Dynamics of Genetic Polymorphisms

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PREFACE

This project was funded by the Norwegian Research Council as a three years personal doctoral scholarship (grant no. 14336/432). An additional five-month financial support was received from the department of Biology, Norwegian University of Science and Technology (NTNU).

The main objective of this project was to explore the within and between generation dynamics of the polymorphism at a set of genetic markers commonly in use in Atlantic cod. A time series of cod, including 15 annual samples from a local spawning area was used to explore the temporal stability of allele frequencies at four different genetic markers; *Gmo132*, *Gmo2*, *PanI*, and mtDNA cytochrome *b* sequence. Temporal stability is an important assumption of the theoretical reference models underlying genetic data analyses and an informative indicator of which evolutionary forces are most heavily affecting the polymorphisms. Furthermore, the project material and design allowed testing potential genotypic performance at individual loci with respect to individual biological data, such as sex, body length, gonadal stage, and age.

The work has been carried out at Trondhjem Biological Station (TBS), Department of Biology, NTNU, during the period July 2001 to November 2004. My supervisor has been Prof. Jarle Mork, TBS, Department of Biology, NTNU.

I am indebted to Jarle Mork, who put the materials of the time series and laboratory facilities to my disposal. I wish to thank Jarle for all help, advice, and for the many interesting discussions. The DNA sequencing could not have been conducted without excellent help from Hlynur Sigurgislason during my stay at Prof. Einar Arnasons laboratory (Intitute of Biology, University of Iceland). I also thank Prof. Arnason for valuable advice in the analyses of sequence data. Prof. Bjørn Ivar Honne (The

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Paper I

Karlsson S. & Mork J. Evidence of natural selection at the Synaptophysin locus (*SypI*) in a natural population of cod (*Gadus morhua* L.). *ICES Annual Science Conference* C.M. 2001/L:11.

Paper II

Karlsson S. & Mork J. (2003). Selection induced variation at the pantophysin locus (*PanI*) in a Norwegian fjord population of cod (*Gadus morhua* L.). *Molecular Ecology*, **12**, 3265-3274.

Paper III (Poster)

Karlsson S. & Mork J. (2003). Effects of different selection regimes between sexes on the genotypic composition of offspring; observations in a Norwegian fjord cod population. Poster presentation at the 133rd Annual meeting, *American Fisheries Society,* August 10-14, 2003, Quebec City, Canada.

Paper IV

Karlsson S. & Mork J. Temporal instability in microsatellite allele frequencies in Atlantic cod. *ICES Journal of Marine Science,* Submitted.

Paper V

Karlsson S. & Mork J. An 18-year time series of mtDNA cytochrome *b* sequence in cod. *Genetics,* Submitted.

In the following text these papers are referred to by their roman numerals.

ABSTRACT

The dynamics of four commonly used genetic markers in cod (*Gadus morhua* L.); *PanI*, *Gmo132*, *Gmo2*, and mtDNA cytochrome *b* sequence were studied. A total of 1455 individuals of cod, sampled in 1985-2002 from a local spawning area were assayed for *Gmo132*, *Gmo2*, and *PanI.* In addition 391 individuals were sequenced for the mtDNA cytochrome *b*. The *Pan*I and *Gmo132* locus showed significant variation in allele frequencies among annual samples as well as among cohorts. At *PanI* there was also a significant difference in allele frequency between males and females, and an excess of heterozygotes, especially pronounced among females. Contrary to the *PanI* locus, *Gmo132* and *Gmo2* showed deficiencies of heterozygotes. The *Gmo2* locus did not show temporal instability in allele frequencies. The presumably neutral polymorphism due to silent site substitutions of the cytochrome *b* did not show significant variation in haplotype frequencies among annual samples and cohort. There was evidence that the *PanI* polymorphism is affected by natural selection. The observed heterozygote deficiencies at *Gmo132* and *Gmo2* locus is suggested to be caused by null alleles and not by Wahlund effects. The *PanI* and the *Gmo132* loci appear to be unreliable genetic markers for characterising populations of cod. The *PanI* appears to violate the assumption of selective neutrality, while the explanation of the observed temporal instability of *Gmo132* remains uncertain. The *Gmo2* is suggested to suffer from null alleles, which makes it a poor genetic marker, although its allele frequencies might serve as a fairly consistent population characteristic. The mtDNA cytochrome *b* sequence appears to be the only genetic marker that does not violate the assumption of selective neutrality or null alleles in this study and hence the allele frequency dynamics at this locus appears to be driven solely by genetic drift, gene flow, and mutation.

INTRODUCTION

In essence, there are four evolutionary forces that can change population allele frequencies; genetic drift, gene flow, mutation, and natural selection. However, due to different nature of these forces their impact is heavily depending on the general biology of the species under study. On a short evolutionary time scale, mutations will normally not change allele frequencies noticeably. Genetic drift and gene flow, on the other hand, can potentially change allele frequencies rapidly. The impact of genetic drift is large in a population with low effective population size. Gene flow is expected to have large impact on allele frequencies within a population if there is a significant amount of immigrants with large differences in allele frequencies as compared to the recipient population. Most genetic polymorphisms are assumed selective neutrality and the maintenance of the neutral alleles occur by a balance between mutation and genetic drift, the so-called neutral theory (Kimura 1968). Polymorphic loci that do not satisfy the assumption of selective neutrality are in addition affected by natural selection. The direction as well as the strength of natural selection may vary among loci and among populations. This is because different loci have different functional roles and the performance of the genotypes at a locus depend on the environment, which often differs among populations as well as within populations through time. The evolutionary effect of natural selection is therefore often highly unpredictable.

During the last decades population genetics has been revolutionised by the development of new types of genetic markers and by computerised analysis and modelling. Different types of genetic markers now in routine use have different evolutionary rates, depending on mutation rates and the effective population size associated with them. Mitochondrial DNA (mtDNA) is in general maternally inherited and exists in only one copy.

Hence, the associated effective population size at this genetic marker is only one fourth of that of nuclear DNA. Because the effective population size is smaller, the effect of genetic drift, and thereby the evolutionary rate, is expected to be higher than for nuclear encoded loci. In addition, the mutation rate is comparatively high in mtDNA, which contributes to a high evolutionary rate. Microsatellites are also fast evolving because of high mutation rates.

Studies on different evolutionary scales require different types of genetic markers. Detection of recent isolation between relatively large populations requires a fast evolving genetic marker (high mutation rate and/or associated low effective population size like that for mtDNA). The types of genetic markers so far used in cod (*Gadus morhua* L.) include *i. a.* haemoglobins (Sick 1961), isozymes (Mork *et al.* 1985), mtDNA RFLP (Smith *et al.* 1989), mtDNA cytochrome *b* sequence (Arnason *et al.* 1992), cDNA RFLP (Pogson *et al.* 1995), minisatellites (Galvin *et al.* 1995a), and microsatellites (Bentzen *et al.* 1996).

Cod is widely distributed in the North Atlantic, with a large potential for migrations and gene flow. Adults often migrate long distances between spawning and feeding areas (Harden Jones 1970). Because cod eggs and larvae are pelagic they can potentially drift long distances with ocean currents before settling (Bjørke 1984; Lough *et al.* 1989). The migration potential of cod means a large potential for gene flow among populations. Furthermore, some cod stocks spawn in large schools that on some spawning grounds may count millions of spawners (ICES 2004, Report of the ICES Advisory Committee on Fishery Management, Extract on Northeast Arctic Cod, ICES, Copenhagen, http://www.ices.dk). The allele changing effect of genetic drift is expected to be small in such large populations. The impact of gene flow will tend to homogenise genetic differences among populations, while a large impact of genetic drift tends to

increase the genetic differences. Most marine species like cod, with large population sizes and large potential for migration, are expected to show low levels of genetic differentiation among populations (Gyllensten 1985; Ward *et al.* 1994; DeWoody & Avise 2000). In general, this has been confirmed by population genetic studies of cod (Table 1). In a study by Mork *et al.* (1985), including nine cod populations covering most of the distribution range of cod, only 2.1% (F_{ST}=0.021) of the total genetic variability at 19 isozyme loci could be ascribed to the genetic variability among populations. For mtDNA cytochrome *b* Arnason (2004) showed that 8% of the total genetic variability observed could be ascribed to genetic variability among cod from Newfoundland, Greenland, Iceland, Faeroes, Norway, and the Baltic. Another study, also including cod from almost the entire distribution range showed an F_{ST} estimate of 0.069 based on 11 nuclear encoded RFLP loci (Pogson *et al.* 1995). A study by Galvin *et al.* (1995b) on one minisatellite locus revealed an F_{ST} value of 0.03 among cod sampled north east of Newfoundland, the Scotian Shelf, the Irish Sea, and Northern Norway. Finally, six microsatellites revealed an F_{ST} value of 0.015 among six populations in the northwest Atlantic and the Barents Sea (Bentzen *et al.* 1996).

Table 1. Summary of F_{ST} estimates among populations of cod on a macrogeographical scale from four independent studies using four different types of genetic markers.

Populations	Genetic markers	F_{ST}	Reference
North America, Greenland, Iceland, Barents Sea, Norwegian Coastal waters, the North Sea, and the Baltic Sea	*AAT-1, AAT-2, AAT-3, CK-1, GAP-1, G3P-1, IDH-1, IDH-2, LDH-1, LDH-2, LDH-3, MDH-3, MP, PGI-1, PGI-2, PGM-1, RE, SDH-1, SOD*	0.021	Mork *et al.* 1985
Newfoundland, Greenland, Iceland, Faeroes, Norway, and Baltic	Mitochondrial DNA, Cytochrome *b* sequence (250bp)	0.081	Arnason (2004)
Northern cod (north east of Newfoundland), Scotian Shelf, Irish Sea, North Norway	*Mmer-AMP2*	0.030	Galvin *et al.* (1995)
South (North Cape, Grand Bank, Nose of the Bank), North (Hamilton, Belle Isle, Funk Island banks), Northeast Spur, Scotian Shelf, Flemish Cap, and Barents Sea	*Gmo2, Gmo4, Gmo120, Gmo132, Gmo141, Gmo145*	0.015	Bentzen *et al.* (1996)

Studies of the population structure of cod have shown extensive differences in the level of genetic differentiation depending, on the genetic marker used. These differences have been observed between various classes of markers, e.g. mtDNA cyt. *b* sequences (Carr *et al.* 1995) *versus* micosatellites (Ruzzante *et al.* 2000a), and isozymes *versus* cDNA RFLP (Pogson *et al.* 1995), but notably also between genetic markers within the same class. In a study of cod populations sampled from the entire distribution range, eleven nuclear encoded RFLP loci were included. F_{ST} estimate from one of these were 0.309, while the average F_{ST} of the remaining ten RFLP loci were 0.034 (Pogson *et al.* 1995). F_{ST} estimates based on this locus (GM798, renamed *SypI* by Fevolden & Pogson 1997 and again later renamed *PanI* by Pogson 2001) has generally been the largest compared to other RFLP loci

included (Pogson *et al.* 1995; Jónsdóttir *et al.* 1999; Pogson *et al.* 2001). Also, among isozymes there are specific loci (*LDH-3* and *PGI-1*) that have frequently shown the highest level of differentiation (Jørstad 1984; Mork *et al.* 1985; Jørstad & Nævdal 1989; Mork & Giæver 1999). Similarly, the microsatellite locus *Gmo132* is an outlier among microsatellite loci. This locus has been included in several studies and has in most of these shown the highest level of differentiation among cod populations (Bentzen *et al.* 1996; Ruzzante *et al.* 1998; Ruzzante *et al.* 2000a; Ruzzante *et al.* 2000b; Ruzzante *et al.* 2001; Knutsen *et al.* 2003; Tuula *et al.* 2003; Lage *et al.* 2004). From these studies, the average F_{ST} estimate from the *Gmo132* locus was 0.0304, while the average F_{ST} estimate from the remaining pooled microsatellite loci were 0.0040. Although these studies cover different geographical scales, the F_{ST} values are comparable, because the *Gmo132* locus was included in all studies and all other microsatellite loci were pooled.

Because genetic markers have shown such large differences with respect to genetic differentiation, models suggested for the genetic structure of the Atlantic cod has varied all the way from no real structure at all (e.g. Arnason *et al.* 1992; Arnason & Palsson 1996), through a population structure maintained by isolation by distance (Mork *et al.* 1985), and to a complete population isolation maintained by behavioural mechanisms (Fevolden & Pogson 1997; Jónsdóttir *et al.* 1999). Because all these models can not be true, it is appropriate to question how well the various markers fulfil the assumptions for their use as such. One strict demand for genetic markers is selective neutrality. Violation of this assumption can lead to gross misconclusions about evolutionary relationships (e.g. gene flow), because selection is so effective in changing allele frequencies. In order to achieve accurate estimates of the degree of gene flow and/or time since divergence among populations or higher taxonomic units, it is essential that

the diverging force is genetic drift only. However, there are many examples of polymorphisms that do not satisfy the assumption of selective neutrality. Actually, the polymorphism of the isozyme *LDH-3* (Mork & Sundnes 1985b; Mork *et al.* 1985; Mork & Giæver 1999), and the haemoglobin in cod has been shown to be influenced by natural selection (Karpov & Novikov 1980; Mork *et al.* 1983; Mork *et al.* 1984; Mork & Sundnes 1985a; Nævdal *et al.* 1992). There is also substantial evidence that the *PanI* polymorphism is affected by natural selection (Pogson & Fevolden 1998; Pogson 2001; Pogson & Fevolden 2003; Karlsson & Mork 2003).

Polymorphisms at the third codon position, so called ”silent” substitutions are however *a priori* regarded as selectively neutral. This applies to the mtDNA cytochrome *b* sequence used in this study, because these substitutions do not alter the amino acid sequences of the protein coded for.

OBJECTIVES AND PROJECT DESIGN

The main objective of this project was to explore the within and between generation dynamics of the polymorphism at a set of genetic markers commonly in use in Atlantic cod. ”Dynamics” refers, in essence, to variation in allele frequency through time, within generations and among generations in one population. ”Explore” refers to evaluation of the impact of the evolutionary forces on the variation in allele frequency through time.

The genetic markers chosen are; a nuclear encoded RFLP named *PanI*, two microsatellite loci *Gmo132* and *Gmo2*, and a sequence of mtDNA cytochrome *b*. The *PanI* locus and the microsatellite *Gmo132* were chosen because they have shown extremely high levels of geographic differentiation among cod population as compared to other genetic markers. The *Gmo2* microsatellite locus, on the other hand, was chosen because it has shown a moderate level of geographic differentiation very similar to isozymes. The polymorphism of the mtDNA cytochrome *b* region is presumably neutral, and hence represents a genetic marker affected solely by genetic drift, gene flow, and mutation.

Neutral polymorphisms are expected to be temporarily stable in large panmictic populations with no immigration from populations with different allele frequencies. The design and material of this study allowed testing whether the various polymorphisms fulfilled this assumption. The material consists of 15 annual samples of cod from a very well defined spawning area (Verrasundet) in the Trondheimsfjord (Fig. 1), with a collection of individual tissue samples stored frozen or in ethanol.

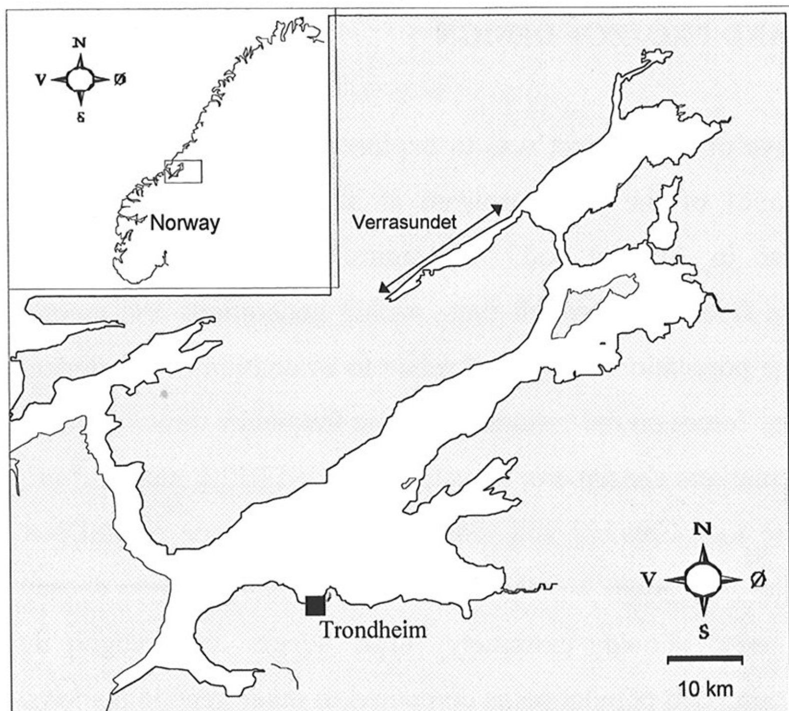

Figure 1. Map of the Trondheimsfjord showing the sidearm Verrasundet, where all samples of spawning cod were taken (map by Jo Forthun).

The time series includes individual biological data such as body length, body weight, gonadal stage, age, and sex. Hence, analyses of potential associations between genotype and phenotypic traits were possible. In order to discriminate among the evolutionary forces possibly responsible for the observed patterns, it is also important to have knowledge of the effective population size, migratory pattern of adults, and current transportation of eggs and larvae. The local cod stock in the Trondheimsfjord is, biologically and genetically, probably the most thoroughly studied of its kind. It is characterised in considerable detail with respect to spawning time and areas, drift pattern of pelagic eggs and larvae, nursery areas, age composition, maturation age, genetic characteristics, migration pattern (from tagging experiments) outside and within the fjord, population size, and year class strength. Tagging experiments and annual landings during spawning

fisheries suggest that the Trondheimsfjord cod population is large enough to prevent large impacts of genetic drift, and relatively isolated preventing large impact of gene flow. These characteristics make the Trondheimsfjord cod population well suited as a model population for testing temporal stability of allele frequencies.

DYNAMICS OF THE GENETIC POLYMORPHISMS: *PanI, Gmo132, Gmo2*, AND mtDNA cytochrome *b* sequence IN A NATURAL POPULATION OF ATLANTIC COD.

Gene flow and genetic drift are expected to affect all loci to a similar extent, while the effect of natural selection is locus dependent. It is in practice impossible to correctly evaluate the degree of genetic isolation and time since divergence among populations from genotypic data of non-neutral loci. This is because natural selection can potentially change allele frequencies on a very short evolutionary time scale. Also, the directional effect of natural selection is often unpredictable, while that is not the case for gene flow. Hence, there are some expectations regarding the dynamics of polymorphisms that makes it possible to evaluate the relative importance of the evolutionary forces within a population. In the following, observations in this project are arranged in sections according to type of effect. General annual sample information is compiled in Table 2.

Temporal variation

In the time series of cod from Verrasundet the *PanI* (Paper II) and the *Gmo132* (Paper IV) polymorphisms showed temporal variation in allele frequencies, while the polymorphism at *Gmo2* (Paper IV) and mtDNA cytochrome *b* (Paper V) did not. The larger variation in allele frequencies at *PanI* and *Gmo132* compared to *Gmo2* is clearly seen in Figure 2. The variation in frequency of the most common haplotype at mtDNA cytochrome *b* was even larger than at the other loci. However, the sample variance was expected to be larger at this locus, because the sample sizes at this locus were effectively only one fourth of that at the other loci. This is because the number of individuals analysed for mtDNA were about half of that analysed for the other loci (Fig. 2). In addition, the mtDNA exists as

one copy (haploid), meaning that the number of haplotypes analysed is the same as the number of individuals analysed, while the nuclear loci exist in two copies (diploid).

Table 2. Summary data annual samples

Year	N	*Gmo132*		*Gmo2*		*PanI*	MtDNA Cyt. *b*		Females	Length	Gonad	Age
		$n_{alleles}$	H_e	$n_{alleles}$	H_e	H_e	n_{haplo}	$\hat{h}$	(%)	(mm)		
2002	42 (42)	17(112-156)	0.677	12(104-140)	0.845	0.230	9	0.808	55	260-840	I-IV	2-10
2001	87 (49)	24(108-166)	0.766	15(104-140)	0.868	0.119	8	0.808	17	299-753	I-IV	3-10
2000	76	23(112-156)	0.822	12(104-138)	0.871	0.088			17	310-775	I-III	2-7
1999	146 (50)	24(98-156)	0.718	17(102-140)	0.867	0.174	7	0.744	44	285-970	I-IV	2-11
1998	100	22(108-154)	0.714	14(104-140)	0.856	0.220			35	345-960	I-IV	3-10
1997	96	23(108-154)	0.785	15(104-140)	0.852	0.136			17	330-830	I-IV	3-10
1996	96 (50)	24(102-156)	0.675	16(102-140)	0.848	0.306	10	0.779	77	273-975	I-IV	2-14
1995	96	22(112-160)	0.745	14(104-140)	0.842	0.136			25	370-910	I-III	3-11
1994	91	21(98-154)	0.695	14(104-142)	0.872	0.143			13	261-990	I-IV	2-13
1992	100 (50)	21(98-152)	0.596	14(104-140)	0.866	0.235	11	0.788	40	192-714	I-IV	1-11
1991	80	21(98-156)	0.694	15(104-142)	0.859	0.220			16	331-935	I-IV	2-13
1990	96 (50)	26(96-162)	0.718	17(104-142)	0.865	0.162	9	0.744	-	387-699	-	3-13
1989	94	22(108-156)	0.694	15(104-140)	0.865	0.289			40	218-825	I-IV	2-12
1986	96 (50)	20(98-154)	0.632	13(104-140)	0.863	0.332	9	0.755	39	95-970	I-IV	1-12
1985	159 (50)	26(98-162)	0.637	20(102-146)	0.856	0.192	11	0.790	9	370-940	I-IV	3-13
Total	1455 (391)	33(96-166)	0.705	22(102-146)	0.860	0.199	20	0.778	31	95-990	I-IV	1-14

N, sample sizes, numbers in parenthesis specify the numbers of individuals analysed for mtDNA cytochrome *b*; $n_{alleles}$, numbers of different alleles observed; H_e, expected heterozygosity; n_{haplo}, number of different haplotypes observed; $\hat{h}$, haplotype diversity. Percentage of females, range of body length, gonadal stage, and age are also presented for each annual sample.

