Utilizing Genetic Techniques to Discriminate Atlantic Cod Spawning Stocks in U.S. Waters: a Pilot Project

Northeast Consortium Award #: P5UZE293

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Period of performance:
December 2004: NEC tentative project approval, contingent on NEPA approvals. Project partners began genetic analysis and sample collection in good faith.
April 2005: Official NEPA approval received, NEC project contract signed
July 2005: Project completed, final report submitted

Permits and Approvals timeline:
12/8/2005: NEC staff (Troy Hartley) requested additional project details for NEPA process
12/15/2004: Heather Deese supplied project details to Troy Hartley for NEPA consideration (including 2 days of dedicated catch and release sampling for tissue samples from spawning cod within Massachusetts Bay during December 2004 or January 2005)
12/22/2004: David Berlinsky receives NMFS Letter of Acknowledgement to conduct sampling.
2/17/2005: Heather Deese submitted revised project plans to NEC staff (cancellation of dedicated at-sea sampling due to delay in approval process – past time for spawning aggregation)
4/4/2005: Official NEPA approval and NEC contract for project

Abstract
This pilot project involved commercial fishermen, recreational fishermen, and U.S and Canadian researchers in a partnership to determine the utility of specific genetic techniques for detecting significant differentiation between cod stocks in the region and to its south. Microsatellite and single nucleotide polymorphism (SNP) DNA analyses were tested on the specific question of differentiating Atlantic cod from Georges Bank and the inshore Gulf of Maine - are cod in these two regions a single or two genetic stocks? Where possible, we further addressed the question of whether stock subdivisions exist within the Gulf of Maine and south of Georges Bank. Unlike previous efforts, this study focused on actively spawning cod, with spawning state confirmed through analysis of gonad biopsies. Additionally, the focus was on spawning aggregations thought most likely to display genetic differentiation, based on the best available information regarding ecological differences, movements, and expected stock delineations, and within the constraints of collecting new samples during winter 2004/2005. The sampling strategy focused primarily on comparing winter-early spring spawning cod from Georges Bank with both winter and spring spawning cod from inshore, western Gulf of Maine.

Based on experience with genetic cod stock identification in Atlantic Canada, Europe, and recent work within U.S. waters, a total of six informative microsatellite loci and three SNP loci (*Pan I, AHR2, and ARNT2*) were tested for usefulness in distinguishing stock structure in this region. Two of the polymorphic SNP loci (*AHR2* and *ARNT2*) were newly isolated and characterized in this project. The project also focused on collecting, recording and synthesizing information on the location and timing of cod spawning aggregations in the Gulf of Maine, Georges Bank, and south of Georges Bank, as a resource for future collaborative research projects, including potentially a broader, longer-term project to identify stock structure utilizing genetic techniques. We found highly significant stock differences among many, but not all, collections made for this study. Cod from Georges Bank were significantly different from those collected in Ipswich Bay during the spring months but not from those collected in the winter. Most interestingly, the spring collection from Ipswich Bay was significantly different from that made at the same location during the winter along with all other collections made in the study. Fish collected from wrecks off Long Island, New York, were significantly different from the Georges Bank collection, but not those from Chatham, MA, or the Stellwagen Bank. In summary, for the first time we have demonstrated a highly significant genetic difference between a collection of spawning cod from Georges Bank and one collection of spawning cod from the inshore Gulf of Maine and our genetic results support the morphological observations of stock differences between cod collected during the winter and spring months in Ipswich Bay.

Introduction

Atlantic cod (*Gadus morhua*) is a principal component of the Northeast groundfish assemblage and has supported tremendously productive commercial and recreational fisheries in the region for years. As recently as 1994, however, cod in the Northeast U.S. region reached historic low numbers, and is currently diagnosed as overfished and experiencing overfishing on Georges Bank and in the Gulf of Maine (NEFSC 2002). Assessments of Atlantic cod have been key driving factors in tightening fishery regulations as implemented in Amendment 13 to the Northeast Multi-species Fishery Management Plan.

Despite the imposition over the past decade of stringent management regimes for cod in U.S. waters, the species has generally not rebounded to the desired levels of abundance. Thus, the need still exists for highly restricted harvest for many, if not all stocks. But, there is considerable evidence that trends in abundance of individual stocks may vary dramatically. However, management of cod in the U.S. waters has, and still occurs, in the absence of detailed and current data of its stock structure. The depressed state of cod populations necessitates stock-specific management plans to maintain fishery sustainability.

Informed management of exploited resources, such as Atlantic cod *Gadus morhua*, should be conducted on the stock level and should be based on discrete unit stocks (Cushing 1968). Individual stocks within the range of widely distributed species often exhibit different life history characteristics and are subject to varying levels of harvest. For example, fecundity, growth rate, and natural mortality rate may differ among individual stocks, including those in cod (Pentilla and Gifford 1976). For widely ranging stocks, exploitation can occur within the confines of their natal spawning areas or at far distant sites where they may co-occur with representatives of other stocks. Identification of stock structure can allow for management that is specifically tailored for individual stocks and reflects their status rather than that of the species as a whole. Thus, threatened stocks can be afforded restrictive harvest while those that are judged to be in better health may be more liberally harvested. Elucidation of stock structure and identification of stock-specific markers can also allow for robust quantitative estimates of the contribution of individual stocks to mixed fisheries that harvest fish from multiple co-occurring stocks. But, design of specific management regimes is especially problematic for marine fishes which rarely display sharp stock divisions, such as those exhibited by anadromous species, and may be caught hundreds of miles from their spawning grounds (Ovenden 1990, Wirgin and Waldman 1994).

