



Settling-depth vs. genotype and size vs. genotype correlations at the *Pan I* locus in 0-group Atlantic cod *Gadus morhua*

Svein-Erik Fevolden^{1,*}, Jon-Ivar Westgaard^{1,2}, Torstein Pedersen¹, Kim Præbel¹

¹Department of Arctic and Marine Biology, Faculty of BioSciences, Fisheries and Economics, University of Tromsø, 9037 Tromsø, Norway

²Institute of Marine Research, PO Box 6404, 9294 Tromsø, Norway

ABSTRACT: We sampled 0-group juvenile Atlantic cod *Gadus morhua* L. within fjords and off-shore in northern Norway from 1994 to 2008 using different gears for the 3 sampling depths: shore seine (0–3 m), pelagic trawl (various depths), and bottom trawl (>80 m). Frequencies of alleles at the *Pan I* locus (4218 fish analysed) showed highly significant differences among samples collected in the different habitats. The *Pan I*^A allele showed a mean frequency of ~80% in the shore seine samples, 12% in the bottom trawl samples, and between 5 and 57% in the pelagic samples. These differences are thought to reflect the co-occurrence of different populations of cod in the area with different settling regimes. Shallow-water settlers are thought to represent the stationary Norwegian coastal cod (NCC), the deep-water settlers represent the migratory Arcto-Norwegian cod (ANC), and the non-settled individuals represent a variable mixture of the 2 populations. For designated samples, we analyzed 16 microsatellites (non-neutral and neutral) that supported a genetic divergence between shallow-water and deep-water settlers. Correlations between length and *Pan I* genotypes within selected samples showed that the *Pan I*^{BB} homozygotes (typical of ANC) were significantly longer than the *Pan I*^{AA} homozygotes (typical of NCC). This could reflect differences in spawning time and growth conditions between ANC and NCC, just as well as one genotype being superior to the others in terms of growth performance. Finally, we argue that the *Pan I* difference between 0-group NCC and ANC is not caused by contemporary selection but reflects adaptation on an ecological post-glacial time scale.

KEY WORDS: Arcto-Norwegian cod · Norwegian coastal cod · 0-group juveniles · *Pan I* locus · Settling regimes · Population divergence

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INTRODUCTION

Following the very first reports on variation in Atlantic cod *Gadus morhua* L. at the *Pan I* (pantophysin) locus (Pogson et al. 1995, Fevolden & Pogson 1995; *Pan I* is identical to the GM798 clone in those papers), a discussion evolved about the power of this locus as a marker for distinguishing populations due to its non-neutral character. Pantophysin

is a membrane protein found in small synaptic transmitter vesicles (Haass et al. 1996), but its function is yet not fully understood, nor are the mechanisms underlying the positive Darwinian selection acting on the gene coding for it (Pogson 2001, Pogson & Mesa 2004). Various physical and oceanographic features have been suggested to act as selective constraints on the locus, with depth and temperature being frequently mentioned candidates

*Email: svein-erik.fevolden@uit.no

(Case et al. 2005, Sarvas & Fevolden 2005a, Pampoulie et al. 2008). One reason for the initial dispute was that *Pan I* exhibited profound divergence among populations of Atlantic cod, e.g. over its total distributional area in the North Atlantic (Pogson et al. 1995), where neutral markers like allozymes (Mork et al. 1985) or mtDNA (Árnason 2004) had disclosed only shallow genealogies. Currently there seems to be a general consensus that *Pan I* is indeed a valid first hand indicator of population divergence in Atlantic cod. The claimed weakness of this locus as being influenced by selection was early on considered its strength, since it can reveal adaptation to local environmental changes earlier than strictly neutral loci (Fevolden & Pogson 1997, Carvalho & Hauser 1998). After Atlantic cod in the NE Atlantic and adjacent waters have been studied for variation at an array of microsatellite loci (e.g. Nielsen et al. 2006, Skarstein et al. 2007, Westgaard & Fevolden 2007, Pampoulie et al. 2011) and single nucleotide polymorphisms (SNPs; e.g. Moen et al. 2008, Nielsen et al. 2009a, Poulsen et al. 2011), the important role of selected markers to reveal adaptive population divergence on ecological time scales seems no longer disputable (see also Waples & Gaggiotti 2006, Helyar et al. 2011, and reviews by Kirk & Freeland 2011 and Salmenkova 2011). The power of assigning individuals to populations of origin is also strengthened by exploiting loci under selection (Nielsen et al. 2009b).

The most striking divergence at the *Pan I* locus exhibited over very short distances has been reported from northern Norway (e.g. Fevolden & Pogson 1997, locus denoted *Syp I*; Pogson & Fevolden 2003, Sarvas & Fevolden 2005a,b). Two major populations of Atlantic cod inhabit coastal waters of northern Norway and the Barents Sea. Norwegian coastal cod (NCC; alternatively called just coastal cod) exhibit very high frequencies of the *Pan I*^A allele class (~80%), whereas Arcto-Norwegian cod (ANC; identical to Northeast Arctic cod) display similar or even higher frequencies of the *Pan I*^B allele (~90%). F_{ST} values of ~0.4 have been reported between NCC and ANC at this locus (Fevolden & Pogson 1997, Sarvas & Fevolden 2005a), meaning that 40% of the total variance observed at the *Pan I* is attributable to differences between the 2 populations. Despite these findings and the growing recognition of the advantages of implementing non-neutral loci in studies of genetic population structure, a recent review (Nordeide et al. 2011, p. 269) questioned whether ANC and NCC make up 1 large population or >1 non-interbreeding group. The authors claimed that differ-

ences between NCC and ANC first and foremost have been reported using markers that are generally agreed to be subject to selection, and thus considered '... less suited to assessing population connectivity'. Such a presumption may be correct if the purpose is to study neutral evolutionary processes (gene flow, genetic drift), but not ecological adaptations on more recent time scales.