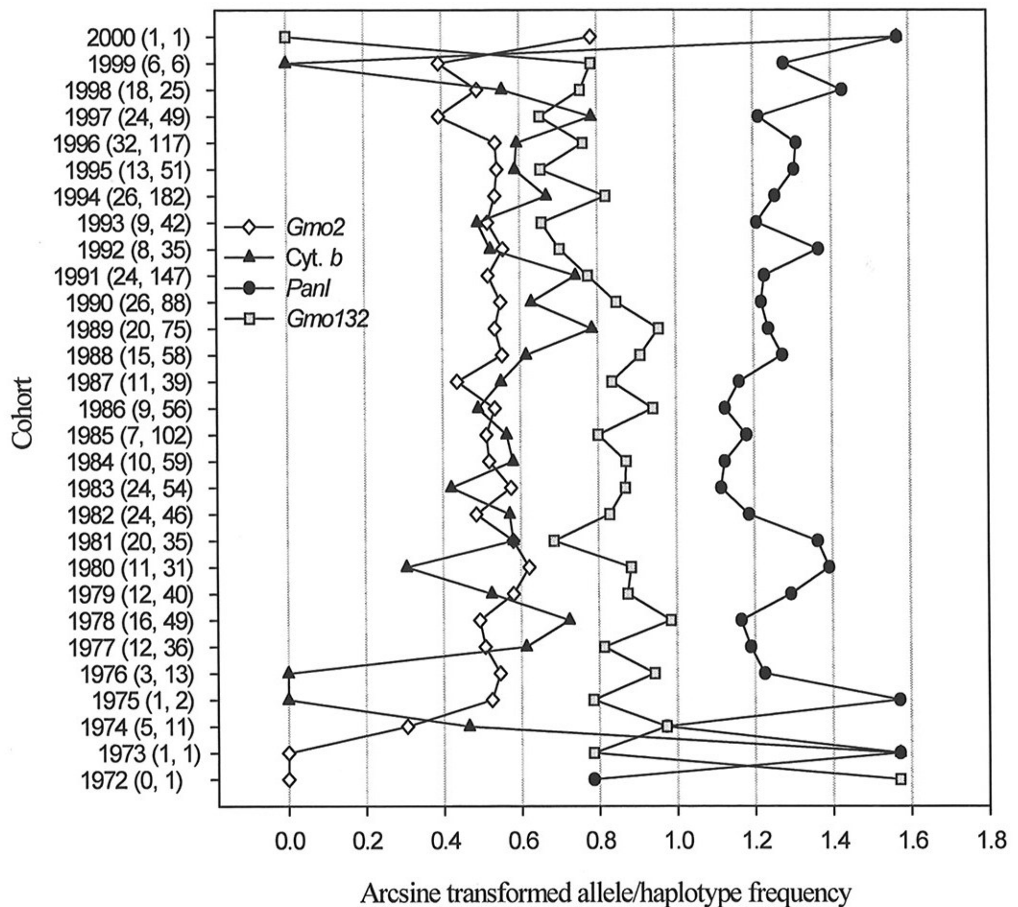

Figure 2. Arcsine transformed allele frequencies at *PanI*, *Gmo132*, *Gmo2*, and the E haplotype frequency by cohort of cod in Verrasundet. At *Gmo132* and *Gmo2* only the most frequent allele are presented; the 112bp and the 106bp fragments respectively and at cytochrome *b* the most frequent haplotype; the E haplotype. The A-allele is presented at *PanI*. The first number in parenthesis represent the sample size of individuals analysed for mtDNA cytochrome *b* sequence, and the second number in parenthesis represent sample size of individuals analysed for *Gmo132*, *Gmo2*, and *Pan*I.

The *Gmo132,* but none of the other three loci showed a significant decrease of the most frequent allele (112bp fragment) with time as revealed by a correlation test (P=0.008, correlation coefficient = - 0.55). Because a correlation test does not account for sample sizes, the smallest cohort samples were not included in the test (c.f. Fig. 2). A cline in allele frequency

of the most common allele at *Gmo132* has been observed along the Norwegian coast, manifested as an increase in allele frequency (0.08-0.7) from Denmark to the Barents Sea (personal communication, Tuula Sarvas, University of Tromsø, The Norwegian College of Fishery Science). This cline coincides with a downward south-north cline in temperature along the Norwegian coast (Aure & Strand 2001). Existing temperature data from the inner parts of the Trondheimsfjord show an increase in temperature from 1977 to 1998, i.e. the period with down going trend in *Gmo132* allele frequency. However, the trend was not significant by a linear regression. Hence, the reason for the temporal trend in allele frequency remains elusive. Similar to allele frequencies of the most common allele at *Gmo132,* allele frequencies at *PanI* also appear to show a geographical south-north cline (Pogson *et al.* 2001). However, unlike the *Gmo132* locus the *PanI* locus did not show a significant correlation between allele frequency and cohort. Although it has been hypothesied that temperature migth be an important selection agent on the *PanI* polymorphism (Paper II), it is not supported by the temperature data at hand.

The variation in allele frequencies among annual samples and cohorts at *PanI* and *Gmo132* (Table 3) is actually similar to estimates of F_{ST} values reported among populations of cod on an oceanical geographic scale (Mork *et al.* 1985; Galvin *et al.* 1995b), while F_{ST} estimates for *Gmo2* and cytochrome *b* were considerable lower and not significant (Table 3). Hence, it seems that allele frequencies at loci that do not show high levels of geographic differentiation are more temporally stable than those that do in the present materials. A logical conclusion of this is that point estimates of allele frequencies at *Gmo132* and *PanI* are not reliable as characteristics of the Trondheimsfjord cod.

Table 3. Trondheimsfjord cod. Single locus homogeneity test and F_{ST}-values, among annual samples and among cohorts.

Locus	Alleles/ haplotypes	Annual Samples P	Cohort P	F_{ST} annual samples (P)	F_{ST} cohorts (P)
Gmo132	33	0.009	~0	0.0039 (P=0.077)	0.0064 (P=0.081)
Gmo2	22	0.346	0.095	0.0004 (P~1)	0.0003 (P~1)
PanI	2	~0	~0	0.0164 (P~0)	0.0136 (P~0)
Cyt. *b*	20	0.106	0.415	0.0071 (P=0.124)	0.0021 (P=0.379)

Genetic variability and impact of genetic drift

The genetic variability, in terms of heterozygosity and number of different alleles/haplotypes, of cod in Verrasundet (Table 2) is at the same level as reported for cod in the large commercial stocks, such as the northeast arctic cod, cod stocks around Iceland, and the North Sea cod. The heterozygosity at the *PanI* locus was on the average 0.199 (Paper II) in Verrasundet, which is at the same level as that reported for northeast arctic cod (Fevolden & Pogson 1997). Furthermore, the observed nucleotide and haplotype diversity at cytochrome *b* (Paper V) were at the same level as that reported from cod in Iceland and Greenland (Arnason *et al.* 2000), in the Faeroe Islands (Sigurgislason & Arnason 2003), and in Barents Sea cod (Arnason & Palsson 1996). The *Gmo132* and the *Gmo2* microsatellites were highly polymorphic. Among 1455 individuals, 33 and 22 different alleles were found and the expected heterozygosities were 0.707 and 0.861, respectively (Paper IV), which matches the values observed among > 650 cod in the Northwest Atlantic shelf and the Barents Sea pooled (Bentzen *et al.* 1996). Evidently the high level of genetic variability observed among cod in Verrasundet implies that the effective population size of the Trondheimsfjord cod stock is large enough to prevent loss of genetic variance due to genetic drift, given that there are no regular immigration.

Deviation from Hardy-Weinberg equilibrium and impact of gene flow

The number of spawners in Verrasundet is estimated (from commercial landings) at >100 000 (J. Mork unpublished). It has been suggested that populations of highly fecund marine species like cod can have effective population sizes several orders of magnitude lower than the actual population sizes (Turner *et al.* 2002; Hauser *et al.* 2002). If the effective population size really is several orders of magnitude lower than the estimated spawning population size in Verrasundet, genetic drift could potentially be an important evolutionary force that would lower the genetic variability. In order to maintain the high genetic variability observed in Verrasundet an ongoing immigration of cod from other populations would thus be necessary. However, if there were a considerable immigration of spawning cod from other populations, one would expect to observe a Wahlund effect (deficit of heterozygotes) in the annual samples. On the contrary, there was an excess of heterozygotes at the *PanI* locus (Paper I; Paper II). However, a significant difference in allele frequency between males and females was also observed (Paper II). A difference in allele frequency among spawning males and females is expected to produce an excess of heterozygotes among their offspring as compared to Hardy-Weinberg. However, only a part of the observed excess of heterozygotes could be ascribed to the observed difference in allele frequency between males and females (Paper III). The observations at the *Pan*I locus could therefore not support a Wahlund type hypothesis.

Populations along the Norwegian coast show large differences in allele frequencies at the *Gmo132* locus but almost no differences at the *Gmo2* locus. (pers. comm. Tuula Sarvas, University of Tromsø, The Norwegian College of Fishery Science). This would suggest that potential immigrants to Verrasundet are more likely to have larger differences in allele

frequencies at *Gmo132* than at *Gmo2* compared to the Trondheimsfjord cod. Thus, a Wahlund effect would expectedly be more pronounced at the *Gmo132* locus compared to the *Gmo2* locus. The two microsatellite loci did show deficiencies of heterozygotes. However, the deficiency was not more pronounced at the *Gmo132* locus. Therefore, the observed deficiencies of heterozygotes are more likely to be caused by null-alleles, a not uncommon artefact in microsatellites.

Five new haplotypes of the mtDNA cytochrome *b* sequence were observed in Verrasundet (Paper V). These haplotypes have previously not been found in any cod population, including 1278 individuals sampled from almost the whole distribution range (Arnason 2004). One of these haplotypes was found in four individuals, while many other and previously described haplotypes were found as singletons. This indicates that the Trondheimsfjord cod is relatively isolated.

The migration pattern of cod in and outside the Trondheimsfjord is well studied by extensive tagging experiments. These have shown that the Trondheimfjord receives none or very few individuals from Norwegian coastal and oceanic areas (O.R. Godø, Institute of Marine Research, Bergen, pers. comm.). Also, only a small (~1.5%) proportion of cod tagged in Trondheimsfjord were recaptured outside the fjord within 5 years after release, and these were exclusively recaptured north of the fjord i.e. in the direction of the coastal current (Sundnes 1980).

Cod eggs and larvae are pelagic, and therefore the gene flow from coastal areas is potentially high at these stages. Eggs and larvae are likely to be transported out of the fjord by the estuarine circulation. Possibly, some eggs and larvae from the coastal current outside the fjord are transported into the fjord by the estuarine counter current and tidal currents (Jacobson 1976). The general ocean current pattern along the Norwegian coast is northward bound (Breen 1980). It is therefore likely that most eggs and

larvae potentially drifting into the fjord would come from more southerly spawning locations. Such directional and persistent type of gene flow would presumably homogenise allele frequencies among Norwegian cod populations, which is supported by observations at most isozyme loci (Mork *et al.* 1985) and most microsatellite loci (Tuula *et al.* 2003). However, the *LDH-3*, *PGI-1*, *Gmo132*, and *PanI* loci that have shown large genetic differentiation among Norwegian cod populations do not support such a scenario. Considering that current Norwegian cod populations have had some 10 000 years since the last glaciation to get homogenised by gene flow. Therefore, it appears contradictious that such homogeneity is shown by most loci but not by the *Gmo132* and the *PanI* locus, unless a locus specific evolutionary force, such as natural selection affects these two loci.

CONCLUDING REMARKS

This project has explored the dynamics of four genetic markers that have frequently been used in studies of population structure in cod. The nuclear encoded RFLP, *PanI*, and the microsatellite locus *Gmo132* were chosen because they in several independent studies have shown the highest estimates of genetic differentiation among geographically separated groups of cod. This raised the question whether this high geographic variation was also reflected in high temporal variability in a local population. The *Gmo2* locus on the other hand was included because it has, as most microsatellite loci, shown moderate levels of genetic differentiation. The mtDNA cytochrome *b* locus was chosen as a presumably neutral reference marker to which the temporal variation at the other loci could be compared.

The time series of cod used in this project has proved very powerful in detecting violations of general assumptions for genetic markers. Not only has it been possible to check temporal variation, but also potential genotypic association with phenotypic traits, which proved important for the *PanI* polymorphism (Paper I & II). The highly significant difference in allele frequency observed between males and females at *PanI* but not at the other loci (Paper IV & Paper V), was an important indication of the actual dynamics of the natural selection at *PanI*. The substantial knowledge about the population dynamics of the cod in the Trondheimsfjord (Ekli 1997), the current system of the fjord (Jacobson 1976), the migration pattern of cod in the fjord (Sundnes 1980), and the apparent lack of adult immigrants from outside the fjord (O. R. Godø, IMR, pers. comm.) proved to be important information in evaluating the relative importance of the evolutionary forces acting on the genetic markers.

The *Gmo132* locus and the *PanI* locus were *a priori* hypothesised to potentially violate the assumptions of temporal stability because they in

previous studies have shown extremely high levels of differentiations compared to other loci of the same type. The present project confirmed that these two loci are temporally unstable with respect to allele frequencies. There is substantial evidence that the variation at the *PanI* locus is not neutral, which is the most likely reason for the observed temporal variation in allele frequencies at this locus. Contrary to what is stated in many contemporary scientific papers, there are in fact no *a priori* reasons to assume that microsatellites are selectively neutral. It has been shown that microsatellites can have in fact important functions in the genome (Kashi & Soller 1999). This project revealed no hard evidence of selection at *Gmo132*. At any rate, however, *Gmo132* and *PanI* are poor genetic markers in characterising the Trondheimsfjord cod, because reliable estimates of allele frequencies cannot be achieved. The *Gmo2* microsatellite locus did not show significant temporal variation in allele frequencies. There were however significant deficiencies of heterozygotes that could not be ascribed to Wahlund effects. With respect to allele frequencies thus, *Gmo2* is probably reliable in characterising the Trondheimsfjord cod. However, interpretation of the deviation from Hardy-Weinberg equilibrium at this locus is maybe of little biological meaning. Among the four loci included in the present project, the mtDNA cytochrome *b* locus was the only one that did not show any violation from assumptions of temporal stability, or lab artefacts.

FUTURE PERSPECTIVES

Allele frequencies at the *PanI* locus show a conspicuous correlation with the south-north temperature cline along the Norwegian coast. In order to achieve a deeper understanding of the action of selection at the *PanI* locus, a crossing experiment with full sib offspring reared at different temperatures could be desirable. From tagging experiments there is extensive knowledge of the migration pattern of adult cod in the Trondheimsfjord and outside the fjord. However, with respect to possible effects of a gene flow it is important to estimate the amount of immigrants into the Trondheimsfjord, not only of adult cod, but also at the pelagic egg and larval stages. Computer simulation of drift pattern at different depth outside and inside the Trondheimsfjord, as well as pelagic sampling in these areas during the time of pelagic cod eggs and larvae might shed some light on this question.

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Paper I

Karlsson S. & Mork J. (2001) Evidence of natural selection at the Synaptophysin locus (*Syp*I) in a natural population of cod (*Gadus morhua* L.). *ICES Annual Science Conference* C.M. 2001/L:11.

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Evidence of natural selection at the Synaptophysin locus (*Syp*I) in a natural population of cod (*Gadus morhua* L.)

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1245 individuals of cod (*Gadus morhua* L.) caught in 12 spawning seasons during 1985-1999 from a well defined spawning area in the Trondheimsfjord (Norway) were genotyped for the polymorphic cDNA RFLP marker *Syp*I (synaptophysin) and (for the same individuals plus 1080 additional specimens) for the polymorphic haemoglobin locus *HbI**. A highly significant temporal instability in *Syp*I allele frequencies was observed, as well as highly significant differences in allele frequencies between males and females. The females showed a significant trend of heterozygote excess at *Syp*I. *Syp*I genotypic differences in body length and within spawning season gonadic development stage were also observed and a significant heterogeneity in allele frequencies between age groups. None of these effects were observed at *HbI**. Genetic drift, immigration and mutation were rejected as sufficient explanatory factors for the observations made at the *Syp*I locus. There were on the other hand strong indications that the *Syp*I locus is strongly influenced by natural selection, involving overdominance in the females and directional selection in the males in the Trondheimsfjord cod stock.

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INTRODUCTION

Haemoglobin (Sick 1961, 1965a & 1965b) and Transferrin (Møller 1966) were the first population genetic markers used to characterise population structure of cod (*Gadus morhua* L.). Later on, several other types of markers have been developed. The most frequently used so far are isozymes (Mork *et al.* 1985), mtDNA RFLP (Smith *et al.* 1989), mtDNA sequencing (Àrnason *et al.* 1992), minisatellites (Galvin *et al.* 1995 b), cDNA RFLP (Pogson *et al.* 1995) and microsatellites (Bentzen *et al.* 1996). Altogether, more than 40 different loci/markers have been employed in distribution wide or in oceanic scale studies on the genetic population structure of cod. In general these studies have shown moderate level of genetic differentiation. There are however some single loci which consistently have shown higher genetic differentiation between cod populations than others. These loci are Haemoglobin (Sick 1965 a, b), *LDH-3** (Mork *et al.* 1985), , *Syp*I (Pogson *et al.* 1995) and the *GMO-132** (Bentzen *et al.* 1996). Also on finer geographic scales some of these loci are distinguished in exposing a high genetic differentiation between groups of cod, as exemplified by studies on North-East Arctic cod and coastal cod in Norway (*HbI**; Frydenberg *et al.* 1965, *Syp*I; Fevolden & Pogson 1997), on cod from different off-shore spawning grounds in Iceland (*HbI**; Jamieson & Jonsson 1971, *Syp*I; Jònsdòttir *et al.* 2000) and on western Atlantic cod (*GMO-132**; Ruzzante *et al.* 1998).

Genetic drift is expected to affect loci differently in a differentiation process between populations. It is however not expected that the same loci shows the largest genetic drift in all comparisons between populations. Loci that consistently show larger heterogeneity than others must for this reason have characteristics that separate them from other loci and hence affect the way one should interpret results based on them. These characteristics can for example be effects of natural selection, as reported for the *HbI** locus (Karpov & Novikov 1980; Mork *et al.* 1983, 1984a, b; Mork & Sundnes 1985a; Nævdal *et al.* 1992), for the *LDH-3** locus (Mork & Giæver 1999 and references therein) and for the *Syp*I locus (Pogson & Fevolden 1998; Pogson 2001). Observed allele frequency differences at these loci (*HbI**; Sick 1965, Møller 1966, Karpov *et al.* 1984, Jørstad 1984, Dahle & Jørstad 1993, *LDH-3**; Mork *et al.* 1985, Jørstad & Nævdal 1989, *SypI*; Pogson *et al.* 1995, Fevolden & Pogson 1997, Jònsdòttir *et al.* 1999) are doubtful as evidence for genetic isolation.

In general, any short term temporal instability of allele frequencies which can not be attributed to genetic drift will reduce the particular marker's reliability as a population characteristic. Markers with inadequate temporal

stability might be detected by their performance in time series from one population, provided it is sufficiently large and its allele frequencies are unaffected by immigration for the loci under study.

The aim of this study was to investigate the temporal stability of the cDNA RFLP locus *Syp*I (Pogson *et al.* 1995) and the *HbI** locus (Sick 1961) in a time series of 15 years (3-4 generations (Ekli 1997)) of samples from a well defined spawning area of cod (Verrasundet) in the innermost part of Trondheimsfjorden, Norway. Based on individual biological and genotypic data the type of evolutionary force acting on *Syp*I and *HbI** were explored.

MATERIAL AND METHODS

Spawning stock samples of cod (*Gadus morhua*) were taken over a 22 year period in Verrasundet, a well defined spawning area for cod in Trondheimsfjorden (Dahl 1899, Sundnes 1980, Mork et al. 1985, Mork & Giæver 1999). In total 1295 individuals were scored for *Syp*I genotypes and 2195 for *HbI** genotypes.

