Age distribution and MHC2 alfa variation in arctic charr from Lake Thingvallavatn

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Útdráttur

Bleikja (*Salvelinus alpinus*) er ferskvatns og sjógöngu af ætt laxfiska. Meðal bleikjunnar má finna margskonar form, sérstaklega í stærð og lögun höfuðs. Mestur fjölbreytileiki í útliti finnst í Þingvallavatni, þar sem fjögur afbrigði finnast. Um er að ræða tvö botnlæg afbrigði kuðungableikju (Planktivorous, PL) og dvergbleikju (small benthic, SB), og tvö sviflæg afbrigði, murtu (small pelagic, SP) og sílableikju (Piscivorous, PI). Hér einbeitum við okkur að því að skoða aldursdreifingu murtunar, og sérstaklega að skoða tíðni MHCIIα (Major histocompatibility complex II α chain) samsæta í stofninum. Tilgátan var sú að tíðni samsæta sé háð aldri.

Við notuðum sýni af murtum sem safnað var 2004, 2010 og 2011. Til aldursgreiningar voru kvarnir einangraðar (með krufningum) eða fundnar í sýnabönkum Líffræðistofnunar Háskólans. Einstaklingar sem safnað var árið 2011 voru greindir til útlitsgerða, myndir af þeim voru einnig teknar til frekari greininga. DNA var einangrað úr þessum einstaklingum og hluti af MHCIIα geninu magnaður upp með PCR. Hreinsaðir bútar voru raðgreindir með Sanger aðferð og einstaklingar greindir til arfgerða (A eða B)

Niðurstöðurnar voru misvísandi, merkið sem fannst í fiskunum frá 2010 var ekki til staðar í eldri fiskum. Því er ekki hægt að álykta að aldur hafi áhrif á tíðni B samsætunnar í murtunni í Þingvallavatni.

Abstract

Salvelinus alpinus, also referred as Arctic charr, is a freshwater salmonid that inhabits the circumpolar region. One of the most remarkable features associated with this species is the amazing variability in different aspects concerning their morphology, life history and behavior. Although four different morphs coexist in Lake Thingvallavatn Iceland (two limnetic and two benthic), this research has dealt with two of them: Planktivorous (PL) and small benthic (SB), limnetic and benthic respectively. Nonetheless, it will be mainly focused on PL morph, especially on the characterization of MHCIIα gene in this population. MHCIIα (*Major histocompatibility complex II α chain)* can be used as a marker of divergence between morphs due to the high rate of polymorphism it shows. In this case, we will focus on two alleles, named as allele A and allele B. Different frequencies of these two alleles are being observed within and between both, PL and SB populations.

Samples used in this study come from Lake Thingvallavatn, and they belong to different years (2004, 2010 and 2011). To determine the age of the fish, otoliths were scored. Pictures were taken in order to differentiate between morphs according to phenotypic features described in other studies. DNA isolation, MHCIIα region amplification by PCR and DNA sequences were obtained. I wanted to test if the genotype distribution of allele A and B in PL population

were related to age. Also, I tested for a correlation between the presence of allele B on PL population and the age.

In conclusion, this study shows that allele A frequency differs between morphs (SB and PL). However, a negative (but not significant correlation) may exist between allele B frequency and age, which may suggests selection against this allele on PL populations.

A toda mi familia, en especial a mi hermano cuyo admirable trabajo es un ejemplo a seguir.
A mis amigos por apoyarme y preocuparse tanto. A los "islandeses" que han estado conmigo cada día.

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1 INTRODUCTION

> "In the broadest sense, evolution is merely change, and so is all-pervasive; galaxies, languages, and political systems all evolve. Biological evolution ... is change in the properties of populations of organisms that transcend the lifetime of a single individual. The ontogeny of an individual is not considered evolution; individual organisms do not evolve. The changes in populations that are considered evolutionary are those that are inheritable via the genetic material from one generation to the next. Biological evolution may be slight or substantial; it embraces everything from slight changes in the proportion of different alleles within a population (such as those determining blood types) to the successive alterations that led from the earliest protoorganism to snails, bees, giraffes, and dandelions."
>
> **Douglas J. Futuyma 1986.**

1.1 EVOLUTION

When referring or discussing what evolution is, some problems arise due to the complexity of the concept itself. However, some definitions have been created in order to clarify it. Different theories of evolution keep being also under discussion and different definitions are settled on them, see for instance the definition by Douglas Futuyma above. This study is not a discussion about what evolution means but what it entails.

> Consequently and according with the Futuymas definition, evolution depends on changes, variations. Nevertheless, different variations occur and that is the reason why an infinite number of characteristics that vary are found among life organisms.

Typically, a regular variation can be defined as: Continuous variation, which corresponds to those features that can be measured, like weight. They are quantitative features. The different phenotypes are distributed in a continuous level. By contrast, there is another type which changes in a qualitative or discontinuous way. That is the reason why discrete phenotypes can be distinguished (Falconer and MacKay 1996).

The nature of discrete phenotypic differences.

Some phenotypic (morphological) differences are so subtle that most of them are overlooked. However, other differences are so dramatic between individuals that they were misidentified as distinct species. Genotypic and phenotypic variation can be correlated, (Pierce 2010), and it is our interest to find and describe such correlations.

1.2 RESOURCE POLYMORPHISMS LEADING TO SPECIATION.

Where do species come from and in which direction they are evolving to, are among the most major questions ever studied? **Speciation** *is defined as the process of multiplication of distinctly different species through reproductive isolation* (Jonsson and Jonsson 2000). The

question is: What are the sources of variation? Ecological factors can lead to speciation (Mayr 1947) as well as genetics ones.

A **polymorphism** can be defined as *the simultaneous occurrence of more than one discontinuous genetically controlled phenotypes in a population* (Jonsson and Jonsson 2000). Thus, Resource polymorphisms are, indeed, a significant force that can lead to speciation (Smith and Skulason 1995).

Several examples of species evolving because of the existence of polymorphisms have been described along history. Darwin´s finches (Grant and Grant 2006) or fishes cichlid (Smith and Skúlason 1996) are great examples. Thus, the existence of different alleles in the genome has made possible the fact that phenotypic variations have been expressed in order to survive.

Arctic charr *(Salvelinus alpinus)*

Arctic charr (*Salvelinus alpinus*) is a northern freshwater fish belonging to the Salmonidae family. Arctic charr inhabits lakes and rivers of the circumpolar region (Noakes 2008). This region has been colonized by Arctic charr after the last glaciation,10.000-15.000/20.000 years ago (Conejeros et al. 2008; Smith and Skúlason 1996; Brunner et al. 1998). The importance of these lakes is based on the fact that after this last glaciation, they have comparable rates of population differentiation (Brunner et al. 1998). This species occupies an important place of interest regarding the study of population differentiation in the northern fishes (Brunner et al. 1998).

Arctic charr has up to four sympatric morphs, found in lake Thingvallavatn, Iceland (Skúlason et al. 1989). The Planktivorous (PL) and Piscivorous (PI) morphs are limnetic and the Small benthic (SB) and large benthic (LB) dwell on the bottom (Figure 1.1)

Thingvallavatn (64°11′N 21°09′O), is Iceland's largest lake. It is located in the neovolcanic zone which causes constant changes on its shape and size (Adalsteinsson 1992). The area covered by the lake is 83 km^2 and it is from 34 to 114 km deep (Adalsteinsson 1992).

Several studies support the hypothesis that all the morphs of Arctic charr come from a single linage that colonized the lakes of circumpolar region (Brunner et al. 1998; Wilson et al. 2004) at the same time.

However it has been demonstrated using neutral microsatellite markers, that SB, PL and LB are genetically differentiated (Kapralova 2008).

The four morphs in Thingvallavatn, iceland.

They differ in characteristics such as body size and spawning coloration, external and internal morphological structures, parasite fauna growth rate, feeding habitat and diet, life history traits and behavior (Sandlund et al. 1987; Malmquist et al. 1992; Skúlason et al. 1989). All of these features help explain why these morphs are so different, although they all are considered the same species (Skúlason et al. 1989). For example, feeding adaptation is an important evolutionary mechanism that may generate this diversification (Komiya et al. 2011). In this case, it is has exhibited an advanced state of trophic diversification (Jonsson and Jonsson 2000). However, these characters are not remarkable enough to classify the morphs as different species. For this reason, it is said that Arctic charr presents a great degree of polymorphism within the populations (Sandlund et al. 1987), which, of course, inhabits

different niches. Thus, it seems like incipient species are arising due to the utilization of different resources which makes possible adaptation (Klemetsen et al. 2006). A small but significant genetic variation on the population level may contribute to this phenotypic differentiation.

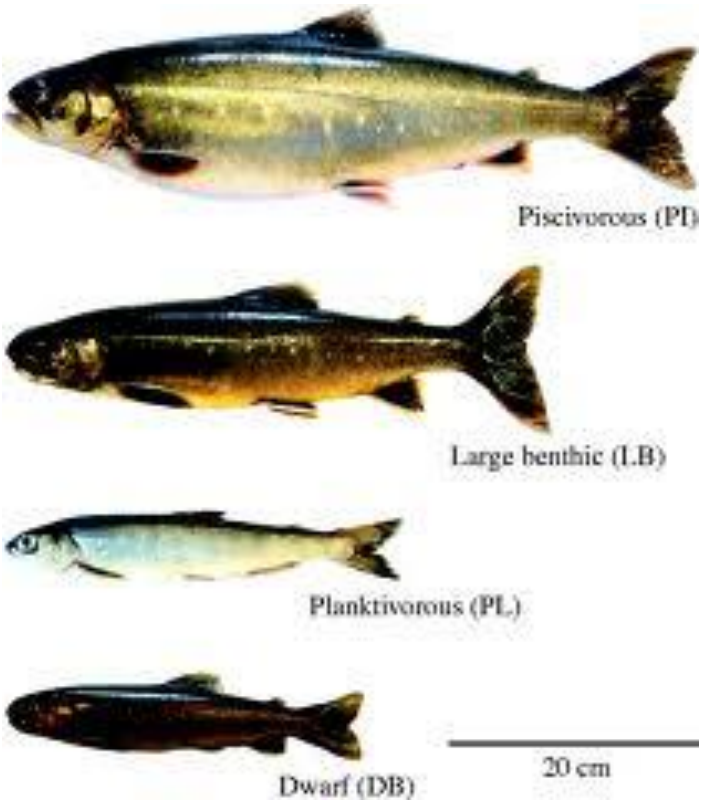


Figure 1.1 *Four morphs of Arctic charr from Lake Thingvallavatn (Jonhston et al 2004)*

