

Genetic differentiation between inshore and offshore Atlantic cod (*Gadus morhua*) off Newfoundland: a test and evidence of temporal stability

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Abstract: The genetic difference between inshore overwintering Atlantic cod (*Gadus morhua*) from Trinity Bay, Newfoundland, and offshore overwintering cod from the Grand Bank region (Ruzzante et al. 1996; Can. J. Fish. Aquat. Sci. **53**: 634–645) has remained stable during 1992–1995. Cod collected inshore during 1995 in Trinity Bay ($N = 150$) were again genetically distinguishable (using $(\delta\mu)^2$ and D_{SW}) from offshore cod ($N = 140$) collected between 1992 and 1994 and were genetically indistinguishable from inshore cod ($N = 123$) also collected between 1992 and 1994. Farm-held cod ($N = 30$), captured inshore in 1992 and pen reared until 1995 were most likely to have been drawn from an inshore overwintering population; they were genetically different from offshore cod and were indistinguishable from wild inshore cod showing high antifreeze activity and from cod collected in relatively cold ($\sim 0.2^\circ\text{C}$) water in December 1995. The farm-held cod were genetically different from cod collected in relatively warm ($\sim 3.3^\circ\text{C}$) water but were indistinguishable from cod with low antifreeze activity. Despite evidence of weak genetic heterogeneity within the pool of wild cod collected inshore in 1995, which was not associated with antifreeze activity nor with water temperature, the magnitude of the genetic differences between inshore and offshore cod has remained unchanged during the period 1992–1995.

Résumé : Les différences génétiques entre la morue franche (*Gadus morhua*) hivernant dans les eaux côtières de la baie Trinité, à Terre-Neuve, et celles hivernant dans les eaux du large dans la région du Grand Banc (Ruzzante et al. 1996; J. Can. Sci. Halieut. Aquat. **53**: 634–645) sont demeurées stables entre 1992 et 1995. Les morues capturées dans les eaux côtières en 1995 dans la baie Trinité ($N = 150$) se distinguaient encore sur le plan génétique (à l'aide de $(\delta\mu)^2$ et de D_{SW}) des morues du large ($N = 140$) capturées entre 1992 et 1994, et ne se distinguaient pas génétiquement des morues des eaux côtières ($N = 123$) également prélevées entre 1992 et 1994. Des morues maintenues en captivité ($N = 30$), capturées dans les eaux côtières en 1992 et élevées dans des parcs de filets jusqu'en 1995, provenaient très vraisemblablement d'une population qui hiverne dans les eaux côtières; elles étaient génétiquement différentes des morues du large, et ne se distinguaient pas des morues sauvages des eaux côtières présentant une forte activité antigél, et des morues capturées dans une eau relativement froide (environ $0,2^\circ\text{C}$) en décembre 1995. Les morues gardées en captivité étaient génétiquement différentes d'autres morues capturées dans une eau relativement chaude (environ $3,3^\circ\text{C}$), mais ne se différenciaient pas des morues présentant une faible activité antigél. Malgré des indications d'une faible hétérogénéité génétique sur l'ensemble des morues sauvages capturées dans les eaux côtières en 1995, qui n'était pas associée avec l'activité antigél ni avec la température de l'eau, l'ampleur des différences génétiques entre les morues des eaux côtières et du large est demeurée identique pendant la période 1992–1995.

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Introduction

Determining whether or not the genetic structure of a stock complex remains stable over time is of primary importance for the proper management and conservation of genetic resources

in a marine fishery. This is of particular importance when dealing with local, or neighbouring, fish populations among which there may not be any readily apparent geographic or oceanographic barrier to mixing. A temporally stable genetic structure implies that existing, separate breeding components persist over time and are thus likely to experience independent population dynamics. For structured natural populations (a stock complex) under exploitation, failure to estimate parameters such as population-specific growth rates and fishing mortality rates separately for each component (e.g., Myers et al. 1997) can lead to the rapid elimination of those components most easily captured. This can result in detrimental effects, genetic and otherwise, not only to the different components, but to the stock complex as a whole.

Ruzzante et al. (1996a) described evidence of population structure within the northern cod (*Gadus morhua*) complex; specifically between cod overwintering inshore in Trinity Bay, Newfoundland, and those overwintering offshore in the North

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Cape region of the Grand Bank. The two subpopulations were genetically distinct despite the fact that individuals from both regions intermingle during most of summer and fall as a result of the inshore feeding migration of offshore cod. However, their study, and that of Bentzen et al. (1996) using virtually identical techniques, provided very limited evidence of temporal stability in the structures resolved within the northern cod complex. The dissemination of results in both of these studies led critics to rightly challenge us to demonstrate temporal stability in the defined structure. Thus, in this paper we address the question of temporal stability by comparing genetic information from new cod samples collected inshore in 1995 in the same general region of Trinity Bay as in Ruzzante et al. (1996a) with three different sets of samples: (i) those collected inshore during the period 1992–1994; (ii) those collected offshore during the same period, both of which were reported in Ruzzante et al. (1996a); and (iii) those more recently collected in 1995 from cod that had been caught in 1992 in the inshore region of Trinity Bay and had been pen reared in Gooseberry Cove since then (see Wroblewski et al. 1996). To maintain consistency with our earlier study we again employ the same nuclear DNA microsatellite loci as the genetic markers. For cod collected inshore we again used antifreeze protein activity in the blood (expressed as thermal hysteresis), time of sampling, and the available thermal habitat as indicators of overwintering grounds. We subsequently distinguish among the populations using the $(\delta\mu)^2$ and D_{SW} estimates of genetic distance (Goldstein et al. 1995; and Shriver et al. 1995, respectively), as well as R_{ST} (Slatkin 1995) and F_{ST} estimates of population structure.

