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Spawning stock identification of Atlantic cod (Gadus morhua) in US waters using Pan I and microsatellite genetic markers

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Abstract
Most Atlantic cod (Gadus morhua) stocks within U.S. waters are currently in decline as a result of overexploitation and fishing pressure from commercial fisheries. A better understanding of the genetic structure of cod populations is essential to identify stocks for successful fisheries management. In this study, the genetic structure of cod from major temporally and geographically distinct spawning grounds in U.S. waters was investigated. Adult and juvenile cod were sampled from aggregations within the Gulf of Maine, Georges Bank, and southern New England waters in cooperation with commercial fishermen and state fishery biologists. Caudal fin clips were collected and analyzed using 11 microsatellite markers and the Pan I locus. Two spawning complexes of cod were identified. The northern spring complex was comprised of cod from coastal Gulf of Maine regions during the spring and summer seasons. The southern complex was comprised of cod collected during the winter months in the Gulf of Maine, on Stellwagen Bank in early spring, and at all southern locations. Georges Bank spawners were identified as a possible intermediate population between the complexes; they exhibited significant divergence from southern New England spawning aggregations, but not from cod within the Gulf of Maine. Differentiation among these populations was stable over a two year study period and was consistent with previous results for Atlantic cod reported by Lage et al. 2004 and Wirgin et al. 2007. Juveniles collected from within the Gulf of Maine could be assigned as mixtures to parental spawning complexes. Divergence among adult and juvenile cod was primarily dependent on differentiation at the Gmo132 and Pan I markers, which are suspected to be under natural selection pressures. Local adaptation to environmental factors such as water temperature and salinity may therefore be the driving force of population differentiation. Natal homing and water currents that limit larval dispersal may also influence the stock structure, but cod migrations and dispersions among spawning aggregations likely limit reproductive isolation and neutral genetic drift of populations within US waters.

Keywords
Biology, Zoology, Biology, Molecular, Agriculture, Fisheries and Aquaculture

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SPAWNING STOCK IDENTIFICATION OF ATLANTIC COD (GADUS MORHUA)
IN U.S. WATERS USING
PAN I AND MICROSATELLITE GENETIC MARKERS

BY

TIMOTHY S. BRETON

B.S., Suffolk University, 2006

THESIS

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ABSTRACT

SPAWNING STOCK IDENTIFICATION OF ATLANTIC COD (*GADUS MORHUA*) IN U.S. WATERS USING *PAN* I AND MICROSATELLITE GENETIC MARKERS

By

Timothy S. Breton

University of New Hampshire, December 2008

Most Atlantic cod (*Gadus morhua*) stocks within U.S. waters are currently in decline as a result of overexploitation and fishing pressure from commercial fisheries. A better understanding of the genetic structure of cod populations is essential to identify stocks for successful fisheries management. In this study, the genetic structure of cod from major temporally and geographically distinct spawning grounds in U.S. waters was investigated. Adult and juvenile cod were sampled from aggregations within the Gulf of Maine, Georges Bank, and southern New England waters in cooperation with commercial fishermen and state fishery biologists. Caudal fin clips were collected and analyzed using 11 microsatellite markers and the *Pan* I locus. Two spawning complexes of cod were identified. The northern spring complex was comprised of cod from coastal Gulf of Maine regions during the spring and summer seasons. The southern complex was comprised of cod collected during the winter months in the Gulf of Maine, on Stellwagen Bank in early spring, and at all southern locations. Georges Bank spawners were identified as a possible intermediate population between the complexes; they exhibited significant divergence from southern New England spawning aggregations, but not from
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limit larval dispersal may also influence the stock structure, but cod migrations and
dispersions among spawning aggregations likely limit reproductive isolation and neutral
genetic drift of populations within U.S. waters.
INTRODUCTION

Atlantic Cod Fishery and Management

The Atlantic cod (*Gadus morhua* L.) is one of the most commercially important marine fishes in the world and comprises a principal component of the northeastern United States (U.S.) groundfish assemblage. Cod are distributed throughout the temperate continental shelf waters of both the northwest and northeast Atlantic Ocean (Collette and Klein-MacPhee 2002). This species was once one of the most plentiful food fishes in the Gulf of Maine and acted as a mainstay of commercial fisheries since the 17th century (Mayo and Col 2006). The high desirability of cod led to dramatic over harvesting of the species, and recent fishing pressures have resulted in significant decline of most stocks across its range (Collette and Klein-MacPhee 2002).

Most cod stocks of the northwestern Atlantic are overexploited and are currently at low biomass levels (Mayo and Col 2006, O’Brien et al. 2006). For example, total stock biomass of the Gulf of Maine has decreased from 41,966 metric tons (mt) in 1990 to approximately 29,000 mt in 2004 (Mayo and Col 2006). Ames (2004) used anecdotal evidence to suggest that nearly half of the coastal spawning grounds for cod in the Gulf of Maine are now abandoned. Attempts to rebuild cod populations resulted in little to no increase in reproductive biomass despite 15 years of reduced fishing (Hutchings 2000). Successful restoration efforts may need to focus on identifying cod distributions and the characteristics of various stocks (Ames 2004). By gaining insight into cod
population structure in U.S. waters, it may be possible to more effectively manage this fishery.

Management plans for cod in U.S. waters are currently based on a two stock model: (1) a Gulf of Maine stock and (2) a Georges Bank and southward stock. Each management zone covers a broad distributional area with many habitats that may sustain multiple smaller and unique cod populations. Studies on cod movements found evidence of different migratory behaviors within the northwest Atlantic, including the existence of sedentary groups (Howell et al. 2008, Lindholm et al. 2007) that suggest a more complex population structure than previously believed. By tailoring fisheries guidelines to a flawed two stock model, management practices may be further depleting these smaller and more vulnerable cod stocks instead of restoring them (Ruzzante et al. 1999).

Differentiation among cod populations has been suggested to result from a variety of factors. Geographic features such as channels or trenches may serve as physical barriers to gene flow that limit dispersal and larval movements (Bentzen et al. 1996, Ruzzante et al. 1996a). Ocean currents and gyres may also be responsible for shaping population structure and retaining individuals within a geographic area (Ruzzante et al. 1998, Pampoulie et al. 2006b). The retention of larvae and juveniles can act as an imprinting episode for adult natal homing back to natal spawning grounds (Ruzzante et al. 1998). Some evidence of spawning site fidelity has been found within the Gulf of Maine (Howell et al. 2008). Even though groups may intermingle during summer and fall feeding migrations, stocks can remain genetically distinct through spawning site fidelity to unique spawning sites (Ruzzante et al. 1996b). Populations may also be separated on a temporal scale, where differences in peak spawning times can result in the
presence of two or more unique populations at a single geographic location (Wirgin et al. 2007). Genetic differentiation in marine fishes can also be the result of local adaptation at fine spatial scales (Conover et al. 2006). Environmental selection pressures such as differences in water temperature or salinity may act to overcome high levels of gene flow in the marine environment (Ward et al. 1994).

Molecular Markers for Genetic Stock Identification

Genetic stock identification provides a sensitive approach to evaluate and understand population structure. Many classes of molecular genetic markers have been developed, including allozymes, mitochondrial DNA markers (mtDNA), microsatellites, and single nucleotide polymorphisms (SNPs). These markers have been used extensively in many historical and recent studies (Avise 2004) to assay individuals and screen populations. Population variation is often greater in microsatellites and SNP loci than other genetic markers such as allozymes, mainly due to higher mutation rates (Avise 2004).

Microsatellites have become the most popular and most sensitive of the current methods in identifying population structure. Microsatellites are codominant molecular markers and most commonly consist of di-, tri-, and tetranucleotide tandem repeats within nuclear DNA. Polymorphisms in microsatellites are due to variations in these repeats, resulting in different allele lengths. Most microsatellites are assumed to be neutral to environmental selection pressures and significant differences among populations are the result of neutral genetic drift as opposed to local adaptation (Conover et al. 2006). Recent evidence suggests some Atlantic cod microsatellites may be under selection pressures (Westgaard and Fevolden 2007). Microsatellites in marine teleost
fishes often display longer allelic lengths and more variation than mammalian species
(Brooker et al. 1994). This may reflect larger evolutionary effective population sizes and
fewer barriers to gene flow in the more continuous marine environment (DeWoody and
Avise 2000).

Many microsatellites have been developed for Atlantic cod over the past decade
(Brooker et al. 1994, Miller et al. 2000, Jakobsdóttir et al. 2006). Although primer
development is laborious and time consuming, microsatellites are easy to use in PCR-
based assays and represent an efficient method of genotyping large numbers of
individuals. A variety of studies used these markers to great effect in cod, including
studies on larval aggregation (Ruzzante et al. 1996a), fishery stocks (Ruzzante et al.
1997), and aquaculture (Pampoulie et al. 2006a). Recent studies using microsatellites
also utilized SNP markers (Wirgin et al. 2007, Pampoulie et al. 2008) as a
complementary approach to identify stock structure.

Single nucleotide polymorphisms (SNPs) are a recently developed class of
molecular markers that have also been useful in evaluating stock structure. SNPs are bi-
allelic loci that represent specific base-pair variants, and they are abundant in most
genomes. The Pantophysin I (Pan I) locus (Pogson 2001) has been used in many cod
stock identification studies, often revealing several times greater levels of differentiation
than microsatellites (Pampoulie et al. 2006a, 2006b, Wirgin et al. 2007, Pampoulie et al.
2008). The Pan I locus encodes a four domain integral membrane protein (Pantophysin)
found in microvesicles and likely fills a role of basic structural functions (Haass et al.
1996). This marker has been suspected to be under a complex set of selection pressures
and salinity have been correlated with *Pan* I allelic frequencies in cod (Case et al. 2005) and a latitudinal cline has been detected within the northwest Atlantic (Sarvas and Fevolden 2005). Although selection at *Pan* I is a likely cause of its high levels of differentiation compared to microsatellites, this locus may still provide insight into population differences on both local (Wirgin et al. 2007) and broad geographic scales (Pampoulie et al. 2008).

Recent cod genomics programs in Canada (Genome Canada, Ottawa, Ontario, Canada) and Norway (National Programme for Research in Functional Genomics – FUGE, Oslo, Norway) have been implemented for quantitative trait loci (QTL) development for cod breeding programs. This genomics research has increased the number of microsatellites (Stenvik et al. 2006b, Wesmajervi et al. 2007, Delghandi et al. 2008) and SNP markers (Moen et al. 2008) available for population genetic studies. These newly developed markers for Atlantic cod will provide more resolution of the stock structure and more precise estimates of effective population sizes and rates of gene flow (Moen et al. 2008).

