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Macro- and micro-geographic variation in pantophysin (*Pan*I) allele frequencies in NE Atlantic cod *Gadus morhua*

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ABSTRACT: Using samples of Atlantic cod *Gadus morhua* L. from the North Sea, and previously published genetic data from the Irish and Celtic Seas, Iceland, and Norwegian fjord and offshore populations, we describe striking macro- and micro-geographic patterns in pantophysin (*Pan*I) allele frequencies. The relatively abrupt discontinuity in *Pan*I allele frequency distribution at 2 different locations is not congruent with standard patterns of isolation by distance and could arise from population admixtures, historical or contemporary natural selection, behavioural segregation or a combination of these factors. Here, we examined the relationships between the distributions of *Pan*I alleles and temperature, salinity and depth. In the northeast Atlantic, temperature was highly correlated with *Pan*I allele frequency, even when the effect of geographic distance was removed. In the Norwegian fjords, partial Mantel tests indicated that temperature, salinity and depth all had a significant effect on *Pan*I allele frequency in juvenile fish. However, a sample from the brackish waters of the eastern Baltic Sea suggested that salinity may be linked to *Pan*I allele frequency distribution and that the relationship with temperature was weaker in areas of low salinity. Strong correlations between *Pan*I allele frequencies and key environmental variables, together with evidence from the available literature, suggested that environmental conditions play an important role in determining the distribution of different *Pan*I genotypes. The combined use of environmental data, *Pan*I genotyping and neutral markers may provide a valuable approach to examine local adaptation, levels of gene flow and stock structuring.

KEY WORDS: Cod · *Gadus morhua* · Pantophysin · Natural selection · Cline

INTRODUCTION

Identifying the forces shaping genetic variation within and among wild populations has been one of the primary aims of molecular population genetics. Historically, such efforts have concentrated on presumably neutral genetic variation to investigate the effects of population size, demographic history and migratory exchange. However studies in which individual loci were shown to be affected by environmental factors have provided the foundation for many classical investigations of adaptive variation; well known examples include alcohol dehydrogenase (*Adh*) in *Drosophila melanogaster* (Oakeshott et al. 1982, Berry & Kreitman 1993, Hey 1999, Verrelli & Eanes 2001), leucine aminopeptidase (*Lap*) in *Mytilus edulis* (Hilbish et al. 1982, Koehn et al. 1983) and lactate dehydrogenase (*Ldh*) in *Fundulus heteroclitus* (Powers & Place 1978, Schulte et al. 1997, Powers & Schulte 1998, Schulte 2001). Such studies have facilitated the application of loci likely to be under selection to questions of population substructure (Ford 2000, Miller et al. 2001) and aquaculture (Moran 2002). Such applications require an understanding of the interaction between environmental factors and genetic variation, although such knowledge is currently limited. One promising

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genetic marker under selection is the pantophysin I (*Pan*I) locus in gadoid fishes, where locus-specific positive Darwinian selection has been demonstrated (Canino & Bentzen 2004, Pogson & Mesa 2004), although relatively little is known about the selection pressures involved. Here, we examine genetic variability at this locus in relation to latitudinal and small-scale heterogeneity in salinity, water depth and temperature.

*Pan*I, known initially as anonymous cDNA clone GM798 (Pogson et al. 1995), was sequenced and identified as synaptophysin, though this has subsequently been revised to pantophysin (Pogson 2001). Pantophysin is an integral membrane protein expressed in both neuroendocrine and nonneuroendocrine cytoplasmic transport vesicles, though its exact function is unknown. It is composed of 4 membrane-spanning domains separated by 2 intravesicular (IV) loops and 2 cytoplasmic tails (Haass et al. 1996, Windoffer et al. 1999, Brooks et al. 2000). In cod, the *Pan*I^{A} and *Pan*I^{B} alleles differ by 4 amino acid substitutions that cluster in the first IV loop and can be differentiated using a single *Dra*I restriction site. Additional sequence variation exists within each of the allelic groups, most notably in the form of a 12 bp intron deletion in the *Pan*I^{B} allele (the ∇2 *Pan*I^{B} allele) and a mutation from aspartic acid to lysine in the *Pan*I^{A} allele (the *Pan*I$^{A'}$ allele), both of which suggest that 2 selective sweeps may be occurring in cod (Pogson 2001). However, as the average number of nucleotide differences between the *Pan*I^{A} and *Pan*I^{B} alleles far exceeds that within the allelic groups (Pogson 2001), this study focuses upon variation between groups.

Since the initial study of Pogson et al. (1995), which found markedly higher levels of among-population differentiation at this locus compared to other loci, evidence for selection at the *Pan*I locus in cod has accumulated. Fevolden & Pogson (1995) and Jónsdóttir et al. (2002) identified a correlation between *Pan*I genotype and growth rate in wild northeast Arctic cod and Icelandic cod, respectively. Fevolden & Pogson (1997) detected highly significant *Pan*I allele frequency differences between populations of Norwegian coastal and northeast Arctic cod at the *Pan*I locus. In contrast, mitochondrial DNA studies on these populations are equivocal, revealing evidence for differentiation between coastal and northeast Arctic cod in Dahle's (1991) study but not in that by Árnason & Pálsson (1996). In a *Pan*I study of cod in a Norwegian fjord, Karlsson & Mork (2003) proposed that significant deviations from Hardy-Weinberg equilibrium may be due to contemporary selection. Although the presence of selection at *Pan*I is well-established, it is less certain whether patterns of differentiation reflect historical selection or contemporary selection. Pogson (2001) examined *Pan*I sequence variation and found strong evidence for both balancing and directional selection, though subsequent analysis of neutral variation failed to differentiate historical from contemporary selection (Pogson & Fevolden 2003). The comparison of nonsynonymous and synonymous substitutions provided clear evidence for the occurrence of strong selection (d_N/d_S = 5.35) (Pogson & Mesa 2004). However, distinguishing between historical isolation and contemporary selection unequivocally at protein loci is notoriously difficult (Powers & Schulte 1996). Establishing the relationships between genetic variation, geography and environmental variables is a key step, and determination of the extent to which environmental variables covary with *Pan*I allele frequencies may help to elucidate the factor(s) responsible for the observed gradients in *Pan*I allele frequencies. Such data may offer powerful insights into the constraints and opportunities afforded by the analysis of adaptive variation (Moran 2002, Carvalho et al. 2003, Manel et al. 2003).