The generally recognized NCC inhabit fjords and near-coastal areas and have spawning sites located well inside fjords, but to some extent also in outer coastal areas. Peak spawning normally occurs in March and April (Skreslet & Danes 1978). NCC are considered relatively stationary, and there seems to be a high fidelity to spawning sites (Godø 1986, Jakobsen 1987, Skjæraasen et al. 2011). The migratory ANC have nursery and feeding grounds in the Barents Sea. After maturation, they perform yearly spawning migrations down to the coast of Norway, where major spawning areas in northern Norway are found in the Lofoten and Vesterålen area and on the inside of relatively shallow near-shore banks off Troms and Finnmark counties (Bergstad et al. 1987). Some of these sites overlap with spawning sites of NCC, allowing for potential intermingling of spawning adults, eggs, and larvae of ANC and NCC. Various mechanisms have been suggested to hamper this intermingling and potential for hybridization between the 2 populations, like differences in peak time of spawning, possible differences in temperature and depth preferences for spawning, and differences in spawning behavior (Nordeide 1998, Nordeide & Folstad 2000, Sarvas & Fevolden 2005a; cf. also Grabowski et al. 2011 for similar features for spawning Icelandic cod). Eggs and larvae spawned offshore will drift northeast-ward with the prevailing Norwegian coastal current. By June to July, larvae and small juveniles are found in the southern Barents Sea as well as along the coast of Troms and Finnmark counties. Due to complex near-shore current features (Pedersen et al. 2005), one would expect some larvae to drift into fjords along the coast in the same way that larvae from fjord-spawning cod to some extent can drift out of fjords, again causing pelagic intermingling of the 2 populations before the 0-group juveniles settle.

The major focus on *Pan I* variation in Atlantic cod has been on adult harvestable year-classes. To assess whether differences at the *Pan I* locus could be linked to life history changes, it is crucial to include studies of the young. Here we summarize *Pan I* data of 0-group juvenile cod sampled in coastal areas of northern Norway in the period 1994

to 2008. The main objectives were to ascertain whether 0-group juveniles of NCC and ANC are genetically as divergent as their parents, and if the 0-group juveniles of the 2 groups behave differently, intermingling as they may in the pelagic egg or larval stage, but segregating at the time of settling. Using vertebrae numbers to discriminate the 2 populations, NCC juveniles were reported to settle in shallow water, whereas ANC juveniles settled in deeper water (Løken et al. 1994). Therefore, those that settle in shallow water would be expected to be dominated by the most common genotype of *Pan I* found in adult coastal cod (*Pan I*^{AA}). The juveniles that settle in deep water would be dominated by the most common *Pan I* genotype found in adult ANC (*Pan I*^{BB}). 0-group juveniles sampled pelagically, and thus not yet settled, could represent a mixture of ANC and NCC in areas where they co-occur. To lend support to our hypothesis that differences in *Pan I* allele frequencies in settled juveniles actually reflect the presence of 2 genetically distinct populations, shallow-water settled juveniles, pelagic non-settled juveniles, and deep-water settlers that were caught at the same location within a few hours' time interval were analyzed for variation at 16 microsatellite loci.

It has been suggested that the *Pan I* signature of Atlantic cod affects growth. In Norwegian (Fevolden & Pogson 1995) and Icelandic waters (Jónsdóttir et al. 2002, Imsland & Jónsdóttir 2003, Jakobsdóttir et al. 2011), the *Pan I*^{AA} homozygotes of post-juvenile cod have been reported to exhibit higher length at age than the *Pan I*^{BB} homozygotes. Jónsdóttir et al. (2008), however, suggested that the genotype that grew fastest around Iceland varied with spawning sites, rendering data on possible growth and *Pan I* genotype correlations for post-juvenile cod inconclusive. There is a lack of studies relating growth and *Pan I* genotypes in 0-group cod. A study comparing the growth of different *Pan I* genotypes within families held under semi-natural conditions showed that 10 wk old larvae of *Pan I*^{AB} heterozygotes grew faster than *Pan I*^{BB} homozygotes (Case et al. 2006). *Pan I*^{AA} homozygotes were not available. In selected samples, we compared the length of 0-group juveniles in the 3 different *Pan I* genotypes to check for evidence of different growth performance among them.

MATERIALS AND METHODS

Juvenile 0-group cod were sampled in fjords and offshore waters of northern Norway (Fig. 1) in the period 1994 to 2008. The total 4218 fish analyzed for *Pan I* were caught in shallow water, 0 to ~3 m, using a shore seine (number of samples: N = 53, number of fish analyzed: n = 2743), pelagically (inshore and offshore), at various depths by a pelagic trawl (N = 21, n = 1128), and at the deep bottom habitat (depth >80 m) using a bottom trawl (N = 11, n = 347). Details of the different samples are given in Table S1 in the supplement at www.int-res.com/articles/suppl/m468p267_supp.pdf. All sampling was done in August and September to enable catching newly settled juveniles. Immediately after being caught, the fish were put in 96% ethanol and stored until being analyzed. A number of samples caught up until 2001 were included in the study of Sarvas & Fevolden (2005a), who aimed at describing geographic variation of cod in the Northeast Atlantic (their sample number is given in Table S1).

DNA from samples up until 2007 was obtained by a modified salt lysis extraction (Fevolden & Pogson 1997). DNA from the 2008 samples was isolated using an E-Z96 Tissue DNA Kit (OMEGA Bio-tek) following the manufacturer's instructions. The 2-allele-class (A and B) *Pan I* locus is in itself a SNP, since a 1-nucleotide substitution decides the presence or absence of a *Dra1* restriction site. The locus was analyzed by the polymerase chain reaction (PCR) based method (Fevolden & Pogson 1997), and only the 2008 samples were analyzed including *Pan I* on a multiplex of mi-

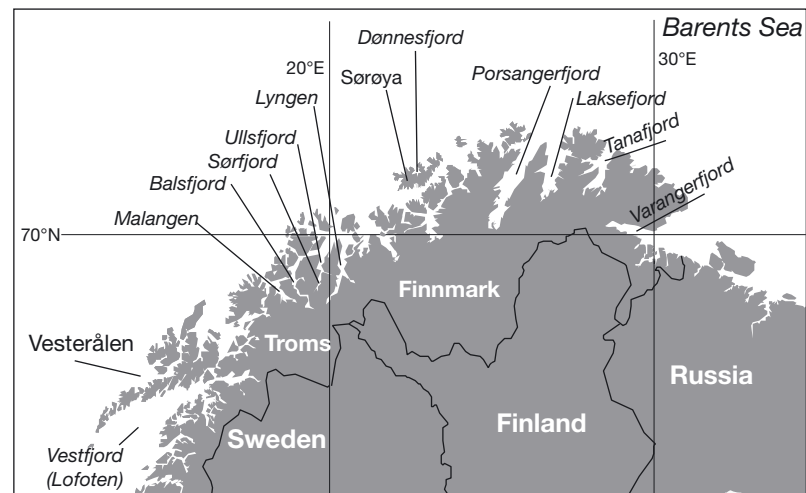


Fig. 1. *Gadus morhua*. Various fjords in northern Norway where 0-group Atlantic cod were sampled. For detailed locations see Table S1 in the supplement at www.int-res.com/articles/suppl/m468p267_supp.pdf

crossatellites and genotyped upon fragment analyses on an ABI 3130XL sequencer (Applied Biosystems) according to Stenvik et al. (2006).

