Genetic Population Structure of Summer Flounder *Paralichthys dentatus* using Microsatellite DNA Analysis

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Abstract

Summer flounder *Paralichthys dentatus* supports one of the most valuable commercial and recreational fisheries along the Atlantic Coast of the U.S. However, in recent decades the management of this species has proven to be one of the most contentious for any exploited marine resource in the region. A coastwide catch quota is imposed annually for summer flounder of which 60% is allocated to the commercial fishery and 40% to the recreational fishery. The allocation is further divided among the individual coastal states from North Carolina to Massachusetts based on their landings in the 1980s. This process, based on political jurisdictions, does not consider the species' biological stock structure. Previous genetic studies (allozyme, mtDNA, and SNPs) provided contradictory results regarding the possible population structure of summer. To address this issue, we used DNA microsatellite analysis at 9 loci to define the coastwide population structure of summer flounder. In total, 1,182 specimens were analyzed from 18 collection sites. Most collections were from the continental shelf during the fall-winter spawning season. These were supplemented with additional samples from inshore waters from North Carolina to Florida, and inshore sites which support significant recreational fisheries at Nantucket Shoals, Massachusetts and Fire Island, New York. The overall level of genetic differentiation in pairwise comparison between collections was very low, mean F_{ST}= 0.001. There was no evidence of genetic differentiation between collections from north and south of Cape Hatteras. Our microsatellite results are consistent with an earlier SNP study which failed to find significant allelic heterogeneity among coastwide collections of summer flounder. However, a subset of pairwise F_{ST} comparisons between some collections proved statistically significant. Furthermore, in STRUCTURE analysis we found evidence of two genetic clusters within the species' northern landings area, however, this finding was not supported by DPAC analysis. We conclude that summer flounder most likely constitute a single population along their entire Atlantic Coast distribution.

Introduction

Management of summer flounder *Paralichthys dentatus* (also known as fluke) has proven to be one of the most contentious for any harvested species along the U.S. Atlantic Coast (Terceiro 2002). Management of summer flounder has pitted recreational versus commercial fishers, states vs. states, and fisherman groups against management agencies and non-governmental organizations (Lavelle 2014). Management of summer flounder has been further complicated by a warming ocean which resulted in a recent significant northward and eastward shift in the distribution of its biomass and the fisheries that target them (Dubik et al. 2019).

Both in weight landed and monetary value, summer flounder supports the most important commercial and recreational flatfish fisheries in the mid-Atlantic and southern New England regions (NOAA 2000). Approximately 70% of the commercial summer flounder harvest comes from the Exclusive Economic Zone (EEZ) (3-200 miles offshore), while more than 90% of recreational landings come from nearshore and estuarine waters. Summer flounder is managed by the Atlantic States Marine Fisheries Commission (ASMFC) in state waters (0-3 miles), and by the Mid-Atlantic Fishery Management Council and NOAA Fisheries in Federal EEZ waters. The most recent Benchmark Stock Assessment (Northeast Fisheries Science Center, 2019) concluded that the summer flounder stock was not overfished, that overfishing was not occurring, and that the stock was rebuilt in 2010.

A coastwide quota on summer flounder harvest through a target fishing mortality level is determined annually based on assessments of spawning stock biomass and recruitment. Once an annual quota is set coastwide, landings are divided on the basis of 60% to the commercial and 40% to the recreational fisheries; however, in some years the recreational harvest has exceeded that of the commercial fishery. Furthermore, the catch allocation among the states is based on the proportion of state landings to coastwide landings reported in the 1980s. Thus, in 2019, New York state's allocation was less than 50% of neighboring New Jersey's and slightly more than 25% of North Carolina's, the highest of any state (27.5% of total coastwide allocation). Recently, it has been suggested that allocation of harvest be partitioned on a regional rather than statewide basis with either three (Massachusetts and Rhode Island; Connecticut to New Jersey; Delaware to North Carolina) or four (Massachusetts; Rhode Island to New Jersey; Delaware to

Virginia; North Carolina) regions proposed. In none of these management scenarios is the actual biological stock structure of summer flounder considered. Furthermore, recent evidence suggests that the coastwide distribution and center of biomass of summer flounder and its fisheries is shifting northward and eastward because of climate change (Lavelle 2014, Dubik 2019), further casting doubt on the reliance of the allocation process based on catch data that is almost four decades old.

The distribution of summer flounder extends from Florida to Nova Scotia, in estuaries, nearshore coastal waters, and the inner and outer Continental shelves. However, the vast majority of the fishery is located between Cape Cod, MA, and Cape Hatteras, NC. The recreational fishery occurs within estuaries and nearshore coastal waters while the commercial fishery is prosecuted both in inshore coastal and continental shelf waters. Summer flounder are usually sexually mature by age 2 at total lengths (TL) of 24-27 cm for males and 30-33 cm for females. Females exhibit faster growth rates and a maximum life expectancy of at least 14 years, versus 12 years for males (Terceiro, 2009). Females dominate the length interval greater than 45 cm TL and all individuals larger than 55 cm TL are believed to be female.

Adult summer flounder undertake offshore spawning migrations beginning in late summer-early fall and extending into early winter. Individuals from southern New England and the mid-Atlantic Bight exhibit strong inshore-offshore movements. Juveniles and adults occur in estuaries and shallow coastal waters during the late spring and summer, followed by movement to the Continental Shelf at depths from 70 to 155 m during the early fall where they remain through winter (Grosslein and Azarovitz 1982). Winter distributions on the Shelf extend from the Norfolk Canyon to Georges Bank. During late winter and early spring, adults migrate shoreward, reaching estuaries and inshore coastal waters by late April-May cued by increasing temperatures during spring (Able and Kaiser 1994). Individuals from the southern portion of the species' range may exhibit less inshore-offshore movement than those from the north; some may be resident year-round in some southern estuaries.