**Methods of Population Genetic Analysis**

Population genetic analysis involves a variety of mathematical approaches, including traditional F-statistics (Wright 1951) and the latest Bayesian clustering probabilities. One of the most commonly applied method of estimating genetic differentiation between subpopulations is the measure of \( F_{ST} \). \( F_{ST} \) is a population level approach that offers a convenient method of summarizing population structure (Weir and Cockerham 1984). \( F_{ST} \) estimates for populations of marine species are often lower than those observed in freshwater and terrestrial species because of larger effective population
sizes that increase the genetic variation at microsatellite markers (DeWoody and Avise 2000). Many cod genetic studies using microsatellite markers report low $F_{ST}$ values (<0.01), which is characteristic of high gene flow and low differentiation among marine fish populations (Brooker et al. 1994). Although these population comparisons represent weak differentiation, they are often highly significant and may reveal important information about migration and larval dispersal over fine scales (Knutsen et al. 2003). Other measures such as Fisher’s exact tests of allelic differentiation have been used as complementary statistics to $F_{ST}$ (Hutchinson et al. 2001, Jorde et al. 2007, Wirgin et al. 2007) and offer a similar population level approach to examine stock differences. Fisher’s exact tests were found to provide high resolving power when using microsatellite markers (Ryman et al. 2006).

One disadvantage to population level approaches and their applications is the \textit{a priori} definition of populations. Population based estimates such as $F_{ST}$ require the user to sort groups of individuals into predefined populations set by sampling regimes. These assumptions will introduce bias into the analysis if the real population structure is different from that of the predefined groups. Recently developed individual based analyses avoid \textit{a priori} bias by comparing each individual to one another without the need for assumed population data (Manel et al. 2003). Individual based approaches offer an advantage over traditional genetic measures that may not detect hidden population structure.

Bayesian clustering programs are individual based analyses designed to identify the number of potential populations within a dataset without \textit{a priori} bias. The most widely used program for clustering analysis is STRUCTURE (Pritchard et al. 2000).
STRUCTURE uses individual multilocus genotype data to minimize linkage disequilibrium and produce a probability that each individual originates from a potential population. STRUCTURE calculates the probability of different numbers of populations (K) in the dataset and determines the most likely K value. STRUCTURE has performed well in simulated tests (Waples and Gaggiotti 2006), complex population structure, and in populations with relatively high gene flow ($F_{ST} \geq 0.03$) among populations (Latch et al. 2006).

Assignment tests are another individual based approach and are most commonly used to assign individuals of unknown origin to previously identified populations. These statistical techniques do not avoid the a priori bias of population assumptions and depend on these definitions to calculate a probability value that each individual originated from a putative population. The program GENECLASS (Piry et al. 2004) is capable of using multiple Bayesian criteria and simulation algorithms to assign individuals to populations, but it may offer less power for resolving differences than other assignment methods when differentiation is low (Koljonen et al. 2005). Pure assignment tests such as GENECLASS assign genotypes based on highest likelihood ratios to certain populations, regardless of the assignment of other individuals in the collection. Newly developed conditional maximum likelihood programs such as ONCOR (Andersen et al. 2008) and the Bayesian program BAYES (Pella and Masuda 2001) add power to these assignments by incorporating mixture modeling prior to direct assignment. Mixture analysis provides a proportional assignment of the unknown collection to the populations, which influences the assignment and gives higher percent correct values by comparison (Koljonen et al. 2005). Low confidence in these assignment tests, however, may still be
present in cases where differentiation is extremely low ($F_{ST} \leq 0.01$), and individual assignments are only as valid as their respective mixture analyses.

Mixture analysis has also been used independently of individual assignments to aid biologists in stock assessment, conservation, and migration. Mixture analysis has given managers insights into the success of hatchery released brown trout ($Salmo trutta$) in Denmark (Ruzzante et al. 2004) and the juvenile migrations of hawksbill turtles ($Eretmochelys imbricata$) in the Caribbean Sea (Bowen et al. 2007). This method is also used in salmon ($Salmo$ spp.) management in northwestern North America. Proportions of salmon stocks in a fishery can be estimated on a monthly or weekly basis (Beacham et al. 2005) to guide management on fishery closures. A maximum likelihood approach was used by Ruzzante et al. (2000a) to identify large contributions of summer Atlantic cod in the Gulf of St. Lawrence to overwintering mixed stock populations on both sides of the Laurentian Channel. The variety of applications offered by mixture analysis provides fishery biologists with a valuable tool to identify both juvenile and adult movements.

**Previous Studies in Cod Stock Identification**

Many studies have been conducted on cod genetic population structure within both the northwest and northeast Atlantic Ocean. Although gene flow is expected to be high in the marine environment (Ward et al. 1994), genetically distinct populations have been found within current management models (Ruzzante et al. 1998, Hutchinson et al. 2001) and within fine spatial and temporal scales (Knutsen et al. 2003, Wirgin et al. 2007). Fine scale population structure has been suggested to originate from oceanographic features (Ruzzante et al. 1998), current fronts of mixing warm and cold water (Pampoulie et al. 2006b), and isolation of relic populations (Hardie et al. 2006).
Differences in larval aggregations (Ruzzante et al. 1996a) and distinctions of farmed strains from wild populations (Pampoulie et al. 2006a) also provide evidence that Atlantic cod are not genetically homogeneous across their ranges.

Within Canadian waters, spatial variation in cod populations has been extensively studied. Weak but significant genetic differentiation has been found in several locations, including differences between cod on Hamilton Bank and Grand Bank (Bentzen et al. 1996), and between an inshore population in Trinity Bay, Newfoundland and offshore overwintering populations (Ruzzante et al. 1996b). The significant divergence among winter populations was temporally stable (Ruzzante et al. 1997) and persisted despite the mixing of these populations during summer and fall feeding migrations (Ruzzante et al. 1996b). Genetic differences have also been found over continental shelf scales and bay scales (Ruzzante et al. 1998, Ruzzante et al. 2000b). Cod from southern sites such as Georges Bank and the Scotian Shelf were genetically divergent from the cod in the Gulf of St. Lawrence (Ruzzante et al. 2000a), Grand Bank, and other northern Canadian waters (Ruzzante et al. 1998). Bay scale population structure has been revealed between aggregations in Gilbert Bay off Labrador and Trinity Bay on the coast of Newfoundland. Ruzzante et al. (2000b) suggested a link between these genetic differences and physiological features, citing that Gilbert Bay cod were smaller and less fecund for their age in comparison to cod from Trinity Bay. Beacham et al. (2002) further studied inshore and offshore locations around Newfoundland, and also found cod in Gilbert Bay to be unique from adjacent bays. Distinct populations have been found in extreme northern ranges, where relic populations of cod in the Canadian Arctic Lakes were strongly differentiated from Gilbert Bay cod and other southern waters (Hardie et al. 9
Arctic populations exhibited the lowest genetic diversity of all studied sites, and the level of differentiation was two orders of magnitude higher in arctic populations than in southern Canada (Hardie et al. 2006).

Similar cases of population divergence have been observed in European waters. Broad scale differences were identified among Baltic Sea cod and other European samples using both microsatellite markers and the Pan I SNP locus (Pampoulie et al. 2008). Four genetically distinct populations of spawning cod were identified within the North Sea alone (Hutchinson et al. 2001), and fine scale structuring of cod was also found to be present within a 300 km area of the Skagerrak ocean basin off the coast of Norway (Knutsen et al. 2003). Observed levels of differentiation were weak in most cases ($F_{ST} < 0.01$) but statistically significant. Knutsen et al. (2003) found no spatial pattern to the genetic differentiation over such fine scales, and they suggested that the low levels of differentiation were due to differences in passive transport of eggs or larvae on ocean currents. Currents have also been suggested as a cause of divergence between populations of cod off Iceland, where clear separation was observed between northeastern and southwestern regions (Pampoulie et al. 2006b). This regional structuring was found to follow current fronts along the island, where mixing cold and warm water masses may act as a genetic barrier to dispersal. A tagging experiment of spawning cod in the region corroborated this observation and found that southwestern cod rarely mixed with those from the northeast (Pampoulie et al. 2006b).

Stock structure of Atlantic cod within U.S. waters has not yet been completely investigated, but two studies demonstrated differentiation involving the Nantucket Shoals, Long Island, and Ipswich Bay populations. Lage et al. (2004) found significant
differentiation between Georges Bank cod and a Nantucket Shoals population. Differences on a temporal scale within Ipswich Bay were detected by Wirgin et al. (2007), where an Ipswich Bay spring aggregation exhibited significant differentiation from both Ipswich Bay winter spawning cod and all other studied sites. This finding raised the question of whether or not the Ipswich Bay spring aggregation should be treated as a separate management unit from its winter counterpart. It also suggested that the Gulf of Maine may represent more than one stock and could be comprised of multiple unique populations separated on both spatial and temporal scales. These studies provided some evidence that stock structure within U.S. waters was highly complex, but more research was needed to fully investigate the fine scale population structuring. Unlike the plethora of genetic studies conducted in Canada and Europe, no study conducted in U.S. waters has examined the temporal stability of these differences. A multi-year sampling regime of spawning aggregations in this region was necessary to properly identify Atlantic cod stocks for successful fishery management. To this end, the present study was initiated to characterize the spawning stock structure of Atlantic cod in U.S. waters and to test for temporal stability among the aggregations over a two year study period.

**Objectives**

The purpose of this study was to examine the fine scale spatial and temporal genetic structuring of Atlantic cod populations within U.S. waters. All known major spawning aggregations in this region were sampled to more fully characterize the stock differences and to test for temporal stability over a two year period. Stability of the stock differences were further investigated by directly comparing the adult aggregations
sampled by Wirgin et al. (2007) to cod from the present study. Four main hypotheses were tested:

1) There is a consistent genetic difference between winter and spring aggregations in Ipswich Bay. Temporal stability of the seasonal variation should be evident in Ipswich Bay cod, and the genetic differences will be similar to previous results by Wirgin et al. (2007).

2) There is a consistent genetic difference between aggregations on Nantucket Shoals and Georges Bank. Lage et al. (2004) detected genetic differences between these populations that should be similar to data in the present study.

3) Further genetic structuring of cod in U.S. waters is present that has not been investigated by Lage et al. 2004 and Wirgin et al. 2007. These previous studies did not sample cod from all known major spawning aggregations in this region.

4) A subset of juvenile cod can be assigned to different adult spawning stocks. These assignments will be similar to expectations of larval drift based on the major water current patterns in the region.
CHAPTER 2

MATERIALS AND METHODS

Sample Collection

Cod were sampled over a two year study period from all known spawning locations in U.S. waters. Sampling was conducted in association with commercial fishermen and state fishery representatives (Massachusetts Division of Marine Fisheries) using bottom trawls and gill nets. Live adult cod were caught, and females in identifiable spawning condition were sampled whenever possible. Spawning condition was assessed by either visual inspection of the gonads or by observations of running milt or eggs. Female spawning condition was assessed using the National Marine Fisheries Service (Gloucester, Massachusetts, U.S.) ovarian staging criteria. Caudal fin clip samples (1 cm²) were taken from each fish and preserved in 95-100% ethanol until later genetic analysis in the laboratory.

