Genetic Variation in the haemoglobin of the Atlantic cod(*GADUS MORHUA*) in trans-atlantic populations

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Ås, September, 2012

PrakashYadav

Abbreviations

3D	Three-dimensional
A	Adenine
C	Cytosine
CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid
dNTPs	Dinucleotidetriphosphates
FC	Feed consumption
G	Guanine
GCE	Gross Conversion Efficiency
GE	Gastric Evacuation
Hb-β1	Haemoglobin β1
HRM	High Resolution Melting
HSI	Hepatosomatic index
K	Lysine
M	Methionine
O_2	Oxygen
NEAC	North East Arctic Cod
PanI	Pantophysin I
PCR	Polymerase Chain Reaction
R	Relaxed
Rpm	Rotation per minute
SNP	Single Nucleotide Polymorphism
SGR	Specific growth rate
T	Tense state
V	Valine

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Summary

Haemoglobin is one of the most studied proteins in nature. Evolutionary modification of this oxygen transporting molecule may affect the oxygen binding affinity and capacity. There are three polymorphism of the Atlantic cod haemoglobin Hb-β1, named as Met55Val, Lys62Ala and Leu122Met. These amino acid substitutions have impact on the function of haemoglobin and their biogeographically distribution depends on temperature. Three dimensional modelling of the tetrameric haemoglobin structure show that a Leu residue is well accommodated in β122 position and the amino acid replacement of Leu with Met brings about a slight increase of interface contacts that probably reduce oxygen binding capacity. In Canadian populations, the frequency of Met122 variant is higher than the Leu122 variant. This might be due to high temperature in Canadian populations and all other remaining populations predominate for Val55 and Ala62 variants except Østersjøen Outer and Skagerrak/Kattegat (North Sea) where Met55 and Lys62 predominate. The results indicate that the Atlantic cod (*Gadus morhua*) Hb- β1 gene is under adaptive changes.

1. Introduction

1.1 Atlantic cod biology

Atlantic cod (*Gadus morhua*) is the most important marine resources in the north Atlantic and is an emerging aquaculture species. It has been identified as an excellent candidate for aquaculture at the northern latitudes because of high worldwide demand, their good market value, declining wild stocks and relatively fast growth at low temperature. It is distributed in the areas of the eastern North America, at Newfoundland, Greenland, and Iceland, in the Barents Sea and along the coast of Norway, in the North Sea, and in the Baltic Sea (Mork *et al.*, 1984).

Atlantic cod is bottom fish species mainly distributed from shoreline and down to continental shelf. They spawn normally winter or spring in well known spawning sites (*ICES, 2005*). It has high fecundity with number of eggs ranging from thousands through to 20-30 millions per female (Lambert *et al*, 2003). The eggs are pelagic, and the hydrographical and egg buoyancy conditions play important role for determining the geographical distributions (Sundby, 2000; Sundby *et al.*, 1989). Cod can reach 2 metres in length (Cohen *et al*., 1990) and 100 kg in weight (Frimodt, 1995). Coastal cod is possibly more vulnerable to human impact. The Lofoten Island on the Norwegian coast is the main spawning site for the North east arctic cod (NEAC) population. The eggs and larvae are migrating north with coastal current and are distributed over large area of Barents Sea and Spitsbergen, and cod has to migrate from Barents Sea and back to Lofoten for spawning (*ICES,* 2005).

Cod farming has been highly developed in Norway, Canada and the United Kingdom as well as in Iceland and the northeast USA and is predicted the second most profitable marine finfish species after Atlantic salmon farming in the Europe. Norway and Iceland have also initiated selective breeding programmes for Atlantic cod. Quantity of catch Atlantic cod fishing was 340,099 tonnes by live weight with value of 3,885 million NOK in 2011 in Norway. (http://www.ssb.no/fiskeri_en). Worldwide farmed cod production is expected to increase from 8000 tonnes per annum in 2005 (FAO, 2007) to 400,000 tons per annum in 2020 (Solsletten, 2001). However there are several challenges that need to be solved such as early sexual

maturation and diseases which can reduce the flesh quality and cause mortality, leading to high economic loss. Besides these, the release of gametes and escaped fish from sea cages may result in interbreeding of farmed fish with wild stocks that may cause genetic contamination, potentially reducing the genetic health of these wild stocks. These factors may prevent the rapid establishment of a sustainable cod-farming industry.

Genetic studies of cod were started during the 1960s and haemoglobin was the first genetic marker to be studied in cod populations (Sick, 1961). As observed in other species, the availability of reference genome sequences has accelerated genomic research on cod. Genomic information can be used for genetic improvement as well as assist management to make decisions (Moen, *et al.*, 2009). SNP genotyping is rapidly becoming a powerful tool for assessing genetic variation in natural populations (Brumfield *et al*., 2003). Studies into the genetic background of complex traits are enhanced by correlating SNP genotype with phenotype and structure-function relationship. Advances in both molecular genetics and statistical methods have made it possible to perform genetic analysis of organisms and hence correlate genetic polymorphism with phenotypic variations within and between fish populations.

Temperature has major effect on geographical distribution of cod population and their behaviour. Rogers *et al.* (2011) reported that 3.1% decrease in length for every 2°C increase in summer temperatures. The Atlantic cod is a marine species whose thermal biology has received attention (Claireaux *et al*., 2000; Petersen and Steffensen, 2003). Atlantic cod is listed as "vulnerable" on the International Union for Conservation of Nature and Natural Resources' Red List of threatened species (Cosewic, 2003; Dulvy *et al.*, 2005; Reynolds *et al*., 2005). The pattern of haemoglobin variants of cod indicates that biogeography of this ecologically and economically important species might be affected by global warming (Andersen *et al.*, 2009).

Three polymorphisms have been identified in the cod haemoglobin Hb-β1 at the positions Met55Val, Lys62Ala and Leu122Met which probably have functional impact on the oxygen binding affinity and capacity. Andersen *et al*. 2009 analyzed the distribution of the substitutions Met55Lys62 and Val55/Ala62 and concluded that positions 55 and 62 are strongly linked and that the biogeographical distribution

of these polymorphisms depends on the temperature variation. The third polymorphism Leu122Met is associated in the allele as either Val-Ala-Leu or Val-Ala-Met (Borza et al., 2009). In this study, we focused mainly on how the Leu122Met SNP is distributed in different populations and how their allelic distribution is related with respect to temperature and behaviour.

1.2 Aims

The objectives of thesis are as follows:

I. Genotyping trans-Atlantic cod populations for the three non-synonymous SNPs identified in Hb-β1 gene with emphasize on the Leu122Met substitution.

II. Relate the geographical distribution Hb-β1 alleles to behaviour and temperature conditions.

III. Modeling the 3D structure of the cod Hb- β1 proteins for studying structure-function relationship of the Leu122Met substitution.

2. Literature Review

2.1 Population Genetics

Population genetics is about microevolution and is a discipline about genetic variation in populations. The genetic variation includes the change in allele frequencies, genotype frequencies and phenotype frequencies in a population. Population genetics predicts diversity that is determined by a number of factors including selection, recombination, mutation rate, genetic drift and effective population size. There are different kinds of genetic markers that can be used to measure genetic diversity. SNPs are a class of genetic markers that is well suited to a broad range of research and management applications, and has recently been used in various fields of conservation, evolution, and aquaculture science (Brumfield *et al.*, 2003; Morin *et al.*, 2004). Haemoglobin and Pantophysin (Pan) are the major genetic markers used to characterise the Atlantic cod populations. The PanI marker can be used to distinguish the migratory NEAC population from the stationary coastal cod populations (Pogson, 2001; Pogson and Fevolden, 2003). Population genetics play prominent role for measuring the genetic variation under adaptation. This significance can be used in selection of individuals for future generation for breeding purpose by measuring the fitness of population over evolutionary period.

2.2 Basic structure and function of haemoglobin

Haemoglobin is an intracellular molecule of erythrocytes, found in all vertebrates except the Antarctic icefishes, and is responsible for carrying O_2 between respiratory organs and metabolic tissues (Jensen *et al.*, 1988). It is one of the most intensively studied proteins which has made possible to understand the structure-function relationships. Vertebrate haemoglobin is a globular protein with quaternary structure composed of 2α and 2β globin chains. Each globin chain is attached to one prosthetic group called heme. Most fish haemoglobins are structurally tetrameric; however, some species such as lamprey and hagfish shows monomeric and oligomeric haemoglobins when they are oxygenated and deoxygenated, respectively (Souza; Bonilla Rodriguez, 2007). The α and β globins are located on different chromosomes in mammals, but on same chromosome in amphibians (Souza, 2007; Bonilla Rodriguez, 2007). In case of teleosts, α and β globins are adjacently attached to each other. In human, stable tetramer is formed by 2 α and 2 β globin chains with 141 and

146 amino acid residues, respectively. The cod haemoglobin genes reveal the characteristic structure of 3 exons and 2 introns encoding α and β globins of 143 and 147 amino acids respectively, except for α3. There are total nine haemoglobin genes-organized in two unlinked clusters designated as $\beta_5\alpha_1\beta_1\alpha_4$ and $\beta_3\beta_4\alpha_2\alpha_3\beta_2$, respectively (Wetten *et al*., 2010).

Expression of globin genes changes during the life stages of fish and is therefore divided into embryonic and adult globins (Maruyama, 2004). In comparison to most mammals and birds, fish have often multiple haemoglobin multiplicity whereas some Antarctic species have no haemoglobins (Ruud, 1954; Powers, 1980).

Haemoglobin has two alternative conformations and there is equilibrium between the two alternative structures, the tense (T) state characterized with deoxy-Hb and relaxed (R) state characterized with oxy-Hb. The T state is thermodynamically stable in absence of ligand because of presence of extra salt bridges and noncovalent bonds in the interface between the two α- β dimers. These bonds are loosened when oxygen binds to the haemoglobin and the T conformation switches to the high affinity R state (Perutz, 1970). During transition between the T and R states, one dimer rotates to another by 15°, and transition occurs when at least one of the hemes in each dimer is oxygenated (Jensen *et al.*, 1998).

Haemoglobin reveals the properties of allostery which is defined as the possibility of interaction among subunits of haemoglobin and the emergence of new properties. The most accepted model about allosteric properties of haemoglobin was proposed by Monod *et al*. (1965). The structural and functional diversity of the multiple haemoglobins in teleosts reveal that they have experienced a major evolutionary pressure to execute their O_2 transporting function under physico-chemical conditions (Perutz, 1983; Weber and Fago, 2004; Weber, 2003).