Geographic behavioral differences may be indicative of stock structure. Tagging studies show that juveniles that summer in New York (Poole 1962) and New Jersey estuaries (Hamer and Lux

1962) return as adults to the same embayments during subsequent summers. Tagging studies also suggest that summer flounder from New York and New Jersey winter in the Hudson Canyon, whereas those from southern New England winter at Georges Bank (Holland 1991). Thus, there may be spatial segregation of spawning summer flounder from different areas during the winter months.

Early life-stage density differences also suggest the possibility of stock differentiation. Peak egg densities occur during October-November at Georges Bank, southern New England, and New York, with spawning occurring slightly later during these months off New Jersey (Able and Kaiser 1994). A December peak off Cape Hatteras was also noted (Smith 1973). Eggs are buoyant, ascending the water column shortly after fertilization. Distributions and densities of larvae essentially mirror those of eggs. Larvae are planktonic and are carried shoreward and enter estuarine nurseries from January to March in the mid-Atlantic Bight where their vertical-to-horizontal transformation and settlement to the benthos occurs during mid-summer. There is no evidence of larval settlement on the Continental shelf, although studies addressing this issue have not occurred.

The stock is the primary unit of fisheries management, which may be composed of one or more discrete populations. Reproductive isolation among stocks maintains their integrity and allows for the development of stock-specific characteristics in response to differing environmental variables. The presence of more than one stock within a species' distribution implies that each be afforded protection consistent with its particular abundance and threats. Historically, a variety of phenotype-based and genetics approaches were used to elucidate the stock structure of wide-ranging and exploitable fishes. Today, DNA-based techniques such as microsatellite and single nucleotide polymorphism (SNP) analyses are frequently used to sensitively plumb for stock structure in widely distributed species such as summer flounder.

Summer flounder is presently managed as a single stock, but evidence for this designation is limited and contradictory. The single stock model was mainly founded on the observation that during fall-early winter summer flounder spawn in a seemingly continuous aggregation on the edge of the Continental shelf from Georges Bank to North Carolina. If stock division does exist,

it was hypothesized to occur at the zoogeographic boundary of Cape Hatteras where the Gulf Stream diverges from southwesterly flowing coastal currents (reviewed in Burke et al. 2000).

Marine fishes such as summer flounder had long been viewed as highly connected demographically because of their vagile pelagic egg and larval life stages, extended duration of these early life-stages, and high adult vagility (Hedgecock 1986; Siegel et al. 2003). But recent genetic evidence suggests that may not always be the case (Knutsen et al. 2010; Benestan et al. 2015; Therkildsen et al. 2013; Clukas et al. 2019). With sensitive techniques such as microsatellite analysis and genotyping at SNP loci identified with next generation sequencing, sophisticated bioinformatic analyses, and faster processing of specimens leading to larger sample size, the ability to detect genetic population structure in marine fishes has increased in recent times. Furthermore, the analysis of a subset of next generation SNP loci allows for the screening of not only adaptively neutral loci but also introduces analyses of loci that may be under selective pressure. Employment of these approaches revealed that rather than being demographically open, marine fishes sometimes exhibit stock structure on finer geographic and temporal scales due to processes that limit dispersal and promote self-replenishment of local populations and spawning site fidelity, egg and larval retention at those sites, and local adaptation (Hauser and Carvalho 2008). Not only have molecular techniques revealed greater levels of heterogeneity of stock structure in marine fishes than previously thought, concomitant variation in ecologically important traits sometimes indicate the presence of extensive adaptive differentiation.

Several earlier studies have investigated the genetic population structure of summer flounder with conflicting results. Using protein electrophoresis at 17 isozyme loci, Van Housen (1984) reported significant allelic differentiation among summer flounder collections from north and south of Cape Lookout, North Carolina. In contrast, using a more sensitive genetic approach, sequence analysis of the mitochondrial DNA (mtDNA) control region, Jones and Quattro (1999) reported the absence of significant haplotype frequency differences between composite collections from north and south of Cape Hatteras, North Carolina. Results of this study are frequently cited as supportive of the single stock model for management of the U.S. summer flounder fishery (Kraus and Musick 2003). However, Jones and Quattro (1999) also reported

significant haplotype heterogeneity between their two most extreme northern samples from Rhode Island Sound, Rhode Island, and Buzzard's Bay, Massachusetts, although, sample sizes were small. Finally, using even a more sensitive genetic approach, Hoey and Pinsky (2018), failed to detect significant allelic frequency heterogeneity at 1,137 single nucleotide polymorphism (SNP) loci in summer flounder that were collected from throughout their distribution although samples sizes were small and many were not collected from offshore spawning locales.

Our objective was to use a sensitive genetic approach, microsatellite DNA analysis, to further assess whether summer flounder constitute a single stock, or multiple stocks over their coastwide distribution and, most importantly, within the Cape Cod to Cape Hatteras management area. If multiple stocks were identified, we sought to determine the boundaries of their units. Our sampling strategy was to focus our collections on the winter spawning months and at offshore spawning locales. Our null hypothesis was that summer flounder constitute a single genetic stock within their almost complete coastwide distribution from Cape Cod to south of Cape Hatteras as evidenced by a homogeneity of microsatellite DNA allelic frequencies.