Figure 1.2 *Four morphs of Arctic charr from Lake Thingvallavatn. Picture taken by V.C. Baltanás*

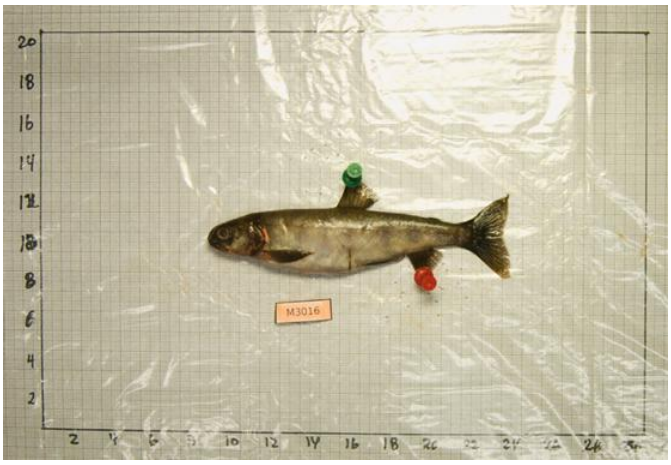


Figure 1.3 *SB from Lake Thinvallavatn, morph recognition photography set up. X and Y scales indicate centimeters. Picture taken by V.C. Baltanás*

1.3 PARASITE-MEDIATED SELECTION PARASITES IN ARCTIC CHARR.

Parasites are one of the strongest forces that can lead to evolutionary change. Considering that it is quite likely that all organisms have parasites (Maynard Smith 1976), genetically defense mechanisms must be coded in order to protect the organism. As parasites evolve, so these mechanisms do to provide hosts a defense against them (Eizaguirre and Lenz 2010). Populations of parasites vary depending on the niche organisms inhabit: It is a biotic factor that plays a major role in natural selection. Therefore, a parasite-mediated selection exists and must be considered as a significant factor in evolution (Eizaguirre and Lenz 2010; Conejeros 2008).

Different morphs of *Arctic charr* inhabit different ecological niches, so they are exposed to different species of parasites. As a result of this selection pressure, host must adapt (Eizaguirre and Lenz 2010).

The co-evolutionary cycle keeps going ahead, and parasites have to evolve in order to develop mechanisms to get through these defenses. Thus, there is a correlation in the direction in which both, hosts and parasites, evolve. In addition, not only parasites are different among species, but different parasites are located in different parts of the same organism; hence, they affect different tissues and the different functions of the organism (Eizaguirre and Lenz 2010).

Focusing on Arctic charr, different populations of parasites have been studied in order to determine how they affect the different morphs and also different tissues (Robertsen et al. 2010).

Some examples of parasites species in *Arctic charr* in Thingvallavatn, Iceland:

-*Diphyllobothrium sp.*

-*Dyplostomum sp*

-Nematodes.

Comparing data analysis in 3 of the 4 morphs *(Planktivorous, Small benthic and Large Benthic)* confirms differences depending on the morph and other features as sex or weight (Frandsen et al. 1989).

1.4 THE GENETIC BASIS FOR THE MORPHOLOGICAL AND ECOLOGICAL VARIATION WITHIN ARCTIC CHARR AND THE *MHC* POLYMORPHISM.

The adaptive immune system has different types of cells that are involved in antigen recognition. They have different pattern of procedure as well as several feature differences: T-cells and B-cells and antibodies (Murphy 2008).

Antigen recognition on T-cells differs clearly from B-cells and antibodies. While B-cells and antibodies bind to the surface of protein antigens intact, T-cells need to make contact with a complex formed by protein antigen processed and a molecule of MHC. Thus, Major histocompatibility complex (MHC) refers to a region of DNA that contains over 200 genes and it encodes for the membrane molecules responsible of presenting to the T-cells the specific antigen so that immune response can be initiated. (Murphy 2008).

There are three classes of MHC molecules: MHC class I, class II and class III. They have different subunit structure and expression as different pattern on the tissues (Murphy 2008). Although they are related to each other, proteins expressed belong to different families. The third class of MHC molecules is non-related with MHC I and II. (Wood 2011).

In fishes, in contrast to tetrapods, the two major histocompatibility complexes are non-linked to each other (Sato et al. 2000; Eizaguirre and Lenz 2010).

MHC II glycoproteins consist in two chains, non-covalently linked, named as α and β. Subsequently, both of them contain two domains (α1, α2 and β1, β2). Five different exons encode the five different domains for each chain. The exon two, encoding α1 and β1 domains, is the exception. The exon 3 for the MHC I and the exon 2 for the MHC II show the highest polymorphism because they encode the part that will form the binding groove afterwards (Eizaguirre and Lenz, 2010).

Another difference between MHC I and II is that MHC II genes are less expressed than class I. They have transmembrane regions and cytoplasmic tails. They also are able to deliver intracellular signals (Murphy 2008; Wood 2011). It also seems that rare MHC II B alleles are associated with a high rate of surviving (Eizaguirre and Lenz 2010).

A unique feature of these genes is the extremely high degree of polymorphism they show (Schenning et al. 1984). Not only do they express numerous alleles but they are also capable

of maintaining a great rate of variability. Therefore, a single population expresses the highest number of different proteins from the same gene ever described in jaw vertebrates and fishes. (Conejeros et al. 2008; Wood 2011; Skulason and Smith 1995).

Due to this incredible rate of polymorphism, MHC has been used as a marker to study divergence among species and also among and within populations (Conejeros et al. 2008).

Because of the high plasticity and variability among populations, it is complicated to characterize and define constituent populations of *Arctic charr* (Conejeros et al. 2008). MHC will be used in this study as a marker of divergence, focusing on the α chain. Although highest variability occurs on β-sequence, (Holland et al. 2008) some authors have settled on MHC II-α variability.

Unifying some premises like different niches implies several parasite populations and different morphs of Arctic charr inhabit those niches, it can be inferred that MCH molecules could be different depending on the parasite load for a determinate morph.

1.5 OTOLITHS, BONES THAT TELL STORIES.

Otoliths (Figure 1.4) are bones located into the vestibular labyrinth (inner ear) of all vertebrates. They are sensitive to gravity and linear acceleration and have a secondary function in sound detection in higher aquatic and terrestrial vertebrates (Das, 1994)

Shape and size are quite variable depending on the species and it seems to be related also with the size of the fish; bigger and older the fish is, bigger the otoliths are. They are composed of calcium carbonate ($CaCO_3$), and, in fishes, they also contain traces from the water the fish inhabits. Therefore this structure is also used as pollution indicator (Shawney and Johal 1999). As the fish grows, also do the ototliths and more $CaCO_3$ is added to the previous structure.

Otoliths are a valuable piece of information to estimate the age (Khan and Khan 2009; Hubert et al. 1984). This structure presents several rings according to the age of the fish. Bright and dark rings can be distinguished. Each one corresponds to a season of growing. Thus, scoring the number of dark rings (number of winters that the fish has survived) is possible to estimate the approximate age. They also offer information about the pattern of growing. A thick ring, composed by a main ring and narrower ones, usually indicates that the fish has grown quite fast that year. (Sigurdur S.Snorrason, personal communication)

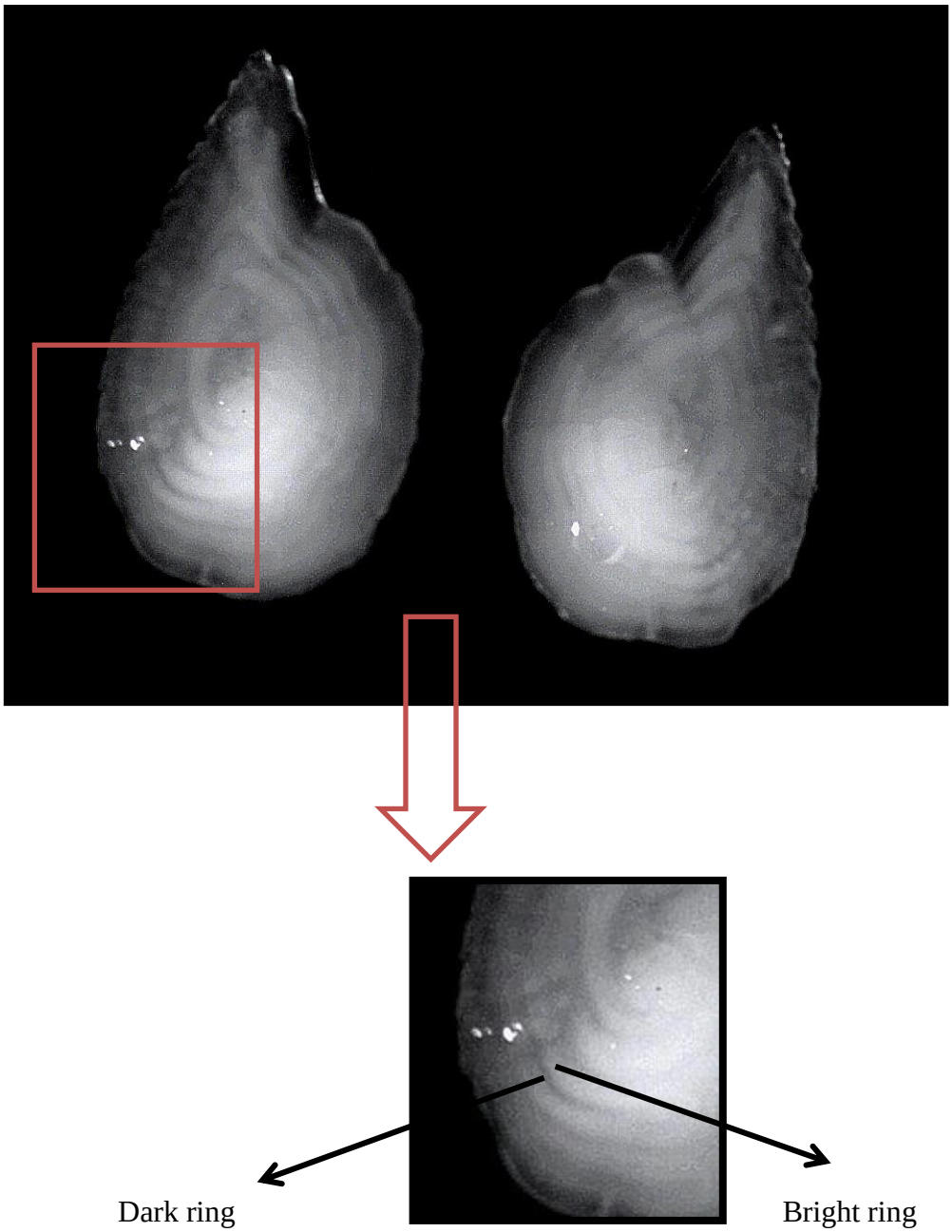


Figure 1.4 *Otolithes from PL. Magnification below. Picture taken by V.C. Baltanás*

1.6 AIMS

The main aim of this study is to elucidate some of the mechanisms relating to the differences between and among morphs in Arctic charr. This is done by using genetic markers such as MHCIIα, which presents an incredible rate of polymorphism (Penn et al, 2002).