In 3 cases when samples were taken within a few hours' interval at different depths for the same locality (cf. Fig. 3 and samples in Table S1 marked by ^{ms}), the fish ($n = 521$) were also analyzed for 18 microsatellite loci (see Table 2 plus PGM108 and pGMO55). These were organized in 4 multiplex PCRs. PCRs were carried out in a 10 μ l reaction containing 50 mM KCl, 10 mM Tris-HCl, 400 μ M dNTP, 1.5 mM MgCl₂, 0.1 to 1.0 μ M primer, and 0.5 units *Taq* polymerase. The PCR profile for the multiplex PCR was an initial denaturation step of 94°C for 10 min followed by 25 cycles of 94°C for 20 s, 56°C (51°C for multiplex 3) for 30 s, and 72°C for 1 min, ending with an elongation step of 72°C for 10 min. Electrophoresis of the amplified PCR products was performed using an ABI 3100 automated sequencer (Applied Biosystems). Alleles were scored using Genemapper 3.7 software (Applied Biosystems), using the automated binning function and subsequent manual verification. All microsatellite loci were initially run through the Micro-checker software (Van Oosterhout et al. 2004) for detection of possible null alleles or scoring errors.

For statistical analyses, the samples were grouped into gear (habitat) categories (shore seine: SS, pelagic trawl: PT, and deep bottom trawl: BT). The overall means of allele frequencies in each category were calculated both as arithmetic means of the frequencies in each individual sample of the different categories and from the total number of genotypes in the pooled sample set for each gear category. To examine possible variation in allele frequencies over years, all samples of 0-group cod taken at a specific year and habitat, independent of locality, were summarized, and average allele frequencies for each year were calculated both as arithmetic means over samples as well as from the total number of genotypes sampled for that year. The PT samples and in particular the BT samples were low in numbers compared to the SS samples (mainly due to lower sampling effort using those gear types), and frequencies for a particular year are occasionally only from 1 sample.

F_{IS} for individual samples (Tables S1 and S2) and pairwise F_{ST} between samples analyzed for microsatellites (see Table 2) were calculated using GenePop 4.0 (Rousset 2008), following Weir & Cockerham (1984). F_{IT} and F_{ST} for pooled samples within each sampling gear (*Pan I* data; Table 1) and their corresponding p values were calculated using the analysis of molecular variance (AMOVA) option in the soft-

ware Arlequin 3.5 (Excoffier & Lischer 2010). Corresponding SE were estimated using the software FS-TAT 2.9.3 (Goudet 2001). To visualize relationships among the 7 samples analyzed for microsatellites (Fig. 3), a multi-dimensional scaling (MDS) plot was produced using XLSTAT (Addinsoft). The MDS plot was based on Nei's D_A genetic distance (Nei et al. 1983). To detect possible outlier loci among the microsatellites, we used the Bayesian likelihood method implemented via reversible jump Markov Chain Monte Carlo in BAYESCAN (Foll & Gaggiotti 2008).

The 3 Varangerfjord samples of Fig. 3 were used in a mixed stock analysis (MSA) with GeneClass 2.0 software (Piry et al. 2004). The SS and BT juveniles were used as reference samples, with the PT juveniles classified as unknown. The distribution of individuals in the pelagic sample representing each of the 2 reference groups was estimated from 2 non-neutral microsatellites (GMO34 and GMO132; see below) plus *Pan I*. A linear regression method based on the proportion of *Pan I*^B alleles in a sample (G. Dahle unpublished) was used to estimate the proportion of NCC and ANC in all 3 pelagic samples of Fig. 3.

Length in mm was measured for juveniles in 3 designated samples, either as standard length (excluding the caudal fin) or total length. The length was compared between the different *Pan I* genotypes. Some of the length-frequency distributions differed significantly from normality when tested with a Lilliefors test. Thus, significance of differences between length distributions of the different *Pan I* genotypes was tested by the nonparametric Mann-Whitney 2-sample test.

RESULTS

Pan I allele frequencies and depth

The frequency of the 2 *Pan I* alleles varied drastically with sampling gear and thus habitat (Fig. 2), from 79% *Pan I*^A in SS samples (means of the fre-

Table 1. *Gadus morhua*. Wright's F_{IT} and F_{ST} values \pm SE for the *Pan I* locus for all samples caught with each of the 3 sampling gears (shore seine: SS; pelagic trawl: PT; bottom trawl: BT). ns: not significant

Gear	F_{IT}	p	F_{ST}	p
SS	0.177 \pm 0.034	<0.001	0.062 \pm 0.014	<0.001
PT	0.286 \pm 0.058	<0.001	0.118 \pm 0.038	<0.001
BT	0.131 \pm 0.091	<0.05	0.020 \pm 0.016	ns