Methods

Sample collections: We focused our collecting efforts and analysis on adult specimens of summer flounder from their spawning locales on the Continental Shelf during the fall-early winter spawning season (Table 1 and Figure 1). Many were obtained from the Northeast Fisheries Sciences Center's (NEFSC) fall and winter trawl surveys. These surveys use a stratified-random design from eastern Georges Bank to Cape Hatteras. The winter survey provided us with late spawners (February) from the Cape Hatteras area that were missed in the fall surveys. A second source was the commercial fall and winter offshore fishery that was sampled by the NEFSC's port observers. Similar to the trawl surveys, this shelf fishery extends from Georges Bank to North Carolina in 100-200 fathoms. Dried scales, otoliths and typical biological data were collected for age and growth studies and were archived at the NEFSC. We also obtained a subset of offshore samples (fin clips stored in EtOH) that were collected from commercial fishermen through collaboration with the Marine Program of Cornell Cooperative

Extension, Riverhead, NY. These samples complemented earlier collections by providing specimens from some distinct locations and times that were not available through the two NEFSC archives. We also analyzed samples from locales south of Cape Hatteras made available through the South Carolina SEAMAP program which three times a year trawls nearshore coastal waters (15-30 ft depths) from Cape Hatteras to Cape Canaveral, FL. We also obtained a collection from inshore waters of North Carolina through assistance of the North Carolina Division of Marine Fisheries (NCDMF). These two southern samples allowed us to address the lingering question of the genetic distinctiveness of collections south of Cape Hatteras as well as provide an "outgroup" for our analysis of more northern collections. Our final two sources of specimens were late summer recreational fisheries on Nantucket Shoals, Massachusetts and in Fire Island Inlet, New York.

DNA Isolations

Dried uncleaned scales from the NEFSC archives and fin clips in EtOH were the two sources of DNAs for this study. In fact, dried scales provided a reliable source of high quality DNA at sufficiently high concentrations for analyses. Total DNA was isolated from 2-5 scales from each specimen and individual fin clips by their incubation in CTAB buffer (Saghai-Maroof et al. 1984), digestion with proteinase K, standard phenol-chloroform extraction, and alcohol precipitations. DNA concentrations and purities were determined with a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). All DNAs were diluted to a final concentration of 50 ng/µl for standardization of subsequent PCR reactions.

Microsatellite DNA analysis

Microsatellite analysis was conducted at nine loci that were previously isolated from summer flounder by Shao et al. (2009), including Pade01, Pade10, Pade11, Pade12, Pade15, Pade26, Pade30, Pade32, and Pade34. PCR reactions were in 12.5-µl total volumes that contained 50 ng of template DNA, 1 x PCR KlenTaq1 buffer, 0.1 µl of dNTPs (25 mM stocks) (GE Healthcare, Piscataway, NJ), 0.5 µl (0.8 µl when using D2-PA dye) of forward and reverse primers (1 µM stock) (Integrated DNA Technologies, Coralville, IA), one of which was labeled with one of three Beckman Coulter dyes D2-PA, D4-PA or D3-PA (Sigma, St. Louis, MO) and 0.025 µl KlenTaq1 polymerase (25 U/µl) (Ab Peptides, Inc., St. Louis, MO). PCR reactions were done

singly and pooled prior to analysis. Cycling parameters were 65 cycles at 95° C for 5 min, denaturation at 95° C for 30 s, annealing at 64° C (except for Pade34 at 66° C) for 30 s, extension at 72° C for 30 sec, and final extension at 72° C for 7 min.

Characterizations of microsatellite genotypes were done on a Beckman Coulter (Fullerton, CA) CEQ^{TM}8000 capillary-based DNA sequencer. Multi-pooled PCR reactions of 0.4 to 2.2 µl were diluted with 33 µl of a mixture (100:1) of A.C.E formamide (Amresco, LLC, Solon, Ohio) and CEQ DNA Size Standard-400 (Beckman Coulter) and run with the FRAG 1 program (Beckman Coulter).

Statistical Analyses

MICRO-CHECKER (Oosterhout et al. 2004) was used to test for the presence of null alleles, errors due to microsatellite stuttering, and large-allele dropout. Multi-locus microsatellite genotypes were compiled for each specimen. Measures of diversity, including mean number of alleles per locus, allelic richness, $F_{ST,}$ and observed and expected heterozygosities (H_O and H_e) were determined for all collections using FSTAT version 2.9.3 (Goudet et al. 1995; Goudet 2001) and GenoDive V.20b27 (Meirman and Van Tienderen 2004). Deviations from Hardy-Weinberg proportions and linkage equilibrium were evaluated in GENEPOP v4.0.6 (Rousset 2007) using the Markov chain method with the default parameters: 10,000 iterations and 10,000 batches (Raymond and Rousset, 1995).

Tests of population differentiation were performed in GenoDive V.20b27 (Meirman and Van Tienderen 2004) using pairwise F_{ST} comparisons at single loci and across all loci using the F_{ST} estimator θ of Weir and Cockerham (1984). F_{ST} is highly dependent on within-population diversity (Hedrick 1999; Balloux and Lugon-Moulin 2002; Meirmans and Hedrick 2011). When loci with large numbers of alleles are examined and population diversity is high the maximum value of F_{ST} is severely deflated, complicating comparisons between populations or different loci. Thus, F'_{ST} tests that corrected the F_{ST} estimates for heterozygosity within populations were conducted using GenoDive. Allele frequency heterogeneity among pairs of collections was also evaluated using loglikelihood G-statistics with 999 permutations implemented in GenoDive (Meirmans and Van Tienderen, 2004). Because

