Genetic differentiation between inshore and offshore Atlantic cod (*Gadus morhua*) off Newfoundland: microsatellite DNA variation and antifreeze level

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Abstract: Microsatellite DNA provided evidence that Atlantic cod overwintering in inshore Newfoundland are genetically distinguishable from cod overwintering offshore. We compared variation in five loci in samples from inshore locations around Trinity Bay, Newfoundland, and from an offshore region on the northern Grand Bank (North Cape). Cod collected inshore were divided into two groups on the basis of antifreeze level in blood: those with high antifreeze levels presumed to have overwintered in cold (<0°C) inshore waters and those with low antifreeze levels presumed to have overwintered offshore in warmer (>2°C) waters. Fish overwintering inshore differed from offshore fish in allele sharing and in $(\delta\mu)^2$ distances, while fish with low antifreeze levels did not. Subpopulation structure (R_{st}) was detected when offshore cod were compared with inshore cod with high levels of antifreeze but not when compared with those with low levels of antifreeze. These results suggest that cod overwintering inshore are genetically distinct from offshore cod. Inshore and offshore cod from the areas studied remain genetically distinct despite the fact that individuals from the two populations intermingle inshore during the summer and fall feeding migration. Thus, we found evidence of population structure at a finer geographical scale than has been shown to date for this species.

Résumé : L'étude de l'ADN microsatellite a permis d'établir qu'on peut distinguer la morue de l'Atlantique hivernant dans les eaux côtières de Terre-Neuve de celle qui hiverne en haute mer. Nous avons comparé les variations à cinq locus chez des échantillons prélevés à des sites côtiers situés près de la baie Trinity, Terre-Neuve, et dans une région de haute mer dans la partie nord du Grand Banc (cap Nord). On a divisé les morues prélevées dans les eaux côtières en deux groupes selon la teneur en antigel de leur sang : les poissons à forte teneur en antigel, soupçonnés d'avoir hiverné dans les eaux côtières plus froides (<0°C), et les poissons à faible teneur en antigel, soupçonnés d'avoir hiverné dans les eaux du large plus chaudes (>2°C). Les poissons hivernant dans les eaux côtières différaient de ceux hivernant en haute mer pour ce qui est du partage des allèles et des distances $(\delta\mu)^2$, contrairement aux poissons au sang à faible teneur en antigel. Une comparaison de la morue du large avec celle des eaux côtières à forte teneur en antigel a permis de détecter une structure de sous-population (R_{st}), mais ce n'était pas le cas avec celle à faible teneur en antigel. Ces résultats suggèrent que la morue hivernant dans les eaux côtières est génétiquement distincte de celle qui hiverne en haute mer. Dans les régions à l'étude, la morue des eaux côtières reste génétiquement distincte de celle de la haute mer, en dépit du fait que les individus des deux populations se mélangent dans les eaux côtières au cours des migrations trophiques d'été et d'automne. Ainsi, nous avons pu mettre en évidence une structure de population à une échelle géographique plus fine que celles indiquées jusqu'ici pour cette espèce. [Traduit par la Rédaction]

Introduction

Knowledge of population structure is essential for the conservation of genetic resources and for an adequate analysis of population dynamics. For natural populations under exploitation, an incorrect interpretation of the genetic structure can lead to overexploitation and to erosion of genetic resources via depletion of some or all of the populations' spawning components. This problem is particularly relevant to marine fish species, which for political or administrative convenience are often managed under assumptions of single, homogeneous, and large breeding populations. If indeed there are separate breeding components, parameters such as population growth rate and fishing mortality should be estimated separately for

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each component (e.g., Myers et al. 1996). When the assumptions of homogeneous and large breeding populations are not met and there are a number of discrete or semidiscrete spawning components within management units, those components most easily captured are likely to be eliminated with often detrimental effects on the stock as a whole.

Atlantic cod (*Gadus morhua*) are distributed in the western Atlantic from Labrador (~63°N) to Cape Hatteras (~35°N). Cod populations inhabiting the region off Labrador and Newfoundland, referred to as Northern cod, exhibit an annual pattern of inshore–offshore migration (Lear 1984, 1986), with most mature individuals overwintering in warm (>2°C) slope water along the margins of the continental shelf. Spawning is protracted (3–4 months from late winter through early summer), generally beginning earlier in the north than in the south (Myers et al. 1993). Evidence to date shows spawning to take place throughout the continental shelf and slope (see review in Taggart et al. 1994), although Hutchings et al. (1993) suggested that inshore spawning may be more pronounced than previously thought. Following offshore spawning, adult cod migrate inshore to the summer feeding grounds. They then return offshore in late autumn – early winter (Templeman 1966) and are joined in this migration by cod maturing for the first time (reviewed in Lear and Green 1984). By age 4, cod are beginning to adopt the distributional and migratory behaviour of the older age-classes (Rose 1993).

While most cod show the above pattern of migration, it has long been known that some fish remain inshore through the winter (Fletcher et al. 1987). The extent to which fish overwintering inshore versus offshore constitute distinct stocks remains largely unknown (Lear 1984; Hutchings et al. 1993). Distinct offshore spawning components do appear to exist, on the basis of analysis of variation in vertebral complement (Templeman 1981; Lear and Wells 1984), the geographic distribution of spawning (Hutchings et al. 1993), and tag recovery data (Templeman 1974, 1979; Lear 1984; reviewed in Lear and Green 1984 and in Taggart et al. 1995). The regular occurrence of juvenile and adult cod overwintering in cold (<0°C) coastal waters (Fletcher et al. 1987; Valerio et al. 1992; Goddard et al. 1992, 1994; Wroblewski et al. 1994) coupled with evidence of inshore spawning (Wroblewski et al. 1995; Smedbol and Wroblewski 1997) suggest that inshore population(s), distinct from the offshore population(s), may exist (Templeman 1966). However, to date there is no published evidence that these populations differ genetically.

Different genetic markers (e.g., allozymes, mitochondrial or nuclear DNA) vary in their ability to detect population structure. Previous studies on the genetic structure of Atlantic cod populations based on allozyme loci have produced ambiguous results. They tend to show significant differences among neighbouring populations when a limited number of blood protein loci are examined (Møller 1968; Jamieson and Otterlind 1971; Jamieson 1975; Cross and Payne 1978; Dahle and Jørstad 1993), but these differences disappear when a larger number of conventional electrophoretic loci are examined over the distributional range of the species (Mork et al. 1982, 1985; see also Grant et al. 1987 for similar results on Pacific cod, *Gadus macrocephalus*). More recent studies based on mitochondrial DNA variation have shown limited or no differentiation of populations throughout most of the species' range (Smith et al. 1989; Carr and Marshall 1991*a*; Árnason and Rand 1992; but see Dahle 1991) or within smaller geographic areas such as around the coast of Iceland (Árnason et al. 1992) or among management divisions within the range of northern cod (Carr and Marshall 1991*a*, 1991*b*; Pepin and Carr 1993). Most recently, in a study comparing cod populations across the Atlantic Ocean, Pogson et al. (1995) demonstrated that nuclear DNA restriction fragment length polymorphism (RFLP) loci are capable of detecting genetic population structure at ocean basin scales where allozyme loci do not.