In Year 1 of the study, 701 individuals were sampled from December 2005 to December 2006. Adult cod were sampled from known spawning aggregations within Georges Bank, Ipswich Bay, Massachusetts Bay, Nantucket Shoals and Stellwagen Bank. Adult cod not in spawning condition (identified as resting individuals) were sampled from aggregations in Ipswich Bay and Platts Bank (Table 1, Fig. 1). In Year 2 of the study, 787 individuals were sampled from January 2007 to January 2008. Adult cod were sampled from known spawning aggregations within Bigelow Bight, Cox Ledge, Georges Bank, Ipswich Bay, Jeffrey’s Ledge, Massachusetts Bay, Ipswich Bay, and Stellwagen.
Table 1. Sample sites, dates collected, sample sizes (n), and condition of Atlantic cod adult and juvenile aggregations sampled during years 1 and 2 of the study.

<table>
<thead>
<tr>
<th>Site</th>
<th>Name</th>
<th>Date</th>
<th>n</th>
<th>Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Year 1 Adults</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Massachusetts Bay</td>
<td>MBW1</td>
<td>12/05-1/06</td>
<td>140</td>
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</tr>
<tr>
<td>Georges Bank</td>
<td>GBW1</td>
<td>2/06</td>
<td>152</td>
<td>Spawning adults</td>
</tr>
<tr>
<td>Massachusetts Bay</td>
<td>MBS1</td>
<td>4/06</td>
<td>36</td>
<td>Spawning females</td>
</tr>
<tr>
<td>Ipswich Bay</td>
<td>IPS1</td>
<td>4/06-5/06</td>
<td>122</td>
<td>Spawning adults</td>
</tr>
<tr>
<td>Stellwagen Bank</td>
<td>SWS1</td>
<td>5/06</td>
<td>41</td>
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</tr>
<tr>
<td>Platts Bank</td>
<td>PBS</td>
<td>8/06</td>
<td>70</td>
<td>Resting adults</td>
</tr>
<tr>
<td>Nantucket Shoals</td>
<td>NTW</td>
<td>11/06</td>
<td>109</td>
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</tr>
<tr>
<td>Ipswich Bay</td>
<td>IPW</td>
<td>12/06</td>
<td>31</td>
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</tr>
<tr>
<td><strong>Year 2 Adults</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
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<td>MBW2</td>
<td>1/07</td>
<td>77</td>
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</tr>
<tr>
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<td>1/07</td>
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</tr>
<tr>
<td>Georges Bank</td>
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<td>2/07-3/07</td>
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<tr>
<td>New York Bight</td>
<td>NYS</td>
<td>3/07-4/07</td>
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<td>4/07</td>
<td>118</td>
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</tr>
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</tr>
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<td>Ipswich Bay</td>
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<td>6/07</td>
<td>78</td>
<td>Spent females</td>
</tr>
<tr>
<td>Bigelow Bight</td>
<td>BBS1</td>
<td>7/07</td>
<td>70</td>
<td>Spent females</td>
</tr>
<tr>
<td>Jeffrey’s Ledge</td>
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<td>12/07</td>
<td>73</td>
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</tr>
<tr>
<td>New York Bight</td>
<td>NYW</td>
<td>11/07-1/08</td>
<td>47</td>
<td>Resting adults</td>
</tr>
<tr>
<td>Massachusetts Bay</td>
<td>MBS2</td>
<td>6/08</td>
<td>50</td>
<td>Ripe females</td>
</tr>
<tr>
<td>Bigelow Bight</td>
<td>BBS2</td>
<td>7/08</td>
<td>47</td>
<td>Spawning adults</td>
</tr>
<tr>
<td><strong>Juveniles</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Massachusetts Bay</td>
<td>MBWJ1</td>
<td>10/06</td>
<td>34</td>
<td>Age-0 juveniles</td>
</tr>
<tr>
<td>Cape Cod</td>
<td>CCWJ</td>
<td>10/06</td>
<td>69</td>
<td>Age-0 juveniles</td>
</tr>
<tr>
<td>Casco Bay</td>
<td>CBSJ</td>
<td>4/07</td>
<td>45</td>
<td>Age-1 immature</td>
</tr>
<tr>
<td>Massachusetts Bay</td>
<td>MBSJ</td>
<td>4/07</td>
<td>46</td>
<td>Age-0 juveniles</td>
</tr>
<tr>
<td>Massachusetts Bay</td>
<td>MBWJ2</td>
<td>10/07</td>
<td>48</td>
<td>Age-0 juveniles</td>
</tr>
</tbody>
</table>
Fig. 1. Sample site locations for all Atlantic cod collected in the present study.
Bank. Resting adult cod were also sampled from seasonal aggregations within the New York Bight (Table 1, Fig. 1). To increase the sample size of the Massachusetts Bay spring population, an additional spawning aggregation was sampled in June 2008. The Bigelow Bight spawning aggregation was sampled again in July 2008 to provide an additional year of samples to test for temporal stability. These two summer 2008 aggregations were added to Year 2 (Table 1).

Young of year (age-0) juvenile cod were sampled by bottom trawl from Massachusetts Bay and the waters off Cape Cod by the Massachusetts Division of Marine Fisheries. Individuals from Massachusetts Bay were sampled in October 2006, April 2007, and October 2007. Individuals from Cape Cod were sampled in October 2006 (Table 1). Whole juveniles were frozen at -20°C and later thawed at University of New Hampshire (UNH) laboratories and fin clips were removed for analysis. A collection of immature (age-1) cod were sampled from Casco Bay in April 2007 by commercial fishermen using bottom trawls, and fin clips were taken from individuals prior to release. All juvenile fin clips (242 total samples) were preserved in 95-100% ethanol.

**Genetic Analyses**

Genetic analyses were conducted in collaboration with Dr. Isaac I. Wirgin at the New York University (NYU) School of Medicine (Tuxedo, NY, U.S.). Adult fin clip samples for DNA extraction and microsatellite analyses were divided between NYU and UNH. Juvenile fin clip analyses were conducted solely at UNH laboratories. DNA extractions at UNH and NYU were preformed using Qiagen DNeasy tissue kits (Qiagen, Valencia, CA, U.S.), and standard phenol/chloroform procedures (see Wirgin et al. 2007), respectively.
A total of 11 diagnostic microsatellite markers and the Pantophysin I (Pan I) SNP locus (Pogson et al. 2001) were used in the genetic analysis. Genotyping at 11 microsatellite markers was split between UNH and NYU laboratories. Analysis of Gmo02 (Brooker et al. 1994), Gmo19, Gmo36 (Miller et al. 2000), PGmo34, PGmo56, and PGmo58 (Jakobsdóttir et al. 2006) was conducted at UNH, while analysis of Gmo132 (Brooker et al. 1994), Gmo35, Gmo37 (Miller et al. 2000), PGmo32, and PGmo38 (Jakobsdóttir et al. 2006) was conducted at NYU. The Gmo37 locus was genotyped using newly developed primers (see Wirgin et al. 2007) and all microsatellites at NYU laboratories were amplified using a polymerase chain reaction (PCR) approach as described in Wirgin et al. 2007.

PCR reactions for microsatellites at UNH laboratories were conducted in 12 µl total volumes containing 2 µl DNA template (50-200 ng/ml), 0.5 µM of each primer, 1X GoTaq Flexi PCR Buffer (Promega Corp., Madison, WI, U.S.), 0.2mg/ml bovine serum albumin, 1 mM MgCl₂, 100 µM dNTPs, and 0.2 U GoTaq Flexi DNA Polymerase (Promega). The forward or reverse primer of each microsatellite was fluorescently labeled with FAM, NED, or HEX, and amplification parameters were followed as described in the literature for each primer set. Pan I genotyping was conducted at UNH using the method of Stenvik et al. (2006a). Three multiplex PCRs were conducted that shared annealing temperatures within 2°C. Multiplex PCR consisted of: 1) Gmo02, Gmo19, and Gmo36, 2) PGmo34 and PGmo56, and 3) Pan I and PGmo58. PCR products were diluted (15 to 20X) and electrophoresed in an automated capillary sequencer (ABI3130, Applied Biosystems, Foster City, CA, U.S.) at the UNH Hubbard Center for Genome Studies. Sample genotyping was conducted manually using PeakScanner v.1.0
(Applied Biosystems) and checked for the presence of null alleles, errors due to microsatellite stuttering, and large-allele dropout using MICRO-CHECKER software (Oosterhout et al. 2004).

**Statistical Analyses of Population Structure**

To characterize population structure, microsatellite and Pan I genotypes from all individuals were compiled together into their respective Year 1 and Year 2 groups and subjected to a variety of population and individual-based analytical methods. Mean number of alleles per locus and observed heterozygosities were calculated using FSTAT 2.9.3 (Goudet et al. 1995). Locus specific $F_{ST}$ values and Fisher's exact tests of allelic differentiation were calculated in GENEPOP version 3.4 (Raymond and Rousset 1995). Tests of deviations from Hardy-Weinberg equilibrium and linkage disequilibrium were conducted in GENEPOP. Markov chain Monte Carlo (MCMC) parameters for all calculations in GENEPOP were increased to 10,000 iterations and 10,000 batches prior to analysis.

To test for genetic differences among the populations, Year 1 and Year 2 collections were subject independently to pairwise allelic differentiation tests in GENEPOP and $F_{ST}$ calculations using the estimator $\theta$ of Weir and Cockerham (1984) in FSTAT. Spawning aggregations sampled in both years of the study were compared using tests of allelic differentiation in GENEPOP and $F_{ST}$ estimations in FSTAT to test for temporal stability from year to year. When no significant differentiation was observed from the same sample site, Year 1 and Year 2 samples were combined into a single population for analysis. Aggregations from Bigelow Bight in July 2007 and 2008 were also tested for temporal stability and combined into a single population. All spawning
populations from Year 1 and Year 2 were then combined into one matrix and subject to calculations of pairwise population $F_{ST}$ and allelic differentiation. $F_{ST}$ values were also used in principal component analysis (PCA) using GENALEX 6.1 (Peakall and Smouse 2006) to visualize the clustering of populations.

To test for differences among populations without a priori assumptions of population structure, individual genotypes of the combined spawning Year 1 and Year 2 groups were input into the Bayesian clustering program STRUCTURE v.2.2. The parameter set used in the analysis consisted of default admixture and independent allele frequency models with a Markov chain Monte Carlo (MCMC) burn-in length of 500,000 and a run length of 1,000,000. Clustering analysis was performed five times for each K value ranging 1-5. Individual ln(Pr X | K) values for each K were averaged over the five runs. Probabilities of K were calculated from the average ln(Pr X | K) values using the method of Pritchard et al. 2007.