Unfortunately, management of Atlantic cod is complicated by uncertainty in their stock structure (i.e., number of stocks within a region, boundaries, and extent of mixing). The distribution of cod along the Atlantic coast of North America is almost continuous, extending from Labrador to New Jersey (Fahay et al 1999). Despite their distribution and agility, it is probable that cod is represented by multiple distinct genetic stocks within U.S. waters. Adult cod are highly migratory but tagging studies suggest that they demonstrate spawning site

fidelity and may over-winter in close proximity to their spawning grounds (Taggart 1997; Perkins et al 1997). But, because their eggs and larvae are planktonic, considerable mixing of embryos and early larvae from geographically distinct spawning aggregations is likely. While these distributional, behavioral, and life history characteristics may limit genetic isolation, geographic or temporal barriers can serve as impediments to gene flow among spawning aggregations. Geographic barriers to gene flow may include seafloor topography and circulation patterns. For instance, it has been demonstrated that the Laurentian Channel, that separates the Scotian Shelf/Banquereau Bank from the Grand Bank serves as an effective barrier to gene flow between northern and southern stocks of Canadian cod (Bentzen et al 1996). It is possible that the Great South Channel similarly isolates fish from Georges Bank and Nantucket Shoals (Lage et al 2004). Browns Bank and Georges Bank are each characterized by distinct and relatively persistent gyre-like circulation patterns that may serve as retention mechanisms to minimize dispersal of young life stages between these banks (Ruzzante et al 1998).

Temporal barriers to gene flow may also exist and result from spawning occurring at different times in different areas. Reproductive activity in northwest Atlantic cod occurs throughout much of the year, extending from early fall to late spring (Colton et al. 1979, David Goethel, Proctor Wells, pers.com.) Within this extended time period, however, spawning peaks differ by location. As a general rule, within the Northwest Atlantic cod complex, spawning of northern stocks occurs earlier than that of stocks to the south (Myers et al 1993). Within southern stocks, there is variation in the onset of peak spawning, with egg concentrations appearing first on Georges Bank in Jan-Feb and then on Brown Bank in Mar-April (Ruzzante et al 1998). In addition, cod may be found spawning at different times, at a specific location. Spawning in Ipswich Bay, for example, occurs during the spring (May-June) as well as fall-winter (November-January).

Genetic stock identification is often a method of choice in the elucidation of stock structure and in mixed stock analysis (Wirgin and Waldman 2004). A variety of genetic approaches are currently available to describe the architecture of fish stocks, including allozymes, mitochondrial DNA, microsatellite DNA, and single nucleotide polymorphism (SNP) analyses. These approaches offer varying levels of sensitivity in elucidating stock structure, primarily because their target DNA sequences vary in their rates of evolutionary change. It is generally acknowledged that microsatellites present the highest levels of genetic diversity, although SNP loci are most frequently observed in the genomes of organisms surveyed to date and can provide a wealth of informative polymorphisms.

The development and maintenance of genetic differences among individual stocks requires that they be reproductively isolated historically and that levels of contemporary gene flow among them be very low. Genetic stock identification provides a sensitive approach to evaluate the stock structure of species in which spawning populations have been reproductively isolated for considerable times and between which contemporary gene flow is very low or non-existent (i.e. where spawning site fidelity is high and there is little mixing during spawning).

Genetic techniques have become a tool of choice for contemporary stock identification research because of their ability to describe temporally stable diagnostic markers that are particularly relevant to reproductive isolation and cohesiveness. Unlike in their early days, these techniques can now be used to handle large numbers of samples, resulting in statistical robustness in evaluating stock structure. Successful use of genetic approaches in defining stock structure is dependent on the existence of reproductively isolated spawning aggregations. Statistically distinctive allelic frequencies among spawning aggregations at selectively neutral loci can only occur in the absence of evolutionarily recent or contemporary gene flow. Distinctive allelic frequencies at loci that are not neutral can occur by strong selection within a single generation.

Genetic techniques have been used, although only recently and to a limited extent, on Atlantic cod within U.S. waters, with mixed results. Wirgin and Waldman (2004) using seven microsatellite DNA loci observed only slight, although sometimes statistically significant, differences in allelic frequencies between cod from Georges Bank and sites in the inshore Gulf of Maine or among sites in Cape Cod Bay and Massachusetts Bay. This study suffered from inadequate sample sizes from some locations and also depended on inappropriate life stages for analysis at others. Additionally, no significant differences in microsatellite allelic frequencies were observed between winter and spring spawning aggregations of cod from an inshore site in the Gulf of Maine (Kovach and Berlinsky, 2004). In contrast, Lage et al. (2004) reported significant genetic divergence between adult, although not necessarily spawning, cod from Nantucket Shoals and those from Georges Bank or Browns Bank, but not between cod from Georges Bank and Browns Bank. The absence of genetic differentiation between Georges Bank and Brown Bank collections was in contrast to results previously reported by Canadian groups (Ruzzante et al. 1998).