2.3 Cod haemoglobin genotypes and temperature

Atlantic cod is a temperate species and distributed throughout the Northern Atlantic, the Barents Sea and the Baltic Sea (Cohen *et al.*, 1990). They are physiologically adapted to temperate and the arctic region of North Atlantic, the North Sea and its

adjoining seas (Jamieson and Thomson, 1972). Cod's physiology and their production are highly influenced by temperature variation. There is significant interaction between haemoglobin genotype and temperature (Imsland *et al.,* 2004). This interaction might have strong influences on the growth pattern, ultimate size and age at sexual maturity. Several studies have been conducted to show the correlation between the HbI-isomers and growth rates but contrasting results exist (Nævdal *et al.*, 1992; Jørstad and Nævdal, 1994; Glover *et al*., 1997).

In Atlantic cod, haemoglobin is polymorphic with three different main genotypes i.e. two homozygotes HbI-1/1 and Hb-I-2/2 and one heterozygote HbI-1/2 (Sick, 1961; Frydenberg, 1965**).** Different biogeographical distributions of these genotypes are associated with seawater temperatures. Frydenberg *et al.* (1965) reported that frequency of genotype HbI-1/1 is more present in warmer regions (North Sea, western part of the Baltic Sea, southern part of the Norwegian Sea, and around the British Isles) while the occurrence of Hb-I-2/2 is higher in northern regions, colder regions (Greenland, Iceland, Canada, the northern part of the Baltic and Sea northern Norway). Karpov and Novikov (1980) reported on the functional properties of the HbI-1/1, HbI-2/2 and HbI-1/2 components. The oxygen affinity for haemoglobin of HbI-2/2 cod is higher at low temperatures (<10 °C) and the affinity of oxygen affinity of HbI-1/1 cod is higher at high temperatures (>14 °C) (Karpov and Novikov, 1981; Brix *et al*., 1998, 2004). Fyhn *et al*. (1994) found new subtypes using isoelectric focusing electrophoresis (IEF), which is used for separating different molecules by their electric charge differences. These subtype genotypes were named as HbI-1/2b and HbI-2/2b and were characterized by an extra set of double bands compared to those found in the corresponding main genotype types, i.e., HbI-1/2 and HbI-2/2. The heterozygote haemoglobin genotype HbI-1/2 has oxygen affinity values which are intermediate values to HbI-1/1 and HbI-2/2 genotypes. This information implies that temperature would be the selective parameter for the distribution of Atlantic cod with different genotypes. Petersen and Steffensen (2003) found that the preferred temperature for two homozygotes HbI-1/1 and HbI-2/2 are 15.4±1.1 °C and 8.2±1.5 °C, respectively. HbI-2/2 cod was shown higher growth than HbI-1/1 cod (Mork *et al*., 1984; Nævdal *et al.,* 1992; Imsland *et al.,* 2004), while Gamperl *et al.* (2009) reported that cod HbI-1/1 juveniles grew faster than heterozygotes (HbI-1/2. Other studies failed to show Hb dependency on

growth (Nævdal *et al.* 1992; Jørstad and Nævdal, 1994; Jordan *et al.* 2006). The homologous genotypes HbI-1/1 and HbI-2/2 fish showed higher feed efficiency than heterozygote HbI-1/2 (Jordan *et al.*, 2006) and Johnston *et al.* (2006) found that HbI-2/2 fish have greater muscle fibre diameter than HbI-1/1 fish.

Haemoglobin genotype might also be associated with behavioural traits like feed acceptance (Kadri *et al.*, 1991; Metcalfe *et al.*, 1992; Stradmeyer, 1992). Behavioural observations can play a vital role in fish growth studies. Salvanes and Hart (2000) found in their feeding trail experiment that homozygous HbI-2/2 fish captured the greater proportion of food given, and suggested that their behaviour upon feeding reflects success in natural hunting for food.

2.4 Cod haemoglobin genes and mutations

Haemoglobin is an important respiratory pigment for the fish adaptation because they constitute interface between organism and environment (Landini, 2004). Atlantic cod expresses polymorphic haemoglobin like some other species, such as birds and human. The mutations can results in amino acid substitutions that may causes structural changes and might affect oxygen binding capacity or affinity to haemoglobin. Cod haemoglobins comprise the three tetramers Hb1 (α1, α1, β1, β1), Hb2 (α2, α2, β2, β2) and Hb3 (α1, α1, β2, β2) (Verde *et al.,* 2006). The polymorphic β1 globin is mostly expressed in the juvenile and adult cod while the level of β1 globin gene has determined very low in the hatched larvae (Wetten *et al.*, 2010).There are three mutations identified in the β_1 haemoglon gene which causes three amino acid substitutions, Met55Val, Lys62Ala and Leu122Met (Andersen *et. al.*, 2009 and Borza *et. al.*, 2009). β_1-Met55Val is a single non-synonymous A/G mutation and β_1-Lys62Ala resulted from AA/GC mutations which are shown in fig 1 and 2. The third amino acid substitution is referred as β_1-Leu122Met which is originated from T/A substitution. First two polymorphisms can constitute four haplotypes: Met55-Lys62 (MK), Val55-Ala62 (VA), and the recombinants Val55-Lys62 (VK) and Met55-Ala62 (MA). A variable number of cod haemoglobin genes and allelic variants have been reported in Norwegian, Icelandic and Canadian cod populations (Andersen *et al*, 2009; Halldorsdottir and Arnason, 2009; Borza *et al.*, 2009).

Andersen *et al*. (2009) found the unambiguous relationship between the isomers and two allele of the β1 globin gene. HbI-1 phenotype is accompanied by methionine at residue 55 and lysine at residue 62 while HbI-2 phenotype is followed by valine at residue 55 and alanine at residue 62. Borza *et al.* (2009) found there is no relationship between the isomers and the β1-Leu122Met polymorphism. There is no any proof that how MA and VK recombinants are associated with HbI-Isomers, but Andersen *et al.* 2009 suggested that these recombinants are connected to rare HbI subtypes which have been reported (Fyhn *et al.*, 1994; Brix *et al.*, 2004; Husebo *et al*., 2004; Imsland *et al*., 2007). There are four homozygote genotypes (VA/VA, MK/MK, MA/MA and VK/VK), six heterozygotes (MK/VA, MK/VK, MK/MA, VA/VK, VA/MA and MA/VK) from four haplotypes (VA, MK, MA and VK) and among six heterozygotes, MK/VA and MA/ KV are called coupling and repulsive heterozygotes respectively (Wetten et al., 2011). High Resolution Melting (HRM) method can be used to distinguish between coupling and repulsive heterozygotes (Tindall *et al.*, 2009; Vossen *et al*., 2010). The geographical distribution of the β_1 haplotypes is correlated with sea water temperature. Generally, it revealed that dominance of the MK haplotype (HbI-1) and VA haplotype (HbI-2) are in temperate waters and cold waters respectively (Frydenberg *et al.* 1965; Sick 1965a; b; Moller 1966; Andersen *et al.*, 2009; Wetten *et al.*, 2011).

2.5 Structural polymorphisms of cod β_1 globin

The molecular mechanisms underlying the functional properties of the cod haemoglobin variants were revealed by studying structure-function relationship in the 3D modelled quaternary structure of the β1-containing tetramer (Andersen *et. al.*, 2009). The 3D model of the cod β_1 globin protein revealed that the distance between residue 55 of β_1 and residue 120 of α_1 at the $\alpha_1\beta_1$ dimer interface increased by 0.77Å replacing Met with Val at locus β_1-55 (Andersen *et al*., 2009)(Fig.1).

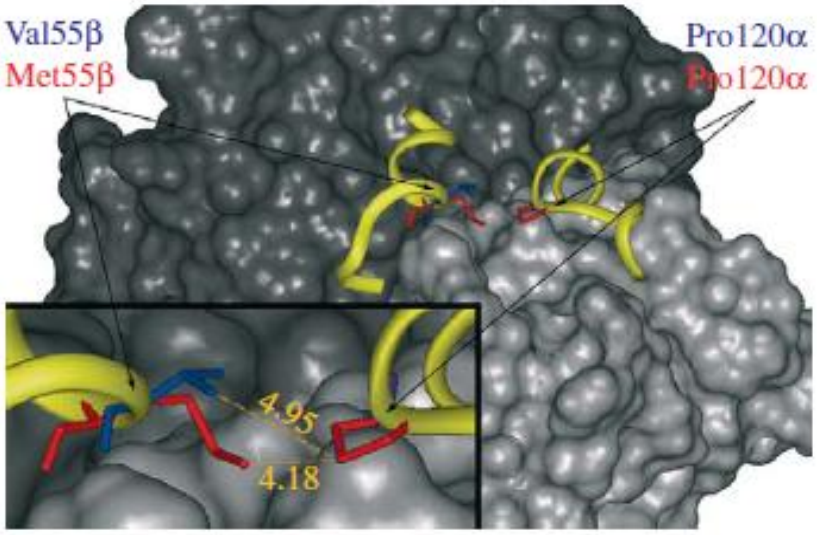


Figure 1. The amino acid substitution Met55Val at the subunit of α1β1 interface of Atlantic cod haemoglobin. (Cited from Andersen *et al.*, 2009).

Owing to increased distance at the interface, there is destabilization of the low oxygen affinity deoxy T-state and transition from T- state to the more stable high-oxygen affinity oxy R-state occurs (Abbasi and Lutfullah, 2002; Shikama and Matsuoka, 2003). In case of human haemoglobin, destabilization of it resulting from similar mutations increased oxygen affinity to haemoglobin (Jessen *et al*., 1991). Brix *et al.* (1998) reported that HbI isomer (VA genotype) have higher oxygen affinity than HbI isomer (MK genotype) is partially because of increased distance between the subunits of the two dimers of the haemoglobin tetramer. The Lys62Ala substitution of the cod β_1 globin did not show any significant steric effects on the adjacent His63 (Andersen *et al*., 2009), which plays prominent role in regulating the access of ligands to the haem pocket (Olsen *et al*., 1988). However there is interaction between Lys62Ala and water in the haem pocket.

The binding energy of a water probe in the haem pocket of the Lys and Ala residues is -14.8 and -11.6 $kcalmol^{-1,}$ respectively and indicates stronger water interaction with the polar Lys compared with the Ala residue(Andersen *et al.*, 2009) (Fig. 2).