Here we make use of the fact that adult northern cod make antifreeze glycoproteins in response to the cold winter temperatures characteristic of coastal Newfoundland and Labrador (Fletcher et al. 1987) to identify fish that overwinter in the cold inshore waters (Goddard et al. 1994). As unprotected blood and the extracellular fluids of cod will freeze at temperatures between –0.5 and –0.8°C (Fletcher et al. 1982), the ability of cod to produce antifreeze glycoproteins appears to be essential for overwintering in coastal waters where temperatures can approach the freezing point of seawater (near –1.8°C) for several months each year. Antifreeze appears in cod plasma as temperatures fall below 0°C, reaches a maximum level after about 75 days, and remains high until water temperatures rise above 0°C (Fletcher et al. 1987; Goddard et al. 1994). In adult cod, antifreeze levels are thus a function of length of continuous exposure to subzero temperatures and can be used as a physiological time tag to infer the recent thermal history of the fish.

In this paper we show that cod populations overwintering in inshore Newfoundland are genetically distinguishable from cod populations overwintering offshore along the edge of the continental shelf of the Grand Bank. We use nuclear DNA microsatellite loci as genetic markers and antifreeze protein levels (expressed as blood plasma thermal hysteresis) and time of sampling as indicators of inshore overwintering. We distinguish populations genetically on the basis of evidence of heterogeneity of allele frequency distributions, allele sharing (Bowcock et al. 1994), and $(\delta\mu)^2$ ($(\delta\mu)^2 = (\mu_A - \mu_B)^2$ where μ_A and μ_B are mean allele sizes in populations *A* and *B*, respectively)(Goldstein et al. 1995) distances, and population structure (R_{st}) (Slatkin 1995).

Materials and methods

Cod sample collections

Cod were collected between January 1992 and June 1994 and were drawn from 14 different samples (Table 1). Four of these samples are broadly categorized as being from offshore (North Cape region of the northern Grand Bank) winter and early summer aggregations, while 10 are from winter and early summer aggregations found inshore in the immediate vicinity of the Random Island region of Trinity Bay (Southwest (SW) Arm, Northwest Arm, Smith Sound, Heart's Ease Ledge (HEL)), elsewhere in Trinity Bay (Bellevue), and Bonavista Bay (Table 1, Fig. 1). Water temperatures at all collection depths were recorded using a calibrated trawl-mounted or wire-mounted profiling conductivity–temperature–depth probe (CTD).

Offshore (North Cape) collections of cod were made using an otter trawl deployed between 400 and 1000 m, where the average bottom temperature among collection dates, locations, and depths ranged from 2.5 to 3.4°C and where the average fork lengths of the cod collected for genetic and antifreeze analysis ranged from 33 to 54 cm (Table 1). The offshore collections made in January and

Table 1. Summary statistics for cod samples analyzed using microsatellite loci.

Sample identifier	Date	Location	Lat. (°N)	Long. (°W)	Depth range (m)	Temperature (°C) Mean	Range	N_1	Length (cm) Mean±SD	Range	N_2	Thermal hysteresis (°C) Mean±SD	Range
SWA	June 1992	SW Arm, Trinity Bay	48.02	–53.90	152–154	–1.4	–1.4–(–1.4)	24	48.1±9.9	32–67	17	0.43±0.11	0.24–0.63
BoB	June 1992	Bonavista Bay	48.58	–53.90	31–37	3.2	0.8–4.8	18	34.2±6.9	26–53	7	0.09±0.09	0.04–0.28
Bvu	July 1992	Bellevue, Trinity Bay	47.65	–53.74	20–20	5.3	5.3–5.3	26	27.1±3.9	24–36			
SWA	April 1993	SW Arm, Trinity Bay	48.02	–53.93	15–32	–0.7	–0.7–(–0.7)	63	61.7±10.4	39–106	61	0.31±0.14	0.07–0.65
SWA	June 1993	SW Arm, Trinity Bay	48.02	–53.90	139–216	–0.9	–1.2–(–0.6)	61	38.5±6.3	27–52	59	0.44±0.13	0.09–0.76
Sms	July 1993	Smith Sound, Trinity Bay	48.17	–53.67	14–45	1.1	1.1–1.1	34	45.1±8.9	33–74	34	0.34±0.11	0.07–0.61
HEL	July 1993	Heart's Ease Ledge, Trinity Bay	48.06	–53.59	40–40	2.4	2.4–2.4	25	49.8±6.5	38–64			
NWA	June 1994	NW Arm, Trinity Bay	48.09	–53.74	18–150	0.6	–1.0–0.9	28	48.5±7.6	30–72	28	0.14±0.09	0.06–0.43
SmS	June 1994	Smith Sound, Trinity Bay	48.22	–53.56	25–25	5.5	5.5–5.5	39	50.1±5.0	43–67	39	0.08±0.01	0.06–0.13
SWA	June 1994	SW Arm, Trinity Bay	48.02	–53.87	163–275	–1.1	–1.3–(–0.5)	34	42.1±8.4	24–58	34		
NCp	Jan. 1992	North Cape, Grand Bank	48.50	–49.46	439–482	3.1	2.8–3.8	82	53.9±6.5	44–75			
NCp	Jan. 1993	North Cape, Grand Bank	48.50	–49.50	380–380	2.8	2.8–2.8	30	34.3±7.1	21–47	19	0.18±0.18	0.07–0.63
NCp	Feb. 1993	North Cape, Grand Bank	48.10	–47.40	935–935	3.4	3.4–3.4	30	49.0±3.4	42–57			
NCp	June 1994	North Cape, Grand Bank	48.30	–49.10	436–436	2.5	2.5–2.5	50	33.3±7.2	21–48	41	0.07±0.01	0.06–0.11

Note: Data are provided by date and location of sample collection for the depth range of sample collections, the water temperature at collection depths (mean and range, i.e., minimum and maximum temperatures), fish length (N_1 is the number of fish collected), and thermal hysteresis (N_2 is the number of fish assayed). Data on thermal hysteresis were available for individuals in 9 (7 inshore + 2 offshore) of the 14 samples.

February were from what are presumed to be prespawning aggregations, while the June collections are from assumed postspawning aggregations. Of the offshore collections, only those cod collected during January 1993 and June 1994 were assessed for blood plasma thermal hysteresis.

Inshore collections of cod were made using a combination of gear (otter trawl, cod trap, gill net, and jigger) deployed at depths of between 140 and 200 m in the deeper channel regions of SW Arm and at shallower depths of between 14 and 150 m elsewhere inshore (Table 1). Average water temperatures at depth associated with collections in SW Arm were consistently subzero and ranged from –0.5 to –1.4°C, while collections from other locations, depths, and dates were associated with higher water temperatures that averaged 0.6–5.5°C (Table 1). The average fork lengths of cod collected among dates and locations in the vicinity of Random Island ranged from 38 to 62 cm, while collections made at Bellevue and Bonavista Bay ranged in length from 27 to 34 cm (Table 1). Cod collected from HEL in July 1993 were from an aggregation that was known to be spawning at that location (Wroblewski et al. 1995; Smedbol and Wroblewski 1996). Blood plasma thermal hysteresis was determined for 7 of the 10 inshore samples (Table 1).