There are two basins in Verrasundet. The inner basin has a maximum depth of 67m, and is separated from the outer basin by a threshold at 15m deep The outer basin has a maximum depth of 110m and is in turn separated from the main fjord by a 70m threshold. Samples were taken at both localities, named Verrabotn and Skalvik, respectively. The samples were taken by R/V "Harry Borthen", from Trondhjem biological station (NTNU), using a bottom trawl (Mesh size: 35mm stretched mesh in the cod end). Individual biological data such as sex, gonadic stage according to Sivertsen (1935), length and weigh were recorded (Table 1). Age determination were performed by Ekli (1997) according to the method of Rollefsen (1933). Blood was drawn in heparinized syringes from newly slaughtered cod, and stored at 4°C. After haemolysis (>30 min.) in double volumes distilled water, the individual samples were genotyped according to Sick's (1961) nomenclature by isoelectric focusing in polyacrylamide gel (Mork & Sundnes 1983) within 10 hours while onboard the research vessel. Samples of one or several of the following different types of tissues were taken and stored frozen or in alcohol: blood, white muscle, gills, heart, liver and kidney.

Table 1. Number of cod (N) and biological data included (X) and not included (-) for each sample season (S=spring, F=fall) and locality. Dates are in the form Year-Month-Day

Season	Locality	Date	N	Age	Weigh	Length	Sex	Gonad	*Syp*I	HbI*
99-S	Verrabotn	990428	92	X	X	X	X	X	X	X
99-S	Skalvik	990428	54	X	X	X	X	X	X	X
98-S	Verrabotn	980422	73	X	X	X	X	X	X	X
98-S	Skalvik	980422	27	X	X	X	X	X	X	X
98-F	Verrabotn	980924	50	X	X	X	X	X	X	X
97-S	Verrabotn	970409	96	X	X	X	X	X	X	X
97-F	Verrabotn	970917	93	X	X	X	X	X	-	X
96-S	Verrabotn	960417	24	X	X	X	X	X	X	X
96-S	Skalvik	960417	72	X	X	X	X	X	X	X
95-S	Verrabotn	950405	76	X	X	X	X	-	X	X
95-S	Skalvik	950405	20	X	X	X	X	-	X	X
94-S	Verrabotn	940420	91	X	X	X	X	-	-	X
92-S	Verrabotn	920422	100	X	X	X	X	-	X	-
91-S	Verrabotn	910422	72	X	X	X	X	-	X	-
91-S	Skalvik	910422	8	X	X	X	X	-	X	-
90-S	Verrabotn	900403	96	X	-	-	-	-	X	X
89-S	Verrabotn	890418	65	X	X	X	X	X	X	X
89-S	Skalvik	890420	24	X	-	X	X	X	X	X
89-F	Verrabotn	890921	161	X	X	X	X	X	-	X
86-S	Skalvik	860423	96	X	X	X	X	X	X	X
85-S	Verrabotn	850415	100	X	X	X	X	X	X	X
85-S	Skalvik	850417	59	X	X	X	X	X	X	X
84-S	Verrabotn	840424	90	X	X	X	X	X	-	X
84-S	Skalvik	840425	120	X	X	X	X	X	-	X
81-S	Verrabotn	810423	123	X	X	X	X	X	-	X
81-S	Skalvik	810424	28	X	X	X	X	X	-	X
80-F	Verrabotn	801016	96	X	X	X	X	X	-	X
80-F	Skalvik	801025	52	X	X	X	X	X	-	X
79-S	Verrabotn	790419	89	X	X	X	X	-	-	X
79-S	Skalvik	790426	186	X	X	X	X	-	-	X
78-S	Skalvik	780508	42	X	X	X	X	-	-	X
Sum			2375							

DNA was isolated from various types of tissues. Approximately 70mg of tissue was crushed by a glass rod in 2ml plastic tubes. 0.75ml reaction buffer (0.1M Tris (pH 7.8), 0.005M EDTA, 0.5% SDS) and 6μl proteinase-K (stock solution 20mg/ml) was added. The reaction mixture was incubated

over night at 50ºC followed by the phenol-chloroform extraction of the DNA described in Sambrook *et al.* (1989), with some modifications. The extraction step was only performed once and instead of an equal volume of phenol and chloroform 1.5 times the volume of chloroform was used. The mixture was centrifuged at 5000*g* for 15 minutes instead of the 12000*g* for 15 seconds.

The *Syp*I gene was amplified as described by Fevolden & Pogson (1997), but with a PCR protocol that closely followed the one described by Galvin *et al.* (1995a). To a total reaction volume of 20µl, containing 1X reaction buffer IV, 3.75 µM $MgCl_2$, 0.25mM dNTP, 0.0705 µM forward primer (B), 0.0720 µM reverse primer (Syn 7) and 1 unit of Taq, 2ng-5µg of template was added. The PCR was run in a Hybaid® Omn-E with the following program: (94°C-5.0 min.) * 1 cycle (94 °C-30 sec.; 55°C-30 sec.; 72°C-30 sec.) * 30 cycles. The PCR was ended by a 7 minutes extension period in 72 °C.

The *Syp*I polymorphism (Fevolden & Pogson 1997) was detected by digestion of the PCR product with the restriction enzyme *Dra*I. To 10µl of PCR product, 8µl of a mastermix was added, which contained 2.92 X restriction enzyme buffer and 6.67 units of *Dra*I. The mixture was incubated in 37°C for 90 minutes. The digestion was stopped by adding 1µl of EDTA and 4µl of loading dye.

The fragments corresponding to the two alleles (A and B) of the *Syp*I locus were separated in a 2% agarose gel (1 X TBE buffer) and stained with ethidium bromide. The electrophorese apparatus (Advanced Biotechnologies, Electro-Fast System; AB-0679) was adjusted to allow a separation distance of 2cm.

Most of the statistical analysis were performed by cross tabulating and one way ANOVA tests in STATGRAPHIC Plus 2.1 (STSC, Inc.) as well as Chi-square tests and trend analysis (one- and two ways tests). In this text, results from trend analysis are noted in the form of $b(x;\ n,\ p)$, where b means binomial distribution, x is the number of events from *n* number of trials, each with a likelihood of *p*. In order to compare body length independently of age, the individual body lengths were multiplied by a factor derived from the overall relationship between age and mean length in the material. The fixation index (F_{ST}) (Weir & Cockerham 1984), conformance to Hardy-Weinberg distribution and calculation of genetic disequilibrium between *Syp*I and *HbI** were performed with the Genepop (version 2) package (Raymond & Rousset 1995).

RESULTS

Except for 5 individuals, all analysed specimens could be genotyped for *Syp*I. The spring sample of 1996 was in 100% concordance with a control genotyping of the same individuals performed at the Icelandic department of marine science (Olof Jònsdòttir Bartes, pers. com.). All individuals except the 1991 and 1992 spring samples were genotyped for *HbI**.
All samples were in Hardy-Weinberg equilibrium. 9 out of the 13 samples had however a nominal excess of heterozygtes for *Syp*I where the females had a significant trend of an excess of heterozygotes ($b(11; 12, 0.5)=0.006$) (Table 2).

Table 2. *Syp*I F_{IS}-values for each sampling year (total) and for females and males in each sampling year in Verrasundet.

Sample	F_{IS} total	F_{IS} females	F_{IS} males
99-s	0.027	0.101	-0.031
98-f	-0.145	0.031	-0.146
98-s	0.051	0.071	0.092
97-s	-0.229	-0.333	0.039
96-s	0.162	0.146	0.222
95-s	0.079	0.143	0.051
94-s	0.083	0.263	0.060
92-s	0.156	0.159	0.156
91-s	0.029	0.238	-0.025
90-s	-0.032	-	-
89-s	0.211	0.307	0.157
86-s	0.074	0.231	0.025
85-s	-0.012	0.166	-0.034

The *Syp*I allele frequencies showed a highly significant heterogeneity between sampling years ($\chi^2=73.01$, df=11, P~0) and between year classes ($\chi^2=45.85$, df=20, P~0). The difference in allele frequency between sampling year and its corresponding year class was not significant. The observed differences in allele frequency between sampling years correspond to a F_{ST} value of 0.014, which is in the same magnitude as F_{ST} values reported from cod populations throughout the specie´s distribution range by Mork *et al.* (1985) and Galvin *et al.* (1995 b) but not by Pogson *et al.* (1995) who reported of a larger F_{ST} value, however mainly caused by the GM798 locus.

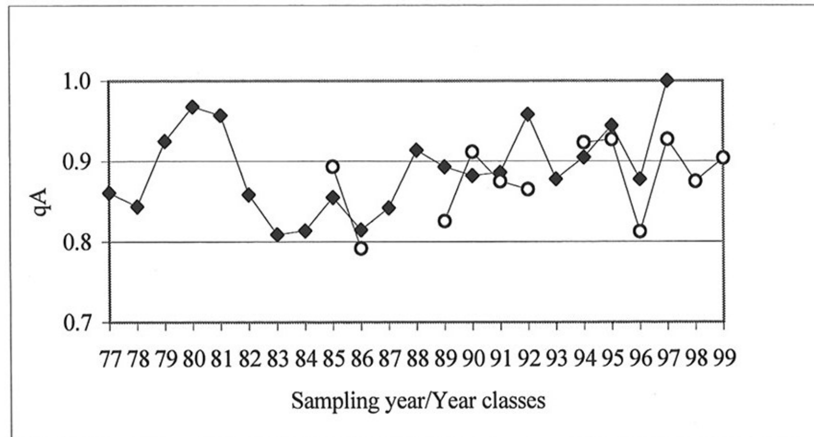

Figure 1. *SypI* allele frequency (qA) for each sampling year (○) and year class (♦) of cod in Verrasundet

When the samples were split into males and females, there were still a highly significant variation in *Syp*I allele frequency between sampling years for both females (χ^2=25.84 df=10, P<0.005) and males (χ^2=22.56, df=10, P=0.0125). In 10 out of 11 sampling years, the males had higher allele frequency than the females (*b*(10; 11, P=0.5)=0.012) (Figure 2.). With all sampling years pooled, the females had an average *Syp*I^A allele frequency of 0.788 and the males 0.903 (χ^2=59.16, df=1, P~0).

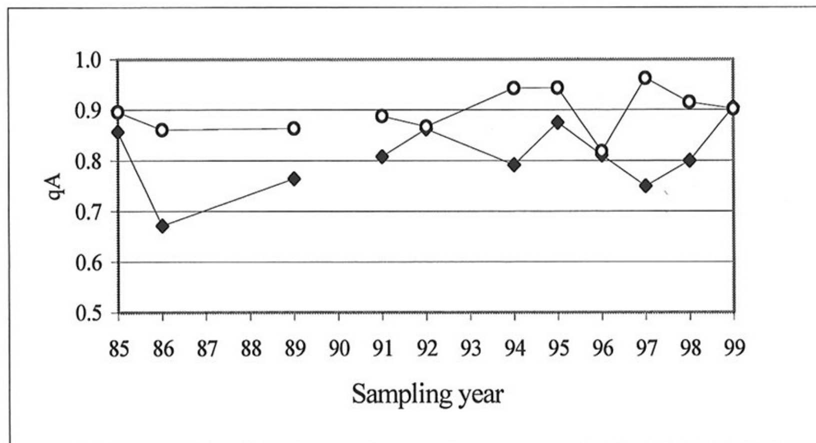

Figure 2. *SypI* allele frequency (qA) for each sampling year of cod in Verrasundet for females (♦) and males (○).

The *Syp*I allele frequency varied also significantly between sampling years within each locality (P=0.0028 in Verrabotn and P=0.0048 in Skalvik). Verrabotn had a significantly higher *Syp*I^A allele frequency than Skalvik (pooled sexes: P~0, males: P~0, females: P=0.08). Both sexes showed a higher excess of heterozygotes at Skalvik (males: F_{IS}=0.100, females: F_{IS}=0.130) than at Verrabotn (males: F_{IS}=0.004, females: F_{IS}=0.093).
Significant *Syp*I genotypic differences were observed for average gonadic stage, average corrected body length and average age for mature individuals (Table 3). Mature individuals had a significantly higher *Syp*I^A allele frequency than immatures (χ^2=21.89, df=1, P~0). The allele frequency in age groups varied significantly (χ^2=45,70 df=13, P~0). This heterogeneity was however only significant for the males (χ^2=40.50, df=12, P~0). There was an increase in *Syp*I^A allele frequency up to 3 years of age, followed by a steady decrease in allele frequency up to 14 years old individuals. Based on this observation the age groups were pooled into three classes (1-2, 3-6, 7-14 years old), where the *Syp*I allele frequency was homogenous within each class but heterogeneous between the classes (summed χ^2=39.50, df=18, P=0.002; over all sampling years). The age class of 3-6 years old individuals showed the lowest excess of heterozygotes.

Table 3. *Syp*I genotypic differences in average gonadic stage, age for mature individuals and body length.

	AA	AB	BB	ANOVA F(df)	P
Gonadic stage	2.64	2.32	2.18	8.30 (2, 781)	0.0003
Age	6.48	6.86	5.50	10.39 (2, 595)	~0
Length	65.40	64.00	72.60	4.20 (2, 1192)	0.009

The *HbI** showed no heterogeneity in allele frequency between sampling year, year classes, sex or sampling locality. Nor were there any genotypic differences in gonadic stage, length or age. No significant genotypic disequilibrium was detected between the *Syp*I locus and the *HbI** locus (summed χ^2=18.23, df=20, P=0.57, over all comparable sampling years). Figure 3 shows the pronounced difference in variation of allele frequency for *Syp*I and *HbI**. The variation in allele frequency at the *Syp*I locus was significantly higher than at the *HbI** locus (F(11,))=5.90, P=0.0064).

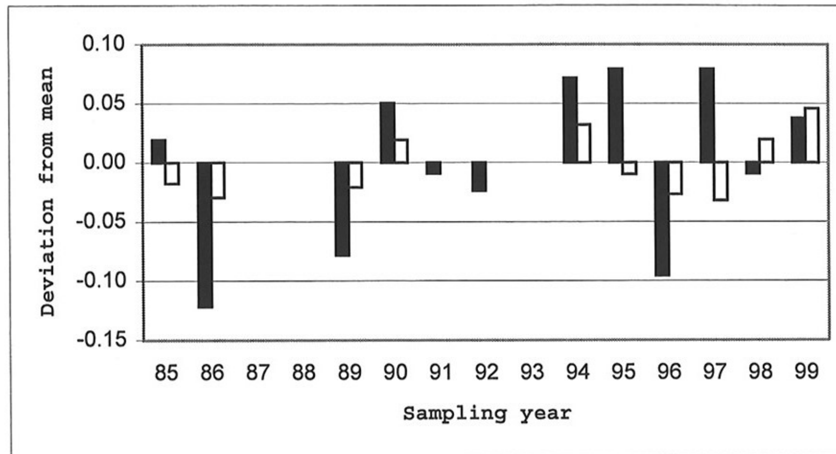

Figure 3. Annual deviations from overall mean of arcsine transformed allele frequencies for *HbI*^{1} (□) and *SypI*^{A} (■).

DISCUSSION

Genetic drift, mutation, gene flow and natural selection are the four evolutionary forces that potentially can affect the allele frequencies in a Mendelian population. How likely each of these are to affect the allele frequencies at the *HbI** and *Syp*I locus in the natural spawning population of Verrasundet is explored in this study.

Genetic drift

From annual commercial landings and tagging experiments, the well defined and extensively studied spawning area of Verrasundet (Sundnes1980 and Ekli 1997) is calculated to count at least 100 000 annual spawners (J. Mork unpublished). If the effective population size is not substantially smaller than this number, genetic drift can not be main responsible for the observed annual variation in *Syp*I allele frequency. Within a single population genetic drift is expected to cause a similar average amount of variation between generations in allele frequency at all loci. However, there was a significantly larger variability at *Syp*I than at *HbI** in the present time series.

Mutation

If mutation were the cause, the annual variation in allele frequency would correspond to a mutation rate of 0.072, which would be a very unusually high rate.

Gene flow

An immigration regime capable of affecting the allele frequency at sypI to the extent observed in this study would be expected to rapidly homogenize the allele frequencies of the populations involved. The study area has been populated by cod since the last glaciation, leaving plenty of time for homogeneity to be reached.

Tagging experiments have shown that the spawning cod disperse in the main fjord and that only a small part (~1.5%) will migrate out of the fjord during 5 years after release (Sundnes 1980). Tagging experiments performed by the Institute of Marine Research (IMR, Bergen, Norway) have shown that the Trondheims fjord received none or very few individuals from outside the fjord (O. R. Godø, IMR. pers. com.). Neither do the observations of an excess of heterozygotes in most of the sampling years (especially among the females) indicate immigration. Furthermore, if there is a significant effect of immigration, despite of the contraindications above, the immigrants must presumably have a *Syp*I A-allele frequency much lower than the local population (~0.2 which is near that reported for the North East Arctic cod by Fevolden & Pogson (1997)). If their allele frequency were higher than that of the local, the annual proportion of immigrants would have to be extremely high (even if they were fixed for the A-allele the necessary proportion would be 40%). So, from this assumption, less excess of heterozygotes would be expected in situations were low A-allele frequency were observed. This was however not the case, since the females showed lower A-allele frequency but more frequently an excess of heterozygotes. 3-6 years old individuals showed the highest A-allele frequency but a smaller excess of heterozygotes than the younger and older ones. Verrabotn showed higher A-allele frequency than Skalvik but a smaller excess of heterozygotes. Immigrants going to Verrasundet are expected to be mature, and from the arguments above have a lower A-allele frequency than the immature (local cod). On the contrary, however, the mature ones showed higher A-allele frequency.

The observed excess of heterozygotes could only to some extent be explained by the observed difference in allele frequency between the males and the females. Different allele frequency between the sexes is expected to give an excess of heterozygotes among the offspring (Falconer & Mackay 1996), and as expected there was a significant correlation between the expected and the observed excess of heterozygotes over the year classes ($F(1, 6)=6.79$, $P=0.04$). The difference in allele frequency and excess of heterozygotes between the sexes can however not be explained from this.

Natural selection
The most reasonable explanation for the observations made at *Syp*I is that natural selection is affecting this locus. The heterozygotes showed the highest average age among the mature individuals, but at the same time the smallest corrected body length. Since higher age at maturation usually results in larger size fish, this result may seems contradictory. It is however fully possible that the heterozygotes have a higher viability compared to the homozygotes among the in general small and slow growing individuals, leading to a higher representation of small individuals among the heterozygotes. The steady decrease in A-allele frequency from three years old individuals to older individuals is compatible with the observation of genotypic differences in average age for mature individuals and a larger excess of heterozygotes among the oldest individuals.
The strong contraindications of genetic drift and immigration as main explanatory factors, the high excess of heterozygotes, the unexpected different behaviour of *Syp*I and *HbI** and the genotypic differences give strong evidence that *Syp*I is a population genetic marker strongly affected by natural selection in Verrasundet. In addition *Syp*I (GM 798) has shown in several independent studies extremely high F_{ST} values between cod populations compared to other cDNA RFLP loci (Figure 4).

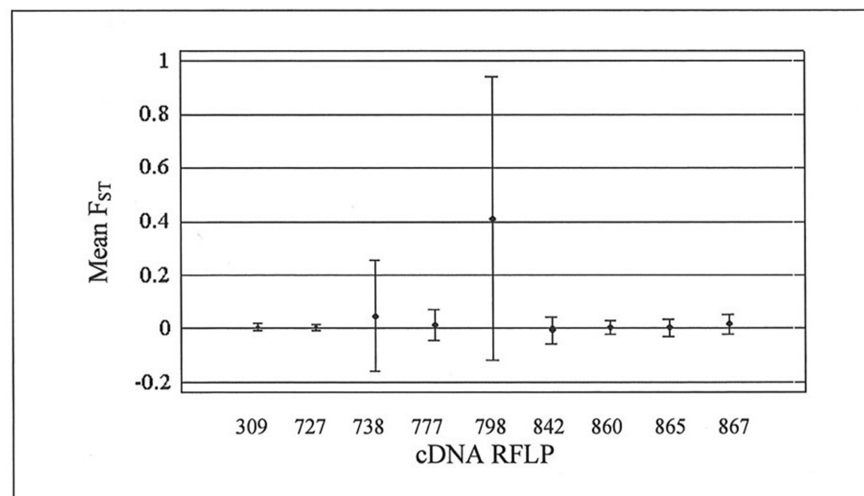

Figure 4. Mean F_{ST} with 95% confidence interval for 9 cDNA RFLP loci, common in three independent investigations of the population structure of cod (Fevolden & Pogson 1995; Pogson *et al.* 1995; Staub 1997). *SypI* is based on and developed from the GM 798 locus.