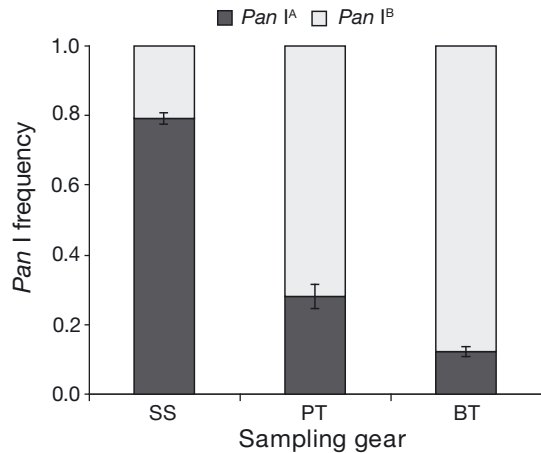


Fig. 2. *Gadus morhua*. Frequencies of the 2 *Pan I* alleles of 0-group Atlantic cod of all shore seine (SS), pelagic trawl (PT), and bottom trawl (BT) samples. Means \pm SEM

quencies in each sample; 80% when calculated from the pooled number of genotypes) to only 12% *Pan I*^A (both estimates) for 0-group juveniles caught by BT. The more intermediate mean frequencies for juveniles caught pelagically, 28% (29% estimated from the number of pooled genotypes), exhibited the largest among-sample variation, with *Pan I*^A frequencies ranging from 5 to 57% (Table S1). No correlation between sampling depth and *Pan I*^A frequencies was found for the pelagic samples ($R^2 = 0.031$, $p = 0.445$). We also found no differences in mean frequencies of the *Pan I*^A allele between pelagic juveniles sampled offshore (0.28 ± 0.17) and inshore (0.29 ± 0.16). The distribution of genotypes relative to Hardy-Weinberg expectations showed that both the PT samples (6 out of 21; 28.6%) and the SS samples (8 out of 53; 15.1%) displayed significant deficits of heterozygotes, indicative of having sampled populations of different genetic origin (Wahlund effect). One of the 11 BT samples (9.1%) deviated significantly from Hardy-Weinberg expectations, showing a deficit of heterozygotes. None of the samples displayed significant excesses of heterozygotes. Both F_{ST} and F_{IT} values within habitats were larger (and highly significant) for the PT samples than for SS and BT samples (Table 1).

Samples that were taken within few hours' time intervals from different habitats in 3 different fjords were compared (Fig. 3). In Varangerfjord, we observed *Pan I* allele frequencies very similar to the total sample set (Fig. 2). In Porsangerfjord and Laksefjord, only SS and PT samples were available. The *Pan I*^A allele frequency in the PT samples differed between the 2 fjords, but in both, a significantly

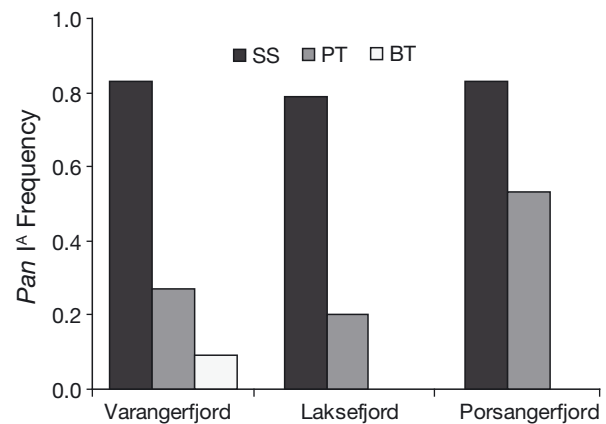


Fig. 3. *Gadus morhua*. *Pan I*^A allele frequency of 0-group Atlantic cod sampled within a few hours' time interval with different gear (see Fig. 2) in 3 fjords. Varangerfjord: sampled on 31 August and 1 September 1996, Laksefjord: 2 September 1996, Porsanger: 4 October 2000

lower *Pan I*^A allele frequency was observed in the PT sample as compared to the SS sample (Fig. 3; pairwise F_{ST} , $p < 0.001$).

The 7 sample sets of Fig. 3 were initially analyzed for variation at 18 microsatellite loci. Microchecker indicated that the locus GMA108 was severely under the influence of null alleles, and pGMO55 showed large heterozygote excess, indicative of scoring errors. Thus, these 2 loci were subsequently excluded from further analysis. Basic genetic data for the remaining 16 microsatellites are given in Table S2. Estimates of pairwise F_{ST} over all loci (Table 2; total ms) were in general low, but highest when comparing shore and deep bottom samples of Varangerfjord ($F_{ST} = 0.0122$, $p < 0.001$). Significant differences between shore and pelagic samples were found in Varangerfjord and Laksefjord, but not in Porsangerfjord (Table 2). Large contributors to the significant differences were loci GMO34 and GMO132, which both showed posterior probabilities >0.99 in the Bayesian selection test, thus being considered non-neutral. When excluding those loci and estimating F_{ST} values based on the neutral loci only, significant differences were still seen between bottom and SS samples in Varangerfjord ($F_{ST} = 0.003$, $p < 0.05$), and between pelagic and SS samples in Laksefjord ($F_{ST} = 0.003$, $p < 0.001$). F_{ST} values from the *Pan I* locus were, not surprisingly, significant for all pairwise comparisons of Table 2.

The MDS plot of microsatellite data (Fig. 4) shows that the SS samples of Fig. 3 are clustered in 1 dimension, whereas the pelagic trawl samples are grouped across dimensions. The 1 BT sample appears as an outgroup. Mixed stock analysis of the 3 Varanger-

Table 2. *Gadus morhua*. Pairwise F_{ST} comparison over 16 microsatellite loci plus *Pan I* for samples from 3 fjords (Porsanger: P, Lakse: L, and Varanger: V) taken with shore seine (SS), pelagic trawl (PT), or bottom trawl (BT). Dates of sampling are as in Fig. 3. P values are from exact tests. Significant p-values are in **bold**. Total ms = all 16 microsatellites. Neutral ms = exclusion of non-neutral microsatellites (GMO34 and GMO132). SE in () for F_{ST} estimates across loci. Superscripts 1 to 4: Multiplex 1 to 4