284 temporally separate collections were made at some locales (616, 621, 622, 626), we also
285 evaluated temporal stability of allelic frequencies at these locales using G-statistics. F_{ST} and
286 GST values were considered significant at the $p \leq 0.01$ level.
287
288 Population structure was also analyzed without *a priori* designation of populations as an
289 exploration of population architecture using STRUCTURE v.2.3 (Pritchard 2000; Falush et al
290 2005; Pritchard et al. 2010). This enabled us to infer the number of genetically homogenous
291 clusters within our collections and allowed assignment of individuals to designated genetic
292 clusters. For STRUCTURE, we used the admixture model with sampling locations as a prior and
293 correlated allelic frequencies among collections. In all instances, we used burn-in lengths of
294 100,000 and run lengths of 100,000. Ten replicates were run for each *K* at *K*=1 to 16. The
295 best value of *K* was determined from optimum values of lnP(D) (Pritchard et al. 2000), ΔK
296 (Evanno et al. 2005), MedMeaK', MaxMeakK', MedMedK', and MaxMedK' (Puechmaille
297 2016) that were all determined in StructureSelector (Li and Liu 2018). STRUCTURE figures
298 were generated in StructureSelector (Li and Liu 2018).
299
300 The R package 3.6.2 was used to perform Discriminant Analysis of Principal Components
301 (DAPC) using the adegenet (v2.1.1) package (Jombart 2008). DAPC identifies clustering by
302 transforming genetic data using a principal component analysis (PCA) that has the largest
303 between-group variance and smallest within-group variance (Jombart and Collins 2015).
304 Bayesian Information Criterion (BIC) values were used to determine the most appropriate
305 range of clusters. The a-score function of adegenet was used to determine the optimal
306 range of principal components (PCs) that must be retained to have sufficient power of
307 discrimination while avoiding the retention of too many dimensions that lead to over-
308 fitting of the data set. The optimal number of PCs to retain was calculated by measuring the
309 difference between the proportion of successful reassignments and values obtained using
310 random groups (Jombart and Collins 2015). DAPC analyses were run on the summer
311 flounder data set with the lower number of PCs retained in each analysis. (Wirgin et al.
312 2020)
313
314 **Results**

We successfully analyzed DNA from 1,182 specimens in this study. The mean level of missing data across all loci was 1.15%, and at individual loci ranged between 0.25% at Pade26 to 3.1% at Pade15 (Table 1 Supplementary Information). Archived scales provided a reliable source of DNA for our analysis. Across all loci, 1.4% did not provide data from DNA isolated from the archived scale samples compared to 0.78% from DNA isolated from fin clips. No data was missing across all 43 specimens for which DNA was isolated from archived otoliths. Seven of the nine loci did not show significant Hardy-Weinberg disequilibrium after Bonferroni correction. Additionally, none of the 36 binary combinations of the nine diagnostic loci showed consistent patterns of significant linkage disequilibrium across all 18 sample collections after Bonferroni correction. However, for two of the collections, locales 539 and 525, there were 11 and 12 loci pairs that exhibited significant disequilibrium at the p <0.01 level. None of the other collections exhibited more than two loci pairs that displayed significant disequilibrium at the p<0.01 level.

In total, we screened 283 polymorphic alleles with a mean of 30.8 alleles/locus with Pade 12 exhibiting the highest number of alleles (40) and Pade32 revealing the lowest number of alleles (17) (Table 1 Supplementary Information). Mean allelic richness across the nine loci was 13.82. Furthermore, mean observed and expected heterozygosity at these nine loci was high at 0.851 and 0.874, respectively.

We found sufficient levels of genetic variation at all 9 loci to potentially aid in identifying differentiation of collections. Using GST analysis, 23 of 153 pairwise comparisons proved significant at the $p \leq 0.01$ level (Table 2 Supplementary Information). The most consistent pattern of significant allelic differentiation at the $p < 0.01$ level was between sampling locale 539 and 9 of the other 17 locales. The next most consistent difference of allelic differentiation at the $p \leq 0.01$ level was between sampling locale 616 and 6 of the other 17 locales. Using FST analysis, we found no evidence of temporal instability of allelic frequencies across up to 9 years within the 4 collection locales tested (616, 621, 622, 626).

Use of F_{ST} analysis was more conservative than GST in distinguishing sampling locales of summer flounder. Mean F_{ST} across all loci and collection sites was low at 0.001. Thirteen of

the 153 pairwise F_{ST} comparisons proved significant at the $p \leq 0.01$ level (Table 2). Similar to GST analysis, the most consistent pattern of significant allelic differentiation at the $p \leq 0.01$ level was between sampling locale 539 and 7 of the other 17 locales. The next most consistent difference of allelic differentiation at the $p \leq 0.01$ level was between the Nantucket Shoals sampling and 4 of the other 17 locales.

Using STRUCTURE analysis (K=1-18) we found some evidence of significant genetic structure among our collections. The use of lnP(D), ΔK, MaxMed K, and MaxMean K analyses suggested the presence of two genetic clusters among these collections while MedMedK and MedMeanK indicated the presence of a single genetic cluster. With STRUCTURE analysis (Fig. 2), adjacent sample locales 613 and 616, south of eastern Long Island, New York, supported a unique genetic cluster of specimens that was absent elsewhere except for a small number of specimens from Pamlico Sound, North Carolina. There was no evidence of this second genetic cluster in specimens from another of the other collections.

DAPC analysis for the summer flounder data is illustrated in Figure 3. BIC values supported a range of 4 to 8 clusters as reasonable explanations of the data set. The number of PCs retained varied from 40 to 50 and final analyses were run with the lowest number as indicated by the a-score. Figure 3 illustrates the DAPC analysis of the data set using 48 PCs and 4 clusters. The cluster analysis supports a high degree of overlap among the 18 summer flounder sample sites with no distinct population segments.