F_{ST} values for neutral genetic markers are expected to belong to the same statistical distribution and to show the same mean value for a given set of

populations as pointed out by Lewontin & Kraukauer (1973). However, Beaumont & Nichols (1996) identified two of the cDNA RFLP markers introduced by Pogson *et al.* (1995) as showing extremely high F_{ST} values by plotting the F_{ST} values against heterozygosity. They concluded that natural selection was responsible for the observations.
Different scenarios of natural selection can explain the persistence of the polymorphism at the *Syp*I locus. The most striking observations made in the present study are the differences in allele frequency and heterozygote proportion between the sexes. These observations may indicate different strength and/or type of selection between the sexes. If so, the females would be responsible for maintaining the polymorphism by overdominance. These observations are very interesting relative to the conclusion of an unusual mix of balancing and directional selection made by Pogson (2001) at the same locus (but now defined as Pantophysin *Pan*I). It is however very well possible that the direction as well as the strength of a selection can vary between years, due to some unknown variable environmental factor. The observed difference between Skalvik and Verrabotn may indicate that cod from these two areas have had different life histories prior to spawning. There is a wide variability of habitats for immature cod in the Trondheimsfjord. Fish from different areas could have experienced different pressures and directions of selection during their young stages. Groups of spawning cod migrating into Verrasundet may choose between Skalvik and Verrabotn based on life history/habitate experiences. Furthermore, it is a fact that the strength of the yearclasses vary considerably in the Trondheimsfjord (Ekli 1997). If we assume that this is mediated by total natural mortality, then the allele changing affects of selection may vary accordingly.

Pogson (2001) explored 9 radical amino acid substitutions within the A and B allele, suggesting that the alleles encode for proteins with considerable different physiological properties. Controlled crossing experiments with full sibs exposed for different environments could perhaps help us to understand the selective mechanisms which affect the *Syp*I polymorphism.

In conclusion, the present study has demonstrated a highly significant temporal instability in *Syp*I allele frequency during 3-4 generations in a local cod population. The females had significant lower allele frequency compared to the males as well as a significant trend of heterozygote excess which was not observed in males. Genotypic differences in growth, gonadic stage and age composition were observed in both sexes. Genetic drift, immigration and mutation were rejected as main explanatory factors. There was however strong evidence that *Syp*I is affected by natural selection in the

Trondheims fjord cod. The conclusion imply that a point estimate of *Syp*I allele frequency is an unreliable population characteristic of the cod population in the Trondheims fjord.

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Paper II

Karlsson S. & Mork J. (2003). Selection induced variation at the pantophysin locus (*Pan*I) in a Norwegian fjord population of cod (*Gadus morhua* L.). *Molecular Ecology*, **12**, 3265-3274.

Paper II is not included due to copyright.

Paper III (Poster)

Karlsson S. & Mork J. (2003). Effects of different selection regimes between sexes on the genotypic composition of offspring; observations in a Norwegian fjord cod population. Poster presentation at the 133rd Annual meeting, *American Fisheries Society,* August 10-14, 2003, Quebec City, Canada.

EFFECTS OF DIFFERENT SELECTION REGIMES BETWEEN SEXES ON THE GENOTYPIC COMPOSITION OF OFFSPRING; OBSERVATIONS IN A NORWEGIAN FJORD COD POPULATION

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Abstract

Natural selection acting on the genotypes at *Syp*I (recently renamed *Pan*I, a cDNA RFLP locus first described as GM 798 by Pogson et al. 1995) is well established (Pogson 2001; 2003). We studied the manifestations of this selection in a time series of spawning cod from the local population in Trondheimsfjorden, Norway. All specimens were sexed and aged, enableing a genetic characterisation of both the parental groups and the cohorts they created. An unusual selection regime was revealed, manifested, *inter alia*, as a heterozygote excess in females and different allele frequencies in males and females. This pattern was consistent over a 15 year period (3-4 cod generations) and statistically significant.

From population genetics theory it is known that different allele frequencies between males and females will produce an excess of heterozygotes in the offspring. This effect was observed in the present materials. The correlation between the magnitude of the sexual allele frequency difference and the heterozygote excess in the offspring was significant. This type of selection regime will, similar to overdominance, contribute to the preservation of alleles. To our knowledge it has not previously been reported for natural fish populations.

Introduction

The polymorphism at the nuclear-encoded *Syp*I locus has frequently been used in studies of population genetic structure in cod (Pogson et al. 1995, Fevolden & Pogson 1995, Fevolden & Pogson 1997, Jonsdottir et al. 1999). Several resent studies have suggested that natural selection is acting upon this locus (Karlsson & Mork 2001, Pogson 2001, Pogson & Fevolden 2003). In the present study we examined variation in *Syp*I genotypes in a time series of a cod stock in the Trondheims fjord, Norway, including 22 different cohorts represented by both males and females.

Material and methods

Samples from spawning cod were collected over a 15 year period with bottom trawl by R/V "Harry Borthen I" from Trondhjem Biological Station, NTNU. In total, 1290 individuals were assayed for genotypes at *Syp*I (Fevolden & Pogson 1997). Age determination from otholiths were performed by the method of Rollefsen (1933). Sex and gonadal stage was recorded for each individual.
Different allele frequencies in mating males and females is expected to produce an excess of heterozygotes among the offspring compared to Hardy-Weinberg proportion (Hedrick 1985, Falconer & Mackay 1996). Fig. 1 depicts this relationship graphicaly (the sexual difference in allele frequency in parents is delta q, and the expected increase in heterozygosity in offspring is Z).

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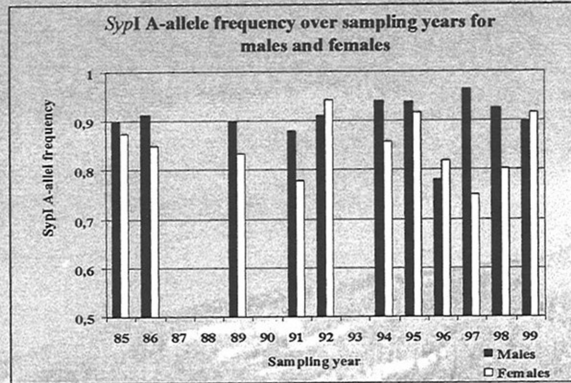

Figure 2. SypI A-allele frequency for mature male and female cod presented for each sampling year.

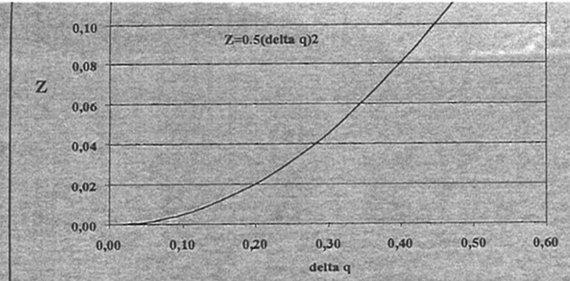

Figure 1. Theoretical relationship between difference in allele frequency between spawning males and females (delta q) and deviation in heterozygosity/homozygosity (Z) among offspring, as compared to Hardy-Weinberg with an equal allele frequency.

Statistical analysis of allelic and genotypic frequencies were performed by chi-square and sign tests. In situations with low expected values, chi-square tests were replaced by Monte Carlo-based exact tests (Zaykin & Pudovkin 1993).

Result

There was a significant difference in *Syp*I A-allele frequency between mature females and males in most sampling years (Summed chi-square=27.11, df=11, P=0.004; cf Fig. 2). The offsprings of these spawning groups showed an excess of heterozygotes that was particularly pronounced in the females. Assuming that our samples are representative of spawning groups and cohorts, the expected effect of sexual allele frequency differences can be compared to the observed deviation in heterozygosity/homozygosity (Figure 3). The males, but not the females, showed a significant correlation between expected and observed deviation from the heterozygosity/homozygosity predicted by Hardy-Weinberg (R-squared=58%, P=0.046). As showned by Fig. 1, the sexual difference in allele frequency must be relatively large in order to result in a notiseable heterozygote excess. This could explain why the relatively large excess of heterozygotes observed among the females could not be explained by this effect.

Conclusions

We observed significant different *Syp*I A-allele frequency between spawning males and females of cod. From population genetics theory this is known to produce an excess of heterozygotes among the offspring, which was observed. The size of the sexual allele frequency difference and the amount of heterozygote excess was significantly correlated among the males. This effect was not sufficient to explain the large excess of heterozygotes observed among the females, which could presumably have been created during their own lifespan. This study revealed evidence of a heterozygote excess caused by different allele frequencies in pawning males and females. This scenario will, similar to balancing selection contribute to the preservation of alleles. Although known from population genetics theory, this effect has to our knowledge not previously been reported from natural fish populations.

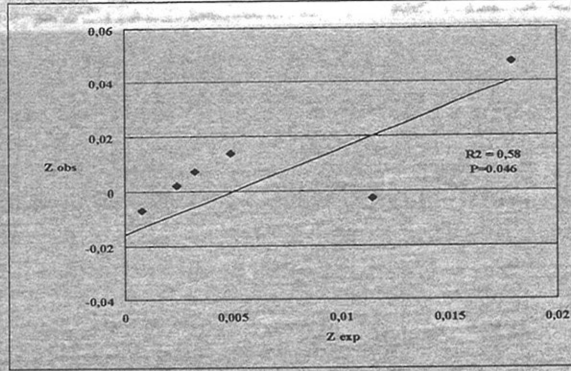

Figure 3. Simple regression analysis of observed (Zobs) and expected (Zexp) deviation from expected heterozygosity/homozygosity according to Hardy-Weinberg, due to different allele frequency between spawning males and females.

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Paper IV

Karlsson S. & Mork J. Temporal instability in microsatellite allele frequencies in Atlantic cod. *ICES Journal of Marine Science,* Submitted.

Temporal instability in microsatellite allele frequencies in Atlantic cod

Sten Karlsson and Jarle Mork

A total of 1455 spawning cod, sampled from a local spawning area in Trondheimsfjord (Norway) between 1985 and 2002 were assayed for the microsatellites Gmo132 and Gmo2. Samples from 15 spawning years comprising 29 consecutive cohorts were analyzed. At the Gmo132, but not the Gmo2 locus, allele frequencies varied significantly among sampling years as well as cohorts, corresponding to Fst values of 0.004 and 0.006 respectively, for Gmo132. Both loci showed significant deviations from Hardy-Weinberg expectation within sampling years as well as cohorts, mostly manifested as deficiencies of heterozygotes. For the Gmo132 locus a heterozygote deficiency was significant also in pooled samples. Possible reasons for the deficency were discussed; *inter alia* the existence of null alleles, or a form of pseudo Wahlund effect due to a patchy distribution of habitats for settling O-group cod in the Trondheimsfjord. It was noted that there might be a relation between the relatively high temporal within-population variability of allele frequencies at Gmo132 and the fact that among microsatellite loci studied so far, Gmo132 is the one that usually shows the highest genetic differentiation geographically in cod.

Keywords: Microsatellite, Gmo132, Gmo2, Atlantic cod

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Introduction

Microsatellites are increasingly used for the study of genetic population structure of natural populations. In Atlantic cod, results from such studies at different geographic scales have been reported (Bentzen *et al.*, 1996; Ruzzante *et al.*, 1998; Hutchinson *et al.*, 2001; Knutsen *et al.*, 2003). Most studies were based on point estimates in time (i. e. sampling each location only once), while a few also consider the temporal aspect, i. e. whether the local genetic characteristics were stable in time in each population (Ruzzante *et al.*, 1996; Ruzzante *et al.*, 2001; Hutchinson *et al.*, 2003). More than theoretically expected, the level of genetic differentiation among geographic regions differs depending on the microsatellite locus used (Bentzen *et al.*, 1996; Ruzzante *et al.*, 1998; Lage *et al.*, 2004). The Gmo132 locus, which has been frequently used, appears to show the highest degree of genetic differentiation compared to other microsatellite loci (Bentzen *et al.*, 1996; Ruzzante *et al.*, 1998; Ruzzante *et al.*, 2000a; Ruzzante *et al.*, 2000b; Ruzzante *et al.*, 2001; Knutsen *et al.*, 2003; Tuula *et al.*, 2003; Lage *et al.*, 2004). In six out of these eigth studies it showed the largest F_{ST} values among the microsatellite loci included (Table 1), and the mean Fst of Gmo132 was significantly higher than the mean of the other microsatellites, lumped in each study (ANOVA, $F_{1, 14}=5.88$, $P=0.029$).). A similar phenomenon has also been observed among cDNA RFLP loci, where *Pan*I showed much higher F_{ST}-values compared to the other loci included (Pogson *et al.*, 1995). The dynamics of the *Pan*I polymorphism were studied by Karlsson and Mork (2003) using a time series of cod collected from the same spawning population as in this study. The same approach is used in this study where we test the allele frequency stability over sampling years and cohorts, as well as the Hardy-Weinberg conformance at two microsatellite loci in cod: Gmo132 and Gmo2.

Materials and methods

Cod were collected during spawning season in Verrasundet, a relatively narrow side-fjord in the inner part of Trondheimsfjord, Norway (Figure 1). Verrasundet is a long known and well defined spawning area in Trondheimsfjord (Dahl, 1899; Sundnes, 1980; Mork *et al.*, 1985; Mork and Giæver, 1999).

Samples from spawning cod were collected over a 18-year period with a bottom trawl (35mm streched mesh in the cod-end) by the R/V "Harry Borthen I" from Trondhjem Biological Station, NTNU (The Norwegian University of Science and Technology). In total 1455 individuals were assayed for genotype at Gmo132 and Gmo2 (Table 1). Except for the sample taken in 1990, sex, age, gonadal stage and total body length were recorded for each individual. Age determination was performed by Ekli (1997 and unpublished) according to the breakage-and-side-illumination method of Rollefsen (1933). Determination of gonadal maturation stage followed Sivertsen (1935): stage I, immature; stage II, maturing; stage III, running and stage IV, spent.

Samples of blood, white muscle, gills, heart, liver, and kidney were stored frozen or in ethanol and DNA was extracted from one of these tissue types, according to the phenol chloroform extraction procedure described in Sambrook *et al.* (1989) with some modifications described in Karlsson and Mork (2003).

PCR analysis of Gmo132 and Gmo2 were first described by Brooker et al. (1994). The reverse primers were end-labeled with IRD800 (MWG-biotech AG). The PCR reaction mixture contained 2ng-5µg template DNA, 0.26µM labeled reverse primer, 0.26µM forward primer, 53mM KCl, 10.5 mM Tris-HCl (pH 9.0), 1.6mM MgCl, 217 mM dNTP, 0.12% Tween, 38ng BSA, and 1 unit of Taq Polymerase (Amersham pharmacia biotech) in a 19-

μl volume. PCR was carried out in a Hybaid® Omni-E thermal cycler programmed for 30 cycles of denaturation at 95°C (1 min), primer annealing at 52°C for Gmo132 and 50°C for Gmo2 (2 min), primer extension at 72°C (20 s). Depending on the strength of the amplification, the PCR products were diluted 1:10-1:50 with formamide loading dye (98% formamide, 10μM EDTA, 1mg/ml bromophenol blue). The diluted PCR product was denatured at 95°C for 5 min. After denaturing, the samples were directly put on ice and 0.5μl of the PCR product were subjected to gel electrophoresis on a 6% denaturing polyacrylamide gel. The electrophoresis was carried out on a LI-COR 4000 DNA sequencer with 25-cm electrophoresis plates. For each tenth well we loaded, adjecently, a commercial ladder (50-350 bp sizing standard, LI-COR, Inc) and a self-made ladder of mixed fragments achieved from Gmo132 and Gmo2 respectively. The lengths of the fragments were determined in relation to the commercial ladder and the lengths of following fragments were determined accordingly. All gel images were analysed manually.

Statistical analyses of continuous variables were performed by one-way analysis of variance (ANOVA) as implemented in STATGRAPHIC Plus 2.1 (STSC, Inc.). For discrete variables, χ^2 and sign tests were used as appropriate. In situations with low expected values (i.e. more than 25% of cells with expected numbers < 5), the traditional χ^2 tests were replaced by the Monte Carlo based exact test (1000 iterations) of Zaykin and Pudovkin (1993). Probabilities that were estimated from individual exact tests were combined into an overall probability by the "omnibus" test of Fisher (1954). In order to compare body length independently of age, individual body length was multiplied by a factor derived from the relationship (linear regression in a double logarithmic plot) between age and mean length in the total material. Homogeneity tests based on allele frequencies over sampling

year and cohort, as well as tests of conformance to Hardy-Weinberg genotypic expectations were carried out with GENEPOP ver.3 (Raymond and Rousset, 1995). Estimates of fixation index θ, the F_{ST} analogue of Weir and Cockerham (1984) were carried out with the GDA program of Lewis and Zaykin (2001) with the option of jackknife across populations, giving the mean and standard deviation of θ. From the mean and standard deviation of θ we were able to test if estimates of θ from Gmo132 and Gmo2 were significantly different from each other. We used MICRO-CHECKER, a free software designed to check microsatellite data for null alleles and scoring errors (available at http://www.microchecker.hull.ac.uk/). In principal, the program explores genotypic data by testing if there is an excess of homozygotes caused by null alleles, stuttering or large allele dropout.

Gmo132 and Gmo2 are highly polymorphic with large numbers of alleles. Analyses performed on these loci will require large sample sizes (Chakraborty, 1992). One procedure to compensate for low sample sizes, and thus large confidence intervals, is binning of alleles (Bentzen *et al.*, 1991; O'Connell and Wright, 1997; Shaw *et al.*, 1999). Even though the sample sizes in the present study were relatively large as compared to recommendations by Ruzzante (1998), analyses were performed with both unbinned and binned alleles. The many and skewly distributed alleles observed at Gmo132 and Gmo2 result in samples where many alleles are represented by very few individuals and hence allele frequency homogeneity test using all alleles will have relatively low statistical power. At both loci, all but the most frequent allele were binned into one allele, resulting in a 2-allele-system. For Gmo2 we additionally binned all alleles except the 8 most frequent one. The binning procedure helped us to explore the temporal variation more extensively without having any effects of large variation in statistical power.

Results

The annual sample sizes ranged from 42 to 159 individuals, with an overrepresentation of males. There was a large span of ages and body lengths. A majority of individuals had running gonads, but immature and spent speciments were also present (Table 2).

The total number of cod analyzed were 1456 for Gmo132 and 1455 for Gmo2. Both the Gmo132 and the Gmo2 locus were highly polymorphic with 33 and 22 alleles and expected heterozygosities of 0.707 and 0.861 respectively. The lower expected heterozygosity at Gmo132 despite the large number of alleles was due to a highly skewed allele frequency distribution at this locus compared to Gmo2 (Figure 2). The cohort sample sizes were fairly large except for the four oldest cohorts and the two youngest cohorts. As expected, the number of alleles represented in a cohort increased with increasing sample size. There were larger number of singleton alleles at Gmo132 compared to Gmo2 due to the skewer distribution of allele frequencies and larger number of alleles at Gmo132 (Table 3).

We did not observe any significant association of genotypes or allele frequency at Gmo132 or Gmo2 with phenotypic traits, such as body length, age, gonadal stage, and sex. These analyses were performed within annual samples and within cohorts as well as on pooled materials.

Here, results from analysis of conformance to Hardy-Weinberg expectation at Gmo132 and Gmo2 are first presented, followed by presentation of variance observed among annual samples and among cohorts.

Conformance to Hardy-Weinberg expectations

Table 4 summarizes the features of Gmo132 and Gmo2 in the total material of cod analyzed in this study. At the Gmo132 locus, 4 out of 15 annual samples showed significant departures from Hardy-Weinberg equilibrium and overall the departure was highly significant. At the Gmo2 locus, 3 out of 15 annual samples showed significant departures from Hardy-Weinberg equilibrium and overall the departure was not significant (P=0.074). At both Gmo132 and Gmo2 the departure was mainly due to a deficit of heterozygotes, because at both loci, 12 out of 15 annual samples showed a deficit of heterozygotes, which is a significant trend (P=0.036, two-way binomial sign test). The deficiencies at the two loci did however only coincide in 10 out of the 15 samples.

The result was similar when analyzes were performed on cohorts instead of annual samples. At Gmo132, 4 out of 26 cohorts showed significant departures from Hardy-Weinberg equilibrium but overall the departure was highly significant ($\chi^2_{[52]}=\infty$, P~0). At Gmo2, 3 out of 26 cohorts showed significant departures from Hardy-Weinberg equilibrium and overall the departure was not significant ($\chi^2_{[52]}=67.6$, P=0.072). Compared to the annual samples, some of the cohort sample sizes were relatively small (Table 2) and hence a lower statistical power was achieved. At both loci the departure was mainly due to a deficit of heterozygotes. At Gmo2 18 out of 26 cohorts showed a deficit of heterozygotes (P=0.076, two-way binomial sign test). At Gmo132 17 out of 26 cohorts showed deficits of heterozygotes (P=0.168, two-way binomial sign test). The deficiencies at the 2 loci did however only coincide in 12 cohorts.

The MICRO-CHECKER software revealed evidence of a general excess of homozygotes for most allele size classes at Gmo2 in the 1999 sample and at Gmo132 in the 1995 sample. When all annual samples were pooled, there was a general excess of homozygotes of most allele size

classes at both Gmo2 and Gmo132 (pooling annual samples was however not appropriate regarding Gmo132, because as shown below, there was significant heterogeneity in allele frequencies among annual samples at this locus). There were no indications of scoring errors due to stuttering or large allele dropout. The general excess of homozygotes for most allele size classes may hence indicate the presence of null alleles or it may indicate a Wahlund effect.