Materials and methods

Tissue collections

Blood from cod was used for estimating antifreeze activity and as the primary source of nuclear DNA for genetic analysis. Blood (1 mL) was collected from live or recently dead cod using a sterile 2-mL 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. If the sample was destined entirely for genetic analysis, it was preserved immediately in 5 mL of 95% ethanol and stored. Alternatively, approximately one half was preserved in ethanol, and the remainder, 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 $4000 \times g$, the blood plasma was removed, placed in 1.5-mL Eppendorf tubes, and stored until analysed. Soft muscle tissue taken from the posterior of the tongue and preserved in alcohol was employed for DNA extraction when blood was unavailable.

Blood plasma antifreeze and thermal hysteresis

Data on antifreeze activity within each plasma sample were obtained using the protocol described in Goddard et al. (1994). Briefly, the blood plasma freezing and melting points were determined by microscopic observation of the freezing and melting behavior of a single ice crystal within the plasma. The difference is termed thermal hysteresis and is a direct measure of antifreeze activity (Kao et al. 1986). In previous studies of adult cod, antifreeze levels have been used in conjunction with details of location and time of capture, to provide evidence of overwintering behavior. Ruzzante et al. (1996a) used thermal hysteresis in individual plasma samples to infer the overwintering behavior of the individual cod based on the comprehensive

study by Goddard et al. (1994). In adult cod, high antifreeze activity ($\geq 0.2^\circ\text{C}$ thermal hysteresis) is indicative of overwintering in cold ($< 0^\circ\text{C}$) water, which was generally inshore, while low or nonexistent winter antifreeze levels ($\leq 0.09^\circ\text{C}$) are indicative of overwintering in warm water, which was generally offshore.

Genetic techniques

DNA extraction from alcohol-preserved tissue from the farm-held cod samples and from wild cod samples collected between 1992 and 1994 was as described elsewhere (Ruzzante et al. 1996a, 1996b; Bentzen et al. 1996). Samples collected from wild cod in 1995 were extracted by the following procedure. An aliquot (50–75 μL) of blood–ethanol was diluted in 750 μL of high TE (100 mM Tris–HCl, 40 mM EDTA) spun for 10 s to pellet the cells, and the supernatant was discarded. The cell pellet was then resuspended in 250 μL of MGPL (Marine Gene Probe Laboratory) lysis buffer (10 mM Tris–HCl, 1 mM EDTA, 200 mM LiCl (pH 8.0), and 0.8% SDS), containing 200 $\mu\text{g}/\text{mL}$ proteinase K. The sample was incubated at 45°C for 15 min, vortexed, and further incubated until the cells were completely lysed (10–20 min). Following incubation, 500 μL of TE (10 mM Tris–HCl, 1 mM EDTA (pH 8.0)) and 750 μL of cold isopropanol was added and the sample was mixed by vortexing. The DNA was pelleted by pulse spinning of the sample at $14\,000 \times g$, and the supernatant was discarded. The sample was then air-dried for 10 min and resuspended in 100 μL of TE. Following resuspension, the sample was diluted to an appropriate concentration for polymerase chain reaction (PCR).

PCR analysis was carried out as described by Brooker et al. (1994) using five dinucleotide microsatellite primers, Gmo2, Gmo132, and Gmo145 (Brooker et al. 1994), Gmo4 (Wright 1993), and Gmo120 (Ruzzante et al. 1996a). Gmo2, Gmo4, Gmo120, and Gmo132 are perfect GT repeats, and Gmo145 is a compound $G_x(GA)_x$ repeat as defined by Tautz (1989). The PCR products were resolved on 6.5% denaturing polyacrylamide gels, and the alleles were sized relative to a sequence ladder generated from M13mp18 (Yanisch-Perron et al. 1985).

Sample collections

Wild cod inshore: April 1995

Tissue samples were taken from 100 wild cod collected between 24 and 27 April 1995 in the Smith Sound region of Trinity Bay, Newfoundland (48.18°N , 53.80°W). These samples were used for genetic and antifreeze protein analysis (Table 1). One half (50) of the cod were tagged and released following blood sampling, and the remainder were killed for aging and maturity inspections. These collections were made as part of a Department of Fisheries and Oceans (DFO) research survey trip Shamook-239 using an otter trawl at a nominal depth of 152 m where the bottom temperature was nominally -0.98°C . These wild cod (35 females and 14 males in the sacrificed subsample) ranged from 45 to 85 cm (median 56 cm) fork length and from 0.70 to 5.73 kg (median 1.54 kg) fresh mass representing ages 5–9 years (median 6 years).

Plasma antifreeze levels in these cod were generally lower than those in the farm-held cod (see below). Values of thermal hysteresis ranged from 0.065°C to 0.483°C , and the majority (72%) had values $< 0.2^\circ\text{C}$. Previous studies have shown that winter antifreeze levels in adult cod are positively correlated with the length of time spent in subzero water and that wild cod sampled in cold water in Trinity Bay in winter have moderate to high antifreeze levels in their plasma by the beginning of May (Goddard et al. 1994). Thus, the unusual antifreeze data obtained in this study led us to suspect that cod in the April 1995 inshore group had been exposed to two different thermal environments in the recent past: some cod had been resident in cold water for several weeks or months, and some had only recently entered, or returned to, subzero water.