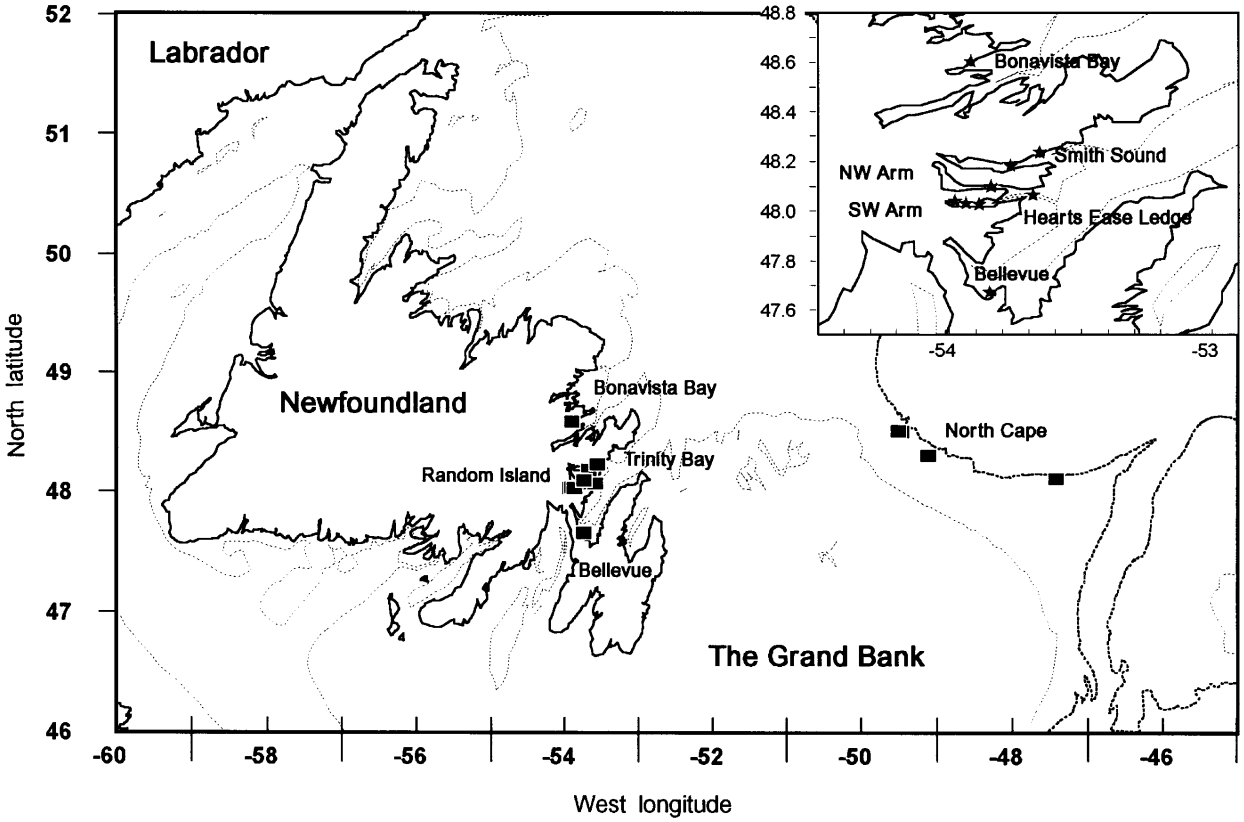
Cod tissue collections

Blood samples were collected from live cod (which were measured for fork length and weight) using a sterile 2-mL syringe and hypodermic needle (21 gauge) inserted ventrally into the blood vessels that run in the haemal arches of the vertebrae between the anal fin and the caudal peduncle (Nielsen and Johnson 1983), and approximately 1 mL of blood was withdrawn. If the sample was destined entirely for genetic analysis, it was preserved immediately in 5 mL of 95% ethanol and was stored. Alternatively, about half was preserved in this manner and the rest, to be used for antifreeze analysis, was injected directly into Vacutainers (Becton Dickinson) containing sodium heparin, mixed gently, and held on ice until centrifuged. Samples were centrifuged for 10 min at 4000 × *g* and the blood plasma was removed, placed in 1.5-mL Eppendorf tubes, and stored until analyzed. Muscle tissue samples for genetic analysis were taken from the anterior of the tongue of dead cod (also measured for length and weight).

Genetic analysis

DNA extraction from alcohol-preserved tissue was as described in Bentzen et al. (1990). Briefly, a sample of about 100 mg of muscle, or 100 µL of blood in ethanol, was washed in high TE (100 mM Tris–HCl, 40 mM EDTA (ethylenediaminetetraacetic acid), pH 8.0) to remove ethanol. The samples were then placed in 250 µL of lysis buffer (10 mM Tris–HCl, pH 8.3, 1 mM EDTA, 400 mM NaCl containing 0.8% sodium dodecyl sulfate (SDS), and 400 µg/mL proteinase K). Samples were digested at 65°C for 18 h and transferred to an SST tube (Becton Dickinson). The samples were extracted twice with 2.5 mL of 0.1 M Tris (pH 8.0) buffered phenol and once with 2.5 mL of chloroform. The supernatant containing the purified nucleic acids was transferred to a 1.5-mL Eppendorf tube and precipitated with 0.2 M NaCl and 1 volume of isopropanol. The DNA pellet was resuspended in 100 µL of TE (10 mM Tris, 0.1 mM EDTA, pH 8.0) and adjusted to 25 ng/µL as a polymerase chain reaction (PCR) template. PCR analysis was as previously described by Brooker et al. (1994) using five sets of cod microsatellite primers, Gmo2, Gmo132, and Gmo145 (Brooker et al. 1994), Gmo4 (Wright 1993), and Gmo120, with the following sequences (5′ to 3′): GAG CAA ACA TGC TCA GAG TG; GAC TGA TCT CCA TGA GAG G (annealing temperature 54°C). Gmo2, Gmo4, Gmo120, and Gmo132 are perfect GT repeats, and Gmo145 is a compound $G_x(GA)_x$ repeat as defined by Tautz (1989). For all loci, except Gmo145, the allele sizes varied by increments of two base pairs (bp). Gmo145 showed occasional alleles that differed by a single bp change. The PCR products were resolved on 6.5% sequencing gels and the alleles were sized relative

Fig. 1. Bathymetric chart (coastline and 200-m and 1000-m isobaths) of Newfoundland and the northern Grand Bank showing cod collection locations. Offshore samples are from the North Cape region; inshore samples are from the Random Island and Bellevue regions of Trinity Bay and Bonavista Bay (enlarged inset). Cod from both regions are considered part of the Northern cod complex (i.e., NAFO divisions 2J, 3K, and 3L).



to a sequence ladder generated from M13mp18. The maximum number of cod assayed for microsatellite DNA was never larger than 40 individuals per sample; in total, we assayed 448 individuals out of the 543 collected from among the 14 samples.

Blood plasma antifreeze analysis

Antifreeze activity within each plasma sample was determined using a nanolitre osmometer (Clifton Technical Physics, Hartford, N.Y.) and the protocol described in Goddard et al. (1994). In brief, the blood plasma freezing and melting points were determined by microscopic observation of the freezing and melting behaviour of a single ice crystal within the plasma. The antifreeze glycoproteins act in a noncolligative manner to inhibit ice propagation by binding to embryonic ice crystals and preventing the addition of water molecules to the ice lattice (DeVries 1983). However, the effect on the melting point is purely colligative. This results in a difference between the freezing and melting points of a solution containing antifreeze proportional to the amount of antifreeze present. The difference (measured in degrees Celcius), the thermal hysteresis, is commonly used as a direct measure of antifreeze activity (Kao et al. 1986).

Antifreeze activity, as measured by thermal hysteresis in the individual plasma samples, was used to infer the overwintering behaviour of the individual cod. High antifreeze levels (≥0.20°C thermal hysteresis) are indicative of cold-water (inshore) overwintering, while low or nonexistent winter antifreeze levels (≤0.09°C thermal hysteresis) are indicative of warm-water (offshore) overwintering.

