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Title: Stock structure of southern flounder (*Paralichthys lethostigma*) in North Carolina and US South Atlantic waters

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Summary

Population structure of southern flounder (Paralichthys lethostigma) in the US South Atlantic was evaluated through examination of population genetics and otolith morphometrics throughout the species Atlantic coast range. Tissue and otolith samples were obtained from fish collected in multiple locations between North Carolina and Florida, and also from selected locations in the Gulf of Mexico to allow for outgroup analysis. Within North Carolina, sampling was subdivided at a regional scale to identify the potential existence of any differences in genetic or morphometric traits among fish inhabiting different regions of the state. Both population genetic and otolith morphometric analyses detected clear differences between southern flounder populations occupying Gulf of Mexico versus US South Atlantic waters. Within the US South Atlantic basin, however, both analyses detected evidence for only weak population structure and a high likelihood for extensive mixing of southern flounder from all locales within the basin. In each case, the pattern of structure observed was not highly resolved, and was not well aligned with geographical features or state boundaries. Further, no strong evidence of population structure within North Carolina (i.e., differences in genetic or morphometric traits among regions) was found. Importantly, southern flounder collected from North Carolina waters do not appear to be genetically or morphmetrically unique within the US South Atlantic basin; an indication of mixing with fish from southern locations. Our findings are suggestive of a single, well-mixed stock of southern flounder occupying the US South Atlantic basin, with only limited evidence for finer-scale population structure likely related to geographical variations in environmental conditions in coastal habitats. Effective management of southern flounder fisheries should include the consideration of population dynamic processes at broader spatial scales to best interpret observed changes in local stock dynamics.

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Part I: Southern flounder (*Paralichthys lethostigma*) stock structure inferred from otolith shape analysis

Abstract

The examination of otolith morphometric variation has been shown to provide improved descriptions of stock structure for several marine fish species. In this investigation, we examined spatial variation in the shape of southern flounder (*Paralichthys lethostigma*) otoliths to better understand population structure at the basin, state, and local levels. Southern flounder were collected from state waters in the Gulf of Mexico (Texas and Florida) and U.S. South Atlantic (North Carolina, South Carolina, and Florida). To reduce sources of variability, we considered only age-1 female fish. From digitized otolith images, we extracted descriptions for common shape indices, as well as elliptical Fourier coefficients, and found strong evidence for differences at the spatial scale of the ocean basin (between fish from either the South Atlantic Ocean or the Gulf of Mexico) but only weak evidence for structure at either within-basin (i.e., among states) or within-state (local) spatial scales. Our finding of differences between the ocean basins aligns with the geographic break in species distribution, in which this species is absent from the southern portion of Florida, as well as with recent genetic findings. Currently, state-level management of southern flounder in both areas does not account for any basin-wide mixing and, therefore, by default, assumes unit stocks in each state, although our findings indicate that mixing could be extensive. Additional sources of information (e.g., genetics, life history traits) collected at appropriate spatial scales should be examined to confirm suspected levels of mixing and to determine suitable management strategies for the conservation of southern flounder stocks throughout their range.

Introduction

Although many marine populations once were considered to be panmictic on the basis of large geographical ranges and larval dispersal over long distances, results from improved stock identification methods (e.g., mtDNA, parasite community, and shape analyses) are calling into question some initial assumptions of population homogeneity (Cadrin et al., 2005). For example, although only very small amounts of gene flow may be required to homogenize considerable genetic variation without selection (Palumbi, 2003), evidence is accumulating from multiple species to support the existence of fine-scale geographic structure in several adaptive traits (Conover et al., 2006).

Regardless of their genetic similarities or differences, fish stocks possessing variable traits that can affect their responses to harvest still must be delineated clearly to achieve management objectives related to yield maximization and biomass conservation (Ricker, 1958; Begg et al., 1999; Conover et al., 2006). Spatial structure of fish stocks and the allocation of fishing effort, therefore, shouldbe considerations in the management of any species (Stephenson, 1999; Ying et al., 2011) because ignoring population structure can lead to negative outcomes, such as loss of genetic diversity and reduction in the yield-generating potential of a stock (Pawson and Jennings, 1996; Bailey, 1997; Booke, 1999). Within fisheries science, stock definitions vary but are focused largely on consistency of unique traits—the characteristics that distinguish a stock should remain constant through time and be unique to that stock (Ihssen et al., 1981; Booke, 1999) for both conservation and harvest purposes (Cadrin, 2000).

Methods of phenotypic stock identification have expanded greatly from abundance and meristic approaches to now include the use of both natural and artificial tags, examination of life history traits, population genetics, and morphometric outlines (reviewed in Cadrin et al., 2005).

Recently, the study of closed-form structures, such as otoliths and scales, has increased with the advent of computers that are able to rapidly analyze large amounts of data. In addition, otoliths are collected routinely for age determination purposes, making large sample sizes available for shape analyses. Consequently, numerous examples exist of successful discrimination of fish stocks based on otolith morphometrics and shape descriptors.

Campana and Casselman (1993) were among the first to use otolith shape as an indicator of stock variation. They conducted an exhaustive study of all 3 types of otolith pairs in which they found evidence of structuring among spawning groups of Atlantic cod (*Gadus morhua*) in the Northwest Atlantic Ocean, in addition to differences in otolith shape among age groups, sexes, and year classes. Begg and Brown (2000) used otolith shapes to challenge successfully the assumption of a single stock of Haddock (*Melanogrammus aeglefinus*) at Georges Bank, and DeVries et al. (2002) clarified previous tag and genetic data when they used otoliths to successfully distinguish stocks of king mackerel (*Scomberomorus cavalla*) from the Gulf of Mexico and the Atlantic that were sampled during their winter mixing off southern Florida. More recently, otolith shape analysis has been done at varying spatial scales for dolphinfish (*Coryphaena hippurus* [Duarte-Neto et al., 2008]), North Atlantic Saury (*Scomberesox saurus saurus* [Agüera and Brophy, 2011]), and anglerfish (*Lophius piscatorius* [Cañas et al., 2012]) to help clarify questions about geographic population structure.

The southern flounder (*Paralichthys lethostigma*) occurs in the Northwest Atlantic Ocean and Gulf of Mexico from North Carolina to Texas; however, this species does not occur around the southern tip of the Florida peninsula (Gilbert[1]). Southern flounder in the South Atlantic and

[1] Gilbert, C. 1986. Species profiles: life histories and environmental requirements of coastal fishes and invertebrates (South Florida)—southern, gulf, and summer flounders. Biol. Rep. U.S. Fish Wildl. Serv. 82(11.54). U.S. Army Corps Eng. Tech. Rep. TR EL-82-4, 27 p. [Available from http://www.nwrc.usgs.gov/wdb/pub/species_profiles/82_11-054.pdf.]

Gulf of Mexico basins are considered separate genetic stocks (Anderson et al., 2012). Management throughout the range of this species occurs generally at the individual state level, despite a high likelihood of within-basin mixing during offshore spawning migrations of adults and the possibility of year-round offshore residents (Watterson and Alexander[2]; Taylor et al.[3]).

Southern flounder support important commercial and recreational fisheries throughout their range, with females contributing most to the landings because growth is greater in females than in males. In 1990–2010, more than 30,000 metric tons were landed commercially, with the vast majority (~98%) of these landings taking place in North Carolina. Over the same period, recreational landings were about 50% lower in magnitude and were more evenly distributed among states between the Gulf of Mexico and South Atlantic basins (NMFS[4]). Additionally, high rates of recreational harvest may be a primary factor that is contributing to population declines in the Gulf of Mexico (Froeschke et al., 2011), which has prompted a new stock enhancement program in Texas aimed at supplementing natural reproduction.

Because no directed fishery exists for southern flounder in offshore habitats and exchange of individuals among states is not well understood, state management agencies assume unit stocks on the basis of state boundaries. However, population structure that does not coincide with state boundaries has been demonstrated in other flounders in the Northwest Atlantic that share geographic ranges of a similar size and life history characteristics with the southern flounder. These flounders include the southern flounder congener summer flounder (*P. dentatus*

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[2] Watterson, J., and J. Alexander. 2004. Southern flounder escapement in North Carolina. Final performance report F-73, Segments 1–3, 41 p. [Available from North Carolina Department of Environment and Natural Resources, Division of Marine Fisheries, P.O. Box 769, Morehead City, NC 28557.]

[3] Taylor, J. C., J. M. Miller, and D. Hilton. 2008. Inferring southern flounder migration from otolith microchemistry. Final report for Fishery Resource Grant 05-FEG-06, 27 p. North Carolina Sea Grant, Raleigh, N.C. [Available from http://www.ncseagrant.org/images/stories/ncsg_pdf/documents/research/05FEG06.pdf]

[4] NMFS (National Marine Fisheries Service). 2011. Personal commun. Fisheries Statistics Division, NMFS, Silver Spring, MD 20910.

[Burke et al., 2000]), winter flounder (*Pseudopleuronectes americanus* [DeCelles and Cadrin, 2011]), and yellowtail flounder (*Limanda ferruginea* [Cadrin, 2010]).

Interestingly, each of these studies reported structuring at varying scales. Summer flounder were found to have structure relative to the biogeographic boundary of Cape Hatteras in North Carolina, whereas evidence indicated that winter flounder and yellowtail flounder had population structures of much finer scales, including the existence of up to 3 stocks within New England waters. Yet despite evidence for within-basin genetic homogeneity (Anderson et al., 2012), flounder residency within specific estuaries for the first few years of life may create regional phenotypic differences that reflect local adaptation. For example, estimates of the von Bertalanffy growth coefficient (*K*) for female southern flounder vary considerably among states. For fish in Texas, Stunz et al. (2000) estimated *K* at 0.75, and Fischer and Thompson (2004) estimated *K* at 0.51 for Louisiana fish. Within the South Atlantic basin, *K* estimates have been lower: 0.23 for fish in South Carolina (Wenner et al.[5]) and 0.28 for fish in North Carolina (Takade-Heumacher and Batsavage[6]).

Mean sizes at age also varied among these studies—mean sizes at age-1 were 288 mm in South Carolina and 396 mm in Louisiana (Wenner et al.[5]; Fischer and Thompson, 2004). Emergent patterns from tagging studies of southern flounder also provide evidence that supports the possibility of phenotypically distinct stocks. Several tagging studies initiated in North Carolina waters have converged on the same 2 general conclusions. First, the majority of tagged

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[5] Wenner, C. A., W. A. Roumillat, J. E. Moran, Jr., M. B. Maddox, L. B. Daniel, III, and J. W. Smith. 1990. Investigations on the life history and population dynamics of marine recreational fishes in South Carolina: Part 1. Final report for project F-37, 187 p. Marine Resources Research Institute, South Carolina Wildlife and Marine Resources Department. Charleston, S.C. [Available from the Marine Resources Research Institute, South Carolina Department of Natural Resources, 217 Fort Johnson Rd., Charleston, S.C. 29422]

[6] Takade-Heumacher, H., and C. Batsavage. 2009. Stock status of North Carolina southern flounder (*Paralichthys lethostigma*), 91 p. North Carolina Division of Marine Fisheries, Morehead City, N.C. [Available from http://00de17f.netsolhost.com/fmps/downloads/souflounderSA.pdf.]

southern flounder were recaptured very close to the tag site (although this result could be more of a reflection of sampling effort than an actual movement and distribution). Second, those fish that were captured away (>20 km) from the tag site were collected in locations exclusively south of the release location (Monaghan[7]; Craig and Rice[8]).

These studies indicate that at least among younger age classes (i.e., age-1 and age-2 fish that dominate commercial and recreational landings), southern flounder have restricted home ranges and may be isolated geographically from other stocks. Such site fidelity to certain locations and subsequent environmental conditions could contribute to phenotypic differences. Additionally, the documented migration south may occur over considerable distances (individuals tagged in North Carolina have been recaptured in Florida), and even small numbers of migrating individuals could suffice to genetically homogenize basin populations (Palumbi, 2003).

Genetic differentiation of southern flounder at the basin level has been established previously (Anderson et al., 2012), and the objective of this study was to examine variation in otolith shape throughout the range of this species—with particular interest in the South Atlantic— to identify possible phenotypic stocks at the within-basin scale. Although other methods of phenotypic stock identification (e.g., testing for spatial variation in growth rates or meristics) also have utility, powerful statistical methods to evaluate variation in otolith shape have developed rapidly in recent years (Stransky, 2014). Further, some of the variation in otolith

[7] Monaghan, J. P., Jr. 1996. Migration of paralichthid flounders tagged in North Carolina. Study 2. *In* Life history aspects of selected marine recreational fishes in North Carolina. Completion Report Grant F-43, Segments 1–5, p. 2.1–2.44. North Carolina Department of Environment and Natural Resources, Division of Marine Fisheries, P.O. Box 769, Morehead City, N.C..

[8] Craig, J. K., and J. A. Rice. 2008. Estuarine residency, movements, and exploitation of southern flounder (*Paralichthys lethostigma*) in North Carolina. Final Report Fishery Resource Grant 05-FEG-15, 39 p. North Carolina Sea Grant, Raleigh, N.C. [Available from http://www.ncseagrant.org/ images/stories/ncsg_pdf/documents/research/05-FEG-15%20Final%20Report.pdf.]

shape is genetically determined, meaning that it should be comparatively less sensitive to short-term changes in environmental conditions. Three spatial scales were examined for possible population structure—between-basin (Gulf of Mexico versus South Atlantic), within-basin (among sites within the South Atlantic and Gulf of Mexico), and within-state (among sites within North Carolina). Any descriptions of geographic stocks (or lack thereof) will be useful both in current management and for the design of studies to examine differences in stock production that could inform future regional management of the southern flounder.

Materials and methods

Data collection

Southern flounder were collected in the South Atlantic from the state waters (≤3 nautical miles, or 5.6 km, from the coasts) of North Carolina, South Carolina, Georgia, and Florida (Fig. 1). In the analysis for this study, 289 right otoliths from age-1 southern flounders were used. Sixty-five samples were from the Gulf of Mexico, and the remaining 224 came from the Atlantic states (Figs. 1 and 2). Because of low sample sizes from both Florida (Atlantic Ocean waters) and Georgia and the close proximity of the sampling locations from which fish were obtained in those 2 states (≤161 km between locations), Florida and Georgia samples were pooled to represent the southern extent of the range in the U.S. South Atlantic. Additionally, where available, samples from the Gulf of Mexico (sites off both Florida and Texas) were included to enable between-basin comparisons.

Given the previously documented genetic differences (Anderson et al., 2012), we expected to also detect sufficient contrast in otolith shape between the basins that would aid our interpretation of variation at finer spatial scales. We explored 3 levels of spatial resolution for

possible stock differentiation. The between-basin scale was explored to compare otolith shape differences with established genetic differences. Within each basin, we examined variation among states, because state boundaries delineate current “stocks” for management of southern flounder. We examined variation between 2 states within the Gulf of Mexico and 3 states in the U.S. South Atlantic. Finally, we investigated the possible existence of shape differences at a local spatial scale by examining fish from 3 distinct regions within North Carolina (Fig. 2), among which it is unlikely that juveniles would have moved.