Table 1. Summary statistics of wild (SH-239, SH-250) and farm-held (Gooseberry Cove rearing pen; FOG) cod samples collected in 1995 in the inshore region of Trinity Bay, Newfoundland, and used for genetic analysis using microsatellite loci and for comparison with similar samples collected during 1992–1994 and analyzed elsewhere (see table 1, Ruzzante et al. 1996a).

Sample identifier	Date	Location	Latitude (°N)	Longitude (°W)	Nominal depth (m)	Nominal water temperature at depth (°C)	Fork-length <i>N</i>	Fork-length range (cm)	Fork-length mean (cm)	Thermal hysteresis range (°C)	Thermal hysteresis mean (°C)
SH-239	April 1995	Smith Sound, Trinity Bay	48.18	53.80	152	-1.0	100	45–85	57.4	0.065–0.483	0.169
SH-250	December 1995	Smith Sound, Trinity Bay	(48.13) ^a	(53.80)	(45)	(2.2)	(50)	(52–65)	(56.9)	—	—
	Set-8		48.07	53.73	46	0.2	17	52–65	57.2	—	—
	Set-10		48.19	53.87	44	3.3	33	52–64	56.7	—	—
FOG	Collected June 1992, farm held until April 1995	East Random Head and Gooseberry Cove, Trinity Bay	48.05	53.34	5	—	30	48–71	64.8	0.167–0.576	0.350
			48.03	53.65							

Note: The farm-held cod (FOG) were collected in June 1992 in the area of East Random Head and were moved to Gooseberry Cove where they were pen-reared until April 1995, when they were sampled for this study.

^aNumber in parentheses represents the average of set-8 and set-10.

Wild cod inshore: December 1995

Tissue samples for genetic analysis were drawn from a total of $N = 50$ wild cod caught between 8 and 13 December 1995 in the Smith Sound region of Trinity Bay, Newfoundland (Table 1). Seventeen of these fish were collected during set-8 (48.07°N, 53.73°W) at a nominal depth of 46 m, where water temperature was 0.2°C. The remaining 33 cod were collected during set-10 (48.19°N, 53.87°W) at a nominal depth of 44 m, and the water mass was warmer at 3.3°C. These collections were made as part of a DFO research survey trip Shamook-250 using hand jigs. These wild cod (37 females and 13 males) were generally the same size (and thus, presumably the same age) as the wild cod collected in April and ranged from 52 to 65 cm (median 57 cm) fork length and from 1.28 to 2.49 kg (median 1.65 kg) fresh weight.

Farm-held cod: June 1992 – April 1995

A total of 31 farm-held cod from the Gooseberry Cove, Trinity Bay, Newfoundland, holding pens were collected on 17 April 1995 for genetic and blood antifreeze protein analysis (Table 1). These cod had been originally trapped in June and July of 1992 in the vicinity of East Random Head, Trinity Bay, and had been transferred to a holding pen where they were fed, more or less ad libitum, until sampled as above in 1995. The farm-held cod collection (23 females and 8 males) were on average 10 cm longer and 1 year older than the wild cod (above) and ranged from 48 to 71 cm (median 66 cm) fork length and from 1.75 to 5.20 kg (median 3.48 kg) whole fresh mass representing ages 6–9 years (median 7 years).

Thermal hysteresis values ranged from 0.167 to 0.576°C, and the vast majority exceeded the value of 0.2°C. These values of antifreeze activity reflect recent and prolonged exposure to subzero water temperatures and are consistent with the thermal regime that the cod were exposed to while held in the pen. Further details on the farm-held cod, their growth, etc., are found in Wroblewski et al. (1996).

Wild cod inshore and offshore as reported in Ruzzante et al. (1996a)

Details of the cod sample collections made between January 1992 and

June 1994 are found in Ruzzante et al. (1996a, their table 1) and are briefly summarized here. Four of the collections were from offshore (North Cape region of the northern Grand Bank) winter and early summer aggregations, and 10 were from winter and early summer aggregations found inshore in the immediate vicinity of the Random Island region of Trinity Bay and elsewhere in Trinity Bay (Bellevue) and in Bonavista Bay. The offshore collections were from depths between 400 and 1000 m, at temperatures of between 2.5 and 3.4°C; the average fork lengths ranged between 33 and 54 cm. Inshore collections of cod were made at depths between 140 and 200 m in the Southwest Arm region of Trinity Bay and at depths of between 14 and 150 m elsewhere inshore.

Average water temperatures at depth associated with collections in the Southwest Arm were consistently subzero (–0.5 to –1.4°C) while collections from other locations, depths, and dates were associated with warmer water (0.6–5.5°C). The average lengths of cod in these inshore collections in the Random Island region ranged between 38 and 62 cm, while those from Bellevue and Bonavista Bay ranged between 27 and 34 cm (juveniles). Those collected from Heart's Ease Ledge in July 1993 were from an aggregation that was known to be spawning at that location (Wroblewski et al. 1996; Smedbol and Wroblewski 1996). Blood plasma thermal hysteresis was determined for 7 of the 10 inshore samples and were in general inversely related to water temperatures (elevated thermal hysteresis at subzero temperatures and depressed or nonexistent thermal hysteresis at temperatures >0°C).

