



T.R.
USKUDAR UNIVERSITY
INSTITUTE OF SCIENCE

DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS PROGRAM

MASTER' S THESIS

**“GENETIC - PHENOTYPIC DIVERGENCES AND EVOLUTION
AMONG THREESPINE STICKLEBACK FISH POPULATIONS
(*Gasterosteus aculeatus*) IN ICELAND”**

Salsabile GHAZALI

Thesis Advisors: Professor Muhsin KONUK

Professor Zophonias Oddur JÓNSSON

ISTANBUL - 2024

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TEXT OF THE OATH

I declare that this study is my thesis study, that I have no unethical behavior at any stage from planning to writing, that I have obtained all the information in the thesis within academic and ethical rules, and that I resource all the information and comments that are not obtained through the thesis research.

21.06.2024
Salsabile GHAZALI



FOREWORD or ACKNOWLEDGEMENTS

First, I would like to express my deepest gratitude to my two supervisors: **Pr. Muhsin Konuk** for his trust in allowing me to conduct my thesis research abroad and granting me the freedom to pursue a project aligned with my interests, and **Pr. Zophonías Jónsson** for inviting me to join his research project and believing in my commitment to research in evolutionary Biology regardless of my background. This opportunity has significantly enhanced my laboratory skills and provided invaluable experience among esteemed experts in aquatic biology, such as **Pr. Bjarni Kristófer Kristjánsson** at Hólar University whom I thank for his guidance and support concerning the phenotypic part of the project, along with his laboratory members in Sauðárkròkur/ Iceland.

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ABSTRACT

This thesis investigates the genotype and phenotype variations in Threespine stickleback fish focusing on their evolution along with adaptation to freshwater environments. The objective is to, first, determine genetic diversity by analyzing inversion chromosomes 1, 11, and 21 and assess morphological variations among different populations. We use Hotshot DNA extraction protocol and simple PCR genotyping assay with the three designed PCR primer pairs to examine samples collected from five locations. Our results indicate a lack of inversion polymorphism in Chromosome 1 in any of the populations as they all display an inverted Marine homozygote genotype, likely due to ancestral traits. In contrast, Chromosomes 11 and 21 manifest inversion polymorphism suggesting local adaptations. Secondly, we assess phenotypic variations by studying defensive traits such as lateral plates, dorsal and pelvic spines, along with examination of gill rakers. These studied variables measurements are via IMAGEJ Software and the results are through ANOVA and Regression model tests using STATA 14.2 software. Thus, regression model tests reveal significant correlations between a few morphological traits and genomic inversions, pointing to the crucial role of genetic variation for ecological adaptation. Finally, the comparison of data from freshwater population with marine population shed light on differences in genotype frequency, reflecting distinct selection in each environment. This study emphasizes the close relationship between these particular inversions and morphology of stickleback shaping their evolution and adaptation in their habitats.

Keywords: Threespine stickleback, chromosomal inversion polymorphism, Defensive traits, genetic divergence, phenotypic variation, freshwater populations.

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ABBREVIATIONS

PCR: The Polymerase Chain Reaction

SP 1: The Dorsal spine number 1 of Threespine stickleback fish
SP 2: The Dorsal spine number 2 of Threespine stickleback fish
PS: The Pelvic spine of Threespine stickleback fish

PL: The Lateral plates of Threespine stickleback fish

GR2: The Gill Raker number 2 of Threespine stickleback fish

GR3: The Gill Raker number 3 of Threespine stickleback fish

GapGR2/GR3: The gap or space between the Gill Raker 2 and the Gill Raker 3

GR_number: The Gill rakers total number in each gill arch of samples

Chr 1: The Chromosome 1

Chr 11: The Chromosome 11

Chr 21: The Chromosome 21

Inv: The Inversion

DNA: DeoxyriboNucleic Acid

mm: The SI unit symbol of millimeter measure

UV: The ultraviolet light

β: The beta sign representing variable on software

c: The constant

ANOVA: The Analysis Of Variance test

The M genotype: The Marine/ marine homozygote genotype

The H genotype: The Marine/ Freshwater heterozygote genotype

The F genotype: The Freshwater/ Freshwater homozygote genotype

CHAPTER 1 INTRODUCTION

1. INTRODUCTION

1.1 Presentation of the hosting universities

I did my thesis research project at the University of Iceland in Reykjavik, at the Faculty of Life and Environmental Sciences where various fields of biology are studied. The frequent topics researched are related to Marine or Arctic biology, but also others related to ecology and molecular biology.

Similarly, to perform analysis on the morphology of the fish, I joined the laboratory of Professor Bjarni K. Kristjánsson in Saudákrókur at Hólar University, Department of Fish Biology and Aquaculture.

1.2 Threespine stickleback Fish

Threespine stickleback fish (*Gasterosteus aculeatus*) are teleost species usually found in the Northern Hemisphere, highly abundant with a short lifespan, able to survive in temperatures approximately from 4 to 21 °C due to extensive geological distribution. (Reid et al, 2001)

Originally from a Marine environment, sticklebacks have, following the glaciation epoch, occupied various freshwater habitats and adapted rapidly to environmental changes and conditions, making them one of the remarkable animal models in multiple domains of science, such as evolutionary biology and genetics. These fish have a wide range of environments from saline to freshwater ecosystems, realizing significant genetic and morphological variations reflecting their evolution and adaptation to different habitats (Ostlund-Nilsson et al, 2006). This genetic diversity leads to morphological changes during sticklebacks evolution in a short time period. (Peichel et al, 2001; Reid et al, 2001).

The stickleback's body length varies between 31 and 70 mm, the range of body size differs from population to another. Stickleback's belly is either silver, white, gray, or yellow sometimes, and the back green, brown to black color according to the environment they are in (Millet et al, 2013; Norton and Gutiérrez, 2019). In the reproduction period, the mature males develop blue eyes with an orange to red color of their bellies to their throats

to attract females. In this case, the male sticklebacks are the ones protecting and maintaining the nest. (Wootton, 1984)

These species studies provide a prevalent insights on the role of chromosomal inversions and phenotypic plasticity in evolution of sticklebacks and their adaptation to various environments (Ostlund-Nilsson et al, 2006).

1.3 Genetic Variation

Numerous molecular techniques and genetic protocols have been developed for performing analysis to characterize inversion polymorphisms within populations.

An inversion is the recombination of chromosomes where a region of that chromosome becomes inverted in its initial position. However, inversions usually do not cause deletion or addition of genetic materials, simply they rearrange DNA. Moreover, inversion polymorphism is settled through selection, due to this, in known inversion chromosomes I, XI, and XXI, an underdominance aspect might occur, promoting the inverted homozygote genotype while inferiorizing the heterozygote genotype. (Doolittle, Donald, 1987)

The first chromosomal inversion evidence was discovered by Alfred Sturtevant (1921) in *Drosophila Melanogaster*, which led to more discoveries of inversions in other models, one of those was the study of the scientist, Kirkpatrick, highlighting the effectiveness of natural selection in inducing frequency of inversion genotypes in populations. (Kirkpatrick, 2010)

PCR BASED GENOTYPING

PCR genotyping is the simple common molecular method of polymerase chain reaction, specific for genetic examinations. The Extracted DNA through the Hotshot DNA extraction protocol will be amplified with designed primers to analyze the difference in length of PCR products through agarose gel electrophoresis.

GEL DOCUMENTATION SYSTEMS

The visualization of the gel, stained with ethidium bromide, occurs in this machine also

called gel imager, showing the length of polymorphism. It contains a digital camera, a platform facing the camera consisting of UV light, the camera is connected to the computer downloading applications of the software depending on the company the machine came from, then can take images of nucleic acid bands.

1.4 Phenotype variation

Phenotype variation refers to the observation of physiological and/or behavioral differences among individuals within populations. These differences result from the interactions between genetic variations and environmental factors. For Threespine stickleback fish, this phenotypic variation is crucial as it can be the results of phenotypic plasticity, which may evolve and be adaptive or/and spontaneous in evolution. They are vertebrates that adapted rapidly to lentic systems, leading to a change in their morphological traits. These traits include Lateral Plates, Dorsal Spines, and Pelvic Spine including Gill Arches.

The studied variables might depend on various factors having significant influence on their changes. (Figure 1(a) and (b)).

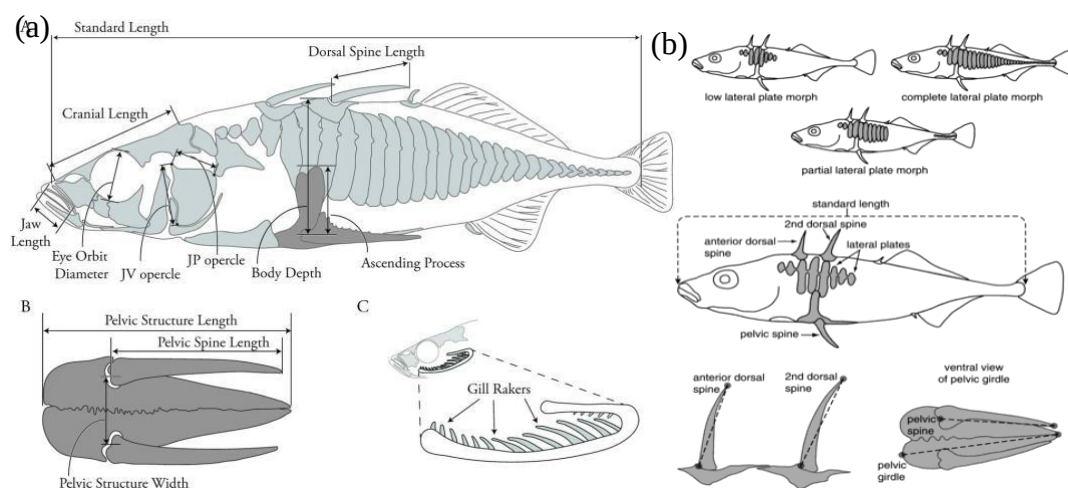


Figure 1: (a) The left image, representation of three spine stickleback fish structure along with gill arch and pelvic structures (Currey, M, 2019). (b) The right image, representation of lateral plates and spines measurement details (Marchinko, 2009)

Nowadays, it has become possible to understand through data collected from personal studies and other research articles about Threespine stickleback fish genetic differentiation and morphological changes, such information is explained in various studies with different methods used but mainly to study sticklebacks' evolution genetically, phenotypically.

Hence, choosing precise and simple molecular methods is necessary for accurate results in each part of the study. Following this reason, suitable methods were selected for our project from multiple research papers of reflecting studies on Threespine stickleback fish.



Figure 2: Threespine stickleback specimens obtained from different locations

1.5 Project Aims

It has been confirmed that genetic variation has an impact on defensive traits representing phenotype variation, resulting in changes in the evolutionary and adaptation of Threespine stickleback populations within freshwater environments.(Peichel et al, 2001; Kitano et al,2008)

We aim to achieve inversion frequencies and morphological variability from freshwater stickleback population, with the primary goal to better understand morphological variation that could be associated with genetic inversions.

A key focus will be on interpreting the significant changes occurring in phenotypic traits due to rapid adaptation mechanisms for surviving in freshwater environments. These

traits include the development and differences in mostly armor plates, dorsal spines, pelvic spine, and gill rakers . Thus, the second purpose is to identify the specific factors such as chromosomal inversion polymorphisms, body size, sex,, and environmental factor locality that drive these adaptive changes. Consequently, in the project, we will consider to what degree, according to *p-values*, the studied traits are correlated with these independent variables within populations.

Ultimately, our hypotheses claim firstly, that through all chromosomal inversions, the three identified genotypes would be present. Meaning, that polymorphism occurs on studied chromosomes due to evolution of stickleback in a new habitat, also the assumption that the allele frequency of M vs F genotypes will be different in different habitats.

The second hypothesis is that the prevalent influence of all factors on morphological traits.

The last hypothesis is the correlation between different inversions genotypes and phenotypic traits.

These hypotheses were constructed based on previous research studies, exposing genetic and phenotypic variations, each individually as well as the correlation between them.

Therefore, the study aims to provide comprehensive insights into the evolution of threespine sticklebacks for a broader understanding of rapid phenotypic evolution and the role of genetic inversions on environmental adaptation.

1.6 Overview of research Chapters

In Chapter 1, we began by giving a brief presentation of receiving university where the Master thesis was achieved, then introducing the host of the study, which is the model our experiments were about. Continuing by describing inversions, the analytical methods,, and principles of molecular biology and genetics. Furthermore, we explained the phenotypic variation part as the order of the thesis research was based on. Lastly, Chapter 1 offers an overview of the project aims adding to it the potential hypotheses claimed for this study and its purpose, including this overview of the chapters.

In Chapter 2, a literature review of the stickleback studies previously achieved, proving the schematic experiments of our research.

Materials and methods were interpreted in Chapter 3. It is the most crucial chapter where all important methodology and full laboratory work of the thesis are described in this part.

Chapter 4 involves the findings of the research, where graphs and tables are explained as results of genetic and phenotypic variations. All goes in the given order previously. Moreover, these findings are well elaborated and explained in Chapter 5, discussing and comparing it to previous studies and giving professional limitations confronted through this study.

The ending of the thesis is in Chapter 6, providing the conclusion of the whole chapter.

CHAPTER 2 GENERAL INFORMATION

2. GENERAL INFORMATION

The study of Threespine Stickleback fish offers a rich example and model for exploring phenotypic variation and genetic divergence concerning the evolution and adaptation of the fish. Early foundational studies have contributed greatly to our understanding of evolutionary dynamics driving phenotypic variation. Wootton (1984) uncovered the life history of sticklebacks while highlighting their adaptive flexibility along with Doolittle, (1987) and Gillespie (2004), introducing the basis of genetics within the population, leading the path for futuristic research on genetic diversity.

2.1 Genetic and Phenotypic variation

Investigating genetic divergence between Threespine stickleback species, Peichel et al (2001) pointed out the importance of genetic factors in regulating phenotypic traits. Later on, Archalbeault and her colleagues (2020) through their examination of Eda Locus, approved Peichel's theory by revealing that genetics is required for adaptation. Moreover, the research of Chan Yingguang Frank et al in 2010, studied the the phenotypic changes, especially the pelvic loss in diverse populations, and found that genetic changes had aa significant impact on morphological traits, illustrating the natural adaptation via genetic regulations.

2.2 Methodology

Molecular techniques and protocols had been enhanced for more accessibility and discoveries in the field of Biology, as for my study, the DNA extraction method had been developed specifically for fish experiments by Meeker and other scientists in 2007, making a rapid process for further examinations. Followed by simple PCR-based genotyping settled by Peichel and Roesti (2022), making genetic analysis easy and operating in my project.

consequently, defensive traits and gill rakers experimental studies were elaborated in student reports by Gautier (2021), Montjaux(2022), Mendes(2022), and,

Requillard(2022), using simple laboratory materials like laboratory forceps, Leica Microscopy, and staining protocol described in the research article of Strickland et al(2023). For image analysis, ImageJ software is used for such a purpose.

2.3 Genetic variation

While providing the historical basis from Kirkpatrick(2010) of the evolution of chromosomal inversions. Similarly, the investigation of Peichel et al (2001) was focusing on inversions occurring on chromosomes 1,11, and 21 in threespine stickleback fish, as they identified valuable genetic divergence and demonstrating that chromosomal inversions on these chromosomes were the key factors of phenotypic divergence in threespine stickleback fish.

Moreover, Roesti et al (2012) found positive differences in Chromosome 11. These variations are held by inversion polymorphisms conserving adaptive genes for lentic ecosystems.

Reid et al (2021) focused specifically on chromosome 21, exploring the inversions occurring on that chromosome were correlated with adaptation to diverse environments. Kusakabe et al (2021) indicated the contribution of chromosomal inversions for genetic divergence of marine and freshwater stickleback populations. Adding the fact that inversions promote adaptation.

2.4 Phenotypic variation

Kitano and his colleagues (2008) studied the evolution of armor plates in sticklebacks and stated that environmental changes can contribute to the loss or reduction of morphological traits. Meanwhile, Howes et al. (2017) and Chan et al.(2010) found out that genetic changes can lead to absence or regression of spines and lateral plates. Moreover, Ólafsdóttir et al.(2007) examined Icelandic threespine stickleback populations and discovered a strong correlation between changes occurring on gill rakers and their location.

In conclusion, Peichel and Marques (2017) evaluated through their study, the genetic structure of morphological changes in sticklebacks, giving a general image of how

phenotypic traits may be influenced by genetic variation. In consequence, the changes happening on the phenotypic scale through defensive traits are the outcome of complicated interactions between genetic variations and ecological pressure. The repeated evolution of morphological traits according to variant environmental conditions, suggests a potential adaptation of the vertebrates.



CHAPTER 3 MATERIALS and METHODS

3. MATERIALS and METHODS

3.1. Origin of samples

Three-spined stickleback fish (*Gasterosteus aculeatus*), were collected from five location, ponds and lakes, in the Reykjavik regions in Iceland, at each location 30 fish were collected. The locations were; Two stations in Askja pond (East and West), Kópavogslækur, Ástjörn and Vífilstaðavatn.



Figure 3: Representation of one of the locations (Askja), along with the laboratory building at the University of Iceland.

3.2. Collection and preparation of samples

At each location, minnow traps (Dynamic Aqua-Supply Ltd, Surrey, BC, Canada, mesh size 3.2 mm) (Figure 4(a)) were deployed at five meters intervals. These traps were left for two to three hours, usually overnight. Once collected, the fish from each location were collected into plastic zipper bags with labels (Figure 4 (b) and (c)) and placed immediately in to a cooler with ice blocks, which resulted in rapid death from “Hypothermia”. The samples were then frozen.



Figure 4: (a) unbaited minnow traps and (b)(c) collection of specimens captured in freezer bags with their labels.

In laboratory, the fish were thawed, the total length of sticklebacks, from snout to the end of the caudal fin, were measured to the nearest millimeter on a measuring board criterion (Figure 5(a) and (b)) and weighted on an analytical scale with a precision of 0,0001g. To preserve images of the fish before staining and dissecting experiments, the left side of each fish was photographed, in a light box providing no shadow and good lighting, with high resolution camera, CANON EOS 600D, along with an identification code and scale (Figure 5(a)(c)).