Temporal variation among annual samples

Figure 3 shows the arcsine transformed allele frequency of the most common allele at Gmo132 and Gmo2 for each annual sample. The figure shows a more pronounced fluctuation in allele frequency at Gmo132 as compared to Gmo2. Homogeneity tests of allele frequencies among annual samples were performed both from binned and unbinned alleles (Table 5). When alleles were not binned there was a highly significant variation among annual samples at Gmo132 but not at Gmo2. When all alleles except the most frequent ones were binned the heterogeneity was even more pronounced at Gmo132 but still not significant at Gmo2. The Gmo2 locus was further explored by binning all except the 8 most frequent alleles. This 9-allele-system did however not reveal any significant heterogeneity among annual samples.

Estimates of the among annual samples genetic variance (F_{ST}) revealed almost significant heterogeneity at the Gmo132 locus but not at the Gmo2 locus (Table 5). The estimates of F_{ST} from Gmo132 and Gmo2 differed significantly from each other ($F_{1,26}=29.93$, $P=0.0001$).

Temporal variation among cohorts

Homogeneity tests of allele frequencies among cohorts showed similar results as tests among annual samples (Table 5). When alleles were not binned there was a highly significant variation among cohorts at Gmo132 but not at Gmo2. When all alleles except the most frequent ones were binned the highly significant heterogeneity remained at Gmo132 but still not significant at Gmo2. When all except the 8 most frequent alleles were binned there was no significant heterogeneity in allele frequencies among cohort at the Gmo2 locus.

Arcsine transformed allele frequency of the most common allele at Gmo132 and Gmo2 for each cohort shows a more pronounced fluctuation in allele frequency at Gmo132 as compared to Gmo2 (Figure 4). The diverging allele frequencies of cohort 72 and 73 are probably due to few individuals represented (Table 3). Estimates of the among cohort genetic variance (F_{ST}) revealed almost significant heterogeneity at the Gmo132 locus but not at the Gmo2 locus (Table 5). The estimates of F_{ST} from Gmo132 and Gmo2 differed significantly from each other ($F_{1,54}$=116.40, P~0).

Discussion

In a large panmictic and isolated population, neutral genetic markers are expected to show no change in allele frequency through generations on a short evolutionary time scale. In the present study we did however find significant variation in allele frequency over time at one locus and also significant departures from Hardy-Weinberg expectation, mainly caused by heterozygote deficiencies.

Departure from Hardy-Weinberg equilibrium

The observed deficit of heterozygotes as compared to Hardy-Weinberg equilibrium observed at both Gmo132 and Gmo2 could be a result of physical mix of populations with different allele frequencies (Wahlund effect) or it can be caused by natural selection, phenotypic assortative mating, inbreeding, and (or) null alleles. We believe that Wahlund effects and/or null alleles are the most reasonable explanations for the observed deficiencies. It is reasonable to assume that a Wahlund effect would be expressed in a sample of spawning individuals like in our annual samples but not in the cohorts if immigrants are spawning individuals. This is because immigrants coming into the spawning grounds and mix with the local cod will result in a Wahlund effect but the resulting cohort would be in Hardy-Weinberg equilibrium. There was however almost the same deviation from Hardy-Weinberg expectation in the cohorts as compared to annual samples, with respect to trend of heterozygote deficiency and overall depature from Hardy-Weinberg equilibrium. Also, there was no significant temporal trend in allele frequencies as would be expected by a frequent effect of immigrants.

Compared to several other microsatellite loci in cod, Gmo2 has in several independent studies shown the largest or among the largest

heterozygotes deficiency, but also relatively low or moderate levels of differentiation among populations (Ruzzante *et al.*, 2000a; Bentzen *et al.*, 1996; Ruzzante *et al.*, 2000b; Ruzzante *et al.*, 1996). If this tendency of Gmo2 to show deficits of heterozygotes is caused by Wahlund effects, then it should correspond to a relatively large level of differentiation among populations as well, which is not the case. This is because potential immigrants are expected to create a greater Wahlund effect in the resident population with greater difference in allele frequency between the resident and the donor population. The Gmo132 locus has on the other hand not shown a tendency of Whalund effects as compared to other loci included in the referred studies, although Gmo132 shows the highest level of differentiation. Potential donor populations along the Norwegian coast of immigrants to the Trondheimsfjord/Verrasundet show low level of differentiation at Gmo2 but almost one order of magnitude higher differentiation at Gmo132, as measured by F_{ST} (personal communication, Tuula Sarvas, University of Tromsø, The Norwegian College of Fishery Science). These observations predict that a Wahlund effect would be more pronounced at Gmo132 compared to Gmo2 if Trondheimsfjord receives immigrants from one or several populations outside the fjord. We did however only observe modest difference in the level of heterozygote deficiency between Gmo132 and Gmo2. Also, there was no significant temporal trend in local allele frequencies at Gmo132 as would be expected by a frequent effect of immigrants.

Observations in the present study and previous studies of cod in Trondheimsfjord (e.g. Mork and Giæver, 1999; Karlsson and Mork, 2003) are not consistent relative to a Wahlund effect hypothesis. An alternative or additional explanation to the observed heterozygote deficiencies might be the presence of null alleles (O'Connel and Wright, 1997, and references therein). An attempt to check for null alleles in our microsatellite data did

not give any clear-cut conclusion because the general trend of excess of homozygotes was seen at both loci. Such an observation could be ascribed to both null alleles and Wahlund effects. Besides from sequencing the flanking regions of Gmo2 and Gmo132, the only possibly observations that could support the presence of null alleles at these two loci are the absence of heterozygote deficiencies at other loci. Previous isozyme studies on this spawning ground did not show any heterozygote defieciencies at *PGM-1**, *MDH-3**, *IDHP-1**, and *PGI-2** (Mork and Giæver, 1999; Mork *et al.*, 1985). On the countrary, an excess of heterozygotes was observed at the *Pan*I locus (Karlsson and Mork, 2003) among cod including the same individuals as in the present study. The *Pan*I is however an unreliable marker in detecting a Wahlund effect because it is suggested to be affected by natural selection.

Temporal variation

The unusually high genetic differentiation among geographic populations at Gmo132 (Bentzen *et al.*, 1996; Ruzzante *et al.*, 1996; Ruzzante *et al.*, 1998; Ruzzante *et al.*, 2000b; Knutsen *et al.*, 2003, Tuula *et al.*, 2003, Lage *et al.*, 2004) prompted our study of the temporal stability of allele frequencies at this locus in one population. Our observation indicates that there might be a relation between high local temporal variability and large geographic variability at a locus. This gets some support from observations at the *Pan*I locus in a previous study (Karlsson and Mork, 2003). The Gmo2 locus on the other hand has shown low or moderate level of geographic differentiation and does not show local temporal variation.

If one or more relatively large populations of cod were giving off immigrants to the spawning population in Verrasundet, causing the observed variation in allele frequency at Gmo132, one would expect the allele frequencies to homogenize among the donor and the recipient

population in a relatively short evolutionary perspective, as well as a directional trend over time in allele frequences in the local population. Alternatively, if populations of cod giving off immigrants to Verrasundet were relatively small and influenced by strong genetic drift there could be a balance between gene flow and genetic drift that could maintain the high genetic variation observed in Verrasundet and also contribute to the observed variation in allele frequencies. Such a system would be very complicated and the definition of the populations would be very difficult. If one, on the other hand, tries to explain our observations by events taking place in the Trondheimsfjord population proper, one may utilize information from some previous studies performed on this stock.

The Trondheimfjord cod is very well studied. Results of tagging experiments (Sundnes, 1980) do not indicate the existence of more than one population of cod in the Trondheimsfjord. Sexually mature cod show a cyclical migration pattern within the fjord. After spawning in Verrasundet, cod disperse into the main fjord and remain there until the next spawning season. Only a small proportion (~1.5%) of tagged fish have been recaptured outside Trondheimsfjord 5 years after release (Sundnes, 1980). Furthermore, tagging experiments performed by the Institute of Marine Research (IMR, Bergen, Norway) have shown that Trondheimsfjord receives virtually nil individuals from coastal or oceanic areas outside the fjord (O.R. Godø, IMR, personal communication). We find it therefor reasonable to regard cod in Trondheimsfjord as a self-recruiting unit (local population). Verrasundet is the most important spawning area for cod in Trondheimsfjord, but there are several other spawning areas as well (Mork, 2000). It is reasonable to believe that cod from the different areas intermingle and that cod sampled in Verrasundet consist of groups that grew up in different areas of the fjord. Cod that settle in one area may by chance have a slightly different genetic composition then other areas, and when

these grow up they may not be representative for the Trondheimsfjord as such when sampled in Verrasundet. Such a dynamic system could explain the maintenance of the high genetic variation, heterozygote deficiency, and temporal variation in allele frequencies observed in Verrasundet, both for neutral and non-neutral markers (see Karlsson and Mork, 2003). However, Mork *et al.* (1985) and Mork and Giæver (1999) did not observe deviations from Hardy-Weinberg equilibrium in Trondheimsfjord cod at isozyme loci. There is no *a priori* support to assume that microsatellites are non-functional or selectively neutral (Kashi and Soller, 1999). We can therefore not exclude that the apparent differences observed between Gmo132 and Gmo2 are due to natural selection at Gmo132. It is however too early to draw such a conclusion at this stage. Instead, further investigations are needed to understand the dynamics of the Gmo132 polymorphisms in Trondheimsfjord. For example, information is needed on the genotypic composition of the young of the year cod and the extent of genetic heterogeneity between young cod habitats.

In conclusion, the allele frequencies at Gmo132 in the Trondheimsfjord cod show so much temporal heterogeneity that a characterization of the population by this locus must be unreliable. This suggests that conclusions about genetic differentiation among geographically separated groups of cod based on this locus should be drawn with care.

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Table 1. Rewiev of F_{ST} estimates reported from 8 different studies of population structure of cod where the Gmo132 were included. F_{ST} values in bold are those included in statistical testing. *F_{ST} values were obtained by taking the mean of estimates from three time periodes each including the same geographic areas.

Locus	F_{ST}	Reference	Mean F_{ST} (Gmo132 not included)
Gmo132	**0.0044**	Ruzzante et al. 2000b	
Gmo151	0.0024		
Gmo145	0.0013		**0.0012**
Gmo120	0.0012		
Gmo4	0.0007		
Gmo2	0.0005		
Gmo132	**0.092**	Bentzen et al. 1996	
Gmo2	0.003		
Gmo145	0.003		**0.0024**
Gmo4	0.003		
Gmo141	0.003		
Gmo120	0		
Gmo132	**0.043**	Ruzzante et al. 1998	
Gmo145	0.003		
Gmo120	0.001		**0.0015**
Gmo4	0.001		
Gmo2	<0.001		
Gmo2	0.017	Ruzzante et al. 2000a	
Gmo4	0.011		
Gmo120	0.011		**0.0118**
Gmo145	0.008		
Gmo132	**0.005**		
Gmo132	**0.051**	Tuula et al. 2003	
Gmo37	0.023		
Tch11	0.008		
Gmo2	0.006		**0.0069**
Tch13	0.005		
Gmo8	0.004		
Gmo19	0.002		
Gmo35	0		
Gmo36	0.0051	Knutsen et al. 2003	
Gmo132	**0.0047**		
Gmo3	0.0045		
Gmo37	0.0036		
Gmo19	0.0022		**0.0024**
Gmo35	0.0020		
Gmo34	0.0009		
Tch13	0.0006		
Tch12	0.0002		
Gmo132	**0.01777**	Ruzzante et al. 2001*	
Gmo1	0.00685		
Gmo2	0.00413		**0.0034**
Gmo4	0.00277		
Gmo145	0.00180		
Gmo120	0.00165		
Gmo132	**0.0255**	Lage et al. 2004	
Gmo1	0.0019		
Gmo8	0.0001		**0.0005**
Gmo19	0		
Gmo34	0		
MEAN	**0.0304**		**0.0040**

Table 2. Summary data annual samples

Year	N	Gmo132^{112}	Gmo02^{106}	Females (%)	Length (mm)	Gonad	Age
2002	42	0.670	0.262	55	260-840	I-IV	2-10
2001	87	0.466	0.230	17	299-753	I-IV	3-10
2000	76	0.395	0.237	17	310-775	I-III	2-7
1999	146	0.517	0.236	44	285-970	I-IV	2-11
1998	100	0.520	0.235	35	345-960	I-IV	3-10
1997	96	0.443	0.307	17	330-830	I-IV	3-10
1996	96	0.562	0.302	77	273-975	I-IV	2-14
1995	96	0.490	0.307	25	370-910	I-III	3-11
1994	91	0.538	0.209	13	261-990	I-IV	2-13
1992	100	0.630	0.235	40	192-714	I-IV	1-11
1991	80	0.544	0.238	16	331-935	I-IV	2-13
1990	96	0.521	0.266	-	387-699	-	3-13
1989	94	0.543	0.197	40	218-825	I-IV	2-12
1986	96	0.599	0.240	39	95-970	I-IV	1-12
1985	159	0.594	0.272	9	370-940	I-IV	3-13
Total	1455	0.532	0.252	31			

N, sample size; Gmo132^{112}, frequency of the most common allele at Gmo132 locus 112bp in size; Gmo2^{106}, frequency of the most common allele at Gmo2 locus 106 bp in size. Percentage of females, range of body length, gonadal stage, and age are also presented for each annual sample.

Table 3. Summary data cohorts

Cohort	N	Gmo132 $n_{alleles}$	Gmo132 $n_{singletons}$	Gmo2 $n_{alleles}$	Gmo2 $n_{singletons}$
72	1	1	0	2	2
73	1	2	2	2	2
74	11	6	3	8	2
75	2	3	2	3	2
76	13	7	4	11	6
77	36	17	9	11	2
78	49	15	4	17	8
79	40	17	8	13	4
80	31	18	11	12	3
81	35	19	4	12	4
82	46	18	7	16	6
83	54	18	5	13	4
84	59	18	7	17	6
85	102	25	5	13	0
86	56	16	3	10	0
87	39	17	6	10	1
88	58	20	5	12	3
89	75	19	7	15	4
90	88	20	4	14	3
91	147	23	4	15	3
92	35	19	6	13	3
93	42	17	5	12	4
94	182	23	1	15	1
95	51	21	3	13	1
96	117	27	9	17	4
97	49	18	3	13	4
98	25	15	7	12	3
99	6	6	4	4	1
100	1	2	2	2	2
Total	1451	33	3	22	1

Sample sizes (N), number of alleles ($n_{alleles}$), and number of singletons alleles ($n_{singletons}$) in cohorts at Gmo132and Gmo2. Total number of individuals differ from Table 1because four individuals were not aged.

Table 4. Single locus statistics of genetic variation overall annual samples.

Locus	N (ind.)	n (alleles)	Range (bp)	Het_{obs}	Het_{exp}	D	HWE $\chi^2_{[df]}$, P
Gmo132	1456	33	96-166	0.682	0.707	-0.035	$\chi^2_{[30]}=\infty$, P~0
Gmo2	1455	22	102-146	0.835	0.861	-0.030	$\chi^2_{[30]}=41.8$, P=0.074

N, samples size; n, number of alleles; allele range given in basepairs; mean observed and mean expected heterozygosity; D, deficiency of heterozygotes measured as $(H_{obs}-H_{exp})/H_{exp}$; HWE, Hardy-Weinberg equilibrium test presented by χ^2, degres of freedom, and corresponding P-value as implemented in Genepop ver.2, Fisher's over all test.

Table 5. Single locus homogeneity test and F_{ST}-values, among annual samples and among cohorts, with unbinned alleles, binning into 2-allele-system, and a 9-allele-system at Gmo2.

Locus	alleles	Annual Samples P	Cohort P	F_{ST} annual samples (P)	F_{ST} cohorts (P)
Gmo132	33	0.009	~0	0.00392 (P=0.077)	0.0064 (P=0.081)
Gmo2	22	0.346	0.095	0.00043 (P~1)	0.0003 (P~1)
Gmo132	2	~0	~0	0.00928 (P=0.002)	0.01687 (P~0)
Gmo2	2	0.388	0.520	0.00096 (P=0.260)	-0.00149 (P=0.640)
Gmo2	9	0.388	0.302	0.00016 (P=0.220)	0.00024 (P=0.613)

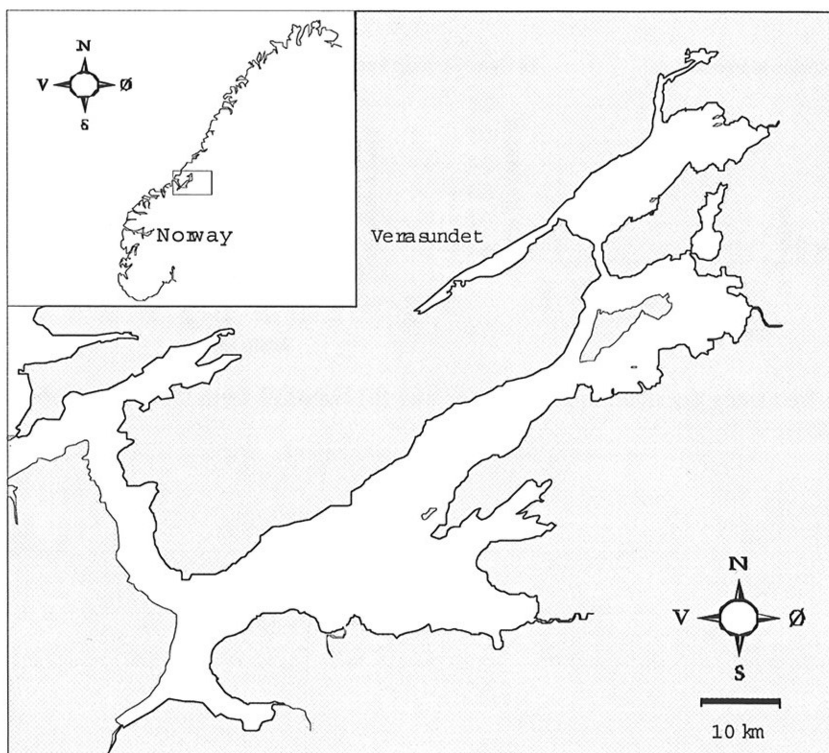
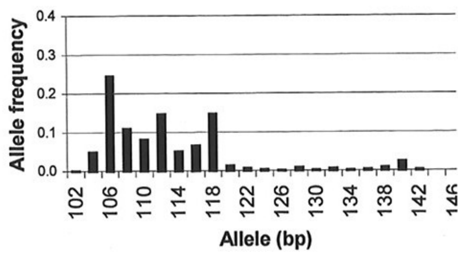


Figure 1. Map of the Trondheimsfjord showing the sidearm Verrasundet, where all samples of spawning cod were taken.



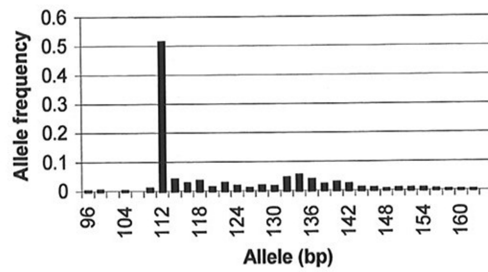


Figure 2. Allele frequency distribution at (a) Gmo2 and (b) Gmo132 from the total number of individuals assayed.

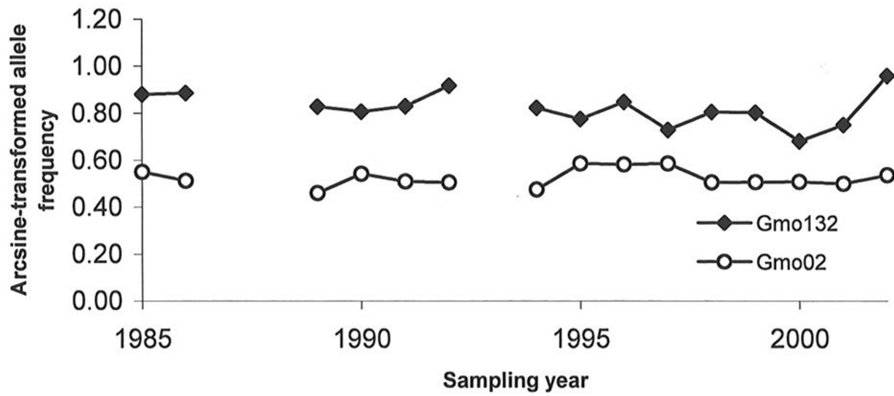


Figure 3. Arcsine transformed allele frequencies of the most common allele at Gmo2 (106bp) and Gmo132 (112bp) by annual samples (for sample sizes, refer to Table 2).