Management of the cod fishery in U.S. waters usually assumes a two stock model; 1) Gulf of Maine, and 2) Georges Bank and south (Mayo, 1995). The empirical verification of this model is limited and the studies upon which it is founded are decades-old. Yet, the potential exists for far more extensive stock sculpting than afforded by this model. This research project was designed to bring us one step closer to updating the stock model for cod in U.S. waters, by testing the feasibility of differentiating stocks using a suite of specific genetic markers, some existing and some newly developed in this project. The current research complimented recent and ongoing studies by utilizing a suite of genetic markers to compare winter spawning aggregations occurring on northeast Georges Bank and with winter and spring spawning aggregations within the western Gulf of Maine. Additional specimens of non-spawning cod collected during winter in the New York Bight region were also included in the analysis. These locations were chosen in order to add the most value to previous studies, while working within the constraints of a project development grant budget and a short project timeline (See methods section, below for details on sample locations).

Thus, the objective of this study was to provide a preliminary investigation of genetic stock structure of cod in U.S. waters using a combination of microsatellite and SNP analyses. We were particularly interested in determining if a collection of spawning fish from Georges

Bank was genetically distinct from those in the inshore Gulf of Maine and if winter and spring collections of spawning fish from an inshore site in Ipswich Bay were genetically distinct. Two batteries of microsatellite loci from Atlantic cod had previously been isolated by Canadian investigators and had been applied to stock identification issues in Canada, Europe, and on two occasions in U.S. waters. We used a subset of these microsatellite loci and isolated 11 single nucleotide polymorphism (SNP) in cod at three gene loci, cytochrome P4501A1 (CYP1A1), the aryl hydrocarbon receptor (AHR2), and the aryl receptor nuclear translocator (ARNT2), and used two of these to in our overall stock identification survey.

The project also focused on collecting, recording and synthesizing information on the location and timing of cod spawning aggregations in the Gulf of Maine, Georges Bank, and south of Georges Bank, in order to inform design of a sampling strategy for a broader, longer-term project to identify stock structure utilizing genetic techniques.

The research contributed directly to all of the Northeast Consortium's (NEC) programmatic goals and addressed the topic area "commercial harvest and species sampling." The project also contributed to a number of the priorities outlined in the Northeast Fisheries Science Center section of the NMFS FY 2004-2009 strategic research plan (NMFS, 2004):
"Determine the biological, environmental, and habitat processes controlling the reproductive success of important fishery resources."
"An outgrowth of the stock assessment peer review is continuing investigations of the appropriate assessment methodologies for Georges Bank/Gulf of Maine cod stocks."

Project objectives and scientific hypotheses

Our overall goal was to test the ability of genetic techniques to describe the spatial and temporal stock structure of Atlantic Cod (*Gadus morhua*) in U.S. waters. Our specific objectives were to:

- Collect tissue samples from approximately 100 spawning cod from multiple sites in U.S. waters including Georges Bank and locales in the Gulf of Maine.
- Characterize allelic/genotype diversity at 6 microsatellite and 3 SNP loci in these and previously collected samples
- Collect and present information from a range of sources outlining the expected timing and location of spawning aggregations throughout the region.

Our overall hypothesis was that cod in U.S. waters are comprised of a single genetic stock.

Participants

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A number of other individuals contributed ideas during the planning stages of NAMA's 2004 cod genetics proposals, including both the June 2004 full proposal and September 2004 project development proposal. We would like to thankfully acknowledge the time and expertise of: Russell Sherman, F/V Lady Jane, Gloucester, MA; Ted Ames, Stonington, Maine; Proctor Wells, F/V Tenacious, Phippsburg, Maine; Jim O'Grady, F/V Iron Horse, Pt. Judith, RI. We are also grateful to Captains Rob Andresen of the Captree Princess and Steve Fromm of the Big M Express who collected cod from New York waters.

Methods

The primary focus of this project was on collecting fin clips from spawning cod and analyzing genetic markers for the fish based on these samples. Genetic analysis was conducted on samples from a total of 350 fish from five sites throughout the region of interest. Of these,

270 fish where positively identified as spawning or recently spent at the time of sampling (including all fish from 4 of the locations). This project resulted in new sample collections of 210 fish (130 spawning or spent, 80 non-spawning). The project also included 140 previously collected fish (all spawning). Table 1, below, summarized the locations, numbers, spawning state, and collection methods for each sample site. The choice of sites and further details are discussed below.