Data analysis

We employed $(\delta\mu)^2$ (Goldstein et al. 1995) and the more recent D_{SW} (Shriver et al. 1995) to estimate genetic distances. Estimates of population structure were obtained using R_{ST} (Slatkin 1995), and for comparative purposes, we also employed the F_{ST} statistic following Weir and Cockerham (1984). Most of these measures were used in our recent studies on Atlantic cod (Bentzen et al. 1996; Ruzzante et al. 1996a, 1996b). Significance values for all tests (i.e., homogeneity of allele frequency distributions, genetic distances, estimates of population 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 by reshuffling alleles or genotypes at each locus separately. Bootstrapping each locus independently enables comparison of estimates across loci. Bootstrapping across individuals and populations enables the estimation of significance values for the test under consideration. We assume 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).

Results

Variability in microsatellite loci within and among 1995 samples

The three sets of cod collected in 1995 vary in sample size by up to a factor of three (Table 1). Consistent with this difference, the largest sample (SH-239, $N = 100$) had the largest number of private alleles (Table 2). Despite the difference in sample size, however, the total number of alleles present in the smallest sample is only approximately 71% of those present in the largest sample (i.e., 84 vs. 119). As expected for microsatellite loci, observed (H_{obs}) and expected heterozygosities (H_{exp}) were high and varied between 0.877 and 0.942 and 0.852 and 0.870, respectively.

Single-locus analysis for the combined data ($N = 180$) indicates that, again as expected, the three loci with the highest number of alleles (i.e., Gmo4, Gmo120, and Gmo145) were also the loci with the highest observed and expected heterozygosities (Table 3). There was no evidence for deficiency or excess of heterozygotes as measured by $d = (H_{obs} - H_{exp})/H_{exp}$ over all loci ($d = 0.0107$, $P = 0.337$; Table 3).

In the following sections we (i) analyse the two wild cod samples collected inshore in 1995, (ii) compare them to inshore and offshore samples collected between 1992 and 1994 and reported in Ruzzante et al. (1996a), and (iii) compare the farm-held cod originally captured in 1992 with various subsets of the 1995 wild inshore samples and with the group of offshore cod.

(i) Comparison between wild cod samples collected inshore in 1995

Comparison of the samples collected inshore in April and December 1995 indicated a marginal degree of genetic difference between them when all individuals in both samples were considered ($(\delta\mu)^2 = 4.17$, $P = 0.07$; $D_{SW} = 0.031$, $P = 0.044$, $N_1 = 100$, $N_2 = 50$; Table 4).

This result led us to consider the potential heterogeneity within each of the April and December 1995 samples using information on antifreeze activity, as well as water temperature at the sampling depth. We first distinguished among individuals from the April 1995 collection according to whether they exhibited high ($>0.20^\circ\text{C}$) or low ($<0.09^\circ\text{C}$) thermal hysteresis (level of antifreeze activity) as detailed in Ruzzante et al. (1996a). We similarly distinguished among individuals from the December 1995 collection on the basis of water temperature at sampling depth. There was no significant difference between the subset collected in April 1995 that showed high thermal hysteresis ($>0.20^\circ\text{C}$, $N = 29$) and that subset collected

in the same month that showed low ($<0.09^\circ\text{C}$, $N = 31$) thermal hysteresis ($(\delta\mu)^2 = 0.373$, $P = 0.948$; $D_{SW} = -0.0507$). Similarly, there was no significant difference between the subset of fish collected in April 1995 that showed high thermal hysteresis ($>0.20^\circ\text{C}$, $N = 29$) and that subset of fish from the December 1995 collection from relatively cold water (0.2°C , set-8, $N = 17$; Table 1) using either $(\delta\mu)^2$ (0.897 , $P = 0.92$) or D_{SW} (-0.024). We also found no evidence of population structure in contrasts between those cod showing high antifreeze activity (April 1995) and those sampled in relatively warm (3.3°C) water in December (set-10, $N = 33$; Table 1) nor between those fish with low antifreeze activity (April 1995, $N = 31$) and cod sampled in relatively cold water in December (set-8, $N = 17$; Table 1). We did find that cod with low antifreeze activity in April ($N = 31$) differed significantly from cod sampled in relatively warm water in December ($N = 33$). The results of these contrasts, coupled with the heterogeneity detected above when all fish in both samples (i.e., April and December 1995) were considered, suggest that some of these subsets, or perhaps all of them, may be constituted by cod of heterogeneous origin, an issue we address later in the Discussion. It should be noted that sample sizes among these secondary comparisons are low, and frequently unequal, making it unlikely for statistically significant genetic distances to be detected when true distances may be small (Ruzzante 1998).

To summarize thus far, both $(\delta\mu)^2$ and D_{SW} provided evidence of weak differences between the two wild inshore samples collected in 1995, but these differences did not appear to be correlated with antifreeze activity nor with water temperature at the depth of sampling. In the following analysis we ignore this potential heterogeneity and pool the two sets of inshore data collected in 1995, hereafter referred to as IN-95, and proceed to compare them with data from the inshore and offshore samples collected between 1992 and 1994 and analyzed elsewhere (Ruzzante et al. 1996a).