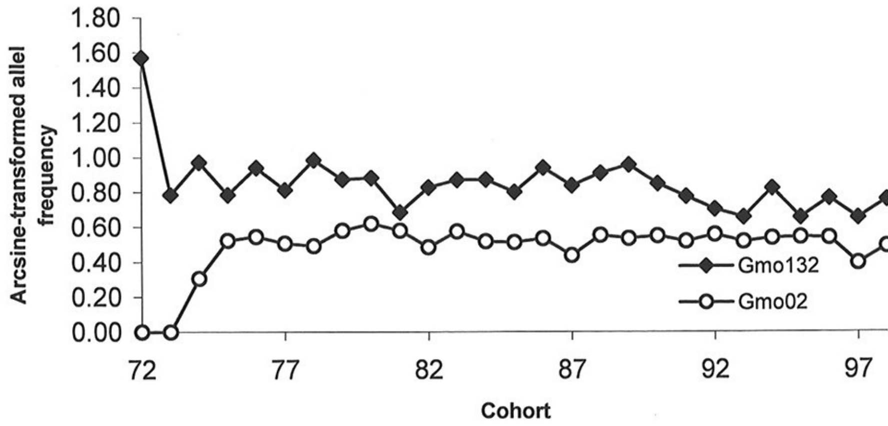


Figure 4. Arcsine transformed allele frequencies of the most common allele at Gmo2 (106bp) and Gmo132 (112bp) by cohort (for sample sizes, refer to Table 3).

Paper V

Karlsson S. & Mork J. An 18-year time series of mtDNA cytochrome *b* sequence in cod. *Genetics,* Submitted.

An 18-year time series of mtDNA cytochrome *b* sequence in cod (*Gadus morhua* L.)

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Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession nos. AY837880-AY838270.

A time series of cod, cyt. *b* sequence

Keyword: mtDNA, cytochrome *b,* sequencing, temporal variation, Atlantic cod

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ABSTRACT

A total of 391 cod from a time series in the local population of Trondheimsfjord, Norway, were assayed for sequencies of the mtDNA cytochrome *b*. The time series included approximately equal numbers of speciemens from eight sampling years in the period 1985 to 2002. No significant variability or trend in haplotype frequencies was detected among sampling years, cohorts, age groups, or between sexes, and no significant association between cyt *b* haplotype and growth was found. Hence the variation at this silent site appears to be neutral. A total of 20 different haplotypes were scored, of which five are not previously reported. The mean haplotype diversity was estimated at 0.778 (SE=0.012), and the nucleotide diversity at 0.57%. The haplotype frequencies in the Trondheimsfjord cod fit well into previously reported clines for the A and E haplotypes across the North Atlantic. A relatively high frequency of a haplotype (ET) not reported from elsewhere in the distribution range may indicate a relative isolation of this cod population. The relatively rich variety of different haplotypes in this fjord population is comparable with that in large, commercial cod stocks and appears not to be indicative of severe bottlenecks, a generally low effective population size or sweepstake reproduction events.

INTRODUCTION

The genetic population structure of the Atlantic cod (*Gadus morhua* L.) has been explored for more than four decades using a wide range of genetic markers; haemoglobins (Sick 1961, 1965a, 1965b), serum transferrins (Møller 1966), isozymes (Odense *et al.* 1969; Lush 1970; Mork *et al.* 1980, 1985), microsatellites (Brooker *et al.* 1994; Bentzen *et al.* 1996; Ruzzante *et al.* 1998; Hutchinson *et al.* 2001; Knutsen *et al.* 2003, minisatellites (Galvin *et al.* 1995), mtDNA RFLP (Smith *et al.*1989; Dahle 1991), nuclear RFLP (Pogson *et al.* 1995; Fevolden and Pogson 1997), mtDNA sequences (Arnason *et al.* 1992; Carr *et al.*1995; Arnason *et al.* 1998; Arnason *et al.* 2000). Various studies and markers have indicated very different levels of genetic differentiation among current cod stocks. However the fact that several of the markers used have been shown to be affected by strong natural selection (haemoglobins: Karpov and Novikov 1980; Mork *et al.* 1983; Mork *et al.* 1984, isozymes *LDH-3** and *PGI-1**: Mork and Sundnes 1985, nuclear RFLP Pan*I*: Pogson and Fevolden 1998; Pogson 2001; Karlsson and Mork 2003) may have contributed to the heterogeneous results in different studies.

The studies using synonymous mutations in sequences of mtDNA cyt. *b* (e.g. Arnason *et al.* 1992) have some important advantages over others, as they allow a fairly safe assumption of selective neutrality of the

marker. Also, the effective population size of mtDNA is only one fourth of that in nuclear DNA and the mutation rates are comparatively high (Carvalho and Pitcher 1995; Sigurgíslason 2003). Thus differentiation by genetic drift and mutation is expected to be higher in mtDNA than at nuclear loci.

However, extensive studies of sequences of the cytochrome *b* region in cod mtDNA have detected only modest genetic differences among populations on a large geographic scale (Arnason *et al.* 1998; Arnason *et al.* 2000; Arnason 2004) and nil differences on a small scale (Carr *et al.* 1995; Arnason and Pálsson 1996; Sigurgíslason and Arnason 2003; Arnason 2004). There are, on the other hand, clines across the North-Atlantic in the frequencies of the most common haplotypes. The E haplotype is the most common one in the Baltic Sea and its frequency is steadily decreasing westwards through Norway, Faroe islands, Iceland, Greenland, and Newfoundland, for the benefit of the A haplotype (Arnason 2004). The evolutionary mechanisms responsible for this pattern are unresolved, but several hypotheses are discussed by Arnason (2004), who regards the most likely one to be demic selection where relatively few, highly fit (for other causes than their cytochrome *b* haplotype) females win reproductive sweepstakes. The random genetic drift of neutral mtDNA would be high with this scenario, hence the hypothesis predicts large temporal changes in haplotype frequencies to occur. As pointed out by Arnason (2004), studies

of temporal variation in haplotype frequences are crucial for testing this hypothesis.

This study reports from an exploration of the temporal variation of mtDNA cytochrome *b* haplotype frequencies in a time series from the local cod population in Trondheimsfjord, Norway.

MATERIALS AND METHODS

Sampling: Cod were collected during spawning season in Verrasundet, a narrow side arm in the inner part of Trondheimsfjord (Fig. 1) and a long known and well-defined spawning area in Trondheimsfjord (Dahl 1899; Sundnes 1980; Mork *et al.* 1985; Mork and Giæver 1999).

Samples of spawning cod were collected with bottom trawl (35mm stretched mesh in the cod end). From a total of 15 annual samples, a subset of 8 were chosen in such a way as to include the entire time period (Table 1).

The number of individuals analysed in the 8 samples ranged between 42 and 50. They were sequenced for a 250 base pair fragment in the cytochrome *b* (cyt *b*) region in mtDNA (Carr and Marshall 1991a; Sigurgíslason and Arnason 2003).

Biological measurements: Sex, gonadal stage, total body length, and age were recorded for each individual, except in the 1990 sample were only the age was determined. Ageing by otoliths was performed by Ekli (1997 and unpublished) according to the breakage-and side-illumination method of Rollefsen (1933). Gonadal maturation stage was determined according to Sivertsen (1935): stage I; immature, stage II; maturing, stage III; running, and stage IV; spent. Total body length was measured to the nearest mm.

Molecular analysis: Samples of blood, white muscle, gills, and liver were stored either frozen or in ethanol, and DNA was extracted from one of these tissue types, according to the phenol-chloroform extraction procedure described in Sambrook *et al.* (1989), with some modifications described in Karlsson and Mork (2003).

PCR amplification, DNA purification, and DNA sequencing followed closely the procedures described earlier (Carr and Marshall 1991a; Sigurgíslason and Arnason 2003). A 359 base pair fragment was amplified using the primers L14413 and H14771 (MWG-Biotech AG). The numbers refer to the 5' end of the primers and correspond to the base pair numbers in the complete sequenced mtDNA in cod (Johansen and Bakke 1996). The PCR mixture contained 2μl template (2ng-5μg), 0.357μM of each primer, 53mM KCl, 10.5 mM Tris-HCl (pH 9.0), 1.6 mgCl, 174μM dNTPs, 0.12% Tween 20, 38 ng BSA, and one unit of Taq polymerase in a total volume of 19μl. PCR was carried out in a Hybaid® Omni-E thermal cycler programmed for 5 min at 94° followed by 30 cycles of denaturation (40s at 94°), annealing (30s at 52°), extension (30s at 72°), and ended by a 7 min. extension period.

The PCR products were checked by running them on an agarose gel and visualised with ethidium bromide. PCR amplifications that gave strong

and sharp bands on the agarose gel were further analysed, while weak amplifications were reanalysed.

Prior to sequencing the PCR products were enzymatically purified from excess primers and nucleotides by the use of Exonuclease and Shrimp alkaline phosphatase (ExoSAP). The reaction mixture contained 5 μl of PCR product, 10 units of Exonuclease I, and 1 unit of Shrimp alkaline phosphatase in a total volume of 7μl. The mixture was incubated in 20 min at 38° followed by a 15 min. step at 80°. Sequencing was carried out using Thermosequenase (Amersham Pharmacia Biotech) and a 5' IRD labeled H14739 primer (MWG-Biotech AG). The sequencing mixture for each of the four bases contained: 1.75 μl ExoSAP treated PCR product, 0.620 μM primer, 0.34X reaction buffer, 0.79X Thermo sequenase dilution buffer, 100 μM dNTP, 0.337μM ddNTP (A, C, G, or T), 1.14 units Thermo sequenase in a total volume of 6.7 μl. The sequencing was carried out in a Hybaid® Omni-E thermal cycler programmed for 5 min at 94° followed by 30 cycles of denaturation (30s at 94°), annealing (30s at 52°), extension (40s at 72°), and ended by a 7 min. extension period. Finally, 4.2 μl of a stop solution was added containing 85% formamide, 20mM EDTA, and 0.1% bromo-phenol-blue.

The sequenced products were denatured at 95° for 5 min. and put directly on ice. 0.7 μl of the sequenced product was subjected to a 6%

denaturing polyacrylamide gel. The electrophoresis was carried out on a LI-COR 4000 DNA sequencer with 41-cm electrophoresis plates.

Data from the electrophoresis were collected using the Base ImageIR Data Collection software. The sequences were further analysed with the Image Analysis software. The achieved sequences were imported to the software program AlignIR Version 2.0, where the sequences were aligned to a known A-haplotype. Ambiguities and mutations were critically checked and corrected. Haplotypes were named with one to two letter code according to (Carr and Marshall 1991a, b; Arnasson 2004).

Statistical analysis: Statistical analyses of continuos variables were performed by one-way analysis of variance (ANOVA) as implemented in STATGRAPHIC Plus 2.1 (STSC, Inc.). For discrete variables, χ^2-tests were used when appropriate. In cases with low expected values, Monte-Carlo based exact tests (1000 iterations) were used (Zaykin and Pudovkin 1993). Probabilities achieved from individual tests were combined into an overall probability by the omnibus test of Fisher (1954). In order to compare body length independently of age, body length were multiplied by a factor derived from the relationship between age and body length in the total material. Estimates of haplotype diversity and nucleotide diversity within annual samples and cohorts as well as nucleotide divergence among annual samples and cohorts (Stephens and Nei 1985; Nei 1987) were carried out using the Arlequin software package (Schneider *et al.* 2000). The Arlequin

package was also used for analysis of molecular covariance (AMOVA) (Excoffier *et al.* 1992) and for exact tests of homogeneity of haplotype frequencies among annual samples and cohorts. In order to increase the statistical power of these tests, all haplotypes except the four most frequent ones were pooled. Tests were also performed with all haplotypes pooled except the most frequent one.

RESULTS

General sample information is compiled in Table 1. The annual sample sizes varied from 42 to 50 individuals with a considerable age span and a significantly skewed sex ratio. All gonadal stages were represented in each sample except in 1985 where there were no immature individuals (Table 1).

The number of different haplotypes in each sample ranged from 7 to 11. In total 20 different haplotypes were found, defined by 15 polymorphic sites (Table 2). Among 20 different substitutions 12 were g↔a purine transitions, 7 c↔t pyrimidine transition, and one was a g↔t transversion. The transition transversion rate was thus 19:1 and the purine:pyrimidine transition ratio was 1.7:1. All observed substitutions were synonymous (i.e. no change in amino acid). Haplotypes differed from each other by one to five substitutions, and they differed by one substitution to their nearest neighbour, except the NL haplotype that differed by two substitutions, because the N haplotype was not found.

Five haplotypes not previously described were found. These were named accordingly to phylogenetic maximum-parsimony networks drawn in previous studies (Arnason *et al.* 2000, Fig. 3; Arnason 2004, Fig 2.). The five new haplotypes were ET, AX, NL, CM, and HK. ET is derived from E by one step, AX is derived from A by one step, NL is derived from N by

one step, CM is derived from C and is the intermediate between C and two earlier described haplotypes, M and MC (Arnason 2004, his figure 2), HK is derived from H by one step (Table 2). Three of the new haplotypes were singletons (NL, CM, and HK), while ET was found in four individuals and AX in two individuals.

Four haplotypes (A, E, G, NI) were polymorphic by the 5% criterion and five (D, C, DI, H, ET) by the 1% criterion. In total 9 singletons were found. The haplotype diversity and the nucleotide diversity ranged from 0.774 to 0.808 and 0.50% to 0.63% respectively among annual samples. Overall, the haplotype diversity was 0.778 and the nucleotide diversity 0.57% (Table 1). Considering the sample sizes, the number of haplotypes, haplotype diversity, and nucleotide diversity were comparable with those found in other populations of cod (Arnason and Palsson 1996; Arnason *et al.* 2000; Sigurgíslason and Arnason 2003).

Analysis of variation among annual samples: Annual frequencies of the four most frequent haplotypes are shown in Figure 2. The haplotype frequencies varied considerably but not significantly among annual samples ($P=0.106 \pm 0.047$, 20 000 Markov steps). Zero or very few individuals were represented in many cells and hence the test had low statistical power. In order to increase the statistical power, all haplotypes except the most frequent haplotype were pooled and an ordinary chi-square homogeneity test was performed. This was done for both the E and A haplotype. Neither

of them showed significant variation among annual samples (A: χ^2=4.78, df=7, P=0.70; E: χ^2=7.81, df=7, P=0.36).

Partitioning of the molecular variance by AMOVA revealed that 0.71% of the total variance was due to variance among annual samples. This is not significantly different from zero (Table 3). Net nucleotide divergence between annual samples were very small and mostly with negative sign, and should be interpreted as nil (Table 4). The VB86 annual sample stood out from the rest by showing a positive sign in comparison with the others. This sample also differed from other samples as revealed by pairwise heterogeneity tests (VB86:VB90, P=0.029; VB86:VB99, P=0.020; VB86:VB02, P=0.042, Markov chain steps=10 000). These P-values are however interrelated and also not formally significant after Bonferroni adjustment.

Analysis of variation among cohorts: Because four individuals were not aged, only 387 specimens were represented in the cohorts. From the period 1973 to 2000, 28 different cohorts were represented. Frequencies of the four most frequent haplotypes are shown in Fig. 2 as well as the sample sizes in each cohort. Haplotype frequencies varied considerably among cohorts, but so did also the number of specimens. An exact test of differentiation among cohorts based on all haplotype frequencies did not reveal significant heterogeneity (P=0.415, 20 000 Markov chain steps). Considering the few individuals represented in some of the cohorts this test

has low statistical power. Additional tests were therefore performed where the variation of the A and E haplotype among cohorts were tested separately by pooling all other haplotypes. Neither of these haplotype frequencies varied significantly among cohorts (A: P=0.334; E: P=0.505, 1000 Monte Carlo simulations).

Partitioning of the molecular variance (AMOVA) into among and within cohorts revealed that 0.21% of the total variance was explained by variance among cohorts. The corresponding F_{ST}-value of 0.0021 is not significantly different from zero (Table 3). Net nucleotide divergence between pairs of cohorts (not shown) showed the same pattern as between annual samples, with very low and mostly negative net divergence, except that no single cohort stood out by showing higher divergence than others. Among the 378 possible pairs, 10 showed unadjusted P-values < 0.05 (10 000 Markov chain steps). After Bonferroni adjustment this is not significant.

Analysis of phenotypic traits: The annual samples were divided into males and females and the contribution by sex to the molecular variance was tested in an AMOVA. The contribution by sex had negative sign and should therefore be interpreted as nil. Also, no significant heterogeneity in haplotype frequencies between the sexes were detected, as revealed by the exact test of Zaykin and Pudovkin (1993), neither with all haplotypes included, nor when only the A and E haplotype were tested separately.

When the annual samples were divided into matures (gonadal stage >I) and immatures (gonadal stage=I), the contribution to the molecular variance was nil (negative sign) with respect to mature or immature stage as revealed by an AMOVA. Homogeneity exact tests (Zaykin and Pudovkin 1993) did not detect any significant heterogeneity in haplotype frequencies between immatures and matures or among all four gonadal stages. This applied both when all haplotypes were included, and when the A and E haplotypes were tested separately.

Twelve different age groups were represented in the total material. No significant variations in haplotype frequencies were detected among these age groups, as revealed by an exact test (Zaykin and Pudovkin 1993).

Mean body length (corrected) of haplotypes were compared by a one-way ANOVA, first by including all observed haplotypes, and then by pooling all haplotypes except the A and E haplotype. None of these two tests detected significant differences in body length among haplotypes.

DISCUSSION

There was no significant heterogeneity of haplotype frequencies among annual samples, cohorts, age groups, or between sexes in the present materials. Hence, cyt *b* variation appears to be the result from random genetic drift and sampling error in one single population.

The observed frequency of the E haplotype in the present study is higher than the E haplotype frequency in Norway reported by Arnason (2004). Nevertheless, the E haplotype frequency reported in the present study fits very well into the frequency cline of this haplotype, from the Baltic in the east to Newfoundland in the west (Arnason 2004). The haplotype frequency data of Norway used in Arnason (2004) is however a collection of data from several locations along the Norwegian coast, with an overweight of locations in northern Norway (Arnason and Pálsson 1996). If there is a cline along the Norwegian coast in agreement with the global cline, our data fit well into such a pattern.

Gene flow seems to be the most plausible explanation for creating the observed cline across the Atlantic. Given the actual sample sizes in previous studies it may therefore not be surprising that genetic differentiation is detected on a large geographic scale (among countries) but not on a small scale (among areas within countries) (Arnason 2004, his table 1). The important questions already raised by Arnason (2004) are: how can

this cline be explained in an evolutionary perspective? Giving the shallow gene genealogy of cytochrome *b* haplotypes, the large frequencies of closely related haplotypes must have increased rapidly (Sigurgíslason and Arnason 2003), so which mechanisms are responsible? Is the pattern observed today an equilibrium situation, will there eventually become larger or smaller genetic distances among cod populations, or will there ever be an equilibrium situation?

In light of the observations in the present study, we will in the following sections try to evaluate the relative importance of natural selection, genetic drift, and gene flow in explaining the previous observed trans-Atlantic cline in haplotype frequencies reported by Arnason (2004).

Natural selection: Previous studies have extensively discussed and concluded that the assumption of neutrality at the cytochrome *b* region is safe (Sigurgíslason and Arnason 2003 and references therein). The main arguments for this have been that the substitutions are mainly synonymous, and those that are not, are conservative changes that are not expected to affect the function of the proteins. Occasionally, significant deviation from expected neutral variation has been observed (Arnason 2004) by neutrality tests (Tajima 1989; Fu 1997). However, these tests are very weak in testing neutrality when that is the intention (Rand 1996; Ford 2002; Ramos-Onsins and Rozas 2002). Probably a more powerful test for the presence of natural selection at a locus is to look for potential associations between

genotype/haplotype and phenotypic traits, as were done in the present study and in a previous study (Karlsson and Mork 2003), or to study genotype performance among full-sibs.

The substitutions observed in the present study were solely at silent site third positions. No association among haplotypes and various phenotypic traits were detected. Hence, the results in the present study support those in previous studies that the variation at this cyt. *b* region is probably neutral.

Genetic drift: Arnason (2004) suggested that large and rapid changes in haplotype frequencies might arise in populations due to sweepstake reproduction, where only a few females, having by chance a specific cyt. b haplotype, contribute to the next generation. This hypothesis is predicting relatively large random temporal changes in haplotype frequencies. The present study does not support this, because no significant temporal variation was found. At the same time it does not reject the hypothesis. However, low effective population size as a result of sweepstake reproduction does not have to be the case in all cod populations.

A few studies have suggested that the effective population size (N_e) may be lower than the census size (N_a) in high fecundity marine species (Turner *et al.* Hauser *et al.* 2002). The N_e/N_a ratio is suggested to be as low as 10^{-6}. These estimates have however very large standard errors and should be used cautiously. What is more interesting with respect to the effect of

genetic drift, however, is the effective population size and not the ratio, because even though the ratio is low, the actual population size might be large. Furthermore, logically the N_a/Ne ratio is not independent of the actual population size, because it would require an actual population size larger than 2 000 000 individuals to have an effective population size of two. Although cod is a highly fecund species with the potential of highly skewed reproductive success among families, there are other features that tend to increase the effective population size, such as multiple spawning and overlapping generations. There are no indications of low effective population size in the Trondheimsfjord cod population. From commercial landings the number of spawners in the present study area (Verrasundet) is estimated at >100 000 (J Mork pers. comm.). If the effective population size was several orders of magnitude lower that this, we would expect to observe a much more extensive temporal variation and lower haplotype and nucleotide diversity than we did. The observed haplotype- and nucleotide diversity among these spawners are actually comparable with those reported from other and larger populations of cod around Iceland and Greenland (Arnason *et al.* 2000) and Norway (Arnason and Pálsson 1996), which further indicates a low impact by genetic drift. A high genetic variability in Trondheimsfjord cod is also observed at microsatellite loci (Karlsson and Mork, submitted.).