Table 1: Cod samples included in genetic analysis

Location	new/ existing	#	Spawning state	Date	Collection method
Northeast Georges Bank	new	100	Spawning confirmed with gonad biopsy	Feb 2005	Collected by DFO research trawl survey, targets large aggregations of fish. Samples and data supplied free of charge to project.
New York Bight	new	80	Not spawning	Jan-Feb 2005	Collected by several party boats that fish wrecks (50-80 miles offshore) targeting cod
Stellwagen Bank	new	29	Ripe or spent biopsy	Jan 2005	Collected by David Berlinsky, during regular fishing operations with Frank Mirarchi, free of charge to project.
Ipswich Bay	existing	50	Spawning biopsy	Jan 2003	Collected by David Berlinsky, during regular fishing operations with David Goethel, free of charge to project.
Ipswich Bay	existing	50	Spawning biopsy	May 2003	Collected by David Berlinsky, during regular fishing operations with David Goethel, free of charge to project.
E. of Chatham, MA (50 fathom edge, NW of Great South Channel)	existing	~40	Spawning confirmed with gonad biopsy	Nov 2003	Jason Link during cooperative research sampling trips with Ted Ligenza (studying trophic ecology of cod), free of charge to project.

The sampling sites for this project were designed to compliment recent and ongoing studies within the region, while working within the confines of the budget and timeline of a pilot project. Because recent genetics research has compared cod from Brown's Bank, Georges Bank, and Nantucket Shoals (Lage, et al. 2004), we chose to focus on the primary question of whether cod from Georges Bank and Gulf of Maine can be differentiated using the best available genetic markers. The focus on the Gulf of Maine/Georges Bank question was based on expected stock delineations (Gulf of Maine and Georges Bank stock as defined by National

Marine Fisheries Service), as well as preliminary indications from tag-recapture sites of the Northeast Regional Cod Tagging Project which display considerable movement of tagged cod along the coast within the western Gulf of Maine, as well as north-south movement of fish between Georges Bank, Brown's Bank and Bay of Fundy, but less movement from west to east between Gulf of Maine and Georges Bank (http://codresearch.org/). Sampling strategies were designed in consultation with R. May and L. O'Brien of the NE Fisheries Center of NMFS. Unfortunately, the NEFSC did not have at hand appropriate samples for our analysis.

Through an arrangement with colleagues at the Department of Fisheries and Oceans, Canada, we were able to obtain approximately 100 samples from actively spawning cod caught during the DFO February 2005 trawl survey. We also had at our disposal existing collection of samples from spawning fish where approximately 50 fish were collected simultaneously. These existing samples were from Ipswich Bay and east of Chatham, Massachusetts (southeast corner of Cape Cod). The project plan therefore involved dedicated sampling within Massachusetts Bay during an expected December spawning aggregation to provide winter 2005 samples from inshore, western Gulf of Maine to compare with winter 2005 samples from Georges Bank. Additionally, through arrangements with charter/party boat captains fishing out of Captree, Long Island, New York and Sheepshead Bay, Brooklyn, New York, we were able to obtain 100 samples from cod wintering 50-80 miles offshore in the New York Bight region. While these New York Bight fish were not spawning, they nevertheless provide a basis for comparing samples collected from geographically separated areas during the same season (winter 2005). This is an important aspect of project design, as temporal (year-to-year) variation in genetic markers from samples collected in the same location during different years can be significant. A number of other sites and existing collections may be appropriate for a larger scale study but were not included in this pilot project.

Full genetic and biopsy analysis was required on all samples, with the exception of the Ipswich Bay samples from January 2003 and May 2003 for which microsatellite analysis was already done but at different loci than used in the current study.

Unfortunately, due to a combination of circumstances, we were unable to undertake the planned dedicated sampling trips in Massachusetts Bay during December 2004 - January 2005. Our project plan called for two dedicated sampling trips with Russell Sherman, F/V Lady Jane or Frank Mirarchi, F/V Christopher Andrew, supervising the choice of specific location, time and catch and release fishing for spawning cod. David Berlinsky (co-PI) would participate in sampling trips to collect fin clips and gonad biopsies from 100 ripe and running fish. Two problems combined to create this issue: 1) delay in approval of the project from NEC and receipt of Letter of Acknowledgement for sampling trips from NMFS until late December, and 2) lack of major spawning aggregations in Massachusetts Bay by that time. (There was a spawning aggregation in the Boston Harbor area in early December 2004, but approvals for sampling trips were not in place at this time). David Berlinsky did accompany Frank Mirarchi during a day of regular fishing operations in early January 2005 and collected samples from 29 ripe or spent cod from Stellwagen Bank, which were included in the genetic analysis.

A suite of previously identified and two newly developed genetic markers were analyzed to compare cod tissue samples from Georges Bank, Gulf of Maine, and New York Bight. Six cod-specific microsatellite loci were analyzed in this project including four loci isolated and characterized by Miller et al. (2000) and two by Brooker et al. (1994). This allowed for direct comparison of results with previous work on western Atlantic populations of cod. The pantophysin (*PanI*) single nucleotide polymorphism (SNP) was included for analysis. Previous studies have identified a marker in the pantophysin gene as the most effective marker for distinguishing European Atlantic Cod populations.

