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ARTICLE

Population Genetic Structure of Southern Flounder Inferred from Multilocus DNA Profiles

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Abstract

Determination of stock structure is an important component of fisheries management; incorporation of molecular genetic data is an effective method for assessing differentiation among putative populations. We examined genetic variation in Southern Flounder *Paralichthys lethostigma* within and between the U.S. South Atlantic and Gulf of Mexico basins to improve our understanding of the scale of population structure in this wide-ranging species. Analysis of amplified fragment length polymorphism (AFLP) fingerprints and analysis of mitochondrial DNA (mtDNA) control region sequences found clear divergence between ocean basins. Based on mtDNA sequences, no genetic differentiation was detected within the U.S. South Atlantic at spatial scales that were broad (among states: North Carolina, South Carolina, Georgia, and Florida) or fine (among estuarine regions within North Carolina). Increased genetic resolution was observed with AFLP fingerprint data, and we found significant subdivision between nearly all Southern Flounder geographic populations, suggesting the presence of finer-scale genetic population structure within the U.S. South Atlantic. However, AFLP genetic cluster analysis also revealed evidence for a high degree of mixing within the Atlantic basin; patterns of variation, which included genetic similarity between South Carolina and Gulf of Mexico samples, were not aligned closely with geography. We examined the partitioning of genetic variation among groups by using analyses of molecular variance and found no evidence that North Carolina Southern Flounder, which are managed on the state level as a unit stock, are differentiated from the remainder of U.S. South Atlantic Southern Flounder. Our findings indicate only weak structure and the potential for basinwide mixing among Atlantic Southern Flounder, suggesting that cooperation among U.S. South Atlantic states will be essential for the effective assessment of stock dynamics and future management plans.

Accurate identification of stock structure is essential for effective fisheries management but remains a challenge for both managers and scientists (Begg et al. 1999; NCDMF 2005, 2013). Examination of genetic variation across a species' range is a commonly used approach for defining

interbreeding populations, and recent advances in molecular techniques have contributed to an expanded interest in the field of fisheries genetics (Ward 2000; Hauser and Carvalho 2008; Waples et al. 2008). Marine species were once assumed to be panmictic, with long-distance dispersal capability and a lack

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of obvious barriers to gene flow in the ocean resulting in low genetic differentiation among putative populations (Ward et al. 1994; Waples 1998). However, highly polymorphic molecular markers have revealed significant genetic structure in marine fish at multiple geographic scales (Ruzzante et al. 1999; Knutsen et al. 2003; O'Reilly et al. 2004; McCairns and Bernatchez 2008), and Knutsen et al. (2011) suggested that even low levels of population subdivision in marine organisms can be biologically meaningful. Maintenance of subtle genetic stock differentiation may be important in conserving local diversity and adaptive variation. Consequently, exploitation of fish populations can lead to reductions in genetic diversity, which may impact the ability of depleted stocks to recover (Hutchinson et al. 2003). Incorporating population genetic analyses into fisheries management and considering both historical and contemporary patterns of molecular diversity can help to promote sustainability and preserve evolutionary processes in the face of increasing anthropogenic impacts (Avisé 1992; Crandall et al. 2000; Conover et al. 2006; Hauser and Carvalho 2008).

The Southern Flounder *Paralichthys lethostigma* is an economically important flatfish that occurs throughout estuarine and coastal ocean waters in the northern Gulf of Mexico (hereafter, Gulf) from northern Mexico to Florida (FL) and in the U.S. South Atlantic (hereafter, Atlantic) from FL to North Carolina (NC). The species supports a thriving recreational fishery across its range and is also a valuable commercial finfish resource in some areas, particularly in NC, where commercial landings averaged 1.5 million kg (3.3 million lb) annually between 1991 and 2007 (NCDMF 2013). In fact, the NC stock has experienced elevated commercial harvest rates since at least 1991; although a fishery management plan (NCDMF 2005, 2013) was first established in 2005, the most recent stock assessment concluded that NC Southern Flounder remain overfished and are still undergoing overfishing (Takade-Heumacher and Batsavage 2009).

Mature Southern Flounder inhabiting estuarine waters migrate offshore annually to spawn during the late-fall and winter months, with recruitment of larvae into estuarine nursery grounds occurring in late winter and early spring (Wenner et al. 1990). Adults typically return to estuarine and nearshore coastal waters after spawning (Monaghan 1996), but recent studies have found that Southern Flounder migration patterns are more variable, as some adults remain offshore through the late-spring and summer months (Watterson and Alexander 2004; Taylor et al. 2008). In addition, previous tagging studies indicate that most individuals display limited movement when occupying estuarine habitats and remain in close proximity to their tagging sites (Wenner et al. 1990; Monaghan 1996; Craig and Rice 2008). Based mainly on the estuarine tagging results, the current fisheries management plan considers Southern Flounder in NC to be a unit stock (NCDMF 2013). However, there is also evidence of extensive southward movement, as some individuals tagged in NC estuaries were found in other

Atlantic states (Monaghan 1996; Craig and Rice 2008). Prior to estuarine recruitment, Southern Flounder remain in a pelagic larval stage for a moderately long duration (~45 d), which increases the potential for long-range dispersal. The unknown level of adult mixing in the offshore environment combined with the long duration of the larval stage implies that the genetic population structure of Southern Flounder could be more homogeneous than currently assumed. The use of molecular methods should provide increased knowledge of Southern Flounder population structure and connectivity, thereby contributing to effective management decisions for the long-term sustainability of the species.

Previous population genetics studies of Southern Flounder have been focused on the Gulf, but there has been no comprehensive study spanning the species' range within the Atlantic. When examining allozyme variation in Southern Flounder, Blandon et al. (2001) found that Gulf populations were significantly differentiated from Atlantic populations, and a break in allele frequencies on the coast of Texas between Galveston Bay and Matagorda Bay indicated the presence of population structure within the Gulf. Updated studies using mitochondrial DNA (mtDNA) and microsatellites again revealed high divergence between Gulf and Atlantic populations of Southern Flounder, but in contrast to the allozyme survey, little differentiation was detected within the Gulf (Anderson and Karel 2012; Anderson et al. 2012). Given the low levels of within-basin variability detected in previous studies, we applied a powerful genetic tool in an attempt to better characterize diversity and stock structure of Southern Flounder in the Atlantic basin.