(ii) Comparison of 1995 wild inshore cod with 1992–1994 wild inshore and offshore cod

The IN-95 pool ($N = 150$) did not differ from the 1992–1994 cod collected inshore in cold water with high ($>0.20^\circ\text{C}$) antifreeze activity (inshore overwintering) ($(\delta\mu)^2 = 1.26$, $P = 0.240$; $D_{SW} = 0.0039$, $P = 0.270$; $R_{ST} = 0.028$, $P = 0.217$; Table 4, but see F_{ST} result). However, it did differ significantly from the 1992–1994 cod collected offshore during winter with three of the four genetic measures ($(\delta\mu)^2 = 2.096$, $P = 0.047$; $D_{SW} = 0.0192$, $P = 0.021$; and $F_{ST} = 0.0014$, $P = 0.0049$; Table 4). There was no statistical evidence with any of the four measures that the 1995 inshore pool differed from low ($<0.09^\circ\text{C}$) antifreeze cod collected between 1992 and 1994 from warm inshore water in summer (Table 4).

The estimates for $(\delta\mu)^2$ and D_{SW} (but not those for R_{ST} and F_{ST} , see Discussion) were 2- to 5- and 10-fold larger, respectively, in the comparison of IN-95 with offshore cod than they were for comparisons involving the different inshore pools (Table 4). Thus, despite the internal heterogeneity detected within the pool of cod collected inshore in April and December 1995, these cod, collectively, were different from offshore overwintering cod and were indistinguishable from cod that had been collected inshore at different times of the year between 1992 and 1994 and that showed either high or low antifreeze activity.

Table 2. Degree of genetic variation (sample size: N (indiv.), mean sample size per locus, number of private alleles, total number of alleles, mean number of alleles per locus, mean observed (H_{obs}) and expected (H_{exp}) heterozygosity, and mean heterozygote deficiency (d) among three samples of northern cod (*Gadus morhua*) at five microsatellite loci and collected from the inshore regions of Trinity Bay, Newfoundland, in 1995: Shamook-239, April 1995 (SH-239); Shamook-250, December 1995 (SH-250); and farm-held cod from the Gooseberry Cove rearing pen (FOG).

Sample	N (indiv.)	Mean sample size per locus	No. of private alleles	Total no. of alleles	Mean no. of alleles per locus	Mean H_{obs}	Mean H_{exp}	Mean d
April 1995	100	97.4	23	119	23.8	0.877	0.870	0.0058
December 1995	50	49.4	9	98	19.6	0.862	0.852	0.0022
FOG	30	28.2	6	84	16.8	0.942	0.870	0.0877

(iii) Comparison of farm-held cod (captured in 1992) with wild cod inshore

We now consider the farm-held cod ($N = 30$) originally captured inshore in 1992 in relation to various subsets of wild cod collected inshore in 1995. Estimates of both $(\delta\mu)^2$ and D_{SW} indicate we cannot reject the hypothesis that the farm-held cod were drawn from the same population of inshore wild cod showing high ($>0.20^\circ\text{C}$) thermal hysteresis in April 1995 ($N = 29$) nor from the same population of cod collected inshore in relatively cold (0.2°C) water in December 1995 ($N = 17$), even when the latter two subsets were combined ($N = 46$) and the analysis was repeated (Table 4). The estimates of genetic distance between the farm-held cod and wild cod collected inshore in April 1995 and showing low thermal hysteresis ($<0.093^\circ\text{C}$, $N = 31$) were twofold larger than in the previous set of comparisons, but they were not significant ($P \geq 0.15$). The estimates of genetic distance between the farm-held cod and the wild cod collected inshore in December 1995 in relatively warm water ($N = 33$) were sixfold larger than in the first set of comparisons and were significant or marginally so (Table 4). Thus, the farm-held cod were more different from the low-antifreeze cod from April 1995 than they were from the high-antifreeze cod also from April 1995, and they were significantly different from the cod collected in December 1995 in warm water. In addition, the farm-held cod were significantly different from the pool of offshore cod ($(\delta\mu)^2 = 8.10$, $P = 0.025$; $D_{SW} = 0.054$, $P = 0.029$; Table 4, line 10). However, interpretation of these results may be difficult because of the difference in sample size between these two groups (Ruzzante 1998). We therefore pooled the farm-held cod with the group of wild cod collected inshore in 1995 and compared this group (hereafter IN-95 + FOG; $N = 180$) against the offshore cod ($N = 140$). The pool of inshore and farm-held cod differed genetically from the offshore cod with all four measures used ($(\delta\mu)^2 = 2.32$, $P = 0.031$; $D_{SW} = 0.0188$, $P = 0.018$; $R_{ST} = 0.0016$, $P = 0.078$; $F_{ST} = 0.0015$, $P = 0.030$), and all estimates were of the same magnitude as (if not slightly larger than) when the farm-held cod were not included in the inshore pool (cf. Table 4, lines 4 and 11).

Comparison of farm-held cod with wild, inshore cod from Bellevue and Hearts Ease Ledge

Estimates of genetic distance are relatively large and significant for the comparison between the farm-held fish ($N = 30$) and fish collected more or less simultaneously in July 1992 in the area of Bellevue, Trinity Bay ($N = 26$; $(\delta\mu)^2 = 17.62$, $P = 0.004$; $D_{SW} = 0.1240$, $P = 0.013$; Table 4, line 12). In contrast, no differences were found between the farm-held fish and fish

collected in July 1993 in the area of Heart's Ease Ledge, Trinity Bay ($N = 25$), from an aggregation that was known to be spawning at the time ($(\delta\mu)^2 = 4.47$, $P = 0.355$; $D_{SW} = 0.0259$, $P = 0.252$; Table 4, line 13).