Because Wirgin's lab has characterized a variety of toxicologically relevant genes in closely related Atlantic tomcod (*Microgadus tomcod*), a subset of these were investigated for diagnostic intron-located SNPs. Three genes were investigated, from which 11 introns were tested in limited population screens for diagnostic polymorphisms of which SNPs at two were added to the battery of markers.

Methods

Characterization of SNP sites

PCR primers for AHR2, ARNT2, and CYP1A1 (Table 2) were designed based on Atlantic tomcod *Microgadus tomcod* sequences that we had previously determined for these genes. In total, we attempted to amplify 11 intronic sequences from these genes of which 4 were from AHR2, 3 were from ARNT2, and 4 were in CYP1A1. Each amplicon from twelve fish (six from NE Georges Bank and six from Long Island, NY) was sequenced using a Beckman Coulter CEQ8000 automated DNA Sequencer using Beckman Coulter DTCS DNA Sequencing Kits. The twelve sequences at each gene locus were aligned using the Pileup Program in the GCG computer analysis package to identify polymorphic nucleotide sites. Of these, two were selected for use in our full-scale stock identification study, intron 6 in AHR and intron 8 in ARNT.

Sample collections

Genetic analyses were conducted on a total of 350 samples, including 140 existing samples (previously collected) and 270 newly collected samples (collected during this study). The sampling sites included Ipswich Bay (both a winter and a spring-spawning population), Stellwagen Bank in Massachusetts Bay, southeastern Cape Cod (east of Chatham and northwest of the Great South Channel), northeast Georges Bank, and western Long Island (New York Bight). All but the Long Island samples consisted of spawning fish (see Table 1).

Fin clips (approximately $1cm^2$) were taken from fish and stored in 1.5 ml plastic microcentrifuge tubes with 95% EtOH for subsequent DNA extraction. To verify reproductive status, ovarian biopsies were taken from fish that appeared ripe but were not visibly running. No biopsies were taken from fish that were running ripe or obviously recently spawned. Data collected for each fish included a standard set of descriptors, including latitude/longitude of capture, start and finish time of tow, number of fish sampled from each tow.

Population screening at microsatellite and SNP loci

A suite of previously identified and newly developed genetic markers was used to test for differentiation among the sampling locations. Six cod-specific microsatellite loci were analyzed, including, *Gmo*19, *Gmo*35, *Gmo*36 and *Gmo*37 (Miller et al. 2000), and *Gmo*02 and *Gmo*132 (Brooker et al. 1994). Three single nucleotide polymorphism (SNP) markers were also analyzed, including pantophysin (*Pan*I; Pogson 2001) and two new SNPs, *AHR6* and *ARNT8*, developed during this study. The latter markers were identified by investigation of toxicologically relevant genes (the aryl hyrocarbon receptor, AHR, and the aryl hydrocarbon receptor nuclear translocator, ARNT) in the closely related Atlantic tomcod (*Microgadus tomcod*) in Wirgin's lab.

DNA was extracted from fin clips by using standard phenol-chloroform-alcohol isolations (NYU) or Qiagen DNeasy tissue kits (Qiagen, Valencia, CA) (UNH). Multiplex PCR was used to generate microsatellite or SNP DNA products. Microsatellites were multi-pooled prior to separation on a Beckman Coulter CEQ8000, capillary-based, automated sequencer (NYU) or an ABI377 automated sequencer (UNH). Alleles were sized by comparison with internal size standards and scored manually with the aid of Genescan software (Applied Biosystems). *Pan*I PCR products were digested with the restriction endonuclease *Dra*I for 2 hrs at 37°C and visualized on 2% agarose gels. The two other SNPs were characterized on a Pyrosequencer™ (Biotage) at NYU. Interlaboratory comparisons of a subset of samples at microsatellite and *Pan* I loci were done to ensure the veracity of the data.

Data analysis

Multi-locus genotypes were compiled for all individuals, and allele and genotype frequencies were estimated for each sampling location. Deviations from Hardy Weinberg expectations were tested for all loci using exact tests (Guo & Thompson 1992); the null hypothesis tested was the random union of gametes. P-values were generated using the Markov chain method by resampling 2000 iterations per batch for 500 batches with Genepop version 3.4 (Raymond and Rousset, 1995). Genetic variation was assessed as the observed number of alleles and heterozygosities per locus. The level of genetic differentiation among samples was characterized using Wright's F_{ST}, estimated by Weir and Cockerham's (1984) θ, as calculated using Genepop software. Tests of allelic and genotypic differentiation among population samples were conducted to test the null hypothesis of a homogenous distribution across samples. Comparisons were also made between all pairs of sampling locations and P-values were generated using the Markov chain method by resampling 1000 iterations per batch for 100 batches with Genepop. In addition, pair-wise F_{ST}s were calculated between all pairs of sampling locations, using FSTAT (Goudet 1995). All tests were performed for single loci as well as all loci combined. Significance of F_{ST}s was determined with 300 permutations. For all tests with simultaneous multiple comparisons, significance was adjusted with Bonferroni corrections.