The dataset from Wirgin et al. (2007) was also compared to samples in the present study to address the question of temporal stability of the stock structure over a longer time period (2003 to 2007). Spawning aggregations from Wirgin et al. 2007 were sampled from Ipswich Bay in January 2003 (48 samples) and May 2003 (48 samples), off of Chatham, Massachusetts in November 2003 (40 samples), on Stellwagen Bank in January 2005 (27 samples), and on northeast Georges Bank in February 2005 (100 samples). The resting adult aggregation sampled from Long Island in January-February 2005 (80 samples) was also included in the analysis. To make comparisons between the two studies, the microsatellite data were calibrated to correct for differences in electrophoresis platforms between years. The Gmo02, Gmo132, Gmo35, Gmo36, and
Gmo37 microsatellites were calibrated to the data from the present study by re-analyzing a subset of samples from Wirgin et al. 2007 using the above methods. Gmo19 could not be calibrated due to high levels of variability between studies and was removed prior to analysis. The Gmo19 locus was the least informative marker in either study (see Wirgin et al. 2007 and Table 2) and its removal was unlikely to affect the comparative analysis. The Pan I SNP locus was not calibrated between studies because the identification of the two alleles was not dependent on the electrophoresis platform. Microsatellite markers used in the present study but not in Wirgin et al. 2007 (PGmo32, PGmo34, PGmo38, PGmo56, PGmo58) were not included in the analysis. A PCA was conducted using pairwise population F_{ST} values to visualize the genetic data.

To test for genetic differences among the juvenile collections, population F_{ST} estimates in FSTAT and allelic differentiation tests in GENEPOP were used. The genotype data were also tested for conformity to Hardy-Weinberg equilibrium in GENEPOP. F_{ST} values were calculated with a combined dataset of juvenile and spawning adult collections in GENALEX and used in a PCA. To assign the juvenile collections to adult spawning stocks, mixture analysis was performed using the conditional maximum likelihood approach of ONCOR. Confidence intervals of mixture proportions were determined through 10,000 bootstraps. The accuracy of the genetic stock identification was assessed through self-assignment of adult individuals to their respective populations using the ‘leave-one-out’ procedure (Andersen et al. 2008) and through 100% fishery simulations of 50 individuals and 1,000 simulations. The ONCOR mixture analysis program was the preferred method for assigning juvenile proportions because it is a maximum likelihood approach that provides more power than pure
assignment tests such as GENECLASS (Koljonen 2005). The Bayesian method of mixture analysis in BAYES was not used because of the inability of the MCMC chains to converge as estimated by the Gelman and Rubin (1992) shrink factor. The failure of the shrink factor to converge indicated that this Bayesian method was not suitable for the weak differentiation present in the dataset. ONCOR does not have this limitation associated with MCMC chains, but assumes baseline allele frequencies without error.
RESULTS

Descriptive Statistics

Observed per locus heterozygosities averaged over all Atlantic cod samples varied from 0.250-0.952 among the microsatellites and 0.093 at the \( \text{Pan} \) I locus (Table 2). The \( \text{PGmo32} \), \( \text{PGmo56} \), and \( \text{PGmo58} \) markers exhibited the lowest heterozygosities values of all assayed microsatellites. The per locus heterozygosities of \( \text{Gmo02} \), \( \text{Gmo19} \), \( \text{Gmo132} \), \( \text{Gmo35} \), \( \text{Gmo36} \), and \( \text{Gmo37} \) were similar to the observed values from Wirgin et al. (2007). Mean number of alleles among the microsatellites ranged from 4.3-60.

The highest levels of differentiation in the present study were observed at the \( \text{Gmo132} \) and \( \text{Pan} \) I markers (Table 2). Per locus tests of allelic differentiation detected highly significant (\( p < 0.0001 \)) and consistent divergence at these markers within the Year 1, Year 2, and juvenile groupings of cod. Per locus \( F_{ST} \) values were also high at these markers relative to the others, ranging from 0.0279-0.0423 in \( \text{Gmo132} \) and 0.0341-0.1091 in \( \text{Pan} \) I. Divergence at the \( \text{Pan} \) I locus was consistently higher than \( \text{Gmo132} \) in all respective groupings. Only two other cases of significant per locus differentiation were observed in the present study, involving \( \text{PGmo56} \) in Year 2 and \( \text{Gmo36} \) in the juvenile analyses. These differences were not consistent among groupings and per locus \( F_{ST} \) values were not as high in comparison to \( \text{Gmo132} \) and \( \text{Pan} \) I. All other \( F_{ST} \) values for microsatellites were extremely low (\( \leq 0.0039 \)) and did not indicate significant differentiation.
Table 2. Per locus mean number of alleles, observed heterozygosities (H₀), F<sub>ST</sub> estimates, and allelic differentiation p-values for Atlantic cod. Mean alleles and mean H₀ values are averaged over all populations in Years 1, 2, and juvenile groupings of the study. Bold indicates significance following standard Bonferroni correction (p ≤ 0.0042).

<table>
<thead>
<tr>
<th>Locus</th>
<th>Mean alleles</th>
<th>Mean</th>
<th>Year 1</th>
<th>Year 2</th>
<th>Juvenile</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>H₀</td>
<td>F&lt;sub&gt;ST&lt;/sub&gt;</td>
<td>Differentiation</td>
<td>F&lt;sub&gt;ST&lt;/sub&gt;</td>
</tr>
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<td>Gmo02</td>
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<tr>
<td>Gmo19</td>
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<td>0.952</td>
<td>-0.0008</td>
<td>0.7712</td>
<td>0.0000</td>
</tr>
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<td>Gmo132</td>
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<td>0.758</td>
<td>0.0279</td>
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<td>0.0423</td>
</tr>
<tr>
<td>Gmo35</td>
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<td>0.757</td>
<td>-0.0020</td>
<td>0.7298</td>
<td>0.0035</td>
</tr>
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<td>Gmo36</td>
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<td>0.571</td>
<td>-0.0020</td>
<td>0.7298</td>
<td>0.0010</td>
</tr>
<tr>
<td>Gmo37</td>
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<td>0.0011</td>
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<td>-0.0040</td>
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<td>0.577</td>
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<td>0.6474</td>
<td>0.0039</td>
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<td>0.0010</td>
<td>0.4980</td>
<td>0.0013</td>
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<td>PGmo56</td>
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<td>0.250</td>
<td>0.0012</td>
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<td>PGmo58</td>
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<td>0.268</td>
<td>0.0001</td>
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<td>-0.0012</td>
</tr>
<tr>
<td>Pan I</td>
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<td>0.093</td>
<td>0.0341</td>
<td>&lt;0.0001</td>
<td>0.0726</td>
</tr>
</tbody>
</table>
Population Differentiation in Year 1

All populations in Year 1 were in Hardy-Weinberg equilibrium (Appendix B) and no linkage disequilibrium was detected (data not shown). Using MICRO-CHECKER software, no evidence of the presence of null alleles, stuttering, or large allele dropout was detected. Several cases of significant differentiation (Table 3) were detected using pairwise population $F_{ST}$ estimates and tests of allelic differentiation. Significant differentiation was evident between Georges Bank cod and spawning adults on Nantucket Shoals. Spawning cod in Ipswich Bay exhibited significant differentiation from winter spawning females in Massachusetts Bay and spawning cod on Nantucket Shoals. Resting adults on Platts Bank also exhibited significant differentiation from the Nantucket Shoals population. Significant differences by allelic differentiation only were found between resting adults on Platts Bank and spawning females in Massachusetts Bay winter, and between spawning cod on Georges Bank and the Massachusetts Bay winter population. No significant differentiation was detected in these latter cases because pairwise $F_{ST}$ estimates are likely more conservative in comparison to allelic differentiation. Fisher's exact tests of allelic differentiation have previously exhibited the highest power in resolving population differences (Ryman et al. 2006).

No significant differentiation was found among the spawning aggregations sampled from Stellwagen Bank, Massachusetts Bay spring, and the resting collection in Ipswich Bay winter. A high but non-significant pairwise $F_{ST}$ value was found between the Ipswich Bay spring and winter collections (Table 3); the lack of statistical significance in these cases may be the result of low sample sizes of these three collections ($n = 41, 36, \text{ and } 31$, respectively). Ruzzante et al. (1996a) suggested that
Table 3. Population differentiation of Atlantic cod in Year 1. Above diagonal are allelic differentiation p-values. Below diagonal are pairwise population FST values with p-values in parentheses. Abbreviated sample site names refer to Table 1. Bold indicates significance following standard Bonferroni correction (p ≤ 0.0018).

<table>
<thead>
<tr>
<th></th>
<th>MBW1</th>
<th>GBW1</th>
<th>MBS1</th>
<th>IPS1</th>
<th>SWS1</th>
<th>PBS</th>
<th>NTW</th>
<th>IPW</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBW1</td>
<td>---</td>
<td>&lt;0.0001</td>
<td>0.1501</td>
<td>&lt;0.0001</td>
<td>0.5362</td>
<td><strong>0.0012</strong></td>
<td>0.2193</td>
<td>0.4503</td>
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<tr>
<td>GBW1</td>
<td>0.0023</td>
<td>---</td>
<td>0.2535</td>
<td>0.0066</td>
<td>0.6856</td>
<td>0.0183</td>
<td><strong>0.0001</strong></td>
<td>0.0533</td>
</tr>
<tr>
<td>MBS1</td>
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<td>-0.0001</td>
<td>---</td>
<td>0.6376</td>
<td>0.3169</td>
<td>0.8281</td>
<td>0.0030</td>
<td>0.0434</td>
</tr>
<tr>
<td>IPS1</td>
<td><strong>0.0071</strong></td>
<td>0.0024</td>
<td>0.0004</td>
<td>---</td>
<td>0.0168</td>
<td>0.7080</td>
<td>&lt;0.0001</td>
<td>0.0424</td>
</tr>
<tr>
<td>SWS1</td>
<td>-0.0012</td>
<td>0.0010</td>
<td>-0.0003</td>
<td>0.0076</td>
<td>---</td>
<td>0.0224</td>
<td>0.0643</td>
<td>0.0044</td>
</tr>
<tr>
<td>PBS</td>
<td>0.0041</td>
<td>0.0007</td>
<td>-0.0016</td>
<td>-0.0002</td>
<td>0.0050</td>
<td>---</td>
<td>&lt;0.0001</td>
<td>0.0258</td>
</tr>
<tr>
<td>NTW</td>
<td>0.0003</td>
<td><strong>0.0039</strong></td>
<td>0.0032</td>
<td><strong>0.0098</strong></td>
<td>0.0012</td>
<td><strong>0.0086</strong></td>
<td>---</td>
<td>0.2701</td>
</tr>
<tr>
<td>IPW</td>
<td>0.0042</td>
<td>0.0053</td>
<td>0.0057</td>
<td>0.0090</td>
<td>0.0071</td>
<td>0.0064</td>
<td>0.0017</td>
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</tr>
</tbody>
</table>
sample sizes of 50 or more individuals are required for identifying Atlantic cod population structure. Small collections of individuals may introduce sample size bias due to the low levels of differentiation expected for high gene flow marine species.

