populations contained of Clusters A and B (r = 0.87, 0.99, $p < 0.01$, respectively) and a negative relationship between longitude and *q* that populations contained of Cluster C (r = –0.99, $p < 0.01$).

DISCUSSION

Fundulus grandis exhibits significant population genetic structure throughout the Gulf of Mexico. Our data suggest that most dispersal occurs between adjacent estuaries and that several genetic discontinuities exist along the Gulf coast. The Florida populations in particular are significantly differentiated from the more western and northern Gulf populations. Comparisons between the most geographically distant populations (Florida and the western Gulf) revealed a higher similarity in allele frequencies than predicted by distance, which may indicate the occurrence of homoplasy. When effective population sizes are very large, which may be the case for this species, homoplasy is predicted to occur in highly polymorphic markers such as microsatellites (Estoup et al. 2002). Populations at varying scales exhibited a pattern of IBD, and spatial autocorrelation revealed that populations located within 160 km of each other were more similar than expected at random. Similarly, assignment tests placed most individuals at either their place of capture or in adjacent populations. The clusters determined by the STRUCTURE analysis were also consistent with IBD in the western Gulf.

Estuarine-dependent species, like the Gulf killifish, are expected to exhibit stronger genetic population structure due to dispersal limitations than species living in more open marine environments. Although limited in number, studies of estuarine fish in the Gulf of Mexico have found the expected IBD relationship (Gold & Richardson 1998, Blandon et al. 2001, Gold & Turner 2002, present study), whereas studies of reef or pelagic organisms in the Gulf have found broad-scale genetic homogeneity (Herke & Foltz 2002, Ball & Chapman 2003, Purcell et al. 2006).

Fundulus grandis shows very similar levels of population subdivision when compared to its closely related congener *F. heteroclitus* which inhabits salt marshes along the Atlantic coast of North America (mean pairwise $F_{ST} = 0.05 \pm 0.004$ for *F. grandis* vs. 0.042 ± 0.005 for southern populations of *F. heteroclitus*) (Adams et al. 2006, present study). *F. heteroclitus* exhibits concordant clines in allele frequencies at allozyme, mitochondrial, and nuclear microsatellite loci, with a strong transition zone occurring at ~41° N latitude (reviewed in Adams et al. 2006, Duvernell et al. 2008). Strong patterns of IBD across the entire range of *F. heteroclitus* (r = 0.85) as well as within northern (r = 0.70) and southern populations (r = 0.79) suggests that this species was distributed along the entire east coast of North America during the Pleistocene glacial maxima and that subsequent rising sea levels brought previously separated populations into contact and produced the observed transition zone (Adams et al. 2006).

Previous intraspecific studies on a number of taxa have found little evidence for genetic discontinuities in the Gulf of Mexico (but see Gurgel et al. 2004), although this may have resulted from a variety of factors including limited sampling and studies of species with high dispersal potential (e.g. pelagic fish) (Avise et al. 1987, Reeb & Avise 1990, Gold & Richardson 1998, Heist & Gold 2000, Blandon et al. 2001, Gold & Turner 2002, Herke & Foltz 2002, Ball & Chapman 2003). The 2 genetic discontinuities we found occur near previously described biogeographic boundaries (Mobile Bay and Florida). The presence of a vicariance zone has been described for some closely related species of fish and invertebrates near Mobile Bay in the northern Gulf (e.g. Bert 1986, McClure & McEachran 1992, Felder & Staton 1994, Gurgel et al. 2004, Harrison 2004, Bilodeau et al. 2005). The Florida sampling localities are along the northern edge of the Caribbean faunal biogeographic province, which is located just below the more temperate Gulf of Mexico province (Briggs 1974, Engle & Summers 2000).