Data analysis

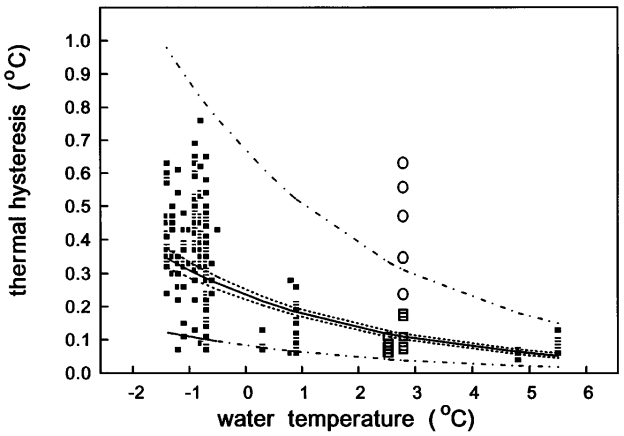
We estimated allele sharing (AS, Bowcock et al. 1994) and $(\delta\mu)^2$ (Goldstein et al. 1995). Subpopulation structure was estimated using R_{st} (Slatkin 1995). For comparative purposes, we also calculated F_{st} values following Weir and Cockerham (1984). Significance values for all tests (i.e., homogeneity of allele frequency distributions, genetic distances, and estimates of subpopulation structure) were determined by Monte Carlo simulations and the bootstrap method (minimum 1000 resampling trials per individual comparison; Manly 1991). In all cases, bootstrapping was conducted across individuals and populations for each locus separately. Bootstrapping each locus independently enables comparison of estimates across loci. Bootstrapping across individuals and populations enables estimation of significance values for the test under consideration (e.g., R_{st}). This method addresses the variance associated with statistical sampling but not that associated with genetic sampling, which occurs in each new generation during the union of gametes chosen from among those produced by the previous generation (Weir 1990). The standard method of accounting for the effects of genetic sampling is to bootstrap across loci, but this method is meaningful only if a large number of loci is available. We assumed that the five microsatellite DNA loci examined in this study constitute a random sample of the genome. Estimates of R_{st} and F_{st} combined over loci were calculated by first averaging numerators and denominators across loci and then taking ratios, as suggested by Weir and Cockerham (1984) and Slatkin (1995). Values are reported as the mean ± SD.

Results

Overwintering grounds as inferred from measures of thermal hysteresis

High antifreeze levels are typical of fish overwintering in cold

Fig. 2. Relationship between cod thermal hysteresis and temperature of the water mass where the samples were collected. The solid heavy line is the fitted relationship (r^2 = 0.60), the fine dotted lines are the upper and lower 95% confidence limits for the population estimates, and the fine dashed lines are the 95% confidence limits for individuals. Each data point represents an individual collected inshore (solid symbol) or offshore (open symbol) where five juvenile cod (open circles) showed anomalously (see text) elevated thermal hysteresis.



(<0°C) inshore water, while near-zero or low levels are typical of fish overwintering in warmer (>2°C) water masses, characteristic of the deep offshore area. Individuals with thermal hysteresis (≥0.20°C) are known to have spent at least 20 days in subzero waters and higher levels are indicative of longer periods in subzero waters (Goddard et al. 1994). Consistent with this evidence, we found that the average measures of thermal hysteresis among the nine samples for which scores were available were significantly correlated (r^2 = 0.60, $p < 0.01$) with water temperatures at the time and location of cod sample collections and declined exponentially with increasing temperature (Fig. 2). High population average measures of thermal hysteresis (>0.20°C) were associated with the subzero temperatures found at collection depths in the SW Arm of Trinity Bay between April and June. Relatively low average levels (<0.20°C) were consistently found in offshore samples (Table 1). However, the North Cape collection (offshore) in January 1993 differed from all other collections in that the mode (0.07°C) and median (0.07°C) values of thermal hysteresis were strikingly dissimilar from the mean (0.18°C), reflecting a strongly skewed distribution driven by 5 of the 19 individuals in the sample with thermal hysteresis values greater than 0.20°C (Fig. 2). These individuals were all less than 40 cm in length and were considered to be juveniles. The physiology of antifreeze production is different in adult and juvenile cod, juveniles being capable of producing antifreeze at temperatures as high as 3°C during the winter. This accounts for the high antifreeze levels present in cod overwintering offshore.

Cod collected inshore were thus separated into two groups: (*i*) individuals with high thermal hysteresis (≥0.20°C), indicating inshore overwintering, and (*ii*) individuals with near-zero thermal hysteresis (≤0.09°C), most of which are expected to be inshore migrants recently arrived from offshore. However, the possibility that this last group also includes inshore overwintering individuals that have been exposed to a warmer (near surface) water mass in the inshore for a period long enough to loose antifreeze (see Fletcher et al. 1987) cannot be eliminated. We excluded from the analysis individual cod with values of thermal hysteresis between 0.09 and <0.20°C because of the uncertainty regarding their overwintering grounds.

In the following sections, we first address the issues of temporal stability and spatial heterogeneity within inshore and offshore locations. We then analyze the genetic distances and population structure between inshore and offshore cod.

Microsatellite DNA variation among locations and sampling dates

Private alleles and heterozygosity

We classified alleles as private if present in a single sample and as common if present in all 14 samples. The number of private alleles per sample ranged from 0 to 6 with a mean of 2.6 ± 1.9 (Table 2). Private alleles represented 22.7% of all alleles present (mean frequency over all individuals, 0.001). Three of the private alleles were present in two individuals, and each of the remaining was present only once. The mean number of common alleles per sample was 5 ± 1.87, representing 15.3% of all alleles present (mean frequency, 0.11; range, 0.04–0.43). On average, each sample contained 66.4% of all alleles detected. Over all samples, the mean number of alleles per locus was 32.6 ± 12.8 with a minimum of 14 for Gmo132 and a maximum of 45 for Gmo4. These were also the loci with the lowest and the highest observed and expected heterozygosities, respectively (Table 3).

As expected from the large number of alleles, observed and expected heterozygosities were high and ranged from 0.845 to 0.951 (Table 2). Coefficients for heterozygote deficiency or excess (i.e., $D = (H_{obs} - H_{exp})/H_{exp}$) averaged over loci were, in general, low (Table 2), reflecting close agreement between observed and expected heterozygosities.

Heterogeneity of allele frequency distributions

Allele frequency distributions were heterogeneous for four of the five loci examined (i.e., Gmo2, Gmo4, Gmo120, and Gmo145) when all 14 samples were compared (Table 3), suggesting that the samples differed substantially from each other. For two loci, the differences were probably due to within-year heterogeneity among inshore locations sampled in 1992 (Gmo2, $p = 0.006$) and 1993 (Gmo145, $p < 0.001$). There was also some evidence (although weaker) of temporal changes (among years) in the allele frequency distributions for all inshore samples pooled within year ($p = 0.011$ for Gmo145 and $p \geq 0.068$ otherwise) and all offshore samples pooled within year ($p \approx 0.050$ for four loci and $p > 0.4$ otherwise). These results suggest some degree of spatial heterogeneity among inshore locations and they also suggest limited temporal changes in both inshore and offshore locations for the 3 years examined.

Genetic distances

The mean allele sharing distance was AS = 0.40 ± 0.0017 (range 0.28–0.53) and 4 of the 91 pairwise comparisons differed from zero ($p < 0.001$ after Bonferroni adjustment for 91

Table 2. Levels of genetic variation in 14 samples of cod (*Gadus morhua*) at five microsatellite loci.