Results

Identification of novel SNPs

SNP loci in Atlantic cod were identified by PCR of in total 11 introns in AHR2, ARNT2, and CYP1A1 using primers that were anchored in neighboring conserved exons of these genes. Of these 11 loci, 10 were successfully amplified and the PCR products usually were of the predicted molecular size based on published Atlantic tomcod sequence. These ten amplicons were fully DNA sequenced and their sequences aligned among twelve individual cod from the two putative populations geographically discrete populations, Northeast Georges Bank and western Long Island, New York. Of these amplicons, three were monomorphic (AHR intron 5, CYP1A1 intron 2, and CYP1A1 intron 3). The remaining 7 introns were polymorphic (ARNT2 intron 1, ARNT2 intron 8, ARNT2 intron 9, AHR intron 6, AHR intron 8, CYP1A1 intron 4, and CYP1A1 intron 5). Several polymorphic nucleotide sites were found within several individual introns. For example, four polymorphic sites were located within ARNT2 intron 1, three sites in ARNT2 intron 8, and two sites in ARNT2 intron 9, AHR2 intron 6, and CYP1A1 intron 5. However, there were no fixed differences between cod at any single polymorphic site except those that had private alleles. Two of the SNP sites were selected for full-scale population screening by Pyrosequencing™, AHR2 intron 6 and ARNT2 intron 8.

Population screening at microatellite and SNP loci

Descriptive statistics, including observed numbers of alleles, heterozygosities, deviations from Hardy-Weinberg equilibrium, F_{ST}s and R_{ST}s are shown in Table 3. The six microsatellite loci were highly polymorphic, with 9-37 alleles per locus and observed heterozygosities ranging from 0.538 – 0.956. Mean number of alleles per locus was 19.5 and mean heterozygosity was 0. 77. Heterozygosities for the SNP loci were lower, as they only had 2 alleles each. In total, 123 alleles were observed among the 9 loci screened in this study. There were no significant deviations from Hardy Weinberg equilibrium, despite a slight trend for heterozygote deficiency at *Gmo*36 for the Georges Bank and Stellwagen Bank populations (these tests were not significant after application of Bonferroni correction for multiple tests; alpha = .05, P<0.00093). F_{ST}s were all positive, and ranged from 0.0005 to 0.0747, with a mean of 0.0068 overall. R_{ST}s ranged from –0.0019 to 0.1134, with 0.0105 overall. F_{ST}s and R_{ST}s were highest for *Gmo*132 and *Pan*I. Tests of population differentiation among samples (allelic differentiation) showed significant divergence at *Gmo*132 (P<0.00001), *Gmo*02 (P<0.05), *Gmo*37 (P<0.05), and *Pan*I (P<0.00001), and were highly significant overall (P<0.00001; Table 3). Results for genotypic differentiation were similar and are not shown.

Results of comparisons among all pairs of sampling locations are shown in Table 3. When Bonferroni corrections are applied, significant differentiation is observed between cod collected in Ipswich Bay in the spring and all other sampling locations including those collected in Ipswich Bay in the winter months, and between the collection of cod made at

northeast Georges Bank and that at Long Island, New York. A substantial, but not statistically significant F_{ST} was also observed between Georges Bank and Chatham samples.

In addition to genetic analysis of cod, this pilot project included an effort to collate and synthesize the best available information on cod spawning aggregation locations and timing

Locus	Alleles	H_o	F_{ST}	R_{ST}	F_{IS}	HWE	Differentiation
Gmo132	22	0.703	0.0246	0.1134	-0.0204	0.561	**<0.00001**
Gmo02	17	0.751	0.0030	0.0066	0.0600	0.151	**0.014**
Gmo19	37	0.956	0.0005	-0.0019	-0.0043	0.663	0.727
Gmo35	12	0.789	0.0017	-0.0071	-0.0016	0.704	0.106
Gmo36	9	0.538	0.0069	0.0006	0.0712	0.029	0.167
Gmo37	20	0.862	0.0023	-0.0021	0.0249	0.393	**0.041**
PanI	2	0.087	0.0747	0.0846	-0.0271	0.892	**<0.00001**
AHR6	2	0.389	0.0043	0.0043	0.1019	0.746	0.159
ARNT8	2	0.293	0.0025	0.0025	0.1236	0.314	0.235
Overall	123	0.596	0.0068	0.0105	0.0313	0.149	**<0.00001**

within U.S. waters of the Gulf of Maine, Georges Bank, and Southern New England. This included queries of spawning fish caught during research trawl surveys (NMFS, DFO, MA, ME-NH) and through the Northeast Regional cod tagging program, as well as active discussion with fishermen and research scientists throughout the region. The resulting synthesis is presented in Appendix A to this report.

Detailed information about the project is presented in this report which will be publicly available through a dedicated section on NAMA's website (www.namanet.org), with electronic links to and from other relevant websites (NEC, FishResearch.org, NMFS). NAMA will take responsibility for retaining public access to the project data into the future (as soon as it is available publicly, pending scientific publication of results), including supplying data and analytical records to NEC for the Fisheries and Oceans Database, while UNH and NYU will maintain physical sample collections and DNA records, so that they are available if required in any future research.