Discussion

We found levels of genetic variation at the 9 microsatellite loci that were screened in our study that were comparable to those typically seen in marine fishes. The mean number of alleles/locus that we report in summer flounder, 30.8, is comparable to the mean rarefied number of alleles/locus of 26 reported across 215 species of marine fishes by Martinez et al. (2018). Furthermore, the magnitude of allelic differentiation among collection locales was also low as evidenced by a mean F_{ST} value of 0.001 across all loci. Despite this, STRUCTURE identified two genetic clusters across the entire collection set, with most of the second cluster centered in collection areas 613 and 616, south of eastern Long Island, New York and north of Hudson

Canyon. GST and F_{ST} analyses also suggested genetic differentiation among several of our collection locales. The collection locale with the most consistent pattern of allelic differentiation from other locales, 539, did not exhibit a second genetic cluster in STRUCTURE analysis. Furthermore, DAPC analysis did not reveal genetic population structuring among our collection sites. We also tested the genetic heterogeneity of a pool of all of collections from north of Cape Hatteras compared to those from Pamlico Sound and the SEAMAP survey and found no evidence of genetic differentiation. In total, our results were consistent with the hypothesis that across almost their entire coastwide distribution, the summer flounder fishery is supported by one, or at most, two stocks. How then do our results compare with earlier phenotypic and genetic analyses of population structure in summer flounder?

Meristic and morphometric studies supportive of two stocks, north and south of Cape Hatteras

Based on meristic characters, Ginsburg (1952) originally proposed the presence of two stocks of summer flounder in U.S. waters; one in Chesapeake Bay and the second off Beaufort, NC. A two-stock model, but with more extensive geographic bounds was proposed by Wilk et al. (1980) who found that of 18 meristic and morphometric variables investigated, 5 morphometric characters were informative in linear discriminant analysis in distinguishing summer flounder from New York to Cape Hatteras compared to those from Cape Hatteras to Florida. They found no significant difference among individual collections made north of Cape Hatteras. An additional, but more limited morphometric study by these same investigators using the same five diagnostic characters, further supported the two-stock model (north and south of Cape Hatteras) (Fogarty et al. 1983).

Studies supportive of more than two stocks in northern waters

Meta-analysis based on different lines of evidence suggested that there are multiple stocks of summer flounder within the North Carolina to Maine management area (Kraus and Musick 2003). Evidence included at least three separate concentrations of eggs on the Continental Shelf off of (1) New Jersey, (2) Virginia-North Carolina, and (3) south of Cape Hatteras (Smith 1973). Furthermore, in controlled laboratory experiments, significant differences in growth rate were observed among summer flounder offspring from North Carolina, Long Island Sound, and Delaware Bay parents (Malloy and Targett 1994; Burke et al. 2000). Based on results from

mark-recapture, Kraus and Musick (2003) postulated the presence of at least three stocks of summer flounder within the northern management unit: (1) one occupying North Carolina estuaries and spawning south of Cape Hatteras, (2) one in New Jersey that moves directly offshore during fall to spawn, and (3) a third group that initially hugs the Virginia-North Carolina coast before moving offshore to spawn. Furthermore, Defosse et al. (1990) hypothesized the existence of two stocks in Virginia based on migration pattern differences of spawning adults. Results from these studies led Kraus and Muscik (2003) to conclude that "collectively these studies suggest that there are multiple stocks within the northern management area."

Genetic studies of stock structure in summer flounder

Genetic studies have provided inconsistent results regarding the stock structure of summer flounder. Initially, using protein electrophoresis at 17 loci of which 5 were polymorphic, Van Housen (1984) reported genetic differentiation between collections from north and south of Cape Hatteras but homogeneity among samples from north of North Carolina. Using a more sensitive genetic approach, sequence analysis of the mitochondrial DNA (mtDNA) control region, Jones and Quattro (1999) found no significant differences in haplotype frequencies between composite collections from north and south of Cape Hatteras nor heterogeneity among collections from north of Cape Hatteras. This led to the conclusion that the phenotypic differentiation in morphometric characters previously observed between collections at the Cape Hatteras barrier probably resulted from environmental influences rather than underlying genetic differentiation of stocks.

Recently, Hoey and Pinsky (2018) used double-digest restriction assisted DNA sequencing (ddRAD), a more powerful genetic approach, to identify single nucleotide polymorphisms (SNPs) in summer flounder and then screened them for collection-level differences in their frequencies. They identified 1,137 SNPs that could be reproducibly genotyped in their collection of 232 summer flounder from Georges Bank to northern Florida. They did not detect a significant break between collections from north and south of Cape Hatteras, nor genetic heterogeneity within the northern collections because of moderate levels of gene flow across this hypothesized boundary. Their STRUCTURE analysis did not support the existence of more than

one genetic cluster coastwide because individuals did not group into separate populations. This conclusion was confirmed by additional analysis with a second landscape genetics package, Geneland, which also supported the existence of a single coastwide population. However, the authors did find that allelic frequencies at 15 of the loci were significantly correlated with environmental variables (bottom salinity, depth, distance along the coast, bottom temperature), including 11 that were correlated with bottom temperature. They concluded that summer flounder constituted a single stock across their complete coastwide distribution, but that despite sufficiently high levels of gene flow among collection sites there was evidence of selective pressure at some loci which may enable them to adapt to variable environmental parameters.

Our results were somewhat, but not in total agreement, with these earlier genetic studies despite differences in study design. We focused on exclusively neutral genetic loci by using a microsatellite approach, whereas Hoey and Pinsky (2018) investigated population structure using far more SNP loci, of which some could be potentially under selective pressure. Our collections were larger and centered on areas on the inner Continental Shelf where spawning is known to occur and which support summer flounder fisheries during the fall and early winter months. We found that overall genetic distance among collections was low, F_{ST}=0.0010 and almost identical to the F_{ST} value of 0.0014 reported by Hoey and Pinsky (2018). Furthermore, both studies failed to find a genetic discontinuity between collections from north and south of Cape Hatteras, as did Jones and Quattro (1999) using a mtDNA sequencing approach. Unlike the SNP and mtDNA studies we found weak evidence of genetic differentiation among collections along the northern edge of the species' range.