We did not find a strong transition zone in the northern Gulf near Mobile Bay, and Bayesian clustering did not reveal a clear signal of admixture in this area arising from 2 differentiated populations coming into secondary contact as is observed in *Fundulus heteroclitus* (Adams et al. 2006). These results suggest that if *F.*

grandis had been forced into southern refugia (e.g. Barnwell & Thurman 1984), it may not have been for long periods of time or, alternatively, there may have been many episodes of extinction-recolonization in the northern Gulf during the Pleistocene. Another possibility is that recolonization of the northern Gulf populations that we sampled may have occurred predominantly from the western Gulf, and a zone of secondary contact with more southeastern populations occurs between Mobile Bay and Tampa Bay. The Florida populations appear to be more highly differentiated from the other Gulf populations, above what is expected by distance alone, suggesting the presence of a transition zone in that region. More continuous sampling is needed to determine whether there is a zone of secondary contact in this region consistent with the southern refugia hypothesis.

The decrease in allelic richness with increasing latitude in the Gulf and the fact that allelic richness is relatively higher in the southwestern and southeastern Gulf compared to the northern populations is consistent with recolonization of the northern Gulf from southern refugia. A decrease in genetic diversity with latitude is usually hypothesized to result from the recolonization of northern habitats after the retreat of the glaciers during the Pleistocene and has been described for a number of taxa (Hewitt 2000, Adams et al. 2006, Haney et al. 2007).

Alternatively, the observed genetic discontinuities in *Fundulus grandis* may simply be located in areas that either have geographic features or selective regimes that limit dispersal and are not indicative of historical transition zones. This may be especially true of the Mobile Bay area that was relatively less differentiated from the western populations. Large bays along the east coast of North America are believed to be effective dispersal barriers for *F. heteroclitus*, which prefer shallow, near-shore water (Duvernell et al. 2008), and may explain the genetic differentiation of *F. grandis* located at Mobile Bay and Tampa Bay. It is currently unknown what potential selective factors might limit gene flow in this species, although adaptations to differing levels of hypoxia, salinity, and temperature are possibilities. A finer scale genetic study of the Mobile and Tampa Bay areas may reveal the nature of the dispersal barriers in these regions (e.g. Duvernell et al. 2008). Recent disturbances such as hurricanes could also affect the population genetic structure of *F. grandis* by altering the distribution of suitable habitat or by killing large numbers of individuals, resulting in decreased population sizes or local extinction and recolonization events. We did not find evidence of recent bottlenecks or founder events in either Mobile Bay or Florida. The effects of hurricanes on near-shore fish populations are believed to be relatively transitory (Greenwood et al. 2006).

The estimation of gene flow between populations using estimators such as F_{ST} assumes the presence of migration–drift equilibrium (e.g. Hutchison & Templeton 1999, Castric & Bernatchez 2003, Austin et al. 2004). Populations with a stepping stone population structure are expected to exhibit a pattern of IBD when they are at migration–drift equilibrium (Kimura & Weiss 1964, Crow & Aoki 1984, Slatkin 1993, Hutchison & Templeton 1999). The relatively strong IBD relationship (r = 0.89) and a *y*-intercept near the origin (0) among the populations west of Mobile Bay suggest these populations may be at migration–drift equilibrium. The genetic discontinuities revealed in Mobile Bay and especially Florida suggest that populations located along the entire Gulf coast are probably not at migration–drift equilibrium; therefore, inferences about the effects of gene flow and selection on population structure must take these patterns into consideration.

As expected, patterns of nuclear microsatellite variation suggest that contemporary gene flow is limited in *Fundulus grandis* and occurs mainly between neighboring sites. Indications of historical influences on the current day genetic structure of *F. grandis* include a negative relationship between genetic diversity and latitude and evidence of genetic discontinuities at hypothesized biogeographic boundaries, although these discontinuities may be related to current day dispersal barriers. Genotyping these populations with a mitochondrial DNA marker and extending the sampling range to include populations from more southern latitudes (i.e. extreme southern and eastern Florida and northern Mexico) could improve our understanding of the patterns we have found in the present study. Although not as dramatic as the Atlantic versus Gulf split seen in many species, results suggest that microsatellite markers can uncover subtle evidence for historical influences and genetic discontinuities within the Gulf of Mexico. More intraspecific studies are needed on other estuarine-dependent species within the Gulf to test the generality of the southern refugia hypothesis.

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