Locus	PSS – PPT		LSS – LPT		VSS – VPT		VSS – VBT		VPT – VBT	
	F_{ST}	p	F_{ST}	p	F_{ST}	p	F_{ST}	p	F_{ST}	p
GMO2 ²	-0.0008	0.265	-0.0010	0.468	0.0060	0.038	0.0051	0.594	0.0333	0.001
GMO3 ²	0.0084	0.849	0.0013	0.701	0.0019	0.344	-0.0069	0.910	-0.0160	0.363
GMO8 ¹			0.0073	0.001	0.0020	0.029	0.0015	0.283	-0.0028	0.921
GMO19 ¹	-0.0013	0.539	-0.0013	0.645	-0.0021	0.854	0.0042	0.087	0.0016	0.169
GMO34 ²	-0.0054	0.118	0.0707	<0.001	0.0499	<0.001	0.1020	<0.001	0.0185	0.101
GMO37 ¹	-0.0066	0.980	0.0044	0.016	-0.0025	0.666	-0.0026	0.110	-0.0051	0.766
GMO132 ²	-0.0068	0.944	0.0379	0.003	0.0508	<0.001	0.0631	<0.001	-0.0044	0.795
PGMO32 ⁴	0.0026	0.320	0.0018	0.188	0.0052	0.195	0.0097	0.104	-0.0071	0.553
GMA107 ⁴	0.0003	0.621	0.0038	0.042	0.0010	0.148	0.0030	0.084	0.0036	0.008
TCH5 ⁴	-0.0001	0.500	0.0043	0.983	-0.0011	0.865	0.0107	0.144	0.0073	0.237
TCH11 ¹	-0.0015	0.661	0.0019	0.064	-0.0020	0.826	-0.0010	0.355	0.0023	0.248
TCH13 ²	0.0085	0.132	0.0063	0.009	-0.0008	0.231	0.0044	0.017	-0.0090	0.697
TCH14 ³	-0.0013	0.285	0.0042	0.127	0.0007	0.557	0.0097	0.050	0.0016	0.294
TCH16 ³	0.0274	0.097	0.0167	0.123	-0.0035	0.622	-0.0050	1.000	-0.0011	1.000
TCH19 ³	-0.0085	0.574	0.0029	0.646	0.0100	0.121	-0.0091	0.571	0.0031	0.619
TCH22 ³	0.0009	0.551	0.0169	0.051	-0.0017	0.909	-0.0015	0.365	-0.0028	0.505
Total ms	-0.0010	0.459	0.0082	<0.001	0.0056	0.001	0.0122	<0.001	0.0032	0.032
	(0.001)		(0.004)		(0.004)		(0.006)		(0.003)	
Neutral ms	-0.0004	0.559	0.0030	<0.001	0.0005	0.305	0.0029	0.023	0.0033	0.059
	(0.001)		(0.001)		(0.001)		(0.001)		(0.003)	
<i>Pan I</i>	0.3798	<0.001	0.2085	<0.001	0.4615	<0.001	0.7071	<0.001	0.1110	<0.001

fjord samples of Fig. 3, using *Pan I*, GMO34, and GMO132 as markers, and shallow settlers (alleged NCC) and bottom settlers (alleged ANC) as reference groups, gives a proportion of 28% NCC and 72% ANC in the pelagic trawl sample. Using the method based on *Pan I*^B frequencies in a sample assumed to be a mixture between NCC and ANC, gives a proportion of NCC in the pelagic sample in Varangerfjord of 24%, Laksefjord 15%, and Porsangerfjord 54% (Table 3).

Pan I allele frequencies over years

Frequencies of *Pan I* alleles in 0-group juveniles settling in shallow water, irrespective of sampling location, were compared over the 14 yr time span of the study. SS samples were not available from 2002 to 2006 due to a temporary stop in sampling activities. As evident from Fig. 5, SS 0-group juveniles that settled in shallow water in northern Norway displayed high *Pan I*^A stability over the total sampling period. The apparent

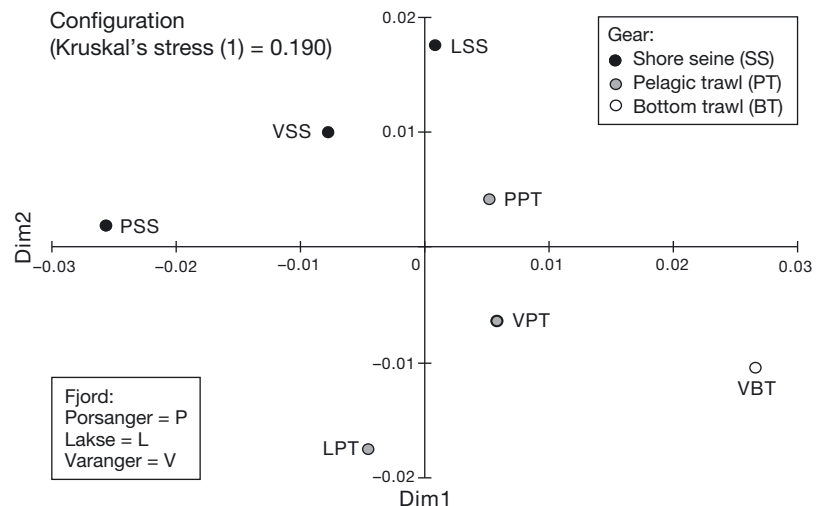


Fig. 4. *Gadus morhua*. Multidimensional scaling (MDS) plot of the 7 samples of Fig. 3 based on the 16 microsatellites of Table 2

lower *Pan I*^A frequencies in 2001 and 2007 are associated with few samples available (1 and 3) in those years. Frequencies of the *Pan I*^A allele of Fig. 5 are means over years. Frequencies calculated by summarizing the different genotypes for each year deviate from the former in 8 of 9 yr by an average of only 1%. In the 3 samples from 2007, the largest (in num-

ber of fish) showed an unusual low frequency of the *Pan I^A* allele (0.62), causing a drop in estimates of *Pan I^A* allele frequencies based on the total number of genotypes by 5%. This particular sample was taken on full high tide, allowing for possible drift of non-settled pelagic larvae into the shore seine. The sample displays a significant deficit of heterozygotes (63 observed versus 81 expected: $p < 0.05$) indicating a Wahlund effect and a mixture of populations. One additional shore seine haul was taken a few hours later at the same location, and the *Pan I^A* allele frequency within this sample (0.78) was close to the total average for SS samples (cf. Fig. 2), albeit still displaying a significant ($p < 0.05$) deficit of heterozygotes. The *Pan I^A* frequencies in samples of juveniles caught pelagically varied considerably among years, whereas they were always low in deep-water bottom samples (Fig. 5).