We examined Southern Flounder populations by using the amplified fragment length polymorphism (AFLP) technique (Vos et al. 1995). This method rapidly generates a genetic fingerprint consisting of hundreds of highly reproducible DNA fragments that are scattered across the genome and that can be compared among individuals and populations without the need for prior characterization of an organism's genome (Bensch and Åkesson 2005). The large number of AFLPs produced allows for assessment of genome-wide variation when analyzing population structure, which can be more informative than targeting mtDNA markers (which are inherited as a single haploid locus). It has been claimed that the information content of an AFLP study is comparable to that obtained from a small number of highly informative microsatellite loci, which are commonly used but require substantial development time and cost (Campbell et al. 2003; Meudt and Clarke 2007; Sønstebo et al. 2007). Although previously underutilized in studies of animals (reviewed by Bensch and Åkesson 2005), AFLPs have become increasingly common in studies of aquatic populations, including commercially important fish and invertebrate species both in aquaculture (e.g., Channel Catfish *Ictalurus punctatus*: Mickett et al. 2003; Olive Flounder *Paralichthys olivaceus*: Liu et al. 2005) and in natural populations (e.g., common shrimp *Crangon crangon*: Weetman

et al. 2007; Striped Mullet *Mugil cephalus*: Liu et al. 2009). For comparison with a more established method, we also sequenced a fragment of the mtDNA control region, which is a commonly used molecular marker. The control region has been identified as highly variable in teleosts (Lee et al. 1995) and was previously used by Anderson et al. (2012) to examine Southern Flounder population structure.

In this study, our main objective was to genotype Southern Flounder individuals from across the species' range by using both AFLPs and mtDNA to address whether distinct genetic populations are present within the Atlantic, particularly whether Southern Flounder in NC represent a unique stock. As evidenced by previous studies, we expected divergence between Gulf and Atlantic populations, but we attempted to determine whether finer structure exists within ocean basins and whether this structure alters our assumptions regarding the level of mixing and warrants a change in the spatial scale of management. In addition, we assessed the viability of the AFLP method in comparison with more traditional mtDNA sequencing for determining stock structure in a widely distributed marine species.

METHODS

Southern Flounder tissue samples were collected from multiple estuarine sites across four states (NC, South Carolina [SC], Georgia [GA], and FL) spanning the current range of the species in the Atlantic (Figure 1). North Carolina was subdivided into three regions (north [NCN], central [NCC], and south [NCS]) to examine genetic population structure within state waters. Samples were obtained from both fishery-independent and fishery-dependent sources in partnership with state fishery management agencies and commercial fishers within each state and were also obtained from licensed seafood dealers. Sampling was conducted from 2010 to 2013, and sampled fish ranged from age 0 to age 3 (96% were age 0–1), with an average TL of 360 mm (range = 166–622 mm). Additional tissue samples, provided by J. D. Anderson (Texas Parks and Wildlife Department), were collected from two sites (Figure 1) in the Gulf (Apalachicola, FL [APFL]; and San Antonio Bay, Texas [SBTX]). Sampling methods for Gulf collections are described by Anderson et al. (2012). Fin clips and gill tissue were excised and preserved in either a salt-saturated dimethyl sulfoxide solution (Atlantic samples) or a 70%

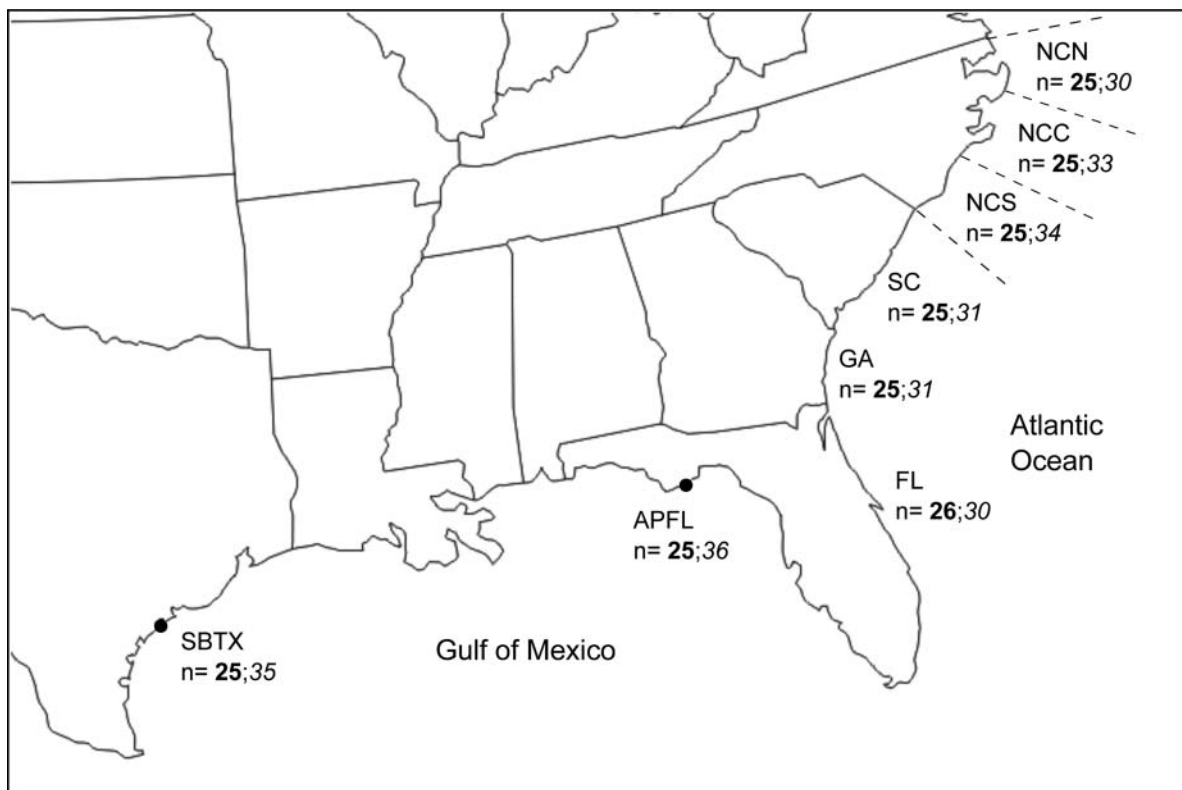


FIGURE 1. Map of Southern Flounder sampling locations in the U.S. South Atlantic and the Gulf of Mexico. Atlantic locations are represented here on a state scale, although multiple sites were sampled within each state (NC = North Carolina; SC = South Carolina; GA = Georgia; FL = Florida). North Carolina was subdivided into three regions (separated by dashed lines; NCN = NC north [Albemarle Sound]; NCC = NC central [Neuse River–Pamlico River estuary]; NCS = NC south [New River and Cape Fear River estuaries]). Gulf of Mexico samples originated from two specific locations (black circles; APFL = Apalachicola, Florida; SBTX = San Antonio Bay, Texas). Sample size (n) for amplified fragment length polymorphisms is shown in bold; n for mitochondrial DNA control region sequences is shown in italics.

solution of ethanol (Gulf samples) and were stored at 4°C. Genomic DNA was extracted from fin clips by using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, California) in accordance with the manufacturer's protocols. Gill tissue was extracted only occasionally, when fin clips were damaged or not readily available.