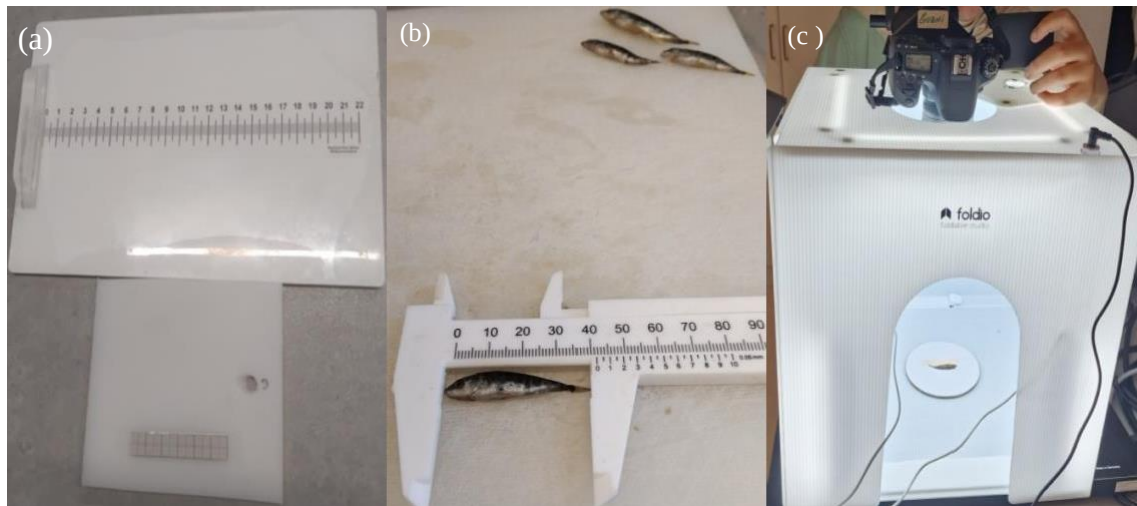


Figure 5: (a) (b) work place for sorting and measuring sticklebacks and (c) Setting the camera and the light box for morphological imaging.

All measurements were collected and written on laboratory paper, including each population and its corresponding fish identification with collection dates, length, weight, and sex. This information was later transferred to an Excel file.

Furthermore, the right fin of each fish was removed and conserved in Eppendorf tubes containing 95% ethanol along with the label given to identify each fish for further DNA analysis.

The fish were then dissected, by cutting the body open ventrally and the stomach and intestines removed and preserved in 96% ethanol.



Figure 6: Eppendorf tubes containing the stomach of each fish with their labels

Finally, each stickleback fish with its identification label is placed in tea bags, then according to its location put in plastic buckets containing 95% ethanol also labeled by the name of the station and date of collection. A total of 30 fish for each station were processed. The tags assigned for each fish have a unique identification number composed of; the initials of the project manager followed by the year of capture (last two digits) and four random digits, which in this case two first digits are specific for each location and the other two are the number of fish sample. For example, ZJ231010 is sample number 10 in Åstjörn population “10” captured in 2023 for the three-spined project.

3.3. Phenotype Variation

3.3.1 Staining Method

To study defensive traits and examine gill rakers, it is difficult to visualize fish in a natural state, thus it requires the staining process to have a clear vision of armor lateral plates number, gill arches, and spines.

3.3.1.1 Preparation of solutions

Table 1: Solutions used for staining protocol

Name of solution	Recipe/ preparation of Solution
Potassium Hydroxide (KOH 1%)	20 g KOH Pellets 2000ml H ₂ O
Alizarin Red stain solution	0.32 g Alizarin Red 2000ml KOH 1% solution

3.3.1.2 Staining Protocol

After fixing the fish in 95% Ethanol, a strict protocol of staining method was followed (Strickland, 2021 according to Millet and Kristjansson, 2013);

First, the fish were rehydrated overnight with water. Then, they were rinsed by KOH1% for 5 minutes (KOH1% solution is collected and re-used two more times). Next, the fish are stained with Alizarin red staining solution for 4 - 5 hours, with a regular checks to prevent overstaining and damage to the specimen. Once sticklebacks are stained, they are bathed for 15 minutes with KOH1% solution. Lastly, they were rinsed overnight by placing the plastic buckets holding each populations samples under light water flow (Figure 7).



Figure 7: Overnight light water flow on Threespine stickleback fish plastic containers. The following morning, they were put in 70% ethanol for fixation.

3.3.2 Sample Processing

The next day, images of the fish on the left side with spines erected are taken along with millimeter paper and the label of each individual(Figure 8).



Figure 8: Photo of a stained stickleback with erected spines with CANON EOS 600D

Once the images were taken, the gill arches were collected. It is required to work under a stereomicroscope linked to the camera on the computer (Figure 9(a)), where the lateral plates (Plates_number) are first, counted and taken into pictures (Figure 9(b)). Secondly, removal of the first longest left side gill arch from each fish (Figure 9(c)), then conserved in 95% ethanol eppendorf tubes with their identification tags.

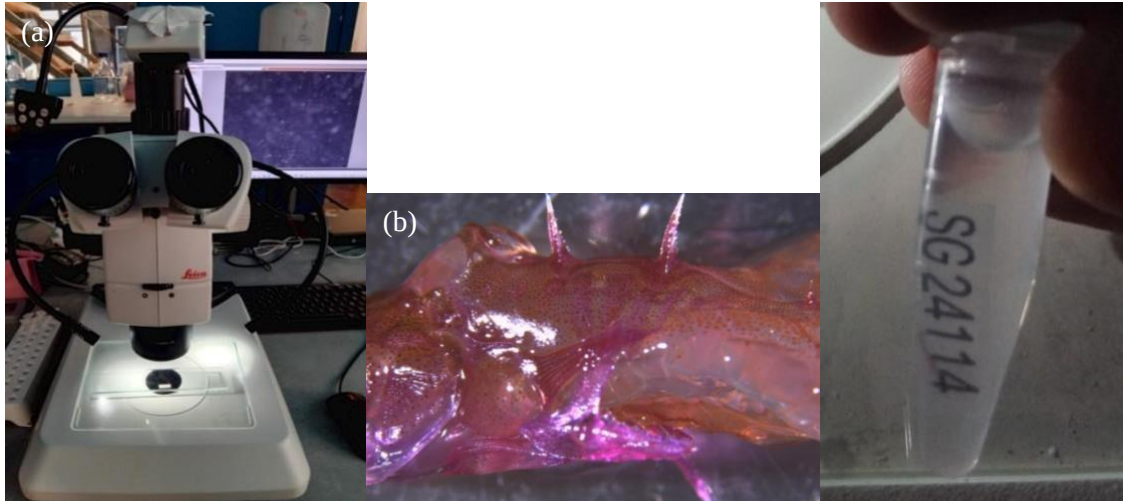


Figure 9: (a) Leica Microscope, (b) image of fish lateral plates under microscope, (c) eppendorf tubewith label tag of stickleback including the gill arch.

Ultimately the gill arches are then photographed individually (Figure 10), the same way as lateral plates through Leica Microscope, placed between two glass slides with a piece of millimeter paper to be able to count how many gill rakers are withing the gill arch, adding to that, the ability to take subsequent measurements.

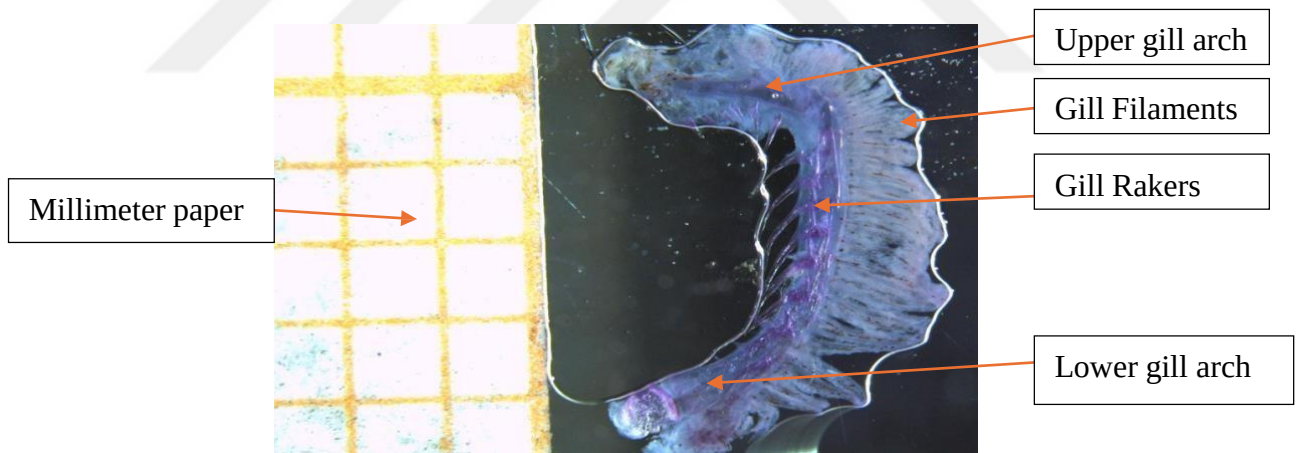


Figure 10: Photo type obtained via Leica Microscope with captions.

The images obtained are then processed with ImageJ software, which permits to measurement of the length of the two dorsal spines (SP1 and SP2) and the pelvic spine (PS). Moreover, the length of the gill raker number 2 (length_GR2), gill raker number 3(length_GR3), the gap between them (Gap_GR2/GR3), and the number of gill rakers (GR_Number) are counted. For the Spines (Figure 11), the gill rakers, and the gap between gill rakers 2 and 3 (Figure 12), 3 measurements were done for each and the mean of those is taken as the final value.

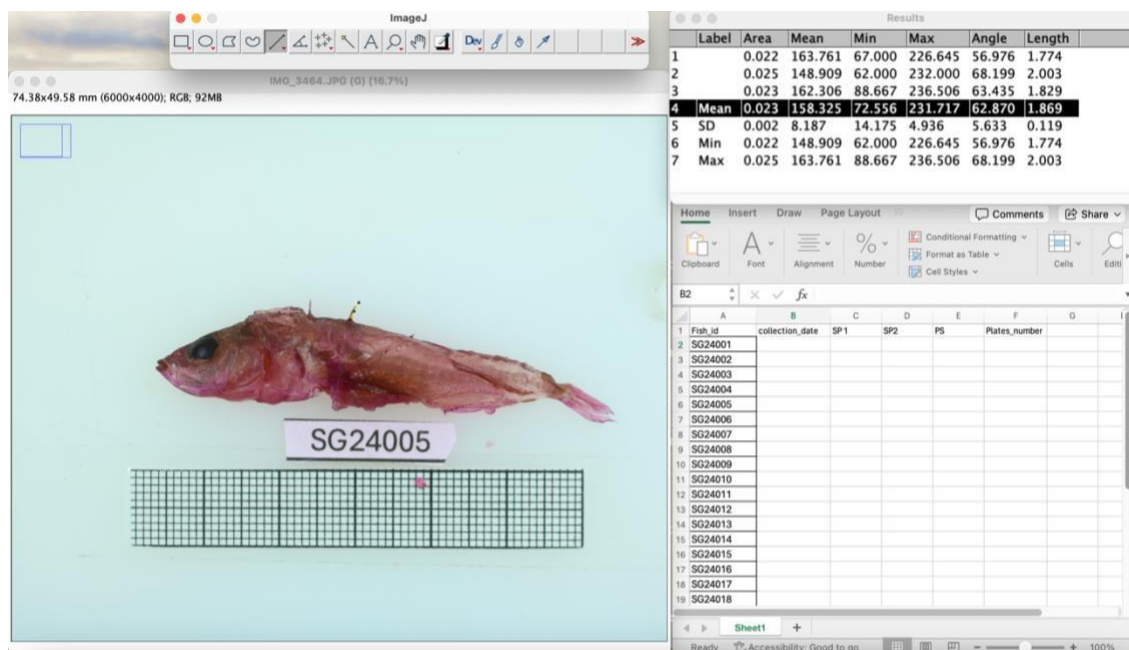


Figure 11: Measuring Dorsal and Pelvic spines length (mm) using ImageJ software.

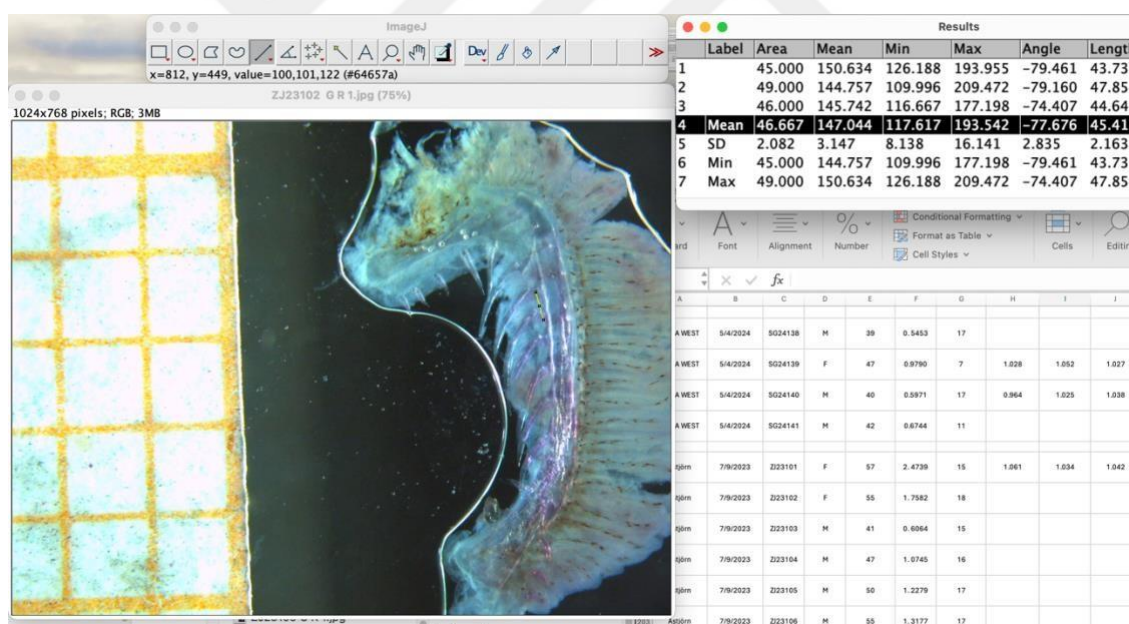


Figure 12: Measuring the Gap between gill raker 2 and 3 and their own length (mm) via ImageJ public software.

The whole data is registered on an excel file for results analysis (Table 2).

Table 2: An excerpt from the measurement data table in Excel.

A	B	C	D	E	F	G	H	I	J	K	L	M	N
Location	Year of collection	Fish_ID	sex	Length	weight	GR_Number	Length_GR2	Length_GR3	Gap_GR2/GR3	SP 1	SP2	PS	Plates_number
ASKJA EAST	5/4/2024	SG24001	M	41	0.5766	16	1,054	1,095	1,047	0,980	1,043	1,082	4
ASKJA EAST	5/4/2024	SG24002	F	49	1,0713	11	1,029	1,016	1,081	0,917	1,068	1,102	7
ASKJA EAST	5/4/2024	SG24003	F	49	1,1144	14	1,063	1,044	1,156	0,929	1,008	1,054	6
ASKJA EAST	5/4/2024	SG24004	F	52	1,5822	12	1,125	1,291	1,108	1,040	1,080	1,348	12
ASKJA EAST	5/4/2024	SG24005	F	62	1,8521	15	0,992	1,013	1,036	1,739	1,869	2,852	5
ASKJA EAST	5/4/2024	SG24006	F	54	1,5714	12	0,974	1,035	0,924	0,895	1,029	1,058	4
ASKJA EAST	5/4/2024	SG24007	F	55	1,7888	12	1,084	1,217	1,066	0,897	1,088	NA	6

3.1 Genotype Variation

Inversions have an important role in evolutionary biology, where species like Threespine stickleback fish show high polymorphism in, for this study, Chromosome 1,11 and 21 with three possible genotypes, marine/marine homozygote (M), marine/freshwater heterozygote (H) and freshwater/freshwater homozygote (F). DNA samples were also used for sex identification of sticklebacks, but for some reason, the male specific band did not amplify on agarose gel after the PCR test, so the sex of the fish was identified through gonads examination when the fish had a ventral dissection.

3.2 DNA Extraction

The protocol used is HOTSHOT(hot sodium hydroxide and Tris) for DNA extraction adapted by Meeker et al (2007) for rapid DNA extraction. First, put the fin tissue cut previously in Eppendorf tubes containing 50 µl of 50 mM NaOH. Samples were placed in a 95°C heated block for 30 minutes. Then, samples were vortex carefully and cooled on ice for 5 minutes. Lastly, 8 µl of 1M Tris--HCl is added to each sample with pH 8.0, then samples are spined down at 4000-5000 rpm for 10 minutes to pellet debris.

Short-term usage is placed at 4 °C, for long-term usage is stored at 20°C.

3.3 PCR TEST

3.4 Protocol for Chromosome 1,11 and 21

Primers and PCR protocol were developed by Catherine Peichel and Marius Roesti (2022).

3.5 Primers

Table 3: PCR primers developed typically for amplifying marine and freshwater inversions.

Chr	Position of indel in v5 genome	Forward primer (5' to 3')	Reverse primer (5' to 3')	Freshwater allele	Marine allele
1	26358387	TCAAAGTGAAGCTCC ACAGC	GGGTGGAGAGCA CAGAGG	242 bp	191 bp
11	6230794	CACATCAGCCTCGGT AGAGC	TGGTCACATAGT GCCAGTGC	376 bp	359 bp
21	10296295	CAGGACCAGTTGTGT TAAATGG	GTACTTGGCTCCT GCTACCG	295 bp	267 bp

3.6 PCR recipe

Table 4: PCR Reaction setup

Component	Volume 10 µl Reaction (ul)
One taq 2X Master Mix with standard buffer	5
10uM Forward primer	1
10 uM Reverse primer	1
Nuclease- Free water	2

A cocktail master mix of One Taq, ddH₂O, and primer pairs according to which inversion, was made for the total number of 152 samples (two are extra samples in two locations) that we had to genotype, without forgetting to do the negative control.

On the other hand, 9 µl of the master mix was distributed in PCR 8 strip-tubes, labelled according to the chromosome of inversion and fish identifier. Each tube was loaded with 1 µl of the DNA extracted previously. The samples were spun down before putting the strip tubes in the PCR machine.

3.7 PCR cycling Program

Table 5: PCR thermocycler steps run in Veriti Applied biosystems

Step	Temperature (°C)	Time (m:ss)
Initial denaturation	94	0:30
Denaturation	94	0:30
Annealing	57	0:35
Extension	68	0:30
Final extension	68	5:00
Hold	4	INF

3.8 Agarose gels

All PCR products were checked on agarose gel for visualization under ultraviolet (UV) light using the Gel Documentation System (Figure 13).



Figure 13: Explanatory Image of Gel Documentation System (SMACgig World,2023)

Once PCR cycling is done, 1µl of loading dye is added to PCR products and spun down. Furthermore, 10 µl of each sample was loaded, including 6µl of reference DNA ladder (50 or 100 bp from NEB)used for size comparison, in 2% agarose gels, electrophoresed in 1X concentration of TAE buffer (Tris base, acetic acid, ethylenediaminetetraacetic

acid). Adding to it, Ethidium Bromide enabled the visualization of DNA fragments under UV-light (Figure 14). The gels were running at 60 V for 70 minutes.