Sample	Mean sample size per locus	Private alleles	Total alleles	Mean no. of alleles per locus	Mean H_{obs}	Mean H_{exp}	Mean D
SWA June 1992	23.0	2	100	20.0	0.888	0.850	0.042
BoB June 1992	17.6	0	91	18.2	0.908	0.845	0.081
Bvu June 1992	25.0	2	102	20.4	0.822	0.837	–0.027
SWA April 1993	40.0	2	110	22.0	0.875	0.854	0.021
SWA June 1993	40.0	5	123	24.6	0.880	0.875	0.001
SmS July 1993	32.0	6	111	22.2	0.951	0.863	0.106
HEL July 1993	23.6	3	103	20.6	0.889	0.855	0.039
NWA June 1994	27.2	1	101	20.2	0.880	0.862	0.023
SmS June 1994	36.3	1	113	22.6	0.856	0.866	–0.018
SWA June 1994	30.6	4	114	22.8	0.853	0.872	–0.031
NCp Jan. 1992	40.0	5	114	22.8	0.850	0.873	–0.025
NCp Jan. 1993	29.8	2	115	23.0	0.887	0.861	0.033
NCp Feb. 1993	24.6	4	103	20.6	0.925	0.853	0.088
NCp Jan. 1994	40.0	0	115	23.0	0.850	0.870	–0.021

Note: H_{obs} and H_{exp} represent observed and expected heterozygosities, respectively. $D = (H_{obs} - H_{exp})/H_{exp}$ averaged over loci. The mean D was calculated by averaging over D values for individual loci. The average disequilibrium coefficient was 0.022.

Table 3. Single locus statistics.

Locus	N	n	Size range	H_{obs}	H_{exp}	14 samples	Inshore vs. offshore	In-high-af vs. offshore	In-low-af vs. offshore
Gmo2	430	26	92–208	0.747	0.804	<0.001**	0.488	0.498	0.238
Gmo4	430	45	153–267	0.986	0.957	0.008*	0.106	0.417	0.321
Gmo120	422	43	134–238	0.955	0.954	0.003*	0.010*	0.015	0.175
Gmo132	438	14	97–131	0.744	0.724	0.339	0.166	0.352	0.800
Gmo145	429	35	137–215	0.953	0.940	0.002**	0.123	0.042	0.251
Overall	448	163		0.877	0.876				

Note: N, number of individuals; n, number of alleles. Size range, allele size range in base pairs; H_{obs}, observed heterozygosity; H_{exp}, expected heterozygosity. Probability of homogeneity of allelic frequency distributions was calculated among 14 samples, between the pooled offshore cod (offshore) and pooled inshore individuals (inshore), between the pooled offshore cod and inshore individuals with thermal hysteresis ≥0.20°C (in-high-af), and between the pooled offshore cod and inshore individuals with antifreeze levels ≤0.09°C (in-low-af). *, $p \leq 0.01$; **, $p \leq 0.002$; with Bonferroni adjustment assuming five simultaneous tests.

simultaneous tests). Three of these four comparisons were between inshore and offshore samples from 1992 and 1993. The fourth comparison involved two of the offshore samples (Fig. 3*a*). The mean $(\delta\mu)^2$ distance was 4.81 ± 2.59 (range 0.29–13.06), and although none of the pairwise comparisons differed from zero ($p \geq 0.015$; Fig. 3*b*), the p value was low ($p < 0.05$) for three pairwise comparisons, all of which involved the Smith Sound July 1993 (SmS July 93) sample with offshore samples.

To summarize thus far, there was some evidence of heterogeneity among inshore locations as well as of limited temporal changes in both inshore and offshore locations but these differences may be an artefact of small sample sizes. Genetic distances were significant for only 4 of 91 pairwise comparisons when measured with the allele sharing method.

Microsatellite DNA variation between inshore and offshore cod

We pooled individuals according to whether they were caught at inshore or offshore locations. For individuals collected inshore, we used the antifreeze level in the plasma, which in cod is correlated with length of exposure to subzero temperature (Goddard and Fletcher 1994; Goddard et al. 1994), as an indicator of overwintering grounds.

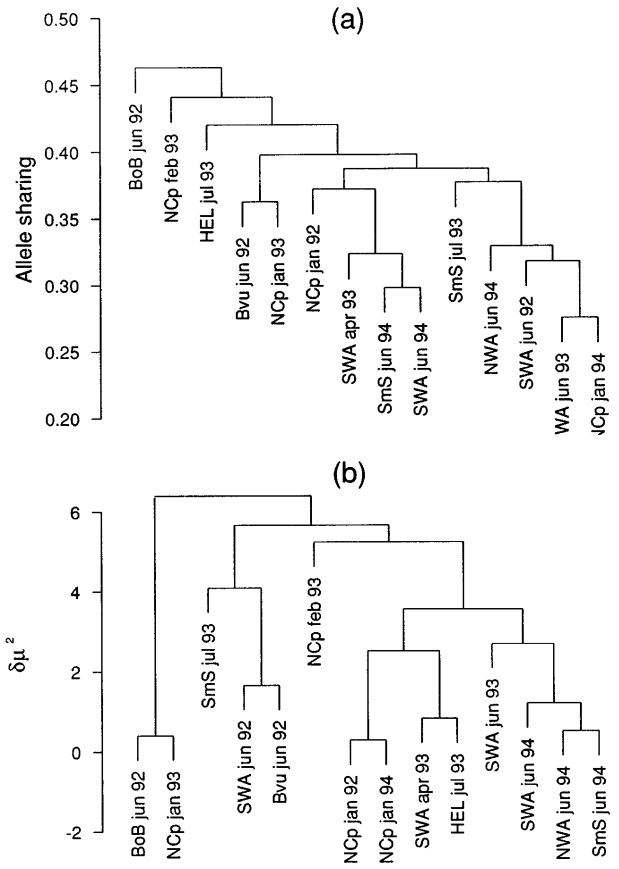
Private alleles

Twenty-six percent (i.e., 39 alleles) of the alleles present inshore were absent offshore (i.e., they were private to the inshore locations) whereas this figure was only 9.6% (i.e., 13 alleles) for offshore samples. In general, however, inshore and offshore private alleles were at very low frequencies. Twenty-four (i.e., 62%) of the inshore private alleles and 10 (i.e., 77%) of the offshore private alleles were represented only once (mean frequency of private alleles in inshore locations, 0.0022 ± 0.0017; and in offshore locations, 0.0015 ± 0.0007).

Heterogeneity of allele frequency distributions

Allele frequency distributions differed between inshore samples (pooled) and offshore samples (pooled) for Gmo120 ($p = 0.010$) but not for the other loci ($p > 0.10$, Table 3). The allele frequency distribution for Gmo120 also differed between inshore and offshore individuals when the inshore group was restricted to cod with high (≥0.20°C) thermal hysteresis ($p = 0.015$; Table 3) but not when it was restricted to cod with low or near-zero (<0.09°C) measures of thermal hys-

Fig. 3. Genetic distance measures among all 14 samples: (*a*) allele sharing distance and (*b*) $(\delta\mu)^2$ distance. The allele sharing distance is based on the proportion, *P*, of alleles that are shared between the multiple locus genotypes of two populations, averaged over loci. The distance measure between two samples is therefore $1 - P$.



teresis ($p = 0.175$, Table 3, and also see below). Gmo145 also showed evidence of heterogeneity when offshore cod were compared with inshore cod with high thermal hysteresis ($p = 0.042$, Table 3) but not when they were compared with inshore cod with low thermal hysteresis ($p = 0.251$, Table 3). Thus, the pool of inshore cod with high thermal hysteresis differs at $\alpha = 0.05$ from the pool of offshore cod for two of the five loci examined despite the fact that inshore samples are themselves somewhat heterogeneous (see above).