The AFLPs were generated by using the AFLP Plant Mapping Protocol (Applied Biosystems, Inc. [ABI], Foster City, California) for regular plant genomes. We produced DNA fingerprints for 12 selective primer pairs (chosen from an earlier screen of 24 primer pairs): *EcoRI*-ACA and *MseI*-CAA; *EcoRI*-ACC and *MseI*-CAC; *EcoRI*-ACC and *MseI*-CAG; *EcoRI*-ACC and *MseI*-CAT; *EcoRI*-ACT and *MseI*-CAA; *EcoRI*-ACT and *MseI*-CAC; *EcoRI*-ACT and *MseI*-CAT; *EcoRI*-ACT and *MseI*-CTC; *EcoRI*-AGC and *MseI*-CAA; *EcoRI*-AGC and *MseI*-CTT; *EcoRI*-AGG and *MseI*-CTA; and *EcoRI*-AGG and *MseI*-CTG. Fragments were sized along with GeneScan 500 ROX size standard (Life Technologies, Carlsbad, California) on an ABI 3130XL Genetic Analyzer at the DNA Analysis Core Facility (Center for Marine Science, University of North Carolina, Wilmington). To test for reproducibility of AFLP fingerprints, one individual from each sampling location was subjected to repeat analysis across all 12 selective primer pairs beginning from genomic DNA. Fragments between 75–500 bp were scored automatically for presence/absence with a default peak calling threshold of 50 relative fluorescence units (rfu) by using ABI GeneMapper version 4.0. Peak heights were normalized across all samples for each primer pair, with only polymorphic loci considered. To further reduce noise and genotyping error, the individual loci in the AFLP profiles were filtered to a locus selection threshold of 200 rfu and a phenotype calling threshold of 20% following Whitlock et al. (2008). In this AFLP profile optimization procedure, loci with an average peak height below 200 rfu are eliminated from analysis, and presence/absence scoring is based on a threshold of 20% of the average peak height for each locus. The complete AFLP profiles (i.e., prior to optimization) were also considered in this analysis but provided similar results and are not reported here.

A fragment of the mtDNA control region was amplified by using the primers L-PROF (5'-AACTCTACCCC-TAGCTCCCAAAG-3'; Meyer et al. 1994) and PL526H (5'-AAAAGAGAACCCCTTACCCG-3'), the latter of which is a Southern Flounder-specific primer designed by one of us (M. A. McCartney, unpublished). The PCR was conducted as follows: an initial denaturation step of 94°C for 1 min, followed by 35 amplification cycles (94°C for 1 min; 58°C for 30 s; and 72°C for 2 min) and a final extension of 72°C for 5 min. Each 25-μL reaction volume contained 1.25 units of MyTaq DNA Polymerase (Bioline, London, UK), 1 × MyTaq Red Reaction Buffer (Bioline), 0.5 μM of each primer, and 1 μL of genomic DNA (5–900 ng). The PCR products were purified by using a StratPrep PCR Purification Kit (Agilent Technologies, Santa Clara, California) and were cycle sequenced in the

forward direction (L-PROF) with ABI BigDye Terminator version 3.1. Sequencing was conducted on the same equipment as described for AFLPs. Sequences were edited, trimmed, and aligned manually in Sequencher version 4.10.1 (Gene Codes Corporation, Ann Arbor, Michigan). Control region sequences were deposited in GenBank under accession numbers KP331548–KP331677.

STRUCTURE version 2.3.4 (Pritchard et al. 2000) was used to assign individual Southern Flounder to an inferred number of genetically distinct clusters (K) present in the AFLP data set. Because AFLPs are dominant markers that are scored as the presence or absence of peaks, there is no distinction between homozygous and heterozygous states. To account for this ambiguity, we implemented the model by Falush et al. (2007) for analyzing dominant markers in STRUCTURE. A recessive AFLP profile (consisting of all peak absences) was input into the data set, and the model evaluated all possible genotypes (i.e., for a peak presence phenotype, the genotypes presence/presence and presence/absence are both considered). Based on previous studies that demonstrated a lack of structure among Southern Flounder populations within ocean basins (Anderson and Karel 2012; Anderson et al. 2012), we assumed that populations were admixed and that allele frequencies across populations were correlated. We did not provide the geographic origin of samples a priori. Five replicates for each K -value from 1 to 8 were tested, with 10,000 burn-in iterations followed by 50,000 Markov-chain Monte Carlo iterations. STRUCTURE HARVESTER (Earl and vonHoldt 2012) was used to implement the Evanno et al. (2005) method for identifying the most likely number of clusters (i.e., K) in a data set. All replicates from each K -value identified by this method were then combined using the "FullSearch" algorithm in CLUMPP version 1.1.2 (Jakobsson and Rosenberg 2007). DISTRICT version 1.1 (Rosenberg 2004) was used to convert CLUMPP outputs into bar plots that showed each individual's probability of assignment to the genetic clusters.