More specifically this study can be divided into the following subsections:

1. To determine the age of the fish by scoring otoliths.
2. To study the weight and length of the fish, as well as other morphological features such as coloration, head morphology or fin size by using morph recognition photographs.
3. To genotype the PL fish for part of the MHCII alpha locus, define the different genotypes present in this morph and estimate the allele frequencies.
4. To test for correlation of the frequency of allele B in the PL population with age of the fish.

2 MATERIALS AND METHODS

2.1 SAMPLING

2.1.1 PL AND SB FROM THINGVALLAVATN, MJÓANES, 2011

Fishes were caught with gill nets in lake Thingvallavatn, Iceland.

A total number of 168 fishes were processed from which 82 were PL (41 males and 41 females) and 86 were SB (52 males and 34 females). The first 151 were caught in 4thof October, 2011 including both, PL and SB. Only 17 SB were sampled on the 16th of November, 2011.

After being kept in separate bags, which were labeled with the correspondent morph, sex and date, fishes were conserved in the freezer at -20 ºC

2.1.2 PL FROM THINGVALLAVATN, REYDARVIKURTANGI, 2004-2005

Gills from PL, caught in Reydarvirkurtangi, lake Thingvallavatn, Iceland, were conserved in 96% Ethanol. A total number of 49 gills belonging to 49 different individuals were selected for this study.

2.2 PROCESSING

2.2.1 TAKING PICTURES AND SELECTION OF THE SAMPLES

Samples from 2011 were processed in four different times. I took pictures of the 168 fish, using a Nikon D3000 positioned on a tripod. 16 of them (8 *PL* and 8 *SB*, 4 males and 4 females from each one) were specifically treated for morphological studies; they were positioned facing left and pinned down. An extra photo was taken from the ventral side to facilitate the morph identification. These samples were labeled as M3001-30016 (Appendix A). I processed all the pictures with the program Lightroom 3 for a better view of the samples.

All samples were weighted, their length was measured and I checked whether the females were mated or not.

For the rest of the 168 samples I only took identification photos and in some occasions fins were cut by Jetty Ramadevi (JR) before taking the picture.

2.2.2 DNA ISOLATION FROM Arctic charr

DNA was isolated from fins of the first 90 samples, (M3001-M3090 Appendix D) by JR.

I isolated DNA from the 49 PL samples. In some cases extraction needed to be repeated because the quantity of DNA was insufficient for our purposes.

2.2.3 REMOVING OTOLITHS FROM PL AND SB AND SCORING OTOLITHS

I removed otoliths from 168 samples (M3001-M3168 Appendix D), 82 PL and 86 SB caught in 2011. All of them were extracted by opening fish´s head cavity. Then, otoliths were cleaned up with paper and 70% ethanol, dried and conserved in individually labeled eppendorfs. In some cases brain was also removed for a better view of the inner ear.

A total of 457 PL otoliths were read. I used a reading procedure described by Kimura and Anderl (2005).

244 (Thingvallavatn, Reydarvikurtangi, 2004-2005) (Appendix A)

131 (Thingvallavatn, Mjóanes,2010) (Appendix B)

82 (Thingvallavatn, Mjóanes, 2011) (Appendix C).

2.3 MOLECULAR WORK

2.3.1 DNA ISOLATION

A/ DNA isolation of fins from PL samples, 2011

DNA from 82 PL samples was isolated by JR using standard phenol-chloroform protocol. Afterwards, stock solutions were kept in the freezer at -20 ºC and DNA was diluted from the initial concentration to 50ng/µl were prepared in order to be used for PCR.

B/ DNA isolation from Gills from PL samples, 2004-2005

DNA was isolated from 49 PL samples using a protocol with salt (NaCl). (Lopera et al. 2007)

Samples were placed in Eppendorf microtubes with 550 µL of lysis buffer (50 mM Tris-HCl, pH 8.0, 50 mM EDTA, 100 mM NaCl), containing 1 % SDS and 7 µL of 200 µg/mL of proteinase K. Tubes were incubated immediately in a heat block at 50ºC for at least 12 hours. Then, 600 µL 5M NaCl were added to each sample before being centrifuged for 10 minutes at 12000 rpm. The supernatant was transferred to new microtubes where the DNA was precipitated with 1000 µL of 96% cold ethanol and incubated later at -20ºC for 2 H. The DNA samples were centrifuged again for 10 min at 12000 rpm. Then, 800 µL of 70 % ethanol was added and samples were centrifuged again. After removing all the ethanol from the samples, the Eppendorfs were left opened until all traces of ethanol had evaporated. Samples were dissolved in 80 µL of ddH_2O and treated with 30 µg/mL of RNAse. The DNA obtained was kept at -20 ºC.

Firstly, the efficiency of this protocol was tested with several samples (fresh tissue conserved in ethanol, fresh frozen tissue and old tissue conserved in 96 % ethanol). Secondly, three different sets of samples from PL gills where separated in order to be extracted.

-Modification of the standard protocol.

Due to the insufficient quantity of DNA of the first set of extractions, this protocol was modified at the following step:

The quantity of proteinase-k was increased on the digestion to improve this process.

Initial proteinase-k added: 7 µL of 200 µg/mL.

First modification: 1 µL of pure proteinase K (20 mg/mL)

Second modification: 2 µL of pure proteinase K (20 mg/mL)

Afterwards, Nano-drop was used to check the quantity of the DNA of every sample. In some cases, DNA quality was visualized on a 1% Agarose gel (1% Agarose + Ethidium bromide).

Because DNA concentration was very low, in some cases different PCR protocols were used.

2.3.2 DNA AMPLIFICATION: PCR

Table 2.1 *PCR procedure*

DNA	Buffer 10x	TAQ	dNTPs	F-primer	R-primer	ddH_2O	TOTAL V.
1 µL	2 µL	0,2 µL	2 µL	0,4 µL	0,4 µL	14 µL	20 µL
2 µL	2 µL	0,2 µL	2 µL	0,4 µL	0,4 µL	13 µL	20 µL
4 µL	2 µL	0,2 µL	2 µL	0,4 µL	0,4 µL	11 µL	20 µL
6 µL	2 µL	0,2 µL	2 µL	0,4 µL	0,4 µL	9 µL	20 µL
15 µL	2 µL	0,4 µL	2 µL	0,4 µL	0,4 µL	0 µL	20 µL

PCR PROGRAM

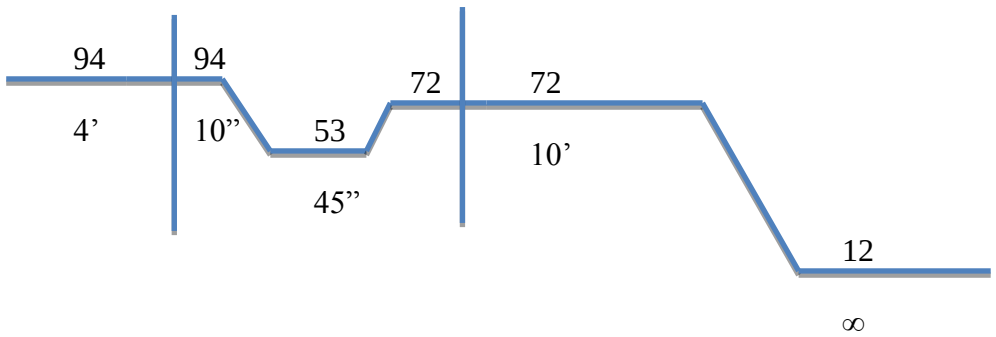


Figure 2.1 *Representation of the sequence program*

The PCR program starts with a denaturation temperature of 94 ºC for 4 minutes followed by the temperatures and times described on the image above; Melting temperature of 53º and extension temperature of 72ºC. The cycle is repeating 35 times and remains at 12º at the end.

PCR product was visualized on a 1,5 % Agarose gel (Agarose + Ethidium bromide). Loading dye was added to each sample and 100 PB ladder was used.

PCR product was stored in the freezer at -20 ºC.

2.3.3 SEQUENCING

A total of 131 samples were sequenced.

Exo-SAP

Firstly, PCR products were purified by Exo-SAP.

Table 2.2 *Exo-SAP procedure*

PCR PRODUCT	ddH_2O	(Exo I) Phospatase buffer	Antarctic phosphatase 0,2x5U/ µl˜ 1U	Exo I 0,1x20U/ µl˜ 1U	Total volume
5 µl	3,7 µl	1 µl	0,2 µl	0,1 µl	10 µl

Exo-SAP program: 35 minutes at 38ºC followed by 20 minutes at 80ºC

SEQUENCING

Table 2.3 *Sequencing protocol*

DdH_2O	VII 5x Buffer	TRR BigDye	R- primer 1pm/ul	Exo-SAP product	Total volume
5,25 µl	2,76 µl	0,49 µl	1,5 µl	5 µl	15 µl

Table 2.4 *Sequencing program characteristics*

Temperature (ºC)	96 ºC	96 ºC	50 ºC	60 ºC	96 ºC
Time (h:m:s)	00:00:10	00:00:10	00:00:05	00:02:00	00:00:10

Cycle is repeated 25 times.

PURIFICATION

I used the following procedure for purifying the sequenced products.