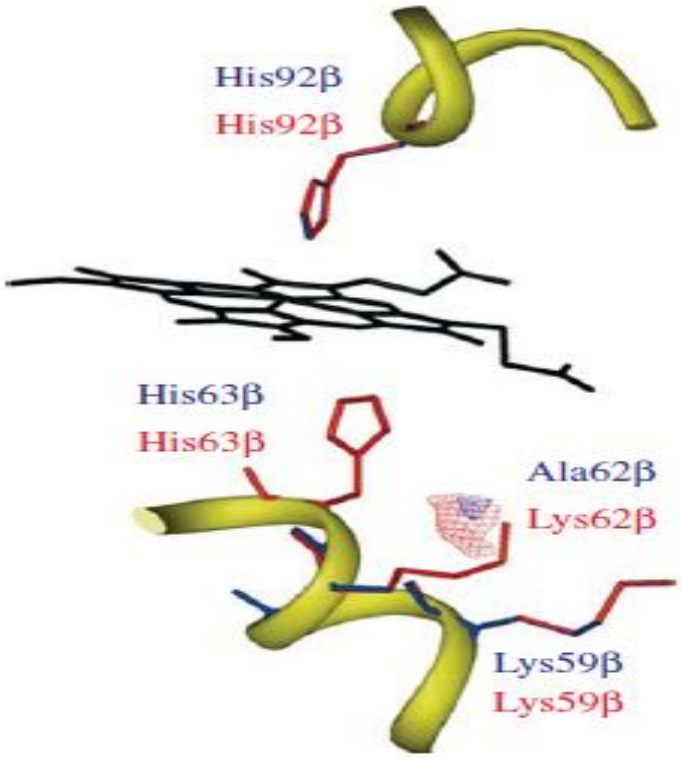


Figure 2. The amino acid substitution Lys62Ala and water interaction in the haem pocket (Cited from Andersen *et al.*, 2009).

3. Materials and Methodology

3.1 DNA samples from different cod populations

Norwegian Atlantic coastal cod cDNA samples (9 cDNA samples) were provided by Dr. Ola Frang Wetten, Hedmark University and North East Arctic Cod (NEAC) cDNA 6 samples were provided by Dr. Hanne Johnsen, University of Tromsø. Norwegian Atlantic coastal cod cDNA samples were prepared from the blood and head kidney cells (eight from blood and 1 from head kidney) and North East Arctic cod cDNA samples was prepared from head kidney and spleen tissue specimens (three males and three females) as mentioned by Johnsen *et al.* (2012). Sample specimens for Icelandic cod were provided by Dr. Christophe Pampoulie, Marine Research Institute, Island. Paul Ragnar Berg, Centre for Ecological and Evolutionary Synthesis (CEES) isolated genomic DNA from Icelandic specimens and while genomic DNA samples from Canadian and Greenland cod populations were kindly provided by Dr. Ola Frang Wetten. Genomic DNA samples from Baltic and Kattegat were provided by Paul Ragnar Berg.

3.2 RNA isolation

Total RNA isolation from samples of North East Arctic Cod (NEAC) was carried out by using Isol-RNA Lysis reagent and Pure Link™ Pro 96 RNA Purification Kit (Invitrogen, Life Technologies Corporation).The Isol reagent lyses cells, removes protein and DNA, and inactivates RNases.

The frozen tissue specimens from head kidney and spleen were transferred from -20 °C and placed on ice until they are thawed at room temperature. Small pieces of tissues were cut with scalpel blade from organ samples and placed in eppendorf tube which contained 800 µL Isol-RNA lysis reagent and 5-10 ceramic beads (1.4 mm zirconium oxide beads). The tissue was homogenized using homogenizer (Precellys® 24 Berlin Technologies) with the speed of 5500 rpm during cycles of 20 seconds with 5 seconds between the cycles. 200 µL of chloroform was added to each tissue specimens and shaked vigorously for 15 sec. Mixed samples with chloroform were incubated at room temperature for 2-3 minutes and centrifuged at 12,000×g for 15 minutes at 4 °C. 350 µL colourless aqueous phases were taken into another 1.5ml eppendorf tube carefully without disturbing the interphase which contains DNA. The

same amount of 350 µL of lysis buffer was added, which helps RNA to attach to membrane in filter plate and 350 µL of 96%-100% ethanol was added to it. The entire volume 1050 µL was transferred into the filter plate which is in the top of the of the receiver plate. The filter plate with receiver plate was centrifugated at 2100×g for 2 minutes at room temperature and was discarded the flow though.

For cleaning, 500 µL of wash buffer II 1x was added and centrifugated at least 2100×g for 2 minutes. 79.9 µL of DNase solution (Appendix I) was added per well. After that, it was incubated 15 minutes at room temperature. Washing buffer I 500 µL was added and centrifugated at 2100×g for 2 minutes and throw the flow though. 750 µL washing buffer II was added to it and centrifugated at 2100×g for 2 minutes and the flow through was thrown. Again 750 µL washing buffer II was added to it and centrifugated at 2100×g for 2 minutes. Membrane was dried by centrifugation at 2100×g for 10 minutes.

For elution, 50 µL RNase free water was added to each well and incubated 1 minute at room temperature and stacked plates were centrifugated at 2100xg for 2 minutes. Eluted RNA was transferred into a new eppendorf tube and finally kept on ice for quality testing.

Quantity and quality of RNA was checked with Nanodrop spectrophotometer (NanoDrop®, Saaven, and Werner AB). Isolated RNA was stored at -80 °C.

3.3 cDNA synthesis

TaqMan® Reverse Transcription Reagents (Applied Biosystems, Roche) was used to synthesize cDNA. It provides all the necessary components to perform the reverse transcription of RNA to cDNA. 500ng concentration of RNA was used for each samples and final total volume 7.1 µL of RNA was made by mixing with water for each samples. TaqMan® Reverse Transcription Reagents PCR mix (Appendix II) 12.9 µL and RNA template with water 7.1 µL was mixed in eppendorf tube, shaked and spinned. The samples were incubated in the MJ thermal cycler under the following conditions for cDNA synthesis.

a. 25°C for 10 minutes,

b. 48°C for 1 minute ,

c. 95°C for 5 minutes and

d. 4°C forever

3.4 Primer design

The PCR primers for haemoglobin β1 gene were designed by Wetten *et al.* (2010).

List of designated primers

Primers name	Primer Sequence	Tm	Amplicon
Hb-β-9_F	5'- GGC CTA AGC TAC ATT GAA CCC -3'	56.5	450
Hb-β-17_R	5'- CAG CCT GCA TCT CCG GGC TC -3'	62.2	450
Hb-β-10_F	5'- AGC GGA GGC ACG TCG AGG -3'	63.4	500
Hb-β-13_R	5'- CAT CCA TTT TGG GTT TGT CAC -3'	56.4	500

3.5 Amplification of cDNA

All 3 exons plus their flanking regions of the cod Hb-β1 gene were amplified by PCR using MJ Thermal Cycler-200. The 9 cDNA samples from Atlantic coastal cod were prepared for amplification. The total PCR reaction was 20 µl with 17.5 µl of master mix and a total input cDNA of 0.5 µl (10 ng). The master mix contained of 2 µl, 10x PCR buffer, 2 µl dNTP (2 mmol/L), and 1 µl (10 mmol/L) of forward primer and reverse primer, and 0.2 µl Taq polymerase. The optimal conditions for PCR reaction was 95°C for 10 minutes followed by 35 cycles of denaturation at 95 °C for 30 sec, followed by annealing step at 59 °C for 0.3 sec and 1 min for elongation step at 72 °C . It was followed by 5 min final elongation at 72 °C and final hold at 10°C for forever. Remaining other cDNA samples from NEAC were amplified by PCR and the sizes of PCR products were confirmed by 1% agarose gel (65V for 30 minutes) gel electrophoresis. The DNA fragments were then visualized with Gel Doc^{TM} EZ Imager and analysed Image Lab (Bio-Rad).

3.5.1 Purification of PCR products

Purification of the PCR products was performed by using Montage ^{TM} PCR Centrifugal Filter Devices and Millipore's Amicon® Ultra-0.5 Centrifugal Filter Devices. Montage PCR devices allow for upto 500 µL sample clean-up of salts, primers and unincorporated dNTPs prior to use of DNA for subsequent applications. It achieves high recoveries of fully functional nucleic acids. It consists of a filtrate collection vial with attached cap and a sample reservoir as shown in the Fig.3.

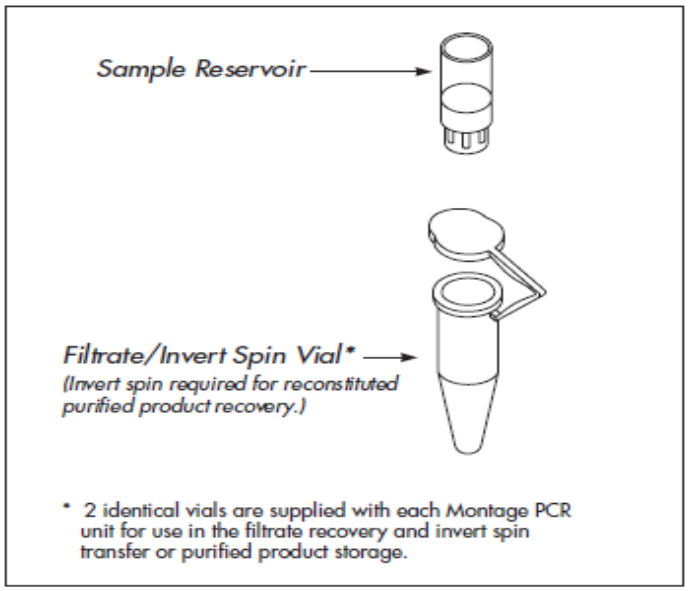


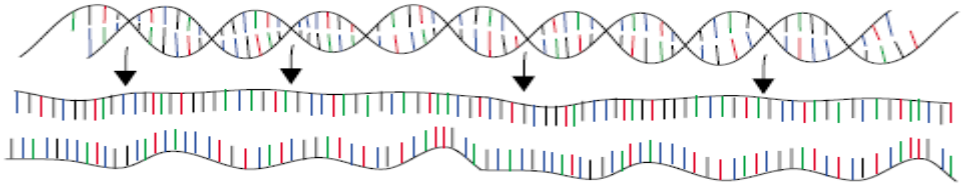
Figure3.Vial with attached cap and sample reservoir (Taken from Millipore Corporation, 2005)

390µl water and 10 µl PCR products were put into the sample reservoir and centrifugated at 1000×g for 15 minutes. After that assembled device was removed from the centrifuge and sample reservoir was separated from the vial. To get the purified cDNA, sample reservoir was placed upright position into a clean vial and 20 µl water was added to the sample reservoir. Sample reservoir was inverted and was spinned at 1000×g for 2 minutes. Purified cDNA was obtained at the bottom of vial. Similar process was applied for purification of PCR product with Millipore's Amicon® Ultra-0.5 centrifugal filter devices except addition of 20 µl water.