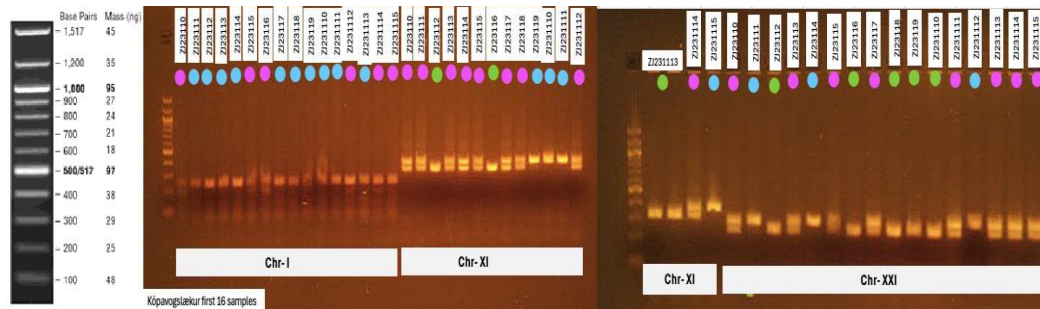


Figure 14: DNA ladder: N3231S (100bp) from New England Biolabs(NEB). 2% agarose gels, loaded with DNA from Kópavogslækur first 16 samples, amplified by PCR, for each chromosome inversion. The green dots are for the M genotype, the blue dots are for the F genotype and pink dots for the H genotype.

3.9 Protocol for Sex identification

Primers and PCR protocol were developed by Theo Bakker et al (2017).

3.10 Primers

Table 6: PCR primers developed to determine the sex of Threespine stickleback fish.

Chr	Primer pair used to determine Sex of threespine stickleback fish:	Forward Primer(5' to 3') Reverse primer (5' to 3')	Position of X chr	Position of Y chr
Sex	<i>N1-1</i>	<i>CATTACAGAAGATGCATTGTTTCAG</i>	271bp	271bp
	<i>Alt-1REV</i>	<i>GTCGTTGCCATCGCCCCG</i>	None	813bp

3.11 PCR recipe

Table 7: PCR Reaction setup

Component	Volume 10µl Reaction (µl)
One taq 2X Master Mix with standard buffer	3.25
10uM Forward primer	0.6
10 uM Reverse primer	0.6
Nuclease- Free water	4.15

A master mix of One Taq, ddH₂O,, and primer pair, was made for the total number of 152 samples (two are extra samples in two locations), without forgetting to do the negative control.

On the other hand, 8.6 µl of the master mix was distributed in PCR 8 strip-tubes, labeled with fish identifier. Each tube is loaded with 1.4 µl of the DNA extracted previously. The samples are spined down before putting the strip tubes in the PCR machine.

3.12 PCR cycling Program

Table 8: PCR thermocycler steps run in Veriti Applied biosystems

Step	Temperature (°C)	Time (m : ss)
Initial denaturation	94	5:00
Denaturation	94	0:30
Annealing	57	0:35
Extension	72	10:00
		40x cycles
Final extension	72	10:00
Hold	12	INF

3.13 Agarose gels

All PCR products were checked on agarose gel for visualization under ultraviolet (UV) light using the Gel Documentation System (Figure 15).

Once PCR cycling is done, 1µl of loading dye is added to PCR products and spun down. Furthermore, 10 µl of each sample was loaded, including 6µl of reference DNA ladder (50 or 100 bp from NEB) used for size comparison, in 2% agarose gels.

The gels were running at 80 V for 30 to 40 minutes.

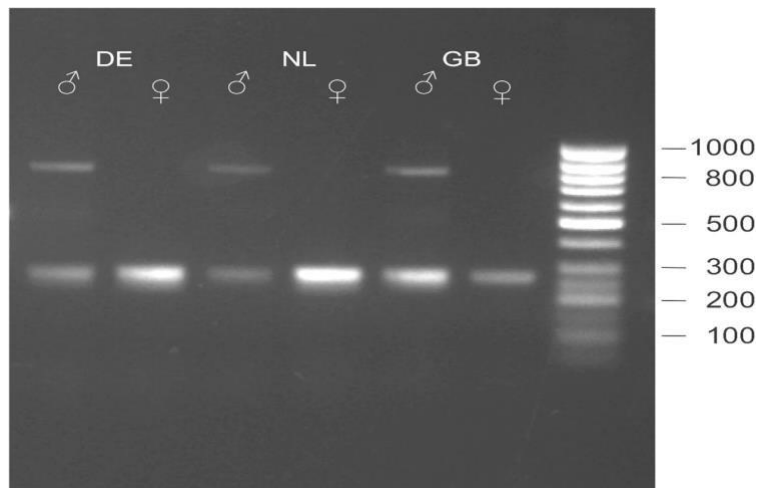


Figure 15: Example of gels from the article of Theo Bakker et al, (2017); Rapid molecular sexing of three-spined sticklebacks *Gasterosteus aculeatus* L., based on large Y-chromosomal Insertions, showing the DNA bands for male and female.

CHAPTER 4 FINDINGS

4. RESULTS

4. 1. Analytical methods

The aim is to achieve and get inversion frequencies along with phenotypic information from Threespine stickleback fish to understand if the samples are polymorphic through inversions as well as their association with phenotype variation through defensive traits. To obtain these results, the data on excel files of 152 in total sticklebacks in 5 locations were processed as follows.

For genotype divergence, Data on inversion percentages was by PCR-based genotyping assay, and the resulting length polymorphism was scored on agarose gel and visualized under UV light.

On the other hand, the traits excel file proceeded through STATA 14.2 software. Histograms and ANOVA tests were used to analyze the studied variables, providing p-values of each morphological trait variation based on one of the independent factors at a time (1:1).

To assure the reliability of the given p-values by ANOVA test, a linear regression model estimation test was run to investigate which linear model, length of the fish, Location, and sex influenced each trait, and which one was symbolic.

Regression Estimations model

Some string variables as sex, location, and so forth, were transformed into numerical forms to allow STATA software to estimate regression outputs. Furthermore, because each variable category was transformed into a dummy variable, meaning (YES= 1, NO= 0), one of each variable category should be eliminated from the equation to permit the (X'X) matrix to be inversible. For example, the location variable has five categories, each category is transformed in the regression equation to a dummy variable (Location 1= 1 if the fish is from Location 1 and Location 1= 0 if the fish is not from Location 1...). Simply, because each individual is from one and only one location, so STATA must take one of the provided locations as a reference case and exclude it from the equation.

Otherwise, STATA software cannot execute the estimations. In all, it is the effect of an independent variable X_1 on Y (dependent) by keeping X_2 and X_3 constant.

In regression terms, the regression equation should be entered in the software as follows:

$$y = \beta_1 * x_1 + \beta_2 * x_2 + \beta_3 * x_3 \text{ as } y = \beta * X, \text{ where } X \text{ is a matrix } (x_1 \ x_2 \ x_3)$$

Thus, Defensive trait $\sim \beta_1 \text{ length} + \beta_2 \text{ location} + \beta_3 \text{ sex} + c$

4.2 Raw Data

In five locations, 152 fish individuals with approximately 46 females more than males. The mean fish length is about 46.03 mm with a minimum of 31 mm and a maximum of 67 mm. Precisely, the Vífilstaðavatn population has the shortest individuals as the highest length is 41, which is below the mean value.

Table 9(a) : Raw data total number of individuals and each sex

Fish_nb	Female_nb	Male_nb
152	99	53

Table 9 (b): Raw data of influencing variables and the studied variables

Variable	Mean	Minimum	Maximum
Sex	1.65131	1	2
Length	46	31	67
Location	3.00657	1	5
Length_GR2	1.03017	0.555	1.611
Length_GR3	1.052028	0.818	1.988
GapGR2_GR3	1.021986	0.531	1.474
GR number	13	7	22
Plates number	49	0	17
SP1	0.9365308	0.656	1.739
SP2	1.035812	0.809	1.869
PS	1.13723	0.931	2.852

4.3 Genomic variation

A total of 152 samples of Three-spined stickleback fish from 5 different locations were chosen for DNA extraction and amplified with a simple PCR assay. The markers amplify inversions on chromosomes one, eleven, and twenty-one, thus the length polymorphism differences of PCR products were assessed by agarose gel separating the size via electrophoresis at 60 voltage under the UV light system. This results in, the possible observation of three genotypes, the M(marine/marine homozygous), the F (freshwater/freshwater homozygous), and the H (marine/ freshwater heterozygous) genotype.

For some reason, the identification of sticklebacks' sex, either male or female, could not be reached through PCR tests, only through gonads while dissecting sticklebacks.

Then analysis were performed to define the three inversion polymorphisms within the locations. The results are shown in graphs, each graph represents a specific chromosome (I, XI, XXI). The x-axis represents locations, and the y-axis represents the frequency of observations per genotype.

Afterwards the data of frequency and percentage of genotypes are set according to chromosomal inversions.

Inversion chromosome I

The collected results after the PCR test and agarose gels run, confirmed that for all the populations, the genotype that was mostly abundant on the gels according to this inversion is the F genotype, specifically for Askja East, Àstjörn and Vífilstaðavatn where it goes beyond 20 observations per population. However, M genotype is absent in Àstjörn and maximum is up to 4 samples as it is in Kópagoslækur population. Thus, chromosomal inversion in these locations is polymorphic as it exhibits all the three genotypes, but with high frequencies for Freshwater/ freshwater homozygote genotype.

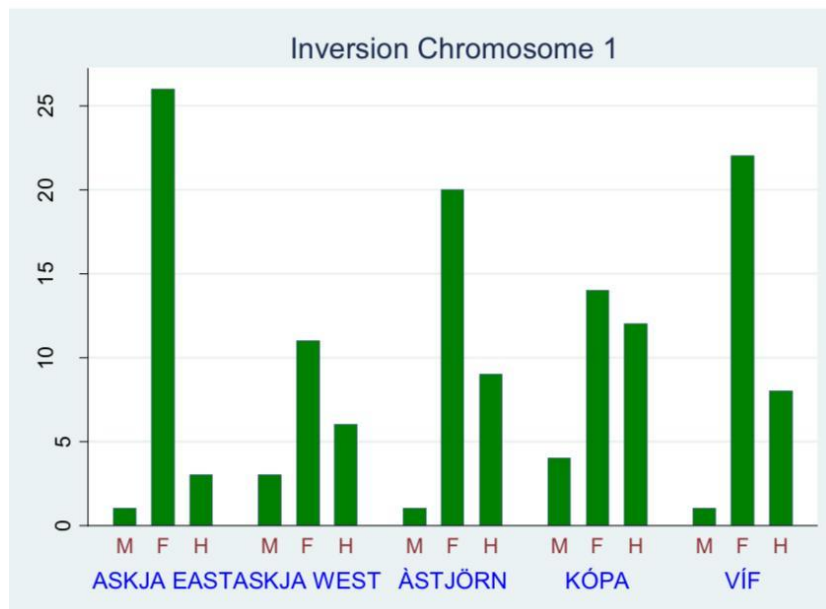


Figure 16: Graph representing the frequency of available genotypes across different locations in chromosome 1.

Table 10: Total data of Frequency and percentage for inversion chromosome 1

Chr 1	Freq.	Percent
M	9	5.92
F	94	61.84
H	38	25
ND	11	7.23

Inversion Chromosome XI

Chromosome 11 is the most polymorphic inversion chromosome and the frequency varies between the three known genotypes. Mainly, M genotype is highly frequent according to the frequencies of all stations, followed by H genotype, and then a small percentage of F genotype (Table 11).

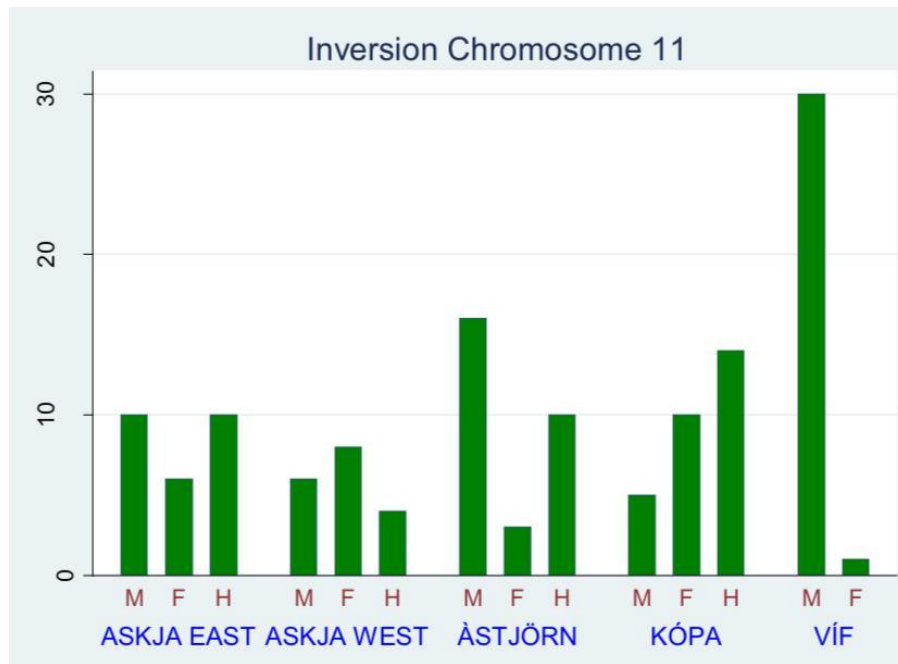


Figure 17: Graph representing the frequency of available genotypes across different locations in chromosome 11.

Table 11: Total data of Frequency, percentage and cumulative sum for chromosome 11

CHR11	Freq.	Percent
M	57	37.5
F	28	18.42
H	38	25
ND	28	18.42
Total	152	100

Inversion Chromosome XXI

Similarly to inversion chromosome XI, chromosome XXI is highly variant in terms of genotypes, but the most abundant one is the M genotype with a frequency of 79 followed by the H genotype 35 and then comes the F genotype with 19 where Vífilstaðavatn has no H and F inverted genotypes. In all, this inversion is polymorphic (Table 12).

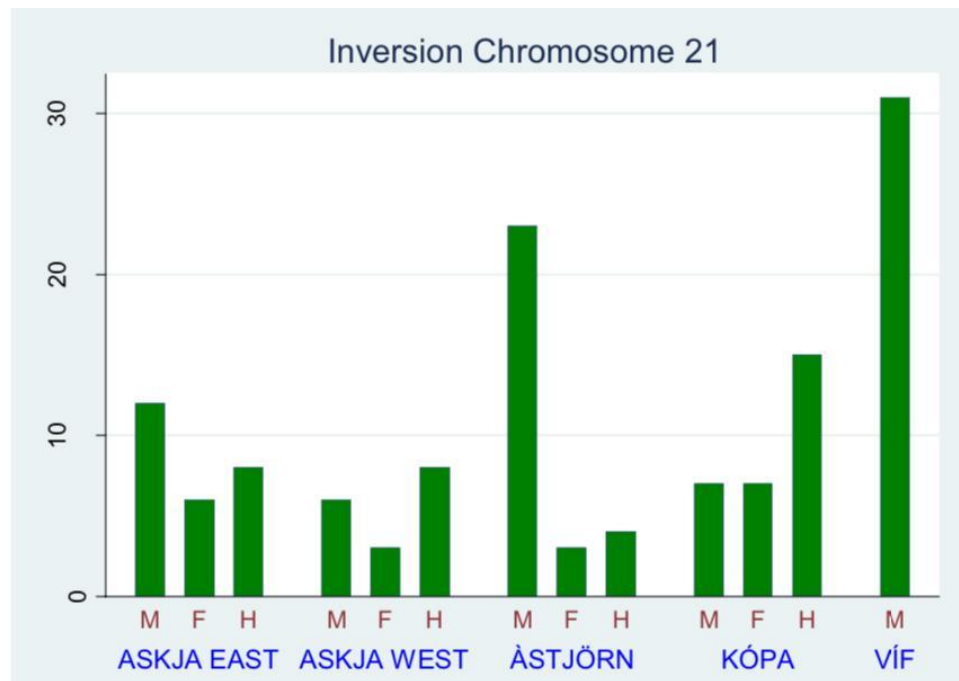


Figure 18: Graph representing the frequency of available genotypes across different locations in Chromosome 21

Table 12: Total data of Frequency, percentage, and cumulative sum for chromosome 21

CHR21	Freq.	Percent
M	79	51.97
F	19	12.5
H	35	23.02
ND	17	11.18
Total	152	100

In the Appendix 3, the raw data of each genotype of chromosomal inversions for each location studies along with the total number of individuals in each determined sex.

4.4 Phenotype variation

STATA 14.2 software was employed to process the data on an Excel file.

Eight observed variables of interest were tested during this research: The number of Lateral plates, the length of the two dorsal spines and the length of the pelvic spine, the number of gills rakers, the length of gill rakers two and three, and the gap between gill raker two and three.

4.4.1 Lateral Plates

The histogram representing the frequency of distribution of lateral plates (Figure 19), does not indicate a normal distribution since the number of plates is a discrete variable, unlike other traits, except the number of gills rakers, it is a variable that can only take integer values.

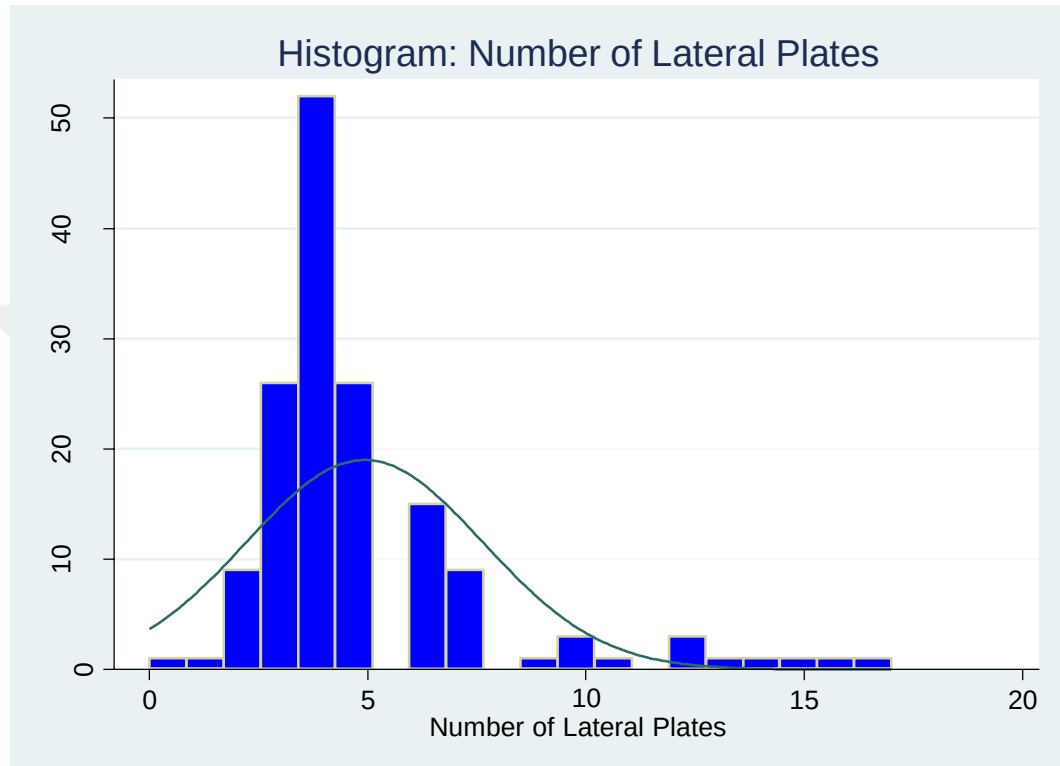


Figure 19: Histogram of the lateral plate number.