To summarize thus far, 26% of the alleles present inshore were absent offshore, and the corresponding figure for offshore samples was less than 10%. Allele frequency distributions for two loci (Gmo120 and Gmo145) were heterogeneous when comparing offshore cod with cod sampled inshore and showing high antifreeze levels (see Fig. 4) but not with those showing low or no antifreeze levels, leading to the conclusion that inshore cod with high antifreeze levels, but not those with low antifreeze levels, may be genetically different at two loci at least from offshore cod.

Genetic distances

Allele sharing and $(\delta\mu)^2$ distances between inshore and offshore sample pools were different from zero in two cases: when offshore cod were compared with all inshore cod (AS, $p < 0.001$; $(\delta\mu)^2$, $p = 0.010$) or with inshore cod with known high (≥0.20°C) thermal hysteresis (AS, $p < 0.001$; $(\delta\mu)^2$, $p = 0.007$; Table 4). Of the 39 inshore private alleles (discussed above), 37 were found among the 228 cod (i.e., 74% of all cod caught inshore) with high thermal hysteresis (≥0.20°C) or for which no thermal hysteresis score was available. Neither allele sharing nor $(\delta\mu)^2$ distances differed significantly from zero for inshore cod with near-zero thermal hysteresis versus offshore cod, or for inshore cod with high thermal hysteresis versus those with near-zero thermal hysteresis (Table 4).

When years were analyzed separately, allele sharing distances between all inshore cod and offshore cod, or between inshore cod with high thermal hysteresis and offshore cod, remained significant for 1992 and 1993. For 1994, *p* values were marginal ($p = 0.061$ and 0.097, respectively; Table 4). $(\delta\mu)^2$ distances were not significant for any of the comparisons ($p \geq 0.088$) when years were analyzed separately (Table 4).

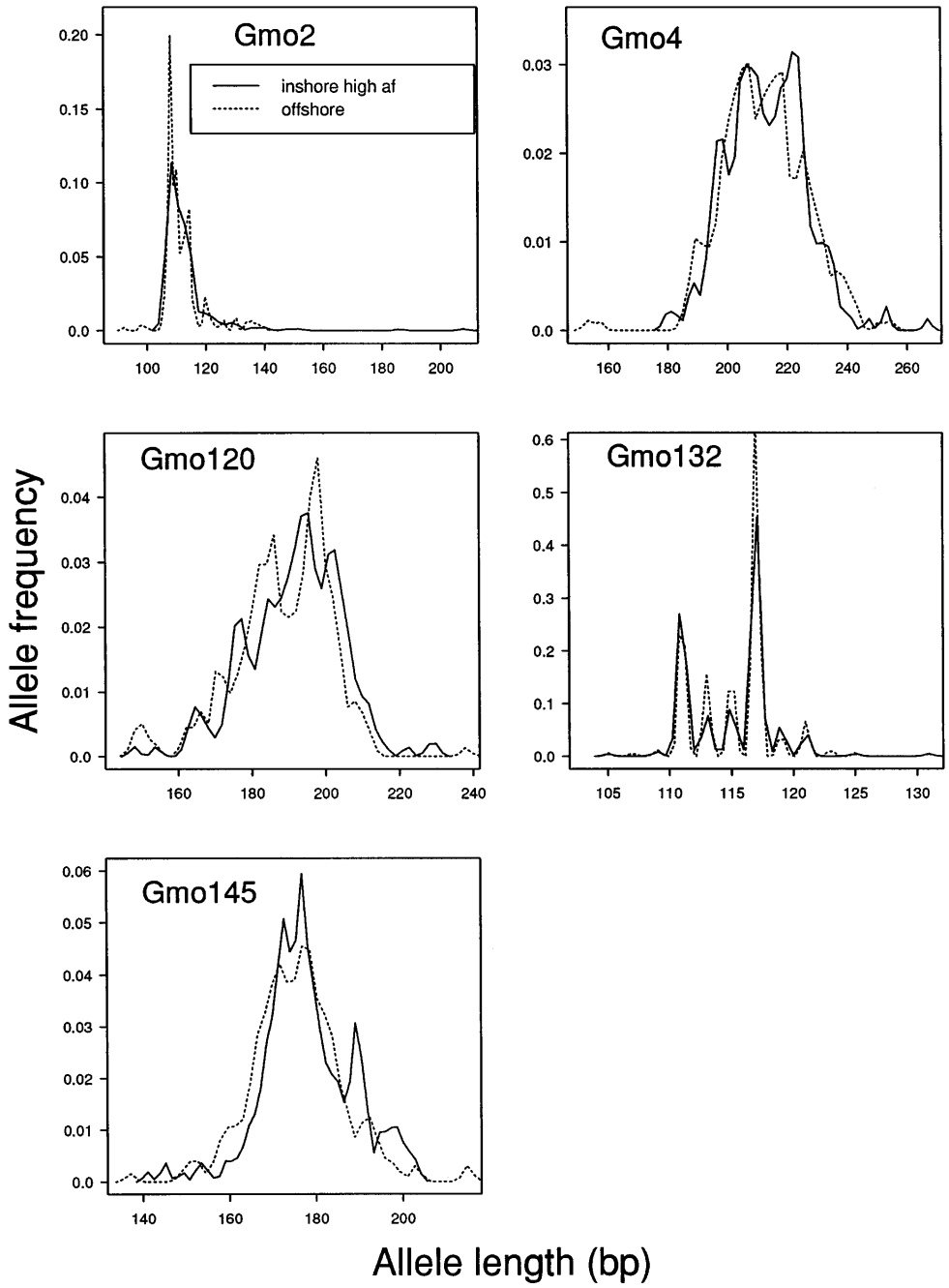
We also compared the group of cod collected from an inshore spawning aggregation on HEL in July 1993, but for which no antifreeze records were available (see Table 1), with offshore cod sampled in 1993 and with the group of inshore cod with high (≥0.20°C) thermal hysteresis sampled in 1993. The cod from the inshore spawning aggregation differed in allele sharing distance from offshore cod (AS = 0.528, $p < 0.001$; Table 4). They were also marginally different from inshore cod known to have high thermal hysteresis (AS = 0.425, $p = 0.036$, Table 4).

The low *p* value for this last comparison (HEL vs. inshore cod with high thermal hysteresis) prompted us to test whether pairs of random samples from the inshore – high-antifreeze group would be different from each other. We found that, on average (test repeated 10 times with different random collections), the two random samples were not different (AS: $p = 0.168$). Thus, these results indicate that individuals from the inshore spawning aggregation (HEL) are clearly different from offshore cod, but they also suggest that they may be somewhat different from other inshore cod aggregations. We do not have sufficient data at present to test this hypothesis further.