We used Arlequin version 3.5.1.2 (Excoffier and Lischer 2010) to compute the genetic differentiation index F_{ST} between all pairs of geographic populations for both AFLP and mtDNA data sets. We applied a Bonferroni adjustment to correct for multiple F_{ST} comparisons by using software from Lesack and Naugler (2011). Arlequin was also used to conduct analyses of molecular variance (AMOVAs; Excoffier et al. 1992). An AMOVA examines the partitioning of genetic differentiation within and among geographical subpopulations. We used this framework to test two different regional groupings for both AFLPs and mtDNA: (1) ocean-basin-scale structure (Gulf versus Atlantic) and (2) structure within the Atlantic (NCN, NCC, and NCS versus SC, GA, and FL). To further investigate the patterns observed from our STRUCTURE results, we also considered a third grouping for AMOVA: subdivision within NC (NCN and NCC versus NCS, SC, GA, and FL). For all analyses conducted in Arlequin, AFLPs were coded as haplotypic data. Arlequin does not

account for dominant marker systems, so the calculated fixation indices for AFLP data are based on peak phenotype (presence/absence) frequencies rather than allele frequencies. Despite this, Arlequin is often used for analysis of AFLPs (Svensson et al. 2004; Bensch and Åkesson 2005; Timmermans et al. 2005; Parchman et al. 2006; Toews and Irwin 2008); the degree of differentiation and their significance values are meaningful, but direct comparisons with F_{ST} values from co-dominant data sets should be avoided.

Mitochondrial DNA control region phylogeny in Southern Flounder was inferred with NETWORK version 4.6.1.2 (Fluxus Engineering; www.fluxus-engineering.com) by using the median joining method (Bandelt et al. 1999) to generate a haplotype network. Network branching patterns were postprocessed with maximum parsimony (Polzin and Daneshmand 2003). The mtDNA control region nucleotide composition and pairwise differences were calculated using MEGA version 6 (Tamura et al. 2013). Pairwise differences for the AFLP data set were also determined with MEGA. DnaSP version 5.10 (Librado and Rozas 2009) was used to assign mtDNA control region haplotypes and to calculate molecular diversity indices and Tajima's D -statistic (Tajima 1989). Sites with gaps were not considered for diversity calculations but were included in all other analyses.

RESULTS

When scored for all 201 samples across the 12 primer pair combinations, 671 polymorphic AFLP loci were identified. In the eight individuals for which the AFLP procedure was repeated, the AFLP profiles were 98.8% reproducible across all loci. Using the method of Evanno et al. (2005) to interpret the AFLP results from STRUCTURE, we identified a strong peak in ΔK at a K -value of 2 and a smaller but distinct secondary peak in ΔK at a K -value of 5. At a K of 2, we observed that each geographic population showed strong membership to one of two distinct genetic clusters representing either the Gulf or the Atlantic (Figure 2). In addition, the Atlantic samples from NCS and southward, particularly SC, displayed partial assignment to the genetic cluster dominating the Gulf locales. At a K of 5, the assignments remained similar to those produced at a K -value of 2, but SC ancestry was instead shared specifically with the western Gulf, and NCS samples displayed some shared assignment with the major genetic cluster found in the Gulf.

Because Southern Flounder from SC unexpectedly showed common ancestry with those sampled in the western Gulf (SBTX), we examined population pairwise distances for individual AFLP primer pairs in an attempt to identify the source of these similarities. For each primer pair, we compared

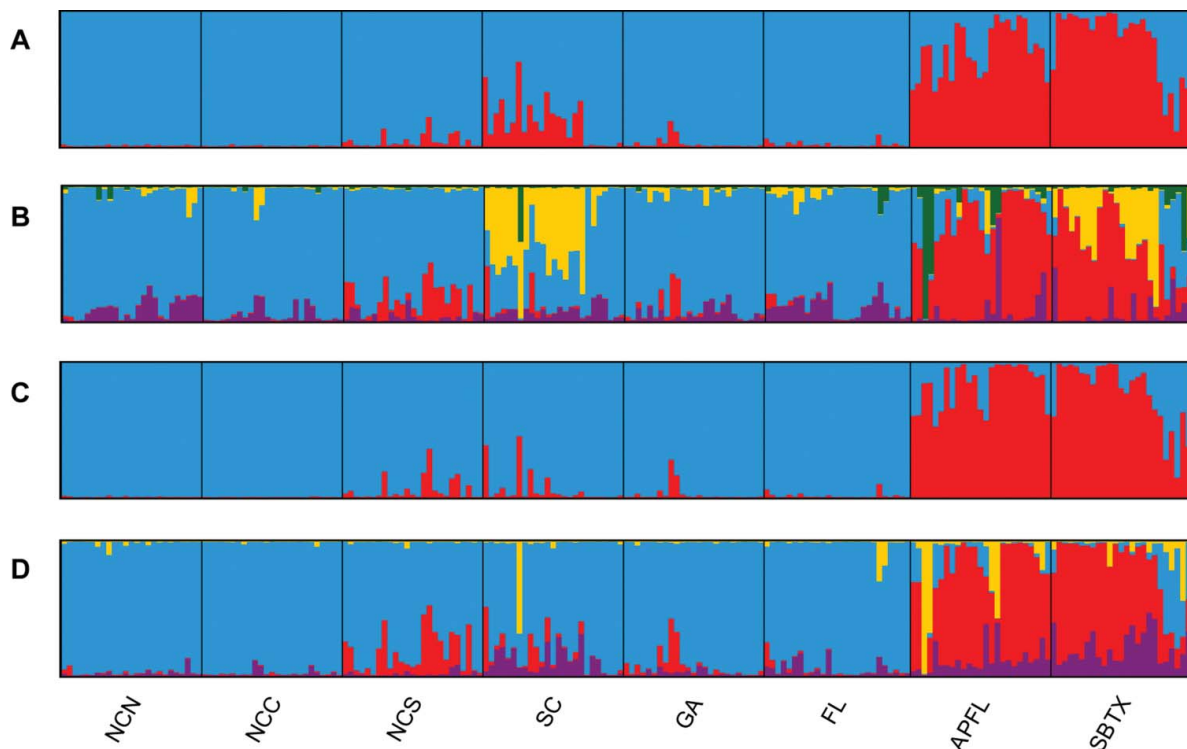


FIGURE 2. Population structure for Southern Flounder as estimated with amplified fragment length polymorphisms (AFLPs). Each individual is represented as a vertical line along the x-axis, and individuals are grouped into geographic populations. Each color represents a different inferred genetic cluster, and the probability of assignment to each of the K genetic clusters is indicated by the proportion of color along the y-axis: (A) K -value of 2 and (B) K -value of 5. Results for the AFLP data set after removing the primer pair *EcoRI*-AGC-*MseI*-CTT are also presented: (C) K -value of 2 and (D) K -value of 4. Location codes are defined in Figure 1.