Following the studies of Fevolden & Pogson (1997), Sarvas & Fevolden (2005) (Norwegian coast and northeast Arctic) and Jónsdóttir et al. (1999) (south and southeast Iceland), the 2 *Pan*I alleles, *Pan*I^{A} and *Pan*I^{B}, have become associated with coastal and offshore populations, respectively. Furthermore, although strong relationships between *Pan*I allele frequency and depth have been described (Sarvas & Fevolden 2005), elucidation of mechanisms that may drive selection at the *Pan*I locus has been impeded by an apparent absence of any relationship between environmental variation and allele frequency.

In the present paper, the geographical and environmental ranges of *Pan*I allele frequency data are extended by the analysis of additional samples from the North Sea and the Baltic Sea, and integrated with existing data from other northeast Atlantic populations (Fevolden & Pogson 1997, Jónsdóttir et al. 1999, 2003, Coughlan 2001) covering a latitudinal transect from 51° 30′ N to 71° 11′ N. This geographical range encompasses a wide variation in temperature, and the Baltic sample incorporated an extreme salinity gradient, offering an opportunity to assess patterns of *Pan*I allele distribution in relation to ecological variation. Additionally, the data set of Fevolden & Pogson (1997) enabled examination of micro-geographic variation in *Pan*I allele frequencies in the Norwegian Fjords in relation to depth, temperature and salinity. Elucidation of genotype-environment relationships at *Pan*I has relevance to predictive recruitment models and aquaculture, particularly given the links between *Pan*I genotype and growth and condition, which support the occurrence of contemporary selection (Fevolden & Pogson 1995, Jónsdóttir et al. 2002, Case et al. in press).

MATERIALS AND METHODS

Sample collection. Samples were collected via research cruises from Bear Island and Gdansk during 1998 and 2002, respectively. Other samples were obtained from commercial catches, which were sampled at the fish markets directly after landing. Six of the main cod spawning grounds in the North Sea (Southern Bight, Flamborough Head, Aberdeen Bank, Moray Firth, Shetland and southwest Norway) were sampled during the spawning seasons of 2002 and 2003 (Fig. 1). Where possible, fish from local inshore boats were used and in all cases, verification of the origin of the fish was obtained from both crews and local fishery offices. Fish were gutted prior to landing and therefore total length measurements were taken to estimate the age and likely maturity from age-length keys (Daan 1974, Hislop 1984). Fish were categorised as mature if greater than 60 cm in total length and where possible, 100 mature fish were sampled from each spawning ground. Pectoral fin clippings were taken and preserved in 70% ethanol for subsequent genetic analysis.

To extend the spatial distribution of the study and enhance statistical power, *Pan*I allele frequency data from both spawning and non-spawning populations were collated from the literature (Fevolden & Pogson 1997, Jónsdóttir et al. 1999, 2003, Coughlan 2001), thereby encompassing a geographical range from 51° 30′ N to 74° 11′ N and 24° 09′ W to 34° 31′ E (Table 1). Within this range lie 2 distinct boundaries between areas in which either *Pan*I^{A} or *Pan*I^{B} alleles are prevalent: one located off southern Iceland (Fig. 1), hereafter known as the northeast Atlantic transect, and the other between Arctic and coastal populations of Norwegian cod, described by Fevolden & Pogson (1997), hereafter known as the Norwegian fjord transect (Fig. 2).

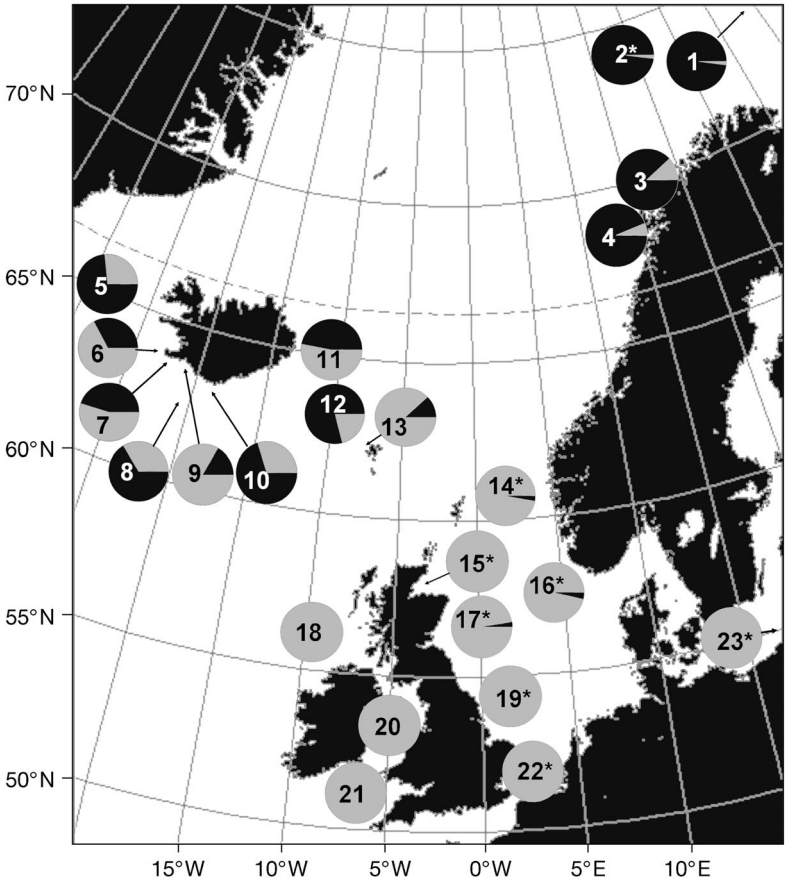

Fig. 1. *Gadus morhua*. Locations of cod samples. Pie charts represent relative allele frequencies. Grey: *Pan*I^{A}; black: *Pan*I^{B}. (1) Barents Sea, (2) Bear Island, (3) Fugleøybanken, (4) Vestfjorden, (5) Jokaldjup, (6) Reykjanesgrun, (7) Eyrabakkabugur, (8) Selvogsbanki, (9) Loftstaðahraun, (10) Kantur, (11) Austfjarðadjup, (12) Iceland-Faeroes Ridge, (13) Faeroes Plateau, (14) Shetland, (15) Moray Firth, (16) Southwest Norway, (17) Aberdeen, (18) North Ireland, (19) Flamborough Head, (20) Irish Sea, (21) Celtic Sea, (22) Southern Bight. Samples 3 and 4: Fevolden & Pogson (1997); Sample 1: Jonsdottir et al. (2003); Samples 5, 8, 12, 13, 18, 20 and 21: Coughlan (2001); Samples 6, 7, 9–11: Jónsdóttir et al. (1999); Samples 2, 14–17, 19, 22: this study. Asterisks denote samples analysed in this study

DNA extraction. DNA was extracted from tissue samples using a method modified from Taggart et al. (1992). A small piece of fin tissue was incubated at 60°C for 3 h in 400 µl of extraction buffer containing 0.1 M Tris HCl, 10 mM disodium EDTA, 0.1 M NaCl, 2% SDS and 0.1 mg ml^{-1} Proteinase K. The protein and other cellular debris were removed with phenol chloroform, and a pellet of DNA was obtained using precipitation with absolute ethanol and centrifugation at 18 000 *g*. The dried DNA was re-suspended in 100 µl of double-distilled H_2O (ddH_2O) and stored at –20°C.