Fish were collected in North Carolina during the fall of 2009 and 2010 as part of the North Carolina Division of Marine Fisheries (NCDMF) fishery-independent gillnet sampling program. Additional samples were purchased from licensed seafood dealers and were obtained through participation in directed commercial trips. Samples from South Carolina, Georgia, and Florida were collected during the fall of 2010 and 2011 as part of existing fishery-independent sampling programs in each state. Additional samples were obtained from Florida during the fall of 2012 through purchases from licensed seafood dealers and participation in directed commercial trips. southern slounder were collected from the Gulf of Mexico in the fall of 1996 from inshore and offshore locations in the vicinity of Panama City, Florida, as part of sampling conducted by the NOAA Southeast Fisheries Science Center. Additional Gulf of Mexico samples were collected in Corpus Christi and Aransas Bays, Texas, during 2009 and 2011 as part of a separate research program.

Because fish age and recent growth histories can have a considerable influence on otolith shape (Campana and Casselman, 1993), the analysis in this study focused on fish captured only during October, November, and December to reduce variability associated with fractional ages (although additional mathematical size corrections are detailed later). Furthermore, assuming a 1

January birthday for all fish, fall sampling meant that fish had completed the majority of their growing for the most recent year, and, therefore, that the translucent zone closest to the otolith edge was approaching maximum width to enable improved annuli identification for aging purposes. Lastly, because the vast majority of individuals collected were age-1 females, our analysis of otolith shape focused on this sex-age group.

Otolith preparation and image analysis

Otoliths were extracted at the time of collection when additional data were collected also (e.g., total length [TL] in millimeters, mass in grams, and sex). Upon excision, otoliths were cleaned with isopropyl alcohol to remove any loose tissue, and then they were dried and stored in paper envelopes. All Atlantic-basin otoliths were aged whole with the NCDMF aging protocol (originally described in Wenner et al.[5]). Otoliths from the Gulf of Mexico were aged previously, and those ages were provided for use in this study (Fitzhugh[9]; Nims[10]). Only right otoliths were made available to us for all agency-collected southern flounder (the left otolith was archived for aging databases). Otoliths were first scanned on an Epson Perfection V500[11] photo scanner (Epson America Inc., Long Beach, CA) at high resolution (1200 dots per inch), yielding images comparable in quality to those from photographs. Use of a flatbed scanner permitted us to scan relatively large batches of samples simultaneously (without bending error, which is a concern in optical microscopy). Although southern flounder otoliths are relatively flat, all otoliths were scanned sulcus-side down in a uniform orientation.

Otolith shapes were described through the use of 1) elliptical Fourier analysis (EFA) and

[9] Fitzhugh, G. TKyear. Personal commun. Panama City Lab, Southeast Fisheries Science Center, National Marine Fisheries Service, NOAA. Panama City, FL 32408.

[10] Nims, M. TKyear. Personal commun. Univ. Texas Austin, Port Aransas, TX 78373.

[11] Mention of trade names or commercial companies is for identification purposes only and does not imply endorsement by the National Marine Fisheries Service, NOAA.

2) calculation of univariate shape indices. Compared with other types of Fourier transforms (e.g., fast Fourier transform), EFA is thought to provide the most consistent results for this type of application (Mérigot et al., 2007). The EFA method decomposes the closed-form contour into separate *x* and *y* coordinates (Kuhl and Giardina, 1982). Generally, a predetermined number of harmonics is defined for analysis, and each harmonic involves 2 parametric functions that describe 4 coefficients (i.e., amplitudes), a_n, b_n, c_n, and d_n for the nth harmonic.

In this study, elliptical Fourier coefficients (EFCs) were calculated with the program SHAPE, vers. 1.3 (Iwata and Ukai, 2002). SHAPE extracts the chain-coded contour of each otolith (Freeman, 1974), and then the software normalizes (i.e., removes any size effects) the chain-code data on the basis of the first 3 coefficients of the first harmonics. As a result of this normalizing, the first 3 coefficients of the first harmonic are degraded ultimately and unfit for analysis; the total number of coefficients = $(4 \times H_n) - 3$, where H_n represents the number of harmonics investigated. This study extracted 10 harmonics, or 37 EFCs, for analysis.

Several size parameters and shape indices (Table 1) also were calculated for each otolith. Otolith area (in square millimeters) was provided as part of the SHAPE output analysis, and perimeter data (in millimeters) were calculated from the chain-code output file. Both otolith length and otolith width were measured (in millimeters) with ImageJ software, vers. 1.45 (National Institutes of Health, Bethesda, MD) following otolith dimension definitions provided in Stevenson and Campana (1992). These size variables were then used to calculate several shape indices, including circularity, rectangularity, ellipticity, aspect ratio, and form function. Each of these variables is a common shape index used routinely in otolith morphometric investigations (Table 1; Russ, 1990).

Statistical procedures

Univariate shape indices were examined for normality through the use of normal quantile–quantile plots, and any non-normal distributions underwent log transformation in an attempt to satisfy the assumption of normality (Cadrin and Fiedland, 2005). To identify and control for the effect of otolith size on the shape indices, an analysis of covariance (ANCOVA) was used with geographic region as the factor and otolith length as the covariate. Both otolith length and fish TL have been used as covariates in similar analyses and would likely yield similar results. However, Campana and Casselman (1993) recommended the use of otolith length because this measured variable is more robust to collection and preservation effects, in addition to its strong correlation with fish TL (in this study, coefficient of correlation [r]=0.90). In the ANCOVA model, if the interaction of region and otolith length was significant (P<0.05), the shape index was excluded from the analysis, as it could not be corrected (Tracey et al., 2006). When the interaction was not significant but the effect of otolith size was significant, the shape index was corrected through the use of the common within-group slope (b), whereby the product of the slope and otolith length was subtracted from the shape variable. One-way analysis of variance (ANOVA), followed by Tukey's honest significant difference (HSD) post-hoc comparisons and Welch's t-tests (with significance for both tests assessed at α=0.05), was used to examine differences in shape indices and to identify which indices could be used in a discriminant analysis.

Linear discriminant analyses were used to examine differences between geographical subsamples (i.e., putative stocks) at all 3 spatial scales. Ideally, the construction of discriminant functions for otolith shape benefits from the inclusion of both EFC and shape indices (Agüera and Brophy, 2011); therefore, any significant shape indices were considered for inclusion in the

discriminant analysis. Finally, jackknife reclassification (i.e., leave-one-out cross validation) was used to examine the classification success of the discriminant functions when classifying known-origin otoliths. Rates of reclassification success were compared with the null classification expectation (i.e., no structure) of $1/g$, where g was the number of groups or putative stocks in the analysis (White and Ruttenberg, 2007).

Results

Shape indices

Circularity was the only shape index that was not normally distributed, nor could it be normalized through transformation, and it was, therefore, dropped from the analysis of this study. Analysis of covariance detected no significant interactions between otolith length and location for any of the shape variables, and, hence, all 4 shape variables were slope adjusted appropriately. At the basin level, all shape variables, except rectangularity, were significantly different (Table 2; Fig. 3). At the within-basin level, form function in the Gulf of Mexico was the only shape index that showed significant differences between states; no differences in shape indices were detected among Atlantic states. At a finer spatial scale comparing regions within North Carolina, differences in otolith shape indices were largely absent, with the exception of form function (Table 2).

Elliptical Fourier analysis

On the basis of the large number of EFCs (n=37) and a lack of high cumulative Fourier power (<70%, including all EFCs; Pothin et al., 2006), we ran cross-validation analyses to explore the descriptive power of harmonics. For this analysis, we started with the first 2 harmonics and

subsequently added harmonics until the rate of jackknife reclassification success declined or plateaued, indicating that the additional harmonics no longer were increasing discriminatory power. In cross-validation analyses for each geographic scale, the rate of jackknife reclassification success plateaued almost immediately, indicating that each additional EFC provided minimal explanatory power (a result that was in agreement with low Fourier power). Although the number of harmonic descriptors varies in otolith shape studies, we selected 10 harmonics (i.e., the first 10) as an appropriate number for each linear discriminant function. We identified 10 harmonics as a compromise between the parsimony of fewer harmonics indicated by cross validation, and a larger number of harmonics based on the notion that fine-scale description is often contained in higher-order harmonics (Cadrin et al., 2005) and that cross validation might not detect these small differences. Ultimately, all 4 discriminant analyses included the first 10 EFCs and any significant shape indices (Table 3). Discriminant function analyses (DFA) were done also with 15 and 20 harmonics to examine the sensitivity of the analysis to the number of harmonics. With 15 and 20 harmonics, the mean (and standard deviation of the mean) reclassification success rate was nearly identical to or slightly worse—although not statistically significant—than the result from our original analysis with 10 harmonics. Because unbalanced sample sizes can be problematic in discriminant function analysis and result in high rate of reclassification success by chance (White and Ruttenberg, 2007), we balanced our sample sizes on the basis of the smallest sample size in each analysis, and then we ran 1000 DFAs with all groups (except the smallest) randomized without replacement. We also conducted randomization tests of samples so that we would have not only a null point estimate ($1/g$, where g = number of groups) but also a distribution (i.e., an expected range) to provide greater inference for our empirical results. Our basin-scale rate of

reclassification success was nearly 80%, the highest level of reclassification success that we detected in any analysis and well outside the upper range of the null distribution (45–56%). Both within-basin reclassifications and the within–North Carolina reclassifications were marginally above—about a 6% improvement—the range of the null expectation distribution (Table 3); however, the distribution of reclassification success rates overlapped with the null distribution of reclassification in each case (Fig. 4).

Discussion

Otolith shape and geographic distribution

Using a combination of univariate shape indices and EFCs, we found strong evidence for the existence of different populations of southern flounder between the 2 basins that we examined and less evidence for population structure as our spatial scope decreased (among states within each basin and among locations within North Carolina). Interestingly (and despite low samples sizes for fish from Florida waters in the Gulf of Mexico), neither of the within-basin analyses detected strong evidence of otolith shape differentiation, although the otoliths represented fish collected from the spatial extremes within each basin distribution. Both of these observations are in agreement with recent genetic findings for this species (Anderson et al., 2012). The use of EFA is considered one of the most reliable methods in otolith morphometric studies (Mérigot et al., 2007), and it took only 10 coefficients to produce a rate of reclassification success of nearly 80% at the basin level (Gulf of Mexico versus South Atlantic). Additionally, because of the resampling aspect of the DFA, this result should be considered particularly robust.

Of greater interest was a lack of evidence for stock differentiation at either the within-basin or within-state spatial scales. Although a number of factors could effectively mix

individuals within each basin (or at finer spatial scales), tagging evidence does not support extensive mixing among or within states for younger, smaller fish, which typically remain within inshore estuarine systems (Monaghan[7]; Craig and Rice[8]). Although phenotypic stocks remain possible on the basis of previous observations of spatial variation in somatic growth rates (e.g., Stunz et al., 2000; Fischer and Thompson, 2004) and differences in otolith size estimates, our analysis of otolith shape did not clearly identify at the sub-basin level stocks of coastal southern flounder within either the South Atlantic or the Gulf of Mexico. We cannot discount the possibility of undetected fine-scale structure; however, the strength with which otolith shape analyses are able to detect both larger-scale genetic stock differences as well as finer-scale environmental differences indicates that within-basin structuring of southern flounder is unlikely.

The interpretation of variation in otolith shapes requires consideration of several factors that can contribute to otolith shape in the context of the species and study design. Otolith growth and shape are controlled dually by genetic and environmental influences (Vignon and Morat, 2010). Although the relative influence of these controls is the subject of ongoing research, early findings indicate that genetic influences determine the overall shape of an otolith and that environmental effects contribute finer morphological details (Hüssy, 2008; Vignon and Morat, 2010; Vignon, 2012).

Almost undoubtedly, southern flounder ranging from Texas to North Carolina experience a wide range of environmental conditions. Geographic gradients in environmental conditions may be expected to differentiate otolith shape sufficiently enough to enable detection of large-scale population structure, but there also may be considerable local environmental variation (e.g., salinity, temperature, and food) that effectively masks larger geographic patterns. Therefore, the ability to discriminate among even broadly spaced locales (e.g., North Carolina versus Florida)

can be compromised.

Broad and fine-scale processes that affect coastal environments, which contribute to fish growth, and potentially otolith shape also can vary independently in time. One way to limit the influence of variable environmental effects is by controlling for year class, although significant year-class effects have been detected in some otolith shape studies (Castonguay et al., 1991) and not in others (Begg and Brown, 2000; Galley et al., 2006). Furthermore, in a study of orange roughy, (*Hoplostethus atlanticus*), Gauldie and Crampton (2002) explored the idea of balancing selection—an alternating, generational morphology—operating to determine fish otolith morphology (on a 2-year cycle). This balancing selection results in a persistent otolith polymorphism in populations that consist of multiple age classes. Polymorphism related to year-class effects could have contributed to within-group variation in our study, as we included fish from multiple year classes, and polymorphism could have made it more difficult to detect broader regional differences in otolith shape.

Our study was improved by removal of possible sex effects, in addition to the fact that our collections were limited to relatively young (age-1) and mostly immature (Midway and Scharf, 2012) individuals. Because variable environmental conditions and growth rates can be major determinants of changes in otolith shape (Campana and Casselman, 1993; Cardinale et al., 2004), we presumed that young southern flounder would most likely reflect the regionally different environments (if sufficient variability existed regionally) and, therefore, would make good candidates with which to detect the effects of those differences on their otolith shape. For instance, if spatial differences existed in growth conditions that were sufficient to generate distinct otolith shapes, then they should be greatest in younger fish at the end of the estuarine phase, when confidence in the spatial segregation of fish is high and they are growing rapidly

while occupying habitats in which local conditions can cause variability in growth. Although variability in fine-scale processes, such as recent feeding history, has been shown to be an important determinant of otolith shape (Gagliano and McCormick, 2004; Hüssy, 2008), it was not practical for us to consider smaller spatial scales given the uncertainty in habitat use beyond the system of capture.

Considerable mixing of individuals from broadly separated areas during some part of the life history also would dampen within-basin and within-state environmental effects on otolith shape. Once they mature, southern flounder emigrate from estuaries to participate in offshore spawning. Within the U.S. South Atlantic, individuals can migrate considerable distances (e.g., North Carolina fish recaptured in Florida; Monaghan[7]; Craig and Rice[8]), meaning that mixing of fish from different regions within the basin may be considerable. Therefore, despite the fact that, prior to maturity, many southern flounder likely occupy small home ranges in specific estuaries that span environmental gradients sufficient to generate differences in growth and otolith shape, a high degree of offshore mixing could result in a level of genetic homogenization that swamps local environmental effects.