Blue bars represent the frequency of observations for each range of lateral plates. Each bar indicates the number of observations within that specific range.

Moreover, the tallest bar found at 5 lateral plates number, shows a frequency of over 50 observations, meaning this is the most common count of lateral plates per sample.

The majority of observations are concentrated between 0 and 10 plates but as the lateral plates number increases, the frequency declines.

Thus, it is required to run a linear regression model test, which gives the p-value of each factor influencing or not on the studied variable with the coefficient sign, either negative or positive.

If the *p-value* is <0.05 and the coefficient of the X variable (independent), the one that influences the Y variable (Defensive traits), is negative, then X has a decreasing effect on

Y. In contrast, if the $p\text{-value} < 0.05$ but the sign- in coefficient is positive, then X has an increasing effect on the Y variable.

The range of number of lateral plates in this study varies between 0 and 17, with an average of 4. The previous studies had mentioned about Marine sticklebacks having fully plated lateral side with approximately more than 30 plates whereas for freshwater fish the number of their lateral plates is around 6 to 10 (Bell and Foster, 1994; Hansson et al, 2016; wiig et al, 2016).

According to ANOVA test results, the size of the fish significantly influenced the number of lateral plates. However, location and sex factors due to the p-value greater than 0.05, indicated that they are not influential variables on this traits.

In contrast, the Regression method provided a weak correlation between the number of lateral plates and location (Table 13). In Åstjörn population, p-value is 0.001, and in Vífilstaðavatn, the p-value is 0.010, i.e. both are less than 0.05.

The results were slightly different from Regression model and ANOVA analysis, because Regression method check all factors together, however, ANOVA test one variable at a time. Furthermore, when correlating one variable at a time for the regression model analysis, it aligns with the ANOVA results, meaning, the p-value of the body size is 0.0021 (< 0.05), affirming the prevalent influence on the number of lateral plates, whereas location has a weak influence, and sex has no influence on the evolution of number of lateral plates.

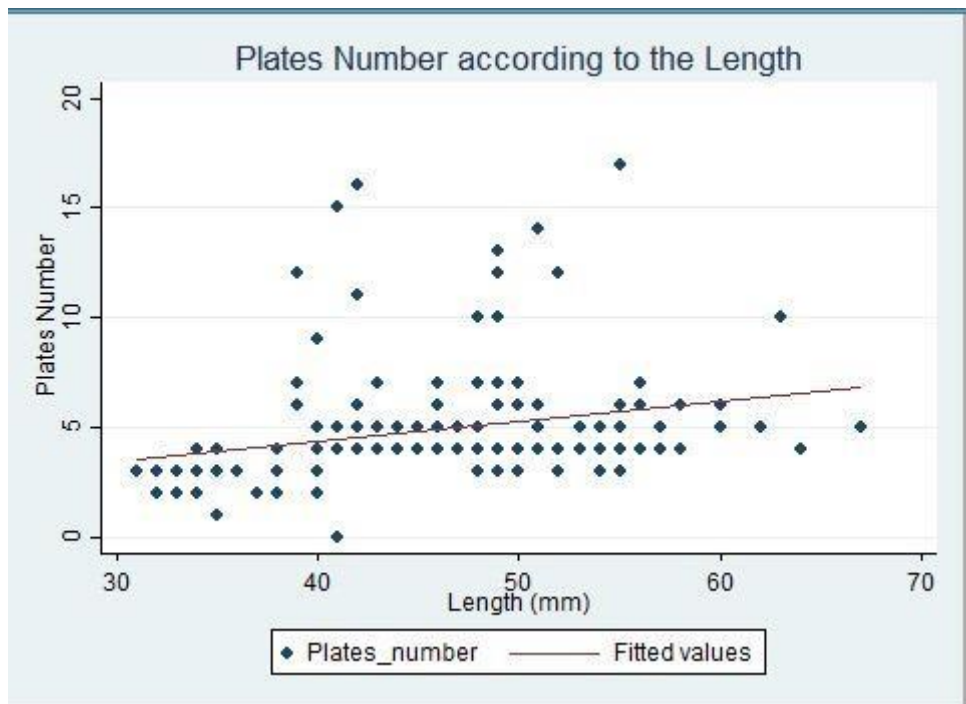


Figure 20: Evolution of the number of side plates according to the length of the fish

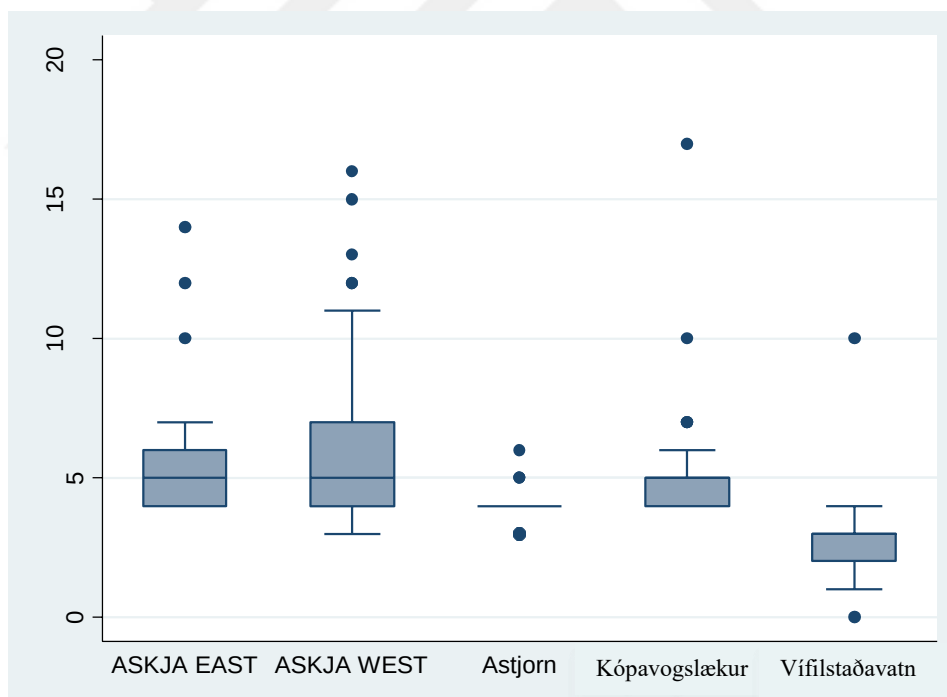


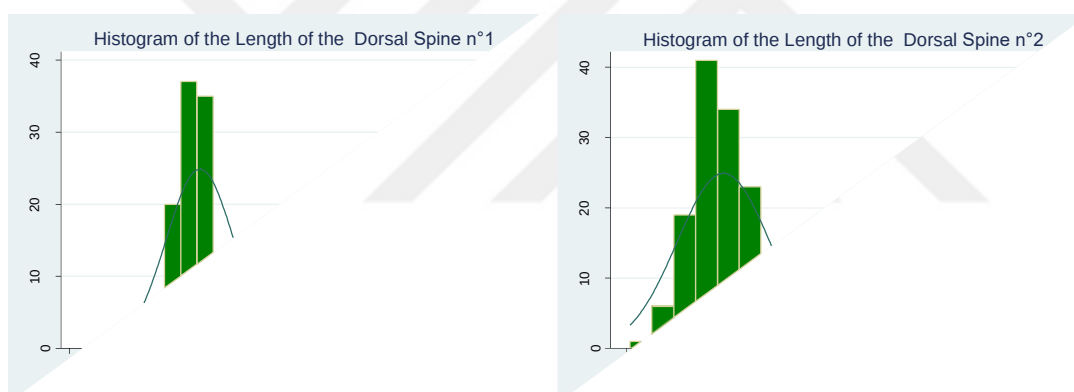
Figure 21: Comparison boxplots graph of plate number with each location

Table 13: STATA14.2 Regression model of plate number

Plates_number	Coef.	Std. Err.	t	P>t	[95% Conf.	Interval]
Sex						
F	0.2499052	0.4517484	0.55	0.581	-0.644601	1.144412
Length	0.0475705	0.0363201	1.31	0.193	-0.024346	0.119488
LocationNum						
ASKJA WEST	1.049283	0.7464164	1.41	0.162	-0.428696	2.527262
Astjorn	-2.3361	0.6826374	-3.42	0.001	-3.68779	-0.984409
Kópavogslækur	-1.049802	0.6226776	-1.69	0.094	-2.282765	0.1831622
Vífilstaðavatn	-2.187135	0.8346973	-2.62	0.01	-3.839919	-0.534350

4.4.2 Dorsal spines

Following results acquired from histograms of dorsal spine n°1 and n° 2, placed together because they evolve in the same way, both exhibit a right-skewed distribution, where most samples have shorter spine lengths with few having longer spines.

**Figure 22:** Histograms of the length of the Dorsal Spines number 1 and number 2

Based on p -values stated by ANOVA and regression estimation test, all done by STATA 14.2 software, both dorsal spines express a correlation with the length of the fish, as the p -value is less than 0.05 and regression coefficient sign is positive, as the length of spines within populations will keep increasing with less p -value (Figure 22). While the sex of the fish had no significant impact on dorsal spines (Tables 14 and 15).

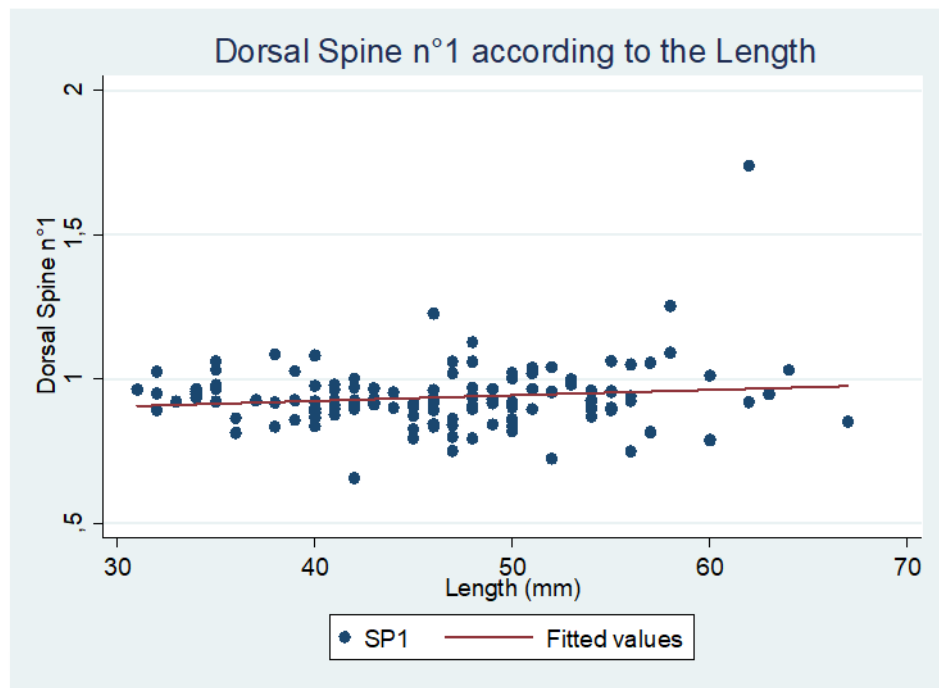


Figure 23: Dorsal spine n°1 in comparison with length (mm)

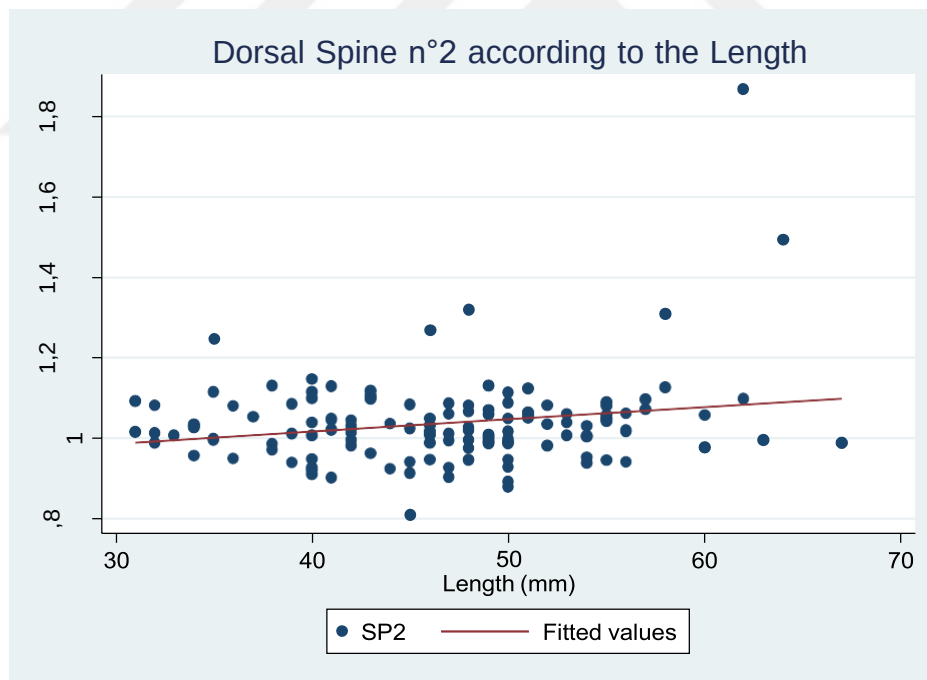


Figure 24: Dorsal spine n°2 in comparison with length (mm)

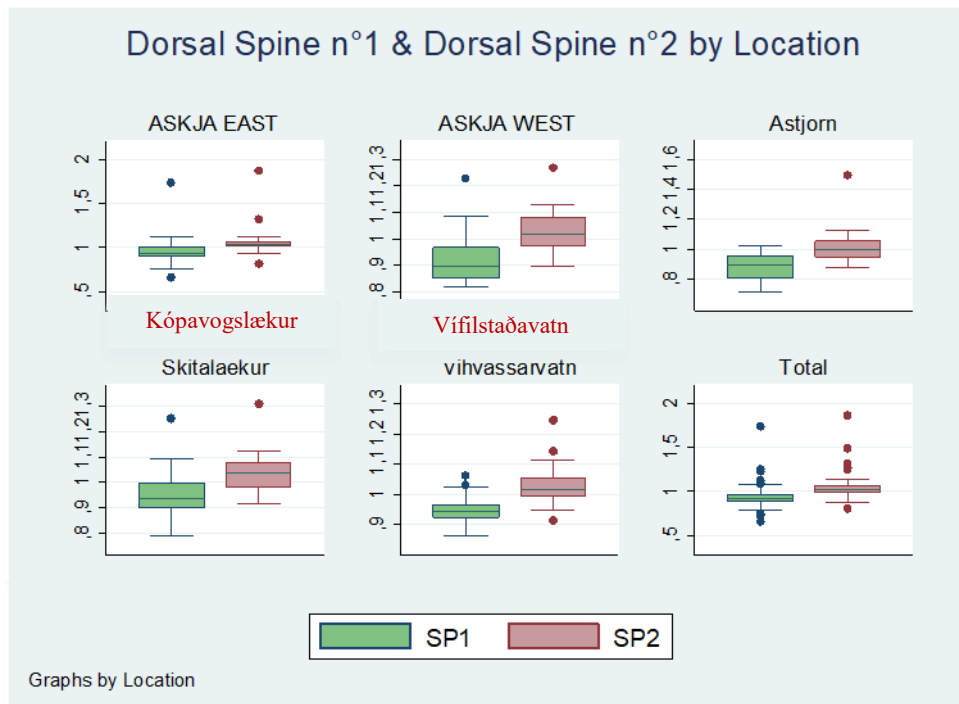


Figure 25: Dorsal spines variation according to locations (mm)

The figure 25 represents location factor effect on dorsal spines, which through the tests in Table 14 and 15, location does not influence these defensive traits.

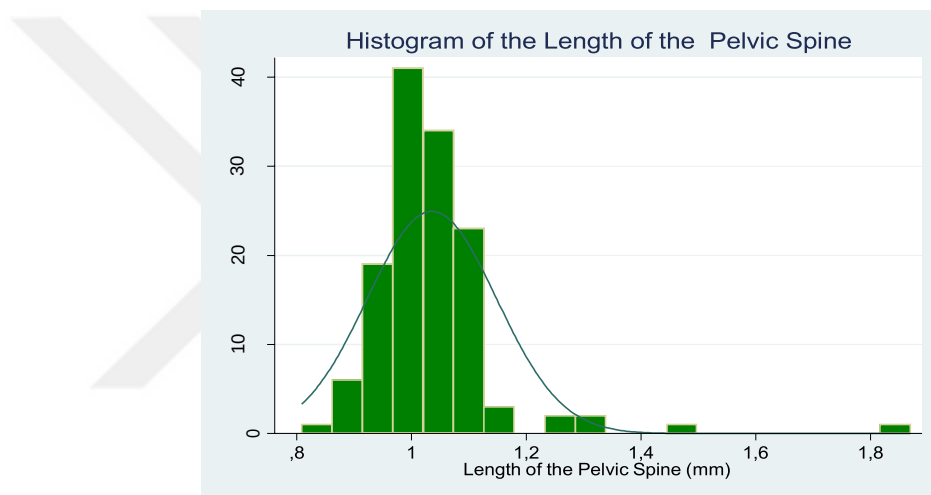
Table 14: STATA 14.2 Regression model of Dorsal Spine n°1 length

SP1	Coef.	Std. Err.	t	P>t	95%Conf	Interval]
Sex						
F	0.0328573	0.0264764	1.24	0.218	-0.019684	0.0853988
Length	0.0023437	0.0020585	1.14	0.054	-0.001741	0.0064288
LocationNum						
Askja West	-0.044878	0.038553	-1.16	0.247	-0.121385	0.031629
Astjorn	-0.094307	0.0399905	-2.36	0.02	-0.173667	-0.0149475
Kópavogslækur	-0.027799	0.0321904	-0.86	0.39	-0.091680	0.0360809
Vífilstadavatn	0.0036259	0.0460415	0.08	0.937	-0.087742	0.0949938

Table 15: STATA 14.2 Regression model of Dorsal Spine n°2 length

SP2	Coef.	Std. Err.	t	P> t	95% Conf	Interval]
Sex						
F	0.0169037	0.0275087	0.61	0.54	-0.037666	0.0714735
Length	0.0042141	0.0020769	2.03	0.045	0.000094	0.0083342
LocationNum						
Askja West	-0.030124	0.0401746	-0.75	0.455	-0.109819	0.0495713
Astjorn	-0.053504	0.0406178	-1.32	0.191	-0.134079	0.0270701
Kópavogslækur	-0.036431	0.0335446	-1.09	0.28	-0.102975	0.0301117
Vífilstadavatn	0.0246465	0.0471638	0.52	0.602	-0.068913	0.1182069

4.4.3 Pelvic Spine

**Figure 26:** Histogram of pelvic spine length (mm)

The evolution of the pelvic spine (PS) goes along with the evolution of dorsal spines. Similar distribution as well with the majority of samples having short pelvic spines or non-existing as it is with almost all fish within the Vífilstadavatn population.