(1) the average pairwise distance among geographic population pairs that included either SC or SBTX to (2) the average pairwise distance among all other geographic population pairs (i.e., those without SC or SBTX). The percent difference between these two metrics was used to assess the unusual levels of genetic differentiation in SC and the western Gulf. The average percent difference was 6.09% (SD = 5.93) across 11 of the 12 primer pairs. The remaining primer pair (*EcoRI*-AGC-*MseI*-CTT) displayed higher levels of differentiation, with a percent difference of 56.77%, and was likely responsible for the similarities observed between SC and SBTX samples. Subsequently, we conducted a second STRUCTURE analysis of the AFLP data set by using the same methods as previously described, except that we excluded the 38 loci from the primer pair *EcoRI*-AGC-*MseI*-CTT. A strong peak in ΔK was identified at a K of 2, and a smaller but distinct peak was observed at a K of 4. At the K -value of 2, the pronounced similarity between SC and SBTX was no longer present, but at the K of 4, a shared partial assignment among SC samples and Gulf samples was again detected (Figure 2). Overall patterns of genetic structure were similar to those observed prior to removal of the *EcoRI*-AGC-*MseI*-CTT primer pair from the AFLP data set.

Comparisons of F_{ST} for Southern Flounder AFLPs showed significant differentiation between all but four pairs of geographic populations ($F_{ST} = 0.020$ – 0.133 , $P < 0.01$; Table 1). The four population pairs that were not significantly differentiated after Bonferroni adjustment were all within-Atlantic comparisons (NCN versus GA; NCN versus FL; NCC versus FL; and GA versus FL). In general, F_{ST} values for Atlantic versus Gulf population pairs were larger than within-basin comparisons, although F_{ST} values for SC versus NCC and for SC versus NCS were comparable to between-basin values. When comparing Southern Flounder from the Gulf and Atlantic basins, AMOVA identified significant differences at all levels

of hierarchical population structure (Table 2). However, within-population variation accounted for 89.9% of the differences ($F_{ST} = 0.101$, $P < 0.0001$). When partitioning differentiation in the Atlantic alone (NCN, NCC, and NCS versus SC, GA, and FL), 96.6% of the variation was due to within-population differences ($F_{ST} = 0.034$, $P < 0.0001$; Table 2). There was no differentiation between these two Atlantic regions. There was also no differentiation between regions when considering NCN and NCC as a separate group relative to NCS, SC, GA, and FL.

Across 260 samples, the final mtDNA control region alignment for Southern Flounder was 480 bp, with 88 segregating sites and a total of 130 unique haplotypes. This included a single-base-pair insertion/deletion that was present in two haplotypes, each composed of one individual from different sites. Excluding sites with gaps, overall haplotype diversity was 0.977 (SD = 0.0036) and nucleotide diversity was 0.011 (SD = 0.0003), with an average of 5.06 nucleotide changes between individuals (Table 3). Tajima's D -statistic was estimated to test for neutrality of nucleotide substitutions; the values were significantly negative for both ocean basins (Atlantic: $D = -2.08$, $P < 0.05$; Gulf: $D = -1.97$, $P < 0.05$), which may be indicative of Southern Flounder population expansion (Fu 1997).

Twenty-nine mtDNA control region haplotypes were common among individual Southern Flounder, and three of the haplotypes were shared between the Gulf and Atlantic. The majority of the haplotypes were singletons, most of which differed from common haplotypes by only one or two nucleotide substitutions. The control region haplotype network showed a pattern of divergence between Southern Flounder in the two ocean basins, although separation was not complete (Figure 3). The majority of Gulf haplotypes were separated from Atlantic haplotypes by at least three nucleotide changes, but there were Atlantic haplotypes present in the Gulf cluster

TABLE 1. Population pairwise F_{ST} comparisons based on amplified fragment length polymorphism profiles (above diagonal) and mitochondrial DNA control region haplotypes (below diagonal) from Southern Flounder sampled in the U.S. South Atlantic (NCN = North Carolina north; NCC = North Carolina central; NCS = North Carolina south; SC = South Carolina; GA = Georgia; FL = Florida) and in the Gulf of Mexico (APFL = Apalachicola, Florida; SBTX = San Antonio Bay, Texas). Values without superscript letters were not significant.

Sample	NCN	NCC	NCS	SC	GA	FL	APFL	SBTX
NCN		0.0218 ^c	0.0436 ^c	0.0506 ^c	0.0122 ^{bd}	0.0062	0.0969 ^c	0.1133 ^c
NCC	0.0049		0.0519 ^c	0.0724 ^c	0.0199 ^b	0.0133 ^{bd}	0.1119 ^c	0.1330 ^c
NCS	0.0052	-0.0002		0.0722 ^c	0.0215 ^c	0.0314 ^c	0.0652 ^c	0.1054 ^c
SC	0.0369 ^{ad}	-0.0079	0.0231		0.0526 ^c	0.0420 ^c	0.1037 ^c	0.0694 ^c
GA	0.0521 ^{ad}	0.0102	0.0367 ^{ad}	-0.0074		0.0099 ^{bd}	0.0771 ^c	0.1105 ^c
FL	0.0215	-0.0095	-0.0029	-0.0036	0.0003		0.0869 ^c	0.1100 ^c
APFL	0.4234 ^c	0.3993 ^c	0.4558 ^c	0.3841 ^c	0.3962 ^c	0.4165 ^c		0.0304 ^c
SBTX	0.3993 ^c	0.3736 ^c	0.4368 ^c	0.3582 ^c	0.3715 ^c	0.3949 ^c	0.0058	

^a $P < 0.05$.

^b $P < 0.01$.

^c $P < 0.0001$.

^dNot significant after Bonferroni correction.

TABLE 2. Results from analyses of molecular variance based on amplified fragment length polymorphisms in Southern Flounder. Populations are grouped at two different geographic scales: (1) ocean basin (Gulf of Mexico versus U.S. South Atlantic) and (2) U.S. South Atlantic (North Carolina versus South Carolina, Georgia, and Florida; * $P < 0.05$; ** $P < 0.0001$).