One additional and less discussed factor that possibly affects otolith shape is the time of capture. To minimize variation in body size, all of our fish were collected during late summer and fall. Each of the age-1 females that we used in this study, therefore, had the opportunity to take advantage of the warm summer growing season, a factor that helped to reduce size variability in our sample. However, this time of capture meant that our samples were taken near the end of a period of rapid otolith growth. In addition to the notion that otolith shape is less variable among older, mature individuals (Campana and Casselman, 1993), Gauldie and Nelson (1990) also found that faster otolith growth (often occurring among the youngest age groups)

resulted in long, thin aragonite crystals. Therefore, it is possible that periods of fast otolith growth can result in a wider variety of otolith shapes present within a stock, making distinctions more difficult among younger age groups. We were not able to address this possibility in our study because southern flounder of older ages are encountered much less frequently in estuarine waters than in offshore waters.

Possible management implications

Our examination of variation of otoliths shape to detect the population structure of southern flounder at 3 geographic scales has possible implications for management. The combination of established genetic differentiation (Anderson et al., 2012) and the high likelihood of differing environmental conditions between the U.S. South Atlantic and the Gulf of Mexico led us to hypothesize the existence of distinct otolith shapes for southern flounder at the spatial scale of the basin, and we did detect it. On the basis of past observations of high site fidelity from tag-return data in estuarine systems (Monaghan[7]; Craig and Rice[8]), we expected to find differences in otolith shape among fish from separate areas within each basin and even potentially among fish from different estuarine systems within North Carolina. We considered North Carolina a good model with which to test for structure at a finer spatial scale because the inshore waters of the state are made up of a range of system types, from large systems in central and northern regions (e.g., Pamlico and Albemarle Sounds) that contain extensive oligohaline reaches to small, river-based estuaries in the southern region.

Given that even relatively fine-scale shifts in habitat have been observed to generate heterogeneity in otolith shape (e.g., Vignon, 2012), we hypothesized that deviation in geography and hydrography among systems within North Carolina might produce enough environmental

variability to influence growth ratesand, therefore, theshape of otoliths from southern flounder. However, we detected only a weak signal of spatial structuring in otolith shape of southern flounder among locales within each basin and among regions within North Carolina. We did achieve a rate of reclassification success that was higher than expected in each case, an outcome suggestive of localized environmental effects, but our findings also indicate that levels of mixing within each basin could be sufficient to largely homogenize these effects. However, we cannot discount the possibility that some fine-scale structure exists at the within-basin spatial scale for southern flounder. Additional investigations to understand the extent of mixing and its impact on spatial variation in vital rates, such as growth, recruitment, and mortality, will be important to fully comprehend harvest effects and to select the appropriate scale for management.

Within both basins, no interjurisdictional management plan for southern flounder exists, and each state managesits fishery separately. In North Carolina, on the basis of a recent stock assessment, southern flounder are considered overfished with overfishing occurring (Takade-Heumacher and Batsavage[6]), but other states (South Carolina, Georgia, and Florida) have yet to conduct comprehensive stock assessments for flounder in their waters. In North Carolina, the southern flounder has long supported a lucrative commercial fishery (NCDMF, http://portal.ncdenr.org/web/mf/commercial-fishing-harvest-statistics), whereas landings by commercial gear have historically been much lower in the other states. At the same time, the recreational fishery has grown considerably throughout the entire basin; with landings more than doubling since the 1980s (National Marine Fisheries Service, http://www.st.nmfs.noaa.gov/recreational-fisheries/index).

Combined with any increase in participation in commercial harvest activities, the steady growth of the recreational fishery for southern flounder may necessitate that other states develop

comprehensive fishery management plans in the near future. In that event, the likelihood that interstate cooperation will be required through a federal fishery management plan (e.g., under the auspices of the Atlantic States or Gulf States Marine Fisheries Commissions) may increase.

Our findings indicate that southern flounder may exist as separate but well-mixed stocks within the South Atlantic and Gulf of Mexico basins. Alternatively, if population structure exists in the form of multiple stocks that differ in their response to harvest, failure to recognize that structure can lead to overexploitation and depletion of less productive and resilient stocks. Therefore, identification of stock structure is essential for accurate estimation of vital rates and stock assessments that promote effective fishery management. Currently, this process is hindered by the lack of understanding of the underlying structure within the population of southern flounder in the U.S. South Atlantic.

Conclusions

Previous studies have found genetic differences between southern flounder in the South Atlantic and southern flounder in the Gulf of Mexico, and the results from analyses of otolith shape variation presented here provide further evidence of basin-level differentiation. At finer spatial scales, we detected only limited evidence of population structure inferred from variation of otolith shape to support the existence of separate stocks within basins or within specific regions of a basin (among estuaries in North Carolina). Additional investigation of stock structure will be necessary, integrating these findings with results from other approaches, such as genetics, acoustic or archival tagging, and analysis of spatial variation in life history traits, to determine the appropriate spatial scales to promote effective management and conservation of southern flounder stock.

Literature Cited

Agüera, A., and D. Brophy. 2011. Use of saggital otolith shape analysis to discriminate Northeast Atlantic and Western Mediterranean stocks of Atlantic saury, *Scomberesox saurus saurus* (Walbaum). Fish. Res. 110:465–471.

Anderson, J. D., W. J. Karel, and A. C. S. Mione. 2012. Population structure and evolutionary history of southern flounder in the Gulf of Mexico and western Atlantic Ocean. Trans. Am. Fish. Soc.141:46–55.

Bailey, K. M. 1997. Structural dynamics and ecology of flatfish populations. J. Sea Res. 37:269–280.

Begg, G. A., and R. W. Brown. 2000. Stock identification of haddock *Melanogrammus aeglefinus* on Georges Bank based on otolith shape analysis. Trans. Am. Fish. Soc. 129:935–945.

Begg, G. A., K. D. Friedland, and J. B. Pearce.1999. Stock identification and its role in stock assessment and fisheries management: an overview. Fish. Res. 43:1–8.

Booke, H. E. 1999. The stock concept revisited: perspectives on its history in fisheries. Fish. Res. 43:9–11.

Burke, J. S., J. P. Monaghan, Jr., and S. Yokoyama. 2000. Efforts to understand stock structure of summer flounder (*Paralichthys dentatus*) in North Carolina, USA. J. Sea Res. 44:111–122.

Cadrin, S. X. 2000. Advances in morphometric identification of fishery stocks. Rev. Fish Biol. Fish. 10:91–112.

Cadrin, S. X. 2010. Interdisciplinary analysis of yellowtail flounder stock structure off New England. Rev. Fish. Sci. 18:281–299.

Cadrin, S. X., K. D. Friedland, and J. R. Waldman. 2005. Stock identification methods—an

overview. *In* Stock identification methods: applications in fishery science (S. X. Cadrin, K. D. Friedland, and J. R. Waldman, eds.), p. 3–6. Elsevier Academic Press, Burlington, MA.

Campana, S. E., and J. M. Casselman. 1993. Stock discrimination using otolith shape analysis. Can. J. Fish. Aquat. Sci. 50:1062–1083.

Cañas, L., C. Stransky, J. Schlickeisen, M. P. Sampedro, and A. C. Fariña. 2012. Use of otolith shape analysis in the stock identification of anglerfish (*Lophius piscatorius*) in the Northeast Atlantic. ICES J. Mar. Sci. 69:250–256.

Cardinale, M., P. Doering-Arjes, M. Kastowsky, and H. Mosegaard. 2004. Effects of sex, stock, and environment on the shape of known-age Atlantic cod (*Gadus morhua*) otoliths. Can. J. Fish. Aquat. Sci. 61:158–167.

Castonguay, M., P. Simard, and P. Gagnon. 1991. Usefulness of Fourier analysis of otolith shape for Atlantic mackerel (*Scomber scombrus*) stock discrimination. Can. J. Fish. Aquat. Sci. 48:296–302.

Conover, D. O., L. M. Clarke, S. B. Munch, and G. N. Wagner. 2006. Spatial and temporal scales of adaptive divergence in marine fishes and the implications for conservation. J. Fish Biol. 69:21–47.

DeCelles, G. R., and S. X. Cadrin. 2011. An interdisciplinary assessment of winter flounder (*Pseudopleuronectes americanus*) stock structure. J. Northwest Atl. Fish. Sci. 43:103–120.

DeVries, D. A., C. B. Grimes, and M. H. Prager. 2002. Using otolith shape analysis to distinguish eastern Gulf of Mexico and Atlantic Ocean stocks of king mackerel. Fish. Res. 57:51–62.

Duarte-Neto, P., R. Lessa, B. Stosic, and E. Morize. 2008. The use of sagittal otoliths in discriminating stocks of common dolphinfish (*Coryphaena hippurus*) off northeastern Brazil

using multishape descriptors. ICES J. Mar. Sci. 65:1144–1152.

Fischer, A. J., and B. A. Thompson. 2004. The age and growth of southern flounder, *Paralichthys lethostigma*, from Louisiana estuarine and offshore waters. Bull. Mar. Sci. 75:63–77.

Freeman, H. 1974. Computer processing of line-drawing images. Computing Surv. 6:57–97.

Froeschke, B. F., B. Sterba-Boatwright, and G. W. Stunz. 2011. Assessing southern flounder (*Paralichthys lethostigma*) long-term population trends in the northern Gulf of Mexico using time series analyses. Fish. Res 108:291–298.

Gagliano, M., and M. I. McCormick. 2004. Feeding history influences otolith shape in tropical fish. Mar. Ecol. Prog. Ser. 278:291–296.

Galley, E. A., P. J. Wright, and F. M. Gibb. 2006. Combined methods of otolith shape analysis improve identification of spawning areas of Atlantic cod. ICES J. Mar. Sci. 63:1710–1717.

Gauldie, R. W., and J. S. Crampton. 2002. An eco-morphological explanation of individual variability in the shape of the fish otolith: comparison of the otolith of *Hoplostethus atlanticus* with other species by depth. J. Fish Biol. 60:1204–1221.

Gauldie, R. W., and D. G. A. Nelson. 1990. Otolith growth in fishes. Comp. Biochem. Physiol. A 97:119–135.

Hüssy, K. 2008. Otolith shape in juvenile cod (*Gadus morhua*): ontogenetic and environmental effects. J. Exp. Mar. Biol. Ecol. 364:35–41.

Ihssen, P. E., H. E. Booke, J. M. Casselman, J. M. McGlade, N. R. Payne, and F. M. Utter. 1981. Stock identification: materials and methods. Can. J. Fish. Aquat. Sci. 38:1838–1855.

Iwata, H., and Y. Ukai. 2002. SHAPE: a computer program package for quantitative evaluation of biological shapes based on elliptic Fourier descriptors. J. Hered. 93:384–385.

Kuhl, F. P., and C. R. Giardina. 1982. Elliptic Fourier features of a closed contour. Comput. Vision Graph. 18:236–258.

Mérigot, B., Y. Letourneur, and R. Lecomte-Finiger. 2007. Characterization of local populations of the common sole *Solea solea* (Pisces, Soleidae) in the NW Mediterranean through otolith morphometrics and shape analysis. Mar. Biol. 151:997–1008.

Midway, S. R., and F. S. Scharf. 2012. Histological analysis reveals larger size at maturity for southern flounder with implications for biological references points. Mar. Coast. Fish. 4:628–638.

Palumbi, S. R. 2003. Population genetics, demographic connectivity, and the design of marine reserves. Ecol. Appl. 13:S146–S158.

Pawson, M. G., and S. Jennings. 1996. A critique of methods for stock identification in marine capture fisheries. Fish. Res. 25:203–217.

Pothin, K., C. Gonzalez–Salas, P. Chabanet, and R. Lecomte–Finiger. 2006. Distinction between *Mulloidichthys flavolineatus* juveniles from Reunion Island and Mauritius Island (south-west Indian Ocean) based on otolith morphometrics. J. Fish Biol. 69:38–53.

Ricker, W. E. 1958. Maximum sustained yield from fluctuating environments and mixed stocks. J. Fish. Res. Board Can. 15:991–1006.

Russ, J. C. 1990. Computer-assisted microscopy: the measurement and analysis of images, 453 p. Plenum Press, New York.

Stephenson, R. L. 1999. Stock complexity in fisheries management: a perspective of emerging

issues related to population sub-units. Fish. Res. 43:247–249.

Stevenson, D. K., and S. E. Campana (eds.). 1992. Otolith microstructure examination and analysis, 126 p. Can. Spec. Publ. Fish. Aquat. Sci. 117.

Stransky, C. 2013. Morphometric outlines. *In* Stock identification methods: applications in fishery science, 2nd ed. (S. X. Cadrin, L. A. Kerr, and S. Mariani, eds.), p. 129–140. Academic Press, London.

Stunz, G. W., T. L. Linton, and R. L. Colura. 2000. Age and growth of southern flounder in Texas waters, with emphasis on Matagorda Bay. Trans. Am. Fish. Soc. 129:119–125.

Tracey, S. R., J. M. Lyle, and G. Duhamel. 2006. Application of elliptical Fourier analysis of otolith form as a tool for stock identification. Fish. Res. 77:138–147.

Vignon, M. 2012. Ontogenetic trajectories of otolith shape during shift in habitat use: interaction between otolith growth and environment. J. Exp. Mar. Biol. Ecol. 420–421:26–32.

Vignon, M., and F. Morat. 2010. Environmental and genetic determinant of otolith shape revealed by a non-indigenous tropical fish. Mar. Ecol. Prog. Ser. 411:231–241.

White, J. W., and B. I. Ruttenberg. 2007. Discriminant function analysis in marine ecology: some oversights and their solutions. Mar. Ecol. Prog. Ser. 329:301–305.

Ying, Y., Y. Chen, L. Lin, and T. Gao. 2011. Risks of ignoring fish population spatial structure in fisheries management. Can. J. Fish. Aquat. Sci. 68:2101–2120.

Table 1

Size parameters, shape indices, and shape formulae used to describe otoliths from female southern flounder (*Paralichthys lethostigma*) collected in the South Atlantic and Gulf of Mexico in 1996 and in 2009–2012 as part of state or federal agency sampling programs and from seafood dealers for this study of the population structure of southern flounder in these basins.

Size parameter	Shape index and formula
Area (A)	Circularity = P^2 / A
Perimeter (P)	Rectangularity = $A / (OL \times OW)$
Otolith length (OL)	Form function = $4\pi A / P^2$
Otolith width (OW)	Aspect ratio = OL / OW
	Ellipticity = $(OL – OW) / (OL + OW)$

Table 2

Results, at 3 spatial scales, of hypothesis testing on univariate shape indices of otoliths from female southern flounder (*Paralichthys lethostigma*) that were collected in 1996 and in 2009–2012 in state waters in 2 basins, the South Atlantic (North Carolina, South Carolina, Georgia, and Florida) and the Gulf of Mexico (Florida, Alabama, Mississippi, Louisiana, and Texas), as part of state or federal agency sampling programs and from seafood dealers..