The results performed by STATA 14.2 p-values table showed that in similarities with dorsal spines, the length of the sticklebacks has a great influence on the pelvic spine of three-spines sticklebacks, as its p-value is equal to 0.0021 (*p-value* < 0.05).

As for the linear regression model test, the *p-value* is indeed less than 0.05 with a regression coefficient sign that is positive, same results previous spines had, more individuals will have increased length of their pelvic spine with less *p-value*.

Station and sex are two non-influencing factors on the length of the pelvic spine.

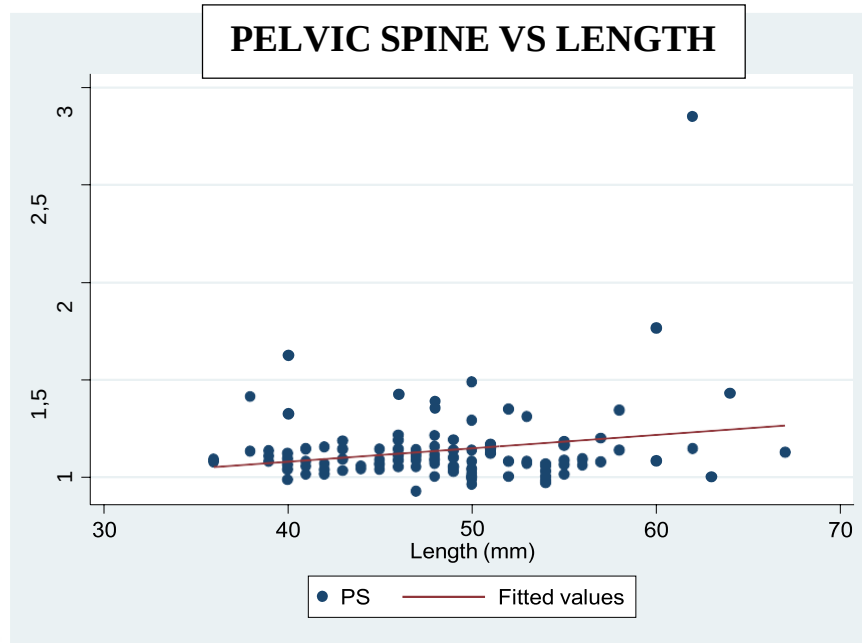


Figure 27: Evolution of Pelvic Spine according to the length (mm)

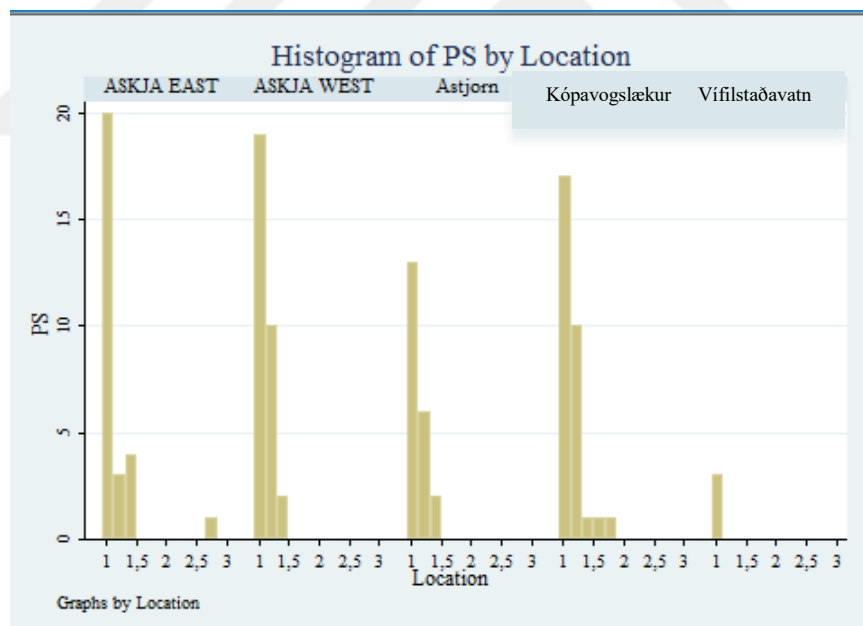


Figure 28: Pelvic spine variation according to stations (mm)

Table 16: STATA 14.2 Regression model of Pelvic spine length

PS	Coef.	Std. Err.	t	P>t	[95% Conf	Interval]
Sex						
F	-0.003848	0.0563257	-0.07	0.946	-0.115918	0.108222
Length	0.0077144	0.0042615	1.81	0.002	-0.000764	0.0161935
LocationNum						
ASKJAWEST	-0.080981	0.0788034	-1.03	0.307	-0.237775	0.0758129
Astjorn	-0.155743	0.0836148	-1.86	0.066	-0.322110	0.0106235
Kópavogslækur	-0.089299	0.0681451	-1.31	0.194	-0.224886	0.0462882
Vífilstadavatn	-0.186008	0.1517719	-1.23	0.224	-0.487986	0.1159702

Table 17: P-values of ANOVA realized models for all variables

Variables	<i>p_values</i>	
	Length	LocationNum
SP1	0.0549	0.3606
SP2	0.0001	0.8219
PS	0.0021	0.9813
PL	0.0020	0.9714

4.4.4 Number of Gill Rakers

The average of the gills rakers number is 13 as the histogram of the number of gills rakers revealed that the observations interval is between 7 to 22 (Figure 29). Statistically speaking, when the count of gills rakers increases, the frequency decreases, thus fewer samples to none with a higher number of GR out of the range provided.

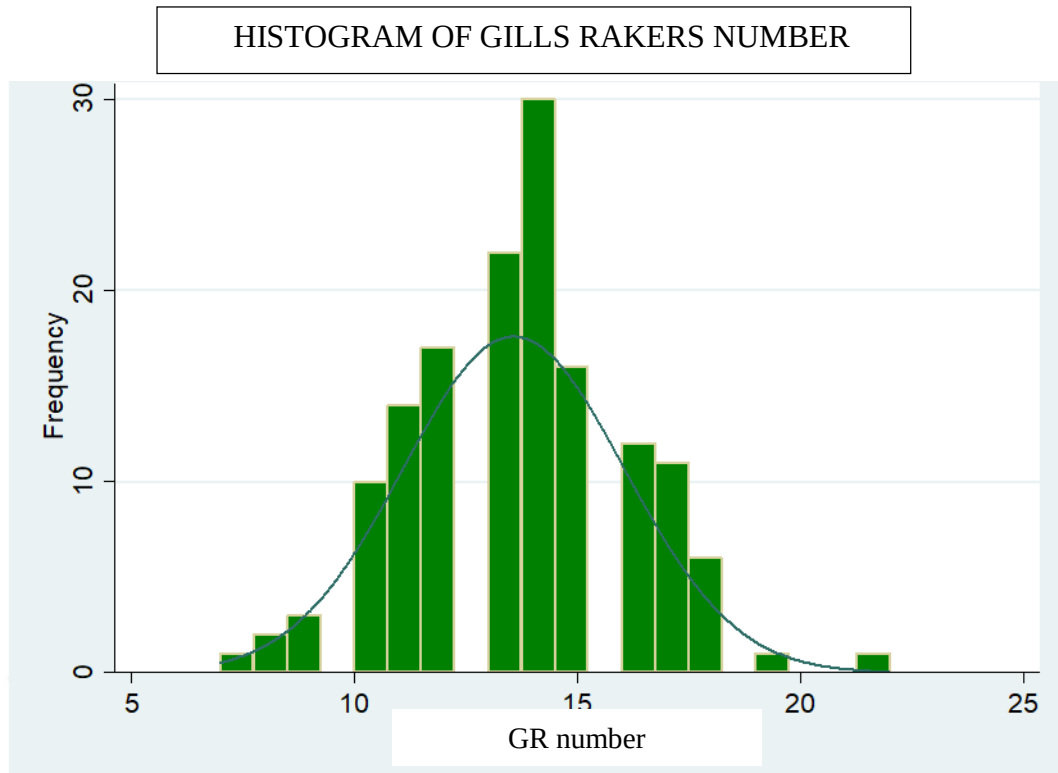


Figure 29: Histogram of gills rakers number

This variable through its histogram, shows a normal distribution of the gills rakers numbers, adding to that, it is a discrete variable as it was for lateral plates number, solely integer values are accepted.

ANOVA analysis determined that location had a significant effect as the p -value is smaller than 0.05 ($= 0.01$), while sex and length do not influence at all on rakers number. Similarly, the Regression model test confirmed a high correlation between location and GR number with a p -value < 0.05 , specifically for Åstjörn and Kópavogslækur. Thus, no proportional relationship between the number of gill rakers and the size of the fish.

Therefore, the gills rakers number varies strongly between populations, i.e. dependent solely on location.

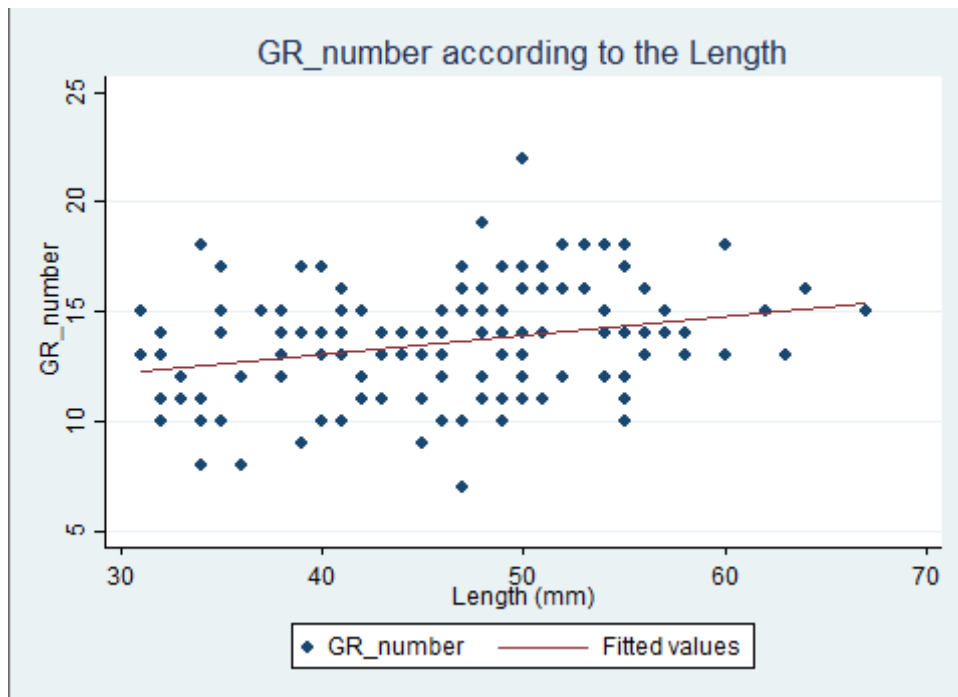


Figure 30: Gills rakers number according to length of the fish (mm)

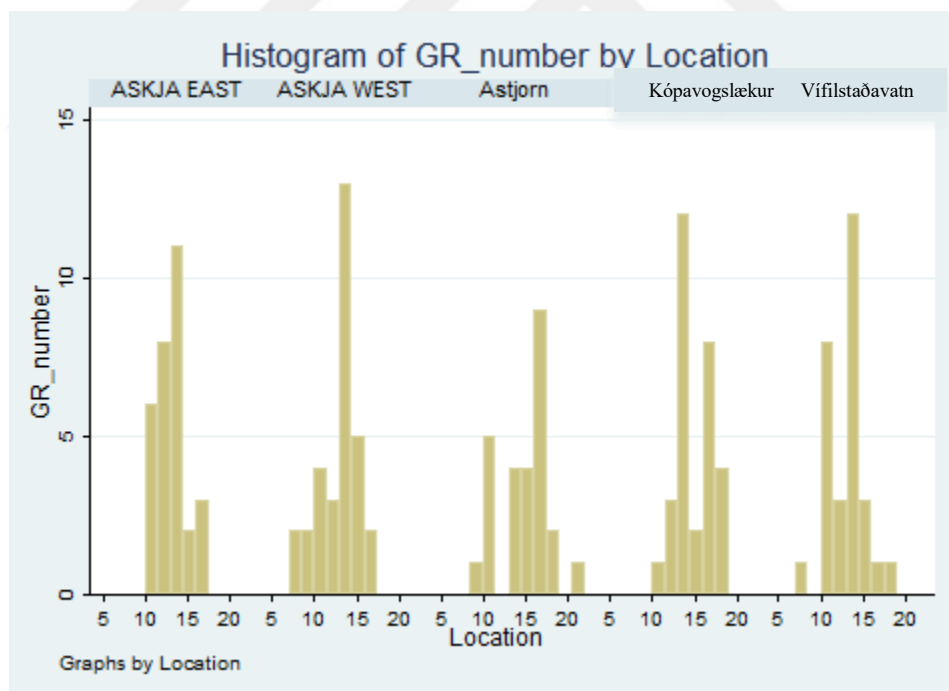


Figure 31: Gills rakers number based on Location

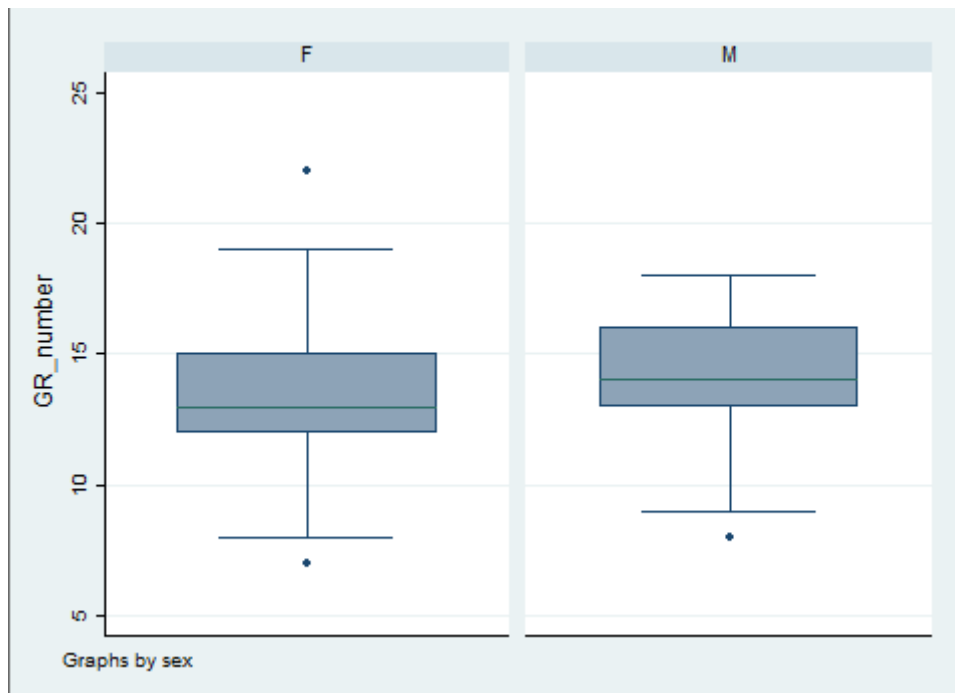


Figure 32: Gills rakers number based on Sex

4.4.5 Second and Third Gill Rakers and the space in between

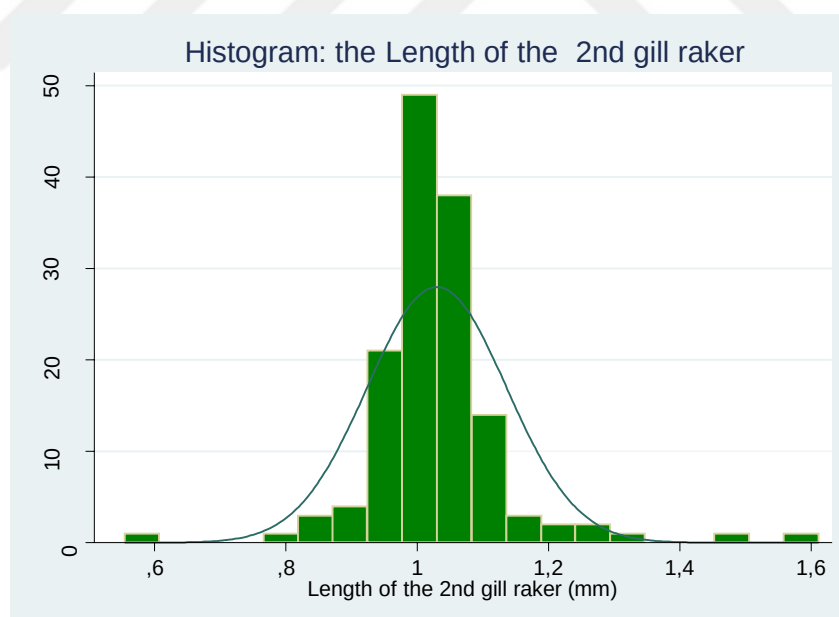


Figure 33: Distribution of length of the gill raker number 2 (mm)

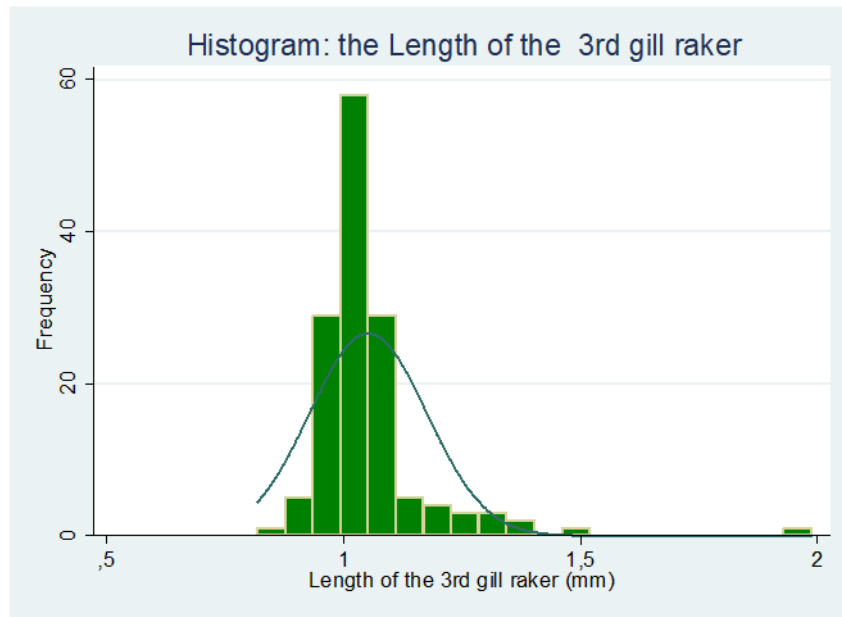


Figure 34: Distribution of length of the gill raker number 3 (mm)

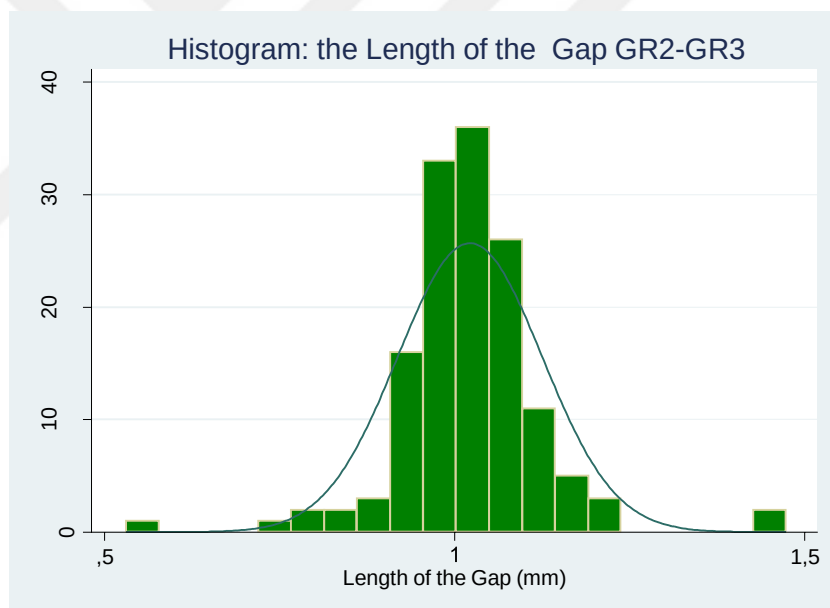


Figure 35: Distribution of the length of the gap between gill raker 2 and 3 (mm)

Through histograms of gill raker numbers 2 and 3, as well as the gap between them, we observed that the distribution of the three variables is towards the center, mostly stable, and observation lengths are within the range of 0.8 and 1.4.