Table 2. Genetic variation in Atlantic cod at 6 microsatellite loci and 3 SNP loci. Number of alleles, observed heterozygosities (H_o), F_{ST}s, R_{ST}s, F_{IS}, p-values for Hardy Weinberg exact tests (HWE), and p-values for allelic differentiation among the 6 sampling locations are listed for each locus. Significant differences are indicated in bold.

Table 3. Population differentiation for all pairs of sampling locations of Atlantic cod. Above diagonal are p-values for pairwise allelic differentiation. Below the diagonal are pairwise F_{ST} values, with p-values in parentheses. Data are for all loci combined. * indicates significance after Bonferroni correction, $\alpha = 0.05$, $p \leq 0.003333$.

	Georges Bank	Long Island	Ipswich-Spring	Ipswich-Winter	Chatham	Stellwagen Bank
Georges Bank	------	0.0008*	<0.00001*	0.06616	0.01084	0.27287
Long Island	0.0050 (0.00333)*	-----	<0.00001*	0.42948	0.59152	0.97701
Ipswich-Spring	0.0121 (0.00333)*	0.0149 (0.00333)*	-----	<0.00001*	<0.00001*	<0.00001*
Ipswich-Winter	0.0027 (0.03667)	0.0002 (0.11000)	0.0095 (0.00333)*	-----	0.08125	0.63774
Chatham	0.0121 (0.09667)	0.0023 (0.29667)	0.0221 (0.00333)*	0.0085 (0.07000)	-----	0.7025
Stellwagen Bank	0.0040 (0.19667)	-0.0035 (0.61333)	0.0126 (0.00667)	-0.0008 (0.31333)	-0.0005 (0.48000)	-----

Conclusions

We successfully characterized microsatellite and SNP polymorphisms in a modest sample of cod collected over most of their distribution in U.S. waters. In total, a complete genetic data set was obtained from six microsatellite and three SNP loci. Overall numbers of alleles at microsatellite loci ranged from 9 to 37 (mean 17.8 alleles/locus) and all three SNP loci revealed biallelic polymorphisms. The overall level of heterozygosity at these loci among our collections was 0.596. Thus, these loci revealed sufficiently high levels of genetic variation to begin to address the issue of genetic stock structure of cod from these collection sites.

Our preliminary results for the first time demonstrate significant genetic differentiation of spawning aggregations of cod collected from Georges Bank and the inshore Gulf of Maine. We also demonstrate that cod collected from the southernmost extent of their current range, western Long Island, NY, are genetically distinct from a spawning aggregation on Georges Bank. Finally, and most intriguingly, we report the existence of a highly distinctive genetic stock of cod collected in the late spring from Ipswich Bay, MA. This stock was highly significantly differentiated from all other collections analyzed in this study.

Cod are managed on a two stock basis in U.S. waters, Gulf of Maine and Georges Bank and to its south. Yet, empirical data supporting this model is ecophenotypic in nature and is outdated in that it was collected at a time in which the size of both stocks was much larger than today. We compared the frequencies of microsatellite and SNP alleles between spawning cod collected in late winter of 2005 from the NE Peak of Georges Bank to those collected in the

winter and spring from Ipswich Bay, MA, off Chatham, MA, Stellwagen Bank, and Long Island, NY. Cod collected from Georges Bank were genetically distinct after Bonferoni correction from those collected in the spring from Ipswich Bay (not the winter) ($p < 0.001$) and Long Island, NY ($p < 0.001$). The collection from Chatham was not genetically distinct from that from Georges Bank after Bonferoni correction ($p = 0.011$) and that from Stellwagen Bank ($p = 0.273$) showed no evidence of genetic differentiation from that made on Georges Bank. Our results for the first time provide the first evidence for discrete genetic stocks on Georges Bank and an inshore locale in the Gulf of Maine.

Most interestingly, our spring sample from Ipswich Bay, MA, collected in May, was highly genetically distinct from that made during the winter at the same locale and at all other locales in this study. This finding is important in that it demonstrates for the first time an absence of gene flow (reproductive isolation) of cod spawning aggregations that are temporally separated at a single spawning locale. It also for the first time demonstrates that cod stocks spawning in the inshore Gulf of Maine are genetically heterogeneous. This points to the need for far more rigorous sampling of spawning aggregations in the inshore Gulf of Maine and probably Georges Bank to fully comprehend the overall architecture of these spatially extensive cod stocks.

Partnerships:

Fishermen and scientists are both interested in using genetic techniques to better understand the relationships between populations of cod with U.S. waters. NAMA's interest in coordinating this project was motivated by strong interest on the part of alliance members and board members, many of whom are commercial fishermen. The interest and enthusiasm of individual fishermen has been evident in the number who provided their expertise on spawning aggregations and sampling methods to inform the initial proposal design for June 2004, and for David Goethel and Frank Mirarchi, in particular, who brought David Berlinsky (co-PI) on their vessels during regular fishing operations so that he could collect samples for analysis. Party boat captains and patrons aboard the Big M Express, Sheepshead Bay, Brooklyn, NY and Captree Queen, Captree, NY, collected New York Bight Samples. This is particularly significant given the low levels of involvement of recreational or party/charter fishermen in cooperative research to date.