Scale	Variable	Test statistic	*P*-value	Test statistic	*P*-value
Basin	Ellipticity	t=3.82	<0.001		
	Rectangularity	t=–1.67	0.100		
	Form function	t=–5.06	<0.001		
	Aspect ratio	t=3.74	<0.001		
		Within Gulf of Mexico		Within South Atlantic	
State	Ellipticity	t=1.48	0.145	F=1.71	0.184
	Rectangularity	t=1.48	0.149	F=0.20	0.815
	Form function	t=–2.06	0.046	F=0.23	0.797
	Aspect ratio	t=1.37	0.178	F=2.09	0.127
North Carolina (regional)	Ellipticity	F=0.55	0.581		
	Rectangularity	F=1.18	0.313		
	Form function	F=8.08	<0.001		
	Aspect ratio	F=0.51	0.600		

Table 3

Rate of jackknife reclassification success, standard error, null expectation, and predictor variables associated with 3 discriminant function analyses that examined otoliths from female southern flounder (*Paralichthys lethostigma*) collected in 1996 and in 2009–2012 in the South Atlantic and the Gulf of Mexico as part of state or federal agency sampling programs and from seafood dealers.

	Basin	within-Atlantic	within-Gulf of Mexico	within-North Carolina
mean reclassification success	0.79	0.47	0.66	0.47
reclassification success range	0.74–0.84	0.38–0.56	0.53–0.77	0.39–0.55
SE	< 0.01	< 0.01	< 0.01	< 0.01
expected mean (1/g)	0.50	0.33	0.50	0.33
expected range	0.45–0.56	0.25–0.41	0.40–0.60	0.26–0.40
predictor variables	ellipticity	10 EFCs	form function	form function
	form function		10 EFCs	10 EFCs
	aspect ratio			
	10 EFCs			

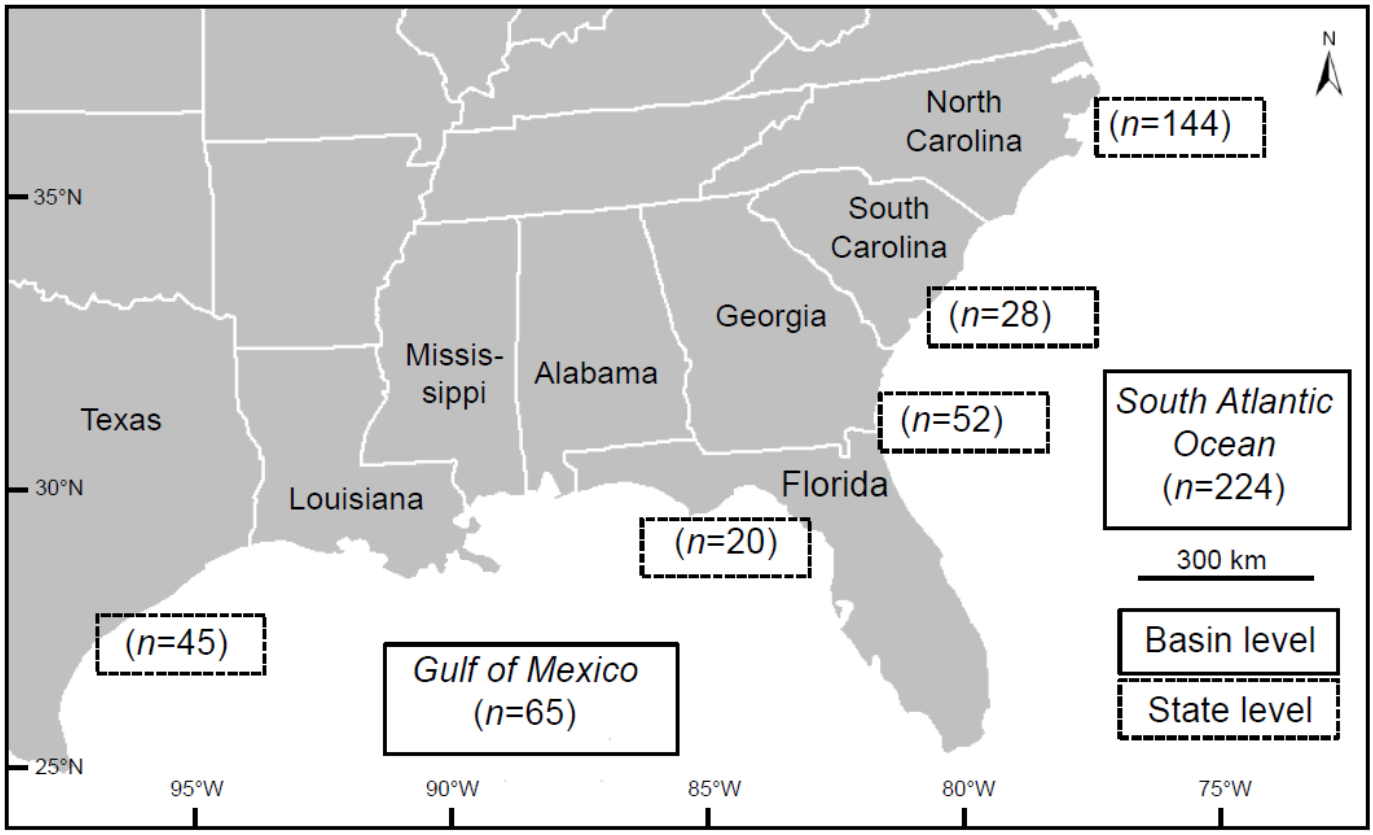

Figure 1 - Map with sample sizes by state and basin for collections of age-1 female southern flounder (*Paralichthys lethostigma*) used in this study of the population structure of this species in the South Atlantic and Gulf of Mexico. Collections occurred in state waters in these 2 basins, the South Atlantic (North Carolina, South Carolina, Georgia, and Florida) and the Gulf of Mexico (Florida, Alabama, Mississippi, Louisiana, and Texas) in 1996 and in 2009–2012 as part of state or federal agency sampling programs and from seafood dealers. Basin sample sizes are the sum of state sample sizes within each respective basin. Although we collected fish from both Georgia and Florida, to increase sample size, the samples from these 2 states were combined on the basis of their close geographic proximity.

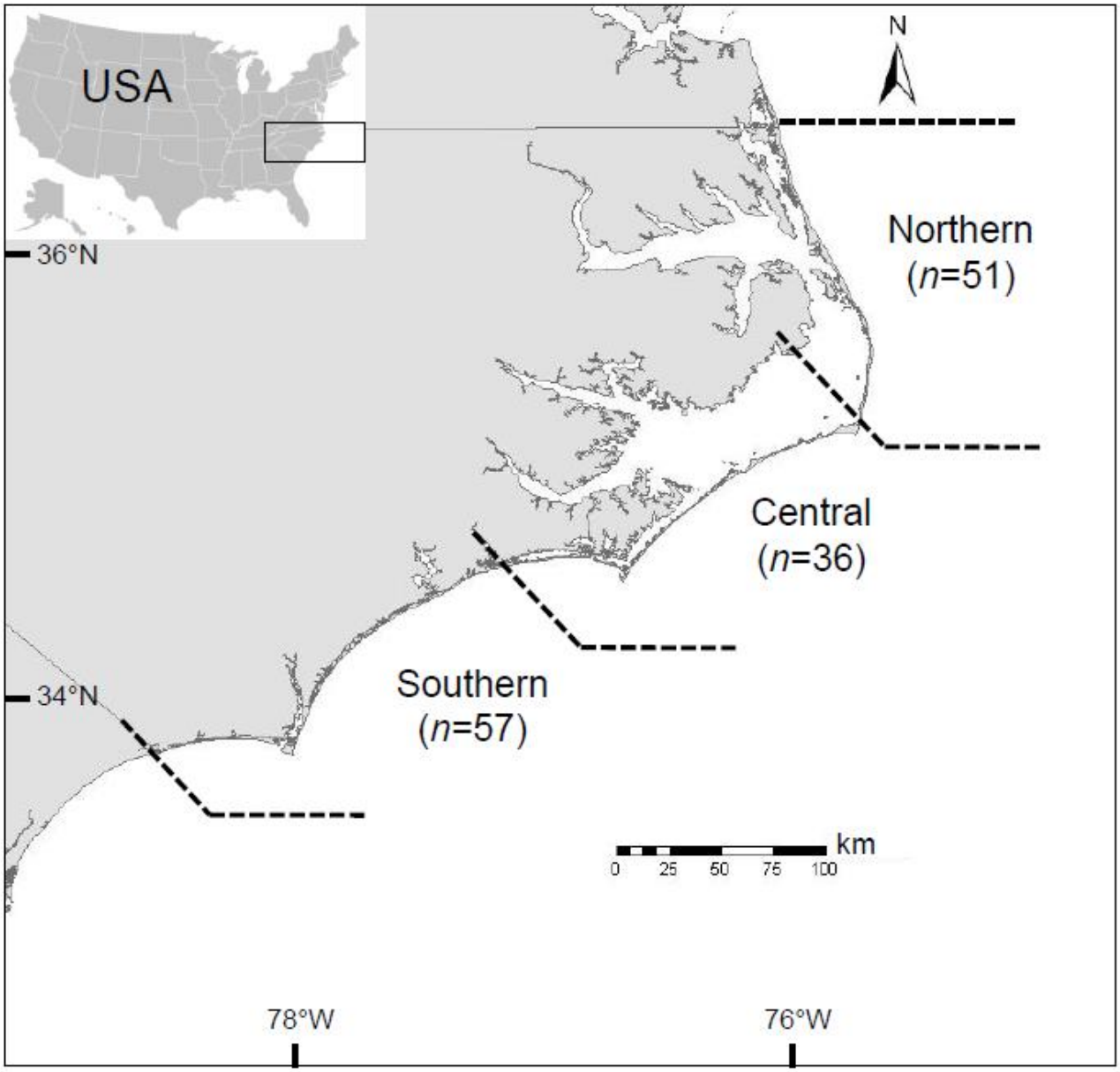

Figure 2 - Map with sample sizes for collections of age-1 female southern flounder (*Paralichthys lethostigma*) that occurred within the state waters of North Carolina in 2009 and 2010 as part of a sampling program of the North Carolina Division of Marine Fisheries (NCDMF) and from seafood dealers. The 3 regions are based on NCDMF sampling areas; however, they are characterized also by different estuaries. The northern region comprises Albemarle Sound, the central region consists of the Neuse–Pamlico Estuary, and the southern region is defined by the Cape Fear and New River estuaries.

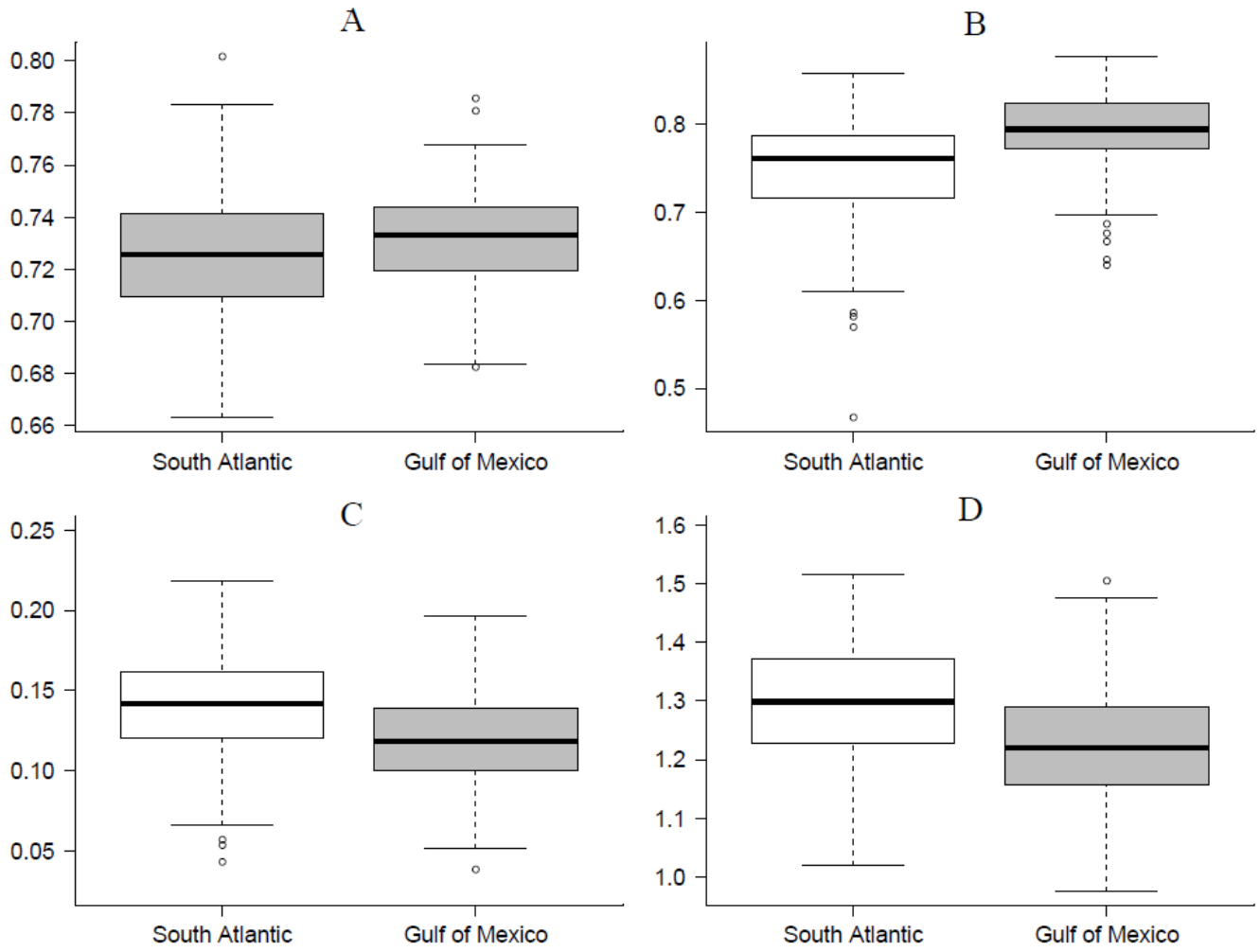

Figure 3 - Boxplots of 4 shape indices for otoliths from female southern flounder (*Paralichthys lethostigma*) collected in 1996 and in 2009–2012 in the South Atlantic and the Gulf of Mexico as part of state or federal agency sampling programs and from seafood dealers. Boxplots for the following shape indices are grouped by basin: (**A**) rectangularity, (**B**) form function, (**C**) ellipticity, and (**D**) aspect ratio. Significant difference (on the basis of Welch's *t*-test and α=0.05) between groups are indicated by different shading of the boxes. Boxes represent the interquartile range; whiskers indicate the first and third quartiles + 1.5 × interquartile range; and dots represent points beyond those defined by the whiskers.