Gene flow: Trondheimsfjord is about 130 km long and thereby the third longest fjord in Norway. The spawning area Verrasundet is situated in the inner part of the fjord. This makes Verrasundet rather isolated as compared to many other spawning areas of cod.

Extensive tagging experiments have been performed along the Norwegian coast as well as in the Trondheimsfjord. These have shown that Trondheimsfjord receives none or very few individuals from coastal or oceanic areas (O.R. Godø, Institute of Marine Research, personal communication). A small proportion (~1.5%) of 1000 cod tagged in Trondheimsfjord were recaptured outside the fjord within 5 years after release, and these were exclusively found north of the fjord (Sundnes 1980).

If there is an extensive gene flow from coastal and oceanic waters outside the Trondheimsfjord to the Trondheimsfjord cod stock one would expect to observe a Wahlund effect at diploid loci. In previous studies this has not been observed (Mork *et al.* 1985; Mork and Giæver 1999; Karlsson and Mork 2003). Depending on the actual differences in allele frequencies in these areas however, this approach might not be sensitive enough to detect population mixture.

This study revealed some haplotypes not previously described in 1278 individuals from almost the whole distribution range of cod (Arnason 2004). One of the new haplotypes was found in four individuals, while some others and previously known haplotypes were found as singletons. These

findings may indicate that the Trondheimsfjord cod is relatively isolated genetically.

Knowledge of the migration pattern of adult cod is good based on extensive tagging experiments. The effect on geneflow from pelagic eggs and larvae, however, must be based on knowledge on e.g. length of pelagic period and oceanic current systems. The potential for a gene flow during the pelagic stage is large, and interchanges of egg and larvae between Trondheimsfjord and the coast is likely to occur, although expectedly mostly one-way due to the estaurine circulation. At least some eggs and larvae are likely to be transported out of the fjord by the estuarine circulation. It is also possible that eggs and larvae from outside the fjord are transported into the fjord by the estuarine compensation current and the tidal current (Jacobson 1976). Along the Norwegian coast outside the fjord, there is a coastal current going northwards. The general drifting pattern of eggs and larvae must therefore be northerly (Breen 1980). Hence, a gene flow set up by (oceanic) current systems appears to be a realistic evolutionary force in most cod stocks.

Concluding remarks: The prediction of Arnason (2004) of temporal variation at the cytochrome *b* sequence polymorphism in natural populations of cod is not supported by the observations in the present study. However, this does not exclude that such effects take place in other cod stocks. Although the present temporal study includes a relatively long time

period, it is very short on an evolutionary time scale. Events such as founder effects, bottlenecks, and sweepstakes that decrease the effective population size may occur frequently in some populations but probably more seldom in large and stable populations. The Trondheimsfjord population is small compared to many other populations but fairly stable in size in the last decades. However cod populations existing in the fringe zones of the distribution range are probably less stable, and may show larger fluctuations in size such as the situation for the Baltic cod (Bagge and Thurow 1994; Nissling and Westin 1991a). The highest frequency of the E haplotype is found in the Baltic (Arnason *et al.* 1998) and is steadily decreasing north and westwards in the Atlantic (Arnason 2004). The present materials fit in this general scheme. A plausible explanation for the observed cline is that the E haplotype frequency has increased rapidly in the Baltic by genetic drift and has spread by gene flow through the Danish belts and further north to Norway by the current system (Breen 1980). Although the Baltic is a marginal habitat for cod it also has a potential of rapid expansion of the stock when the conditions are favourable (Nissling and Westin 1991b). The hypothesis is still speculative, because the magnitude of a possible effect of genetic drift, due to founder effects, bottle necks or sweepstake reproduction is not known. Furthermore, the required amount of eggs and larvae that drift between areas and, capable of setting up the haplotype frequency cline observed is not known. Additional important questions are how old the

observed cline is and whether it is at an equilibrium. Clearly further studies are needed before understanding can be reached of the forces responsible in creating and maintaining the trans-atlantic haplotype frequency cline. It might be fruitful to study the temporal variation of the cytochrome *b* haplotypes in the Baltic and include additional genetic markers. Also, spawning areas of cod in the North Sea remain to be analysed for cytochrome *b*.

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TABLE 1

Summary data

Year	N	Females (%)	Length (mm)	Gonad	Age	n_{haplo}	$\hat{h}$	SE $\hat{h}$	$\hat{\pi}$ (%)
2002	42	55	260-840	I-IV	2-10	9	0.808	0.032	0.66
2001	49	17	299-650	I-IV	3-7	8	0.808	0.025	0.55
1999	50	44	310-970	I-IV	3-11	7	0.744	0.037	0.54
1996	50	77	273-975	I-IV	2-11, 14	10	0.779	0.037	0.52
1992	50	40	255-714	I-IV	2-5, 7-9	11	0.788	0.044	0.62
1990	50	-	-	-	3-9, 11-12	9	0.744	0.036	0.51
1986	50	39	195-970	I-IV	2-10, 12	9	0.755	0.037	0.50
1985	50	9	420-800	II-IV	4-8, 10-12	11	0.790	0.043	0.63
Total	391	42	195-800	I-IV	2-12, 14	20	0.778	0.012	0.57

N=sample size; nhaplo=number of different haplotypes; $\hat{h}$=haplotype diversity, SE $\hat{h}$=Standard error of the haplotype diversity estimate;

TABLE 2

Segregating Site

Haplo-type	487	488	494	496	508	523	541	547	562	631	649	673	685	691	695	697	N	Frequency
A	**A**	**C**	**C**	**A**	**T**	**G**	**A**	**C**	**A**	**G**	**C**	**A**	**C**	**G**	**G**	**C**	117	0.2992
E	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	126	0.3223
G	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	56	0.1432
NI	-	-	-	-	-	-	-	-	-	A	-	-	-	T	-	-	29	0.0742
D	-	-	-	-	-	A	-	-	-	-	-	-	T	-	-	-	17	0.0436
C	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	13	0.0332
DI	-	-	-	-	-	A	-	-	-	-	-	-	T	A	-	-	10	0.0256
H	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	6	0.0153
ET	-	-	-	-	C	-	-	-	-	-	-	G	-	-	-	-	4	0.0102
AX	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	2	0.0051
LJ	G	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	2	0.0051
B	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	1	0.0026
CM	-	-	-	-	-	-	-	-	-	-	-	-	T	-	A	-	1	0.0026
EC	-		-	-	C	-	-	-	-	-	-	-	T	-	-	-	1	0.0026
EK	-		-	-	C	-	-	-	-	-	-	-	-	-	-	T	1	0.0026
HK	-	-	-	-	-	-	G	T	-	-	-	-	-	-	-	-	1	0.0026
LI	G		-	-	-	-	-	-	-	A	-	-	-	T	-	-	1	0.0026
MI	-		-	-	-	-	-	-	-	A	-	-	-	-	-	-	1	0.0026
NL	G	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	0.0026
X	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	1	0.0026

Segregating nucleotide sites of 20 haplotypes in a 250-basepair fragment of mitochondrial sequence from the cyt *b* region in 391 specimens of cod from Verrasundet. Site numbers are as in Johansen & Bakke (1996) minus 14 000. N is the observed number of each haplotype and Frequency is the relative frequency of each haplotype in the total material.

TABLE 3

Analysis of molecular variance (AMOVA)

Source of Variation	d.f	Variance components	Percentage of variation	P-value
Among annual samples	7	V_a=0.0050	0.71	0.124
Within annual samples	383	V_b=0.7060	99.29	
Total	390	0.7110		
Among cohorts	27	V_a=0.0015	0.21	0.379
Within cohorts	360	V_b=0.7068	99.79	
Total	387	0.7360		

Analysis of molecular variance (AMOVA) from a 250 bp sequence variation in the cyt *b* region mtDNA within and among annual samples and cohorts of cod in Verrasundet. The P-values are achieved from 1023 permutations.

TABLE 4

Nucleotide diversity and divergences per 100 base pair from a 250 bp sequence variation in the cyt *b* region in the mtDNA among annual samples of cod in Verrasundet

Area	VB85	VB86	VB90	VB92	VB96	VB99	VB01	VB02
VB85	**0.6635**	*0.0267*	*-0.0038*	*-0.0033*	*-0.0082*	*0.0014*	*-0.0015*	*-0.0028*
VB86	0.6350	**0.5531**	*0.0227*	*0.0331*	*0.0392*	*0.0372*	*0.0171*	*0.0302*
VB90	0.5965	0.5678	**0.5371**	*-0.0035*	*-0.0058*	*-0.0071*	*-0.0077*	*-0.0098*
VB92	0.5869	0.5682	0.5235	**0.5169**	*-0.0027*	*-0.0037*	*-0.0064*	*-0.0008*
VB96	0.6323	0.6245	0.5715	0.5645	**0.6175**	*-0.0054*	*-0.0022*	*-0.0076*
VB99	0.5878	0.5685	0.5162	0.5094	0.5578	**0.5094**	*-0.0054*	*-0.0081*
VB01	0.5807	0.5442	0.5113	0.5025	0.5571	0.4998	**0.5010**	*-0.0057*
VB02	0.6434	0.6213	0.5733	0.5722	0.6156	0.5611	0.5594	**0.6290**

Estimates of inter-annual sample (d_{XY} lower triangle), intra-annual samples (d_X or d_Y; bold on diagonal), and net (dA italics on upper triangle) nucleotide divergence between areas. Entries are per 100 base pairs.

FIGURE LEGENDS

Figure 1. Map of the Trondheimsfjord showing the sidearm Verrasundet, where all samples of spawning cod were taken.

Figure 2. Frequencies of the four most frequent haplotypes and the remaining pooled haplotypes from a 250 bp sequence variation in the cyt *b* region in the mtDNA by annual sample of cod in Verrasundet. Sample sizes are given in parenthesis.

Figure 3. Frequencies of the four most frequent haplotypes and the remaining pooled haplotypes from a 250 bp sequence variation in the cyt *b* region in the mtDNA by cohort of cod in Verrasundet. Sample sizes are given in paranthesis.

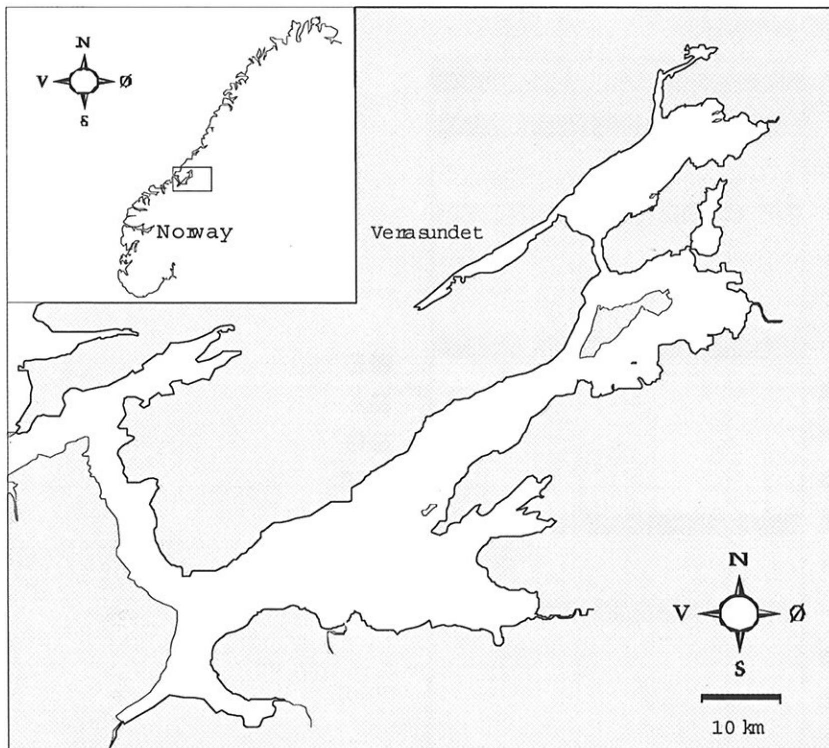

Figure 1. Map of the Trondheimsfjord showing the sidearm Verrasundet, where all samples of spawning cod were taken.

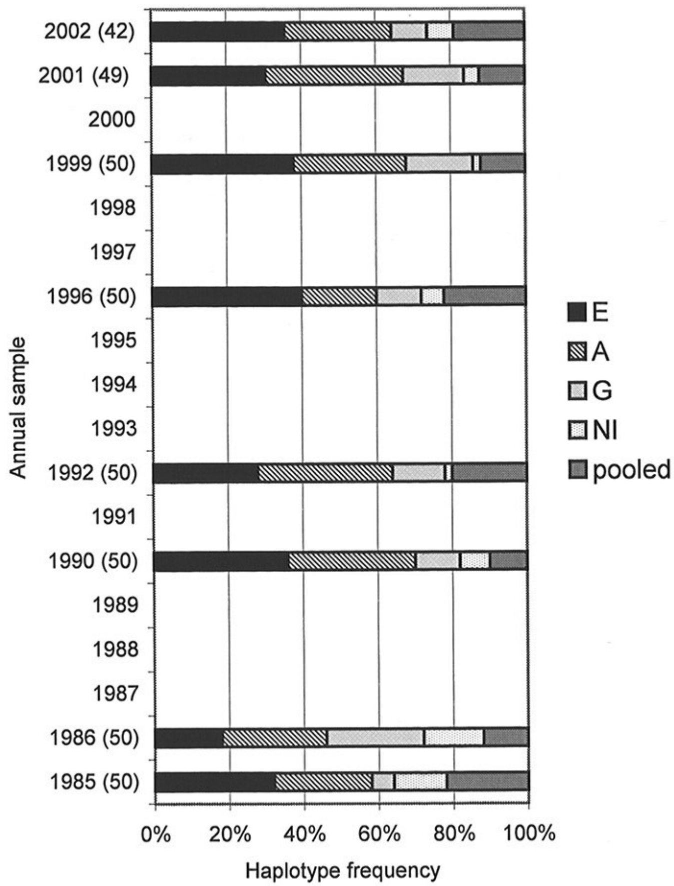

Figure 2. Frequencies of the four most frequent haplotypes and the remaining pooled haplotypes from a 250 bp sequence variation in the cyt *b* region in the mtDNA by annual sample of cod in Verrasundet. Sample sizes are given in paranthesis.

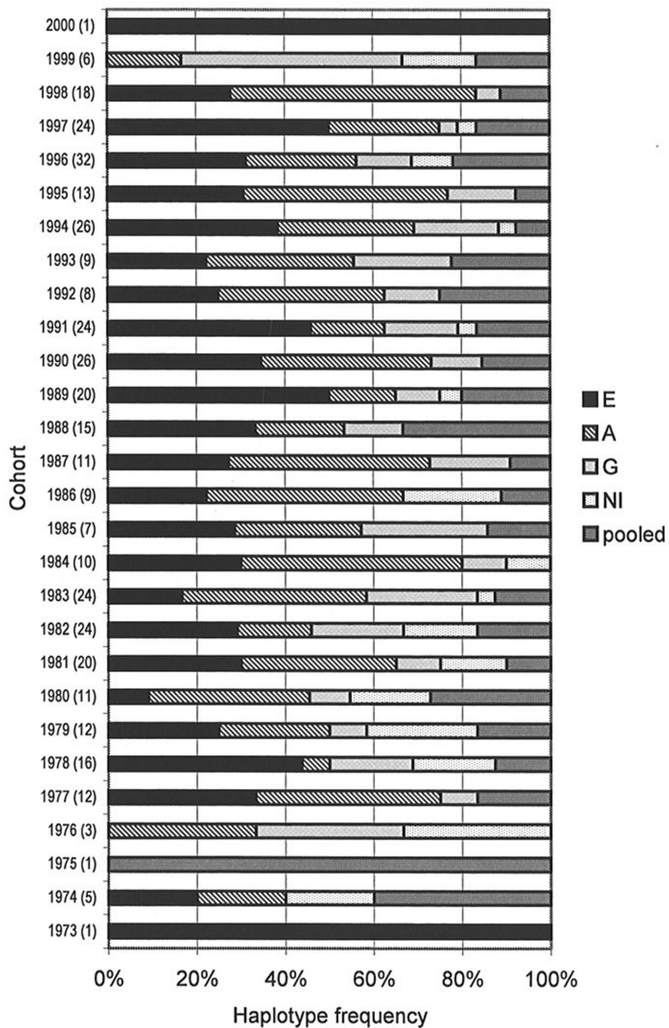

Figure 3. Frequencies of the four most frequent haplotypes and the remaining pooled haplotypes from a 250 bp sequence variation in the cyt *b* region in the mtDNA by cohort of cod in Verrasundet. Sample sizes are given in paranthesis.

Doctoral theses in Biology
Norwegian University of Science and Technology

Year	Name	Degree	Title
1974	Tor-Henning Iversen	Dr. philos Botany	The roles of statholiths, auxin transport, and auxin metabolism in root gravitropism
1978	Tore Slagsvold	Dr. philos. Zoology	Breeding events of birds in relation to spring temperature and environmental phenology.
1978	Egil Sakshaug	Dr.philos Botany	"The influence of environmental factors on the chemical composition of cultivated and natural populations of marine phytoplankton"
1980	Arnfinn Langeland	Dr. philos. Zoology	Interaction between fish and zooplankton populations and their effects on the material utilization in a freshwater lake.
1980	Helge Reinertsen	Dr. philos Botany	The effect of lake fertilization on the dynamics and stability of a limnetic ecosystem with special reference to the phytoplankton
1982	Gunn Mari Olsen	Dr. scient Botany	Gravitropism in roots of *Pisum sativum* and *Arabidopsis thaliana*
1982	Dag Dolmen	Dr. philos. Zoology	Life aspects of two sympartic species of newts (*Triturus, Amphibia*) in Norway, with special emphasis on their ecological niche segregation.
1984	Eivin Røskaft	Dr. philos. Zoology	Sociobiological studies of the rook *Corvus frugilegus.*
1984	Anne Margrethe Cameron	Dr. scient Botany	Effects of alcohol inhalation on levels of circulating testosterone, follicle stimulating hormone and luteinzing hormone in male mature rats
1984	Asbjørn Magne Nilsen	Dr. scient Botany	Alveolar macrophages from expectorates – Biological monitoring of workers exosed to occupational air pollution. An evaluation of the AM-test
1985	Jarle Mork	Dr. philos. Zoology	Biochemical genetic studies in fish.
1985	John Solem	Dr. philos. Zoology	Taxonomy, distribution and ecology of caddisflies (*Trichoptera*) in the Dovrefjell mountains.

1985	Randi E. Reinertsen	Dr. philos. Zoology	Energy strategies in the cold: Metabolic and thermoregulatory adaptations in small northern birds.
1986	Bernt-Erik Sæther	Dr. philos. Zoology	Ecological and evolutionary basis for variation in reproductive traits of some vertebrates: A comparative approach.
1986	Torleif Holthe	Dr. philos. Zoology	Evolution, systematics, nomenclature, and zoogeography in the polychaete orders *Oweniimorpha* and *Terebellomorpha*, with special reference to the Arctic and Scandinavian fauna.
1987	Helene Lampe	Dr. scient. Zoology	The function of bird song in mate attraction and territorial defence, and the importance of song repertoires.
1987	Olav Hogstad	Dr. philos. Zoology	Winter survival strategies of the Willow tit *Parus montanus*.
1987	Jarle Inge Holten	Dr. philos Bothany	Autecological investigations along a coust-inland transect at Nord-Møre, Central Norway
1987	Rita Kumar	Dr. scient Botany	Somaclonal variation in plants regenerated from cell cultures of *Nicotiana sanderae* and *Chrysanthemum morifolium*
1987	Bjørn Åge Tømmerås	Dr. scient. Zoology	Olfaction in bark beetle communities: Interspecific interactions in regulation of colonization density, predator - prey relationship and host attraction.
1988	Hans Christian Pedersen	Dr. philos. Zoology	Reproductive behaviour in willow ptarmigan with special emphasis on territoriality and parental care.
1988	Tor G. Heggberget	Dr. philos. Zoology	Reproduction in Atlantic Salmon (*Salmo salar*): Aspects of spawning, incubation, early life history and population structure.
1988	Marianne V. Nielsen	Dr. scient. Zoology	The effects of selected environmental factors on carbon allocation/growth of larval and juvenile mussels (*Mytilus edulis*).
1988	Ole Kristian Berg	Dr. scient. Zoology	The formation of landlocked Atlantic salmon (*Salmo salar* L.).