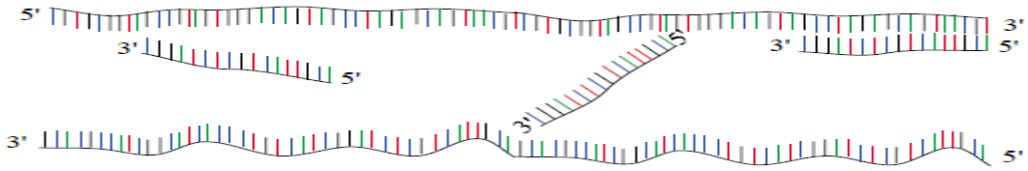
3.6 Sequencing reaction

The BigDye Terminator v3.1 Cycle Sequencing Kit was used for sequencing reaction. This kit is based on the Sanger dideoxy-mediated chain termination method. The BigDye® Terminator v3.1 Sequencing Buffer (5X) is supplied at a 5X concentration. Chain-termination method requires a single-stranded DNA primer, DNA polymerase, deoxynucleotidetrophosphates (dNTPs) and dideoxy-nucleotidetriphsphates (ddNTPs) which lacks of 3-OH group required for formation of phosphodiester bond between nucleotides and thus terminating DNA strand elongation. The ready reaction premix in the sequencing kit consists of a dNTPs, fluorescent-labelled ddNTPs and AmpliTaq Polymerase. The mixture(Appendix III)

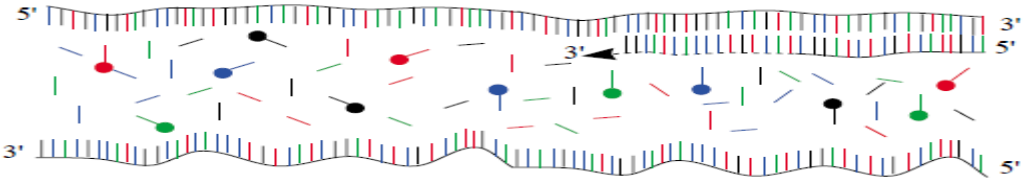
of 5x Big Dye terminator(v3.1) sequencing buffer, Big Dye terminator ready reaction Premix and water was mixed in one eppendorftube and spinned it. Primers at the rate of 0.1 µl are put in each sequencing tube and again spinned it. Finally PCR products 10 µl (10ng) as template was put in each sequencing reaction tubes. Tubes are placed inside on the Gene Amp® PCR System 9700 and sequencing cycle was carried out .There are three major steps in a sequencing reaction (like in PCR), which were repeated for 25 cycles as shown in Fig.4.



A. Denaturation



B. Annealing



C. Extension

Fig.4. Outline of three PCR steps (Andy Vierstraete, 1999)

1. 96°C for 1 minute

2. Repeat the following for 25 cycles: 95 °C for 10 sec, 50 °C for 5 sec and 60 °C for 4 min

3.10°C forever and was held until ready to purify.

Sequencing reaction products were stored at -20 °C until they are purified.

3.7 Sequencing reaction cleanup

For optimal results, sequencing reaction products were purified before sequencing using a vacuum based filtration with Montage-SEQ$_{96}$ Cleanup Kit (Millipore).

Montage SEQ_{96} Sequencing Reaction Cleanup Kit, Montage SEQ kits provide efficient and centrifugation-free sequencing reaction cleanup in 96 and 384-well formats. In addition to eliminating centrifugation steps, Montage-SEQ kits do not require filtrate collection or column packing. This method removes unincorporated dye terminators, enzymes, excess primers and salts to generate high quality DNA sequence data prior to capillary electrophoresis. Montage-SEQ_{96} Cleanup Kit contains membrane where DNA is retained while smaller contaminants are filtered to waste.

20 µl of injection solution that provided in kit was added to each sequencing reactions and mixed gently. After that, mixture was transferred to the SEQ_{96} plate. SEQ_{96} plate was placed on the vacuum manifold and 15Hg pressure was applied for 2-3 minutes until no fluid remains in the wells. SEQ_{96} plate was covered with aluminium foil during this process. Vacuum was shut off and the plate was removed from manifold. Excess fluid was blotted from the bottom of the plate with paper towel. The same procedure was repeated two times again with 25 µl of injection solution. After adding 25 µl injection solution, the DNA was resuspended by pipetting up and down 15-20 times. The resuspended and purified DNA was transferred to the sequencing plate and finally sequencing plate was centrifugated at 1500-2000 rpm for 1 minute at room temperature prior to sequencing.

3.8 Sequencing using ABI *3730*

The DNA sequencing reaction was performed from the amplified and purified cDNA samples and the cleaned sequencing reaction product were placed in the sequencing plate and the plates were loaded into the ABI *3730* DNA analyzer for sequencing (Applied Biosystems). This ABI 3730 DNA analyzer is based on the capillary electrophoresis systems used for analyzing fluorescently labelled DNA fragments (Fig.5). The autosampler brings waste reservoir to capillary array and other processes were carried out as shown below in flow chart. The capillary array is filled with POP-7™ polymer, which separates the DNA fragments and autosampler positions plates to capillary array.

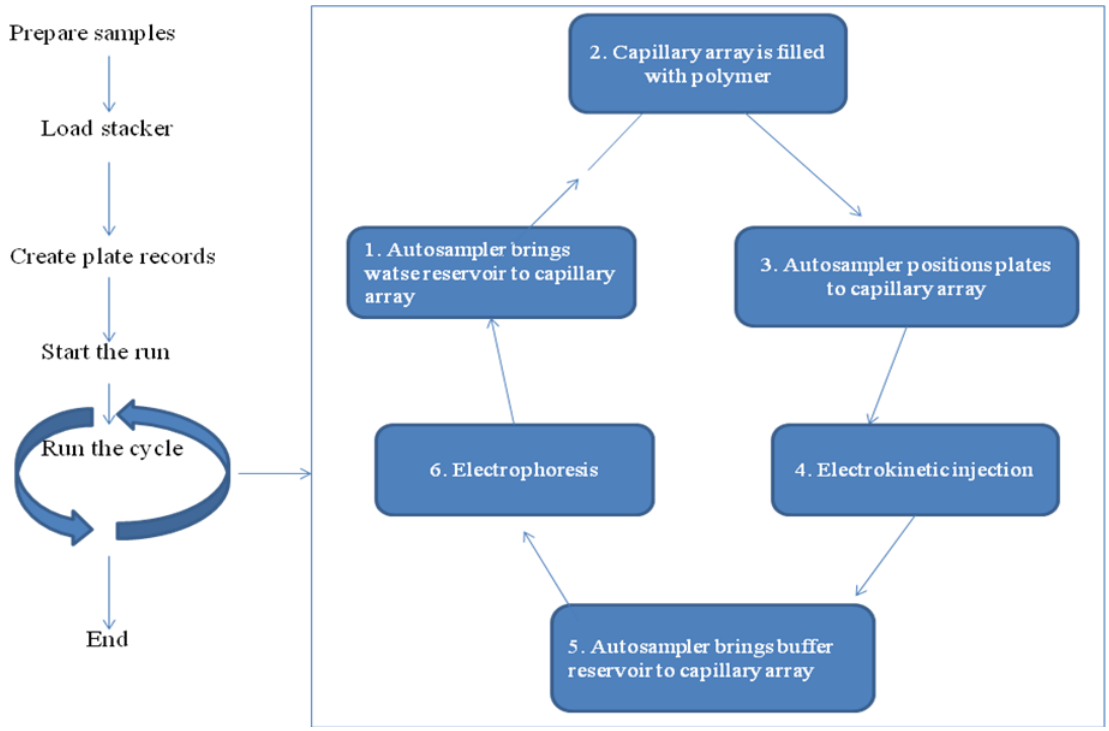


Figure 5. DNA Analyzer Run Cycle

The fluorescently labelled DNA was loaded into the capillary array by a short period of electrophoresis called electrokinetic injection. The autosampler brings the buffer reservoir to the capillary array for electrophoresis and the labelled DNA sequence fragments are separated by size as they travel through the polymer-filled capillary array.

3.9. Multiplex PCR

Multiplex PCR is a modification of simple PCR. It is a widespread molecular biology technique for amplification of multiple targets in a single PCR experiment. It is first described in 1988 to detect the deletions in dystrophin gene (Chamberlain *et al.*, 1988) and later applied for analysis of microsatellite and SNPs (Hayden, 2008). Multiplex PCR reaction can be divided into 2 categories:

a. Single template PCR reaction: This method uses a single template which can be a genomic DNA along with several pairs of forward and reverse primers to amplify specific regions within a template.

b. Multiple template PCR reaction: This technique uses multiple templates and several primer sets in the same reaction tube. The multiplex PCR has several advantages and offers considerable cost and labour savings. It can be used for pathogen identification, high throughput SNP genotyping, mutation analysis, gene deletion analysis, template quantization, linkage analysis etc.

3.10 SNPs genotyping of Hb-β1

Atlantic cod samples from Canada, Greenland, Iceland, Baltic Sea, North Sea and Norwegian Sea were genotyped for Hb- β1 SNPs by Sequenom MassARRY SNP analysis. Samples for Hb- β1 SNPs genotyping were collected from 10 different locations are demonstrated in maps below (Fig.6). Numbers in parentheses represent sample sizes. 50 samples from Greenland (Nuuk and Sisimiut), 69 samples from Canadian populations (George Bank, Labrador and Newfoundland), 78 samples from Icelandic cod populations, 96 samples from Baltic Sea (Østersjøen Inner and Østersjøen Outer), 39 samples from Norwegian Sea (Lofoten NEAC) and 48 samples from North Sea (Skagerrak/Kattegat) were used for Hb- β1 SNPs genotyping.