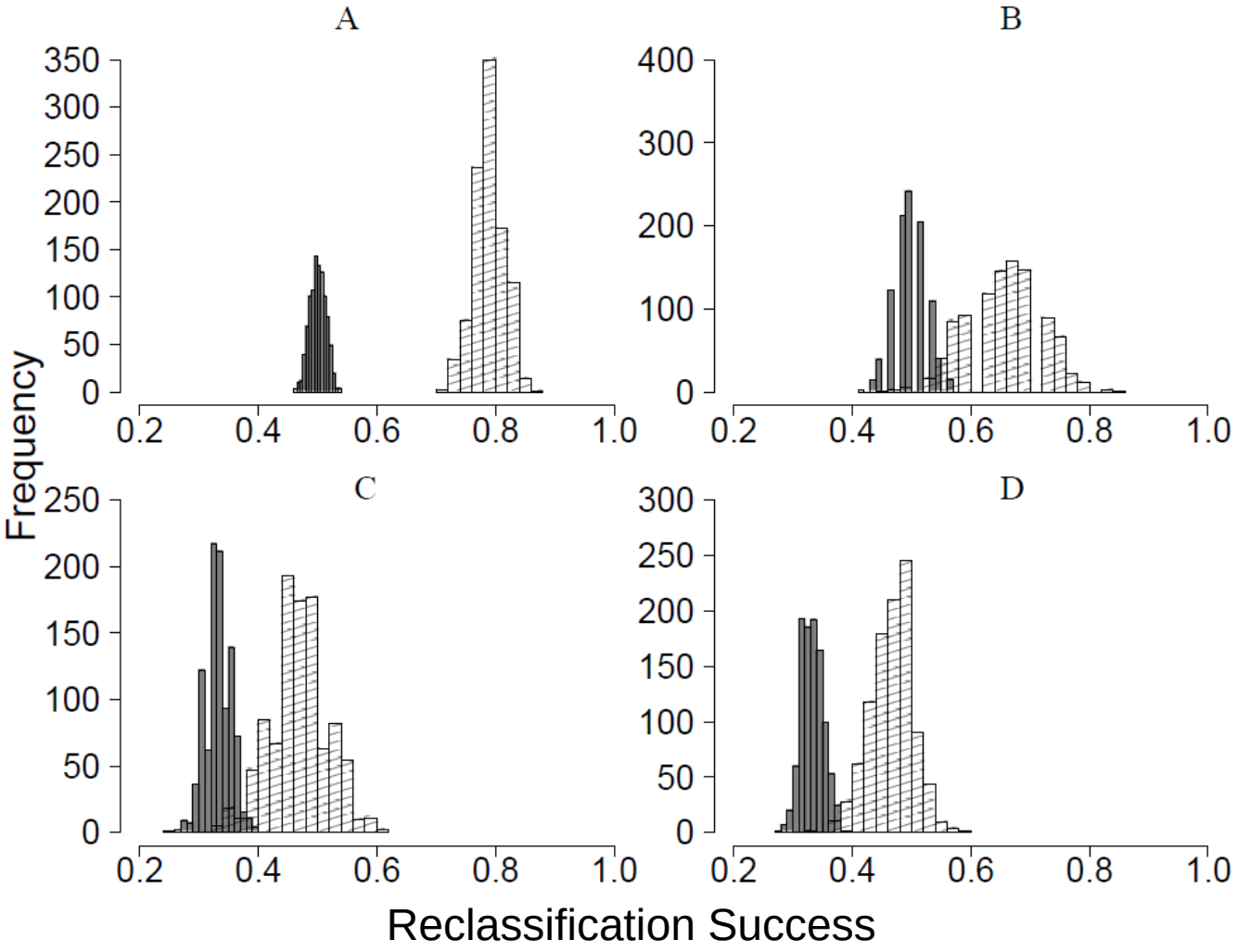

Figure 4 - Distributions of randomized reclassification (i.e., null) success rates (shown as the grey bars on the left in each panel) and actual linear discriminant randomization (shown as the hatched bars on the right) for each of 4 geographic analyses of otolith shapes from female southern flounder (*Paralichthys lethostigma*) collected in 1996 and in 2009–2012 in 2 basins, the South Atlantic (North Carolina, South Carolina, Georgia, and Florida) and the Gulf of Mexico (Florida, Alabama, Mississippi, Louisiana, and Texas). Distributions are shown for 4 geographic groups: (**A**) both basins, (**B**) Gulf of Mexico states, (**C**) Atlantic states, and (**D**) North Carolina regions. Only the basin-level analysis indicated clear separation of distributions; each of the within-basin analyses and the within–North Carolina analysis revealed some degree of distributional overlap (illustrated by the grey, hatched bars).

Part II: Population genetic structure of Southern Flounder inferred from multilocus DNA profiles

Abstract

Determination of stock structure is an important component of fisheries management, and 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 in order to improve our understanding of the scale of population structure in this wide-ranging species. We analyzed amplified fragment length polymorphism (AFLP) fingerprints and mitochondrial control region sequences in Southern Flounder, and found clear divergence between ocean basins with both molecular markers. Within the U.S. South Atlantic, no genetic differentiation was detected at either broad (among states: North Carolina, South Carolina, Georgia, Florida) or finer (among regions within North Carolina) spatial scales when using mitochondrial markers. Increased genetic resolution was observed with AFLP fingerprint data, and we found significant subdivision between nearly all Southern Flounder populations, suggesting that finer scale population structure may be present within the U.S. South Atlantic. However, AFLP genetic cluster analysis also revealed evidence for a high degree of mixing within the Atlantic basin, and patterns of variation, which included a genetic similarity among South Carolina and Gulf of Mexico populations, were not aligned closely with geography. When examining the partitioning of genetic variation among groups using analysis of molecular variance, we found no evidence that the North Carolina populations, which are managed on the state level as a unit stock, are differentiated from the remainder of the U.S. South Atlantic. Our findings indicate only weak

structure and the potential for basin-wide mixing among Atlantic Southern Flounder populations, suggesting that cooperation among U.S. South Atlantic states will be essential for effective assessment of stock dynamics and future management plans.

Introduction

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). Examining genetic variation across a species range is a commonly used approach for defining populations, and recent advances in molecular techniques have contributed to 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 lack of obvious barriers to gene flow in the ocean resulting in low genetic differentiation between 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. Maintaining 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 consideration of both historical and contemporary patterns of molecular diversity, can help to promote sustainability, as well as preserve evolutionary processes in the face of increasing anthropogenic impacts (Avise 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 and the U.S. South Atlantic. It supports a thriving recreational fishery across its range, and is also a

valuable commercial finfish resource in some areas (e.g., commercial landings in North Carolina (NC) averaged 3.3 million pounds annually between 1991-2007; (NCDMF 2013). In fact, the NC stock has experienced elevated commercial harvest rates since at least 1991, and although a fishery management plan (NCDMF 2005, 2013) was first established in 2005, the most recent stock assessment concluded that *P. lethostigma* remains overfished and is still undergoing overfishing (Takade-Heumacher and Batsavage 2009).

Mature *P. lethostigma* inhabiting estuarine waters migrate offshore 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). Adult flounder typically return to estuarine and nearshore coastal waters following spawning (Monaghan 1996), but recent studies have found *P. lethostigma* migration patterns to be more variable, with some adults remaining 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 et al. In review). Based mainly on the estuarine tagging results, the current fisheries management plan considers *P. lethostigma* in NC to be a unit stock (NCDMF 2013). However, there is also evidence of extensive southward movement of some individuals tagged in NC estuaries to other U.S. South Atlantic states (Monaghan 1996; Craig et al. In review). Prior to estuarine recruitment, *P. lethostigma* remain in a pelagic larval stage for a moderately long duration (~45 d), which increases potential for long-range dispersal. The unknown level of adult mixing in the offshore environment combined with a long larval stage duration implies that the population structure of *P. lethostigma* may be more complex than currently assumed. The incorporation of molecular methods should provide increased

knowledge of *P. lethostigma* population structure and connectivity, and contribute to effective management decisions for the long-term sustainability of the species.

Previous *P. lethostigma* population genetics studies have been focused on the Gulf of Mexico (hereafter, Gulf), and there has yet to be a comprehensive study spanning the species range within the U.S. South Atlantic (hereafter, Atlantic). When examining *P. lethostigma* allozyme variation, Blandon et al. (2001) found populations in the Gulf to be significantly differentiated from Atlantic populations, with a break in allele frequencies on the coast of Texas between Galveston Bay and Matagorda Bay indicating the presence of population structure within the Gulf. Updated studies of *P. lethostigma* using mitochondrial DNA (mtDNA) and microsatellites again revealed high divergence between Gulf and Atlantic populations, but in contrast to the allozyme survey, little within-basin differentiation was detected (Anderson and Karel 2012; Anderson et al. 2012). All three prior studies show some evidence of weak genetic structure within the Gulf, but no clear indication of discrete stocks. 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 *P. lethostigma* in the U.S. South Atlantic basin.

We examined *P. lethostigma* populations 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, scattered across the genome, 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 allow for assessment of genome-wide variation when analyzing population structure, which can be more informative than targeting mitochondrial DNA markers (which are

inherited as a single haploid locus). Information content of an AFLP study has been claimed to be comparable to using 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; Sønstebø et al. 2007). Although previously underutilized in studies of animals (reviewed in Bensch & Åkesson 2005), AFLPs have become increasingly common in studies of marine populations, including studies of commercially important fish species, both in aquaculture (e.g., channel catfish, Mickett et al. 2003; olive flounder, Liu et al. 2005) and in natural populations (e.g., common shrimp, Weetman et al. 2007; striped mullet, Liu et al. 2009). For comparison with a more established method, we also sequenced a fragment of the mitochondrial 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 *P. lethostigma* population structure.

In this study, our main objective was to genotype *P. lethostigma* individuals across the range of the species using both AFLPs and mtDNA to address whether distinct populations of *P. lethostigma* are present within the U.S. South Atlantic, and in particular whether North Carolina represents a unique stock. As evidenced by previous studies, we expected divergence between Gulf and Atlantic populations, but 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 to more traditional mtDNA sequencing for determining stock structure in a widely distributed marine species.

Materials and Methods

Paralichthys lethostigma tissue samples were collected from multiple sites across four states (North Carolina [NC], South Carolina [SC], Georgia [GA], Florida [FL]) spanning the current range of the species in the U.S. South Atlantic (Figure 1). North Carolina was subdivided into three regions (north [NCN], central [NCC], south [NCS]) to examine 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, as well as from licensed seafood dealers. Sampling was conducted from 2010 to 2013, and fish collected ranged from age 0-3 (96% were age 0-1) with an average total length of 360mm (range=166-622mm). Additional tissue samples were provided by JD Anderson (Texas Parks and Wildlife), and were collected from two sites in the Gulf of Mexico (Apalachicola, Florida [APFL] and San Antonio Bay, Texas [SBTX]). Sampling methods for Gulf of Mexico collections can be found in Anderson et al. (2012). Fin clips and gill tissue were excised and preserved in either a salt saturated dimethyl sulfoxide solution (Atlantic samples) or 70% ethanol (Gulf samples) and stored at 4°C. Genomic DNA was extracted from fin clips using the DNeasy Blood and Tissue Kit (Qiagen, Valencia CA) following manufacturer's protocols. Gill tissue was extracted only occasionally, when fin clips were damaged or not readily available.

AFLPs were generated using the AFLP Plant Mapping Protocol (Applied Biosystems, Foster City CA) for regular plant genomes, and DNA fingerprints were produced for 12 selective primer pairs that were chosen from an earlier screen of 24 primer pairs: *EcoRI*-ACA/*Mse*I-CAA, *EcoRI*-ACC/*Mse*I-CAC, *EcoRI*-ACC/*Mse*I-CAG, *EcoRI*-ACC/*Mse*I-CAT, *EcoRI*-ACT/*Mse*I-CAA, *EcoRI*-ACT/*Mse*I-CAC, *EcoRI*-ACT/*Mse*I-CAT, *EcoRI*-ACT/*Mse*I-CTC, *EcoRI*-

AGC/*Mse*I-CAA, *EcoRI*-AGC/*Mse*I-CTT, *EcoRI*-AGG/*Mse*I-CTA, and *EcoRI*-AGG/*Mse*I-CTG. Fragments were sized along with GeneScan 500 ROX size standard (Life Technologies, Carlsbad CA) on an ABI3130xl Genetic Analyzer (Applied Biosystems) in the DNA Analysis Core Facility located at the Center for Marine Science, UNCW. To test for reproducibility of AFLP fingerprints, one sample from each population was repeated across all 12 selective primer pairs beginning from genomic DNA. Fragments between 75-500bp were scored automatically with the default 50 rfu (relative fluorescence unit) peak-calling threshold using GeneMapper v4.0 (Applied Biosystems). Peak heights were normalized across all samples for each primer pair, with only polymorphic loci considered (hereafter referred to as the "full" profile). To further reduce noise and genotyping error, the loci in the full AFLP profile were filtered to a locus selection threshold of 200 rfu and a phenotype calling threshold of 20% following Whitlock et al. (2008). Here, 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 average peak height for each locus (hereafter referred to as the "optimized" profile). Both full and optimized AFLP profiles were used for each AFLP analysis described below unless otherwise noted.

A fragment of the mitochondrial control region was amplified using the primers L-PROF 5'- AACTCTCACCCCTAGCTCCCAAAG-3' (Meyer et al. 1994) and PL526H 5'-AAAAGAGAACCCCTTACCCG-3', the latter of which is a *P. lethostigma* specific primer designed by one of the authors (McCartney, unpublished). The polymerase chain reaction was conducted as follows: An initial denaturation step of 94°C for 1 minute, followed by 35 amplification cycles (94°C, 1 min; 58°C, 30 sec; 2°C, 2 min), and a final extension of 72°C for 5 minutes. Each 25ul reaction volume contained 1.25U MyTaq DNA Polymerase (Bioline, London UK), 1X MyTaq Red Reaction Buffer (Bioline), 0.5uM of each primer, and 1ul of

genomic DNA (5-900 ng). PCR products were purified using a StratPrep PCR Purification Kit (Agilent Technologies, Santa Clara CA) and cycle sequenced in the forward direction (L-PROF) with BigDye Terminator v3.1 (Applied Biosystems). Sequencing was conducted on the same equipment as previously described for AFLPs. Sequences were edited, trimmed, and aligned manually in Sequencher 4.10.1 (Gene Codes Corporation, Ann Arbor MI).