Restriction fragment length polymorphism. A 313 bp fragment of the pantophysin gene was PCR-amplified using primers designed from *Pan*I sequences (D. O'Leary pers. comm., GenBank accession nos. AF288943–AF288977). These primers flank the diagnostic *Dra*I restriction site, which differentiates the *Pan*I^{A} and *Pan*I^{B} alleles, and amplify a shorter fragment (1051 bp) than that amplified by Fevolden & Pogson (1997), so enhancing PCR and screening efficiency (Coughlan 2001). Each 12 µl reaction mix contained 20 ng of template DNA, 1× NH_4 reaction buffer (Bioline), 200 µM dNTPs, 50 mM KCl, 100 µg ml^{-1} BSA, 250 nM of each primer (1 primer end-labelled with Cy5 fluorescent dye) and 0.45 U *Taq* polymerase (Bioline). PCR was

Table 1. *Gadus morhua*. Details of offshore samples contributing to the northeast Atlantic transect. Position: location from which the samples were caught. Area of environmental sampling: area inside which temperature and salinity data were taken for each population. –: no data

Population	n	Sample date	Frequency *Pan*I^A allele	Mean June temp. (1980–2002) (±SD)	Mean June salinity (1980–2002) (±SD)	Position	Area of environmental sampling Lat. min.	Lat. max.	Long. min.	Long. max.	Source
(1) Barents Sea	62	Aug 95	0.02	2.41 (1.46)	34.77 (0.39)	74° 11' N, 34°31' E	73° 40' N	74° 40' N	34° 09' E	35° 09' E	Jónsdóttir et al. (2003)
(2) Bear Island	50	Aug 98	0.02	4.42 (1.97)	34.98 (0.12)	74° 00' N, 21° 30' E	73° 30' N	74° 30' N	19° 00' E	22° 00' E	This study
(3) Fugleøybanken	100	Jan 93	0.12	7.59 (0.50)	34.22 (0.39)	69° 41' N, 16° 46' E	69° 00' N	70° 00' N	15° 30' E	17° 00' E	Fevolden & Pogson (1997)
(4) Vestfjorden	32	Feb 93	0.063	8.36 (1.20)	33.23 (0.45)	68° 13' N, 14° 08' E	67° 12' N	68° 12' N	13° 06' E	15° 06' E	Fevolden & Pogson (1997)
(5) Jokaldjup	34	May 96	0.265	8.55 (0.55)	–	64° 06' N, 24° 09' W	63° 00' N	65° 00' N	23° 00' W	25° 00' W	Coughlan (2001)
(6) Reykjanesgrunn	49	Jan 98	0.673	8.85 (0.00)	35.04 (0.00)	63° 47' N, 22° 50' W	63° 17' N	64° 17' N	22° 20' W	23° 20' W	Jónsdóttir et al. (1999)
(7) Eyrabakkabugur	31	Jan 98	0.549	9.36 (1.56)	34.64 (0.49)	63° 47' N, 21° 40' W	63° 17' N	64° 17' N	21° 10' W	22° 10' W	Jónsdóttir et al. (1999)
(8) Selvogsbanki	55	Apr 98	0.336	9.48 (1.38)	–	63° 42' N, 21° 56' W	62° 25' N	64° 25' N	20° 33' W	22° 33' W	Coughlan (2001)
(9) Loftstaðahraun	95	Apr 97	0.833	8.85 (1.39)	–	63° 42' N, 21° 56' W	63° 12' N	64° 12' N	20° 11' W	21° 11' W	Jónsdóttir et al. (1999)
(10) Kantur	87	Mar 97	0.299	9.42 (0.90)	35.13 (0.19)	63° 47' N, 20° 51' W	62° 00' N	63° 00' N	20° 00' W	16° 30' W	Jónsdóttir et al. (1999)
(11) Austfjarðadjup	82	Oct 97	0.47	6.69 (2.29)	34.72 (0.39)	64° 33' N, 12° 20' W	64° 03' N	65° 03' N	11° 50' W	12° 50' W	Jónsdóttir et al. (1999)
(12) Iceland-Faeroes Ridge	50	Oct 97	0.21	8.76 (0.40)	35.22 (0.04)	62° 53' N, 08° 59' W	62° 03' N	63° 03' N	08° 19' W	09° 19' W	Coughlan (2001)
(13) Faeroes Plateau	55	Sep 97	0.88	8.27 (0.15)	35.19 (0.03)	62° 20' N, 07° 10' W	61° 50' N	63° 50' N	06° 40' W	07° 40' W	Coughlan (2001)
(14) Shetland	128	Feb 03	0.965	10.61(1.22)	34.61 (0.88)	60° 75' N, 02° 00' E	60° 15' N	61° 15' N	00° 30' E	03° 30' E	This study
(15) Moray Firth	80	Mar 02	1	12.48 (1.18)	34.59 (0.34)	58° 00' N, 03° 00' W	57° 25' N	58° 13' N	03° 28' W	02° 06' E	This study
(16) Southwest Norway	100	Jan 03	0.965	11.98 (1.59)	32.98 (2.31)	58° 25' N, 03° 05' E	57° 45' N	58° 45' N	02° 30' E	04° 30' E	This study
(17) Aberdeen	100	Jan 03	0.975	10.99 (1.14)	34.70 (0.18)	56° 54' N, 00° 30' W	57° 24' N	56° 24' N	02° 00' W	01° 00' E	This study
(18) North Ireland	50	Aug 00	1	11.13 (0.99)	33.68 (0.915)	56° 00' N, 08° 00' W	56° 18' N	56° 18' N	06° 40' W	07° 40' W	Coughlan (2001)
(19) Flamborough Head	100	Feb 02	0.995	10.63 (1.35)	34.15 (0.63)	54° 07' N, 00° 16' E	55° 18' N	56° 18' N	00° 00' E	05° 00' E	This study
(20) Irish Sea	109	Aug 00	1	12.18 (1.42)	33.29 (1.12)	53° 45' N, 05° 30' W	52° 30' N	54° 30' N	06° 00' W	03° 00' W	Coughlan (2001)
(21) Celtic Sea	110	Aug 00	1	12.57 (1.47)	34.62 (0.74)	51° 30' N, 06° 00' W	50° 30' N	52° 00' N	08° 30' W	05° 30' W	Coughlan (2001)
(22) Southern Bight	100	Feb 02	1	14.06 (1.70)	32.57 (2.87)	51° 48' N, 02° 16' E	51° 00' N	52° 00' N	02° 00' E	04° 00' E	This study
(23) Gdansk Deep	40	Sep 02	1	–	–	54° 51' N, 18° 45' E	–	–	–	–	This study