Pan I genotypes and length frequencies

In 3 sample sets of 0-group juveniles (Fig. 6), length was measured for each analyzed fish and related to the 3 possible genotypes, the 2 homozygotes *Pan I^{AA}* and *Pan I^{BB}*, and the heterozygote *Pan I^{AB}*. In the pelagic sample (Lyngsfjord), where all 3 genotypes were represented in fairly good numbers, there was a clear result of *Pan I^{BB}* representing the largest individuals, *Pan I^{AA}* the smallest, and the heterozygotes intermediate of the 2 homozygotes (Fig. 6a). Pairwise comparisons of length in all 3 genotypes came out highly significant (Table 4). This sample exhibited a highly significant deficit of heterozygotes (29 versus 48, $p < 0.001$), clearly demonstrating the mixed population feature of pelagic juveniles. The SS sample

Table 3. *Gadus morhua*. Percent proportion of Norwegian coastal cod (NCC) and Arcto-Norwegian cod (ANC) in the pelagic samples from Varangerfjord, Laksefjord, and Porsangerfjord (see Fig. 3), estimated from mixed stock analysis (MSA; Varangerfjord only) using the markers GMO34, GMO132, and *Pan I*, or from the *Pan I^B* method (cf. 'Materials and methods')

Fjord	— MSA —		<i>Pan I^B</i> method	
	NCC	ANC	NCC	ANC
Varanger	28.1	71.9	24	76
Lakse	—	—	15	85
Porsanger	—	—	54	46

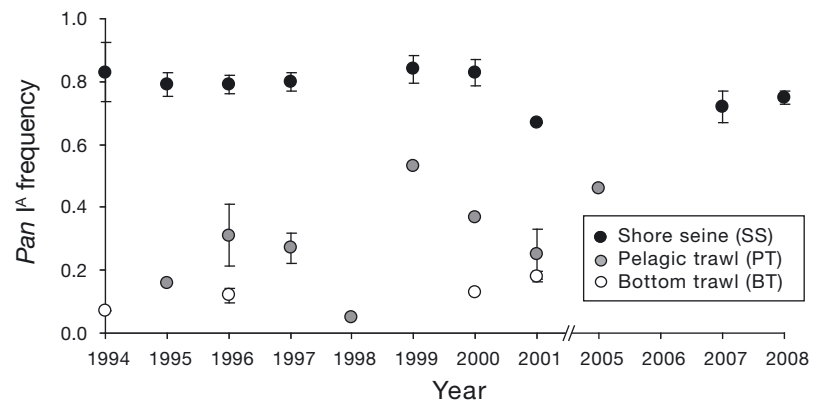


Fig. 5. *Gadus morhua*. *Pan I^A* allele frequency in 0-group cod of samples taken the same year with 3 different sampling gears. Means \pm SEM

from Dønnesfjord showed the same pattern as the pelagic sample (Fig. 6b). *Pan I^{AA}* homozygotes were significantly smaller than both the *Pan I^{BB}* homozygotes and the *Pan I^{AB}* heterozygotes, whereas the *Pan I^{BB}* homozygotes were only insignificantly larger than the heterozygotes (Table 4). The mere 3 *Pan I^{BB}* homozygotes from Malangen made a comparison between those and the other genotypes there inappropriate, but again *Pan I^{AA}* homozygotes were significantly ($p < 0.05$) smaller than the heterozygotes (Fig. 6c).

DISCUSSION

Vertical divergence

The present data clearly show that Atlantic cod 0-group juveniles settling in shallow waters in northern Norway are dominated by the *Pan I^A* allele class. The samples of juveniles collected by bottom trawl (inshore and offshore) had very low frequencies of that allele and thus correspondingly high frequencies of the *Pan I^B* allele. Yet non-settled juveniles caught by the pelagic trawl displayed great variability in frequencies of the 2 alleles, which we take as evidence for variable proportions of 2 populations, ANC and NCC being present pelagically before they segregate to settle. The lack of correlation between *Pan I* allele frequencies and depth within the pelagic habitat, together with lack of differences between *Pan I* allele frequencies inside fjords and off the coast, signify an apparent arbitrary distribution of 0-group individuals before they are ready to settle. The lowest *Pan I^A* frequency (0.05) and thus the highest *Pan I^B* frequency was in fact observed in a sample from the innermost part of Porsangerfjord, clearly

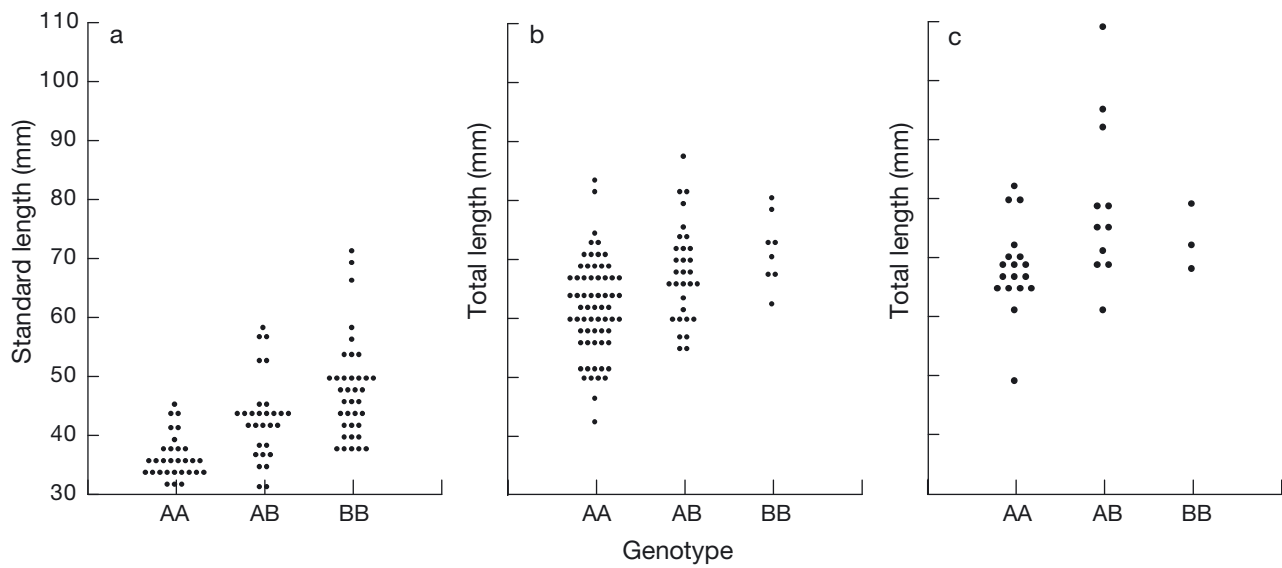


Fig. 6. *Gadus morhua*. Length plot of the 3 different *Pan I* genotypes, *Pan I*^{AA}, *Pan I*^{AB}, and *Pan I*^{BB}, in 3 samples of 0-group Atlantic cod. (a) Pelagic trawl from Lyngsfjord 3 September 2005, (b) shore-seine sample from Dønnesfjord 17 September 2008, (c) shore-seine sample from Malangen (Aursfjord) 16 September 2008