Discussion

We have shown, on the basis of microsatellite DNA variation, that cod sampled inshore in April of 1995, and later in December of 1995 in the area of Smith Sound, western Trinity Bay, were collectively genetically indistinguishable from cod populations overwintering inshore in the same general area during 1992–1994 and were genetically distinguishable from cod populations overwintering offshore along the edge of the continental shelf on the Grand Bank during 1992–1994. These are consistent with very similar results reported in Ruzzante et al. (1996a) and, therefore, provide evidence of temporal stability in the genetic differences between inshore and offshore overwintering cod populations in the region, at least at the scale of 2–3 years. We have further shown that a group of farm-held cod, initially caught inshore in western Trinity Bay in the summer of 1992 and subsequently pen reared for 3 years, were also representative of the inshore overwintering population from which they were presumably drawn originally.

Virtually all of the genetic measures employed above led us to statistically reject the hypothesis of no genetic structure between inshore and offshore overwintering cod. Differences between the complex of wild inshore cod collected in 1995 and that offshore collected between 1992 and 1994 was detected with the $(\delta\mu)^2$ and D_{SW} estimates of genetic distance, as well as with the F_{ST} estimate of population structure (Table 4). Although these results provide evidence of temporal stability in the genetic structure within the northern cod complex overwintering inshore and offshore, they represent only a partial test of temporal stability as no wild cod samples from the northern Grand Bank region (i.e., offshore) were available in 1995. A more rigorous test of the temporal stability hypothesis would require the collection and processing of more or less contemporaneous offshore and inshore samples, the former now being difficult to obtain. What is of particular interest in this study, which is in contrast to the results reported in Ruzzante et al. (1996a), is that the genetic differences detected here between inshore and offshore pools were not as clearly associated with antifreeze activity (thermal hysteresis) among the cod sampled inshore. Antifreeze appears in the plasma of adult cod as a direct result of prolonged residence in subzero water. In previous studies, cod sampled in winter in Trinity Bay have had high antifreeze levels indicating long-term residence in subzero (inshore) water (most likely the Trinity Bay area; Goddard

Table 3. Single-locus statistics (sample size, N ; number of alleles, n ; allele size range in base pairs; observed and expected heterozygosity, H_{obs} , H_{exp} ; and heterozygote deficiency (d) for each of five microsatellite cod loci among three sample sets of northern cod (*Gadus morhua*) collected from the inshore regions of Trinity Bay, Newfoundland, in 1995.

Locus	N	n	Size range	H_{obs}	H_{exp}	d
Gmo2	179	19	104–148	0.782	0.779	0.0047
Gmo4	171	37	115–265	0.994	0.953	0.0437
Gmo120	177	31	152–222	0.983	0.944	0.0415
Gmo132	172	10	105–135	0.698	0.729	–0.0427
Gmo145	176	42	147–207	0.955	0.928	0.0065
Mean over all loci				0.882	0.870	0.0107 ($P = 0.337$)

et al. 1994). In the present study antifreeze levels in cod sampled inshore in winter were remarkably heterogeneous suggesting exposure to thermal regimes with temperatures $>0^{\circ}\text{C}$ (antifreeze low or absent) and $<0^{\circ}\text{C}$ (antifreeze high). The absence of a tight correlation between antifreeze level and genetic difference in this study may thus reflect movement of cod among thermal regimes inshore. Alternatively, this absence of a correlation may be a result of the relatively small sample sizes for the different categories of antifreeze levels used in the present study relative to the previous study. Whatever the reason, the results clearly indicate that, regardless of antifreeze levels, cod sampled inshore in April and December 1995 were genetically distinguishable from offshore cod populations and were genetically indistinguishable from inshore cod populations collected between 1992 and 1994.

The subset of farm-held cod collected in 1992 was statistically most likely to have been drawn from an inshore overwintering population: (i) they were statistically indistinguishable from wild inshore cod with high antifreeze activity collected in April 1995; (ii) they were likewise indistinguishable from cod collected in relatively cold water in December 1995; and (iii) although they were indistinguishable from cod with low antifreeze activity collected in April 1995 (Table 4, line 8), they were different (with $(\delta\mu)^2$ and marginally so with D_{SW} ; Table 4, line 9) from cod collected in relatively warm water in December 1995. These findings suggest that the April and December 1995 inshore cod that appeared to have a preference for warmer overwintering environments may be genetically farther removed from the more homogeneous (farm-held) inshore cod than those April and December 1995 cod collected inshore in cold water with high antifreeze levels. Furthermore, either by themselves, or grouped with the wild cod collected inshore in 1995, the farm-held cod were genetically different from offshore cod (Table 4, lines 10 and 11).