Population structure

There was evidence for significant population structure as measured by R_{st} (Slatkin 1995) when the pool of offshore cod was compared with the pool of inshore cod with high thermal hysteresis (Table 5), i.e., R_{st} differed significantly from zero for Gmo120, Gmo145, and the five loci combined (Table 5). Note that Gmo120 and Gmo145 exhibited marked differences in allele frequencies between the inshore – high-antifreeze group and the offshore group), even for relatively common alleles (Fig. 4). By contrast, there was no evidence that inshore cod with near-zero thermal hysteresis and offshore cod come from separate populations, whether each locus was considered separately or whether all loci were combined (Table 5). Variances of estimates of R_{st} in pairwise comparisons can be relatively large when sample sizes differ (D.E. Ruzzante, unpublished) and this may account for the relatively high (but not significant) value of the overall R_{st} estimate in the last comparison. Inshore cod with high thermal hysteresis did not differ significantly from those with near-zero thermal hysteresis (Table 5), reflecting the likelihood that the group of in-

Fig. 4. Allele frequency distributions for the five loci (plotted as density functions) for inshore overwintering cod (i.e., cod with high (≥0.20°C) thermal hysteresis; solid lines) and offshore cod (dotted lines).



shore cod with near-zero thermal hysteresis can include inshore migrants recently arrived from offshore as well as cod that overwintered in sub-zero inshore water and were later exposed to warmer near-surface water masses. There was also no evidence of significant population structure when all 14 samples were considered or when the pool of offshore cod were compared with the pool of inshore cod regardless of thermal hysteresis ($p \geq 0.422$, data not shown).

Contrary to R_{st}, F_{st} was not significantly different from zero in any of the comparisons tested, indicating that the F_{st} measure does not detect evidence of population structure with these data (Table 5).

Discussion

We have provided evidence, based on microsatellite DNA variation, that cod populations overwintering in inshore Newfoundland are genetically distinguishable from cod populations overwintering offshore along the edge of the continental shelf on the Grand Bank. We were able to provide this

Table 4. Allele sharing and $(\delta\mu)^2$ genetic distances between inshore and offshore samples for all 3 years combined and for each year separately.

Comparison	N_1	N_2	Allele sharing	$(\delta\mu)^2$
Inshore vs. offshore	308	140	0.319**	2.17*
In-high-af vs. offshore	123	124	0.292**	3.46*
In-low-af vs. offshore	58	124	0.292 (0.246)	1.01 (0.602)
In-high-af vs. in-low-af	123	58	0.277 (0.387)	1.33 (0.490)
		1992		
Inshore vs.offshore	68	40	0.333*	3.99 (0.129)
		1993		
Inshore vs. offshore	139	60	0.364**	3.53 (0.088)
In-high-af vs. offshore	69	54	0.383**	4.74 (0.132)
		1994		
Inshore vs. offshore	101	40	0.308 (0.061)	2.38 (0.310)
In-high-af vs. offshore	38	35	0.346 (0.097)	4.30 (0.247)
In-low-af vs. offshore	47	35	0.306 (0.245)	2.25 (0.512)
In-high-af vs. in-low-af	38	47	0.296 (0.272)	0.99 (0.818)
Inshore spawning group HEL vs. offshore (1993)	25	30	0.528**	6.81 (0.275)
Inshore spawning group HEL vs. in-high-af	25	25[a]	0.425 (0.036)	5.44 (0.408)

Note: Inshore, all inshore individuals; in-high-af, inshore individuals with thermal hysteresis ≥0.20°C; in-low-af, inshore individuals with antifreeze levels ≤0.09°C. N_1 and N_2 are sample sizes (numbers of individuals). Significance values were calculated after Bonferroni adjustment for three simultaneous tests (*, $p < 0.017$; **, $p < \sim 0.003$). Other P values are given in parentheses. In 1992, sample sizes for individuals with known antifreeze levels, either high or low, were too low for meaningful comparisons. In 1993, sample sizes for individuals with known low antifreeze levels were too low for meaningful comparisons.

[a]To equalize sample sizes the test was conducted between the 25 individuals from the spawning group (HEL) and a random set of 25 individuals from the in-high-af group ($n = 69$). The test was repeated 10 times, each with a different random set of 25 in-high-af individuals. Results are averages over the 10 tests, each consisting of 1000 bootstrap trials.

Table 5. Population structure as determined by R_{st} and F_{st}.

Locus	In-high-af vs. offshore	In-low-af vs. offshore	In-high-af vs. in-low-af
Gmo2	0.0056 (0.047)	0.0643 (0.156)	0.1704 (0.095)
Gmo4	0.0003 (0.284)	0.0510 (0.103)	0.0297 (0.210)
Gmo120	0.0131* (0.004)	0.0020 (0.479)	−0.0101 (na)
Gmo132	−0.0004 (na)	0.0122 (0.314)	0.0329 (0.162)
Gmo145	0.0102* (0.010)	0.0172 (0.340)	0.0115 (0.378)
Overall R_{st}	0.0069* (0.006)	0.0279 (0.109)	0.0357 (0.096)
Overall F_{st}	0.0002 (0.344)	0.0003 (0.372)	−0.0012 (na)

Note: The following comparisons were made: inshore cod with high (≥0.20°C) thermal hysteresis (in-high-af, $N = 123$) vs. pooled offshore (offshore, $N = 124$) cod; inshore cod with low (i.e., ≤0.09°C) thermal hysteresis (in-low-af, $N = 58$) vs. pooled offshore cod ($N = 124$); and inshore cod with high ($N = 123$) vs. inshore cod with low ($N = 58$) thermal hysteresis. R_{st} estimates are given for each individual locus and overall (overall R_{st}). The F_{st} estimate is overall loci (overall F_{st}). *, $p \leq 0.01$; **, $p \leq 0.002$ with Bonferroni adjustment assuming five simultaneous tests.

evidence by using blood plasma antifreeze levels to categorize individuals into inshore or offshore overwintering aggregations because adult cod produce antifreeze during the winter if they are continuously exposed to sub-zero temperatures for a period of weeks or months (Goddard et al. 1994) but not if they remain in warm (>2°C) water during the winter (Fletcher et al. 1987). The inshore and offshore overwintering environments are generally subzero and >2°C, respectively. Therefore, overwintering in these two different thermal environments results in the antifreeze categories used in this study.

Typically, cod move to their offshore overwintering grounds in early winter and begin to form spawning aggregations on the outer slopes of the continental shelf (Lear and Green 1984). The landward feeding migration of cod that have overwintered and spawned offshore begins in spring, with most adults arriving inshore in June–July (Lear and Green 1984). In this study, samples were collected from offshore areas in January, February, and June and from inshore areas during April, June, and July. Knowledge of northern cod migration patterns coupled with the sampling dates suggests that while the samples collected offshore were from offshore overwintering individuals, the inshore samples could have come from inshore overwintering cod or from individuals newly arrived from offshore in the spring. However, since high antifreeze levels are only seen in cod residing for weeks or months in subzero temperatures, thermal hysteresis is a valid method of discriminating between the two overwintering groups. From May onwards, cod that have overwintered inshore move from cold and deep water to warmer surface waters and gradually lose their plasma antifreeze (Fletcher et al. 1987; Goddard et al. 1994). For this reason, cod caught inshore with thermal hysteresis values between 0.09 and 0.20°C were excluded in some of our analyses.

Five individual cod from offshore samples and classified as juveniles on the basis of length showed elevated antifreeze activity (Fig. 2). This is the result of a difference in physiology between adult and juvenile cod. During the winter when exposed to the same seasonal temperatures and photoperiod, juvenile cod can produce antifreeze at temperatures as high as 3°C (Goddard et al. 1992). It is extremely unlikely that some of these offshore juveniles with high thermal hysteresis would migrate inshore during the winter. Nevertheless, if such juveniles were present in our inshore samples, they would be indistinguishable from the group of inshore cod on the basis of thermal hysteresis alone. Despite this possibility, we detected genetic differences between inshore and offshore cod, which suggests that our estimates of subpopulation structure may be conservative. Clearly, age, length, physiology (e.g., antifreeze, spawning condition, etc.), time of year, and available thermal environment must all be considered when assessing the genetic basis for population structure.