carried out using an MJ Research Tetrad® thermocycler using 1 cycle at 95°C for 60 s, 5 cycles at 94°C for 45 s, 60°C for 30 s, 72°C for 60 s, 35 cycles of 94°C for 45 s, 58°C for 30 s, 72°C for 60 s, and a final annealing step of 72°C for 180 s. Of the PCR product, 3 µl was then digested in a solution containing 5 U *Dra*I restriction endonuclease (Promega), 14.3 µl of ddH_2O, 1× Promega Buffer B and 0.1 mg ml^{-1} BSA at 37°C for 6 h, with a final enzyme-denaturing step of 65°C for 5 min. Fragments were sized using electrophoresis on 1.5% agarose gels and visualised by staining with ethidium bromide.

Northeast Atlantic environmental data. Sea-surface temperature (SST) and salinity data for the period between 1980 and 2002, which correspond to the chosen sampling areas, were obtained from the International Council for the Exploration of the Sea (ICES: www.ices.dk/ocean) and the World Ocean Database (www.nodc.nooa.gov/) websites. Ambient temperature and salinity data at depth were not available, so surface data were used based upon the assumption that high mortality, and likely selection, occurs predominantly during the pelagic stage when cod larvae are drifting in surface waters (Brander 2000). Data were sorted by month, and then mean temperature and salinity for April, May and June (approximately the time of larval development) were calculated across the 22 yr time period, to provide a long-term average that is less susceptible to interannual fluctuation (Planque & Frédou 1999). The relationships of *Pan*I allele frequency to latitude, salinity and temperature were then examined.

Norwegian fjord environmental data. In an attempt to distinguish between the effects of latitude, salinity and temperature, salient data from Norwegian coastal and northeast Arctic cod (Fevolden & Pogson 1997) were reanalysed (Table 2). Ambient temperature and salinity data from the location, depth and date at which the cod were sampled were obtained from the website of the 'Sea Environmental Data from Northern Norwegian Fjords and Coastal Areas' project (http://lupus.nfh.uit.no). Such detailed data are crucial for the fjord analyses given the occurrence of marked seasonal variation and vertical stratification in both temperature and salinity (Ottersen et al. 1998). The relationships between *Pan*I allele frequency and salinity, depth and temperature were examined using samples for which corresponding environmental data were available.

Statistical analysis. *Pan*I allele frequencies could not be transformed adequately to calculate parametric test statistics, and therefore Mantel tests were used to investigate the relationship between environmental variation and genetic variation at the *Pan*I^A locus. A matrix of genetic similarity between

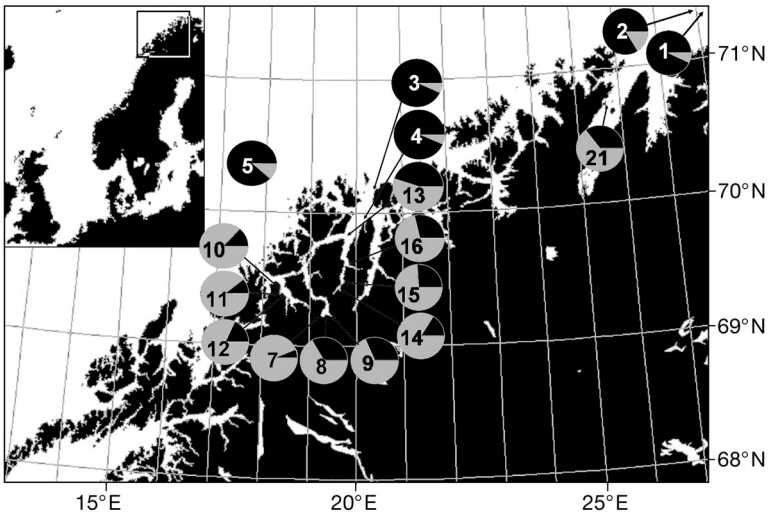

Fig. 2. *Gadus morhua*. Location and *Pan*I allele frequencies of samples analysed by Fevolden & Pogson (1997). Pie charts represent relative allele frequencies. Grey: *Pan*I^{A}; black: *Pan*I^{B}

populations was constructed using Nei et al.'s (1983) genetic distance (D_A), and corresponding matrices of differences in mean June SST, mean June salinity and latitude were also constructed. Simple Mantel tests (Mantel 1967, Mantel & Valand 1970) were used to test correlations between pairs of matrices whilst the use of partial Mantel tests (Manly 1997, Fortin & Gurevitch 2001) allowed the effect of a third variable, such as geographic distance, to be excluded. Differences in mean salinity and temperature for April and May were also analysed. Simple and partial Mantel tests were performed using 'zt' software with 99 999 permutations (Bonnet & Van de Peer 2002).

The Gdansk sample was collected at the eastern extreme of the Baltic Sea hybrid zone reported by Nielsen et al. (2003). Due to the striking halocline and thermal stratification in the Baltic Sea, in addition to clearly limited gene flow between the Baltic and North Seas, surface temperature and salinity data were not applied to this sample and it was not included in the analysis of the northeast Atlantic transect. However, the sample provided a valuable opportunity to observe *Pan*I allele frequency distribution outside the Norwegian fjords in an environment differing strongly in both salinity and temperature from the North Sea.