STRUCTURE v. 2.3.4 (Pritchard et al. 2000) was used to assign individuals to an inferred number of genetically distinct clusters (K) present in the *P. lethostigma* AFLP dataset. Based on previous studies of *P. lethostigma* which demonstrated a lack of structure among 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 each of clusters $1 \leq K \leq 8$ 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 method by Evanno et al. (2005) for identifying the most likely number of K clusters in our dataset. All replicates from each K value identified by this method were then combined using CLUMPP v.1.1.2 (Jakobsson and Rosenberg 2007) using the "FullSearch" algorithm. DISTRUCT v.1.1 (Rosenberg 2004) was used to convert CLUMPP outputs into bar plots showing the probability of assignment of each individual to the genetic clusters.

We used Arlequin v.3.5.1.2 (Excoffier and Lischer 2010) to compute F_{ST}, a measure of population differentiation, between all pairs of populations for both AFLP and mtDNA datasets. We applied a Bonferroni adjustment to correct for multiple F_{ST} comparisons using software from Lesack and Naugler (2011). Arlequin was also used to conduct analyses of molecular variance (AMOVA) (Excoffier et al. 1992). An AMOVA examines the partitioning of genetic

differentiation within and among geographical subpopulations, and we used this framework to test two different regional groupings for both AFLPs and mtDNA: (i) ocean basin scale (Gulf of Mexico vs. U.S. South Atlantic) and (ii) structure within the U.S. South Atlantic (NCN/NCC/NCS vs. SC/GA/FL). To further investigate the patterns observed from our STRUCTURE results, we also considered two additional groupings for AMOVA analysis: (iii) similarity between South Carolina and the Gulf of Mexico (SC/Gulf vs. NCN/NCC/NCS/GA/FL) and (iv) subdivision within North Carolina (NCN/NCC vs. NCS/SC/GA/FL). As per the genetic clustering results, (iii) was conducted using the optimized AFLP profile and (iv) was analyzed with the full AFLP profile.

P. lethostigma mitochondrial control region phylogeny was inferred with the program NETWORK v.4.6.1.2 (Fluxus Engineering, www.fluxus-engineering.com), using the median joining method (Bandelt et al. 1999) to generate a haplotype network. Network branching patterns were post-processed with maximum parsimony (Polzin and Daneshmand 2003). Control region nucleotide composition and pairwise differences were calculated using MEGA 6 (Tamura et al. 2013). Pairwise differences were also determined for the AFLP dataset with MEGA. DnaSP 5.10 (Librado and Rozas 2009) was used to assign 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 AFLPs were scored for all 201 samples across 12 primer pair combinations, the full profile included 1847 polymorphic loci and the optimized profile yielded 671 polymorphic loci. In the eight samples for which we repeated the AFLP procedure, the full profile was 93.6%

reproducible and the optimized profile was 98.8% reproducible across all loci. Using the method by Evanno et al. (2005) to interpret the results from STRUCTURE, we identified a mode of ΔK at K=4 genetic clusters present in our dataset for the full AFLP profile. Assuming four genetic clusters in the full AFLP profile, we observed that populations all showed strong membership to two distinct clusters—one Gulf and the other Atlantic. In addition, Atlantic populations from NCS and southward displayed partial assignment to a cluster that was also present in Gulf populations (Figure 2). With the optimized AFLP profile, we identified a strong peak in ΔK at K=2, and a smaller but distinct secondary peak in ΔK at K=5. At K=2, two major groups were formed separating the Gulf and Atlantic populations, but the SC population appeared to share some ancestry with the Gulf populations. At K=5, the assignments remained similar to those at K=2 and K=4, but SC ancestry was instead shared with the western Gulf population, and the southern NC population displayed some shared assignment with the genetic cluster dominating the Gulf populations.

Because SC unexpectedly showed common ancestry with the western Gulf population (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 (i) average pairwise distance among population pairs that included either SC or SBTX to (ii) average pairwise distance among all other population pairs (those without SC or SBTX). The percent difference between these two metrics was used to assess unusual levels of genetic differentiation in SC and the western Gulf. Average percent difference was 4.09% (SD=3.72) for the full AFLP profile and 6.09% (SD=5.93) for the optimized profile across 11 of the 12 primer pairs. The remaining primer pair, *EcoRI*-AGC/*MseI*-CTT, displayed higher levels of differentiation, with percent differences of 33.02% for the full profile and 56.77% for the

optimized profile, and was likely responsible for the similarities observed between SC and SBTX.

F_{ST} comparisons for *P. lethostigma* AFLPs showed highly significant differentiation between all pairs of populations (F_{ST}=0.032-0.138, P<0.0001) using the full AFLP profile, both within and between ocean basins (Table 1). When examining the optimized AFLP profile, all but three pairs of populations were also highly significantly differentiated (F_{ST}=0.033-0.151, P<0.0001). The three population pairs not significantly differentiated after Bonferroni adjustment were all within-Atlantic comparisons (NCN/GA, NCN/FL, GA/FL). In general, F_{ST} values for Atlantic vs. Gulf population pairs were larger than within-basin comparisons, although in the optimized AFLP profile F_{ST} values for SC/NCC and SC/NCS were comparable to between-basin values. When comparing Gulf and Atlantic regions, AMOVA for the full AFLP profile resulted in significant differences at all levels of population structure (Table 2). However, within-population variation accounted for 88.4% of the differences (F_{ST}=0.116, P<0.05). When partitioning differentiation in the Atlantic alone (NC populations and the remaining Atlantic populations considered as two separate regions) using the full AFLP profile, 94.3% of the variation was due to within-population differences (F_{ST}=0.057, P<0.05) (Table 3). There was no differentiation between these two regions. The AMOVA results for the optimized AFLP profile were very similar to those of the full profile (Table 2, Table 3).

We also investigated other hierarchical structures based on the genetic clustering results. In the STRUCTURE analyses, SC showed membership to the Gulf cluster and NCN and NCC appeared differentiated from the remaining Atlantic populations (Figure 2). When comparing SC/Gulf to the other Atlantic populations with AMOVA, the two groups were significantly

differentiated (F_{CT}=0.047, P<0.05). There was no differentiation between regions when considering NCN and NCC as a separate group from NCS and the remainder of the Atlantic.

Across 260 samples, the final *P. lethostigma* mitochondrial control region alignment was 480 base pairs 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 (π) was 0.011 (SD=0.0003), with an average of 5.06 nucleotide changes between individuals (Table 4). Control region nucleotide composition was A=0.352, T=0.272, C=0.213, and G=0.164. Tajima's D statistic was estimated to test for neutrality of nucleotide substitutions. D values were significantly negative in both ocean basins (Atlantic: D= -2.08, P<0.05; Gulf: D= -1.97, P<0.05), which may be indicative of *P. lethostigma* population expansion.

Twenty-nine *P. lethostigma* control region haplotypes were common among individuals, three of which were shared between Gulf and Atlantic populations. The majority of the haplotypes were singletons, most of which diverged from common haplotypes by only 1-2 nucleotide substitutions. The control region haplotype network showed a pattern of divergence between the two ocean basins, although separation is 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 and vice versa. There is no clear pattern of finer scale structure within the Atlantic cluster based on the haplotype network.

Pairwise population comparisons using the mitochondrial control region showed strong differentiation (F_{ST}=0.37-0.45, P<0.05) between all pairs of cross-basin populations (Table 5). Three pairs of populations within the Atlantic (GA/NCC, GA/NCN, SC/NCN) were also

significantly differentiated, although F_{ST} values were much smaller (F_{ST}=0.037-0.052, P<0.05). None of these within-Atlantic comparisons remained significant after applying a Bonferroni correction. All other pairs of populations within the Atlantic and within the Gulf were never significantly differentiated. When comparing the Gulf and Atlantic, the control region AMOVA showed that the majority of the variation was explained by differences within populations (59.3%, F_{ST}=0.407, P<0.05), although a large portion of differentiation was due to differences between ocean basins (40.2%, F_{CT}=0.402, P<0.05) (Table 6). Within the Atlantic, almost all of the variation was explained by differences within populations (98.2%, FST=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%, FCT=0.018, P>0.05), and these differences were not significant.

Discussion

We observed divergence between Gulf and Atlantic *P. lethostigma* populations with 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 1,2,5,6). 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 *P. lethostigma* allozyme, mtDNA, and microsatellite studies, and correspond with a well-known biogeographic break in marine fauna between the Gulf and Atlantic (Avise 1992; Blandon et al. 2001; Anderson and Karel 2012; Anderson et al. 2012). *P. lethostigma* 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 the species across south Florida.

We predicted that both markers would perform well for evaluating deep divergence, but also wanted to assess how AFLPs and mtDNA would compare when examining subtle structure, as we expected to see in the Atlantic *P. lethostigma* populations. Although mtDNA markers are commonly used in population genetic studies because of their rapid mutation rates and purportedly neutral evolution, the mitochondrial genome is also haploid, maternally inherited effectively as a single locus, and it is very small compared to the nuclear genome. Thus, studies relying solely on mtDNA markers may draw incomplete conclusions when attempting to make inferences about organisms and populations as a whole (reviewed in Ballard & Whitlock 2004). AFLP loci are scattered across the genome and may better reflect demographic, historical, and selective events whose influence on patterns of diversity can vary across loci, so we expected increased genetic resolution with AFLP analysis.

AFLP and mtDNA results were generally in agreement when assessing *P. lethostigma* differentiation between the two ocean basins, but diverged when examining structure within ocean basins. The magnitude of differentiation was much higher for pairwise comparisons between ocean basins with mtDNA (F_{ST}=0.358-0.456) than with AFLPs (F_{ST}=0.084-0.151, optimized profile), although both were significant. The opposite was seen with population comparisons within the Atlantic, where mtDNA values (F_{ST}=0.000-0.052) were low and not significant, compared to values for AFLPs (F_{ST}=0.026-0.091, optimized profile), which showed significant differentiation for nearly all within-Atlantic comparisons. Interpreting the degree of population structure as proposed by Wright (1978), genetic differentiation among *P. lethostigma* populations when comparing ocean basins can be characterized as "very great" (mtDNA) or "moderate" (AFLPs), and comparisons within the Atlantic showed "little" to "moderate" genetic differentiation for AFLPs.

All *P. lethostigma* populations within the Atlantic were significantly differentiated when using the full AFLP dataset and nearly all were differentiated with the optimized AFLP dataset (Table 1), 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 with enough markers (Ryman et al. 2006). Our study compared a single mtDNA marker to AFLP profiles with 1847 loci (full) and 671 loci (optimized), 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 that produced low levels of genetic differentiation. Based on AFLP differentiation, genetic structure appears to be present among Atlantic *P. lethostigma* populations, although this does not necessarily mean that each population represents a discrete stock.

Waples et al. (2008) cautioned that statistical significance is not always correlated with biological significance, and the life history and biology of an organism must be taken into consideration when determining stocks. Thus far, we have no evidence that *P. lethostigma* populations from different locales within the Atlantic are biologically distinct. Midway et al. (In press) also detected only weak evidence for differentiation among *P. lethostigma* populations within the Atlantic and also within NC using otolith morphometric analysis. These results are consistent with our findings. And 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 south Atlantic populations. The same lack of NC regional structure was found with AMOVA using mtDNA.

Given these results, we can submit with confidence that NC does not represent a distinct stock within the Atlantic.

Rather than focusing strictly on patterns of differentiation, it may be more revealing to assess the genetic composition of each *P. lethostigma* population. AFLPs are highly accurate for population assignment tests when sufficient numbers of loci are used, 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 (K) across the most likely values of K for both the full and optimized AFLP profiles (Figure 2). While we examined patterns of variation at K=2, 4, and 5, no major shifts in genetic patterns were observed above K=3 (red, blue, and yellow clusters in Figure 2). All three summary plots show a strong genetic distinction between the Gulf and Atlantic. Within NC, NCN and NCC appear to be homogeneous, while NCS displays a small difference in assignment probabilities, suggesting that a mild barrier to gene flow exists between these regions. The most unexpected observation was the shared clustering of SC, located near the center of the Atlantic populations, with the Gulf, and with SBTX in particular.

By examining pairwise differences in AFLP profiles between 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. While similarity between these two populations was unforeseen, we did not identify any aberrant peaks that may have resulted in erroneous fingerprints. Although this clustering was more pronounced in the optimized AFLP profile than in the full AFLP profile, we do not feel that the procedure of filtering loci skewed our results. Errors in peak scoring are a common source of AFLP genotyping error (Bonin et al. 2004), and optimizing AFLP data by setting peak height thresholds for inclusion of loci and

phenotype calling (presence/absence) is an established method of reducing scoring subjectivity and error (Whitlock et al. 2008). This results in a conservative dataset that performed well in our tests for reproducibility (98.8%). Nonetheless, we included analyses from both datasets in this study for consideration, and the full and optimized AFLP profiles produced essentially the same results in both AMOVA and F_{ST} comparisons.

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

Differences in habitat use may provide a possible explanation for the genetic similarity between SC and Gulf populations. Blandon et al. (2001) found a disruption in *P. lethostigma* allozyme frequencies along the mid-Texas coast corresponding to a salinity gradient. Allozymes, unlike neutral molecular markers, are enzyme polymorphisms that may be subject to selection in varying environmental conditions. AFLP loci, like other genome-scale molecular datasets, are anonymous and consist mostly of neutral loci, but often include loci that are linked to genes under selection (Luikart et al. 2003). A recent study of *P. lethostigma* otolith chemistry revealed that individuals collected from a single bay in the northwest Gulf of Mexico displayed wide variability in habitat use and migratory behavior, with some fish occupying a mixture of

oligohaline and estuarine/marine habitats throughout their lifetime, and others exhibiting evidence of estuarine/marine residency only (Nims and Walther 2014). These findings show that *P. lethostigma* with different environmental histories can inhabit the same location, which in turn suggests the possibility for geographically disjunct individuals to share molecular profiles based on their habitat preferences and physiological tolerances. Whether these differences in habitat use are controlled by genetics, and whether our AFLP survey included loci that are linked to genes responsible for physiological tolerance, is currently unknown. To further examine 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—outliers that are a signature of selection (Beaumont and Balding 2004).

The results of this study confirmed a clear divergence between Gulf and Atlantic *P. lethostigma*. There was also evidence of genetic structure among populations of *P. lethostigma* at a finer spatial scale that were 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 indication of strong regional genetic differences among *P. lethostigma* populations within North Carolina, and there was also no differentiation between North Carolina and the other South Atlantic states. *P. lethostigma* in North Carolina are currently managed as a unit stock. This current molecular analysis suggests that a single stock designation within North Carolina should be adequate, with 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 South Atlantic, as evidenced by the presence of only weak genetic structure within the basin, suggests that inter-state cooperation will be necessary for a comprehensive population assessment of *P. lethostigma* at an appropriate spatial

scale. In order to sustain fishery yields and meet conservation objectives for *P. lethostigma*, the spatial scale of stock assessment will need to be more closely aligned with that of important ecological processes that determine stock dynamics. The findings of this study suggest that these processes likely operate over broad spatial scales, and that future management of *P. lethostigma* could be improved through a synthesis of biological information encompassing the species range.