The genetic evidence suggesting population subdivision between inshore and offshore cod described here is consistent with the morphometric differences detected for these groups by Pepin and Carr (1993) and with the concept of local residency or spawning fidelity at coastal bay scales discussed by Wroblewski et al. (1996). Cod tagged inshore (Trinity Bay) during winter (January to March) exhibit a high probability of being captured in the local area (68% within 30 nautical miles (1 nautical mile = 1.85 km) and 83% within 60 nautical miles) even 3 or more years subsequent to release (Taggart et al. 1995; Wroblewski et al. 1996). Our findings are also consistent with a general pattern of population differentiation exhibited

among distinct offshore cod aggregations within the Northern cod complex (Bentzen et al.[3]), which reflect Lear's (1984, p. 157) observation of cod "homing to specific offshore overwintering and spawning areas, although with some straying." The significant R_{st} estimate obtained in the present study between inshore overwintering and offshore cod (i.e., $R_{st} = 0.0069$, Table 5) was about half the magnitude of the R_{st} estimate between Northern cod (offshore samples) and cod from a distant population on the Scotian Shelf (Bentzen et al.[3]).

Comparison among different genetic measures

Most of the methods that we used showed evidence of population structure. Structure between inshore and offshore cod was detected with the analysis of heterogeneity of allele frequency distributions, allele sharing distance (Bowcock et al. 1994), $(\delta\mu)^2$ distance (Goldstein et al. (1995), and the R_{st} estimate (Slatkin 1995) but not with the F_{st} estimate. The discrepancy between methods is not unexpected given the different underlying assumptions and properties of these methods. Three of the methods that showed evidence of structure, i.e., allele sharing, $(\delta\mu)^2$, and R_{st}, were developed specifically for microsatellites, and R_{st} and $(\delta\mu)^2$ are based on a stepwise mutation model that assumes that the mutation process has a memory of the prior allelic state (Goldstein et al. 1995; Slatkin 1995; see also Valdes et al. 1993; Shriver et al. 1993; Di Rienzo et al. 1994, and references therein).

When years were analyzed separately, we detected differences between inshore and offshore samples with the allele sharing distance but not with $(\delta\mu)^2$. This discrepancy may be explained by the fact that the allele sharing distance is more heavily influenced by alleles at low frequencies (which are more likely to be private or rare) than other methods (e.g., $(\delta\mu)^2$) and may thus be more likely to detect differences between populations than $(\delta\mu)^2$.

It may not be surprising that the genetic difference between inshore overwintering and offshore overwintering cod was detected with R_{st} but not with F_{st}. Slatkin (1995, pp. 461–462) concluded that under the assumptions of a generalized stepwise mutation model at microsatellite loci, "R_{st} will generally provide less biased estimates of demographic parameters . . . than . . . F_{st}." This suggests that if the generalized stepwise mutation model accurately mimics the mutation process at microsatellite loci, it would probably be erroneous to infer high levels of migration or historical association between populations from the lack of detectable variation with the F_{st} measure.

Contrast between microsatellite and mitochondrial DNA evidence

Our results contrast sharply with those derived from the analysis of DNA sequence variation in the cytochrome *b* region of mitochondrial DNA, which did not detect evidence of subpopulation structure within the Northern cod complex (Carr and Marshall 1991*a*, 1991*b*; Pepin and Carr 1993). Carr et al. (1995), in particular, found no evidence that "bay" cod overwintering in Trinity Bay are genetically distinct from offshore cod. In their study, a single common genotype occurred at a frequency greater than 80% and no alternative genotype occurred at frequencies higher than 3%. The contrast between the results of Carr et al. (1995) and ours is all the more remarkable when one considers that individual cod assayed for mitochondrial DNA variation in their study and those assayed for nuclear DNA variation in the present paper were all drawn from the same spawning aggregation at HEL during July 1993 and that those used in this study showed significant allele sharing distances with offshore samples (Table 4).

The fact that subpopulation structure between inshore and offshore cod off Newfoundland can be detected with microsatellite DNA allele length variation but not with sequence variation in the cytochrome *b* region of mitochondrial DNA suggests that the latter technique might not be sufficiently sensitive to address questions of stock structure in cod at small spatial and perhaps temporal scales. There may not be enough variation in the cytochrome *b* region of mitochondrial DNA to distinguish among these populations, as is the case among East African cichlid fish species from Lake Victoria (Meyer et al. 1990; reviewed in Meyer 1993). Our results support the assertion of Park and Moran (1994, p. 278) that the "potential for detecting variation is much greater" in nuclear DNA (e.g., variable number of tandem repeats) than in mitochondrial DNA studies and that, if they exist, genetic differences are more likely to be detected by nuclear than by mitochondrial DNA studies (see also Carvalho and Hauser (1994) and Ward and Grewe (1994) for similar conclusions).

The contrast between mitochondrial DNA and microsatellite DNA analysis in resolving population structure may be related to the fact that mtDNA behaves as a single locus owing to the lack of recombination, whereas many independent loci can be analyzed using microsatellite DNA (Wright and Bentzen 1994). In addition, microsatellite DNA loci can show extremely high levels of allelic variation. For example, Carr et al. (1995) found a maximum of 17 mitochondrial DNA alleles, while the two most variable microsatellite loci in the present study, Gmo4 and Gmo120, had 45 and 43 allelic variants, respectively (Table 3). Furthermore, microsatellite loci are codominant markers inherited in a Mendelian fashion, in contrast to the haploid mitochondrial DNA, which is predominantly maternally inherited. The former can be tested for Hardy–Weinberg expectations if sample sizes are larger than currently expedient to deal with, and can thus provide additional information about population structure (see, e.g., Ruzzante et al.[4]). The difference in the mode of inheritance between mitochondrial and microsatellite DNA suggests that the combination of the two techniques may be very powerful in resolving questions of population structure for species with differential migration or dispersal rates between sexes (see, e.g., Morin et al. 1994 for a study based on single sequence repeat nuclear loci and mitochondrial DNA in chimpanzees).

Collectively, the data presented in this paper suggest the existence of some degree of population structure between inshore overwintering cod from Trinity Bay, Newfoundland,

[3] P. Bentzen, C.T. Taggart, D.E. Ruzzante, and D. Cook. Microsatellite polymorphism and the population structure of cod (*Gadus morhua*) in the North West Atlantic. Submitted for publication.

[4] D.E. Ruzzante, C.T. Taggart, and D. Cook. Spatial and temporal variation in the genetic composition of larval cod (*Gadus morhua*) aggregation: cohort contribution and genetic stability. Submitted for publication.

and offshore overwintering cod on the Grand Bank. These two subpopulations remain genetically distinct despite the fact that individuals from the two regions intermingle during most of summer and fall as a result of the inshore feeding migration by offshore individuals. It is not known if there is any mixing during the formation of spawning aggregations, but our results suggest that there is little genetic mixing. Our results provide evidence of genetic differences among populations within the northern cod complex at a smaller spatial scale than the current management divisions. Although it remains to be shown whether the structure detected in the present paper remains stable over time, future efforts to rebuild the (currently collapsed) cod fishery should consider the possibility of differential contribution to recruitment by inshore and offshore stock components.

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