45 µL of solution A was added to the every sample. Then, 125 µL of 96 % ice cold ethanol were also added and everything was mixed well. The mixture was centrifuged at 4000 RPM for 30 minutes. The supernatant was discarded immediately and the plat was put upside-down onto of 3 layers of Kimwipes. After that, the samples were centrifuged at 300 RPM for 2 minutes. I added 250 µL of 70 % cold ethanol and centrifuged again at 4000 RPM for 5 minutes. Again, the plate is placed upside-down onto of 3 layers of Kimwipes and centrifuged at 300 RPM for 5 minutes. Samples were dried in a dark place for 15-20 minutes minimum. Finally, samples were dissolved in 9,9 HiDi. These samples were sequenced on an AB 3500 xL Applied Biosystems Genetic Analyzer.

2.4 DATA PROCESSING

2.4.1 FISH PROCESSED

Data from 168 PL individuals processed from 2011 was compiled in a excel sheet (Appendix D) where weight, length and mating status (whether the sample is mated or not) and age was indicated.

2.4.2 OTOLITHS PROCESSED

Data from 244 individuals from 2004-2005 was compiled in a excel sheet (Appendix A) where ID and age was indicated. Also data of samples from 2010 (Appendix B) with 131 individuals and samples from 2011 (Appendix C) with 82 individuals was compiled in excel sheets.

2.4.3 DNA SEQUENCES AND STATISTICAL ANYLISIS

Sequences from different 2 sets of samples (2004 and 2011) have been aligned and the frequency of the different alleles belonging to the MHC IIα gen polymorphic has been determinate.

Statistical analysis for samples from 2004, 2010 and 2011 has been obtained using a t tests and linear regression (t-test and ln in R www.r-project.org) Graphs of results were also produced with R.

3 RESULTS

3.1 DNA ISOLATION, PCR AMPLIFICATION AND SEQUENCING.

We first tested the effectiveness of the standard protocol on fins that were conserved under different circumstances:

Fresh fins from samples of 2011:

4 samples conserved in ethanol (1-4.)

4 samples frozen (1F-4F).

Old fins conserved in ethanol (O2001-O2004).

Tale 3.1 *Nanodrop results from the extraction of samples from fresh and old fins tissue treated with standard protocol with salt (NaCl)*

Sample ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
1	12.10.2011	10:51	204,56	4,091	1,991	2,05	2,09	50,00	230	1,961	-0,033
2	12.10.2011	10:52	148,81	2,976	1,443	2,06	2,27	50,00	230	1,313	-0,032
3	12.10.2011	10:53	86,42	1,728	0,842	2,05	1,89	50,00	230	0,912	-0,039
4	12.10.2011	10:54	109,66	2,193	1,029	2,13	1,59	50,00	230	1,379	-0,033
1-F	12.10.2011	10:55	359,42	7,188	3,580	2,01	2,05	50,00	230	3,511	0,027
2-F	12.10.2011	10:56	142,45	2,849	1,393	2,05	2,21	50,00	230	1,292	-0,034
3-F	12.10.2011	10:57	171,37	3,427	1,656	2,07	2,14	50,00	230	1,599	-0,029
4-F	12.10.2011	10:58	207,67	4,153	2,025	2,05	2,04	50,00	230	2,036	0,048
O2001	12.10.2011	10:59	14,31	0,286	0,146	1,97	1,63	50,00	230	0,176	-0,021
O2002	12.10.2011	11:00	5,84	0,117	0,045	2,62	1,05	50,00	230	0,111	-0,044
O2003	12.10.2011	11:01	17,33	0,347	0,177	1,95	2,18	50,00	230	0,159	-0,053
O2004	12.10.2011	11:01	5,30	0,106	0,044	2,40	3,22	50,00	230	0,033	-0,071

Table 3.1 shows that the standard protocol works successfully for new fresh fin tissue but not for old fin tissue (O2001-O2004).
Also fresh tissue gives better results using standard protocol than old tissue which requires a higher amount of proteinase-k on the digestion. Further studies will be necessary to test the effectiveness of the modified protocol with salt on fins.

Results for DNA isolation using Protocol extraction with salt (NaCl) (Lopera et al 2007) were satisfying once the original protocol were modified increasing the original amount of proteinase-k we used on the digestion (First step on the extraction). We changed from 7 µL of 200 µg/mL to µL to 2 µL of pure proteinase k (20 mg/ml). Quantity and quality of genomic DNA are comparable to the DNA obtained with phenol-chloroform protocol, previously tested in other studies in some tissues like fish fins and larvae samples (Lopera et al 2007).

Table 3.2 *Nanodrop results from the extraction of samples from 2004 (gills) treated with standard protocol with salt (NaCl)*

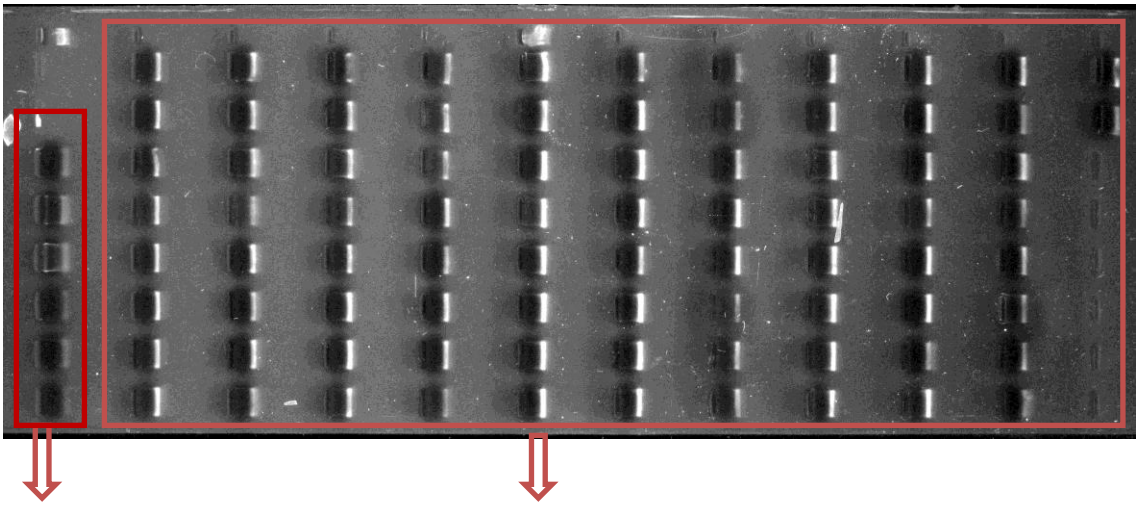
Sample ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
Re-32	7.11.2011	17:41	69,72	1,394	0,683	2,04	2,03	50,00	230	0,687	0,043
Re-36	7.11.2011	17:42	5,42	0,108	0,074	1,47	1,58	50,00	230	0,069	0,008
Re-37	7.11.2011	17:43	2,88	0,058	0,062	0,93	1,33	50,00	230	0,043	0,012
Re-42	7.11.2011	17:43	8,94	0,179	0,096	1,87	1,80	50,00	230	0,099	0,040
Re-43	7.11.2011	17:45	10,42	0,208	0,116	1,79	1,40	50,00	230	0,149	0,009
Re-44	7.11.2011	17:45	10,60	0,212	0,097	2,18	2,26	50,00	230	0,094	-0,017
Re-45	7.11.2011	17:46	9,18	0,184	0,072	2,56	1,99	50,00	230	0,092	-0,005
Re-51	7.11.2011	17:47	21,67	0,433	0,247	1,76	2,32	50,00	230	0,187	-0,005
Re-52	7.11.2011	17:47	34,54	0,691	0,377	1,83	1,68	50,00	230	0,411	0,031
Re-53	7.11.2011	17:48	20,57	0,411	0,221	1,86	2,12	50,00	230	0,194	0,004
Re-57	7.11.2011	17:49	27,10	0,542	0,252	2,15	1,93	50,00	230	0,281	0,003
Re-61	7.11.2011	17:50	8,71	0,174	0,123	1,42	1,91	50,00	230	0,091	0,000
Re-65	7.11.2011	17:50	3,53	0,071	0,023	3,01	3,16	50,00	230	0,022	0,003
Re-67	7.11.2011	17:51	11,08	0,222	0,123	1,80	2,31	50,00	230	0,096	0,012
Re-68	7.11.2011	17:52	11,19	0,224	0,130	1,72	1,32	50,00	230	0,169	0,003
Re-73	7.11.2011	17:52	34,69	0,694	0,415	1,67	2,14	50,00	230	0,324	0,165
Re-74	7.11.2011	17:53	77,33	1,547	0,767	2,02	1,64	50,00	230	0,942	0,006
Re-75	7.11.2011	17:54	10,87	0,217	0,124	1,75	1,91	50,00	230	0,114	0,010
Re-77	7.11.2011	17:54	10,40	0,208	0,113	1,84	2,33	50,00	230	0,089	-0,005
Re-81	7.11.2011	17:55	10,36	0,207	0,121	1,72	2,05	50,00	230	0,101	0,013

On table 3.2 amount of DNA is not as good as we expected at first, so I increased the amount of proteinase-k on the digestion.

Table 3.3 *Nanodrop results from the extraction of samples from 2011 (gills) treated with modified protocol with salt (NaCl)*

Sample ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
Re-36	24.11.2011	16:36	58,08	1,162	0,574	2,02	1,97	50,00	230	0,591	0,025
Re-41	24.11.2011	16:36	433,34	8,667	4,382	1,98	2,05	50,00	230	4,235	0,279
Re-44	24.11.2011	16:37	40,33	0,807	0,403	2,00	1,47	50,00	230	0,549	0,036
Re-47	24.11.2011	16:38	332,81	6,656	3,257	2,04	2,22	50,00	230	2,999	0,153
Re-48	24.11.2011	16:39	48,09	0,962	0,464	2,07	1,95	50,00	230	0,493	0,046
Re-50	24.11.2011	16:40	86,16	1,723	0,831	2,07	2,29	50,00	230	0,754	-0,24
Re-54	24.11.2011	16:40	810,64	16,213	8,227	1,97	2,19	50,00	230	7,410	0,156
Re-55	24.11.2011	16:41	267,85	5,357	2,593	2,07	2,20	50,00	230	2,440	0,099
Re-56	24.11.2011	16:41	1115,07	22,301	11,551	1,93	2,08	50,00	230	10,711	0,562
Re-58	24.11.2011	16:42	1669,25	33,385	17,475	1,91	2,23	50,00	230	14,973	0,199
Re-62	24.11.2011	16:43	1726,81	34,536	17,510	1,97	2,21	50,00	230	15,618	0,353
Re-64	24.11.2011	16:45	197,29	3,946	1,956	2,02	2,27	50,00	230	1,740	0,116
Re-65	24.11.2011	16:45	2914,43	58,289	30,329	1,92	2,03	50,00	230	28,745	0,914
Re-71	24.11.2011	16:46	338,40	6,768	3,474	1,95	1,73	50,00	230	3,908	0,932
Re-76	24.11.2011	16:46	1584,06	31,681	16,436	1,93	2,20	50,00	230	14,413	0,467
Re-79	24.11.2011	16:47	828,54	16,571	8,671	1,91	2,02	50,00	230	8,184	0,427
Re-81	24.11.2011	16:47	125,96	2,519	1,409	1,79	1,39	50,00	230	1,814	0,316
Re-82	24.11.2011	16:48	1599,21	31,984	15,721	2,03	2,07	50,00	230	15,441	0,853

PCR amplification worked successfully for all the samples from 2004 and 2011. A total of 131 samples (49 from 2004 and 82 from 2011) were amplified.