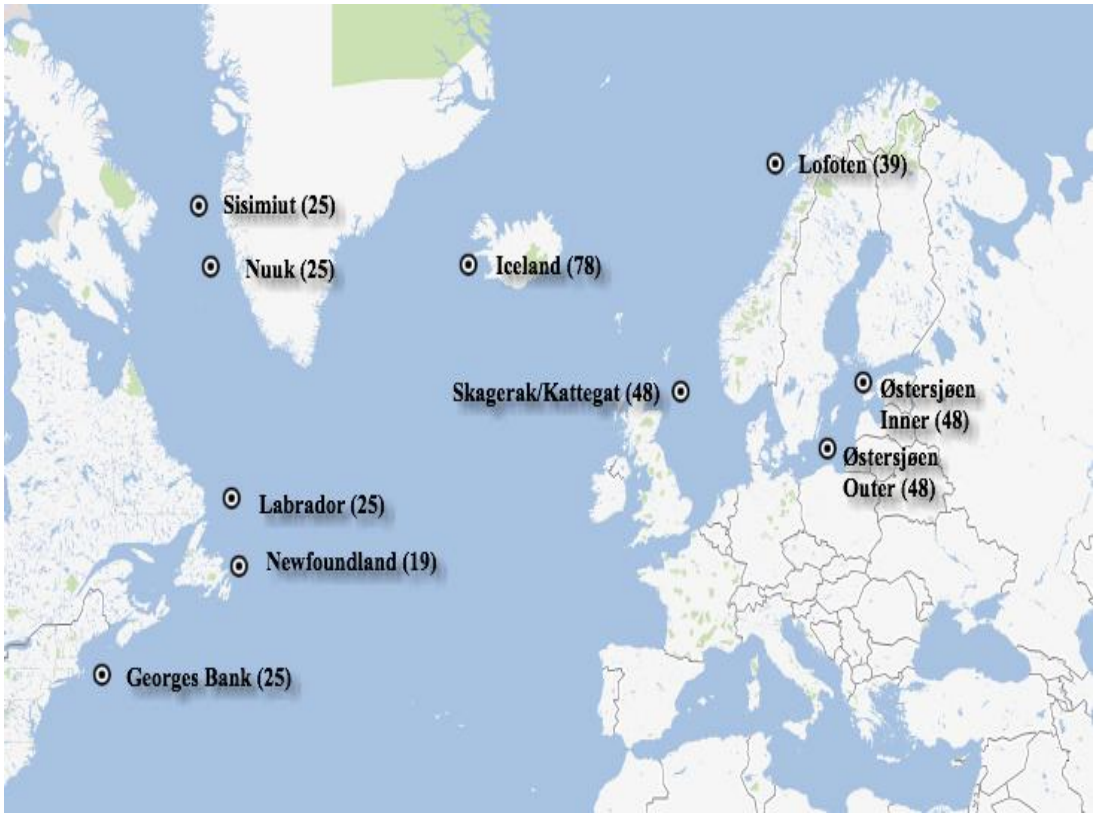


Figure 6. Sample locations for ten Atlantic cod populations. (Sample size is given in brackets.)

3.11 Statistical analysis

Deviation from Hardy-Weinberg proportions within cod samples was calculated as F_{Is} and population differentiation, Fst (Weir and Cockerham, 1984). Calculations and statitistical testing of $F_{IS,}$ Fst and genotypic linkage disequilibrium between loci were performed using Arlequin3.5 (Escoffier & Schneider, 2005) and GENEPOP on web (Raymond and Rousset, 1995).

3.12 Modelling of 3D structure of Hb-β1

Three dimensional (3D) modeled structure of Hb-β1 was built by comparative modeling using the MODELLER program v. 9 as implemented in DiscoveryStudio 3.1 (Accelrys Inc., San Diego, CA, USA) following the same computational protocol previously published (Andersen *et al*., 2009). Fifty models were generated and their consistency was evaluated on the basis of the PDF (probability density function) violations provided by the program.

4. Results and Discussion

4.1 Sequence analysis of Hb-β1

The polymorphic sites of Met55Val, Lys62Ala and Leu122Met were investigated in Norwegian Atlantic coastal cod and North East Arctic Cod (NEAC) by amplifying and sequencing cDNA samples from nine and six individuals, respectively. The sequence analysis revealed the two non-synonymous A/G and AA/GC mutations causing the amino acid substitutions Met55Val (M/V) and Lys62Ala (K/A), respectively, previously identified by (Andersen *et al*., 2009). The individuals analyzed were shown to contain only the "T allele" representing the Leu residue at the third mutation T/A causing the amino acid substitution Leu122Met (L/M) as shown Fig.7.

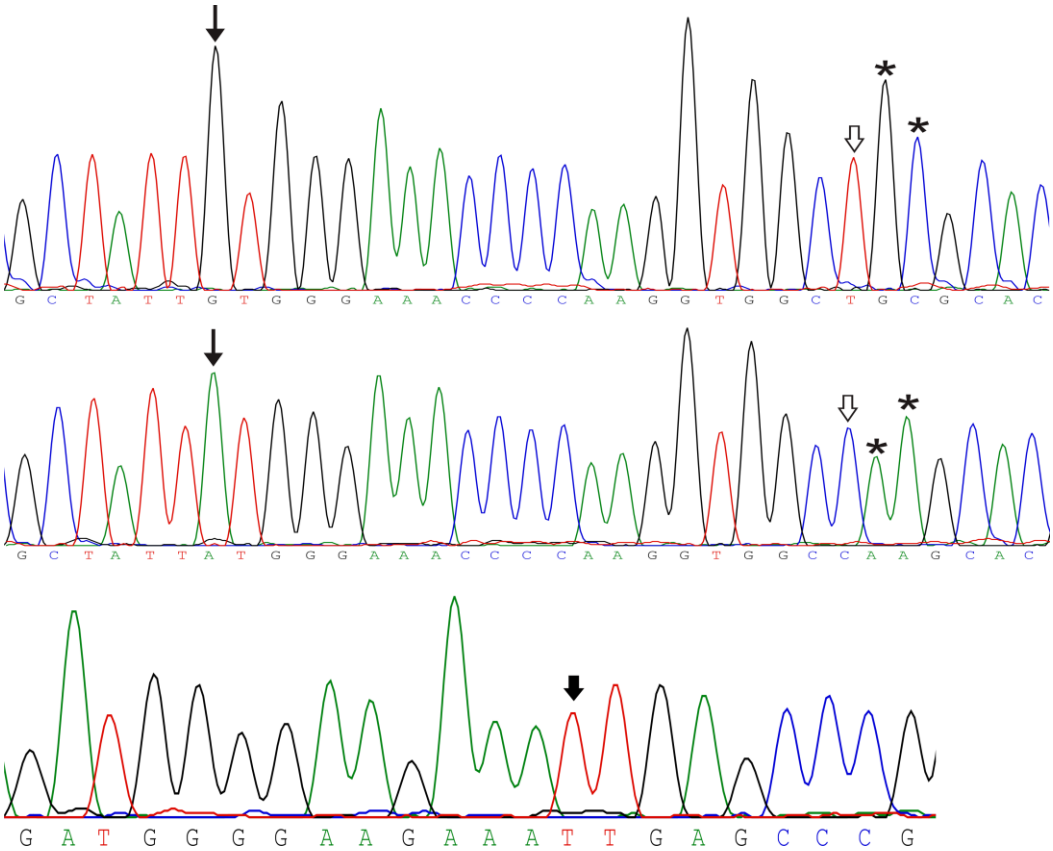


Figure 7. Upper two chromatograms reveal that there are two amino acid substitutions in the mutated β1 region. Downward shaded (↓) and an asterisk (*) show the Met55-Lys62 (upper) and Val55-Ala62 (middle) globin variants, respectively. Non- shaded arrows (⇩) shows synonymous mutation. In third (lower) chromatogram, the bold downward arrows (⬇) show the position of third mutation Leu122Met in the Hb- β1 gene.

4.2 Structure-function analysis of Leu122Met

Met122Leu is the third polymorphic position of the cod Hb-β1 as shown in the 3D modeled structure (Fig. 8 and 9).

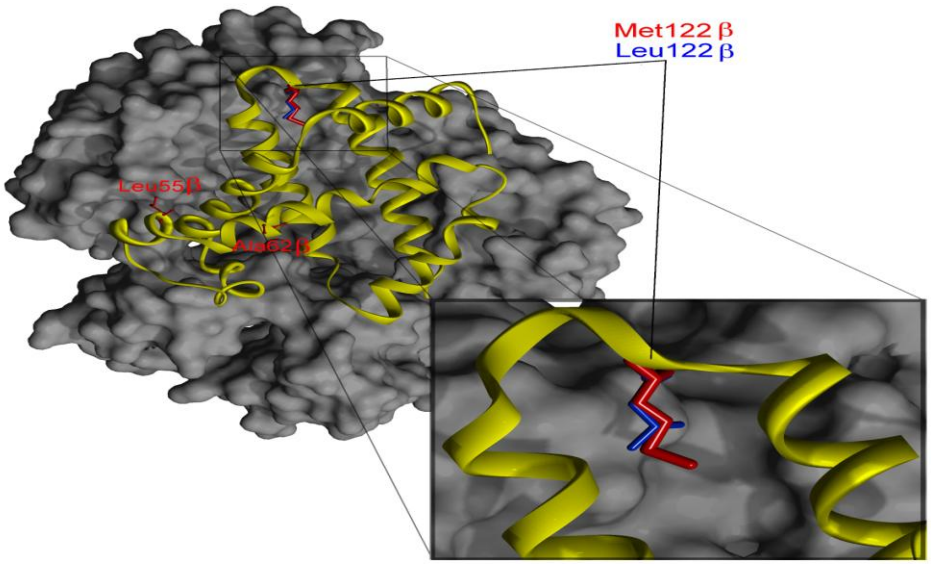


Figure 8. 3D structure of cod Hb- β1 showing the third amino acid substitution - Leu122Met (provided by Dr. Maria Cristina De Rosa, Institute of Chemistry of Molecular Recognition, Catholic University of Rome).