Our results indicate that the genetic differences detected earlier (Ruzzante et al. 1996a) between cod that overwinter inshore in the area of western Trinity Bay and cod that overwinter offshore in the area of the Grand Bank are temporally stable, at least at the scale of 2–3 years. However, it is also important to ask whether the magnitude of the differences remained more or less constant over the period 1992–1995. A comparison of the $(\delta\mu)^2$ estimate for the inshore 1995 pool versus offshore pool in the present study (Table 4, line 4) with that in our earlier study (Table 4, line 1; Ruzzante et al. 1996a) shows that the genetic distance between inshore and offshore pools remained virtually unchanged (i.e., 2.10 vs. 2.17 respectively). Ruzzante et al. (1996a) did not use D_{SW} , but values for

the different contrasts of the 1992–1994 inshore and offshore pools using this measure are reported here in a footnote to Table 4. Again, the D_{SW} estimate for the comparison between the 1995 inshore pool and offshore cod (Table 4, line 4) is of a similar magnitude to that between the inshore and offshore 1992–1994 pools (i.e., 0.0192 vs. 0.0175, respectively). The R_{ST} and F_{ST} estimates for the 1992–1994 inshore–offshore contrast without discrimination of the inshore fish according to antifreeze activity were not reported in Ruzzante et al. (1996a). We calculated these estimates as $R_{ST} = 0.0013$ and $F_{ST} = 0.0007$, which are similar to (in the case of R_{ST}), or smaller than (in the case of F_{ST}) the respective values reported in this study (i.e., $R_{ST} = 0.0012$ and $F_{ST} = 0.0014$; Table 4). We conclude that not only did the structure detected earlier (Ruzzante et al. 1996a) between inshore overwintering cod from Trinity Bay and offshore overwintering cod from the Grand Bank remain in existence, but the magnitude of the genetic distance between these groups, however measured, has remained virtually identical.

The issue of temporal stability in genetic structure has previously been considered for a number of marine species using a variety of genetic techniques and, not surprisingly, conclusions vary with the species under consideration. For example, using mitochondrial DNA haplotype frequencies, Brown et al. (1996) recently demonstrated that, for American shad (*Alosa sapidissima*), there is relatively little temporal variation compared with the existing geographically based variation among populations from different river drainages spanning the distributional range of the species. On the other hand, allozyme genotypic frequencies in Pacific salmon populations can differ markedly over time, in part because of the unique life-history features of salmonids, and single “snapshot samples” are unlikely to adequately define stocks (Waples 1990; Waples and Teel 1990). Therefore, pooling of periodic samples to average out any temporal changes in allele frequencies has been suggested for analyses of mixed-stock fisheries in these species (Waples 1990; Waples and Teel 1990; see also Brown et al. 1996). Significant temporal genetic variation has also been found among populations of queen conch (*Strombus gigas*) in Florida Keys (Campton et al. 1992). For some species, the degree of temporal variation in allele frequencies has been shown to differ among life-history stages. For example, using allozymes, Kordos and Burton (1993) compared the temporal variation in allelic frequencies among larvae, juveniles, and adults of the blue crab (*Callinectes sapidus*). They detected seasonal variation in larval allelic frequencies, with summer samples being more variable than winter samples, which

Table 4. Estimates, with P values given in parentheses, of $(\delta\mu)^2$, D_{SW} , R_{ST} , and F_{ST} .

Sample comparison	N_1	N_2	$(\delta\mu)^2$	D_{SW}^*	R_{ST}	F_{ST}
(1) Apr.-95 vs. Dec.-95	100	50	4.17 (0.070)	0.0310 (0.044)		
(2) IN-95 vs. IN92-94-HI-AF	150	123	1.26 (0.240)	0.0039 (0.270)	0.028 (0.217)	0.0030 (0.059)
(3) IN-95 vs. IN92-94-LO-AF	150	58	0.28 (0.933)	-0.01127 (NA)	0.035(0.229)	0.0006 (0.279)
(4) IN-95 vs. OFFSHORE	150	140	2.10 (0.047)**	0.0192 (0.021)	0.0012(0.250)	0.0014 (0.049)
(5) FOG vs. Apr.-95-HI-AF	30	29	3.58 (0.418)	0.017 (0.262)		
(6) FOG vs. Dec.-95-COLD	30	17	2.48 (0.650)	-0.018 (NA)		
(7) FOG vs. Apr.-95-HI-AF + Dec.-95-COLD	30	46	2.95 (0.440)	0.0092 (0.311)		
(8) FOG vs. Apr.-95-LO-AF	30	30	5.13 (0.258)	0.0397 (0.155)		
(9) FOG vs. Dec.-95-WARM	30	33	12.25 (0.010)	0.0656 (0.076)		
(10) FOG vs. OFFSHORE	30	140	8.10 (0.025)	0.054 (0.029)		
(11) IN-95 + FOG vs. OFFSHORE	180	140	2.32 (0.031)	0.0188 (0.018)	0.0016 (0.078)	0.0015 (0.030)
(12) FOG vs. Bellevue	30	26	17.62 (0.004)	0.1240 (<0.013)		
(13) FOG vs. HEL	30	25	4.47 (0.355)	0.0259 (0.252)		

Note: Comparisons between samples (N_1 , N_2) collected in inshore areas nearby Trinity Bay, Newfoundland, in April and December 1995, and samples collected offshore and inshore between 1992 and 1994 and reported in Ruzzante et al. 1996a. Comparisons of the magnitude of estimates across pairwise contrasts are valid only across contrasts with roughly equivalent sample sizes (see Ruzzante 1998). Apr.-95-HI-AF and Apr.-95-LO-AF, subsets from April 1995 with high and low antifreeze, respectively; Dec.-95-COLD and Dec.-95-WARM, subsets from December 1995 from relatively cold and relatively warm water, respectively; IN-95, April 1995 and December 1995 samples pooled; IN92-94-HI-AF and IN92-94-LO-AF, subsets from the inshore collections from 1992 to 1994 (reported in Ruzzante et al. (1996a)) showing high and low antifreeze levels respectively; FOG, farm-held cod from the Gooseberry Cove rearing pen; HEL, cod from Hearts Ease Ledge reported in Ruzzante et al. (1996a), as were Bellevue cod.