Source of variation	df	Sum of squares	Variance component	Percentage of variation	Fixation index
Ocean basin scale					
Among groups	1	401.326	4.042	6.98	$F_{CT} = 0.0698^*$
Among populations within groups	6	587.121	1.822	3.15	$F_{SC} = 0.0338^{**}$
Within populations	193	10,045.97	52.052	89.88	$F_{ST} = 0.1013^{**}$
Total	200	11,034.42	57.916		
U.S. South Atlantic					
Among groups	1	87.036	-0.179	-0.34	$F_{CT} = -0.0034$
Among populations within groups	4	402.025	1.964	3.71	$F_{SC} = 0.0370^{**}$
Within populations	145	7,407.171	51.084	96.62	$F_{ST} = 0.0338^{**}$
Total	150	7,896.232	52.869		

and vice versa. There was no clear pattern of finer-scale structure within the Atlantic cluster based on the haplotype network.

Pairwise population comparisons using the mtDNA control region showed strong differentiation ($F_{ST} = 0.37\text{--}0.45$, $P < 0.0001$) between all pairs of populations compared across basins (Table 1). Three pairs of populations within the Atlantic (GA and NCS; GA and NCN; and SC and NCN) were also significantly differentiated, although F_{ST} values were much smaller ($F_{ST} = 0.037\text{--}0.052$, $P < 0.05$). None of these within-Atlantic comparisons remained significant after a Bonferroni correction was applied, and no other pairs of populations within

the Atlantic or Gulf were found to be significantly differentiated with any test. When comparing the Gulf and Atlantic, the AMOVA for the mtDNA control region showed that the majority of variation was explained by differences within populations (59.3%; $F_{ST} = 0.407$, $P < 0.0001$), although a large portion of the differentiation was due to differences between ocean basins (40.2%; F_{CT} [genetic differences among groups] = 0.402, $P < 0.05$; Table 4). Within the Atlantic, almost all of the variation was explained by differences within populations (98.2%; $F_{ST} = 0.018$, $P > 0.05$), and only a small portion of the variation was explained by differences between NC and the other Atlantic populations (1.8%; $F_{CT} = 0.018$, $P > 0.05$), but neither of these differences was significant (Table 4).

TABLE 3. Molecular diversity measures for populations of Southern Flounder sequenced at the mitochondrial DNA control region (excluding sites with gaps). Number of samples (n), number of polymorphic sites excluding gaps (N_p), number of haplotypes (N_h), haplotype diversity (H_d), and nucleotide diversity (π) are given for each site. Location codes are defined in Table 1.

Location	n	N_p	N_h	H_d	π
U.S. South Atlantic					
NCN	30	28	19	0.910	0.0082
NCC	33	30	25	0.981	0.0087
NCS	34	26	22	0.934	0.0073
SC	31	31	25	0.981	0.0085
GA	31	28	23	0.974	0.0079
FL	30	28	23	0.972	0.0080
Overall (Atlantic)	189	70	93	0.967	0.0082
Gulf of Mexico					
APFL	36	35	25	0.946	0.0094
SBTX	35	28	19	0.909	0.0080
Overall (Gulf)	71	47	39	0.931	0.0087
Total (Atlantic plus Gulf)	260	87	129	0.977	0.0106

DISCUSSION

We observed divergence between Gulf and Atlantic Southern Flounder populations based on both mtDNA control region sequences and AFLP fingerprints. All population pairwise F_{ST} and AMOVA F_{CT} values were significant when comparing between ocean basins, regardless of the molecular marker used (Tables 1, 2, 4). The same result was observed with AFLP population assignments (Figure 2) and in the mtDNA haplotype network (Figure 3). These findings are in agreement with previous Southern Flounder studies that examined allozymes (Blandon et al. 2001), mtDNA (Anderson et al. 2012), and microsatellites (Anderson and Karel 2012), and they correspond with a well-known biogeographic break in marine fauna between the Gulf and Atlantic (Avice 1992). Southern Flounder are absent from the southern tip of Florida (Ginsburg 1952; Gilbert 1986), so a transition in genetic composition between ocean basins appears to correspond with the habitat discontinuity that is responsible for the disjunct distribution of Southern Flounder across south Florida.

We predicted that both markers would perform well for evaluating deep divergence, but we also wanted to assess