The same data analysis tests were performed on variables of interest as the ones previously studied, the results were as follows:

- For the length of gill raker two, ANOVA *p-values* of the two characteristics sex and length of fish do not have any influence on the length of gill raker number 2. The same goes for linear regression model results, the *p-values* are beyond 0.05. However, location has a weak influence on the length of GR 2.
- For the length of gill raker three, *p-values* acquired by ANOVA analysis, it is observed that only location has a great influence on the length of gill raker three as its *p-value* is equal to $0.04 < 0.05$, similar results given by regression model test, where Kópavogslækur has a *p-value* less than 0.05.
- For the space between gill raker 2 and gill raker 3, sex, length and location of the specimens do not have any relationship with the space, according to ANOVA test and regression model assay.

Table 18: p-values ANOVA analysis results

	p_values	p_values	p_values
Variables	Sex	Length	LocationNum
Length_GR2	0.6359	0.8566	0.0531
Length_GR3	0.4878	0.5695	0.0445
GapGR2-GR3	0.9812	0.8655	0.6459
GR_Number	0.1328	0.8193	0.0196

Table 19: STATA 14.2 Regression model of gill raker 2 length(mm)

Length_GR2	Coef.	Std. Err.	t	P>t	[95% Conf	Interval]
Sex						
F	0.0171477	0.0211583	0.81	0.419	-0.024720	0.059016
Length	-0.000774	0.0017198	-0.45	0.653	-0.004178	0.0026283
LocationNum						
Askja West	0.0196269	0.0353842	0.55	0.58	-0.050392	0.0896458
Astjorn	-0.044918	0.0335382	-1.34	0.018	-0.111284	0.0214471
Kópavogslækur	0.0011905	0.0304283	0.04	0.969	-0.059021	0.0614026
Vífilstadavatn	0.0340014	0.0403942	0.84	0.04	-0.045931	0.1139343

Table 20: Regression test result of length of gill raker 3 (mm)

Length_GR3	Coef.	Std. Err.	t	P>t	[95% Conf	Interval]
Sex						
F	0.0306609	0.0273229	1.12	0.264	-0.023492	0.084814
Length	-0.001277	0.0022171	-0.58	0.566	-0.005672	0.0031165
LocationNum						
Askja West	-0.053991	0.0435327	-1.24	0.218	-0.140271	0.0322888
Astjorn	-0.067940	0.0410056	-1.66	0.1	-0.149212	0.0133316
Kópavogslækur	-0.091158	0.036581	-2.49	0.014	-0.163660	-0.018655
Vífilstadavatn	-0.026967	0.0497645	-0.54	0.589	-0.125599	0.0716641

Table 21: Regression model outcome of the space between gill raker 2 and 3(mm)

GapGR2GR3	Coef.	Std. Err.	t	P>t	[95% Conf	Interval]
Sex						
F	0.0056907	0.0227516	0.25	0.803	-0.039402	0.0507835
Length	0.0009558	0.0018462	0.52	0.606	-0.002703	0.0046149
LocationNum						
Askja West	0.0403397	0.0362493	1.11	0.268	-0.031505	0.1121847
Astjorn	0.0268497	0.0341451	0.79	0.433	-0.040824	0.0945242
Kópavogslækur	0.000755	0.0304608	0.02	0.98	-0.059617	0.0611272
Vífilstadavatn	0.0653455	0.0414386	1.58	0.118	-0.016784	0.1474754

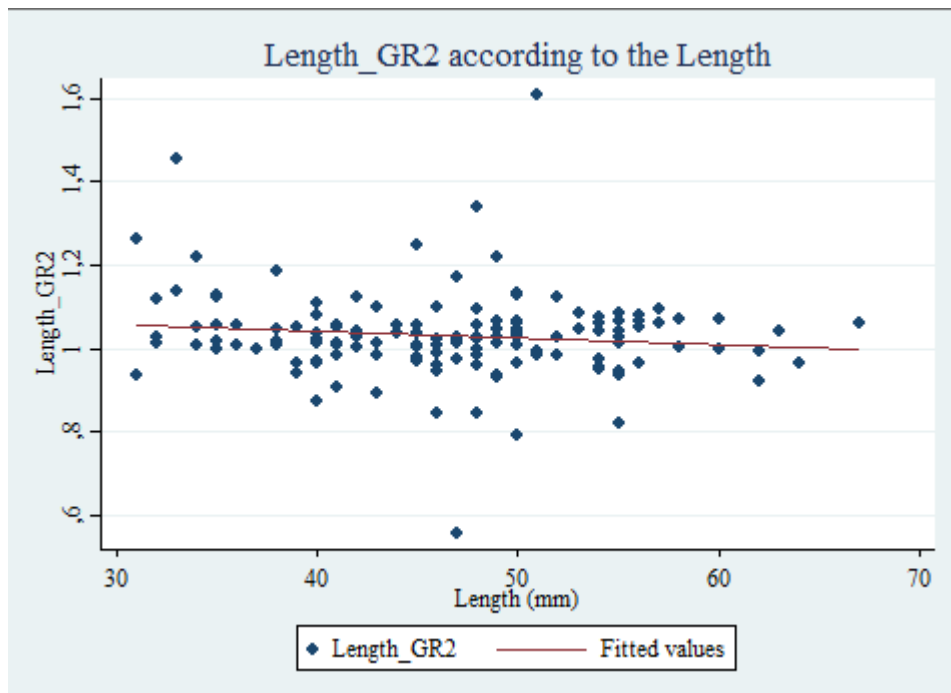


Figure 36: length of gill raker according to the length of the fish (mm)

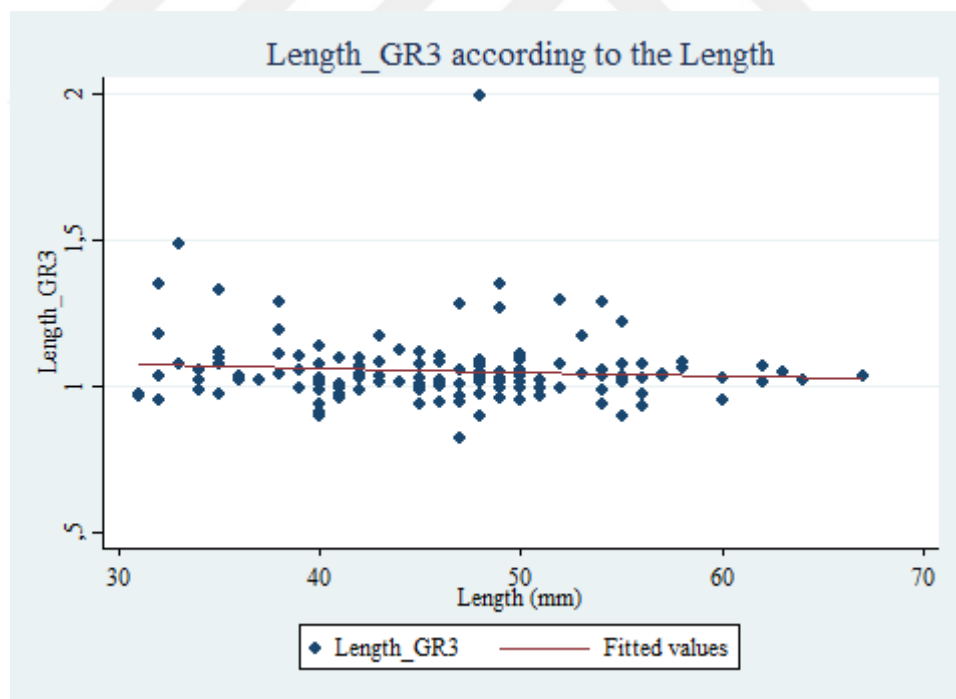


Figure 37: Length of gill raker 3 according to the length of fish (mm)

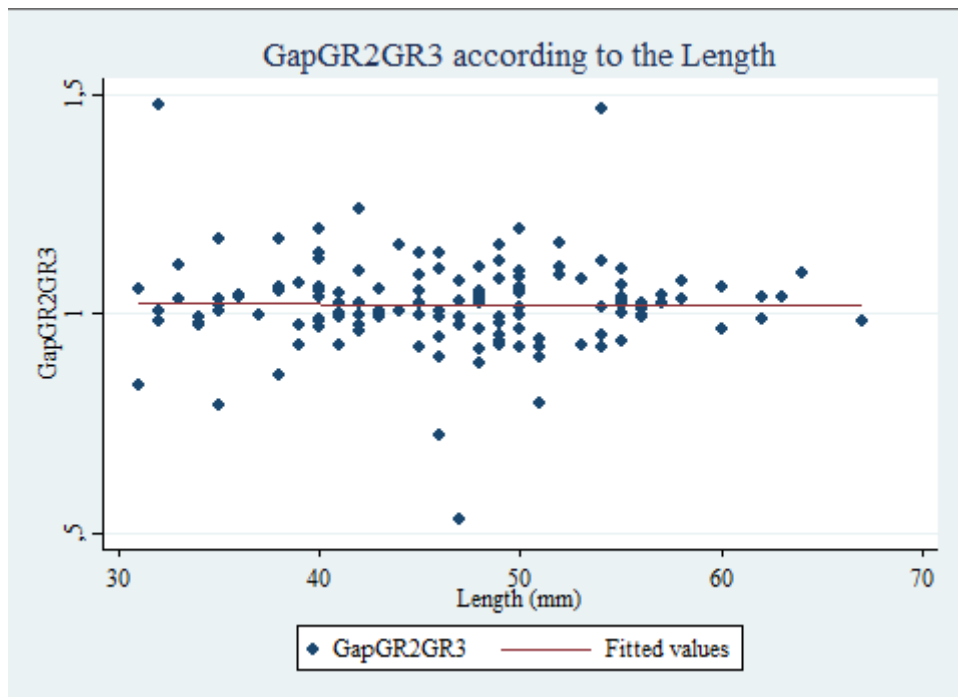


Figure 38: Gap length in accordance with the length of the sticklebacks (mm)

In compliance with the figures above, the graphs exhibit the inexistent relation between the length of the fish and the three dependent variables. Generally, as the fish increases in size, its gill rakers become longer (Felix Mendes, 2022). However, the mean length of total samples is below 50, which can explain the fact that the hypothesis of length might be a strong influence towards Y variables was wronged since most of the gill rakers length is observed within the interval of 30 to 46 mm, and a small number of sticklebacks are available above 50 mm.

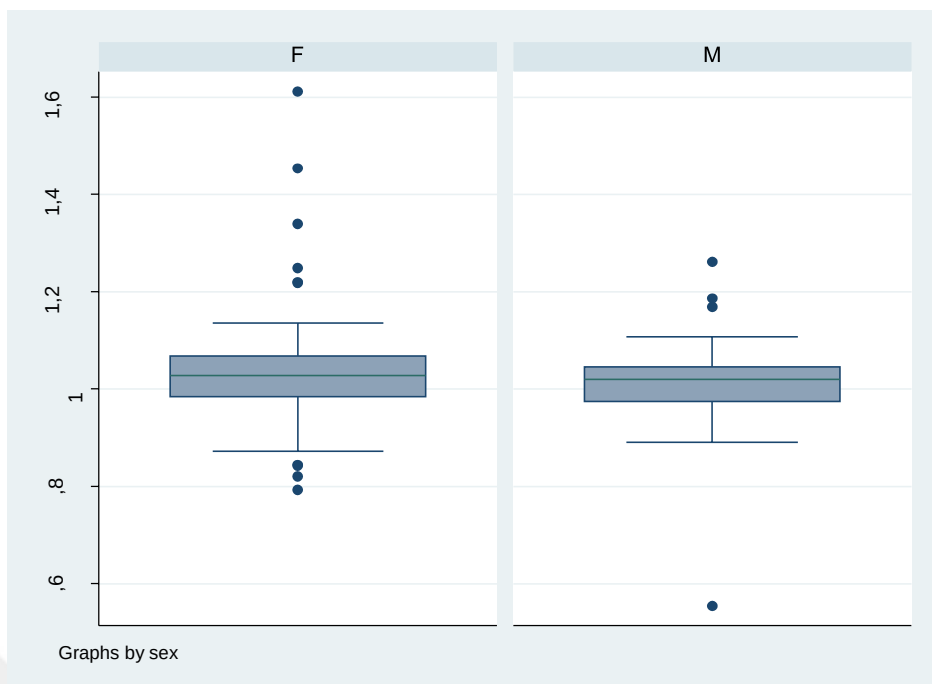


Figure 39: Length of gill raker 2 according to sex (F:female; M: male)

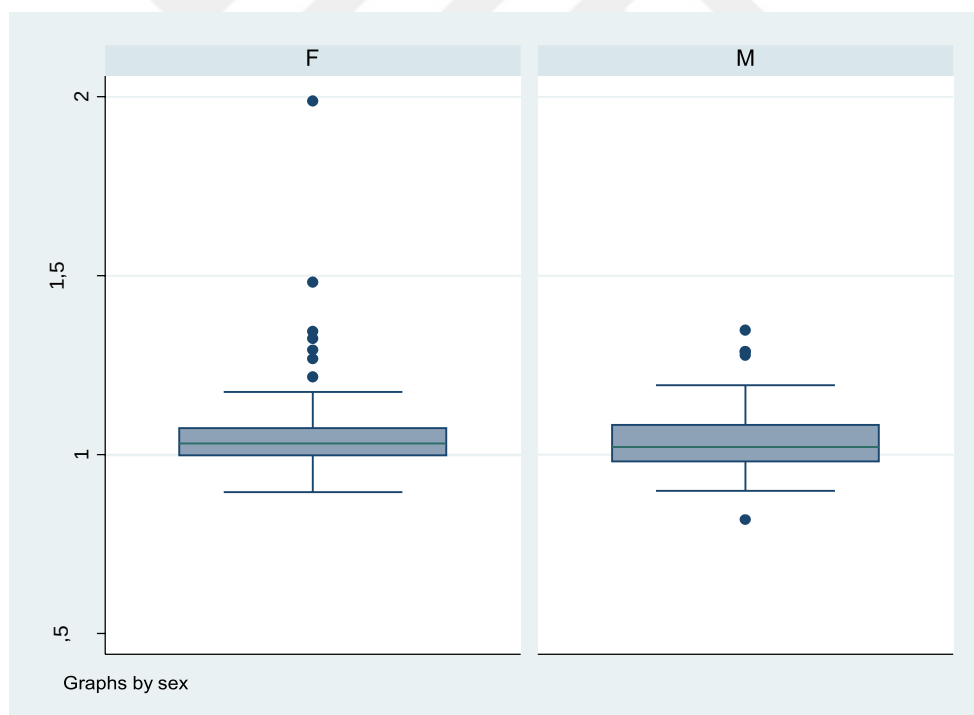


Figure 40: Length of gill raker 3 according to sex (F:female; M: male)

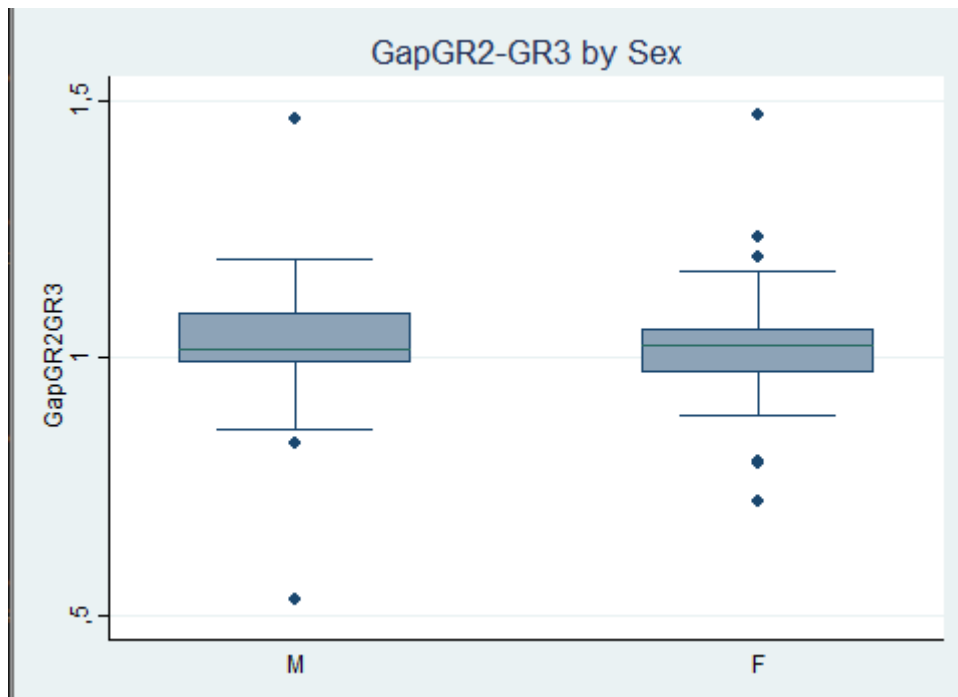


Figure 41: gill rakers gap according to sex (F: female; M: male)

In all, more or less the gill rakers 2 and 3 and the variable of gap in between according to sex, show a similarity between females and males individuals.

With the combination of ANOVA analysis and Regression test, the sex variable of three-spined stickleback fish does not influence at all on the evolution of the three mentioned variables.