Our finding of little to no genetic differentiation of a coastal fish species with its major population bounded by Cape Cod and Cape Hatteras is consistent with other fish species with a similar range. Indeed, in a review of stock structure of 25 Atlantic coastal fishes (McBride 2014) found two relevant patterns among them. Though sampling locations, collection times, and genetic approaches varied, no significant genetic variation was found within mid-Atlantic stocks of species that spawn on the continental shelf, including bluefish *Pomatomus saltatrix*, Atlantic menhaden *Brevoortia tyrannus*, and tautog *Tautoga onitis*. However, some species did show a genetic break north and south of Cape Hatteras. These included black sea bass *Centropristus*

striatus, scup *Stenotomus chrysops*, and red drum *Sciaenops ocellatus.* In his review, McBride favored summer flounder as a species with two stocks divided by Cape Hatteras, although he recognized the data were equivocal.

In the future, a warming ocean in the northeastern U.S. (Wallace et al. 2018) may provide opportunity for differentiation. For example, analysis of standardized trawl data from 1968 to 2007 (Ney et al. 2009) showed a significant increase in maximum latitude of summer flounder at a rate of 0.029 ^{0}lat yr^{-1} and an increase in population size and area occupied. Though summer flounder are still not common north of Cape Cod, eventual establishment of a population north of this major zoogeographic barrier with discrete phenologies and movement patterns might begin to drive genetic stock differences.

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Figure Legends

Figure 1

Map of the Atlantic Coast of North America depicting 18 locales from which summer flounder were collected for microsatellite DNA analysis in this study. Shaded areas are trawl sampling strata designated by the Northeast Fisheries Center of NOAA. Open areas are statistical reporting areas designated by NOAA for monitoring of offshore commercial fisheries in the Northeastern U.S. Samples were also obtained from North Carolina to Florida from the SEAMAP program and from inshore North Carolina waters through the NCDMF. Additionally, collections were made from the recreational fisheries at Nantucket Shoals, Massachusetts and Fire Island Inlet, New York.

Figure 2

STRUCTURE analysis of the coastwide population structure of summer flounder based on microsatellite analysis of nine loci from 18 collection sites encompassing the entire range of the summer flounder fishery along the Atlantic Coast of the U.S. Each vertical bar represents a single individual and different colors represent the contribution of each *K* genetic cluster to each specimen's genotype. The number of clusters depicted include; Panel 1 K=2; Panel 2 K=3.

Figure 3

Discriminant analysis of principal components (DAPC) plot of nine microsatellite loci across 18 collections of summer flounder from the Atlantic coast of North America. The eigenvalue inset shows the relative amount of variance for each discriminant function. Specimens from each collection locale are depicted in different colors. Each dot represents an individual specimen and the line connects the dot to the location at which it was sampled.

Table 1

Collection locales, number of specimens successfully genotyped, dates collected, source of specimens, mean total length (range) of summer flounder analyzed in this study

Collection locale	N specimens	Date collected	Source	Tissue	TL
539	26	11/23/2010	Commercial fishery	Fin clips	ND
537	40	11/22/2010	Commercial fishery	Fin clips	ND
	52	12/21/2010	Commercial fishery	Fin clips	ND
616	48	12/16/2005	Commercial fishery	Scales	55.6 (38-68)
	24	1/24/2005	Trawl survey	Scales	40.4 (33-47)
	30	11/18/2010	Commercial fishery	Fin clips	55.0 (49-61)
613	77	11/28/2010	Commercial fishery	Fin clips	ND
525	28	12/23/02003	Commercial fishery	Scales	49.2 (42-55)
	26	3/17/2003	Commercial fishery	Scales	63.0 (53-73)
526	25	12/19/2005	Commercial fishery	Scales	63.5 (53-81)
621	25	11/20/2010	Commercial fishery	Scales	43.2 (38-51)
	25	1/7/2001	Trawl survey	Scales	38.9 (34-43)
622	25	1/6/2003	Trawl survey	Scales	47.1 (41-54)
	25	11/17/2006	Commercial fishery	Scales	46.5 (37-62)
	24	1/6/2008	Trawl survey	Scales	41.3 (38-46)
	25	11/9/2009	Commercial fishery	Scales	44.6 (39-48)
626	25	1/5/2000	Trawl survey	Scales	40.0 (35-46)
	25	1/13/2001	Commercial fishery	Scales	39.3 (31-49)
	26	1/21/2005	Commercial fishery	Scales	40.2 (35-46)
	24	11/6/2009	Commercial fishery	Scales	39.3 (35-46)
	39	12/1/2010	Trawl survey	Scales	42.5 (37-50)
	22	12/6/2010	Trawl survey	Otoliths	44.9 (41-55)
	21	12/13/2010	Travel survey	Scales	45.5 (41-55)
	19	12/26/2010	Trawl survey	Otoliths	51.3 (47-55)
639	25	11/10/2009	Commercial fishery	Scales	45.5 (41-54)
703	28	12/9/2009	Commercial fishery	Scales	42.1 (37-49)
70	25	2/13/07	Trawl survey	Scales	42.6 (32-55)
	19	2/8/06	Trawl survey	Scales	47.0 (32-56)
6	29	2/23/06	Trawl survey	Scales	41.3 (32-52)
	25	2/26/07	Trawl survey	Scales	51.3 (37-71)

61	32	2/11/06	Trawl survey	Scales	30.3 (25-48)
	32	2/11/07	Trawl survey	Scales	45.3 (35-61)
Pamlico Sound	58	4/08-5/2011	Angling	Scales	34.0 (18-49)
SEAMAP	50	7/9-8/10/2011	Trawl survey	Fin clips	17.8 (16-26)
Nantucket Shoals	68	8/5-8/12/2019	Angling	Fin clips	55.6 (46-69)
Fire Island Inlet	65	8/22/2019	Angling	Fin clips	38.1 (23-65)