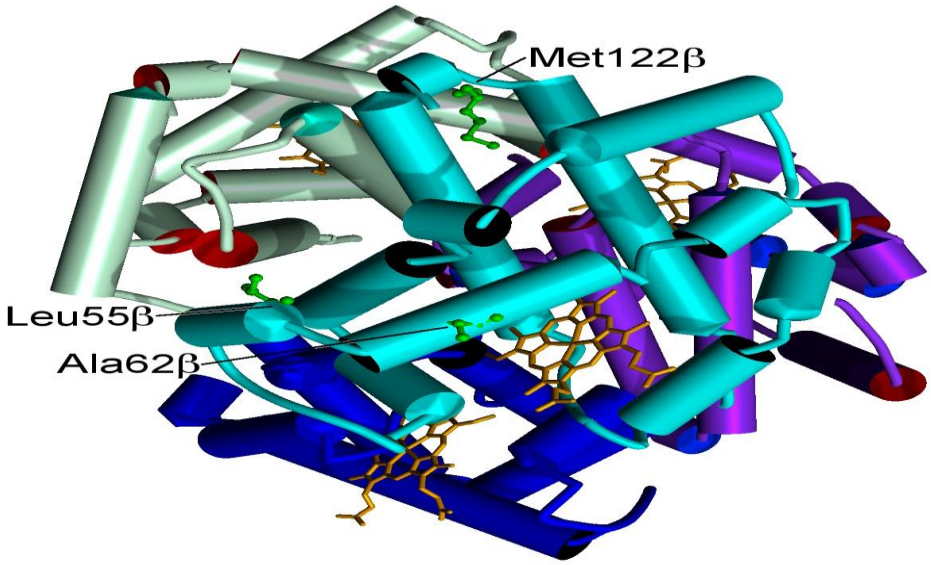


Figure 9. 3D structure of cod Hb- β1 showing the positions three amino acid substitutions (provided by Dr. Maria Cristina De Rosa, Institute of Chemistry of Molecular Recognition, Catholic University of Rome).

Analysis of three-dimensional models shows that a Leu residue is well accommodated in β122 position, but the amino acid replacement of Leu122 with Met122 brings about a slight increase of interface contacts. This is outlined in table 1 showing the number of atoms in contact at position β122.

Table 1. Residues of α chain and number of atoms at a maximum distance of 4 Å from β122 position

Residue	Contacts	No. of atoms
Met122β	Arg31α	3
	Ile112α	1
Leu122β	0	0

In the Atlantic cod, changing of Leu residue with Met residue decreases the gap between the contacts that probably stabilizes the T-structure with a resultant decreases in oxygen binding affinity. As a consequence of the introduction of a Leu residue, a smaller number of intersubunit contacts are formed. Computational studies would therefore predict a slight destabilization of the mutant. Due to formation of intersubunit, distance is probably increased between contacts, there is destabilization of the low oxygen affinity deoxy T-state and transition from T- state to the more stable high- oxygen affinity oxy R-state occurs (Abbasi and Lutfullah, 2002; Shikama and Matsuoka, 2003).

4.3 SNPs genotyping of Hb- β1

Total 382 samples from 10 different populations (Nuuk, Sisimiut, Newfoundland, Labrador, Iceland, NEA (Lofoten outside), Østersjøen Inner, Østersjøen Outer and Skagerrak/ Kattegat) were genotyped for the SNPs underlying the Met55Val, Lys62Ala and Leu122Met of Hb- β1. Due to ambiguous results, 376, 373 and 376 samples for Met55Val, Lys62Ala and Leu122Met loci were genotyped, respectively. Genotype frequencies for the different populations are summarized in Table 2.

Table 2. Genotype frequency in cod populations at three different loci

Sampling Locations	No. of samples	Met55Val			Lys62Ala			Leu122Met		
		Genotype frequency			Genotype frequency			Genotype frequency		
		GG	AG	AA	CC	CA	AA	TT	TA	AA
Nuuk	25	1.000	0.000	0.000	0.916	0.083	0.000	0.75	0.208	0.041
Sisimiut	25	0.96	0.04	0.000	0.791	0.166	0.041	0.44	0.56	0.000
Labrador	25	0.92	0.08	0.000	0.64	0.36	0.000	0.160	0.360	0.480
George bank	25	0.875	0.083	0.041	0.739	0.217	0.043	0.166	0.416	0.416
Newfoundland	19	0.894	0.105	0.000	0.736	0.263	0.000	0.105	0.421	0.473
Iceland	78	0.973	0.026	0.000	0.960	0.039	0.000	0.842	0.157	0.000
Lofoten(Outside)	39	0.794	0.153	0.051	0.769	0.179	0.051	0.923	0.076	0.000
Østersjøen (Inner)	48	1.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000
Østersjøen(Outer)	48	0.145	0.604	0.250	0.125	0.604	0.270	1.000	0.000	0.000
Skagerrak/ Kattegat	48	0.270	0.416	0.312	0.170	0.425	0.404	1.00	0.000	0.000
Average		0.750	0.170	0.079	0.678	0.225	0.096	0.752	0.162	0.085

From genotype distribution in table 2, it can be observed that there is higher average percent of GG (75%), CC (67%) and TT (75%) genotype at loci Met55Val, Lys62Ala and Leu122Met, respectively, where the G, C and T encode the amino acids Val, Ala and Leu, respectively.

Distribution of allele frequency of Hb-β1 in the different populations

Greenland populations (Nuuk and Sisimiut)

In Nuuk and Sisimiut populations, Val55, Ala62 and Leu122 allele frequencies are mostly found (Tab. 3, Fig. 10). The Met55Val locus is monomorphic for the Val55 allele in the Nuuk population.

Table 3. Allele frequency at the three polymorphic loci in different populations

Sampling Locations	No. of samples	(Met55Val)		(Lys62Ala)		(Leu122Met)	
		Allele frequency		Allele frequency		Allele frequency	
		A	G	A	C	T	A
Nuuk (Greenland)	25	0.000	1.000	0.041	0.958	0.854	0.145
Sisimiut(Greenland)	25	0.02	0.98	0.125	0.875	0.72	0.28
Labrador(Canadian)	25	0.04	0.96	0.18	0.82	0.34	0.66
George bank(Canadian)	25	0.083	0.916	0.152	0.847	0.375	0.625
Newfoundland(Canadian)	19	0.052	0.947	0.131	0.868	0.315	0.684
Iceland	78	0.013	0.986	0.019	0.960	0.986	0.078
Lofoten NEA	39	0.128	0.871	0.141	0.858	0.961	0.038
Østersjøen Inner	48	0.000	1.000	0.000	1.000	1.000	0.000
Østersjøen Outer	48	0.552	0.447	0.572	0.427	1.000	0.000
Skagerrak/ Kattegat	48	0.520	0.479	0.617	0.382	1.00	0.000
Average		0.140	0.858	0.197	0.799	0.755	0.251

In table 3 it can be observed that there is higher average frequency of G (0.85), C (0.79) and T (0.75) alleles, while other alleles have minor frequency.

Canadian populations (Labrador, George Bank and Newfoundland)

There was predominance of the Val55, Ala62 and Met122 alleles in the Canadian populations. Interestingly, the frequency of the Met122 allele was almost twice the frequency of the Leu122 allele in all Canadian populations.

Icelandic and NEAC Lofo cod populations

In Icelandic cod populations, the frequency of Val55, Ala62 and Leu122 were 0.98, 0.96, and 0.98, respectively, which were very similar to the frequencies found in the spawning NEAC population outside Lofoten as shown in Fig.10

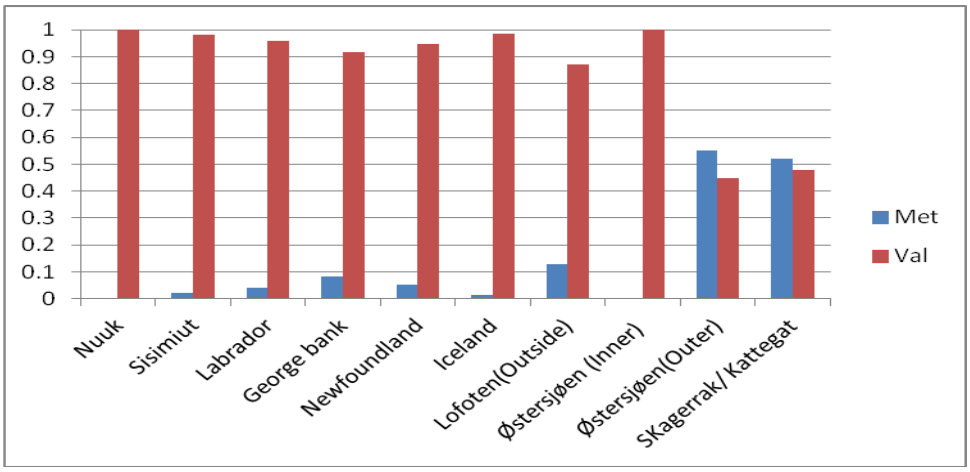


Figure 10a. Allele frequencies at locus1 (Met55Val)

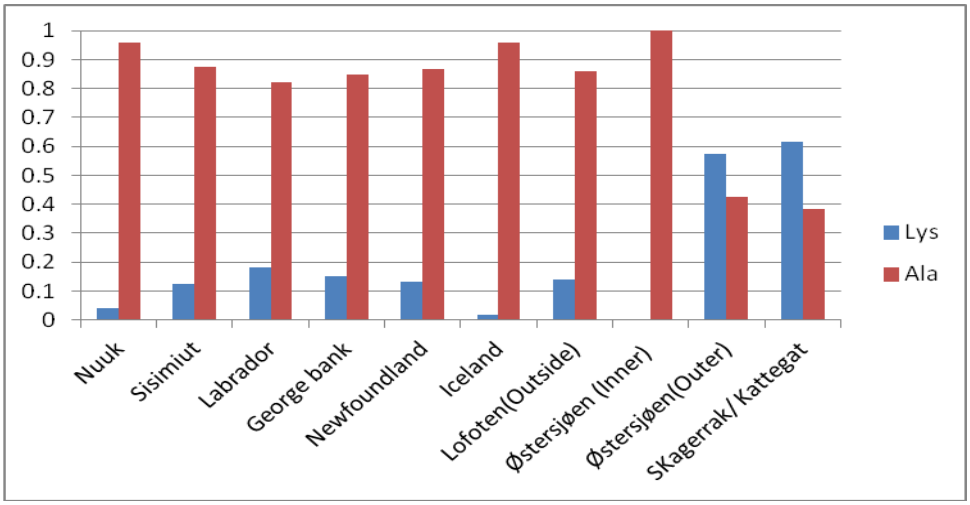


Figure 10b. Allele frequency at locus2 (Lys62Ala)