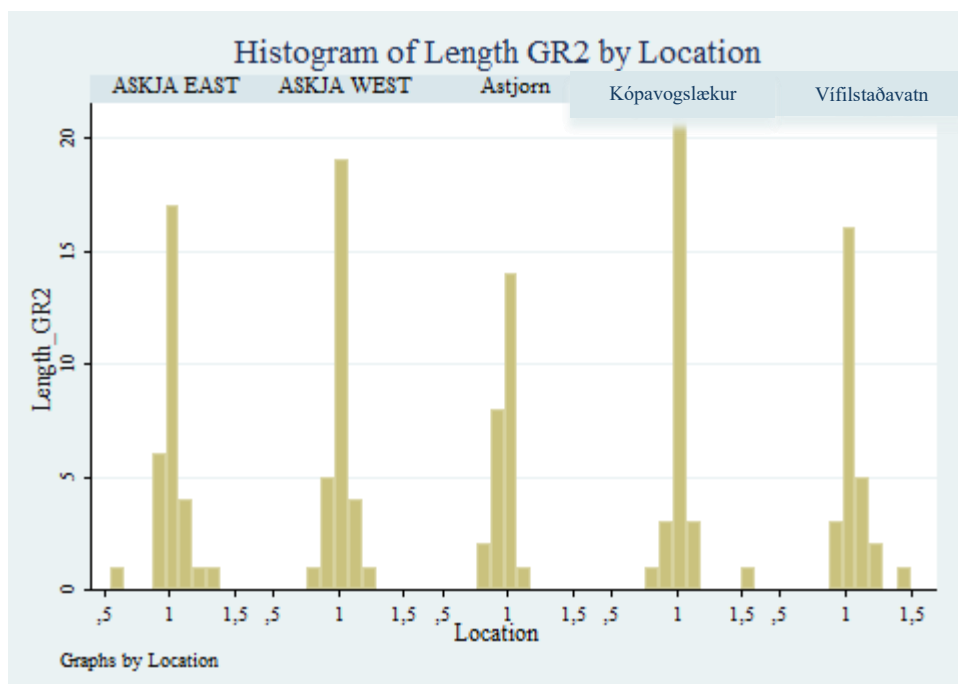


Figure 42: Length of Gill raker 2(mm) according to location

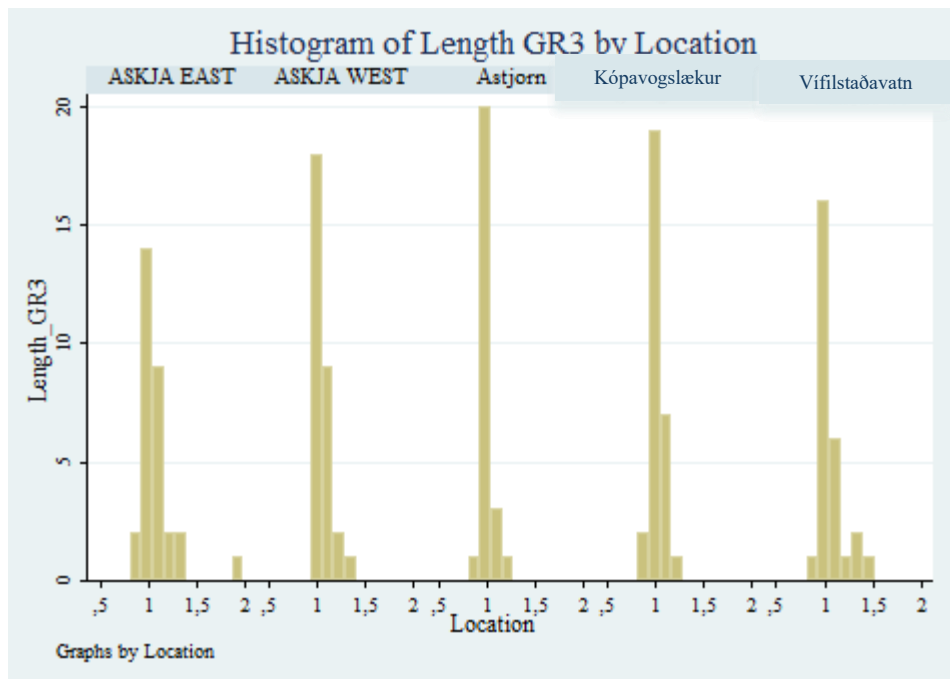


Figure 43: Length of Gill raker 3(mm) according to location

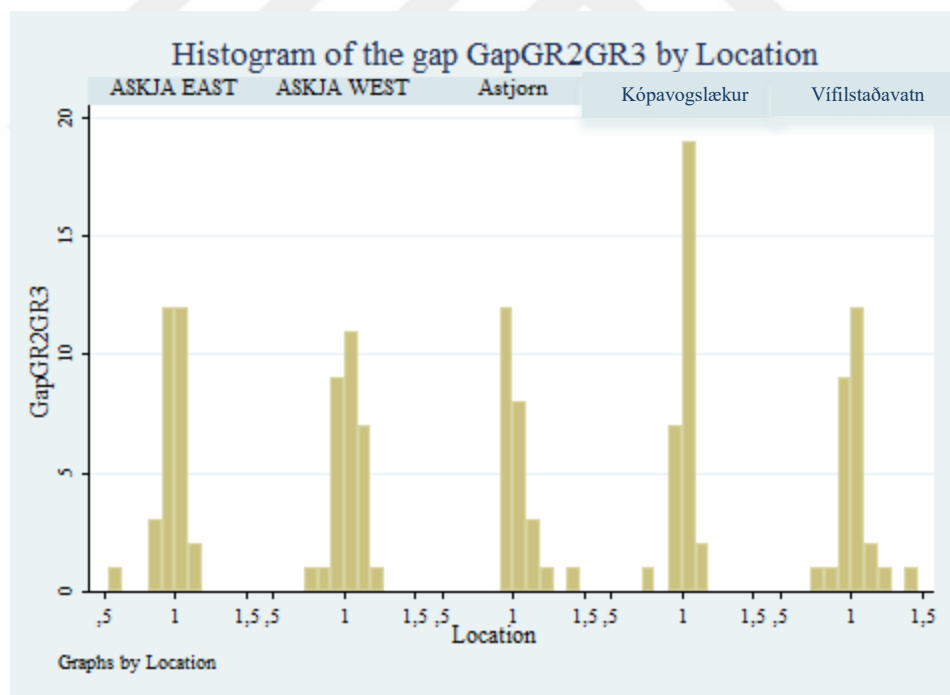


Figure 44: Space between gill raker 2 and 3 in terms of location

The histograms of the gap between gill raker n°2 and n°3, gill rakers n°2 and n°3 in compliance with location, demonstrate a great influence of the latter on gill rakers two

and three but not on the gap between them. To have a more thorough analysis, a sum-up table (Table 22) had been set representing each location and its influence on which location exactly per studied variables and general locations effect.

Table 22: Studied variables versus station occurrence.

	Àstjörn	Kópavogslækur	Vífilstadavatn	Askja east	Askja west	General
LengthGR2	X		X			X
LengthGR3		X				X
GapGR2_GR3						
Gill rakernum	X	X				X

CHAPTER 5 DISCUSSION

5. DISCUSSION

In this project, we aimed to study the variations occurring in genotype and the ones on phenotype related to the evolution of sticklebacks and their adaptation to the lentic system, mainly freshwater. First, we investigated whether Threespine stickleback fish were polymorphic on the given inversion chromosomes 1, 11 and 21 indicating genetic differentiation. Further examinations were on the morphological variation in stickleback populations. Finally, ecological variation through defensive traits associated or not with genomic differences within the fish.

Genomic variation

With 152 samples, 30 from each location (5), the right fin was dissected, providing DNA of samples. This fin tissue went through the Hotshot DNA extraction protocol, which later was added to the master mix containing primer sets adequate for the chromosome studied, One Taq DNA polymerase, and Nuclease-free water in each PCR 8-strip tube per sample, following the protocol previously mentioned in (**chapter 3**).

In total, for inversion chromosome 1 all the amplified markers had determined a small percentage of the Marine Homozygote genotype, but F genotype is highly frequent for this chromosome as it is abundant mostly in Askja East, Vífilstaðavatn and Àstjörn. Thus populations in this chromosome are polymorphic.

Carrying on chromosome 11, in a total of all locations, the Marine homozygote genotype is recurrent with 57 observations. Followed by the Heterozygote genotype with 25%, then 18.42% of the Freshwater homozygote genotype. The recurrence of M genotype is mostly observed in Askja East, Àstjörn and Vífilstaðavatn. However, for Kópavogslækur sticklebacks, the H genotype is the most abundant inversion as 14 samples exhibit this later, followed by the F genotype then few samples had the M genotype observed on gel. Similarly, for Askja west population, the F genotype is the abundant genotype, followed by the M genotype, but a low percentage of the H genotype.

Ultimately, for inversions occurring in Chromosome 21, it seems that Vífilstaðavatn, revealed a fixation of the M genotype and the absence of other inverted genotypes. Subsequently, the inverted Marine genotype is abundant in Àstjörn and Askja East. However, for Kópavogslækur and Askja West, it is the lowest observed genotype.

Through appendix 3, in general, Kópavogslækur stickleback population has the highest percentage and frequency beyond the average of the inverted Heterozygote genotype in all inversion chromosomes, continuing with Askja East, Àstjörn and Askja West populations then a low percentage of the H genotype in Vífilstaðavatn population.

To sum up, the data collected showed a genetic diversity leading to the distribution of different genotypes across various freshwater locations.

Furthermore, comparing the results of Marine habitats population data collected by Catherine Peichel in 2022 from the British Columbia region in Canada, 1151 stickleback fish in total named Oyster Lagoon with my results in this study, the most abundant genotype with the highest observation frequency is mainly the inverted M genotype. Consequently, in the same order for all the three inversion chromosomes, the Marine Homozygote genotype is highly abundant followed by the H genotype then a few observations with the F genotype (table 23).

Table 23: Marine Stickleback Population data of inversions in known chromosomes given by Catherine Peichel, University of Bern in 2022.

Chr1 M	Chr1 H	Chr1 F	Chr1 ND	Chr11 M	Chr 11 H	Chr11 F	Chr11 ND	Chr21 M	Chr 21 H	Chr21 F	Chr21 ND
1060	80	7	4	999	141	8	3	946	176	27	2

Comparing and contrasting freshwater populations of my study with marine populations of Catherine Peichel's Olyster lagoon population, although we do not have a similar total number of fish, overall interpretation, in terms of chromosome 1, the F genotype is greatly frequent in freshwater populations than what it is for marine populations. Similarly, the H genotype is the second recurrent genotype for this inversion, however, few individuals with the M genotype.

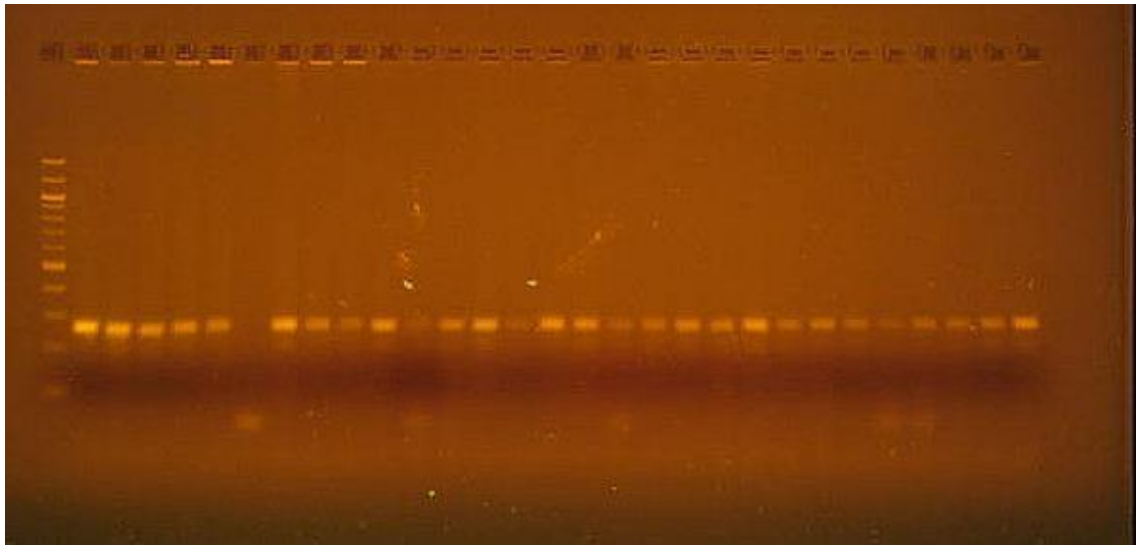
Moreover, in Chromosome 11, the M genotype is frequent in the Oyster Lagoon population suggesting adaptive advantage in marine conditions, as it is in freshwater populations of this research.

Lastly, for chromosome 21, the M genotype is the most abundant in both locations, with the least frequency in the F genotype. However, for all chromosomes, the frequency of the H genotype is similar between the marine population and freshwater populations, showing a higher rate of adaptive polymorphism in both.

Taken altogether, the M genotype occurs naturally on Chromosomes 11 and 21 at a high frequency rate in Stickleback populations, unlike in the inversion Chromosome 1, which is not prevalent. This can be attributed to the anadromous nature of sticklebacks, i.e. migrating from sea to spawn, that this M homozygote genotype provides advantages related to salinity tolerance, retaining ancestral traits crucial for adapting to new habitats and ensuring survival in both marine and freshwater environments.

The identified differences in genotype frequencies between marine and freshwater stickleback fish populations show a difference in the evolution of distinct genetic traits, meaning, each population has adapted to thrive in their specific environment which underlines the system of evolution where fish had to change over time to fit local conditions. These findings helped illustrate how environmental pressures shape the genetic composition of populations and lead to the evolution of traits that improve survival and reproduction in specific habitats.

For the case of the sex chromosome where the Y chromosome does not amplify, so the length of PCR products gave one band instead of two for males as it is in Figure 45. Many trials for solving this issue were taken into consideration, including having the opinions of other professors and the assistance of other students, PhD, and master students, as well as adapting the protocol given to me with theirs or trying different types of PCR, like Touchdown PCR, but all these different speculations proposed were not effective. Thus, it was hard to amplify the bands concluding that it might be due to unsuitable or bad primer pair that should not be used on these populations, even with known male and female sticklebacks as positive controls, however no amplifications.



osome agarose gel showing the DNA bands for females but no upper band formales.

Fortunately, I could detect the sex of sticklebacks through gonad examination while dissecting the fish.

PHENOTYPE DIVERGENCE

Threespine stickleback fish come with a vast interval of phenotypes with various morphological variations, as morphological traits. Left lateral plates, Dorsal spines 1 and 2, pelvic spine, and gill arches were photographed with a digital camera and examined according to those images in Image J software.

Through data collected, the Kópavogslækur population has the greatest positive results in all defensive traits counts as in plates number it goes up to 17, for gill numbers goes to 19 and so forth, followed by Askja West and Askja East, but Àstjörn and Vífilstaðavatn are the lowest in all counts of defensive traits.

As in the Vífilstaðavatn location, few sticklebacks have no lateral plates or go up to 4 plates number maximum. Whereas for dorsal spines, most fish in the population have one dorsal spine or are non-available. Similarly, for pelvic spine, only three samples had pelvic spine while others do not (Figure 46).



Figure 46: Spine deficient with absent pelvic spine stickleback in Vífilstaðavatn population

According to the study of Cheryll Tickle and Nicholas J. Cole (2004), where they focused on a population in freshwater with only one dorsal spine instead of three spines underlining the fact that this morphological change is due to environmental pressures conducting to evolutionary adaptation. For example, in some habitats, which could be the case of Vífilstaðavatn, predators might have a preference to prey with spines either easier to catch or grab by the pelvis, in this case,, this population selects to have fewer spines including unavailable pelvic spine (Shapiro et al. 2004). Secondly, it might be because of habitat characteristics, as having fewer spines reduces the risk of being trapped or enhances the ability to navigate through the environment. Also, it is energy-consuming to have the spines and other studied traits. Finally, it could be that this pond or lake does not have a risk of predation (Kitano J et al. 2008), so the morphology of sticklebacks differs from their ancestors and other freshwater populations with reduced phenotypic traits.

These defensive traits had been reduced after the transition of sticklebacks from marine to freshwater environments, as stickleback fish adapt to environmental changes either by inducing the traits related to morphological evolution or reducing all. So, through the given results, defense traits have diverse sizes and count from one location to another, as left side lateral plates, dorsal spines one and two, and pelvic spine were greatly correlated to the body length of the fish, whereas all, except the gap between gill rakers two and three and the pelvic spine, had a strong association with station they were collected from, either lake or pond. However, the sex factor did not have any interaction with the variation of these phenotypic traits.

ASSOCIATION OF ECOLOGICAL VARIATION WITH GENOMIC INVERSIONS

Interestingly, using the Regression model assay to study the effect of factors such as length, location, and sex on studied variables, which are the morphological traits, one of the influential factors examined in parallel was the chromosomal inversions, according to my findings (Appendix 1) it has been observed that precisely, lateral plates number and the space between gill raker two and three correlate highly with genetic variation as their *p-values* are less than 0.05.

Even though only two out of eight studied traits were influenced by chromosomal inversions, it has still provided significant proof that ecological variation is associated with the selection of these inversions. Chromosomal inversions impact frequently specific genomic regions containing genes that directly control traits essential for thriving in certain environments. In consequence, these genetic variations provide crucial survival and reproductive advantages, for example, in the appearance of predators, the three spine stickleback fish persists in their population through selection, where inversions maintain the beneficial allele and reduce the recombination within an affected region supporting the adaptation of morphological traits according to specific habitat sticklebacks are in. We might suggest through this analysis that the selection influencing these two traits might indirectly improve other traits within populations, thus the presence of inversions contributes highly to phenotypic diversity.

To affirm this hypothesis, many studies were done and are still ongoing all around the world on threespine stickleback species, associating the genetic and morphological adaptation and evolution of this vertebrate. In the article of (Catherine Peichel et al, 2001), researchers stated that these changes occurring on defensive traits, it is due to allelic differences, demonstrating that genetic variation contributes to morphological differentiation and these changes occur with the evolution and adaptation of the fish.

LIMITATIONS:

Concerning the difficulties faced while running this study, mainly the PCR analysis that took most of the time to work efficiently afterward. First, I had the issue of not having any DNA marker amplified or shown on agarose gel, i.e. only the DNA ladder appeared. Then, with the help of my lab mates who suggested changing a few steps in the protocol, PCR products of the characterized chromosomes of inversions perfectly demonstrated differences in their lengths on agarose gel. The second problem was with sex identification through PCR assay, while doing it for the first time, it was with a similar protocol as other chromosomes, however, after receiving the published paper where

initially our primers come from about rapid sexing of sticklebacks through PCR test, I followed the same protocol. The results were the same, so many different methods and trials were considered but without positive outcomes. As explained previously, examination of gonads determined the sex of the fish.

The third problem with PCR, few of the results were unamplified.

Furthermore, we planned with Professor Zophonias to examine parasites on sticklebacks with Professor Haseeb, unfortunately the time was not enough to continue this part of the research.