1989	John W. Jensen	Dr. philos. Zoology	Crustacean plankton and fish during the first decade of the manmade Nesjø reservoir, with special emphasis on the effects of gill nets and salmonid growth.
1989	Helga J. Vivås	Dr. scient. Zoology	Theoretical models of activity pattern and optimal foraging: Predictions for the Moose *Alces alces*.
1989	Reidar Andersen	Dr. scient. Zoology	Interactions between a generalist herbivore, the moose *Alces alces*, and its winter food resources: a study of behavioural variation.
1989	Kurt Ingar Draget	Dr. scient Botany	Alginate gel media for plant tissue culture,
1990	Bengt Finstad	Dr. scient. Zoology	Osmotic and ionic regulation in Atlantic salmon, rainbow trout and Arctic charr: Effect of temperature, salinity and season.
1990	Hege Johannesen	Dr. scient. Zoology	Respiration and temperature regulation in birds with special emphasis on the oxygen extraction by the lung.
1990	Åse Krøkje	Dr. scient Botany	The mutagenic load from air pollution at two work-places with PAH-exposure measured with Ames Salmonella/microsome test
1990	Arne Johan Jensen	Dr. philos. Zoology	Effects of water temperature on early life history, juvenile growth and prespawning migrations of Atlantic salmion (*Salmo salar*) and brown trout (*Salmo trutta*): A summary of studies in Norwegian streams.
1990	Tor Jørgen Almaas	Dr. scient. Zoology	Pheromone reception in moths: Response characteristics of olfactory receptor neurons to intra- and interspecific chemical cues.
1990	Magne Husby	Dr. scient. Zoology	Breeding strategies in birds: Experiments with the Magpie *Pica pica*.
1991	Tor Kvam	Dr. scient. Zoology	Population biology of the European lynx (*Lynx lynx*) in Norway.
1991	Jan Henning L'Abêe Lund	Dr. philos. Zoology	Reproductive biology in freshwater fish, brown trout *Salmo trutta* and roach *Rutilus rutilus* in particular.

1991	Asbjørn Moen	Dr. philos Botany	The plant cover of the boreal uplands of Central Norway. I. Vegetation ecology of Sølendet nature reserve; haymaking fens and birch woodlands
1991	Else Marie Løbersli	Dr. scient Botany	Soil acidification and metal uptake in plants
1991	Trond Nordtug	Dr. scient. Zoology	Reflctometric studies of photomechanical adaptation in superposition eyes of arthropods.
1991	Thyra Solem	Dr. scient Botany	Age, origin and development of blanket mires in Central Norway
1991	Odd Terje Sandlund	Dr. philos. Zoology	The dynamics of habitat use in the salmonid genera *Coregonus* and *Salvelinus*: Ontogenic niche shifts and polymorphism.
1991	Nina Jonsson	Dr. philos.	Aspects of migration and spawning in salmonids.
1991	Atle Bones	Dr. scient Botany	Compartmentation and molecular properties of thioglucoside glucohydrolase (myrosinase)
1992	Torgrim Breiehagen	Dr. scient. Zoology	Mating behaviour and evolutionary aspects of the breeding system of two bird species: the Temminck's stint and the Pied flycatcher.
1992	Anne Kjersti Bakken	Dr. scient Botany	The influence of photoperiod on nitrate assimilation and nitrogen status in timothy (*Phleum pratense* L.)
1992	Tycho Anker-Nilssen	Dr. scient. Zoology	Food supply as a determinant of reproduction and population development in Norwegian Puffins *Fratercula arctica*
1992	Bjørn Munro Jenssen	Dr. philos. Zoology	Thermoregulation in aquatic birds in air and water: With special emphasis on the effects of crude oil, chemically treated oil and cleaning on the thermal balance of ducks.
1992	Arne Vollan Aarset	Dr. philos. Zoology	The ecophysiology of under-ice fauna: Osmotic regulation, low temperature tolerance and metabolism in polar crustaceans.
1993	Geir Slupphaug	Dr. scient Botany	Regulation and expression of uracil-DNA glycosylase and O^6-methylguanine-DNA methyltransferase in mammalian cells
1993	Tor Fredrik Næsje	Dr. scient. Zoology	Habitat shifts in coregonids.

1993	Yngvar Asbjørn Olsen	Dr. scient. Zoology	Cortisol dynamics in Atlantic salmon, *Salmo salar* L.: Basal and stressor-induced variations in plasma levels ans some secondary effects.
1993	Bård Pedersen	Dr. scient Botany	Theoretical studies of life history evolution in modular and clonal organisms
1993	Ole Petter Thangstad	Dr. scient Botany	Molecular studies of myrosinase in Brassicaceae
1993	Thrine L. M. Heggberget	Dr. scient. Zoology	Reproductive strategy and feeding ecology of the Eurasian otter *Lutra lutra*.
1993	Kjetil Bevanger	Dr. scient. Zoology	Avian interactions with utility structures, a biological approach.
1993	Kåre Haugan	Dr. scient Bothany	Mutations in the replication control gene trfA of the broad host-range plasmid RK2
1994	Peder Fiske	Dr. scient. Zoology	Sexual selection in the lekking great snipe (*Gallinago media*): Male mating success and female behaviour at the lek.
1994	Kjell Inge Reitan	Dr. scient Botany	Nutritional effects of algae in first-feeding of marine fish larvae
1994	Nils Røv	Dr. scient. Zoology	Breeding distribution, population status and regulation of breeding numbers in the northeast-Atlantic Great Cormorant *Phalacrocorax carbo carbo*.
1994	Annette-Susanne Hoepfner	Dr. scient Botany	Tissue culture techniques in propagation and breeding of Red Raspberry (*Rubus idaeus* L.)
1994	Inga Elise Bruteig	Dr. scient Bothany	Distribution, ecology and biomonitoring studies of epiphytic lichens on conifers
1994	Geir Johnsen	Dr. scient Botany	Light harvesting and utilization in marine phytoplankton: Species-specific and photoadaptive responses
1994	Morten Bakken	Dr. scient. Zoology	Infanticidal behaviour and reproductive performance in relation to competition capacity among farmed silver fox vixens, *Vulpes vulpes*.
1994	Arne Moksnes	Dr. philos. Zoology	Host adaptations towards brood parasitism by the Cockoo.
1994	Solveig Bakken	Dr. scient Bothany	Growth and nitrogen status in the moss *Dicranum majus* Sm. as influenced by nitrogen supply

1995	Olav Vadstein	Dr. philos Botany	The role of heterotrophic planktonic bacteria in the cycling of phosphorus in lakes: Phosphorus requirement, competitive ability and food web interactions.
1995	Hanne Christensen	Dr. scient. Zoology	Determinants of Otter *Lutra lutra* distribution in Norway: Effects of harvest, polychlorinated biphenyls (PCBs), human population density and competition with mink *Mustela vision*.
1995	Svein Håkon Lorentsen	Dr. scient. Zoology	Reproductive effort in the Antarctic Petrel *Thalassoica antarctica*; the effect of parental body size and condition.
1995	Chris Jørgen Jensen	Dr. scient. Zoology	The surface electromyographic (EMG) amplitude as an estimate of upper trapezius muscle activity
1995	Martha Kold Bakkevig	Dr. scient. Zoology	The impact of clothing textiles and construction in a clothing system on thermoregulatory responses, sweat accumulation and heat transport.
1995	Vidar Moen	Dr. scient. Zoology	Distribution patterns and adaptations to light in newly introduced populations of *Mysis relicta* and constraints on Cladoceran and Char populations.
1995	Hans Haavardsholm Blom	Dr. philos Bothany	A revision of the *Schistidium apocarpum* complex in Norway and Sweden.
1996	Jorun Skjærmo	Dr. scient Botany	Microbial ecology of early stages of cultivated marine fish; inpact fish-bacterial interactions on growth and survival of larvae.
1996	Ola Ugedal	Dr. scient. Zoology	Radiocesium turnover in freshwater fishes
1996	Ingibjørg Einarsdottir	Dr. scient. Zoology	Production of Atlantic salmon (*Salmo salar*) and Arctic charr (*Salvelinus alpinus*): A study of some physiological and immunological responses to rearing routines.
1996	Christina M. S. Pereira	Dr. scient. Zoology	Glucose metabolism in salmonids: Dietary effects and hormonal regulation.
1996	Jan Fredrik Børseth	Dr. scient. Zoology	The sodium energy gradients in muscle cells of *Mytilus edulis* and the effects of organic xenobiotics.

1996	Gunnar Henriksen	Dr. scient. Zoology	Status of Grey seal *Halichoerus grypus* and Harbour seal *Phoca vitulina* in the Barents sea region.
1997	Gunvor Øie	Dr. scient Bothany	Eevalution of rotifer *Brachionus plicatilis* quality in early first feeding of turbot *Scophtalmus maximus* L. larvae.
1997	Håkon Holien	Dr. scient Botany	Studies of lichens in spurce forest of Central Norway. Diversity, old growth species and the relationship to site and stand parameters.
1997	Ole Reitan	Dr. scient. Zoology	Responses of birds to habitat disturbance due to damming.
1997	Jon Arne Grøttum	Dr. scient. Zoology	Physiological effects of reduced water quality on fish in aquaculture.
1997	Per Gustav Thingstad	Dr. scient. Zoology	Birds as indicators for studying natural and human-induced variations in the environment, with special emphasis on the suitability of the Pied Flycatcher.
1997	Torgeir Nygård	Dr. scient. Zoology	Temporal and spatial trends of pollutants in birds in Norway: Birds of prey and Willow Grouse used as Biomonitors.
1997	Signe Nybø	Dr. scient. Zoology	Impacts of long-range transported air pollution on birds with particular reference to the dipper *Cinclus cinclus* in southern Norway.
1997	Atle Wibe	Dr. scient. Zoology	Identification of conifer volatiles detected by receptor neurons in the pine weevil (*Hylobius abietis*), analysed by gas chromatography linked to electrophysiology and to mass spectrometry.
1997	Rolv Lundheim	Dr. scient. Zoology	Adaptive and incidental biological ice nucleators.
1997	Arild Magne Landa	Dr. scient. Zoology	Wolverines in Scandinavia: ecology, sheep depredation and conservation.
1997	Kåre Magne Nielsen	Dr. scient Botany	An evolution of possible horizontal gene transfer from plants to sail bacteria by studies of natural transformation in *Acinetobacter calcoacetius*.

1997	Jarle Tufto	Dr. scient. Zoology	Gene flow and genetic drift in geographically structured populations: Ecological, population genetic, and statistical models
1997	Trygve Hesthagen	Dr. philos. Zoology	Population responces of Arctic charr (*Salvelinus alpinus* (L.)) and brown trout (*Salmo trutta* L.) to acidification in Norwegian inland waters
1997	Trygve Sigholt	Dr. philos. Zoology	Control of Parr-smolt transformation and seawater tolerance in farmed Atlantic Salmon (*Salmo salar*) Effects of photoperiod, temperature, gradual seawater acclimation, NaCl and betaine in the diet
1997	Jan Østnes	Dr. scient. Zoology	Cold sensation in adult and neonate birds
1998	Seethaledsumy Visvalingam	Dr. scient Botany	Influence of environmental factors on myrosinases and myrosinase-binding proteins.
1998	Thor Harald Ringsby	Dr. scient. Zoology	Variation in space and time: The biology of a House sparrow metapopulation
1998	Erling Johan Solberg	Dr. scient. Zoology	Variation in population dynamics and life history in a Norwegian moose (*Alces alces*) population: consequences of harvesting in a variable environment
1998	Sigurd Mjøen Saastad	Dr. scient Botany	Species delimitation and phylogenetic relationships between the Sphagnum recurvum complex (Bryophyta): genetic variation and phenotypic plasticity.
1998	Bjarte Mortensen	Dr. scient Botany	Metabolism of volatile organic chemicals (VOCs) in a head liver S9 vial equilibration system in vitro.
1998	Gunnar Austrheim	Dr. scient Botany	Plant biodiversity and land use in subalpine grasslands. – A conservtaion biological approach.
1998	Bente Gunnveig Berg	Dr. scient. Zoology	Encoding of pheromone information in two related moth species
1999	Kristian Overskaug	Dr. scient. Zoology	Behavioural and morphological characteristics in Northern Tawny Owls *Strix aluco*: An intra- and interspecific comparative approach

1999	Hans Kristen Stenøien	Dr. scient Bothany	Genetic studies of evolutionary processes in various populations of nonvascular plants (mosses, liverworts and hornworts)
1999	Trond Arnesen	Dr. scient Botany	Vegetation dynamics following trampling and burning in the outlying haylands at Sølendet, Central Norway.
1999	Ingvar Stenberg	Dr. scient. Zoology	Habitat selection, reproduction and survival in the White-backed Woodpecker *Dendrocopos leucotos*
1999	Stein Olle Johansen	Dr. scient Botany	A study of driftwood dispersal to the Nordic Seas by dendrochronology and wood anatomical analysis.
1999	Trina Falck Galloway	Dr. scient. Zoology	Muscle development and growth in early life stages of the Atlantic cod (*Gadus morhua* L.) and Halibut (*Hippoglossus hippoglossus* L.)
1999	Torbjørn Forseth	Dr. scient. Zoology	Bioenergetics in ecological and life history studies of fishes.
1999	Marianne Giæver	Dr. scient. Zoology	Population genetic studies in three gadoid species: blue whiting (*Micromisistius poutassou*), haddock (*Melanogrammus aeglefinus*) and cod (*Gradus morhua*) in the North-East Atlantic
1999	Hans Martin Hanslin	Dr. scient Botany	The impact of environmental conditions of density dependent performance in the boreal forest bryophytes *Dicranum majus*, *Hylocomium splendens*, *Plagiochila asplenigides*, *Ptilium crista-castrensis* and *Rhytidiadelphus lokeus*.
1999	Ingrid Bysveen Mjølnerød	Dr. scient. Zoology	Aspects of population genetics, behaviour and performance of wild and farmed Atlantic salmon (*Salmo salar*) revealed by molecular genetic techniques
1999	Else Berit Skagen	Dr. scient Botany	The early regeneration process in protoplasts from *Brassica napus* hypocotyls cultivated under various g-forces
1999	Stein-Are Sæther	Dr. philos. Zoology	Mate choice, competition for mates, and conflicts of interest in the Lekking Great Snipe
1999	Katrine Wangen Rustad	Dr. scient. Zoology	Modulation of glutamatergic neurotransmission related to cognitive dysfunctions and Alzheimer's disease

1999 Per Terje Smiseth	Dr. scient. Zoology	Social evolution in monogamous families: mate choice and conflicts over parental care in the Bluethroat (*Luscinia s. svecica*)
1999 Gunnbjørn Bremset	Dr. scient. Zoology	Young Atlantic salmon (*Salmo salar* L.) and Brown trout (*Salmo trutta* L.) inhabiting the deep pool habitat, with special reference to their habitat use, habitat preferences and competitive interactions
1999 Frode Ødegaard	Dr. scient. Zoology	Host spesificity as parameter in estimates of arhrophod species richness
1999 Sonja Andersen	Dr. scient Bothany	Expressional and functional analyses of human, secretory phospholipase A2
2000 Salvesen, Ingrid	Dr. scient Botany	Microbial ecology in early stages of marine fish: Development and evaluation of methods for microbial management in intensive larviculture
2000 Ingar Jostein Øien	Dr. scient. Zoology	The Cuckoo (*Cuculus canorus*) and its host: adaptions and counteradaptions in a coevolutionary arms race
2000 Pavlos Makridis	Dr. scient Botany	Methods for the microbial econtrol of live food used for the rearing of marine fish larvae
2000 Sigbjørn Stokke	Dr. scient. Zoology	Sexual segregation in the African elephant (*Loxodonta africana*)
2000 Odd A. Gulseth	Dr. philos. Zoology	Seawater tolerance, migratory behaviour and growth of Charr, (*Salvelinus alpinus*), with emphasis on the high Arctic Dieset charr on Spitsbergen, Svalbard
2000 Pål A. Olsvik	Dr. scient. Zoology	Biochemical impacts of Cd, Cu and Zn on brown trout (*Salmo trutta*) in two mining-contaminated rivers in Central Norway
2000 Sigurd Einum	Dr. scient. Zoology	Maternal effects in fish: Implications for the evolution of breeding time and egg size
2001 Jan Ove Evjemo	Dr. scient. Zoology	Production and nutritional adaptation of the brine shrimp *Artemia* sp. as live food organism for larvae of marine cold water fish species

2001	Hilmo, Olga	Dr. scient Botany	Lichen response to environmental changes in the managed boreal forset systems
2001	Ingebrigt Uglem	Dr. scient. Zoology	Male dimorphism and reproductive biology in corkwing wrasse (*Symphodus melops* L.)
2001	Bård Gunnar Stokke	Dr. scient. Zoology	Coevolutionary adaptations in avian brood parasites and their hosts
2002	Ronny Aanes	Dr. scient	Spatio-temporal dynamics in Svalbard reindeer (*Rangifer tarandus platyrhynchus*)
2002	Mariann Sandsund	Dr. scient. Zoology	Exercise- and cold-induced asthma. Respiratory and thermoregulatory responses
2002	Dag-Inge Øien	Dr. scient Botany	Dynamics of plant communities and populations in boreal vegetation influenced by scything at Sølendet, Central Norway
2002	Frank Rosell	Dr. scient. Zoology	The function of scent marking in beaver (*Castor fiber*)
2002	Janne Østvang	Dr. scient Botany	The Role and Regulation of Phospholipase A_2 in Monocytes During Atherosclerosis Development
2002	Terje Thun	Dr. philos Biology	Dendrochronical constructions of Norwegian conifer chronologies providing dating of historical material
2002	Birgit Hafjeld Borgen	Dr. scient Biology	Functional analysis of plant idioblasts (Myrosin cells) and their role in defense, development and growth
2002	Bård Øyvind Solberg	Dr. scient Biology	Effects of climatic change on the growth of dominating tree species along major environmental gradients
2002	Per Winge	Dr. scient Biology	The evolution of small GTP binding proteins in cellular organisms. Studies of RAC GTPases in *Arabidopsis thaliana* and
2002	Henrik Jensen	Dr. scient Biology	Causes and consequenses of individual variation in fitness-related traits in house sparrows
2003	Jens Rohloff	Dr. philos Biology	Cultivation of herbs and medicinal plants in Norway – Essential oil production and quality control

2003	Åsa Maria O. Espmark Wibe	Dr. scient Biology	Behavioural effects of environmental pollution in threespine stickleback *Gasterosteus aculeatur* L.
2003	Dagmar Hagen	Dr. scient Biology	Assisted recovery of disturbed arctic and alpine vegetation – an integrated approach
2003	Bjørn Dahle	Dr. scient Biology	Reproductive strategies in Scandinavian brown bears
2003	Cyril Lebogang Taolo	Dr. scient Biology	Population ecology, seasonal movement and habitat use of the African buffalo (*Syncerus caffer*) in Chobe National Park, Botswana
2003	Marit Stranden	Dr.scient Biology	Olfactory receptor neurones specified for the same odorants in three related Heliothine species (*Helicoverpa armigera, Helicoverpa assulta* and *Heliothis virescens*)
2003	Kristian Hassel	Dr.scient Biology	Life history characteristics and genetic variation in an expanding species, *Pogonatum dentatum*
2003	David Alexander Rae	Dr.scient Biology	Plant- and invertebrate-community responses to species interaction and microclimatic gradients in alpine and Artic environments
2003	Åsa A Borg	Dr.scient Biology	Sex roles and reproductive behaviour in gobies and guppies: a female perspective
2003	Eldar Åsgard Bendiksen	Dr.scient Biology	Environmental effects on lipid nutrition of farmed Atlantic salmon (*Salmo Salar* L.) parr and smolt
2004	Torkild Bakken	Dr.scient Biology	A revision of Nereidinae (Polychaeta, Nereididae)
2004	Ingar Pareliussen	Dr.scient Biology	Natural and Experimental Tree Establishment in a Fragmented Forest, Ambohitantely Forest Reserve, Madagascar
2004	Tore Brembu	Dr.scient Biology	Genetic, molecular and functional studies of RAC GTPases and the WAVE-like regulatory protein complex in *Arabidopsis thaliana*
2004	Liv S. Nilsen	Dr.scient Biology	Coastal heath vegetation on central Norway; recent past, present state and future possibilities

2004 Hanne T. Skiri	Dr.scient Biology	Olfactory coding and olfactory learning of plant odours in heliothine moths. An anatomical, physiological and behavioural study of three related species (*Heliothis virescens, Helicoverpa armigera* and *Helicoverpa assulta*).
2004 Lene Østby	Dr.scient Biology	Cytochrome P4501A (CYP1A) induction and DNA adducts as biomarkers for organic pollution in the natural environment
2004 Emmanuel J. Gerreta	Dr. philos Biology	The Importance of Water Quality and Quantity in the Tropical Ecosystems, Tanzania
2004 Linda Dalen	Dr.scient Biology	Dynamics of Mountain Birch Treelines in the Scandes Mountain Chain, and Effects of Climate Warming
2004 Lisbeth Mehli	Dr.scient Biology	Polygalacturonase-inhibiting protein (PGIP) in cultivated strawberry (*Fragaria* x *ananassa*): characterisation and induction of the gene following fruit infection by *Botrytis cinerea*
2004 Børge Moe	Dr.scient Biology	Energy-Allocation in Avian Nestlings Facing Short-Term Food Shortage
2005 Matilde Skogen Chauton	Dr.scient	Metabolic profiling and species discrimination from High-Resolution Magic Angle Spinning NMR analysis of whole-cell samples