**Population Differentiation in Year 2**

No significant deviations from Hardy-Weinberg equilibrium (Appendix B) and no cases of linkage disequilibrium were detected in Year 2 of the study (data not shown). Using MICRO-CHECKER software, the possible presence of null alleles was detected within the winter spawning cod on Cox Ledge at PGmo56 and within the first Bigelow Bight summer aggregation at Gmo02. However, because these populations conformed to Hardy-Weinberg expectations, this possibility was not considered in further analyses. Several cases of significant differentiation were detected within Year 2 using pairwise population $F_{ST}$ estimates and tests of allelic differentiation (Table 4). All significant $F_{ST}$ comparisons included spring spawning cod within coastal Gulf of Maine, which consisted of cod in Massachusetts Bay, Ipswich Bay, and Bigelow Bight. In most cases differentiation was observed between these spring spawning aggregations and the southern cod on Cox Ledge and within the New York Bight. Several cases of significant differentiation were also apparent between the Massachusetts Bay winter population and the spring populations in Massachusetts Bay and Ipswich Bay. The Stellwagen Bank spring population was more differentiated from the spring coastal Gulf of Maine populations than winter spawning cod in Massachusetts Bay or cod in southern New England waters. No significant differentiation was observed between spawning cod on Cox Ledge and resting adults in the New York Bight. The lack of differentiation in the
Table 4. Population differentiation of Atlantic cod in Year 2. Above diagonal are allelic differentiation p-values. Below diagonal are pairwise population FST values with p-values in parentheses. Abbreviated sample site names refer to Table 1. Bold indicates significance following standard Bonferroni correction (p ≤ 0.0008).

<table>
<thead>
<tr>
<th></th>
<th>MBW2</th>
<th>CLW</th>
<th>GBW2</th>
<th>NYS</th>
<th>CLS</th>
<th>SWS2</th>
<th>IPS2</th>
<th>BBS1</th>
<th>JLW</th>
<th>NYW</th>
<th>MBS2</th>
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<tr>
<td>MBW2</td>
<td>---</td>
<td>0.1777</td>
<td>0.0039</td>
<td>0.0525</td>
<td>0.1363</td>
<td>0.0540</td>
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<td>0.2299</td>
<td>0.0139</td>
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<td>&lt;0.0001</td>
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<tr>
<td>CLW</td>
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<td>---</td>
<td>0.0050</td>
<td>0.0286</td>
<td>0.7491</td>
<td>0.0098</td>
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<td>&lt;0.0001</td>
<td>0.6509</td>
<td>0.0659</td>
<td>&lt;0.0001</td>
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<td>0.0029</td>
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<td>0.0091</td>
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<td>0.0079</td>
<td>-0.0008</td>
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</tr>
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Year 2 Georges Bank spawning population as compared to other aggregations may have been due to low sample size (n = 45).

**Identification of Spawning Complexes**

Spawning aggregations from both Year 1 and Year 2 were tested for temporal stability using allelic differentiation and F_{ST} estimates. No significant differentiation was detected between spawning aggregations sampled in both the Year 1 and Year 2 groupings (Appendix C). The comparison between Massachusetts Bay spring cod from both years exhibited a high but non-significant F_{ST} estimate that was likely due to sample size bias of the Year 1 collection (n = 36). The Ipswich Bay spring, Georges Bank winter, Massachusetts Bay winter, Massachusetts Bay spring, and Stellwagen Bank spring spawning aggregations from both years were then combined for analysis to identify potential spawning complexes in U.S. waters. No significant deviations from Hardy-Weinberg equilibrium conditions were detected at any of the combined populations (Appendix B).

Using cluster analysis of the combined spawning aggregations in STRUCTURE, no significant population structuring was detected, and K = 1 was identified as the most probable number of source populations (Table 5). However, significant differentiation among populations was detected using F_{ST} analysis and allelic differentiation. The combined spawning aggregations mirrored the Year 1 and Year 2 results and again separated the spawning aggregations of Bigelow Bight summer, Ipswich Bay spring, and Massachusetts Bay spring from cod in offshore Gulf of Maine locations and southern New England waters (Table 6). Significant differentiation was also evident between spawning female cod in Massachusetts Bay spring and Massachusetts Bay winter. The
Table 5. Number of sources populations (K) and mean probability of the dataset given K (ln(Pr X | K)) as determined by STRUCTURE. P-values for each K were calculated using the method of Prichard et al. 2007.

| K | ln(Pr X | K) | p-value |
|---|---------|---------|
| 1 | -49578.7 | 1.0     |
| 2 | -49877.3 | 0.0     |
| 3 | -50224.1 | 0.0     |
| 4 | -51413.7 | 0.0     |
| 5 | -53549.5 | 0.0     |
Table 6. Population differentiation of spawning aggregations of Atlantic cod in combined years 1 and 2. Above diagonal are allelic differentiation p-values. Below diagonal are pairwise population $F_{ST}$ values with p-values in parentheses. Abbreviated sample site names refer to Table 1. Bold indicates significance following standard Bonferroni correction ($p \leq 0.0011$).

<table>
<thead>
<tr>
<th></th>
<th>MBW</th>
<th>GBW</th>
<th>MBS</th>
<th>IPS</th>
<th>SWS</th>
<th>NTW</th>
<th>CLW</th>
<th>CLS</th>
<th>BBS</th>
<th>JLW</th>
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<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.0852</td>
<td>0.0192</td>
<td>0.3885</td>
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<td>0.0449</td>
<td>&lt;0.0001</td>
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<td>0.0044 (0.0033)</td>
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<td>&lt;0.0001</td>
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<td>&lt;0.0001</td>
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<td>0.0619</td>
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<td>0.0038 (0.0589)</td>
<td>0.0097 (0.0011)</td>
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<td>0.0032</td>
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<tr>
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<td>0.0148 (0.0011)</td>
<td>0.0107 (0.0011)</td>
<td>0.0017 (0.0044)</td>
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<td>0.0391</td>
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<td>0.0129 (0.0011)</td>
<td>0.0105 (0.0011)</td>
<td>0.0028 (0.0056)</td>
<td>0.0005 (0.0356)</td>
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<td>0.7500</td>
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<td>0.0071 (0.0011)</td>
<td>0.0178 (0.0011)</td>
<td>0.0144 (0.0011)</td>
<td>0.0053 (0.0222)</td>
<td>0.0028 (0.0122)</td>
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<td>0.0201</td>
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<td>0.0014 (0.0233)</td>
<td>0.0081 (0.0011)</td>
<td>0.0116 (0.0011)</td>
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<td>0.0129 (0.0011)</td>
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<td>&lt;0.0001</td>
</tr>
<tr>
<td>JLW</td>
<td>0.0019 (0.2344)</td>
<td>0.0026 (0.0278)</td>
<td>0.0088 (0.0067)</td>
<td>0.0079 (0.0011)</td>
<td>0.0032 (0.2956)</td>
<td>0.0037 (0.0111)</td>
<td>0.0009 (0.6267)</td>
<td>0.0071 (0.0422)</td>
<td>0.0082 (0.0011)</td>
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</table>
combined Georges Bank spawning collection exhibited significant differentiation from the Nantucket Shoals and Cox Ledge spawning collections, but was not differentiated by F<sub>ST</sub> analysis from any Gulf of Maine population. Significant divergence of the Georges Bank cod from the majority of populations in the Gulf of Maine, however, was detected using tests of allelic differentiation. Only the Stellwagen Bank spring and Jeffrey’s Ledge winter spawning aggregations were similar to Georges Bank cod by both differentiation methods.

Using principal coordinate analysis of the spawning aggregations, two main clusters of populations (Fig. 2) were identified: a spring coastal Gulf of Maine population of Bigelow Bight, Ipswich Bay, and Massachusetts Bay and another cluster comprised of spring spawners on Stellwagen Bank, winter spawners on Jeffrey’s Ledge and Massachusetts Bay, and southern aggregations on the Nantucket Shoals and Cox Ledge. This apparent clustering of populations and consistent differentiation suggests the existence of two different spawning complexes of Atlantic cod within this region. The northern spring complex (NSC) is evident within coastal Gulf of Maine waters only in the spring and summer, while a southern complex (SC) is present year round in southern waters and at different locations and seasons within the Gulf of Maine (Fig. 3). The Georges Bank spawning collection was observed as an intermediate population between the two complexes (Fig. 2) and could not be placed in either grouping based on pairwise differentiation values (Table 6). This offshore population was highly distinct from spawning cod on Cox Ledge and the Nantucket Shoals, but showed some level of similarity with both the northern spring complex and southern complex of cod within the Gulf of Maine.
Fig. 2. Principal coordinate analysis of pairwise population $F_{ST}$ values for combined years 1 and 2 spawning aggregations. Circles encompass similar populations with no significant pairwise $F_{ST}$ values (see Table 5). Sample site names refer to Table 1. Percent values refer to the total percentage of variation explained by each axis.
Fig. 3. Proposed Atlantic cod spawning complexes in U.S. waters based on $F_{ST}$ estimates. Georges Bank represented a possible intermediate population and exhibited significant differentiation from southern New England but not within the Gulf of Maine.
Differentiation of Resting Adult Aggregations

The resting adult aggregations from Platts Bank, Ipswich Bay winter, and the New York Bight were found to cluster within the two spawning complexes (Fig. 4). The Platts Bank summer adults were observed to cluster within the northern spring complex. New York Bight spring and winter adults were observed to cluster closely with each other within the southern complex and Georges Bank. The Ipswich Bay winter adults clustered far from the southern complex, which was likely due to a bias of low sample size (n = 31). The large displacement of Ipswich Bay winter cod was largely due to variation along the second axis, which explained far less of the total variation (Fig. 4). Variation along this second axis was roughly one third that of the first and is therefore unlikely to represent a significant population difference. Variation along the first axis alone places the Ipswich Bay winter collection into the southern complex.

Temporal Stability of Spawning Complexes

A comparison with the spawning aggregation dataset of Wirgin et al. (2007) suggests that the population structure was temporally stable from 2003 to 2007 (Fig. 5). The 2003 Ipswich Bay spring spawning aggregation was observed to cluster with the other coastal Gulf of Maine samples into the northern spring complex. The Ipswich Bay winter, Chatham, Stellwagen Bank, Long Island, and Georges Bank cod clustered within the southern complex. Although the Chatham and Long Island cod do not have a temporal counterpart in this study, these additional aggregations are likely subpopulations within the southern complex.
Fig. 4. Principal coordinate analysis of pairwise population $F_{ST}$ values for combined years 1 and 2 spawning (•) and resting adult aggregations (○). Sample site names refer to Table 1. Percent values refer to the total percentage of variation explained by each axis.
Fig. 5. Principal coordinate analysis of pairwise population $F_{ST}$ values for combined years 1 and 2 spawning aggregations (*) and spawning aggregations from Wirgin et al. 2007 (●). Spawning aggregation names from Wirgin et al. 2007 are in italics. Sample site names from the present study refer to Table 1. Percent values refer to the total percentage of variation explained by each axis.
Differentiation of Juvenile Collections

The juvenile collections from Cape Cod, Casco Bay, and Massachusetts Bay were all in Hardy-Weinberg equilibrium conditions except for one significant deviation in the Massachusetts Bay collection from October 2007. This collection deviated from equilibrium conditions at the Gmo37 locus \((p = 0.0006)\), and a significant heterozygote deficiency was detected (Appendix B). No linkage disequilibrium was detected in the juvenile collections (data not shown). Using MICRO-CHECKER software, no evidence were found of the presence of null alleles, stuttering, or large allele dropout.