* D_{SW} estimates between inshore and offshore 1992-1994 pools were not reported in Ruzzante et al. (1996a). D_{SW} estimates (P values) for comparisons between those pools are as follows: IN92-94 vs. OFFSHORE: 0.0175 ($P = 0.016$); IN92-94-HI-AF vs. OFFSHORE: 0.0240 ($P = 0.015$); IN92-94-LO-AF vs. OFFSHORE: -0.0003 (NA); IN92-94-HI-AF vs. IN92-94-LO-AF: -0.0073 (NA).

**A very similar result (i.e., 2.17) was reported in Ruzzante et al. (1996a).

showed relative homogeneity in allele frequencies. The authors attributed these changes to seasonal differences in population sources (Kordos and Burton 1993).

Despite the evidence for the temporal stability we have provided and the nature of variation that might be expected based on other studies, some inconsistencies among our results require explanation. For example, on the one hand there were clear genetic affinities and differences between the farm-held cod and the various subsets of wild inshore cod (e.g., high vs. low antifreeze activity, etc.), while on the other hand there was evidence of genetic heterogeneity within the pool of wild cod collected inshore in 1995, which was neither clearly associated with antifreeze activity nor with water temperature at sampling depth. Despite this heterogeneity, when the 1995 inshore cod were considered as a group they were different from offshore cod. We interpret these results to suggest that the subset of farm-held cod was comprised of fish that were genetically more homogeneous than any of the subsets of wild cod collected in 1995. This suggestion is supported by the fact that, while the farm-held cod were genetically different from cod sampled more or less simultaneously in June of 1992 in the Bellevue area of Trinity Bay (but see below), they were genetically indistinguishable from spawning cod sampled in July 1993 in the immediate vicinity (Hearts Ease Ledge) of their original capture (see Wroblewski et al. 1996). Considered collectively, these observations and results lead us to conclude that the pool of inshore cod is comprised of weakly heterogeneous subsets of inshore cod, which as a group, are distinguishable from offshore cod. Alternatively, the weak heterogeneity detected within the inshore samples collected in 1995 may be a result of what appears to be a very recent (1995 and 1996 following the collapse of northern cod) anomaly in the normal pattern of cod migration, whereby large aggregations of cod

that are normally found offshore are no longer found there (Shelton et al. 1996) and may be remaining inshore (see Bratley 1996; Lilly 1996; Rose 1996; Taggart 1996). These large aggregations appear to be in addition to what are normally inshore overwintering and reproducing populations (see review in Lilly 1996). Nevertheless, despite the internal heterogeneity within the inshore pool of cod we analyzed, this pool remains genetically different from what were known to be offshore cod, at least up until 1994.

The above discussion raises an intriguing question that remains unanswered. We ask, to what extent can different inshore cod aggregations or samples from different, though neighbouring, regions inshore be considered as genetically homogeneous, especially in relation to offshore overwintering cod populations. For example, the farm-held cod were similar to cod from a large spawning aggregation collected 1 year later, in July 1993, in the area of East Random Head but were different from cod collected at the same time, in June 1992, in the area of Bellevue. Note, however, that this last group (Bellevue) was comprised entirely of juveniles (length 27.1 ± 3.9 cm (mean \pm SD), $N = 26$; see Ruzzante et al. 1996a, Table 1). Thus, the possibility that they may have originated offshore, may have drifted inshore as eggs or larvae (Davidson and DeYoung 1995), and are too young to be moving back to the offshore spawning grounds cannot be discounted. Such a scenario is supported by the fact that the juvenile cod sampled in Bellevue ($N = 26$) appear genetically indistinguishable from the pool of offshore cod collected between 1992 and 1994 ($N = 140$, $(\delta\mu)^2 = 3.81$, $P = 0.197$; $D_{SW} = 0.0008$). Whatever the origin of the juvenile cod sampled in Bellevue, the various comparisons within the group of cod collected inshore in April and December 1995 suggest that cod from different inshore aggregations or from neighbouring inshore regions may be

genetically heterogeneous and may thus be reproductively isolated, or at least partially so.

Bentzen et al. (1996, p. 2719) suggested that "... it may be wise to seriously reconsider the current (and historical) management scheme for northern cod, which presumes one stock distributed throughout the inshore and offshore regions stretching from Hamilton Bank to the Grand Bank through NAFO divisions 2J, 3K, and 3L." That suggestion was based primarily on a north-south population structure of offshore northern cod. Our results here further suggest a reconsideration based on what appears to be an inshore-offshore (east-west) structure. The nature of such reconsideration is entirely consistent with Templeman (1962, p. 108) who envisioned that: "Very likely in the future enough differences will be found to indicate a number of north-south and inshore-offshore sub-stocks of this Labrador-Newfoundland stock which either do not intermingle greatly or separate out at certain seasons." From a geographic perspective, the concept of inshore statistical and management units independent of offshore units at least in Division 3L is not new (Halliday and Pinhorn 1990, Figs. 10 and 11, pp. 16 and 17) and might be useful in any management reconsideration, especially given that it is not clear from where "rebuilding" of the northern cod stock complex might stem: inshore, offshore, or both. The feasibility of such a management approach is equally unclear.

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