776 **Table 2**

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778 F_{ST} analysis of allelic diversity at nine microsatellite loci in summer flounder from 18 collection locales along the Atlantic Coast
779 of the U.S. F_{ST} values are above the diagonal and associated *p* values are below. *p* values that are ≤0.001 are indicated in bold
780 and italics. NC=North Carolina, SM=SEAMAP, NS=Nantucket Shoals, FI=Fire Island Inlet

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	539	**537**	**616**	**613**	**525**	**526**	**621**	**622**	**626**	**639**	**703**	**61**	**70**	**6**	**NC**	**SM**	**NS**	**FI**
539		0.006	0.006	0.006	0.001	0.008	0.006	0.006	0.006	-0.001	0.006	0.008	0.002	0.011	0.006	0.004	0.007	0.007
537	0.014		0.001	-0.000	-0.001	0.002	0.001	-0.001	-0.001	-0.004	-0.002	0.000	0.001	0.000	0.000	-0.001	0.003	0.000
616	***0.001***	0.127		0.000	-0.001	0.003	0.001	0.002	0.001	-0.001	-0.001	0.003	0.003	0.004	0.004	0.001	0.003	0.003
613	0.016	0.458	0.318		0.001	0.003	0.002	0.000	0.000	-0.003	-0.001	-0.001	0.002	0.000	0.002	0.000	0.003	0.002
525	0.284	0.640	0.663	0.239		0.002	0.000	0.001	0.000	-0.004	-0.001	0.003	0.001	0.002	0.002	0.000	0.003	0.001
526	0.020	0.232	0.109	0.129	0.179		0.002	0.004	0.001	0.001	0.003	0.001	0.003	0.003	0.003	0.001	0.004	-0.001
621	0.030	0.270	0.231	0.120	0.386	0.250		0.001	-0.001	-0.002	0.001	0.002	0.000	0.001	0.001	0.001	0.001	0.000
622	***0.006***	0.929	0.033	0.403	0.186	0.032	0.128		0.000	-0.002	0.000	0.001	0.002	0.000	0.003	0.000	0.004	0.001
626	***0.005***	0.843	0.049	0.310	0.536	0.241	0.668	0.406		-0.002	-0.001	0.000	0.001	0.000	0.002	0.000	0.001	0.000
639	0.549	0.993	0.674	0.959	0.968	0.346	0.776	0.824	0.888		-0.002	-0.001	-0.004	-0.002	-0.003	-0.004	0.000	-0.002
703	0.052	0.834	0.710	0.566	0.577	0.118	0.235	0.534	0.734	0.661		0.001	0.004	0.002	0.004	0.001	0.001	0.001
61	***0.002***	0.434	0.023	0.662	0.053	0.330	0.153	0.201	0.308	0.656	0.274		0.001	0.000	0.002	0.000	0.003	0.001
70	0.177	0.145	0.033	0.056	0.350	0.107	0.478	0.051	0.196	0.950	0.057	0.183		0.002	0.002	0.001	0.002	0.002
6	***0.002***	0.552	***0.006***	0.449	0.067	0.071	0.200	0.545	0.522	0.844	0.193	0.529	0.097		0.000	0.000	0.002	0.002
NC	***0.008***	0.480	***0.007***	0.047	0.081	0.124	0.170	0.033	0.030	0.944	0.035	0.140	0.094	0.559		-0.001	0.004	0.002
SM	0.039	0.836	0.133	0.454	0.559	0.333	0.337	0.325	0.579	0.980	0.344	0.433	0.239	0.605	0.789		0.002	0.000
NS	***0.008***	***0.005***	0.016	***0.009***	0.037	0.040	0.219	***0.003***	0.058	0.378	0.310	0.028	0.097	0.131	0.013	0.046		0.005
FI	0.013	0.549	0.011	0.052	0.233	0.565	0.455	0.183	0.309	0.840	0.247	0.268	0.141	0.126	0.094	0.620	***0.004***	

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784 Fig 1
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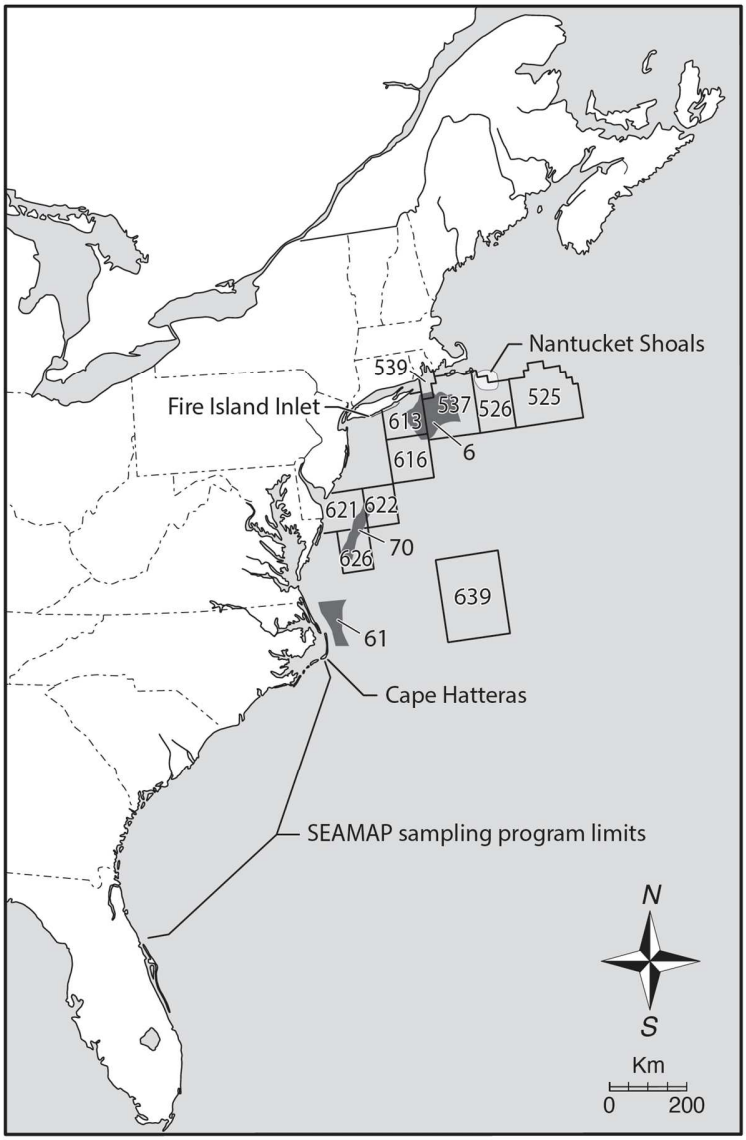