Significant differentiation among the juvenile collections was detected using tests of \(F_{ST}\) and allelic differentiation (Table 7). The Massachusetts Bay cod from October 2006 were genetically distinct from the Cape Cod samples collected in the same month. The Massachusetts Bay cod from October 2007 exhibited significant differentiation from all other juvenile collections except for samples from the same location in the previous year. These two similar Massachusetts Bay collections from October were combined into a single population for further analyses. Significant differentiation between the Casco Bay immature fish and the Cape Cod collection was detect using tests of allelic differentiation, but this differentiation was not significant by \(F_{ST}\) analysis. The combined Massachusetts Bay collection clustered with the spawning adults of the northern spring complex when analyzed using principal coordinate analysis (Fig. 6). The Massachusetts Bay spring juvenile collection, Cape Cod juveniles, and the Casco Bay immature fish clustered within the southern complex.
Table 7. Population differentiation of juvenile Atlantic cod. Above diagonal are allelic differentiation p-values. Below diagonal are pairwise population $F_{ST}$ values with p-values in parentheses. Abbreviated sample site names refer to Table 1. Bold indicates significance following standard Bonferroni correction ($p \leq 0.0050$).

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<tr>
<th></th>
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<th>CBSJ</th>
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<th>MBWJ2</th>
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Fig. 6. Principal coordinate analysis of pairwise population $F_{ST}$ values for combined years 1 and 2 spawning aggregations (•) and juvenile collections (●). Sample site names refer to Table 1. Juvenile sites MBWJ1 and MBWJ2 were combined prior to analysis. Percent values refer to the total percentage of variation explained by each axis.
**Mixture Analysis of Juvenile Collections**

Mixture analysis was used to assign proportions of juvenile collections to spawning complexes. Individual adult populations were not used because of the lack of differentiation among the aggregations within a complex (see Table 6). The Georges Bank spawning population was removed prior to mixture analysis because it was unlikely that young of year juveniles from within the Gulf of Maine could originate from this adult population given ocean currents in the region.

Prior to mixture analysis, a leave-one-out cross validation test (Table 8) and a 100% fishery simulation test (Table 9) were conducted on the genetic data of the spawning complexes. These tests detected moderate power for the assignment of juvenile proportions. The southern complex percent correct scores from the cross validation test ranged from 74.0 to 83.9%, while the northern spring complex scores were lower and ranged from 54.1 to 65.5% (Table 8). These values indicated that a higher percentage of individuals could be assigned correctly to the southern complex than to the northern spring complex. The 100% fishery simulation consisted of simulating a mixture sample composed entirely of one population and then assigning those individuals back to the same population. The percent correct scores in this simulation were greater than the validation test in all cases, but the northern spring complex still produced lower values by comparison (Table 9). The low percent correct scores in these tests indicated that low levels of differentiation were present between the complexes.

Approximately 97% of the combined Massachusetts Bay fall collection was assigned to the northern spring complex through mixture analysis (Fig. 7). The high proportional assignment to this complex indicated that the majority of juveniles from
Table 8. Test of the accuracy of genetic stock identification using a leave-one-out cross validation test in ONCOR. Individuals from spawning Atlantic cod populations (see Table 5 and Fig. 2) were reassigned back to pre-defined reporting groups of northern spring complex (NSC) and southern complex (SC) (see Fig. 3). Percent correct values refer to the percentage of individuals correctly assigned back to each reporting group. The Georges Bank spawning population was removed prior to analysis. Abbreviated sample site names refer to Table 1.

<table>
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<th>Population</th>
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<td>NSC</td>
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<td>SC</td>
<td>80.6</td>
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<tr>
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<td>SC</td>
<td>80.4</td>
</tr>
<tr>
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<td>SC</td>
<td>83.9</td>
</tr>
<tr>
<td>CLS</td>
<td>SC</td>
<td>82.6</td>
</tr>
<tr>
<td>BBS</td>
<td>NSC</td>
<td>54.1</td>
</tr>
<tr>
<td>JLW</td>
<td>SC</td>
<td>74.0</td>
</tr>
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</table>
Table 9. Test of the accuracy of genetic stock identification using a 100% fishery simulation in ONCOR. Spawning Atlantic cod populations (see Table 5 and Fig. 2) were assigned to either the northern spring complex (NSC) or southern complex (SC) reporting groups (see Fig. 3). Mixture samples consisting entirely of single populations were simulated and then assigned to each adult complex. Percent correct refers to the percentage of these 100% mixtures assigned back to their correct reporting group. The Georges Bank spawning population was removed prior to analysis. Abbreviated sample site names refer to Table 1.

<table>
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<tr>
<th>Population</th>
<th>Assigned Reporting Group</th>
<th>Percent Correct</th>
<th>Standard Deviation</th>
<th>95% Confidence Interval</th>
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<td>NSC</td>
<td>86.37</td>
<td>7.97</td>
<td>(68.73, 99.85)</td>
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<td>IPS</td>
<td>NSC</td>
<td>76.75</td>
<td>9.87</td>
<td>(56.18, 94.96)</td>
</tr>
<tr>
<td>SWS</td>
<td>SC</td>
<td>85.89</td>
<td>8.96</td>
<td>(65.95, 99.97)</td>
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<tr>
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<td>SC</td>
<td>91.21</td>
<td>7.25</td>
<td>(74.18, 100.00)</td>
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<tr>
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<td>SC</td>
<td>93.36</td>
<td>5.82</td>
<td>(79.41, 100.00)</td>
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<tr>
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<td>SC</td>
<td>95.35</td>
<td>5.23</td>
<td>(81.98, 100.00)</td>
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<tr>
<td>BBS</td>
<td>NSC</td>
<td>76.49</td>
<td>9.89</td>
<td>(56.07, 94.26)</td>
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<tr>
<td>JLW</td>
<td>SC</td>
<td>83.48</td>
<td>9.52</td>
<td>(63.11, 99.68)</td>
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</table>
Fig. 7. Mixture analysis of juvenile collections to pre-defined spawning Atlantic cod reporting groups of northern spring complex (NSC) and southern complex (SC) (see Fig. 3). Black bars refer to percentages of juveniles assigned to each complex and shaded regions refer to 95% confidence intervals. Abbreviated site names refer to Table 1. Juvenile sites MBWJ1 and MBWJ2 were combined prior to analysis.
October in Massachusetts Bay originated from the northern spring complex. The seasonal shift of spawning complexes within the Gulf of Maine was evident in the Massachusetts Bay juveniles, and the majority (~87%) from the spring season were assigned to the southern complex. Although juveniles off Cape Cod were collected in the same month as the Massachusetts Bay fall collection, the majority in this case (~78%) were assigned instead to the southern complex (Fig. 7). Mixture analysis of the Casco Bay immature collection was equivocal, with roughly equal proportions of each complex present.
CHAPTER 4

DISCUSSION

Stock Structure of Atlantic Cod

Atlantic cod spawning aggregations in U.S. waters exhibited weak but statistically significant differentiation within the Gulf of Maine and Georges Bank management stocks. A pattern of genetic divergence was apparent that separated two main spawning complexes; a northern spring complex within coastal Gulf of Maine, and a southern complex present in both the Gulf of Maine and southern New England waters. The offshore Georges Bank spawning aggregation was genetically heterogeneous and could not be grouped into either complex. Differentiation among the complexes and Georges Bank spawning cod was similar to previous results found by Lage et al. 2004 and Wirgin et al. 2007. Comparisons of adult aggregation genetic data from Wirgin et al. 2007 to the present study indicate temporal stability of the stock structure over a five year period (2003-2007).

The spawning complexes were characterized by low levels of divergence ($F_{ST} < 0.01$) and population structuring was not evident when a priori assumptions of sampling regime were removed. The STRUCTURE clustering program is unable to identify population differentiation at $F_{ST} < 0.03$ (Latch et al. 2006), and the majority of pairwise population $F_{ST}$ values in the present study were less than half of this value (Tables 3, 4, 5, and 7). Very little stabilization of $\ln(Pr X | K)$ during the cluster analyses was evident at high $K$ values (3-5), indicating that no structuring could be detected by the statistical
method. The assumptions of population division made by $F_{ST}$ analysis and allelic differentiation give added power to these methods, but in return preclude the detection of hidden structuring outside the pre-defined sampling locations (see Pritchard et al. 2007).

**Heterogeneity of Spawning Complexes in the Gulf of Maine**

The northern spring complex of Atlantic cod occupied spring and summer coastal regions within the Gulf of Maine and consisted of spawning aggregations from Massachusetts Bay, Ipswich Bay, and the Bigelow Bight off southwestern Maine. These unique spawning aggregations were not present during the winter season and were replaced in Massachusetts Bay and Ipswich Bay by cod from the southern complex. Wirgin et al. (2007) previously studied the genetic variation of cod in Ipswich Bay and documented genetic divergence between spring and winter spawning aggregations in the area. Although few fish were sampled in Ipswich Bay during the winter months in the present study, the distinction between spring and winter remained evident (Fig. 4). This divergence was also temporally stable from 2003 to 2007 when the present study was compared to spawning aggregation data from Wirgin et al. 2007. These data are supported by a tag and recapture study conducted by Howell et al. (2008) that observed two different spawning groups present in Ipswich Bay during the spring and winter seasons. Ipswich Bay experienced a seasonal change in abundance, with strong movements of cod into the region during the spring and winter months for spawning. Spawning site fidelity was also evident in the spring spawning group, where tagged fish dispersed and returned to the released location (within 20 km range) one year later (Howell et al. 2008). The yearly migration events into the Ipswich Bay region and the
separate movements of winter and spring aggregations support the genetic distinctness of the two spawning complexes.