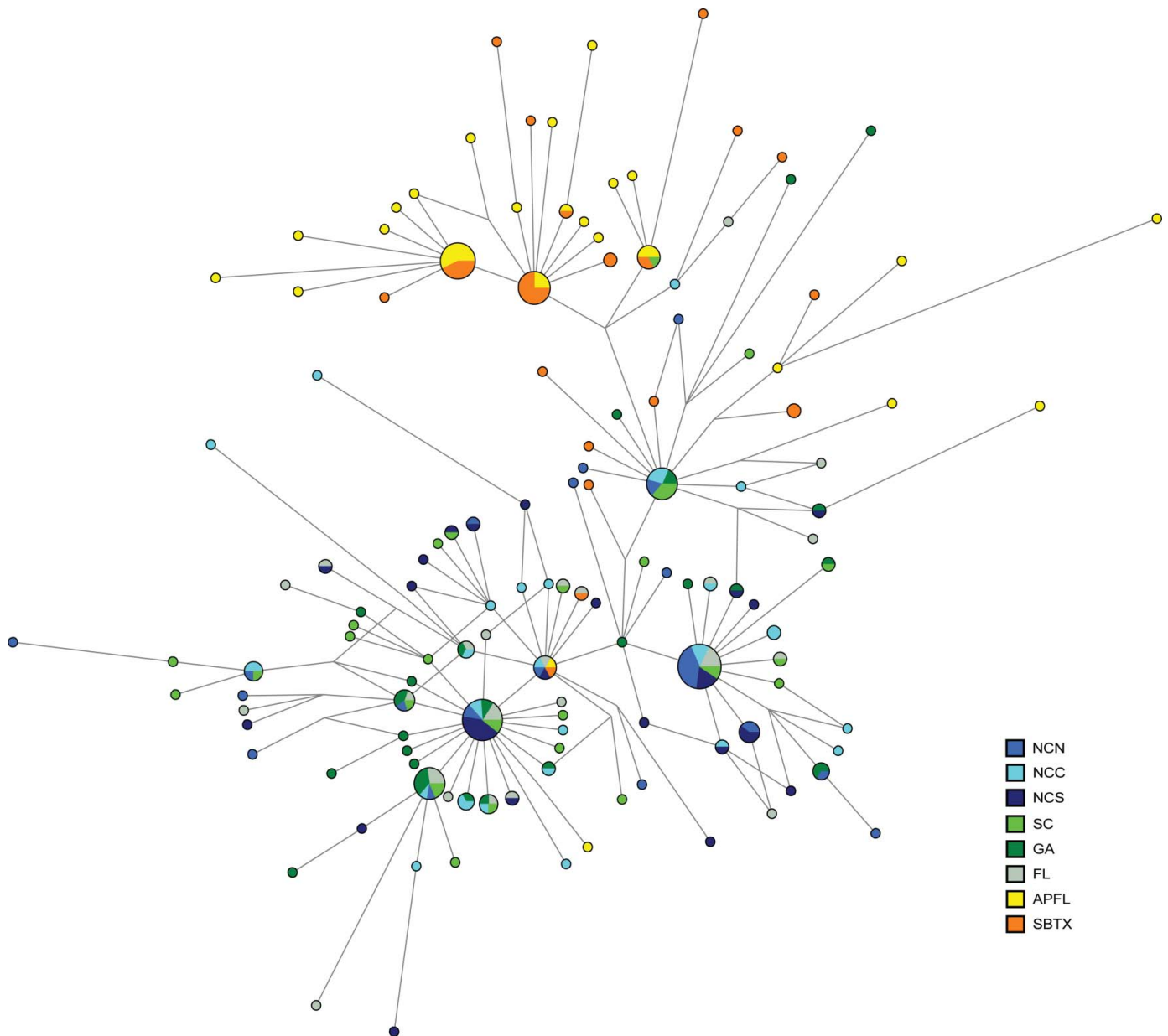


FIGURE 3. Median joining network of mitochondrial DNA control region haplotypes identified in Southern Flounder. Circles represent haplotypes; colored wedges indicate geographic populations. Circles and wedges are proportional to the sample size, and the length of the connection is proportional to the number of nucleotide changes between haplotypes. Location codes are defined in Figure 1.

how AFLPs and mtDNA would compare when examining subtle structure, as we expected to see in the Atlantic Southern Flounder populations. Although mtDNA markers are commonly used in population genetic studies because of their high mutation rates and purportedly neutral evolution, the mitochondrial genome is also haploid (maternally inherited, effectively as a single locus) and is very small in comparison with the nuclear genome. Thus, studies that rely solely on mtDNA markers may draw incomplete conclusions when attempting to make inferences about organisms and populations as a whole (reviewed by Ballard and

Whitlock 2004). Amplified fragment length polymorphism loci are scattered across the genome and may better reflect demographic, historical, and selective events for which influences on diversity patterns can vary across loci, so we expected to obtain increased genetic resolution with AFLP analysis.

The AFLP and mtDNA results were generally in agreement when assessing Southern Flounder differentiation between the two ocean basins, but the results began to diverge when we examined structure within ocean basins. The magnitude of differentiation for pairwise comparisons between ocean basins

TABLE 4. Results from analyses of molecular variance based on mitochondrial DNA control region sequences in Southern Flounder. Populations are grouped at two different geographic scales: (1) ocean basin (Gulf of Mexico versus U.S. South Atlantic) and (2) U.S. South Atlantic (North Carolina versus South Carolina, Georgia, and Florida; * $P < 0.05$; ** $P < 0.0001$).

Source of variation	df	Sum of squares	Variance component	Percentage of variation	Fixation index
Ocean basin scale					
Among groups	1	141.313	1.344	40.15	$F_{CT} = 0.4016^*$
Among populations within groups	6	15.532	0.019	0.56	$F_{SC} = 0.0094$
Within populations	252	499.841	1.983	59.28	$F_{ST} = 0.4072^{**}$
Total	259	656.685	3.346		
U.S. South Atlantic					
Among groups	1	5.263	0.035	1.78	$F_{CT} = 0.0178$
Among populations within groups	4	7.755	0.000	-0.01	$F_{SC} = -0.0001$
Within populations	183	356.284	1.947	98.24	$F_{ST} = 0.0176$
Total	188	369.302	1.982		

was much higher with mtDNA ($F_{ST} = 0.358\text{--}0.456$) than with AFLPs ($F_{ST} = 0.065\text{--}0.133$), although both were highly significant. The opposite was seen for population comparisons within the Atlantic, as mtDNA differentiation values ($F_{ST} = -0.010$ to 0.052) were nonsignificant and lower than AFLP values ($F_{ST} = 0.006\text{--}0.072$), which showed significant differentiation for nearly all within-Atlantic comparisons. If we were to interpret the degree of population structure as proposed by Wright (1978), genetic differentiation among Southern Flounder populations when comparing ocean basins would be characterized as “very great” (mtDNA) or “moderate” (AFLPs), whereas comparisons within the Atlantic basin showed “little” to “moderate” genetic differentiation based on AFLPs.

The levels of genetic differentiation detected in this study were consistent with previously reported F_{ST} values for Southern Flounder mtDNA (Anderson et al. 2012), but we observed markedly higher differentiation using AFLPs than was previously found with microsatellites (within-basin $F_{ST} = -0.002$ to 0.007 ; between-basin $F_{ST} = 0.025\text{--}0.046$; Anderson and Karel 2012). Similar discrepancies between markers have also been observed in recent studies of other species, for which differentiation was both higher (Mock et al. 2002; Whitehead et al. 2003; Gruenthal et al. 2007) and lower (Maguire et al. 2002) when comparing AFLPs to microsatellites. For analyses of highly variable multi-allelic loci, as in the Anderson et al. (2012) study where there were up to 53 alleles per microsatellite locus, very high levels of within-population heterozygosity can result in reduced magnitudes of differentiation between populations (Hedrick 1999). Different molecular markers are subject to different evolutionary processes, which can affect levels of diversity within and between populations (reviewed by Zink and Barrowclough 2008; Toews and Brelsford 2012). Therefore, the lack of correspondence in the magnitude of differentiation estimated with the different markers used to assess Southern Flounder genetic structure thus far is not surprising.

In this study, nearly all Southern Flounder geographic populations within the Atlantic were significantly differentiated (after Bonferroni correction for multiple comparisons) when using the AFLP data set (Table 1), thus contradicting the mtDNA results. The power to detect differentiation increases with the number of loci, and even very small values of F_{ST} can become statistically significant when enough markers are used (Ryman et al. 2006). We compared a single mtDNA marker to an AFLP profile comprising 671 loci, so we expected increased resolution. However, the levels of within-Atlantic AFLP differentiation we detected are not considered low for marine populations. Even very weak but significant levels of genetic differentiation can be biologically relevant; for example, Knutsen et al. (2011) used empirical mark-recapture data to confirm the existence of distinct, localized populations of coastal Atlantic Cod *Gadus morhua* that produced low levels of genetic differentiation. Based on AFLP differentiation, genetic structure appears to be present among geographic populations of Southern Flounder in the Atlantic, although this does not necessarily mean that each locale represents a discrete stock.