RESULTS

When visualised on agarose, individuals homozygous for the absence of the polymorphic *Dra*I restriction site (*Pan*I^{A} allele) produced a single 313 bp band; heterozygotes produced 3 bands (104, 209 and 313 bp); and individuals homozygous for the presence of the restriction site (*Pan*I^{B} allele) produced 2 bands (104 and 209 bp). No statistically significant deviations of genotype frequencies from Hardy-Weinberg equilibrium were detected in any of the samples of the present study or in those taken from the literature.

Northeast Atlantic transect

By combining data from the literature with samples from the southern range of Atlantic cod, the distribution of *Pan*I allele frequencies could be examined across a wide latitudinal range, extending from 51° 30′ N to 74° 11′ N. The mean annual surface temperature across this area ranged from 3.2 to 12.2°C, with a minimum temperature of 0.4°C in the Barents Sea and a maximum temperature of 18.1°C in the Southern Bight, closely corresponding to the temperature ranges reported for cod in previous studies (Björnsson et al. 2001 and references therein). In contrast, variation in surface salinity was low, with the minimum (31.06) and maximum (35.35) values occurring in the Celtic Sea in October and November, respectively.

A striking north–south gradient in allele frequency was apparent (Fig. 1), with southern populations fixed for the *Pan*I^{A} allele and increasing frequencies of the *Pan*I^{B} allele in the more northerly populations. Across all populations, simple Mantel tests (Table 3) indicated that genetic distance was significantly correlated with differences in mean June SST ($r = 0.621$, $p < 0.001$), and differences in latitude ($r = 0.779$, $p < 0.001$) (Fig. 3). Salinity variation showed no correlation with genetic distance ($r = 0.023$, $p = 0.353$) and was excluded from further analysis. Although geographic distance is strongly correlated with genetic distance ($r = 0.603$, $p < 0.001$), temperature differentials nevertheless remained significantly correlated to genetic distance when the effect of geographic distance was controlled ($r = 0.363$, $p < 0.001$). Mean temperature and salinity data for April and May gave similar results to those for June and are not presented.

In common with North Sea samples, the Gdansk sample was fixed for the *Pan*I^{A} allele. Temperature below the Baltic halocline shows limited seasonal variation, and ranges between 4 and 6°C (Kullenberg 1981), which is similar to northern waters in which the *Pan*I^{B} allele is prevalent. On the other hand, the high frequency of the *Pan*I^{A} allele in the low salinity of the eastern Baltic is consistent with Norwegian fjord data.

Table 2. *Gadus morhua*. Details of samples contributing to the Norwegian fjord transect. Data from Fevolden & Pogson (1997) and from http://lupus.nfh.uit.no showing the temperature and salinity readings which most closely match the date, depth and location of sampling sites. Juv: juvenile; Ad: adult

Locality	Data from Fevolden & Pogson (1997)					Temperature and salinity data from http://lupus.nfh.uit.no				
	Stage	Sampling date	Sampling depth (m)	n	Frequency of *Pan*I[A] allele	Station name	Station position	Sample date (d/mo/yr)	Sample depth (m)	Temp. (°C)	Salinity
(1) Barents Sea (70° 49.4′ N, 28° 27′ E)	Juv	Sep 95	150	29	0.069	Helnes1	71° 07′ N, 26° 18′ E	06/09/95	150	6.399	34.833
(2) Barents Sea (70° 42.0′ N, 25° 57.1′ E)	Juv	Sep 95	200	28	0.161	Helnes1	71° 07′ N, 26° 18′ E	06/09/95	200	5.558	34.917
(3) NW Arnøya	Juv	Nov 95	440	37	0.068	Spenna	70° 07′ N, 20° 17′ E	08/11/95	315	6.871	34.658
(4) Eidstranddjupet	Juv	Nov 95	270	23	0.065	Karlsøy	69° 57′ N, 20° 04′ E	08/11/95	234	5.889	34.479
(5) Fugleøybanken	Ad	Jan 93	158	100	0.12	GSDJupet2	69° 50′ N, 17° 13′ E	29/01/93	150	6.779	34.512
(7) Balsfjorden	Juv	Sep 95	2–0	65	0.938	Tennes	69° 17′ N, 19° 22′ E	08/09/95	0	9.486	26.911
(8) Balsfjorden	Ad	Feb 93	100	69	0.667	Tennes	69° 17′ N, 19° 22′ E	22/02/93	100	3.319	33.422
(9) Balsfjorden	Ad	Sep 95	82	27	0.685	Tennes	69° 17′ N, 19° 22′ E	08/09/95	82	4.759	33.232
(10) Malangen	Juv	Sep 94	2–0	78	0.865	Målsnes	69° 21′ N, 18° 35′ E	01/09/94	0	8.983	19.331
(11) Malangen	Juv	Sep 95	2–0	46	0.819	Målsnes	69° 21′ N, 18° 35′ E	07/09/95	0	9.915	26.268
(12) Malangen	Ad	Feb 93	415	30	0.817	Målsnes	69° 21′ N, 18° 35′ E	15/02/93	113	6.493	34.219
(13) Ullsfjorden (Eidstrand)	Juv	Sep 94	2–0	30	0.55	UllsfjordM	69° 49′ N, 19° 50′ E	01/09/94	0	9.161	33.184
(14) Ullsfjorden (Sørfjorden; Skjåberg)	Juv	Sep 95	2–0	40	0.837	Storura	69° 25′ N, 19° 33′ E	07/09/95	0	9.175	24.146
(15) Ullsfjorden (Sørfjorden; Skognes)	Juv	Sep 95	2–0	69	0.739	Njoskejufta	69° 28′ N, 19° 41′ E	07/09/95	0	8.943	28.08
(16) Ullsfjorden (Jøvika)	Juv	Sep 95	2–0	28	0.714	Jøvik	69° 38′ N, 19° 47′ E	07/09/95	0	8.845	31.32
(21) Porsangerfjorden (Reppvåg)	Juv	Sep 95	2–0	31	0.645	Porsanger Ytre V	70° 43′ N, 25° 44′ E	05/09/95	0	8.804	32.649

Norwegian Fjord transect

Fevolden & Pogson (1997) described a strong gradient in *Pan*I allele frequencies between Norwegian coastal and northeast Arctic cod (Fig. 2). Simple Mantel tests examining correlations between genetic distance and differences in temperature, depth and salinity, showed that depth and salinity, but not temperature, had a significant effect (Table 4). However, 4 of the 16 populations, (Fugleøybanken 5, Balsfjorden 8, Balsfjorden 9 and Malangen 12) (Fig. 4A) were composed of adults, while the rest were juveniles. When the analysis was repeated with juveniles only, differences in depth, salinity and temperature all showed highly significant relationships with genetic distance, remaining significant after sequential Bonferroni correction. Partial Mantel correlations were also all significant after sequential Bonferroni correction, with the exception of the correlation between genetic distance and depth, while the effect of temperature was controlled. Controlling for each variable in turn, and testing all possible combinations, temperature, depth and salinity all yielded significant statistical correlations with genetic distance in the juvenile fjord cod. No significant relationship between *Pan*I allele frequency and any environmental variable was detected if the analysis was restricted to the 4 adult samples.