Gills; PCR-product (2004) Fins; PCR-product (2011)

Figure 3.1 *PCR product from gills and fins.*

3.2 MHC POLYMORPHISMS

The following results show sequences of individuals from 2004 and 2011. Frequency of SB and PL alleles on the populations is indicated.

A total number of 131 samples were processed. Two different set of samples are differentiated.

3.2.1 PL FROM 2004, REYDARVIKURTANGI, THINGVALLAVATN (ICELAND)

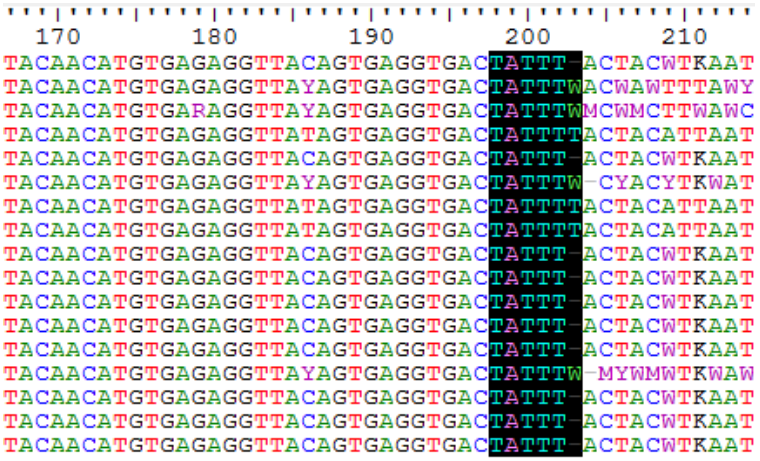


Figure 3.2 *Aligned sequences. Darkened region indicates the polymorphism of interest (2004).*

A total number of 49 samples were extracted, PCR-ed and sequenced. All samples were successively extracted and amplified. However only 34,7% were of them were successively sequenced.

Table 3.4 *Genotype frequency 2004*

Genotype	Homozygous for Allele A (3T/3T)	Homozygous for Allele B (4T/4T)	Heterozygous (3T/4T)
Number of samples	10	3	4
Genotype frequency	58,8 %	17,6 %	23,5 %

Table 3.5 *Allele frequency 2004*

Alleles	Allele A (3T)	Allele B (4T)
Allele frequency	68.00%	32.00%

Results show that the number of homozygous for allele A is more representative within PL samples population than the other genotypes (Table 3.4). Considering just allele frequency, allele A frequency is higher than allele B frequency (Table 3.5).

3.2.2 PL FROM 2011, MJÓANES, THINGVALLAVATN (ICELAND)

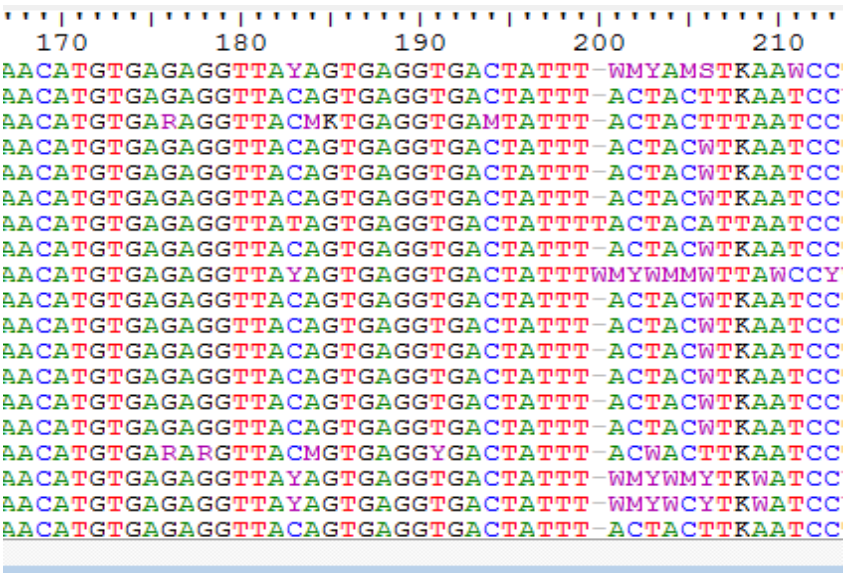


Figure 3.3 *Aligned sequences show the region with the polymorphism of interest (2011)*

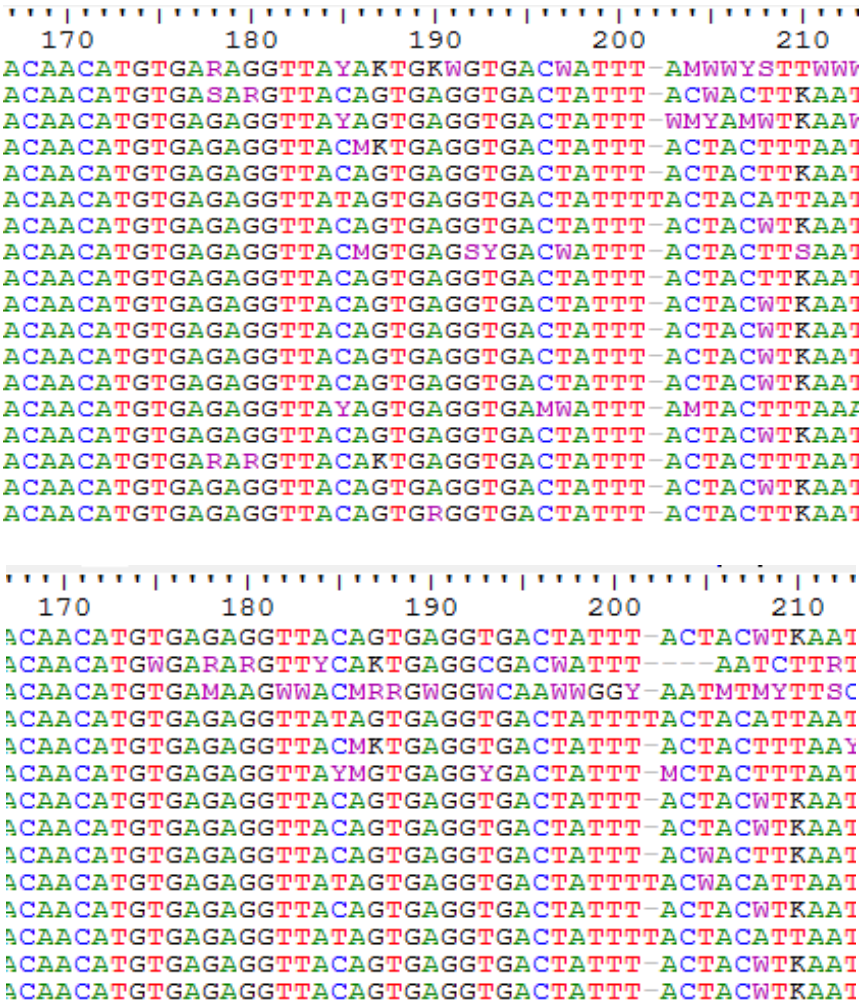


Figure 3.3 *Aligned sequences show the region with the polymorphism of interest (2011)*

A total number of 82 samples were processed. 61% of the sequences were successfully obtained.

Table 3.6 *Genotype frequency 2011.*

Genotype	Homozygous for Allele A (3T/3T)	Homozygous for Allele B (4T/4T)	Heterozygous (3T/4T)
Number of samples	44	5	1
Genotype frequency	88 %	10 %	2 %

Table 3.7 *Allele frequency 2011.*

Alleles	Allele A (3T)	Allele B (4T)
Allele frequency	93.00%	7.00%

Results indicate that homozygous for allele A frequency is quite more representative on PL (Table 3.6). Also allele A frequency is very high comparing to allele B frequency (Table 3.7).

3.3 GENOTYPE DISTRIBUTION REGARDING AGE IN PL POPULATION. ALLELE FREQUENCY AND AGE CORRELATION IN PL POPULATION.

Two graphs shows frequency of different alleles on the population from 2004 and 2010.

3.3.1 MHC FROM PL 2004

Figure 3.4 shows the distribution of the 3 different genotypes related to the age (from 4 to 10 year old). This graph shows that the majority of the samples are aged 5-9 and that the 3T genotype is predominant (X= Number of samples; Y= Age)

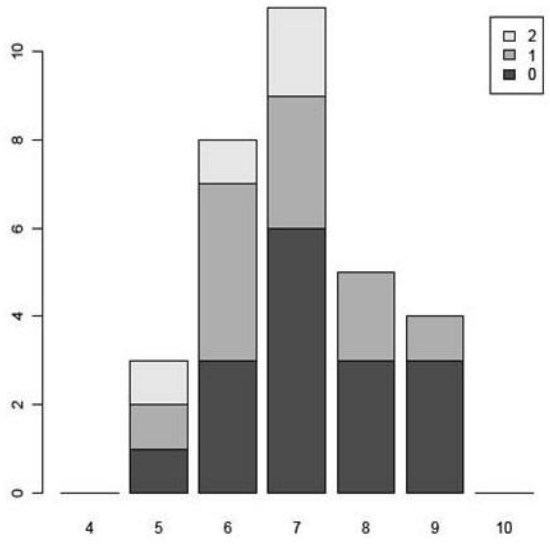


Figure 3.4 *Genotype distribution in PL 2004. 3T/3T = 0, 3T/4T= 1 and 4T/4T= 2. 3T = Allele A; 4T= Allele B*

Figure 3.5 shows the regression of the B allele frequency in the PL sample (2004). For ages 4, 5 and 10 the sample size was very low. At first for age 6 and 7 we observe the frequency of the B allele increasing with age, but after age 8, its frequency decreases. Error bars indicate Wilson confidence intervals.