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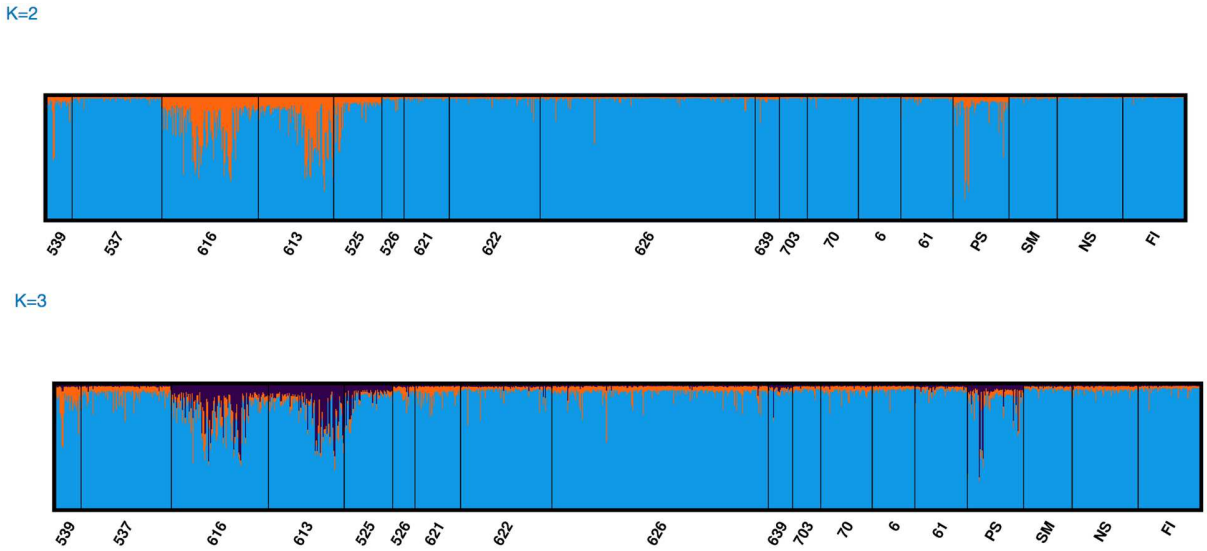
Fig 2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793 **Fig. 3**

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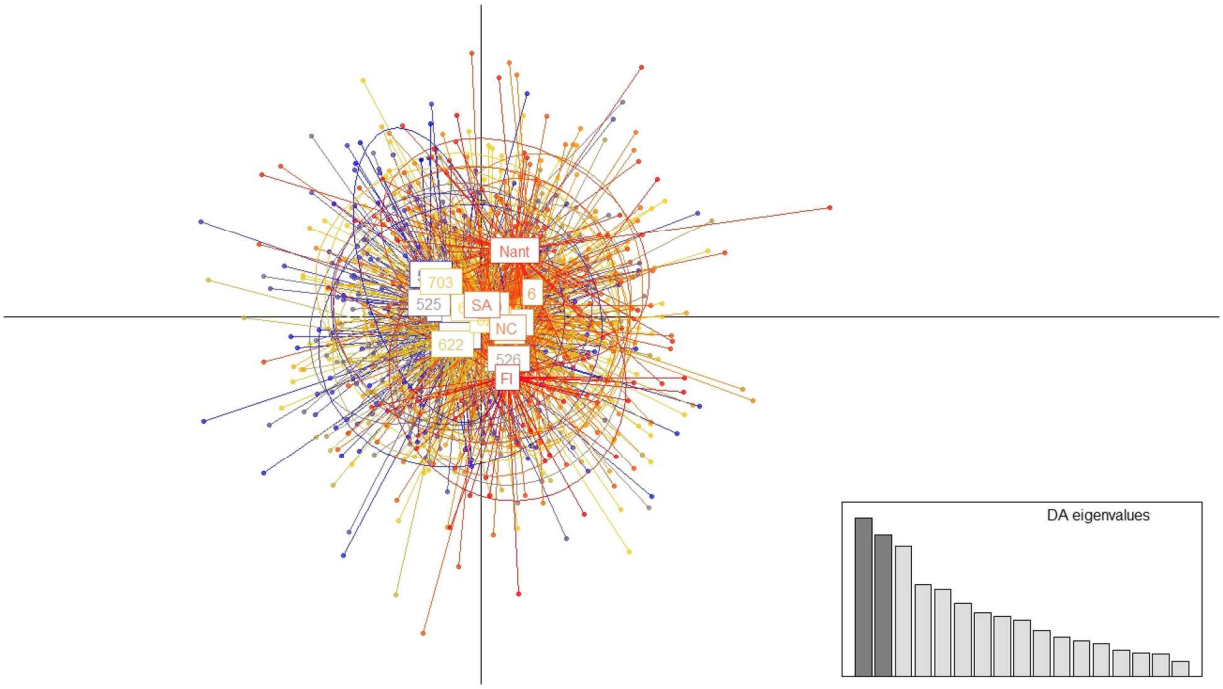

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