DISCUSSION

The data collected here, combined with those collated from the literature, reveal 2 clear zones in which *Pan*I allele frequencies change rapidly over relatively small geographic distances. Several scenarios may explain such findings; past geographical isolation and subsequent secondary contact may result in hybrid zones within the northeast Atlantic transect, around Iceland, and within the Norwegian Fjord transect. The covariation of *Pan*I allele frequency distribution with ecological variation would, however, be unusually coincidental if historical isolation alone was responsible for geographic distribution of *Pan*I alleles. Alternatively, strong contemporary selection limiting gene flow or a combination of historical isolation and selection could yield an allelic frequency cline across an environmental gradient. Such a cline may also be maintained by behavioural segregation into differing environments. Clearly, these potential mechanisms may interact with each other, and conceivably different factors may be responsible for the development and the maintenance of the cline. Strong gradients in *Pan*I allele frequency at the very least suggest limited effective

Table 3. *Gadus morhua*. Analysis of northeast Atlantic transect data. Simple Mantel tests of the correlation between genetic distance (Gendist) and environmental variables, and Partial Mantel tests estimating the correlation between genetic distance and environmental variables while keeping the effect of a third variable (in parentheses) constant. Asterisks denote level of significance (*p < 0.05, **p < 0.01, ***p < 0.001). Tests significant after table-wide sequential Bonferroni correctionare in bold

	Matrices compared	r	p
Simple Mantel tests	Gendist × Temperature	0.621	**<0.001*** **
	Gendist × Latitude	0.779	**<0.001*** **
	Gendist × Salinity	0.023	0.353
	Gendist × Geographic Distance	0.603	**<0.001*** **
Partial Mantel tests	Gendist × Temperature (Geographic distance)	0.363	**<0.001*** **
	Gendist × Geographic distance (Temperature)	0.268	**0.002****

migration, which has important implications for studies of stock structure, regardless of whether contemporary selection or historical isolation is the cause.

Causes of *Pan*I allele frequency transition zones

Although the widely acknowledged natural selection at the *Pan*I locus (Pogson 2001, Pogson & Fevolden 2003, Canino & Bentzen 2004, Pogson & Mesa 2004) could feasibly result in the transition zones described here, the steep allele frequency cline may also be a consequence of historical isolation and secondary contact. *Fundulus heteroclitus*, for which mitochondrial (mt)DNA analysis has identified 2 major haplotype assemblages separated by a transition zone (González-Villaseñor & Powers 1990), is a well-known example of such processes. Indeed, adaptive variation seen at many protein-coding loci (Powers & Schulte 1996) may be a consequence of historical isolation and secondary contact (Hilbish 1996). Hybrid zones have been described in Atlantic cod (Nielsen et al. 2003), but evidence for secondary contact along *Pan*I transition zones from mtDNA is equivocal. Árnason & Pálsson (1996) found little differentiation at the mtDNA cytochrome *b* gene between the proposed Arctic and coastal cod stocks while Dahle (1991) detected major divergence between these 2 groups. The discordance between these studies may be due to the sampling and assignment of fish into Arctic and coastal groups; Árnason & Pálsson (1996) assigned individuals purely on the basis of their capture location, thus potentially sampling mixed populations, while Dahle (1991) differentiated between Arctic and coastal cod using haemoglobin polymorphism. Considerable differences in haplotype diversity also exist among some geographically proximate Icelandic populations, though there is no evidence of any overall geographic pattern (Árnason et al. 2000). The divergence between Norwegian Arctic and coastal cod described by Dahle (1991), together with the patterns seen in Icelandic cod are consistent with the data presented here and may suggest that the transition zones between *Pan*I allelic groups are due to historical isolation and subsequent secondary contact. Such a possibility is further substantiated by Fevolden & Pogson (1997), who found evidence of mixing between genetically divergent populations, indicated by an increase in F_{IS} from 0.002 at the head of Ullsfjorden, to 0.275 near the mouth of the fjord, as the *Pan*I^{B} allele becomes more prevalent.

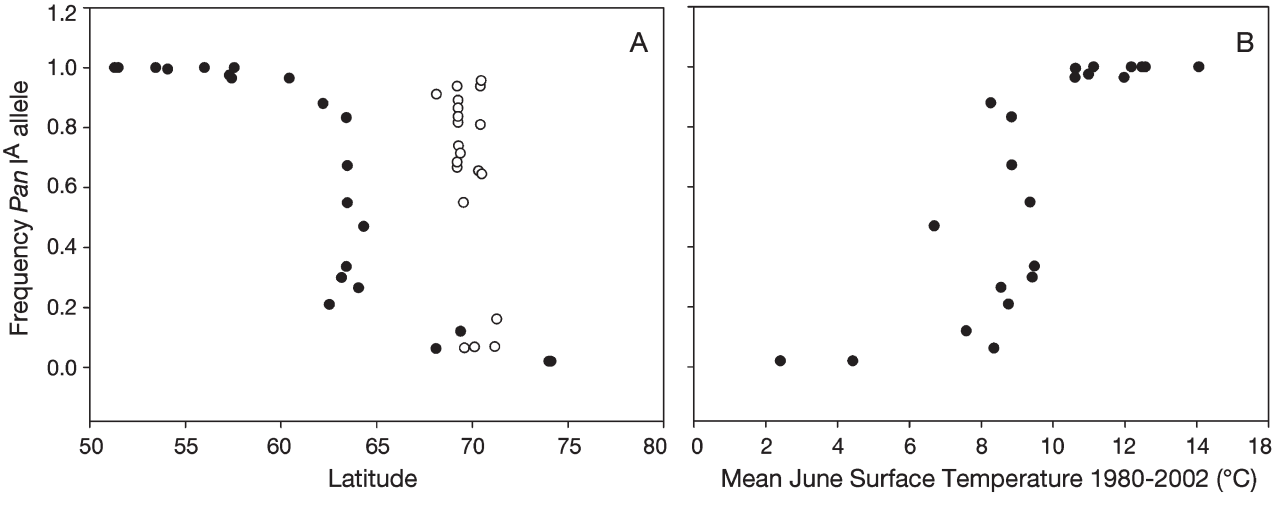

Fig. 3. *Gadus morhua*. (A) *Pan*I^{A} allele frequency and latitude for the northeast Atlantic (●) and the Norwegian fjord transects (○). (B) *Pan*I^{A} allele frequency and mean surface temperature in June in the northeast Atlantic