Table 4. *Gadus morhua*. Mann-Whitney tests for equality of length–frequency distributions of different *Pan I* genotypes (AA, AB, BB) in 0-group Atlantic cod from 3 different fjords (same as in Fig. 4). Lilliefors tests for normality of length–frequency distributions are given; $p < 0.05$ indicates non-normality

Fjord	— AA vs AB —		— AB vs BB —		— AA vs BB —		— Normality Lilliefors —		
	<i>U</i>	<i>p</i>	<i>U</i>	<i>p</i>	<i>U</i>	<i>p</i>	AA	AB	BB
Lyngs	176.5	<0.001	350	0.016	76.5	<0.001	0.009	0.011	0.005
Dønnes	615.5	0.003	85	0.174	82.5	0.002	0.734	0.504	0.491
Malangen	46.0	0.017	13.5	0.173	20.0	0.585	0.016	0.173	0.038

illustrating that ANC juveniles may drift into fjords by means of local current patterns. The relatively frequent significant deficits of heterozygotes in the PT samples (28.6%), together with high and significant F_{ST} and F_{IT} values within the pelagic habitat, support the assumption of a varying degree of intermingling between 2 populations in that habitat. Also among the 53 SS samples, 15.1% exhibited significant deficits of heterozygotes and a high F_{ST} estimate (Table 1), providing evidence that there is not necessarily an absolute segregation of the 2 populations even after settling. Some ANC juveniles may settle in shallow water before they migrate towards deeper water, or they have not actually settled at the time of being caught in the shore seine.

The sharp settling depth stratification shown herein of *Pan I* allele frequencies is extraordinary and to our knowledge is unparalleled elsewhere for adult cod or for juveniles. We believe a different settling strategy is 1 of probably many means to maintain the segregation of 2 populations despite the fact

that they partly overlap in distribution during the spawning season. The peculiarity of NCC and ANC to maintain separate life history strategies, stationary versus migratory, one would assume has a strong genetic component. Thus, the sharp divergence at the *Pan I* locus demonstrated herein between 0-group juveniles settled at different depths would not point to contemporary post-settlement selection acting on cohorts from a common gene pool. On the contrary, we believe the 2 discriminated groups of 0-group juveniles are offspring of parental cod with similar divergent *Pan I* allele frequencies (many examples of which are given by Fevolden & Pogson 1997, Sarvas & Fevolden 2005a,b, Westgaard & Fevolden 2007).

The microsatellite data provide support for the deep bottom-settling juveniles in Varangerfjord being genetically distinguishable from those having settled in shallow water. The pelagic samples of juveniles from Varangerfjord and Laksefjord were also significantly divergent from the shallow-water set-

tlers in the respective fjords, apparently due to the high proportion of ANC juveniles in the pelagic samples (76 and 85%, Table 3). The Porsangerfjord pelagic sample with a higher frequency of the *Pan I*^A allele (Fig. 3), and thus lower proportion of ANC juveniles (46%), was not distinguishable from SS juveniles by means of microsatellites. The MDS plot of microsatellites shows a reasonable grouping of the SS samples in 1 dimension, whereas the pelagic samples are grouped across dimensions, presumably due to their variable proportion of NCC and ANC in them.

The fact that 2 of the microsatellite loci that contribute to the significance in pairwise habitat comparisons were considered non-neutral, in accordance with previous studies (Nielsen et al. 2006, Skarstein et al. 2007, Westgaard & Fevolden 2007), is in itself no disqualification of their capacity to reveal population divergence. Rather, the microsatellite data, in line with *Pan I*, could be suggestive of a relatively recent (post-glacial) diversifying selection having acted on non-neutral loci, whereas the time lag may be insufficiently long to affect neutral loci (cf. Pogson & Fevolden 2003). Notwithstanding, in the present scenario, even when excluding the non-neutral microsatellites, significant differences were found between bottom settlers and shore settlers in Varangerfjord and between shore settlers and non-settled individuals in Laksefjord. Thus, evidence for NCC and ANC being genetically distinguishable is provided both from non-neutral and neutral loci.

Temporal stability

The relative temporal stability in *Pan I* allele frequencies of settled cod juveniles in northern Norway, in both shallow and deep water (Fig. 5), runs counter to reports of adult cod, both in central Norway (Trondheimsfjorden; Karlsson & Mork 2003) and from fjords in northern Norway, e.g. Balsfjord, Ullsfjord, or Varangerfjord (Sarvas & Fevolden 2005a,b). Whereas in Trondheimsfjorden, *Pan I* heterogeneity across years and significant excesses of heterozygotes were ascribed to natural selection (Karlsson & Mork 2003), inter-annual variation among post-juvenile cod in fjords in northern Norway is more plausibly explained by various degrees of intermingling between ANC and NCC. During the spawning season, ANC 'accidentally' penetrate fjords for feeding purposes, whereas there seem to be more or less permanent resident cod in outer fjord and coastal waters, reachable by NCC, with an ANC

Pan I signature (Westgaard & Fevolden 2007). Thus, bottom trawl catches of adult cod taken in fjords both in late winter or early spring and in the fall are apt to contain a variable fraction of ANC, causing an apparent spatial and temporal *Pan I* heterogeneity.

The temporally more homogeneous *Pan I* allele frequencies exhibited by newly settled 0-group juveniles reflects that shallow settling is primarily an adaptation of cod of the NCC type and deep water settling a characteristic of ANC. Slight inter-annual *Pan I* divergence was also observed for the shallow-water settlers (Fig. 5), again likely due to mechanical mixing with ANC juveniles. One example given in the Results shows that the shore seine does not necessarily exclusively catch juveniles that have settled, not precluding the general picture of a significant genetic divergence between shallow- and deep-water settlers.