Literature Cited

Anderson, J. D., and W. J. Karel. 2012. Population genetics of southern flounder with implications for management. North American Journal of Fisheries Management 32:656-663.

Anderson, J. D., W. J. Karel, and A. C. S. Mione. 2012. Population structure and evolutionary history of southern flounder in the Gulf of Mexico and western Atlantic Ocean. Transactions of the American Fisheries Society 141:46-55.

Avise, J. C. 1992. Molecular population structure and the biogeographic history of a regional fauna: a case history with lessons for conservation biology. Oikos 63:62-76.

Ballard, J. W. O., and M. C. Whitlock. 2004. The incomplete natural history of mitochondria. Molecular Ecology 13:729-744.

Bandelt, H. J., P. Forster, and A. Röhl. 1999. Median-joining networks for inferring intraspecific phylogenies. Molecular Biology and Evolution 16:37-48.

Beaumont, M. A., and D. J. Balding. 2004. Identifying adaptive genetic divergence among populations from genome scans. Molecular Ecology 13:969-980.

Begg, G. A., K. D. Friedland, and J. B. Pearce. 1999. Stock identification and its role in stock assessment and fisheries management: an overview. Fisheries Research 43:1-8.

Bensch, S., and M. Åkesson. 2005. Ten years of AFLP in ecology and evolution: why so few animals? Molecular Ecology 14:2899-2914.

Blandon, I. R., R. Ward, T. L. King, W. J. Karel, and J. P. Monaghan, Jr. 2001. Preliminary genetic population structure of southern flounder, *Paralichthys lethostigma*, along the Atlantic Coast and Gulf of Mexico. Fishery Bulletin 99:671-678.

Bonin, A., E. Bellemain, P. Bronken Eidesen, F. Pompanon, C. Brochmann, and P. Taberlet. 2004. How to track and assess genotyping errors in population genetics studies. Molecular Ecology 13:3261-3273.

Campbell, D., P. Duchesne, and L. Bernatchez. 2003. AFLP utility for population assignment studies: analytical investigation and empirical comparison with microsatellites. Molecular Ecology 12:1979-1991.

Conover, D. O., L. M. Clarke, S. B. Munch, and G. N. Wagner. 2006. Spatial and temporal scales of adaptive divergence in marine fishes and the implications for conservation. Journal of Fish Biology 69:21-47.

Craig, J. K., Author, and Author. In review. Estuarine residency and migration of southern flounder (*Paralichthys lethostigma*) inferred from conventional tag returns at multiple spatial scales.

Crandall, K. A., O. R. P. Bininda-Emonds, G. M. Mace, and R. K. Wayne. 2000. Considering evolutionary processes in conservation biology. Trends in Ecology & Evolution 15:290-295.

Earl, D., and B. vonHoldt. 2012. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conservation Genetics Resources 4:359-361.

Evanno, G., S. Regnaut, and J. Goudet. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular Ecology 14:2611-2620.

Excoffier, L., and H. E. L. Lischer. 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Molecular Ecology Resources 10:564-567.

Excoffier, L., P. E. Smouse, and J. M. Quattro. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131:479-491.

Gilbert, C. R. 1986. Species profiles: life histories and environmental requirements of coastal fishes and invertebrates (South Florida)--southern, Gulf, and summer flounders. Coastal Ecology Group, U.S. Army Corps of Engineers, Technical Report 82 (11.54), Vicksburg, MS.

Ginsburg, I. 1952. Flounders of the genus *Paralichthys* and related genera in American waters. Fishery Bulletin 52:267-351.

Hauser, L., and G. R. Carvalho. 2008. Paradigm shifts in marine fisheries genetics: ugly hypotheses slain by beautiful facts. Fish and Fisheries 9:333-362.

Hutchinson, W. F., C. van Oosterhout, S. I. Rogers, and G. R. Carvalho. 2003. Temporal analysis of archived samples indicates marked genetic changes in declining North Sea cod (*Gadus morhua*). Proceedings of the Royal Society of London. Series B: Biological Sciences 270:2125-2132.

Jakobsson, M., and N. A. Rosenberg. 2007. CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. Bioinformatics 23:1801-1806.

Knutsen, H., P. E. Jorde, C. André, and N. C. Stenseth. 2003. Fine-scaled geographical population structuring in a highly mobile marine species: the Atlantic cod. Molecular Ecology 12:385-394.

Knutsen, H., E. M. Olsen, P. E. Jorde, S. H. Espeland, C. André, and N. C. Stenseth. 2011. Are low but statistically significant levels of genetic differentiation in marine fishes 'biologically meaningful'? A case study of coastal Atlantic cod. Molecular Ecology 20:768-783.

Lee, W.-J., J. Conroy, W. H. Howell, and T. Kocher. 1995. Structure and evolution of teleost mitochondrial control regions. Journal of Molecular Evolution 41:54-66.

Lesack, K., and C. Naugler. 2011. An open-source software program for performing Bonferroni and related corrections for multiple comparisons. Journal of Pathology Informatics 2:52-52.

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

Liu, J.-Y., Z.-R. Lun, J.-B. Zhang, and T.-B. Yang. 2009. Population genetic structure of striped mullet, *Mugil cephalus*, along the coast of China, inferred by AFLP fingerprinting. Biochemical Systematics and Ecology 37:266-274.

Liu, Y.-G., S.-L. Chen, B.-F. Li, Z.-J. Wang, and Z. Liu. 2005. Analysis of genetic variation in selected stocks of hatchery flounder, *Paralichthys olivaceus*, using AFLP markers. Biochemical Systematics and Ecology 33:993-1005.

Luikart, G., P. R. England, D. Tallmon, S. Jordan, and P. Taberlet. 2003. The power and promise of population genomics: from genotyping to genome typing. Nature Reviews Genetics 4:981-994.

McCairns, R. J. S., and L. Bernatchez. 2008. Landscape genetic analyses reveal cryptic population structure and putative selection gradients in a large-scale estuarine environment. Molecular Ecology 17:3901-3916.

Meudt, H. M., and A. C. Clarke. 2007. Almost Forgotten or Latest Practice? AFLP applications, analyses and advances. Trends in Plant Science 12:106-117.

Meyer, A., J. M. Morrissey, and M. Schartl. 1994. Recurrent Origin of a Sexually Selected Trait in *Xiphophorus* Fishes Inferred from a Molecular Phylogeny. Nature 368:539-542.

Mickett, K., C. Morton, J. Feng, P. Li, M. Simmons, D. Cao, R. A. Dunham, and Z. Liu. 2003. Assessing genetic diversity of domestic populations of channel catfish (*Ictalurus punctatus*) in Alabama using AFLP markers. Aquaculture 228:91-105.

Midway, S. R., S. X. Cadrin, and F. S. Scharf. In press. Southern flounder (*Paralichthys lethostigma*) stock structure inferred from otolith shape analysis. Fishery Bulletin 112:326-338.

Monaghan, J. P., Jr. 1996. Life History Aspects of Selected Marine Recreational Fishes in North Carolina, Study 2: Migration of Paralichthid Flounders Tagged in North Carolina. North Carolina Division of Marine Fisheries, Completion Report Grant F-43, Segments 1-5, Morehead City.

NCDMF (North Carolina Division of Marine Fisheries). 2005. North Carolina Fishery Management Plan: Southern Flounder (*Paralichthys lethostigma*). North Carolina Department of Environment and Natural Resources, Technical Report, Morehead City.

NCDMF (North Carolina Division of Marine Fisheries). 2013. North Carolina Southern Flounder (*Paralichthys lethostigma*) Fishery Management Plan Amendment 1. North Carolina Department of Environment and Natural Resources, Technical Report, Morehead City.

Nims, M. K., and B. D. Walther. 2014. Contingents of Southern Flounder from Subtropical Estuaries Revealed by Otolith Chemistry. Transactions of the American Fisheries Society 143:721-731.

O'Reilly, P. T., M. F. Canino, K. M. Bailey, and P. Bentzen. 2004. Inverse relationship between FST and microsatellite polymorphism in the marine fish, walleye pollock (*Theragra chalcogramma*): implications for resolving weak population structure. Molecular Ecology 13:1799-1814.

Polzin, T., and S. V. Daneshmand. 2003. On Steiner trees and minimum spanning trees in hypergraphs. Operations Research Letters 31:12-20.

Pritchard, J. K., M. Stephens, and P. Donnelly. 2000. Inference of Population Structure Using Multilocus Genotype Data. Genetics 155:945-959.

Rosenberg, N. A. 2004. DISTRUCT: a program for the graphical display of population structure. Molecular Ecology Notes 4:137-138.

Ruzzante, D. E., C. T. Taggart, and D. Cook. 1999. A review of the evidence for genetic structure of cod (*Gadus morhua*) populations in the NW Atlantic and population affinities of larval cod off Newfoundland and the Gulf of St. Lawrence. Fisheries Research 43:79-97.

Ryman, N., S. Palm, C. André, G. R. Carvalho, T. G. Dahlgren, P. E. Jorde, L. Laikre, L. C. Larsson, A. Palmé, and D. E. Ruzzante. 2006. Power for detecting genetic divergence: differences between statistical methods and marker loci. Molecular Ecology 15:2031-2045.

Sønstebø, J. H., R. Borgstrøm, and M. Heun. 2007. A comparison of AFLPs and microsatellites to identify the population structure of brown trout (*Salmo trutta* L.) populations from Hardangervidda, Norway. Molecular Ecology 16:1427-1438.

Tajima, F. 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123:585-595.

Takade-Heumacher, H., and C. Batsavage. 2009. Stock Status of North Carolina Southern Flounder (*Paralichthys lethostigma*). North Carolina Division of Marine Fisheries, Technical Report, Morehead City.

Tamura, K., G. Stecher, D. Peterson, A. Filipski, and S. Kumar. 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Molecular Biology and Evolution 30:2725-2729.

Taylor, J. C., J. M. Miller, and D. Hilton. 2008. Inferring Southern Flounder Migration from Otolith Microchemistry. North Carolina Sea Grant, Final Report for Fishery Resource Grant 05-FEG-06, Raleigh.

Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. van de Lee, M. Hornes, A. Friters, J. Pot, J. Paleman, M. Kuiper, and M. Zabeau. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research 23:4407-4414.

Waples, R. S. 1998. Separating the wheat from the chaff: patterns of genetic differentiation in high gene flow species. Journal of Heredity 89:438-450.

Waples, R. S., A. E. Punt, and J. M. Cope. 2008. Integrating genetic data into management of marine resources: how can we do it better? Fish and Fisheries 9:423-449.

Ward, R. D. 2000. Genetics in fisheries management. Hydrobiologia 420:191-201.

Ward, R. D., M. Woodwark, and D. O. F. Skibinski. 1994. A comparison of genetic diversity levels in marine, freshwater, and anadromous fishes. Journal of Fish Biology 44:213-232.

Watterson, J. C., and J. L. Alexander. 2004. Southern Flounder Escapement in North Carolina, July 2001-June 2004. North Carolina Division of Marine Fisheries, Final Performance Report F-73, Morehead City.

Weetman, D., A. Ruggiero, S. Mariani, P. W. Shaw, A. R. Lawler, and L. Hauser. 2007. Hierarchical population genetic structure in the commercially exploited shrimp *Crangon crangon* identified by AFLP analysis. Marine Biology 151:565-575.

Wenner, C. A., W. A. Roumillat, J. E. Moran, Jr., M. B. Maddox, L. B. I. Daniel, and J. W. Smith. 1990. Investigations on the Life History and Population Dynamics of Marine Recreational Fishes in South Carolina: Part 1. Marine Resources Research Institute, South Carolina Wildlife and Marine Resources Department, Final Report F-37, Charleston.

Whitlock, R., H. Hipperson, M. Mannarelli, R. K. Butlin, and T. Burke. 2008. An objective, rapid and reproducible method for scoring AFLP peak-height data that minimizes genotyping error. Molecular Ecology Resources 8:725-735.

Wright, S. 1978. Evolution and the genetics of populations. Variability within and among natural populations, volume 4. University of Chicago Press, Chicago.

Tables

Table 1. Population pairwise F_{ST} comparisons for *Paralichthys lethostigma* amplified fragment length polymorphisms. Full AFLP profiles (1847 loci) are represented below the diagonal, optimized AFLP profiles (671 loci) are represented above the diagonal. Italics: P < 0.05; bold: P < 0.01; bold italics: P < 0.0001; ns: not significant after Bonferroni; plain text: not significant. See Figure 1 for population names.

	NCN	NCC	NCS	SC	GA	FL	APFL	SBTX
NCN		***0.0418***	***0.0631***	***0.0700***	**0.0324**^{ns}	0.0261	***0.1153***	***0.1314***
NCC	***0.0325***		***0.0713***	***0.0913***	***0.0399***	***0.0331***	***0.1300***	***0.1507***
NCS	***0.0640***	***0.0712***		***0.0912***	***0.0415***	***0.0508***	***0.0843***	***0.1237***
SC	***0.0608***	***0.0748***	***0.0654***		***0.0719***	***0.0611***	***0.1220***	***0.0883***
GA	***0.0505***	***0.0568***	***0.0552***	***0.0592***		*0.0297*^{ns}	***0.0959***	***0.1287***
FL	***0.0435***	***0.0467***	***0.0629***	***0.0559***	***0.0485***		***0.1051***	***0.1278***
APFL	***0.1165***	***0.1249***	***0.0990***	***0.1068***	***0.1061***	***0.0957***		***0.0502***
SBTX	***0.1285***	***0.1383***	***0.1162***	***0.0895***	***0.1232***	***0.1125***	***0.0392***	

Table 2. Analysis of molecular variance (AMOVA) for *Paralichthys lethostigma* populations grouped into two regions: U.S. South Atlantic and Gulf of Mexico. Results for both full (1847 loci) and optimized (671 loci) AFLP datasets are shown. Significant fixation indicies ($p<0.05$) are indicated with an asterisk.

Source of variation	d.f.	Sum of squares	Variance Components	Percentage of Variation	Fixation indices
Among groups					
Full dataset	1	1742.387	8.664	6.64	F_{CT}=0.0664*
Optimized dataset	1	802.652	4.043	6.98	F_{CT}=0.0698*
Among populations within groups					
Full dataset	6	2651.132	6.496	4.98	F_{SC}=0.0534*
Optimized dataset	6	1174.242	2.878	4.97	F_{SC}=0.0534*
Among individuals within populations					
Full dataset	394	45404.471	115.24	88.37	F_{ST}=0.1163*
Optimized dataset	394	20091.942	50.995	88.05	F_{ST}=0.1195*
Total					
Full dataset	401	49797.99	130.4		
Optimized dataset	401	22068.836	57.916		

Table 3. Analysis of molecular variance (AMOVA) for *Paralichthys lethostigma* populations along the U.S. South Atlantic coast using amplified fragment length polymorphisms. Populations are grouped into two regions: North Carolina and non-North Carolina populations (South Carolina/Georgia/Florida). Results for both full (1847 loci) and optimized (671 loci) AFLP datasets are shown. Significant fixation indices ($p<0.05$) are indicated with an asterisk.