Waples et al. (2008) cautioned that statistical significance is not always correlated with biological significance and that the life history and biology of an organism must be taken into consideration when determining stocks. Thus far, we have no evidence that Southern Flounder from different locales within the Atlantic are biologically distinct. Based on otolith morphometric analysis, Midway et al. (2014) also detected only weak evidence for differentiation among Southern Flounder populations within the Atlantic, including within NC. These results are consistent with our findings. Despite the possibility that many AFLP loci should allow for greater power in the detection of population subdivision, AMOVA did not find significant genetic structure when comparing NC (or portions of NC) to the remaining Atlantic locales. The same lack of NC regional structure was found with AMOVA based on mtDNA.

These results do not support the consideration of NC Southern Flounder as a distinct stock within the Atlantic.

In addition to evaluating patterns of differentiation, we also assessed the genetic composition of each Southern Flounder population. When sufficient numbers of loci are used, AFLPs are highly accurate for population assignment tests and can be particularly informative for systems with weak genetic structure (Campbell et al. 2003). We saw similar patterns in individual assignments to inferred genetic clusters (i.e., K) across the most likely values of K for the AFLP profiles (Figure 2). Although we examined patterns of variation at K -values of 2 and 5, the major genetic patterns remained the same. The two summary plots both showed a strong genetic distinction between Gulf and Atlantic Southern Flounder. Within NC, the NCN and NCC samples appeared to be homogeneous, whereas NCS displayed a small difference in assignment probabilities, suggesting that a mild barrier to gene flow may exist between these regions. The most unexpected observation was the shared clustering of SC samples—obtained near the center of the Atlantic populations—with the Gulf samples, particularly those from SBTX.

Overall, the AFLP genetic cluster analyses suggested high gene flow within the Atlantic, with the possible exception of SC. Of the three mtDNA haplotypes shared between ocean basins, one consisted of individuals from SC and both Gulf locales, although this was a low-frequency haplotype (Figure 3). In addition, the SC samples were differentiated from one of the NC geographic populations in the mtDNA F_{ST} analysis, but this subdivision was no longer significant after Bonferroni adjustment. In contrast, SC was always highly significantly differentiated from both Gulf locales in pairwise F_{ST} comparisons, whether based on mtDNA or AFLPs. With these varying results, the potential distinction of SC genotypes from other Atlantic samples remains inconclusive.

By examining pairwise differences in AFLP profiles between geographic populations, we were able to identify a single primer pair (*EcoRI*-AGC-*MseI*-CTT) as the likely cause for the assignment of SBTX and SC individuals to the same genetic cluster. This was confirmed by a genetic cluster analysis that excluded loci from the primer pair in question, although the similarity between SC and Gulf samples was still present to a smaller degree when the number of assumed genetic clusters was increased (Figure 2). Because one primer pair appears to be responsible for this grouping, it is possible that the pattern may be an artifact of the AFLP procedure. Errors in peak scoring are a common source of AFLP genotyping error (Bonin et al. 2004), so we used an established method to reduce scoring subjectivity and error in our AFLP data by setting peak height thresholds for inclusion of loci and phenotype calling (Whitlock et al. 2008). This resulted in a conservative data set that performed well in our tests for reproducibility (98.8%).

Another concern in AFLP studies is fragment size homoplasy, which can occur as a result of the anonymous nature of

AFLP loci. Equal-length DNA fragments are scored as a presence on the same locus, regardless of whether the fragments are homologous in genomic origin. Amplified fragment length polymorphism homoplasy can result in the underestimation of differentiation between populations and may provide one potential explanation for the unforeseen similarity we observed between SC and SBTX Southern Flounder. Homoplasy bias increases with the number of loci scored per primer pair and with smaller fragment sizes (<125 bp; Vekemans et al. 2002; Caballero et al. 2008). We reduced the numbers of AFLP loci analyzed per primer pair by applying peak height thresholds as previously described (only 38 of 139 loci initially detected were analyzed for *EcoRI*-AGC-*MseI*-CTT), but small fragment sizes were not excluded in this study. The potential influence of AFLP homoplasy on genetic diversity estimates must be considered, but experimental quantification of the degree of homoplasy is not within the scope of most AFLP studies (Meudt and Clarke 2007).

Alternatively, it may be possible for the geographically disjunct individuals from SC and the Gulf to have similar molecular profiles based on shared adaptive traits. Like other genome-scale molecular data sets, AFLP loci are anonymous and consist mostly of neutral loci, but they often include loci that are linked to genes under selection (Luikart et al. 2003). To investigate any patterns of distribution that may be related to adaptive evolution, future studies would benefit from a genome scan, which detects AFLP loci that show abnormally high among-population differentiation; such outliers are a signature of selection (Beaumont and Balding 2004).

The results of this study confirmed a clear divergence between Gulf and Atlantic Southern Flounder. There was also evidence of genetic structure among geographic populations of Southern Flounder at a finer spatial scale, which was only revealed upon analysis of AFLPs. We found intriguing patterns of differentiation and genetic clustering that did not correspond with obvious geographic patterns. However, there was no differentiation between samples from NC and those from the other Atlantic states when examining hierarchical population structure. Southern Flounder in NC are currently managed as a unit stock. The present molecular analysis suggests that a single-stock designation within NC should be adequate, as there is no clear evidence for additional subdivisions at a finer spatial scale (i.e., between regions within the state). However, the potential for high levels of mixing throughout the Atlantic basin, as evidenced by the presence of only weak genetic structure within the basin, suggests that interstate cooperation will be necessary to achieve a comprehensive population assessment of Southern Flounder at an appropriate spatial scale. To sustain fishery yields and meet conservation objectives for Southern Flounder, the spatial scale of stock assessment will need to be more closely aligned with the spatial scale of important ecological processes that determine stock dynamics. Our present findings suggest that these processes likely operate over broad spatial scales and that future

management of Southern Flounder could be improved through a synthesis of biological information encompassing the species' range.

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