Genotype-length correlations

In post-juvenile cod, *Pan I*^{AA} homozygotes were reported to exhibit the fastest growth (length-at-age) both among NCC and among the offshore alleged ANC, where this genotype was rare (Fevolden & Pogson 1995). *Pan I*^{AB} heterozygotes were intermediate in length of the 2 homozygotes. Similar features were recently reported from Icelandic waters (Jakobsdóttir et al. 2011), although growth-genotype relations there have previously been reported to vary among spawning sites (Jónsdóttir et al. 2008). Reports on individual length in NCC and ANC populations, classified by otolith typing, showed no difference in growth between the 2 populations (Berg & Albert 2003). It was claimed, however, that since NCC mature at a younger age than ANC, growth of NCC slows down relative to ANC after maturation.

The present data showing that 0-group juveniles of the commonest coastal cod genotype *Pan I*^{AA} were significantly smaller on average in early fall than the *Pan I*^{BB} genotypes, does not inevitably signify slower growth in coastal cod juveniles. Length-at-age is in itself a crude measure of growth, and due to the long spawning season for cod in general, the age of the juvenile fish studied herein could vary by weeks. The spawning period of coastal cod originating from different fjords has also been shown to vary (Otterå et al. 2006). Thus, the larger average length of the present *Pan I*^{BB} juveniles could mean that they are older on average than the coastal cod *Pan I*^{AA} juveniles. They could also have been spawned from larger ANC females, which has been suggested to influence

larval size (Trippel et al. 1997), and they could have experienced different growth conditions, of which temperature is important for larval cod (Otterlei et al. 1999, Vikebø et al. 2005). Conversely, the mere presence of compelling length–genotype correlations strengthens the evidence that 0-group juveniles of NCC and ANC intermingle during their pelagic phase and are profoundly divergent at the *Pan I* locus even from the earliest life stages. A genetic component to growth performance cannot be dismissed, and the apparent intermediate length of the *Pan I*^{AB} heterozygote as compared to both homozygotes (Fig. 6a,b) could theoretically even be interpreted as an additive genetic effect. A closer look at the length distribution, however, shows that the heterozygotes are spread over the entire length span and could belong to either of the 2 populations.

***Pan I*— validity and selective constraints**

An array of new molecular markers is now available and is being used for studies of population structuring in a variety of organisms, including Atlantic cod. Chips for SNPs are about to become commercially available, and thousands of markers can be used to establish population divergence. Many of these will be proven non-neutral, and some may come up with differences between the 2 major Norwegian stocks of cod that can rival the *Pan I* locus (cf. Moen et al. 2008). This will not preclude the important role that the *Pan I* locus, itself a SNP, has played since it was first identified as a marker with particularly high potential to discriminate populations of cod (Pogson et al. 1995). The great challenge regarding *Pan I* is that the mechanisms underlying its selective character are still unresolved. The recognition that NCC and ANC in Norwegian waters partly overlap in distribution but are still genetically strictly divergent at the *Pan I* locus provides a unique system for a deeper insight into which selective constraints cause this divergence. The coexistence of migratory and stationary stocks of Atlantic cod has been reported from different regions of the North Atlantic (e.g. Robichaud & Rose 2004, Jakobsdóttir et al. 2011), but nowhere so well defined and with such abrupt differences at the *Pan I* locus as between ANC and NCC.

Depth correlates with *Pan I* allele frequencies in adult cod (Case et al. 2005, Sarvas & Fevolden 2005a, Pampoulie et al. 2008). With depth, one could assume that temperature plays an adaptive role, but the ambient temperature in the regions inhabited by

NCC and ANC varies both in time and space. Thus, attempts to correlate *Pan I* frequency distribution with temperature at the time of sampling can be misleading for adult cod. One cannot exclude, however, that at the time of settling for juvenile cod, temperature may contribute to the segregation between the 2 stocks. A characteristic of *Pan I* allele frequency distribution of adult cod in northern Norway is the sharp inshore–offshore divergence (Sarvas & Fevolden 2005a). A similar scenario was recently reported from Greenland waters (Pampoulie et al. 2011). Atlantic cod sampled within fjords of Greenland also had very high frequencies of the *Pan I*^A allele, whereas cod taken well offshore had low frequencies of the same allele. Thus, there is reason to believe that specific features of fjord water contribute to the split between offshore and inshore populations, salinity being one likely factor. The salinity in shallow water in fjords is often low due to freshwater runoff. One could speculate that settling in shallow low-saline water is an adaptive strategy for coastal cod that evolved at a time glacialiation isolated this population from the larger offshore populations in high-saline water. The ANC, with its deep-water settling strategy and migratory behavior, could signify a postglacial recolonization of the Barents Sea from historical offshore populations. The structure of many fish species, both freshwater and marine, is likely to be affected by the period previous to, during, and following the last glacial maximum (LGM), and ice-age refugia were recently suggested as contributing agents for the population structuring of Pacific cod *Gadus macrocephalus* (Canino et al. 2010). Isolated populations of Atlantic cod are reported from various northern meromictic lakes (Hardie et al. 2008), some of which have brackish or even freshwater upper layers. *Pan I* analyzed from 4 specimens in 1 of these, Lake Mogolnye, Kildin Island, on the Kola Peninsula, revealed 3 homozygotes for the *Pan I*^A allele and 1 *Pan I*^{AB} heterozygote. Likewise, 84 specimens of cod caught in the low-saline White Sea (Russia) in 2002 were close to fixed for the *Pan I*^A allele (frequency of 98.2%; our unpublished data). Thus, a closer look into low-salinity and *Pan I* affinities should be encouraged.

The 2 *Pan I* alleles in Atlantic cod appear to have diverged >2 Myr ago (Pogson & Mesa 2004). Moreover, both the *Pan I*^A and the *Pan I*^B lineages show a long residency, in part endemic, in populations of cod both east and west in the North Atlantic (Pogson 2001, Bigg et al. 2008). There is reason to believe that the observed distinct differences in *Pan I* frequencies between ANC and NCC reflect a relatively

recent diversifying adaptation, a structure that has appeared on an ecological rather than evolutionary timescale. The similar *Pan I* divergence for 0-group juveniles as for adults, and thus no indication of life history changes, lends no support to contemporary selection, but an adaptation most likely influenced by the LGM and physical oceanographic features following the gradual disappearance of ice.

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