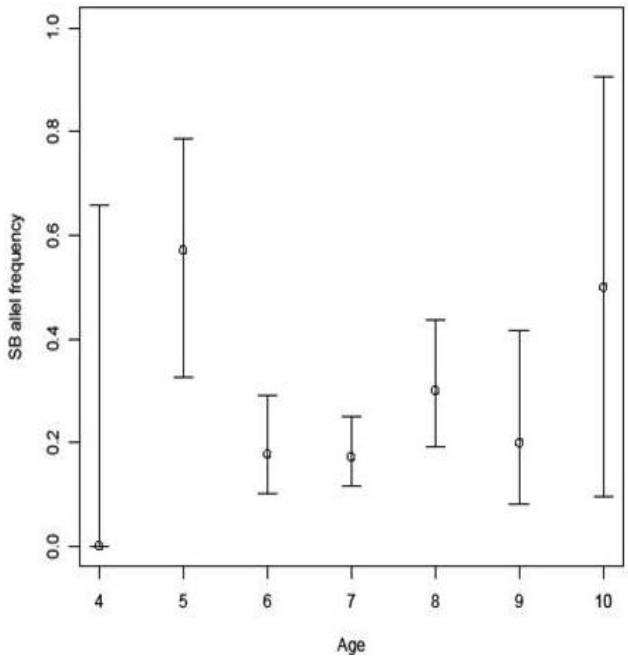


Figure 3.5 *B allele frequency in PL from year 2004*

3.3.2 MHC FROM PL 2010

Figure 3.6 shows the distribution of the 3 different genotypes related to the age (from 4 to 10). This graph shows that PL fish were aged 4-10 and that the 3T genotype is predominant (X= Number of samples; Y= Age).

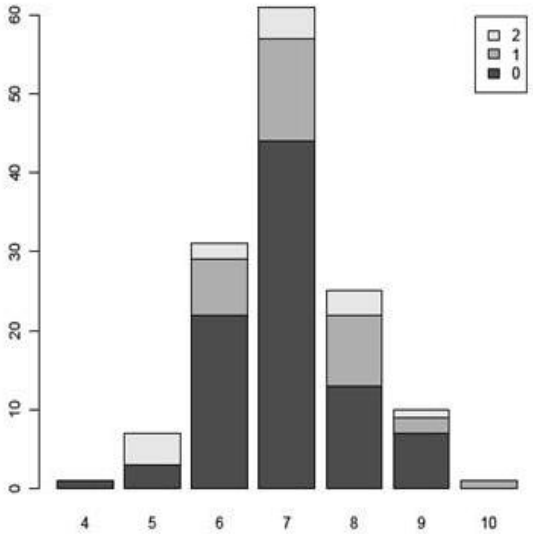


Figure 3.6 *Genotype distribution in PL 2010. 3T/3T = 0, 3T/4T= 1 and 4T/4T= 2. 3T = Allele A; 4T= Allele B*

Figure 3.7 shows the regression of the B allele frequency in the PL sample (2010). For ages 4 and 9 the sample size was very low. Error bars indicate 95% confidence intervals on allele frequencies.

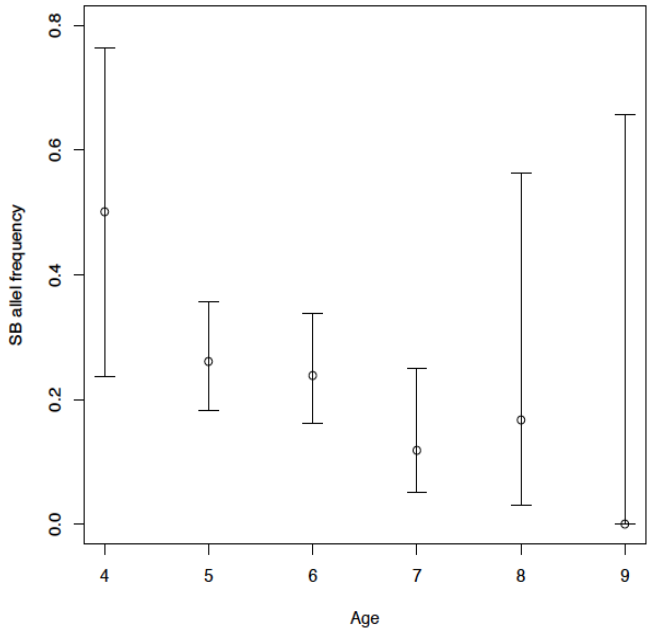


Figure 3.7 *B allele frequency in PL 2010*

To test whether there is a relation between age and allele frequency we conducted linear regression. Tables 3.8-3.11 show the linear regression results for samples from 2010. There is an indication of a negative correlation but results are not significant. The two sets of analyses are done on two summaries of age. AGE= Individuals that have been possible determine their age with an entire number. AGE (+)= Individuals that have not been possible determine their age with an entire number because it was not clear what year they belong to. Those with a plus, where awarded half a year extra (6+ became 6.5 in the data table).

Table 3.8. *Regression coefficients and test statistics for test of AGE(+) on allele frequency.*

	Estimate Std	Error	t-value	Pr (>|t|)
(Intercept)	1,8693	0,7672	2,436	0,0212*
Age (+)	-0,1803	0,1086	-1,660	0,1077

Significant codes; *= 0,01

Table 3.9. *R-squared and F-tests on regression of AGE(+) on allele frequency*

Multiple R-squared	Adjusted-R squared	F-Statistic	P-Value
0.08675	0.05526	2.755 on 1 and 29 DF	0.1077

Table 3.10. *Regression coefficients and test statistics for test of AGE on allele frequency.*

	Estimate Std	Error	t-value	Pr (>|t|)
(Intercept)	2,1376	0,8443	2,532	0,0170 *
Age	-0,2115	0,1158	-1,826	0,0782 .

Significant codes; *=0,01; . =0,05

Table 3.11. *R-squared and F-tests on regression of AGE on allele frequency.*

Multiple R-squared	Adjusted-R squared	F-Statistic	P-Value
0,08675	0,05526	2,755 on 1 and 29 DF	0,1077

Both the t-tests and the F-tests tell the same story, there is not a significant linear relationship between Age of the fish and the frequency of the B allele in SP
(p = 0,1077, and p = 0,0782).

4 DISCUSSION

4.1 OTOLITHS SCORED

The majority of the fish caught was aged 6-8 with a peak at 7. This result is consistent between years and sampling locations. Therefore we can conclude that most of the mature fish present in the sampling sites were aged 6-8.

4.2 DNA ISOLATION, PCR AMPLIFICATION AND SEQUENCYING

Increasing the amount of proteinase-k during the digestion step increases a lot the amount and quality of the extracted DNA (table 3.2 and 3.3)

In table 3.3 the quantity of DNA is larger when the standard protocol is used. Comparisons of some samples that were extracted in this set of samples indicate that the tissue was in good condition and the problem of the low quantity of DNA was the protocol I used. The rest of the variables measured with the nanodrop also show that what concerns to the extraction, this protocol works very well for the gill tissue samples conserved in ethanol.

On Figure 5 some differences between DNA from gills and DNA from fins can be appreciated .The gel image reveals that the quantity of DNA amplified is higher for fins than for gills. It can be inferred that the new fresh samples (2011) are better conserved than the old gills tissues conserved in ethanol (2004). It can be hypothesized that because the gill samples were taken for anatomical studies and not for DNA analysis the tissue sample might have been left outside for a long time in the field, which would have led to tissue degradation and poor DNA quality.

Not all the samples were successively sequenced. The explanation could be that mistakes have been made during the PCR or sequencing procedure. These low quality sequences were in both types of samples gills and fins. Therefore, further studies should be made to determine if the tissue itself is the problem.

4.3 GENOTYPE DISTRUBUTION AND ALLELE FREQUENCY

As the results show, the three possible genotypes appearing on PL population are distributed irregularly. Homozygous for allele A are much more abundant in the PL population from different periods (2004 and 2010) whereas homozygous for allele B and heterozygous are barely represented.

If the sequences are observed, for homozygous for allele A frequency has increased in a 29,2 % from 2004 to 2011. This could be due to incomplete sampling, as the sample from 2004 is rather small.

Also both graphics of genotype distribution indicates that in absolute values, homozygous for allele A are more represented respecting to the others on a same interval of age in 2010 than in 2004. Therefore it indicates that the total presence of allele A has been progressively increasing. The more plausible explanation is this allele is under positive selection and it is being fixed in this population. It can be hypothesized that having the allele A is advantageous in the environment that PL inhabits (Pierce, 2010).

We could not confirm the negative correlation between Age and allele B frequency that we predicted in the 2010 sample. That could mean that age does not affect allele frequency, or that the effects of age on allele frequency change with time. Nonetheless it can be suggested that the detrimental effect that the presence of this allele B in a PL individual it will appear later in life.

However, further studies will be necessary to determine if there is indeed a negative correlation between age and allele B frequency and what effects could it cause on PL population considering the factors that compose the environment that surrounds PL.

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APPENDIX A

Samples from 2004-2005. The table shows ID given to the samples and the age from the scoring.

Symbol + Indicates a half state of growing in between two years (For example: 7+ Indicates that the fish died between the 7th and the 8th year of growing)-
Blank cells belong to the samples which age has not been possible to determine.