Table 4. *Gadus morhua.* Analysis of Nowegian fjord data. Simple Mantel tests of the correlation between genetic distance (Gendist) and environmental variables, and partial Mantel tests estimating the correlation between genetic distance and environmental variables while keeping the effect of a third variable (in parentheses) constant. Asterisks denote levels of significance (*p < 0.05, **p < 0.01, ***p < 0.001. Tests significant after table-wide sequential Bonferroni correction are in bold

	Matrix compared	r	p
Simple Mantel tests			
All samples	Gendist × Temperature	0.168	0.068
	Gendist × Salinity	0.306	0.020*
	Gendist × Depth	0.398	0.008**
Juvenile samples	Gendist × Temperature	0.823	**<0.001***
	Gendist × Salinity	0.502	**0.004****
	Gendist × Depth	0.704	**<0.001***
Partial Mantel tests			
All samples	Gendist × Temperature (Depth)	0.068	0.265
	Gendist × Depth (Temperature)	0.371	0.01**
	Gendist × Temperature (Salinity)	0.111	0.170
	Gendist × Salinity (Temperature)	0.280	0.030*
	Gendist × Depth (Salinity)	0.394	0.013*
	Gendist × Salinity (Depth)	0.300	0.0157*
Juvenile samples	Gendist × Temperature (Depth)	0.662	**<0.001***
	Gendist × Depth (Temperature)	0.352	0.016*
	Gendist × Temperature (Salinity)	0.832	**<0.001***
	Gendist × Salinity (Temperature)	0.539	**<0.001***
	Gendist × Depth (Salinity)	0.753	**<0.001***
	Gendist × Salinity (Depth)	0.599	**<0.001***

Sarvas & Fevolden (2005) also report significant deficits of heterozygotes (possible Wahlund effects) in several Norwegian populations, although none of the samples used in the present study revealed such deviations. The strong correlations between *Pan*I allele frequencies, *Hb*-I[1] and blood type E frequencies led Sarvas & Fevolden (2005) to state that Norwegian coastal cod and northeast Arctic cod represent different breeding units. These markers are clearly under the influence of selection, and in the case of *Hb*, known to influence temperature preference (Petersen & Steffensen 2003), but the results of mtDNA studies, significant Wahlund effects in some mixed populations, and concordance of *Pan*I allele frequencies, *Hb*-I[1] and blood type E frequencies, suggest that historical isolation is a probable cause of differences between Norwegian coastal and Arctic cod.

Maintenance of *Pan*I allele frequency transition zones

There is strong evidence for an unusual mix of balancing and directional selection at the *Pan*I locus. Comparison of neutral and selected mutations in the *Pan*I[A] allele, though inconclusive regarding historical isolation versus contemporary selection, led Pogson & Fevolden (2003) to conclude that Norwegian coastal and Arctic cod have experienced recent diversifying selection at the *Pan*I locus that is not reflected in the slower, neutral evolution of mitochondrial genes. However, they compared their *Pan*I variation with the mtDNA results of Árnason & Pálsson (1996), who may have inadvertently sampled mixed populations, rather than mtDNA haplotypes specific to *Pan*I genotypes.

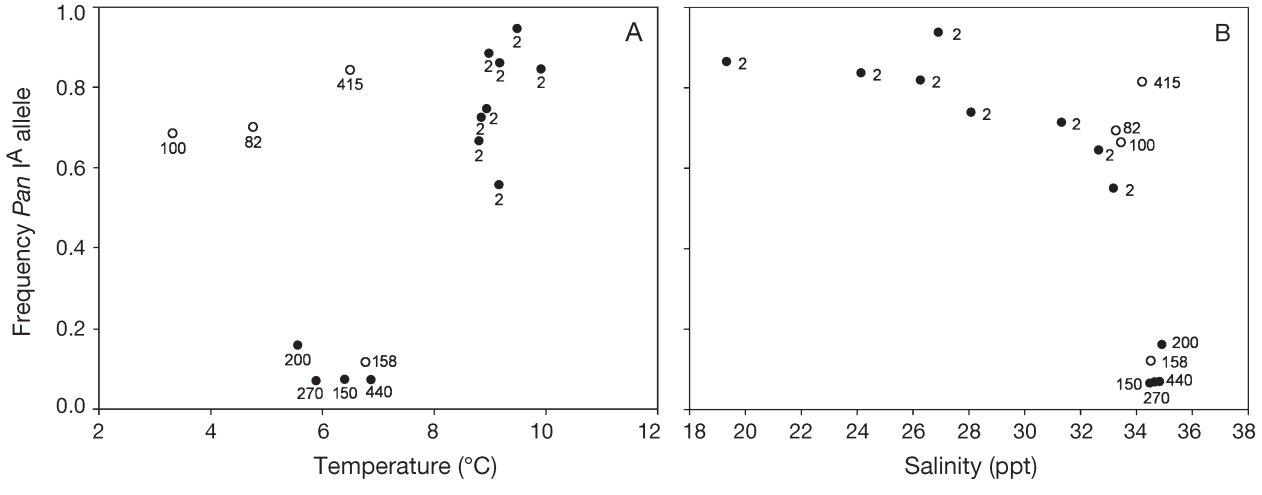

Fig. 4. *Gadus morhua. Pan*I[A] allele frequency to (A) temperature and (B) salinity in the Norwegian fjords for adult (○) and juvenile (●) cod. Allele frequency and depth data (accompanying each point) are from Fevolden & Pogson (1997). Temperature and salinity data are from http://lupus.nfh.uit.no and correspond as closely as possible to the location, date and depth at which the cod were sampled

Where steep gradients in allele frequency occur, correlation with environmental data may provide important information about the possible role of selection in maintaining patterns of genetic differentiation (Powers & Place 1978, Hilbish et al. 1982, Koehn et al. 1983, Schulte et al. 1997, Powers & Schulte 1998, Schulte 2001). On the northeast Atlantic transect, only temperature correlated significantly with *Pan*I allele frequency, and a partial Mantel test indicated that the correlation remained significant after excluding the effect of geographic distance. In the fjords, partial Mantel tests indicated that depth, temperature and salinity had a significant effect on the distribution of *Pan*I allele frequencies in juvenile cod. However, due to stratification in the fjords and the consequent covariation of temperature and salinity with depth, it was not possible to separate the effects of different variables, even with partial Mantel tests.