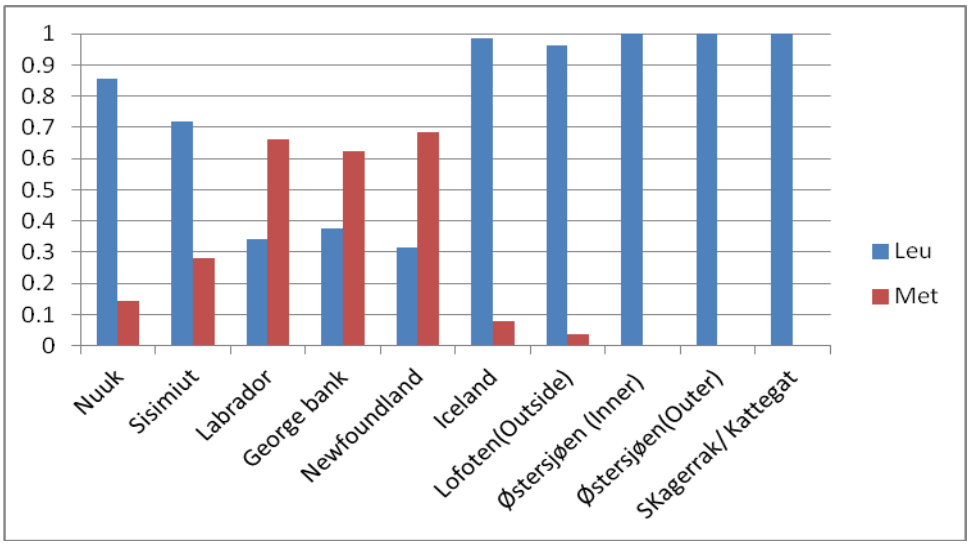


Figure 10c. Allele frequency at Locus3 (Leu122Met)

Baltic Sea (Østersjøen Inner and Østersjøen Outer)

Østersjøen Inner were shown to be monomorphic for the Val55, Ala62 and Leu122 variants, whereas cod from Østersjøen Outer showed higher frequencies of the Met55 and Lys62 variants compared to the Val55 and Ala62 variants. This population was monomorphic for the Leu122 allele.

North Sea (Skagerrak and Kattegat)

In North Sea, the frequency of the Met55 and Lys62 alleles predominated, and Leu122 was the monomorphic allele as in the Østersjøen Outer population. Wetten *et al.* (2011) also reported that Met55-Lys62 is mostly found is the North Sea, Kattegat and southern part of the Norwegian coast.

From allele distribution in different populations, it can be concluded that Greenland, Canadian, Icelandic, Norwegian sea and Baltic sea (Østersjøen Inner) cod populations have higher frequencies of the Val55 and Ala62 alleles, respectively, in contrast to the populations from North sea (Skagerrak and Kattegat) and Baltic sea (Østersjøen Outer).

Geographical distribution of Hb-β1 alleles related to temperature and behavior

Thermal change may result in changes in geographic distribution of the Atlantic cod. It has been proposed that thermal change could also result in a shift in cod distribution towards a more optimal climate (Perry *et al.*, 2005).

For Met55Val locus, there is higher frequency of Met55 allele compared to Val55 in populations having higher temperature like North Sea (Skagerrak and Kattegat) and Baltic Sea (Østersjøen Outer), where average temperature is 17°C in the summer. Norwegian Sea (Lofoten NEA) has allele frequency of 0.128 of Met55. There is average temperature of 10-12°C in Norwegian Sea in the summer. Greenland Sea (Nuuk and Sisimiut), Canadian Sea (Labrador, Newfoundland and George Bank), Icelandic Sea waters temperature ranges 6.5 to 15°C in the summer. These populations predominate in the Val55 alleles. So we can say that higher temperature has preference of Met55 alleles than Val55 alleles.

For Lys62Ala locus, there is higher occurrence of Ala62 alleles in colder regions (Greenland, Iceland, Canada, the northern part of the Baltic and Norwegian northern Sea) while frequency of Lys62 is higher in warmer regions (North Sea and Baltic Sea).

Some of the highest frequencies of the HbI-1/1 genotype (0.4) were found in the North Sea (Skagerrak/Kattegat), where water temperatures are high, and frequencies gradually decline (to 0.00) with declining temperature along Nuuk, Labrador, Newfoundland, Iceland and Østersjøen. Frydenberg *et al.* (1965) reported that the occurrence of Hb-I-2/2 (Val55-Ala62) is higher in northern regions, colder regions (Greenland, Iceland, Canada, the northern part of the Baltic and Norwegian northern Sea) while frequency of genotype HbI-1/1(Met55-Lys62) is more present in warmer regions (North sea, western part of the Baltic Sea, southern part of the Norwegian Sea). Consistently, the oxygen affinity for haemoglobin of HbI-2/2 cod is higher at low temperatures (< 10 °C) and the affinity of oxygen affinity of HbI-1/1 cod is higher at high temperatures (>14 °C) (Karpov and Novikov, 1981; Brix *et al*., 1998, 2004).

For the third Leu122Met loci, Greenland, Iceland, Norwegian Sea (Lofoten NEA), Baltic Sea (Østersjøen Inner and Outer) and North sea (Skagerrak and Kattegat) have higher frequency of Leu122 than Met122 allele, but all Canadian cod populations (Newfoundland, Labrador, George Bank) show less frequency of Leu122 than Met122 variant. Thus, the Canadian cod populations might have less oxygen binding affinity of haemoglobin than the other populations because replacement of Leu with Met increases the contacts of interface as shown in the 3D modelled structure. On the other hand, the proposed lower affinity of the Met122 variant might be opposed by the Val residue at locus Met55Val because replacement of Met with Val loose the oxygen pockets and helps for oxygen binding (Andersen *et al*., 2009). Conversely the proposed low oxygen binding affinity of haemoglobin displaying Met55 and Met122 might explain the very low frequency of this variant in the populations examined.

Atlantic cod physiology is highly influenced by temperature fluctuations and thus affecting fish behaviour. However, knowledge on the effects of temperature on Atlantic cod juvenile behaviour is limited. Temperature affects the specific growth

rate (SGR), hepatosomatic index (HSI), adjusted specific growth rate (SGRA), food consumption (FC), gross conversion efficiency (GCE) and gastric evacuation (GE) of juvenile Atlantic cod and there was a significant decrease in SGR, SGRA and FC (range 60–96%) when they are reared at 2 vs. 11 °C (Pérez-Casanova, 2009). Fluctuating temperature conditions have profound effect on the free swimming behavior of the Atlantic cod. There is marked increased in swimming activity and heart rate due to rise in temperature inside the homogenous water column, but in thermally stratified column, voluntary activities increased and heart rate is not dependent on temperature variation (Brett and Glass, 1973; Wardle, 1977, 1980; He and Wardle, 1988). Acute increases or decreases of temperature through 2.5°C led to marked differences in oxygen consumption, with metabolic rate changes of 15 and 30 %, respectively (Claireaux,1995). HbI-1/1 cod swim faster than HbI-2/2 cod in normoxia at 15 °C (Skjæraasen, 2008). Consequently, Atlantic cod populations from North Sea (Skagerrak and Kattegat) and Østersjøen Outer (Baltic Sea) might have possibly faster swimming ability than Atlantic cod from other locations.

HbI-2/2 (Val55-Ala62) cod prefer a temperature of 8.2±1.5°C while HbI-1 (Met55-Lys62) cod prefer 15.4±1.1°C but during hpoxia condition, HbI-1/1 cod prefer 9.8±1.8°C (Petersen and Steffensen, 2003). Weber (1990) reported that haemoglobin genotype is associated with metabolic rate through ability to carry oxygen and several studies found that there is relationship between metabolic rate and aggression (Nakano, 1994; Yamamoto *et al*., 1998; Cutts *et al*., 1998). HbI-2/2 cod genotype displayed a higher level of aggression than other genotypes (Perutz, 2007) and Salvanes and Hart (2000) reported that the HbI-2/2 genotype was dominant in feeding.

Theodorou (2010) reported in his experiments that Atlantic cod juveniles held at the temperature of 13ºC were more aggressive and higher feeding motivation than juveniles held at 3ºC and he also demonstrated that Atlantic cod held at 3 ºC revealed higher aggregation and Atlantic cod have diminished abilities to alter their behaviour in response to environmental change at higher temperatures owing to consequential increase in activity or higher metabolic demands.

4.4 Population genetics results

There was 5% genetic variation within the populations (F_{IS}) and 37% between the populations (Fst) due to Met55Val polymorphism while Lys62Ala and Leu122Met had no highly significant role in genetic variation within populations (Appendix 4.1).

There is linkage disequilibrium between loci Met55Val and Lys62Ala but there is no linkage disequilibrium between Met55Val and Leu122Met and Lys62Ala and Leu122Met (Appendix 4.2).

There was 34% and 44% of genetic variations between subpopulations (F_{ST}) due to loci Lys62Ala and, Leu122Met respectively (Appendix4.1). There is indication of excess of heterozygote in the samples from Labrador, Iceland, Newfoundland and Østersjøen Outer for Met55Val; Nuuk, Labrador, Newfoundland, Iceland Østersjøen Outer for Lys62Ala and Sisimiut, Iceland and Lofoten NEA for Leu122Met loci, respectively (Appendix 4.3).

For Met55Val locus, there were homozygote excess in the samples from George Bank, Lofoten NEA and Skagerrak/Kattegat. Samples from Sisimiut, Lofoten NEA and Skagerrak/Kattegat revealed also excess of homozygosity for the Lys62Ala locus. All Canadian and Nuuk samples have excess of homozygosity for the Leu122Met locus (Appendix 4.3).

Deviations from Hardy-Weinberg equilibrium were tested using an exact probability test. When testing all samples for all loci, no deviation from the Hardy-Weinberg equilibrium was detected. In this case, null-hypothesis is that populations are in Hardy-Weinberg equilibrium. This hypothesis would be rejected if p-values go below 0.05. For all these populations, there is no any p-value value below 0.05, so it concluded that all samples are in Hardy-Weinberg equilibrium at the investigated markers (Appendix 5).

F_{IS} measures the reduction in heterozygosity due to non-random mating within the subpopulation and helps to detect departures from Hardy-Weinberg by measuring the amount of heterozygote deficiency or excess observed in the sample (Weir and Cockerham, 1984). Values significantly greater than zero indicate an excess of homozygotes possibly resulting from population admixture, inbreeding, or failure to

detect heterozygotes. Conversely, negative F_{IS} indicates an excess of heterozygotes and out breeding.

Østersjøen Inner samples are monomorphic for all three loci. Østersjøen outer, Nuuk, and Skagerrak/ Kattegat samples are monomorphic for loci Leu122Met, Met55Val and Leu122Met, respectively (Appendix 5).