SAMPLE ID	AGE	SAMPLE ID	AGE	SAMPLE ID	AGE	SAMPLE ID	AGE
S-17	7+	**H-33**	7	**H-34**	7+	**M-39**	7+
H-74	9	**S-49**	9+	**O-13**	7	**M-9**	9+
M-35	6	**O-34**	6	**M-12**	6	**H-66**	7+
O-23	7	**H-56**	7	**O-33**	6+	**M-37**	8+
O-16	9	**O-7**	8	**H-61**	7	**O-37**	8+
O-52	6	**H-82**	7+	**M-27**	7+	**M-48**	7
M-20	6	**O-12**	7+	**H-48**	10	**M-26**	7
O-14	7	**H-43**	7	**O-51**	6	**M-23**	8
M-8	8	**H-27**	7	**M-10**	9+	**H-14**	5+
H-45	7	**H-64**	6	**H-41**	8+	**M-25**	7+
H-70	6++	**H-57**	7	**M-13**	8	**M-16**	8
O-55	5+	**H-65**	6+	**O-10**	8+	**O-35**	8
O-30	6	**S-56**	7+	**M-54**	6	**M-28**	
S-38	6+	**S-18**	7+	**M-44**	7	**O-57**	6+
O-17	6	**H-30**	8	**M-59**	8	**M-31**	8
H-75	6+	**S-36**	7+	**M-58**	6	**O-22**	6+
H-58	7+	**H-54**	7	**M-4**	6+	**M-40**	5+
O-25	7+	**M-32**	6	**M-38**	6	**M-33**	7
H-73	6	**O-3**	7+	**M-49**	8+	**M-19**	7
H-35	8+	**M-53**	6	**M-56**	7	**M-55**	5
H-36	7+	**M-6**	7	**M-30**	6+	**M-42**	6
S-19	7	**M-14**	7+	**O-24**	7	**O-18**	7+
H-62	6	**H-42**	7	**O-49**	8	**H-13**	7+
M-51	6+	**H-32**	7	**M-24**	6+	**M-29**	8
H-49	5+	**O-19**	7+	**O-56**	6+	**O-15**	9+
O-31	7+	**M-52**	6	**O-27**	6	**M-46**	7
S-40	7+	**O-11**	9+	**O-28**	7	**O-20**	8+
H-40	8+	**M-34**	7+	**M-45**	7+	**O-5**	6
S-47	9+	**O-54**	8+	**M-47**	7	**H-47**	4
O-48	7+	**M-11**	7+	**M-43**	7	**O-1**	7
H-55	5+	**O-85**	6+	**O-6**	8	**O-38**	7
H17	7+	**O-26**	6	**O-9**	7+	**S-34**	7+

SAMPLE ID	AGE	SAMPLE ID	AGE	SAMPLE ID	AGE	SAMPLE ID	AGE
H-60		**S-42**	9	**Re-60**	8+	**Re-51**	6
M-50	7+	**H-69**	8	**S-22**	7	**H-52**	7
S-31	8+	**S-53**	8	**Re-72**	6	**S-57**	5
H-67	7+	**S-26**	8	**S-41**	6	**Re-46**	6
S-29	6+	**M-57**	7+	**Re-37**	6	**S-20**	7
S-50	7+	**S-35**	7	**H-68**	6+	**S-30**	7
H-53	6+	**S-52**	6+	**Re-70**	8	**Re-49**	7
H-38	9	**Re-67**	7+	**S-39**	8	**H-46**	6
H-59	5	**Re-69**	8	**Re-59**	9	**Re-44**	6
S-24	8	**Re-63**	5+	**Re-38**	8	**Re-74**	6
O-32	6+	**O-37**	5	**Re-80**	7	**H-50**	6+
S-55	7	**S-37**	7+	**Re-41**	8	**S-44**	7
H-72	7	**S-63**	7	**Re-75**	8	**Re-45**	7
S-58	9	**S-66**	9	**S-62**	7	**Re-52**	7
O-8	8	**S-65**	8	**Re-42**	6+	**Re-33**	6
Re-66	8+	**Re-62**	7	**Re-53**	7	**Re-61**	6+
H-39	7	**Re-40**	6	**Re-48**	7	**Re-65**	8
H-76		**S-43**	7	**Re-77**	5+	**S-60**	7+
S-54	8	**Re-50**	7+	**Re-34**	6+	**Re-64**	8
S-32	9+	**S-64**	6+	**Re-73**	8	**Re-78**	7
S-51	7	**Re-76**	8+	**O-2**	7		
O-50	6	**O-21**	7+	**O-36**	7		
S-48	6+	**S-27**	6+	**O-29**	6		
H-63	7+	**S-46**	6	**Re-81**	6		
H-29	7	**Re-35**	8	**Re-43**			
S-33	7+	**Re-58**	7+	**Re-57**	7+		
S-61	7	**S-59**	7	**Re-56**	7		
S-28	7	**M-36**	6	**Re-55**	6		
S-21	7	**Re-54**	7+	**Re-47**	7		
S-23	7+	**H-44**	8	**Re-68**	7		
H-37	7	**O-4**	5	**Re-79**	6		
S-25	6	**H-51**	6	**Re-39**	6		

APPENDIX B

Samples from 2010. The table shows ID given to the samples and the age from the scoring.

Symbol + indicates a half state of growing in between two years (For example: 7+ Indicates that the fish died between the 7th and the 8th year of growing)-

Blank cells belong to the samples which age has not been possible to determine.

SAMPLE ID	AGE	SAMPLE ID	AGE	SAMPLE ID	AGE	SAMPLE ID	AGE
1	5+	**69**	6	**103**	6+	**171**	5+
2	8	**70**	6	**139**	5+	**172**	6+
3	5+	**71**	7+	**140**	6+	**212**	9
4	7	**72**	5+	**141**	5+	**213**	9
5	5+	**73**	9	**142**		**214**	9
6	7	**74**	5	**143**	4+	**215**	7+
7		**75**	7+	**144**	6+	**216**	10
8	6	**76**	5+	**145**		**217**	6
9	7+	**77**	7+	**146**	6	**218**	8+
10	5	**79**	6+	**147**	9	**219**	6+
11	6+	**80**	6+	**148**	5+	**220**	5+
12	7	**81**	6+	**149**	7	**221**	6+
13	6+	**82**	7	**150**	9	**222**	8
14	8+	**83**	8+	**151**	7	**223**	8+
15	7	**84**	8	**152**	6+	**224**	7
16	6+	**85**	7	**153**	5+	**225**	6
17	7	**86**	8	**154**	8	**226**	7+
18		**87**	8+	**155**	5	**227**	7+
54		**88**	8+	**156**	5	**228**	9
55	5	**89**	9	**157**	5+	**229**	7+
56	6	**90**	6+	**158**	6	**230**	8
57	6+	**91**		**159**	6+	**231**	
58	5	**92**	9	**160**	5	**232**	7
59	6+	**93**	9	**161**		**233**	9
60	7+	**94**	4+	**162**	5+	**234**	6+
61	6	**95**	7	**163**		**235**	7
62	7	**96**		**164**	10	**236**	9+
63	6+	**97**	6	**165**	5+	**237**	7
64		**98**	6	**166**	5	**238**	4+
65		**99**	7	**167**	7	**239**	6+
66		**100**	9	**168**	5	**240**	6
67		**101**	9+	**169**	7+	**241**	7
68	5	**102**		**170**	7		

APPENDIX C

Samples from 2011. The table shows ID given to the samples and the age from the scoring.

Symbol + indicates a half state of growing in between two years (For example: 7+ Indicates that the fish died between the 7th and the 8th year of growing)-

Blank cells belong to the samples which age has not been possible to determine.

SAMPLE ID	AGE	SAMPLE ID	AGE	SAMPLE ID	AGE	SAMPLE ID	AGE
3001	7	**3030**	5	**3051**	7+	**3072**	8
3002	7	**3031**	8	**3052**	5+	**3073**	7+
3003	7	**3032**	7	**3053**	7	**3074**	5
3004	5	**3033**	6	**3054**	8	**3075**	7+
3005	7	**3034**	6	**3055**	5	**3076**	5
3006	7	**3035**		**3056**	7	**3077**	5
3007	6	**3036**	6	**3057**	7	**3078**	6+
3008	7	**3037**	7	**3058**	8+	**3079**	5+
3017	6	**3038**	7+	**3059**	5	**3080**	6
3018	7	**3039**	7	**3060**	5	**3081**	5+
3019	6	**3040**	7	**3061**	6	**3082**	6
3020	8	**3041**	7	**3062**	6	**3083**	6+
3021	7	**3042**	6	**3063**	5	**3084**	6+
3022	6	**3043**	6	**3064**	6	**3085**	7+
3023	7	**3044**	6	**3065**	7	**3086**	6
3024	6	**3045**	6	**3066**	8	**3087**	6
3025	6	**3046**	8	**3067**	6	**3088**	6
3026	6+	**3047**	6	**3068**	7	**3089**	6
3027	5	**3048**	6	**3069**	5+	**3090**	7
3028	6	**3049**	7	**3070**	7		
3029	8	**3050**	6	**3071**	4		

APPENDIX D

Samples from 2011, Different features indicated. M*=PL. D**=SB

SAMPLE ID	MORPH	SEX	WEIGHT	MATED	LOCATION	DATE (2011)
M3001	M*	Female	106,8	Yes	Mjóanes	4th OCT
M3002	M	Female	77	Yes	Mjóanes	4th OCT
M3003	M	Female	116,52	Yes	Mjóanes	4th OCT
M3004	M	Female	92,51	Yes	Mjóanes	4th OCT
M3005	M	Male	55,24		Mjóanes	4th OCT
M3006	M	Male	71		Mjóanes	4th OCT
M3007	M	Male	58		Mjóanes	4th OCT
M3008	M	Male	73		Mjóanes	4th OCT
M3009	D**	Male	14,3		Mjóanes	4th OCT
M3010	D	Male	22,74		Mjóanes	4th OCT
M3011	D	Male	15,09		Mjóanes	4th OCT
M3012	D	Male	14,43		Mjóanes	4th OCT
M3013	D	Female	57,14	Yes	Mjóanes	4th OCT
M3014	D	Female	28,37	Yes	Mjóanes	4th OCT
M3015	D	Female	22,24	Yes	Mjóanes	4th OCT
M3016	D	Female	32,96	Yes	Mjóanes	4th OCT
M3017	M	Female	90,02	Yes	Mjóanes	4th OCT
M3018	M	Female	91,3	No	Mjóanes	4th OCT
M3019	M	Female	65,69	Yes	Mjóanes	4th OCT
M3020	M	Female	41,87	Yes	Mjóanes	4th OCT
M3021	M	Female	127,31	Yes	Mjóanes	4th OCT
M3022	M	Female	66,02	Yes	Mjóanes	4th OCT
M3023	M	Female	77,8	No	Mjóanes	4th OCT
M3024	M	Female	72,06	Yes	Mjóanes	4th OCT
M3025	M	Female	101,24	No	Mjóanes	4th OCT
M3026	M	Female	74,34	Yes	Mjóanes	4th OCT
M3027	M	Female	64,22	No	Mjóanes	4th OCT
M3028	M	Female	96,46	No	Mjóanes	4th OCT
M3029	M	Female	75,64	No	Mjóanes	4th OCT
M3030	M	Female	71,61	No	Mjóanes	4th OCT
M3031	M	Female	62	Yes	Mjóanes	4th OCT
M3032	M	Female	79,73	Yes	Mjóanes	4th OCT
M3033	M	Female	58,26	Yes	Mjóanes	4th OCT
M3034	M	Female	70,65	Yes	Mjóanes	4th OCT
M3035	M	Female	120,94	No	Mjóanes	4th OCT
M3036	M	Female	69,71	Yes	Mjóanes	4th OCT
M3037	M	Female	95,11	Yes	Mjóanes	4th OCT
M3038	M	Female	76,26	Yes	Mjóanes	4th OCT
M3039	M	Female	60,4	Yes	Mjóanes	4th OCT