The lack of congruence between the 2 transects in the relationship between salinity and *Pan*I allele frequency may be due to the use of surface salinity data instead of data collected at the salient depth. Alternatively, the greater range of salinity present in the fjords may exert a stronger stock-specific selective pressure on fjord fish than that experienced by the northeast Atlantic fish. Indeed, the Gdansk sample, which originates from a region of the Baltic that does not exceed a salinity of 20 (Kullenberg 1981), yielded *Pan*I allele frequencies most similar to the least saline fjord samples, though the high *Pan*I^{A} allele frequency in this sample may also reflect recent separation from the proximate North Sea populations. There are physiological differences between brackish and open-ocean cod in response to salinity variation (Nelson et al. 1996), and the Baltic salinity gradient may act as an ecological barrier, preventing cod from the Belt Sea spawning in the low-salinity conditions of the eastern Baltic (Nissling & Westin 1997). Furthermore, in the extremely low salinity conditions of the Baltic Sea (7 to 16), cod significantly prefer the highest available salinities (Tomkiewicz et al. 1998). These physiological data support the notion of selective advantage of the *Pan*I^{A} allele in the low-salinity cold Baltic, which is typically associated with warmer waters in the Norwegian transect. Furthermore, they suggest that in areas of exceptionally low salinity close to the tolerance limit of cod, temperature is likely to play a reduced role in determining *Pan*I genotype.

In areas of 'normal' salinity (e.g. the northeast Atlantic transect), on the other hand, temperature may be the determining factor in *Pan*I allele frequencies. Indeed, there is considerable evidence that temperature may determine cod distribution and recruitment (Clark & Green 1990, Rose 1993, Ottersen & Sundby 1995, Ottersen et al. 1998, Castonguay et al. 1999, Planque & Frédou 1999, Petersen & Steffensen 2003), although this may be due more to behavioural segregation than direct selection. It has been shown that cod are extremely sensitive to changes in temperature and will actively maintain their position in water of optimum temperature (Clark & Green 1990, Castonguay et al. 1999). Furthermore, cod with different haemoglobin genotypes not only exhibit considerable clinal variation related to temperature, similar to the pattern for *Pan*I, but they also have markedly different thermal preferences that vary by 7.2°C (Petersen & Steffensen 2003). Vertical segregation according to genotype, regardless of the cause, would explain the striking disjunct distribution of juvenile cod in the Norwegian fjords, as well as some inconsistencies in previous studies of variation at the *Pan*I locus. For example, Fevolden & Pogson (1997) described temporal variation in 3 samples from 1 location in Balsfjord with respective *Pan*I^{A} allele frequencies of 0.667, 0.685 and 0.938. Different sampling depths (100, 82 and 2–0 m, respectively) and associated variation in temperatures (3.32, 4.76 and 9.5°C, respectively) may underlie such observed allelic variation. Furthermore, in the thermally stratified waters, such as those off the coast of Iceland and the Faroes, temperature at a single position can vary considerably with depth, providing an environment in which fish adapted to very different ecological conditions could coexist. Indeed, recent results from cod tagged on the spawning grounds off southwest Iceland indicate that, though sharing a common spawning ground, the fish segregate during feeding migrations into 2 distinct groups which inhabit shallow warm water and deep cold water (Pálsson & Thorsteinsson 2003). It remains to be seen whether variation at the *Pan*I locus (or neutral loci) is partitioned between the 2 groups. However, such findings indicate that vertical structuring does occur, providing a plausible explanation for the extensive allelic variation in Icelandic populations and the Balsfjord samples of Fevolden & Pogson (1997). The results of Jónsdóttir et al. (2001) also support such an assertion, showing considerably higher *Pan*I^{A} allele frequency at Loftstaðahraun (50 to 70 m) compared to Kantur (150 to 450 m). It is noteworthy that these populations were sampled during the spawning season and that depth differences between the populations are likely to be even further accentuated outside the spawning season (Pálsson & Thorsteinsson 2003).

The northwest Atlantic environmental data used here are subject to limitations, notably a lack of depth data, which is crucial in stratified waters. Although SST is significantly correlated to genetic distance, it does not take depth into account, and salinity and temperature measurements taken from the depth at which adult fish are distributed, particularly in the stratified

Icelandic waters, would help to clarify relationships between ecological variation and *Pan*I. Of 4 adult populations sampled by Fevolden & Pogson (1997), 3 are outliers (Balsfjorden 8, Balsfjorden 9 and Malangen 12) (Fig. 4A), highlighting the importance of sampling age groups consistently. It was not possible to discern any relationship between genetic distance and temperature, depth or salinity with only 4 adult populations. However, adults may be expected to behave differently to juveniles, particularly since the temperature preferences of cod and several other species decrease with increasing body size (Björnsson et al. 2001, Lafrance et al. 2005).

Despite these limitations, the data, together with evidence from the literature, suggest that temperature, depth and salinity are linked to selection at the *Pan*I locus, and may maintain clines that have possibly developed due to secondary contact. What is less certain is the relative contribution of selection and limited dispersal to maintenance of these clines. Whether selection and migration are weak or strong, it is clear that gene flow in the Norwegian and Icelandic waters is limited, but without knowledge of the strength of selection occurring at the *Pan*I locus, it is impossible to determine the extent of gene flow in cod populations from *Pan*I data. However, data from a transplant experiment of Arctic cod to southern Norway show differences in growth but not survival among *Pan*I genotypes (Case et al. in press), suggesting that selective effects, although undoubtedly present, are relatively weak. Weak selection would require limited effective migration to explain such rapid shifts in allele frequency. There is good evidence that behavioural segregation may moderate migration and contribute to the maintenance of *Pan*I clines, explaining the strong correlations between *Pan*I allele frequency variation and environmental variation. Further studies estimating selection coefficients in a variety of environments may render *Pan*I a powerful marker to elucidate population structure, local adaptation and temporal analysis of adaptation to global climate change.

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