Robichaud and Rose (2004) reviewed many tagging studies and classified Atlantic cod aggregations according to four migratory behavior categories: sedentary residents, accurate homers, inaccurate homers, and dispersers. Cod exhibiting year round site fidelity were described as sedentary residents. Cod performing seasonal migrations to either small or large geographic areas were identified as accurate or inaccurate homers, respectively. Groups of cod without a clear pattern of migration and spawning were described as dispersers. Cod in Ipswich Bay have previously been assigned to the category of sedentary resident based on tag and recapture data (Howell et al. 2008). Recent data on cod migrations also provide evidence that cod in Ipswich Bay do not undertake extensive offshore movements, but instead stay within coastal Gulf of Maine regions (Tallack and Whitford 2008). Results from the present study were consistent with these findings and identified spring spawning cod in this region as part of a unique northern spring complex. Massachusetts Bay winter and spring aggregations followed similar seasonal variation to cod in Ipswich Bay (Table 5), and the Bigelow Bight summer spawners also cluster within this potential stock. This complex requires separate classification and is possibly composed of accurate homers. The spring spawning cod appear to exhibit spawning site fidelity to the coastal Gulf of Maine region during the spring and summer seasons, and these aggregations are not present during the winter spawning season when the southern complex predominates. The genetic similarity of spring spawning cod along the Gulf of Maine coast supports the idea that fidelity is to the general region and not just specifically to Ipswich Bay. Cod migrations between these
adjacent bays are known to occur (Tallack and Whitford 2008), and it is possible that spawning cod move along the Gulf of Maine coastline during the spring season.

The Platts Bank resting adult collection also clustered within the northern spring complex (Fig. 4). These resting fish were sampled in late August at approximately 58 km offshore, far from the expected coastal spawning aggregations. They may have been spring spawners from inshore areas in the Gulf of Maine that have dispersed away from coastal regions after spawning. If this occurs, then cod from the northern spring complex may be present at other offshore locations in late summer, when spawning events have ended and dispersal is ongoing. Howell et al. (2008) observed local postspawning movements from the Ipswich Bay area over a six to eight month timeframe in which the mean distance from the spawning site was 70-90 km. The summer resting adults on Platts Bank are located within this range and these cod may be one example of local dispersal that is characteristic of the northern spring complex.

Low differentiation at neutral microsatellite loci observed in the present study (Table 2) indicate that moderate to high levels of gene flow are likely ongoing in this region, and dispersers are likely present that homogenize the genetic variation. The non-significant \( F_{ST} \) values observed at neutral microsatellites cannot describe the levels of gene flow (Conover et al. 2006), but they do indicate that reproductive isolation is unlikely to be the driving force of differentiation. Despite the existence of spawning site fidelity and sedentary groups of cod in the western Gulf of Maine (Lindholm et al. 2007, Howell et al. 2008), transient groups or individuals may also be present at spawning times that introduce moderate levels of gene flow. Although Lindholm et al. (2007) were able to identify high site fidelity among cod on boulder reefs, a larger percentage of
tracked individuals were observed to depart the area rapidly upon release. This variability in cod migratory behavior may explain how genetic variation is homogenized between sedentary and more transient groups. The northern spring complex of spawning cod is likely composed of fish having multiple different migration and spawning strategies.

**Heterogeneity of the Southern Complex and Georges Bank**

The southern complex of spawning cod identified in this study (Fig. 3) did not fit the current model of cod stocks in U.S. waters (Mayo and Col 2006, O’Brien et al. 2006). Winter spawning aggregations in the Gulf of Maine, spring spawners on Stellwagen Bank, and aggregations in southern New England waters were all genetically similar. Unlike the northern spring complex, the southern complex consisted of both winter and spring spawning events and exhibited a wide spatial range. This similarity between Gulf of Maine populations and cod in southern New England waters is supported by recent data from tag and recapture studies. Cod from the Cape Cod and Great South Channel region were found to migrate both into the Gulf of Maine and in a southern direction towards the Nantucket Shoals (Tallack and Whitford 2008). Resting aggregations in Long Island waters and within the New York Bight were also similar to the southern complex and do not appear to represent a distinct population. These data suggest that the southern complex of cod extends further down the mid-Atlantic coast than what is presented in Fig. 3.

Significant differentiation between cod on Georges Bank and southern New England waters was consistent with previous studies in U.S. waters (Lage et al. 2004, Wirgin et al. 2007). The consistent and stable divergence between these areas likely
indicates that adult cod in southern New England waters and the mid-Atlantic region are not the product of larval spillover from Georges Bank (Lough 2004), but instead represent a separate stock. Recent data on cod movements support this hypothesis and show limited migration from Georges Bank to southern New England waters (Tallack and Whitford 2008). The stability of this divergence may also indicate that the clockwise gyre on Georges Bank is enough of a retention mechanism to limit larval movements in a southwestern direction towards the mid-Atlantic coast.

Georges Bank spawning cod were only weakly differentiated from adults within the Gulf of Maine, including both aggregations of the northern spring and southern complexes (Table 5). The offshore spawning cod on northeastern Georges Bank did not cluster clearly within either spawning complex (Fig. 2) and appeared to be genetically heterogeneous. This finding may be explained by the drift of eggs and larvae on the western Gulf of Maine current. The counterclockwise gyre of the Gulf of Maine may cause larvae to drift south and eastward towards Georges Bank (Collette and Klein-MacPhee 2002). The spawning aggregations on Stellwagen Bank and Jeffrey’s Ledge exhibited the highest similarity with Georges Bank cod (Table 5), and these offshore Gulf of Maine subpopulations would be likely to have their larvae drift along the western Gulf of Maine current in a southeast direction. Inshore Gulf of Maine cod from both complexes were only weakly differentiated from cod on Georges Bank (Table 5). They may also be connected to Georges Bank via larval drift. Dispersal modeling data provided evidence that larvae from these inshore Gulf of Maine spawning grounds may drift in a southern direction towards eastern Georges Bank and the Great South Channel (Huret et al. 2007).
**Heterogeneity of Juveniles within the Gulf of Maine**

The stock composition of juveniles within the Gulf of Maine was heterogeneous and varied depending on season and location. A large proportion of the Massachusetts Bay fall juvenile collection was assigned to the northern spring complex, while spring juveniles from the same location were assigned to the southern complex (Fig. 7). This seasonal change in mixture proportions reflects the differences in adult spawning complexes present within the Gulf of Maine. Juveniles spawned from the northern spring complex appear to be retained within inshore Gulf of Maine regions through the fall season. The high retention of juveniles within Massachusetts Bay is likely a result of its inshore location that reduces exposure to the western Gulf of Maine current and limits larval dispersal (Huret et al. 2007). This retention may act as an imprinting episode for natal homing during spring spawning events in this region.

A large proportion of the Cape Cod collection was assigned to the southern complex, and it is likely that the majority of these juvenile cod were spawned from spring spawners on nearby Stellwagen Bank. The predominant western Gulf of Maine current in the area would likely be capable of moving larvae and juveniles off Stellwagen Bank and in a southern direction towards Cape Cod.

The mixture proportions of the Casco Bay immature cod cannot be explained in the same manner. These age-1 juvenile fish did not represent a young of year collection and therefore were not dependent on ocean currents for movements. Any number of individuals within this collection could have originated from either stock with equal chance due to their ability to freely migrate and disperse within the region. The equal mixture proportions of the Casco Bay collection (Fig. 7) reflects this potential dispersing
behavior of immature individuals. Age-1 juveniles from both stocks may perform seasonal migrations into inshore locations during the spring season (Hanson 1996). The Casco Bay collection may consist of juveniles moving from overwintering sites in deep waters to more shallow locations in the spring.

**Differentiation at Non-Neutral Loci**

The majority of the genetic differentiation observed in this study was due to two markers (Gmo132 and Pan I) suspected to be under selection pressures (Fevolden and Pogson 1997, Karlsson and Mork 2003, Pogson and Fevolden 2003, Sarvas and Fevolden 2005, Nielsen et al. 2006). The Gmo132 microsatellite marker has been used in many Atlantic cod population studies, exhibiting several times higher levels of differentiation than other assumed neutral markers (e.g. Lage et al. 2004, Wirgin et al. 2007). The high levels of differentiation present at this locus (see Table 2) is inconsistent with neutral expectations that assume neutral genetic drift causes relative similar levels of differentiation across all microsatellite loci. Nielsen et al. (2006) found evidence of hitch-hiking selection at Gmo132, which means that the microsatellite is linked to a gene under selection pressures. Assumed neutrality at this locus is invalid and the levels of differentiation are inflated with respect to neutral genetic drift and reproductive isolation. The high genetic divergence at Gmo132 may be caused by local adaptation that is capable of overcoming the effects of gene flow (Conover et al. 2006). The temporal stability of differentiation at Gmo132 in U.S. waters (Lage et al. 2004, Wirgin et al. 2007) suggests that selection pressures are driving local adaptation in northwest Atlantic cod, despite the presence of at least moderate levels of gene flow acting to homogenize variation at neutral microsatellite markers.
Natural selection is a likely cause of high differentiation at the Pan I locus in relation to other SNP markers and neutral microsatellites (see Wirgin et al. 2007). Nucleotide and protein level differences between the Pan I\textsuperscript{A} and Pan I\textsuperscript{B} alleles have previously been documented, including differences by 19 nucleotide mutations and four amino acids on the protein level (Pogson 2001). These differences may contribute to differential expression or functioning of the protein in certain tissues (Pogson 2001). Local adaptation favoring certain alleles may lead to high levels of genetic divergence. Selection at this locus has been reported to cause both spatial divergence among populations (Case et al. 2005, Sarvas and Fevolden 2005, Pampoulie et al. 2006b, 2008) and differences among cohorts and sexes (Karlsson and Mork 2003). Differences in water temperature, salinity, and depth among spatially and temporally segregated groups may drive selection pressures that favor physiological differences between the two alleles. Selection at this SNP locus has been described as a complex combination of balancing and directional selection (Pogson 2001), and the effects of these pressures on local scales likely produce the high levels of divergence observed among populations, cohorts, and sexes.

Variation at the Pan I locus has been used extensively to discriminate between two divergent groups of cod in the Barents Sea, identified as northeast Arctic cod and Norwegian coastal cod. Recent diversifying selection at Pan I within these populations (Pogson and Fevolden 2003) has resulted in the dominance of the Pan I\textsuperscript{B} allele within Arctic cod while the Pan I\textsuperscript{A} allele is predominant in Norwegian coastal cod (Fevolden and Pogson 1997, Sarvas and Fevolden 2005). These two groups of cod separated by broad geographic scales can also be distinguished by the suspected non-neutral
microsatellite loci Gmo34 and Gmo132 (Westgaard and Fevolden 2007). Divergence of the two groups is probably the product of different environmental conditions found inshore versus offshore in Norwegian waters. Sarvas and Fevolden (2005) observed a strong negative correlation between the frequency of the Pan I^A allele and depth at sampling among combined Arctic and coastal cod. Pampoulie et al. (2006b) found a similar correlation between Pan I^A allele frequency and sampling depth in Icelandic cod. In both cases, the dominance of the Pan I^B allele was evident at greater depths (up to 450 m) and reached average frequencies of approximately 0.90.

Differentiation at the Pan I locus was not as profound in U.S. waters, which may be due to the finer spatial scales analyzed in the present study. A similar trend in Pan I variation, however, was evident between the northern spring complex and the southern complex. The coastal Gulf of Maine spawners of the northern spring complex exhibited higher Pan I^A frequencies (0.06-0.16) than more offshore aggregations of the southern complex (0.00-0.02). Atlantic cod in U.S. and European waters may be under similar selection pressures. The lower levels of divergence in this study relative to northeastern cod stocks may be due to smaller depth and temperature differences in U.S. waters.