Haplotype frequency was calculated by using Arlequin3.5 software in different populations. There are AAT and GCA non-recombinant haplotypes and others are recombinant haplotypes. Recombinant haplotype are ACT, GAA, GAT, GCT and AAA. These recombinant haplotypes showed that there is recombination between loci Met55Val and Lys62Ala alleles and alleles between Lys62Val and Leu122Met loci and alleles between Met55Val and Leu122Met. High resolution melting analysis of trans-Atlantic populations revealed low allelic frequencies of Val-Lys and Met-Ala recombinants (Wetten *et al*., 2011). Borza *et al.* (2009) reported that Leu122 Met was associated in the allele as either Val-Ala-Leu or Val-Ala-Met, but the Met-Lys-Met haplotype was found in two out of 15 offspring examined from the crossing of two heterozygote parents. This study revealed additional recombinations displaying heterozygous frequencies in wild trans-Atlantic populations indicating the combined results of the distributional history of cod and the different selective forces acting in different areas under recent and current environment conditions.

Conclusion

It can be concluded that Val55-Ala62 haplotype predominated in the Greenland, Canadian, Iceland, and Lofoten NEA, Østersjøen Inner while Met55-Lys62 was mainly found in Østersjøen outer and Skagerrak/Kattegat. The Canadian populations showed higher frequency of the Met residue at position 122, while the Leu122 allele was almost fixed in East-Atlantic populations. The 3D modelled structure of the cod haemoglobin showed that the replacement of Leu with Met brings about a slight increase in interface contacts that might cause less oxygen affinity.

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List of Appendices

Appendix 1. DNase Solution

		1x
1.	10xDNase I buffer	8 µl
2.	DNase I	9.9 µl
3.	RNase free water	62 µl

Appendix 2. cDNA synthesis

	1x
10x Buffer	2 µl
25 µM $MgCl_2$	4.4 µl
dNTP	4.0 µl
50 µM Random Hexamers	0.5 µl
50 µM Oligo d (T)	0.5 µl
RNase inhibitor 20 U/ µl	1 µl
MultiScribe™ Reverse Transcriptase 50U/ µl	0.5 µl

Appendix 3. Sequencing reaction

	1x
5x Big Dye terminator (v3.1) sequencing buffer	3.5 µl
Big Dye terminator ready reaction Premix	1 µl
Water	5 µl

Appendix 4. Population genetics results from GENEPOP and Arlequin

4.1. Multilocus estimates for diploid data

Locus	Fwc (IS)	Fwc (st)	Fwc (It)
HbB1_SNP1	0.0561	0.3753	0.4103
HbB1_SNP2	0.0005	0.3480	0.3483
HbB1_SNP3	0.0082	0.4416	0.4462

4.2. Genotypic linkage disequilibrium

P-value for each locus pair across all populations
(Fisher's method)

Locus pair	Chi2	df	P-Value
HbB1_SNP1 & HbB1_SNP2	Infinity	16	Highly sign
HbB1_SNP1 & HbB1_SNP3	14.765741	12	0.254498
HbB1_SNP2 & HbB1_SNP3	20.835672	14	0.105918

4.3. Allele frequency based correlation (F_{IS}) and diversity

Met55Val

	1-Qintra	1-Qinter	F_{IS}
Nuuk	0.0000	0.0000	-
Sisimiut	0.0400	0.0400	0.0000
Labrador	0.0800	0.0783	-0.0213
George Bank	0.0833	0.1576	0.4713
Iceland	0.0263	0.0261	-0.0067
Lofoten NEA	0.1538	0.2274	0.3234
Newfoundland	0.1053	0.1023	-0.0286
Østersjøen Outer	0.6042	0.4987	-0.2116
Østersjøen Inner	0.0000	0.0000	-
Skagerak/Kategatt	0.4167	0.5053	0.1754

Lys62Ala

	1-Qintra	1-Qinter	F_{IS}
Nuuk	0.0833	0.0815	-0.0222
Sisimiut	0.1667	0.2246	0.2581
Labrador	0.3600	0.3000	-0.2000
George Bank	0.2174	0.2648	0.1791
Iceland	0.0395	0.0389	-0.0135
Lofoten NEA	0.1795	0.2463	0.2712
Newfoundland	0.2632	0.2339	-0.1250
Østersjøen Outer	0.6042	0.4934	-0.2246
Østersjøen Inner	0.0000	0.0000	-
Skagerak/Kategatt	0.4255	0.4783	0.1103

Leu122Met

	1-Qintra	1-Qinter	F_{IS}
Nuuk	0.2083	0.2554	0.1844
Sisimiut	0.5600	0.4083	-0.3714
Labrador	0.3600	0.4600	0.2174
George Bank	0.4167	0.4801	0.1321
Iceland	0.1579	0.1463	-0.0791
Lofoten NEA	0.0769	0.0749	-0.0270
Newfoundland	0.4211	0.4444	0.0526
Østersjøen Outer	0.0000	0.0000	-
Østersjøen Inner	0.0000	0.0000	-
Skagerrak/Kattegat	0.0000	0.0000	-

1-Qintra: gene diversity intra-individuals
1-Qinter: gene diversity inter-individuals

Appendix 5. Deviations from Hardy Weinberg expectations

Population	Locus	Genotype	Obs. Het.	Exp. Het.	P-value	s.d.	Steps done
Nuuk	1	This is monomorphic: No test done					
	2	24	0.08333	0.08156	1.00000	0.00000	10100
	3	24	0.20833	0.25443	0.39594	0.00577	10100
Sisimiut	1	25	0.04000	0.04000	1.00000	0.00000	10100
	2	24	0.16667	0.22340	0.30050	0.00505	10100
	3	25	0.56000	0.41143	0.12743	0.00355	10100
Labrador	1	25	0.08000	0.07837	1.00000	0.00000	10100
	2	25	0.36000	0.30122	0.55485	0.00442	10100
	3	25	0.36000	0.45796	0.38050	0.00491	10100
George Bank	1	24	0.08333	0.15603	0.11792	0.00328	10100
	2	23	0.21739	0.26377	0.41257	0.00506	10100
	3	24	0.41667	0.47872	0.66525	0.00484	10100
Newfoundland	1	19	0.10526	0.10242	1.00000	0.00000	10100
	2	19	0.26316	0.23471	1.00000	0.00000	10100
	3	19	0.42105	0.44381	1.00000	0.00000	10100
Iceland	1	76	0.02632	0.02614	1.00000	0.00000	10100
	2	76	0.03947	0.03895	1.00000	0.00000	10100
	3	76	0.15789	0.14639	1.00000	0.00000	10100
Lofoten NEA	1	39	0.15385	0.22644	0.10446	0.00289	10100
	2	39	0.17949	0.24542	0.13782	0.00309	10100
	3	39	0.07692	0.07493	1.00000	0.00000	10100
Østersjøen Inner	1	This locus is monomorphic: no test done.					
	2	This locus is monomorphic: no test done.					
	3	This locus is monomorphic: no test done.					
Østersjøen outer	1	48	0.60417	0.49978	0.15158	0.00310	10100
	2	48	0.60417	0.49452	0.14535	0.00385	10100
	3	This locus is monomorphic: no test done.					
Skagerrak/ Kattegat	1	48	0.41667	0.50439	0.25941	0.00429	10100
	2	47	0.42553	0.47769	0.54554	0.00460	10100
	3	This locus is monomorphic: no test done.					

Here null-hypothesis is that populations are in Hardy-Weinberg equilibrium. This means that we would reject this hypothesis if p-values go below 0.05. For all these populations, there is no any p-value value below 0.05, so we should say that all samples are in Hardy-Weinberg equilibrium at the investigated markers.

Appendix 6. Cod haemoglobin (Hbβ1) SNPs

TTAAGACTACGCCACAATGGTTGAATGGACAGCTGCTGAGCGGAGGCAC
GTCGAGGCGGTCTGGAGCAAGATCGACATTGATGTCTGCGGACCACTCG
CGTTGCAGAGATGCCTGATTGTGTATCCGTGGACGCAGCGCTACTTCGGT
AGCTTTGGCGACCTGAGCACCGACGCCGCTATTA/GTGGGAAACCCCAAG
GTGGCC/TA/GA/CGCACGGCGTCGTGGCCCTGACCGGCCTGAGGACGGCT
CTGGACCACATGGACGAAATCAAGTCCACCTACGCTGCCCTGAGCGTGC
TGCACTCCGAGAAACTGCACGTCGACCCCGACAACTTCCGACTGCTGTGT
GAGTGCCTGACCATTGTCGTCGCCGGGAAGATGGGGAAGAAAT/ATGAG
CCCGGAGATGCAGGCTGCGTGGCAGAAGTACCTGTGCGCGGTGGTTTCC
GCCCTGGGGAGACAGTACCACTAGAAGATGTG

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ttaagactacgccacaatggttgaatggacagctgctgagcggaggcacgtcgaggcggtc
  -  D  Y  A  T  M  V  E  W  T  A  A  E  R  R  H  V  E  A  V
tggagcaagatcgacattgatgtctgcggaccactcgcgttgcagagatgcctgattgtg
 W  S  K  I  D  I  D  V  C  G  P  L  A  L  Q  R  C  L  I  V
tatccgtggacgcagcgctacttcggtagctttggcgacctgagcaccgacgccgctatt
 Y  P  W  T  Q  R  Y  F  G  S  F  G  D  L  S  T  D  A  A  I
a/gtgggaaaccccaaggtggcc/ta/ga/cgcacggcgtcgtggccctgaccggcctgaggacggct
M/V   G  N  P  K  V  A   K/V   H  G  V  V  A  L  T  G  L  R  T  A
ctggaccacatggacgaaatcaagtccacctacgctgccctgagcgtgctgcactccgag
 L  D  H  M  D  E  I  K  S  T  Y  A  A  L  S  V  L  H  S  E
aaactgcacgtcgaccccgacaacttccgactgctgtgtgagtgcctgaccattgtcgtc
 K  L  H  V  D  P  D  N  F  R  L  L  C  E  C  L  T  I  V  V
Gccgggaagatggggaagaaat/atgagcccggagatgcaggctgcgtggcagaagtacctg
 A  G  K  M  G  K  K  L/M  S  P  E  M  Q  A  A  W  Q  K  Y  L
tgcgcggtggtttccgccctggggagacagtaccactagaagatgtg
 C  A  V  V  S  A  L  G  R  Q  Y  H  -  K  M
```