A number of researchers are working on furthering and refining genetics techniques within this and other regions for studying Atlantic cod, and numerous fishery scientists are interested in how genetic techniques can provide support or revision to stock boundaries.

The project fostered partnerships between researchers at two different institutions (NYU and UNH) and fishermen from Maine to Rhode Island. With NAMA's coordination, fishermen and scientists both contributed significantly to the project design. Many of the individuals who met through the initial proposal round and this pilot project are now collaborating on a NEC 2005 proposal for a follow-up project.

Impacts and applications:

The results of this pilot project indicate that genetic techniques are appropriate for stock identification for Atlantic Cod. This is an important result, as genetic analysis can be a powerful tool for informing fisheries management.

NOAA fisheries managers and researchers, the New England Fishery Management Council members and staff, academic researchers, commercial fishermen, and fishing community groups should all be made aware of this result. In fact, Northwest Atlantic Marine Alliance is itself one potential end user, with a particular organizational interest in the results as they relate to determining appropriate spatial scales for managing New England's groundfish.

However, before genetic analysis can be used to inform management of cod stocks in U.S. waters, a broader, longer-term project is required to more sensitively dissect stock structure within Georges Bank and within the Gulf of Maine and to confirm temporally stable differentiation of spawning aggregations. This longer term project should include further analysis of spawning fish and mixed stock analysis of non-spawning fish in other areas at other times. In total, these analyses will potentially provide invaluable insight into stock structure of Atlantic Cod and management efforts likely to be most effective in rebuilding the stocks and managing a sustainable fishery for this species.

A number of individuals are 'key end users' who would benefit by learning the results of this project:

- Paul Howard, Executive Director, New England Fishery Management Council
- Pat Kurkul, Regional Administrator, NOAA Fisheries & other NOAA Fisheries staff (as detemined by Pat Kurkul)
- John Boreman, Northeast Fisheries Science Center
- Loretta O'Brien, Northeast Fisheries Science Center
- Ralph Mayo, Northeast Fisheries Science Center
- Steve Cadrin, Northeast Fisheries Science Center
- New England Fishery Management Council members (George Lapointe, Fred Vincent, Paul Diodati, Eric M. Smith, John Nelson, Rip Cunningham, Dave Goethel, Phil Ruhle, Rodney Avila, Dana Rice, Frank Blount, John W. Pappalardo, SallyMcGee, Anthony "Bud" Fernandes, James Odlin, Thomas R. Hill, John C. Williamson, John Vince O'Shea, Bob Beal, RADM David P. Pekoske, Capt. Mark Landry, Dr. Marvin Moriarty, Dr. James Geiger, Nikki Brajevich)
- New England Fishery Management Council staff

Related projects:

Molecular supplies and analysis in David Berlinsky's lab at UNH were partially supported by a grant from the Marine Protected Areas program of the Cooperative Institute for New England Mariculture and Fisheries.

Presentations:

The PIs have spoken informally with members of the fishing, scientific, and management community about this project, including results from the genetic analysis and utility of the genetic tools for stock identification. Timing of genetic analysis (spring 2005) and limited scope of this pilot project did not allow for presentation of project results at regional forums such as Maine Fishermen's Forum, Massachusetts Fishermen's Partnership meetings, NMFS and Sea Grant sponsored regional workshops.

Student participation:

Matt Lubicky, Undergraduate, UNH with David Berlinsky

Published reports and papers:

PIs intended to develop progress reports for NEC in January 2005 and April 2005, but as project funding was not secured (due to NEPA approval) until April 2005, this final report has been prepared without milestone progress reports. A dedicated webpage on NAMA's website is being developed for the project including project plans and results. Comprehensive project data publicly will be made available through NAMA's website at earliest date possible (although it is not currently available as PIs will be looking to publish results). This final project report will be posted on NAMA's websites, as well as NEC's website and FishResearch.org. and distributed widely by PIs. Project PIs will aim to publish project results in peer-reviewed literature as soon as possible after completion of analysis.

Images:

There were no images associated with this project.

Future research:

This pilot project was designed in response to Northeast Consortium review of a proposal for "Genetic Identification of Atlantic Cod Spawning Stocks in U.S. Waters using Microsatellite DNA Markers" in July 2004. Since this project has demonstrated successful application of microsatellite analysis in distinguishing cod spawning aggregations within U.S waters, a broader, longer-term study including replicated sampling over multiple year classes across many more major spawning sites is an appropriate next step for future research. Such a project will be required in order to fully elucidate the mixing or fidelity of Atlantic cod to spawning sites within our region. This project development pilot has not only to tested the feasibility of these specific genetic techniques, but also to laid the groundwork for a broader, longer-term project by undertaking a series of discussions with fishermen and researchers to identify spawning aggregations throughout the region. The PIs have worked with NEC staff, fishermen, researchers, and managers throughout the final stages of this pilot project to develop a specific research design for a broader, longer-term project which was submitted to NEC in mid July 2005.

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