Water temperature and salinity have been observed to have a significant correlation with Pan I^A allele frequency (Case et al. 2005). These factors may explain the differences between the Norwegian cod stocks at individual fjord scales (Fevolden and Pogson 1997). Case et al. (2005) studied variation at Pan I in northeast Atlantic cod and observed a positive trend between Pan I^A allele frequency and temperature, while a negative trend was evident between Pan I^A allele frequency and salinity. These selection pressures may also be present within coastal U.S. waters, where higher spring
temperatures and lower salinities result in higher Pan I^A allele frequencies in cod. Spring river runoff into the Gulf of Maine likely plays an important role in depressing ocean salinity in coastal regions. Coupled with high inshore spring and summer temperatures, these two selection pressures may drive local adaptation of spring spawners. The exclusion of spring spawners on Stellwagen Bank from the northern spring complex may be explained by its more offshore location. Increased salinity and decreased temperature in relation to inshore Massachusetts Bay and Ipswich Bay may play a role in separating these populations. Steep transition zones between Pan I^A allele frequency and temperature and salinity have been observed (Case et al. 2005), indicating even a small difference in these environmental factors could potentially result in a dramatic increase or decrease in allele frequency. Slightly elevated salinity or lower temperature on Stellwagen Bank could therefore be one reason for the divergence of these cod from the northern spring complex.

Temperature and salinity differences that drive local adaptation at Pan I likely have an effect on the fitness of cod from different populations. The association between Pan I allele frequencies and growth has previously been studied, but the relationship remains unclear and likely depends on several environmental parameters (Jónsdóttir et al. 2008). Case et al. (2006) examined northeast Atlantic cod larvae and found that Pan I^{AB} heterozygotes exhibited significantly higher mean dry weights, standard lengths, and RNA/DNA ratios than Pan I^{BB} homozygotes. These data suggested that the Pan I^A allele was associated with faster growth in natural populations, although a direct comparison with the Pan I^{AA} genotype was not possible in the study (Case et al. 2006). A comparative study between the Norwegian coastal cod and northeast Arctic cod also

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provided evidence that coastal cod larvae grow faster and exhibit greater weight at age than larvae originating from the northeast Arctic stock (Otterlei et al. 1999). This stock-specific growth difference was found across a wide range of temperatures (4-14°C) and suggested that higher growth rates of larvae from Pan I^A dominated populations is not limited to high environmental temperatures. These studies provide evidence that directional selection is present at this locus and that cod with the Pan I^A allele have increased fitness. Selection pressures acting on Pan I, however, are likely complex (Pogson 2001) and directional selection alone cannot explain the presence of Pan I^B dominated cod populations. The maintenance of both Pan I alleles indicates the presence of ongoing balancing selection that favors different alleles under different environmental conditions. Pampoulie et al. (2006b) suggested the presence of divergent selection at the Pan I locus in adult cod around Iceland, where cod with the Pan I^B allele predominate in warmer, highly saline southern waters and cod with the Pan I^A allele are more common in low salinity northern waters. Jónsdóttir et al. (2008) found variations in growth associated with Pan I allele frequencies in different environments and suggested that different physiological properties of the pantophysin proteins could be acting in different areas across Icelandic waters. The relationships between Pan I and fitness of Atlantic cod in U.S. waters has not yet been investigated. Higher spring and summer temperatures in coastal Gulf of Maine regions may contribute to increased fitness of the Pan I^A allele in these populations.

Differential growth rates are not the only fitness effects to result from differences in water temperature. Temperature may also be a selective factor for population differences in antifreeze production (Ruzzante et al. 1996b, Goddard et al. 1999) and the
distribution of polymorphic haemoglobin (Hbl-1 and Hbl-2 alleles) in Atlantic cod. Haemoglobin allele frequencies have been observed to differ substantially across the ranges of Atlantic cod (Sick 1965a, b), especially along the coast of Norway where a clear cline of Hbl-1 frequencies was evident (Frydenberg et al. 1965). Patterns of haemoglobin variation in this region are similar to divergence observed at the Pan I locus (see Sarvas and Fevolden 2005), where Pan I^A and Hbl-1 alleles dominate at warmer temperatures. Jónsdóttir et al. (1999) examined Pan I and haemoglobin variation in southern Icelandic waters and observed that the two markers varied independently but showed similar distinction among populations. The small but significant level of divergence at Pan I within U.S. waters may also indicate that selection pressures from water temperature may vary antifreeze production and haemoglobin genotypes in cod populations within the Gulf of Maine. If differentiation within cod haemoglobin is similar to Pan I variation, then Hbl-1 allele frequencies would be expected to be higher in cod from the northern spring complex than the southern complex. Considering that cod with different haemoglobin genotypes are known to prefer different temperatures (Petersen and Steffensen 2003), the divergence between these two complexes may represent important stock differences if variation in haemoglobin exists. Local adaptation based on selection pressures from water temperature likely plays an important role in shaping Atlantic cod spawning complexes within U.S. waters. Future studies attempting to differentiate between these complexes will need to focus on non-neutral genetic markers and potential physiological differences.
CHAPTER 5

CONCLUSIONS

Two divergent complexes of spawning Atlantic cod were identified in U.S. waters. A northern spring complex occurred in coastal regions of the Gulf of Maine and was present only during the spring and summer seasons. A southern complex of cod occurred in both the Gulf of Maine and southern New England waters during both winter and spring spawning seasons. These two complexes overlap geographically in Massachusetts and Ipswich Bay but are separated temporally by different spawning seasons. Georges Bank was identified as an intermediate population between the two complexes; it was strongly divergent from southern sites but similar to both spring and winter spawning populations in the Gulf of Maine. These differences were temporally stable over a two year period and were consistent with preliminary studies conducted in this region (Lage et al. 2004, Wirgin et al. 2007). The present study differed from previous analyses by sampling all known major spawning aggregations in U.S. waters and testing for temporal stability of the stock differences. Juvenile collections within the Gulf of Maine exhibited similar levels of genetic heterogeneity and mirrored the adult spawning complexes from which they were spawned. Juveniles could be assigned as groups to the spawning complexes from which they originated.

Differentiation among the complexes is likely driven by local adaptation acting to overcome the effects of gene flow. Although spawning site fidelity, larval retention in inshore areas, and limited migration patterns are evident among cod groups, at least
moderate levels of gene flow are present among populations that homogenize the genetic variation at neutral loci. A combination of high water temperatures and a decrease in salinity within spring coastal regions of the Gulf of Maine may drive adaptation in the northern spring complex. Physiological differences may be present in cod from the northern spring complex that increases their fitness under these environmental conditions.

Many unanswered questions concerning the stock structure of Atlantic cod remain. Although there is evidence of spawning site fidelity among cod groups in the Gulf of Maine, the presence of natal homing to spawning grounds remains unknown. Larval retention in inshore areas may allow for imprinting and natal homing as adults, but the exact levels of retention were not tested in the present study. Larvae from within the Gulf of Maine may also drift on ocean currents to more offshore sites such as Georges Bank. Additional cod populations from different spawning grounds on Georges Bank will need to be assayed using genetic markers to more clearly identify stock composition and larval recruitment to this area. Future studies will also need to focus on the physiological differences between the two spawning complexes and Georges Bank. The selection pressures acting on cod in U.S. waters need to be studied further to identify important population differences for effective management and conservation.
LITERATURE CITED


Ruzzante, D.E., C.T. Taggart, S. Lang, and D.Cook. (2000a) Mixed-stock analysis of Atlantic cod near the Gulf of St. Lawrence based on microsatellite DNA. Ecological Applications 10: 1090-1109.


APPENDIX A. ANIMAL CARE AND USE APPROVAL DOCUMENTATION

University of New Hampshire

Research Conduct and Compliance Services, Office of Sponsored Research
Service Building, 51 College Road, Durham, NH 03824-3585
Fax: 603-862-3564

28-Nov-2006

Berlinsky, David
Zoology, Spaulding Life Science Center
Durham, NH 03824

IACUC #: 061006
Project: Genetic Identification of Atlantic Cod Spawning Stocks in U.S. Waters using Microsatellite and SNP DNA Markers
Category: B
Approval Date: 17-Nov-2006

The Institutional Animal Care and Use Committee (IACUC) reviewed and approved the protocol submitted for this study under Category B on Page 5 of the Application for Review of Vertebrab Animal Use in Research or Instruction - the study involves either no pain or potentially involves momentary, slight pain, discomfort or stress.

Approval is granted for a period of three years from the approval date above. Continued approval throughout the three year period is contingent upon completion of annual reports on the use of animals. At the end of the three year approval period you may submit a new application and request for extension to continue this project. Requests for extension must be filed prior to the expiration of the original approval.

Please Note:
1. All cage, pen, or other animal identification records must include your IACUC # listed above.
2. Use of animals in research and instruction is approved contingent upon participation in the UNH Occupational Health Program for persons handling animals. Participation is mandatory for all principal investigators and their affiliated personnel, employees of the University and students alike. A Medical History Questionnaire accompanies this approval; please copy and distribute to all listed project staff who have not completed this form already. Completed questionnaires should be sent to Dr. Gladi Porsche, UNH Health Services.

If you have any questions, please contact either Roger Wells at 862-2726 or Julie Simpson at 862-2003.

For the IACUC,

Jessica Bolker, Ph.D.
Chair

cc: File
APPENDIX B. TESTS OF HARDY-WEINBERG EQUILIBRIUM

P-values of probability of tests of Hardy-Weinberg equilibrium of Year 1 Atlantic cod. Sample site names refer to Table 1. No significant deviations from equilibrium conditions were present (p ≤ 0.0005).

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P-values of probability tests of Hardy-Weinberg equilibrium of Year 2 Atlantic cod. Sample site names refer to Table 1. No significant deviations from equilibrium conditions were present (p ≤ 0.0003).

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P-values of probability tests of Hardy-Weinberg equilibrium of combined Year 1 and Year 2 spawning Atlantic cod. Sample site names refer to Table 1. No significant deviations from equilibrium conditions were present (p ≤ 0.0004).

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P-values of probability tests of Hardy-Weinberg equilibrium of juvenile collections of Atlantic cod. Sample site names refer to Table 1. Bold indicates significance following standard Bonferroni correction ($p \leq 0.0008$).

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P-values of heterozygote deficiency and heterozygote excess tests of Hardy-Weinberg equilibrium of juvenile collections of Atlantic cod. Sample site names refer to Table 1. Bold indicates significance following standard Bonferroni correction (p ≤ 0.0008).

### Heterozygote Deficiency

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APPENDIX C. TESTS OF TEMPORAL STABILITY

Tests of temporal stability of Atlantic cod spawning aggregations sampled in both Year 1 and Year 2 groupings. Sample site names refer to Table 1. No significant differentiation is present among population comparisons ($p \leq 0.0083$).

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