Source of variation	d.f.	Sum of squares	Variance Components	Percentage of Variation	Fixation indices
Among groups					
Full Dataset	1	489.843	0.266	0.22	F_{CT}=0.0022
Optimized Dataset	1	174.072	-0.179	-0.34	F_{CT}=-0.0034
Among populations within groups					
Full Dataset	4	1798.409	6.671	5.52	F_{SC}=0.0554*
Optimized Dataset	4	804.05	3	5.67	F_{SC}=0.0565*
Among individuals within populations					
Full Dataset	296	33697.351	113.842	94.26	F_{ST}=0.0574*
Optimized Dataset	296	14814.342	50.048	94.66	F_{ST}=0.0534*
Total					
Full Dataset	301	35985.603	120.78		
Optimized Dataset	301	15792.464	52.869		

Table 4. Molecular diversity measures for populations of *Paralichthys lethostigma* sequenced at the mitochondrial control region, excluding sites with gaps. Number of samples (n), number of polymorphic sites excluding gaps (Np), number of haplotypes (Nh), haplotype diversity (Hd), and nucleotide diversity (π) are given for each site. See Figure 1 for population names.

Basin	Location	n	Np	Nh	Hd	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	260	87	129	0.977	0.0106

Table 5. Pairwise F_{ST} comparisons for *Paralichthys lethostigma* at the mitochondrial control region. Italics: P < 0.05; bold: P < 0.01; bold italics: P < 0.0001; ns: not significant after Bonferroni; plain text: not significant. See Figure 1 for population names.

	NCN	NCC	NCS	SC	GA	FL	APFL	SBTX
NCN								
NCC	0.0049							
NCS	0.0052	-0.0002						
SC	*0.0369*^{ns}	-0.0079	0.0231					
GA	*0.0521*^{ns}	0.0102	*0.0367*^{ns}	-0.0074				
FL	0.0215	-0.0095	-0.0029	-0.0036	0.0003			
APFL	***0.4234***	***0.3993***	***0.4558***	***0.3841***	***0.3962***	***0.4165***		
SBTX	***0.3993***	***0.3736***	***0.4368***	***0.3582***	***0.3715***	***0.3949***	0.0058	

Table 6. Analysis of molecular variance (AMOVA) for mitochondrial control region sequences of *Paralichthys lethostigma*. Populations are grouped on two different geographic scales: A) Ocean Basin (Gulf of Mexico vs. U.S. South Atlantic) and B) U.S. South Atlantic (North Carolina vs. South Carolina/Georgia/Florida). Significant values ($p<0.05$) are indicated with an asterisk.

Source of Variation	d.f.	Sum of Squares	Variance Components	Percentage of Variation	Fixation indices
A. Ocean Basin					
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		
B. 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		

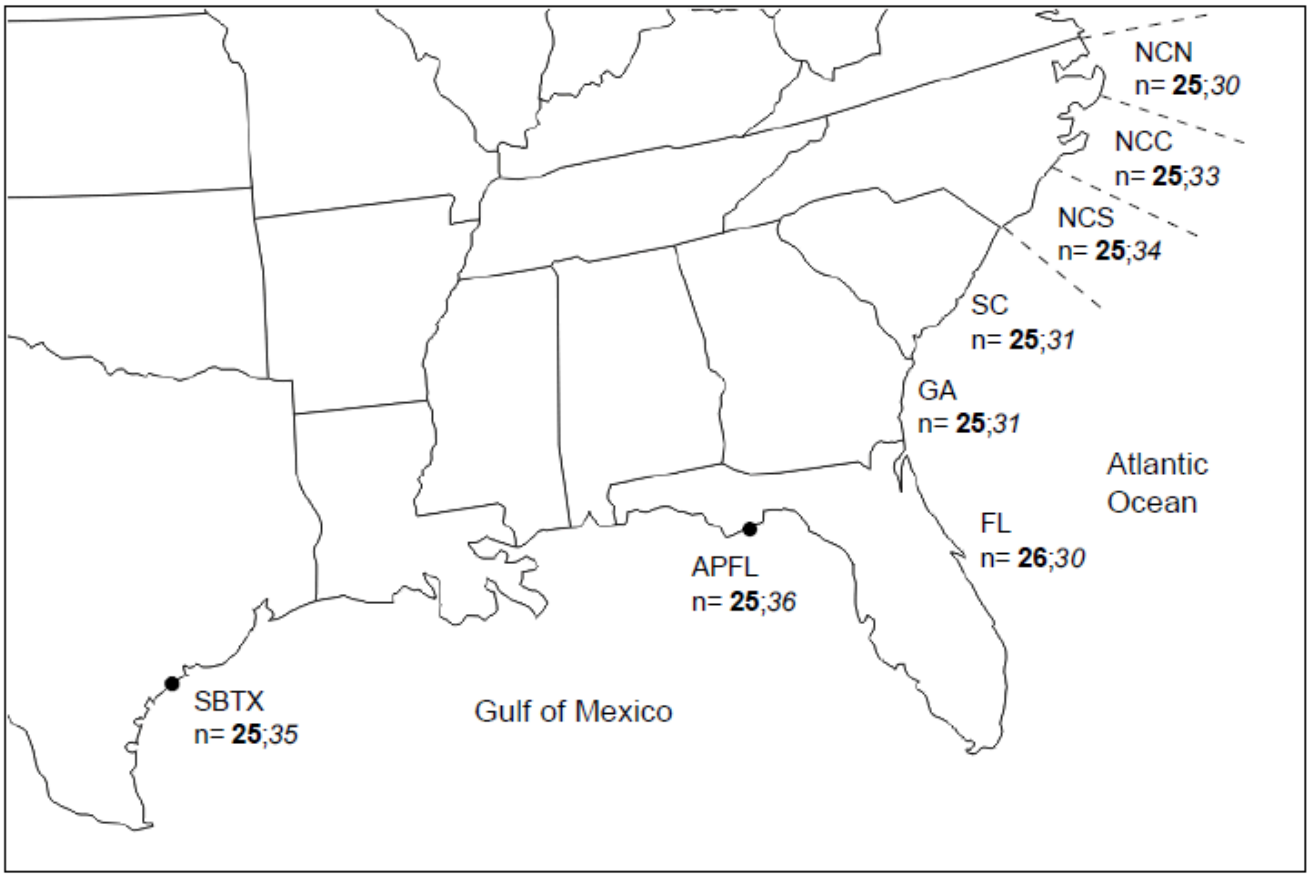

Figure 1 - Map of *Paralichthys lethostigma* sampling locations. U.S. South Atlantic locations are represented here on a state scale, although multiple sites were sampled within each state. North Carolina was subdivided into three regions (separated by dashed lines), and Gulf of Mexico samples originated from two specific locations (black circles). NCN = North Carolina North (Albemarle Sound); NCC = North Carolina Central (Neuse-Pamlico Estuary); NCS = North Carolina South (New River and Cape Fear Estuaries); SC = South Carolina; GA = Georgia; FL = Florida; APFL = Apalachicola, Florida; SBTX = San Antonio Bay, Texas. Number of samples (n) are represented in bold for amplified fragment length polymorphisms, and in italics for mitochondrial control region sequences.

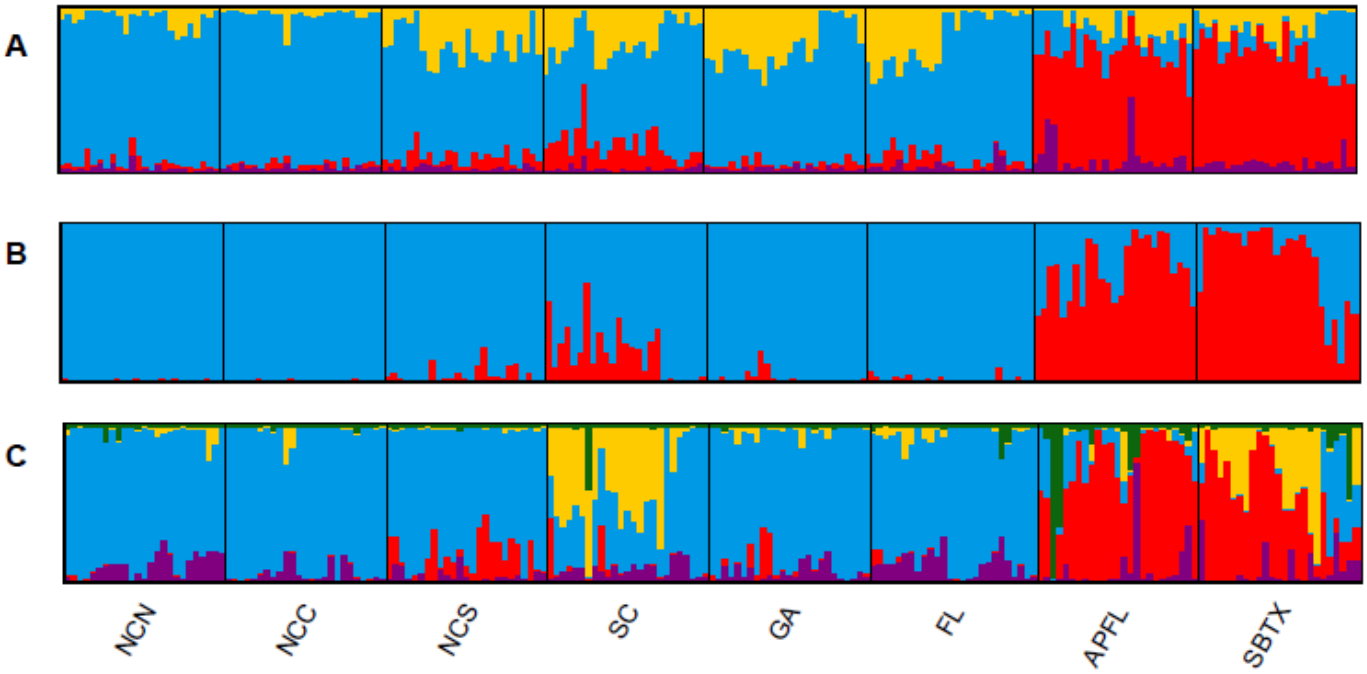

Figure 2 - Population structure for *Paralichthys lethostigma* estimated with amplified fragment length polymorphisms. Individuals are represented as vertical lines along the X-axis and are grouped into geographic populations. Each color represents a different inferred genetic cluster, and probability of assignment to K genetic clusters is shown on the Y-axis. A) K=4, 1847 loci (full AFLP profile); B) K=2, 671 loci (optimized AFLP profile); C) K=5, 671 loci (optimized AFLP profile). See Figure 1 for population names.

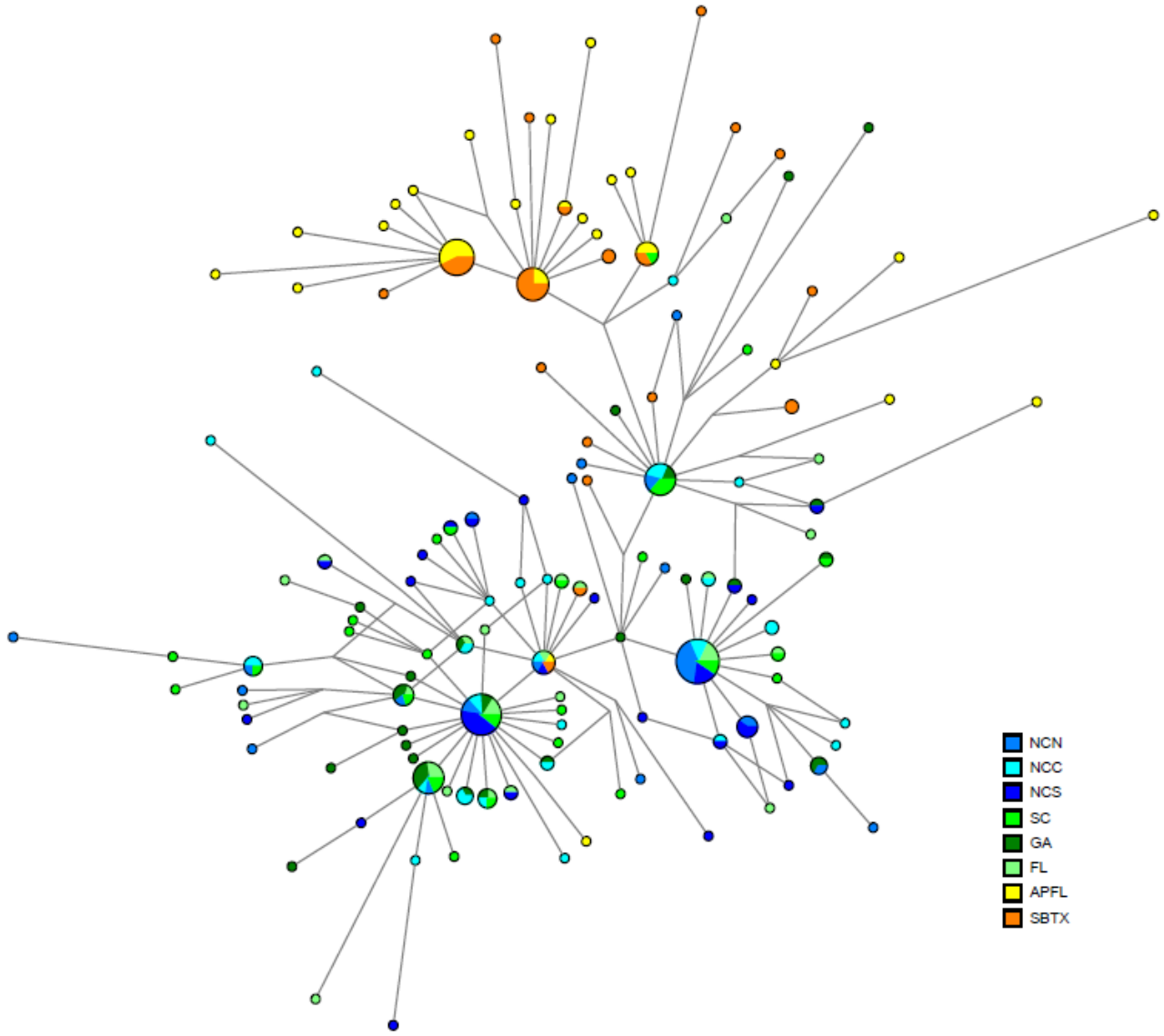

Figure 3 - Median joining network of *Paralichthys lethostigma* mitochondrial control region haplotypes. Circles represent haplotypes and colored wedges indicate geographic population. Circles and wedges are proportional to sample size, and length of connection is proportional to the number of nucleotide changes between haplotypes. See Figure 1 for population names.