SAMPLE ID	MORPH	SEX	WEIGHT	MATED	LOCATION	DATE (2011)
M3040	M	Female	94,68	Yes	Mjóanes	4th OCT
M3041	M	Female	67,93	Yes	Mjóanes	4th OCT
M3042	M	Female	115,94	Yes	Mjóanes	4th OCT
M3043	M	Female	79,12	No	Mjóanes	4th OCT
M3044	M	Female	72,08	Yes	Mjóanes	4th OCT
M3045	M	Female	43,98	Yes	Mjóanes	4th OCT
M3046	M	Female	75,95	Yes	Mjóanes	4th OCT
M3047	M	Female	112,6	Yes	Mjóanes	4th OCT
M3048	M	Female	66,9	Yes	Mjóanes	4th OCT
M3049	M	Female	90,33	Yes	Mjóanes	4th OCT
M3050	M	Female	64,49	Yes	Mjóanes	4th OCT
M3051	M	Female	103,32	No	Mjóanes	4th OCT
M3052	M	Male	75,9		Mjóanes	4th OCT
M3053	M	Male	70,91		Mjóanes	4th OCT
M3054	M	Male	82,45		Mjóanes	4th OCT
M3055	M	Male	44,71		Mjóanes	4th OCT
M3056	M	Male	48,16		Mjóanes	4th OCT
M3057	M	Male	62,43		Mjóanes	4th OCT
M3058	M	Male	93,77		Mjóanes	4th OCT
M3059	M	Male	43,83		Mjóanes	4th OCT
M3060	M	Male	75,13		Mjóanes	4th OCT
M3061	M	Male	54,37		Mjóanes	4th OCT
M3062	M	Male	63,35		Mjóanes	4th OCT
M3063	M	Male	55,98		Mjóanes	4th OCT
M3064	M	Male	64,53		Mjóanes	4th OCT
M3065	M	Male	67,54		Mjóanes	4th OCT
M3066	M	Male	57,97		Mjóanes	4th OCT
M3067	M	Male	86,74		Mjóanes	4th OCT
M3068	M	Male	113,9		Mjóanes	4th OCT
M3069	M	Male	79,62		Mjóanes	4th OCT
M3070	M	Male	55,63		Mjóanes	4th OCT
M3071	M	Male	28,76		Mjóanes	4th OCT
M3072	M	Male	85,25		Mjóanes	4th OCT
M3073	M	Male	64,8		Mjóanes	4th OCT
M3074	M	Male	67,42		Mjóanes	4th OCT
M3075	M	Male	75,93		Mjóanes	4th OCT
M3076	M	Male	74,24		Mjóanes	4th OCT
M3077	M	Male	53,7		Mjóanes	4th OCT
M3078	M	Male	47,51		Mjóanes	4th OCT
M3079	M	Male	43,12		Mjóanes	4th OCT
M3080	M	Male	54,31		Mjóanes	4th OCT
M3081	M	Male	53,97		Mjóanes	4th OCT
M3082	M	Male	75,21		Mjóanes	4th OCT

SAMPLE ID	MORPH	SEX	WEIGHT	MATED	LOCATION	DATE (2011)
M3083	M	Male	77,82		Mjóanes	4th OCT
M3084	M	Male	76,8		Mjóanes	4th OCT
M3085	M	Male	73,31		Mjóanes	4th OCT
M3086	M	Male	107,51		Mjóanes	4th OCT
M3087	M	Male	78,96		Mjóanes	4th OCT
M3088	M	Male	120,51		Mjóanes	4th OCT
M3089	M	Male	94,45		Mjóanes	4th OCT
M3090	M	Male	125,2		Mjóanes	4th OCT
M3091	D	Male	14,05		Mjóanes	4th OCT
M3092	D	Male	19,08		Mjóanes	4th OCT
M3093	D	Male	23,06		Mjóanes	4th OCT
M3094	D	Male	14,82		Mjóanes	4th OCT
M3095	D	Male	43,22		Mjóanes	4th OCT
M3096	D	Male	25,39		Mjóanes	4th OCT
M3097	D	Male	15,18		Mjóanes	4th OCT
M3098	D	Male	15,5		Mjóanes	4th OCT
M3099	D	Male	15,29		Mjóanes	4th OCT
M3100	D	Male	17,59		Mjóanes	4th OCT
M3101	D	Male	14,05		Mjóanes	4th OCT
M3102	D	Male	28,78		Mjóanes	4th OCT
M3103	D	Male	13,33		Mjóanes	4th OCT
M3104	D	Male	12,05		Mjóanes	4th OCT
M3105	D	Male	15,22		Mjóanes	4th OCT
M3106	D	Male	18,5		Mjóanes	4th OCT
M3107	D	Male	26,26		Mjóanes	4th OCT
M3108	D	Male	13,48		Mjóanes	4th OCT
M3109	D	Male	17,15		Mjóanes	4th OCT
M3110	D	Male	12,91		Mjóanes	4th OCT
M3111	D	Male	23,29		Mjóanes	4th OCT
M3112	D	Male	12,62		Mjóanes	4th OCT
M3113	D	Male	11,96		Mjóanes	4th OCT
M3114	D	Male	16,16		Mjóanes	4th OCT
M3115	D	Male	12,38		Mjóanes	4th OCT
M3116	D	Male	17,96		Mjóanes	4th OCT
M3117	D	Male	13,05		Mjóanes	4th OCT
M3118	D	Male	19,58		Mjóanes	4th OCT
M3119	D	Male	21,82		Mjóanes	4th OCT
M3120	D	Male	17,04		Mjóanes	4th OCT
M3121	D	Male	14,13		Mjóanes	4th OCT
M3122	D	Male	12,62		Mjóanes	4th OCT
M3123	D	Male	14,91		Mjóanes	4th OCT
M3124	D	Male	10,48		Mjóanes	4th OCT
M3125	D	Male	12,37		Mjóanes	4th OCT

SAMPLE ID	MORPH	SEX	WEIGHT	MATED	LOCATION	DATE (2011)
M3126	D	Male	30,94		Mjóanes	4th OCT
M3127	D	Male	28,37		Mjóanes	4th OCT
M3128	D	Female	27,42	No	Mjóanes	4th OCT
M3129	D	Female	40,01	No	Mjóanes	4th OCT
M3130	D	Female	33,01	No	Mjóanes	4th OCT
M3131	D	Female	32,27	No	Mjóanes	4th OCT
M3132	D	Female	25,37	Yes	Mjóanes	4th OCT
M3133	D	Female	25,1	Yes	Mjóanes	4th OCT
M3134	D	Female	15,07	No	Mjóanes	4th OCT
M3135	D	Female	17,16	Yes	Mjóanes	4th OCT
M3136	D	Female	78,75	No	Mjóanes	4th OCT
M3137	D	Female	35,65	No	Mjóanes	4th OCT
M3138	D	Female	23,2	Yes	Mjóanes	4th OCT
M3139	D	Female	10,65	Yes	Mjóanes	4th OCT
M3140	D	Female	13,58	Yes	Mjóanes	4th OCT
M3141	D	Female	24,11	No	Mjóanes	4th OCT
M3142	D	Female	22,51	Yes	Mjóanes	4th OCT
M3143	D	Female	31,68	No	Mjóanes	4th OCT
M3144	D	Female	28,4	Yes	Mjóanes	4th OCT
M3145	D	Female	21,2	No	Mjóanes	4th OCT
M3146	D	Female	18,71	Yes	Mjóanes	4th OCT
M3147	D	Female	23,14	No	Mjóanes	4th OCT
M3148	D	Female	26,79	No	Mjóanes	4th OCT
M3149	D	Female	21,11	Yes	Mjóanes	4th OCT
M3150	D	Female	30,69	No	Mjóanes	4th OCT
M3151	D	Female	35,7	No	Mjóanes	4th OCT
M3152	D	Female	20,41	Yes	Mjóanes	4th/16th Nov
M3153	D	Male	27,94		Mjóanes	4th/16th Nov
M3154	D	Female	17,32	Yes	Mjóanes	4th/16th Nov
M3155	D	Female	14,06	Yes	Mjóanes	4th/16th Nov
M3156	D	Female	27,62	Yes	Mjóanes	4th/16th Nov
M3157	D	Male	24,59		Mjóanes	4th/16th Nov
M3158	D	Female	25,31		Mjóanes	4th/16th Nov
M3159	D	Male	31,13		Mjóanes	4th/16th Nov
M3160	D	Male	12,09		Mjóanes	4th/16th Nov
M3161	D	Female	11,35	Yes	Mjóanes	4th/16th Nov
M3162	D	Female	12,97	Yes	Mjóanes	4th/16th Nov
M3163	D	Female	28,81	Yes	Mjóanes	4th/16th Nov
M3164	D	Female	19,18	Yes	Mjóanes	4th/16th Nov
M3165	D	Male	36,32		Mjóanes	4th/16th Nov
M3166	D	Male	77,55		Mjóanes	4th/16th Nov
M3167	D	Male	32,66		Mjóanes	4th/16th Nov
M3168	D	Male	33,33		Mjóanes	4th/16th Nov