In all, it is normal for a scientist to face multiple issues throughout the study, since all this work done is highly subject to human mistakes and miscalculations, due to involving precise and repetitive manipulations. As an example, overstaining the fish because we only have an approximate duration in the protocol, damages the samples and cannot take images after staining of erected spines and perhaps even the lateral plates. Or the differences in measurements as they were done via ImageJ software, it is not precise and. Give the exact length, I had to do three times measurement to have the mean out of the three. Also, staining, pouring water, and so forth on fish, while they are in tea bags with the paper tag in, is not convenient at all, it is advantageous only while staining so the tag does not go far from its appropriate sample but when pouring liquids in general and then I should photograph the fish along with its tag, mostly the labeling paper gets cut within the previous process making it difficult to realize which sample is it, adding to that, the tea bags since they are papers they rip apart, so always need a full stock of tea bags to replace the torn ones.

To sum up, as a student, this experience of working on my master's thesis on a subject completely different from my background and in the laboratory, increased my competencies and pointed out what I mastered and what I am weak at. It was a great opportunity to do my research in another country and to discover different aspects of Molecular Biology and Genetics.

CHAPTER 6 CONCLUSION

6. CONCLUSION and RECOMMENDATIONS

In this project, we investigated the genetic and phenotypic divergence in Threespine stickleback fish across multiple freshwater locations. Our findings revealed that chromosomes 1, 11 and 21 exhibited significant genetic diversity with varying frequencies for inverted Freshwater homozygote, Marine homozygote, and Heterozygote genotypes through PCR-based assay. The variations indicate local adaptation conducted by environmental conditions and genetic historical backgrounds. Meanwhile, the prevalence of certain genotypes in certain chromosomes and/or in specific populations insinuated that these fish have evolved distinct traits according to their respective habitats.

Moreover, the morphological analysis showed a prevalent difference in phenotypical traits among populations, while some had long and complete spines and numerous plates, others had absent spines. Hence, attribution of evolutionary characteristics on stickleback fish. Furthermore, these defensive traits along with demonstrating a strong correlation with body length and location, some of them had a direct correlation with chromosomal inversions, proving the importance of genetic variation in phenotypic diversity and ecological adaptation.

In conclusion, the genetic and phenotypic divergence observed in Threespine stickleback populations highlights the complex relationship between inversions, phenotypic information,, and environment in evolutionary processes.

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APPENDIX 1 **Regression estimation test of studied variables correlated with** **chromosomal inversions**

Length_GR2	Coef.	Std. Err.	t	P>t	[95% Conf.	Interval]
CHR1						
ND	-0.0047092	0.0720359	-0.07	0.948	-0.1472552	0.1378368
CHR11						
F	0.0181757	0.0280112	0.65	0.518	-0.0372535	0.0736049
H	0.0394625	0.0319495	1.24	0.219	-0.0237598	0.1026847
ND	0.0157504	0.0520465	0.3	0.763	-0.0872402	0.118741
CHR21						
F	-0.0078549	0.0313266	-0.25	0.802	-0.0698447	0.0541348
H	-0.0100036	0.0269028	-0.37	0.711	-0.0632394	0.0432321
ND	-0.0441559	0.054084	-0.82	0.416	-0.1511784	0.0628666

Length_GR3	Coef.	Std. Err.	t	P>t	[95% Conf.	Interval]
CHR11						
F	0.0228486	0.03387	0.67	0.501	-0.0442806	0.0899778
H	0.0301978	0.038554	0.78	0.435	-0.046215	0.1066106
CHR21						
F	0.0867713	0.038081	2.28	0.025	0.0112959	0.1622467
H	0.0137616	0.0330864	0.42	0.678	-0.0518145	0.0793377

GapGR2GR3	Coef.	Std. Err.	t	P>t	[95% Conf.	Interval]
CHR11						
F	0.0556064	0.0282033	1.97	0.051	-0.0002916	0.1115044
H	0.0354449	0.0321036	1.1	0.272	-0.0281834	0.0990733
CHR21						
F	0.0439607	0.0317098	1.39	0.168	-0.0188871	0.1068085
H	0.0148604	0.0275508	0.54	0.591	-0.0397443	0.0694652

GR_number	Coef.	Std. Err.	t	P>t	[95% Conf.	Interval]
CHR11						
F	0.8042295	0.5940563	1.35	0.179	-0.3727032	1.981162
H	1.099402	0.6781431	1.62	0.108	-0.2441214	2.442926
CHR21						
F	0.7437091	0.6796204	1.09	0.276	-0.6027415	2.09016
H	0.0279238	0.5880575	0.05	0.962	-1.137124	1.192972

Plates_number	Coef.	Std. Err.	t	P>t	[95% Conf.Interval]
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CHR11						
F	0.8004696	0.5602646	1.43	0.156	-0.3089104	1.909849
H	1.625624	0.6435872	2.53	0.013	0.3512571	2.899991
CHR21						
F	0.348317	0.6479704	0.54	0.592	-0.9347292	1.631363
H	-0.2382402	0.5613181	-0.42	0.672	-1.349706	0.8732257

SP1	Coef.	Std. Err.	t	P>t	[95% Conf.	Interval]
CHR11						
F	0.0375651	0.0318126	1.18	0.241	-0.025566	0.1006962
H	0.0543101	0.034992	1.55	0.124	-0.0151304	0.1237506
CHR21						
F	-0.0169474	0.0340822	-0.5	0.62	-0.0845824	0.0506876
H	-0.0282357	0.0296961	-0.95	0.344	-0.0871666	0.0306952

SP2	Coef.	Std. Err.	t	P>t	[95% Conf.	Interval]
CHR11						
F	0.0247421	0.0334635	0.74	0.461	-0.0416406	0.0911248
H	0.0657691	0.0365881	1.8	0.075	-0.0068118	0.1383501
CHR21						
F	-0.0280911	0.0351322	-0.8	0.426	-0.0977839	0.0416017
H	-0.0347308	0.0305773	-1.14	0.259	-0.0953878	0.0259263

PS	Coef.	Std. Err.	t	P>t	[95% Conf.	Interval]
CHR11						
F	0.1291217	0.0817274	1.58	0.118	-0.0334903	0.2917336
H	0.1493819	0.0806428	1.85	0.068	-0.011072	0.3098357
CHR21						
F	-0.04864	0.0699018	-0.7	0.489	-0.1877227	0.0904427
H	-0.050948	0.0599586	-0.85	0.398	-0.1702469	0.0683508

4.1 APPENDIX 2
Two tables of Variables values and measurements for each sample in each population

Location	Year of collection	Fish_ID	sex	Length	weight	GR_Number	Length_GR2	Length_GR3	GapGR2/3
ASKJA EAST	5/4/2024	SG24001	M	41	0,5766	16	1,054	1,095	1,047
ASKJA EAST	5/4/2024	SG24002	F	49	1,0713	11	1,029	1,016	1,081
ASKJA EAST	5/4/2024	SG24003	F	49	1,1144	14	1,063	1,044	1,156
ASKJA EAST	5/4/2024	SG24004	F	52	1,5822	12	1,125	1,291	1,108
ASKJA EAST	5/4/2024	SG24005	F	62	1,8521	15	0,992	1,013	1,036
ASKJA EAST	5/4/2024	SG24006	F	54	1,5714	12	0,974	1,035	0,924
ASKJA EAST	5/4/2024	SG24007	F	55	1,7888	12	1,084	1,217	1,066
ASKJA EAST	5/4/2024	SG24008	F	55	1,5928	17	1,065	1,073	1,025
ASKJA EAST	5/4/2024	SG24009	F	56	1,5212	13	1,065	1,077	0,995
ASKJA EAST	5/4/2024	SG24010	F	51	1,0864	14	0,991	0,994	0,926
ASKJA EAST	5/4/2024	SG24011	F	62	2,4223	15	0,919	1,070	0,988
ASKJA EAST	5/4/2024	SG24012	M	49	1,0333	10	0,929	1,348	0,952
ASKJA EAST	5/4/2024	SG24013	F	45	0,8329	11	1,248	1,115	1,052
ASKJA EAST	5/4/2024	SG24014	F	50	1,1061	12	1,131	1,107	1,046
ASKJA EAST	5/4/2024	SG24015	F	51	1,2492	11	0,985	0,989	0,903
ASKJA EAST	5/4/2024	SG24016	F	48	1,2488	12	1,339	1,988	1,050
ASKJA EAST	5/4/2024	SG24017	F	42	0,7210	12	1,003	0,983	0,998
ASKJA EAST	5/4/2024	SG24018	F	56	1,9686	14	1,078	1,028	1,011
ASKJA EAST	5/4/2024	SG24019	F	46	0,9349	14	0,987	1,017	0,901
ASKJA EAST	5/4/2024	SG24020	F	48	0,9277	14	0,960	1,033	0,888
ASKJA EAST	5/4/2024	SG24021	F	48	1,1112	11	1,055	1,066	1,036
ASKJA EAST	5/4/2024	SG24022	F	50	1,1267	14	1,033	1,095	1,084
ASKJA EAST	5/4/2024	SG24023	F	42	0,6126	12	1,034	1,092	0,974
ASKJA EAST	5/4/2024	SG24024	M	41	0,6347	14	0,905	0,972	0,927
ASKJA EAST	5/4/2024	SG24025	F	39	0,6102	14	1,053	0,992	0,928
ASKJA EAST	5/4/2024	SG24026	M	47	0,9163	16	0,555	0,818	0,531
ASKJA EAST	5/4/2024	SG24027	F	48	0,9612	12	1,000	1,045	0,967
ASKJA EAST	5/4/2024	SG24028	M	47	0,8276	10	1,169	1,276	1,075
ASKJA EAST	5/4/2024	SG24029	F	40	0,6376	13	0,872	0,908	0,968
ASKJA EAST	5/4/2024	SG24030	F	60	1,9429	13	1,068	1,027	0,964
ASKJA WEST	5/4/2024	SG24111	F	42	0,7553	12	1,025	1,068	1,237
ASKJA WEST	5/4/2024	SG24112	F	46	0,9193	12	1,099	0,998	1,138
ASKJA WEST	5/4/2024	SG24113	M	42	0,7733	15	1,041	1,025	1,096
ASKJA WEST	5/4/2024	SG24114	F	49	1,3502	15	1,219	1,267	0,978

ASKJA WEST	5/4/2024	SG24115	F	50	1,1235	11	1,129	1,031	1,095
ASKJA WEST	5/4/2024	SG24116	F	54	1,2883	14	1,059	1,057	1,121
ASKJA WEST	5/4/2024	SG24117	F	46	0,9347	14	1,007	1,010	1,006
ASKJA WEST	5/4/2024	SG24118	M	45	0,8076	13	1,038	1,027	1,090
ASKJA WEST	5/4/2024	SG24119	F	50	1,1539	11	1,046	1,035	0,999
ASKJA WEST	5/4/2024	SG24120	F	42	0,6484	15	1,121	1,026	0,960
ASKJA WEST	5/4/2024	SG24121	M	40	0,4985	14	1,108	1,133	1,138
ASKJA WEST	5/4/2024	SG24122	M	46	0,8517	15	1,021	1,101	0,991
ASKJA WEST	5/4/2024	SG24123	M	50	1,2737	13	1,027	0,954	1,058





ASKJA WEST	5/4/2024	SG24124	M	45	0,7362	9	1,055	0,987	0,998
ASKJA WEST	5/4/2024	SG24125	F	46	0,8547	10	0,844	1,078	0,722
ASKJA WEST	5/4/2024	SG24126	M	36	0,3757	8	1,054	1,019	1,041
ASKJA WEST	5/4/2024	SG24127	F	41	0,6109	14	0,984	0,990	1,000
ASKJA WEST	5/4/2024	SG24128	M	44	1,1000	13	1,057	1,013	1,158
ASKJA WEST	5/4/2024	SG24129	M	49	0,9234	13	1,028	0,960	0,938
ASKJA WEST	5/4/2024	SG24130	F	50	1,1283	12	NA	NA	NA
ASKJA WEST	5/4/2024	SG24131	F	50	1,1163	14	1,066	0,995	1,047
ASKJA WEST	5/4/2024	SG24132	M	40	0,5886	14	1,020	0,982	1,126
ASKJA WEST	5/4/2024	SG24133	M	38	0,4672	13	1,015	1,193	1,169
ASKJA WEST	5/4/2024	SG24134	M	46	0,7527	13	0,945	0,946	1,103
ASKJA WEST	5/4/2024	SG24135	M	43	0,7297	14	0,890	1,082	0,990
ASKJA WEST	5/4/2024	SG24136	M	38	0,4478	15	1,044	1,288	0,861
ASKJA WEST	5/4/2024	SG24137	M	39	0,4540	9	0,942	1,099	1,070
ASKJA WEST	5/4/2024	SG24138	M	39	0,5453	17	0,965	1,056	0,974
ASKJA WEST	5/4/2024	SG24139	F	47	0,9790	7	1,028	1,052	1,027
ASKJA WEST	5/4/2024	SG24140	M	40	0,5971	17	0,964	1,025	1,038
ASKJA WEST	5/4/2024	SG24141	M	42	0,6744	11	1,035	1,041	1,022
Àstjörn	7/9/2023	ZJ23101	F	57	2,4739	15	1,061	1,034	1,042
Àstjörn	7/9/2023	ZJ23102	F	55	1,7582	18	1,011	1,025	1,018
Àstjörn	7/9/2023	ZJ23103	M	41	0,6064	15	1,010	0,994	0,991
Àstjörn	7/9/2023	ZJ23104	M	47	1,0745	16	1,012	0,942	0,973
Àstjörn	7/9/2023	ZJ23105	M	50	1,2279	17	1,006	1,050	1,013
Àstjörn	7/9/2023	ZJ23106	M	55	1,3177	17	1,042	1,035	1,031
Àstjörn	7/9/2023	ZJ23107	M	54	1,9042	18	1,075	1,284	1,467
Àstjörn	7/9/2023	ZJ23108	F	55	1,6777	10	0,946	1,018	1,002
Àstjörn	7/9/2023	ZJ23109	F	64	2,1667	16	0,964	1,019	1,093
Àstjörn	7/9/2023	ZJ231010	M	49	1,1661	17	1,014	0,989	0,994
Àstjörn	7/9/2023	ZJ231011	F	54	1,4419	15	0,955	0,988	0,952
Àstjörn	7/9/2023	ZJ231012	M	47	1,0539	17	0,974	1,003	0,992
Àstjörn	7/9/2023	ZJ231013	M	56	1,6513	16	1,053	0,975	0,992
Àstjörn	7/9/2023	ZJ231014	M	50	1,0060	16	0,963	1,015	1,194
Àstjörn	7/9/2023	ZJ231015	F	40	0,6323	13	1,022	1,006	1,062
Àstjörn	7/9/2023	ZJ231016	M	52	1,1494	16	0,983	1,075	1,159
Àstjörn	7/9/2023	ZJ231017	M	55	1,5770	11	0,935	1,015	0,936
Àstjörn	7/9/2023	ZJ231018	M	50	0,8890	NA	NA	NA	NA
Àstjörn	7/9/2023	ZJ231019	M	51	1,1444	NA	NA	NA	NA
Àstjörn	7/9/2023	ZJ231020	M	48	0,9444	14	1,094	1,027	1,104
Àstjörn	7/9/2023	ZJ231021	F	50	1,2244	22	0,793	1,088	0,925
Àstjörn	7/9/2023	ZJ231022	F	48	0,8870	15	NA	NA	NA

Àstjörn	7/9/2023	ZJ231023	F	41	0,5500	10	1,005	1,009	1,024
Àstjörn	7/9/2023	ZJ231024	F	48	1,0940	11	0,842	0,898	1,023
Àstjörn	7/9/2023	ZJ231025	F	40	0,5210	NA	NA	NA	NA
Àstjörn	7/9/2023	ZJ231026	M	53	1,5088	NA	NA	NA	NA
Àstjörn	7/9/2023	ZJ231027	F	45	0,6955	14	0,970	1,009	1,137
Àstjörn	7/9/2023	ZJ231028	F	54	1,4180	14	1,042	0,987	0,953
Àstjörn	7/9/2023	ZJ231029	F	49	1,1700	10	0,935	1,024	0,927
Àstjörn	7/9/2023	ZJ231030	F	45	0,8830	9	1,009	1,074	0,926
Kópavogslækur	7/9/2023	ZJ23110	M	48	1,1654	16	0,983	1,084	1,033
Kópavogslækur	7/9/2023	ZJ23111	F	58	1,8440	13	1,069	1,082	1,076
Kópavogslækur	7/9/2023	ZJ23112	F	57	1,6131	14	1,092	1,043	1,023
Kópavogslækur	7/9/2023	ZJ23113	F	50	0,9869	17	1,035	1,053	1,059
Kópavogslækur	7/9/2023	ZJ23114	M	52	1,2458	18	1,029	0,994	1,087
Kópavogslækur	7/9/2023	ZJ23115	F	50	1,1975	12	1,059	1,036	0,963
Kópavogslækur	7/9/2023	ZJ23116	F	48	1,0254	19	0,984	0,973	1,050
Kópavogslækur	7/9/2023	ZJ23117	F	43	0,7241	11	0,985	1,015	1,057
Kópavogslækur	7/9/2023	ZJ23118	F	51	1,2206	16	1,611	1,018	0,943
Kópavogslækur	7/9/2023	ZJ23119	F	48	0,9500	16	1,029	1,089	1,048
Kópavogslækur	7/9/2023	ZJ231110	F	67	2,9356	15	1,061	1,033	0,985
Kópavogslækur	7/9/2023	ZJ231111	M	53	1,3007	16	1,048	1,038	1,078
Kópavogslækur	7/9/2023	ZJ231112	M	48	1,0286	16	1,023	1,012	0,921
Kópavogslækur	7/9/2023	ZJ231113	F	60	1,6563	18	0,999	0,949	1,062
Kópavogslækur	7/9/2023	ZJ231114	F	53	1,4222	18	1,084	1,173	0,930
Kópavogslækur	7/9/2023	ZJ231115	F	58	1,4313	14	1,004	1,059	1,033
Kópavogslækur	7/9/2023	ZJ231116	M	54	1,4290	12	0,951	0,940	1,014
Kópavogslækur	7/9/2023	ZJ231117	F	51	1,0970	17	0,984	0,966	0,798
Kópavogslækur	7/9/2023	ZJ231118	F	40	0,5620	14	NA	NA	NA
Kópavogslækur	7/9/2023	ZJ231119	M	47	0,8090	15	1,017	0,968	0,992
Kópavogslækur	7/9/2023	ZJ231120	M	43	0,6480	14	1,011	1,034	1,004
Kópavogslækur	7/9/2023	ZJ231121	F	55	1,4220	14	0,820	0,896	1,036
Kópavogslækur	7/9/2023	ZJ231122	F	46	1,6240	12	0,959	0,996	0,948
Kópavogslækur	7/9/2023	ZJ231123	F	56	0,8411	13	0,963	0,929	1,022
Kópavogslækur	7/9/2023	ZJ231124	M	49	0,9320	13	1,046	1,011	1,119
Kópavogslækur	7/9/2023	ZJ231125	F	45	0,7520	13	1,002	0,935	1,023
Kópavogslækur	7/9/2023	ZJ231126	F	43	0,6766	13	1,101	1,168	1,003
Kópavogslækur	7/9/2023	ZJ231127	F	45	0,7070	13	0,978	0,992	1,053
Kópavogslækur	7/9/2023	ZJ231128	M	44	0,6611	14	1,037	1,119	1,004
Kópavogslækur	7/9/2023	ZJ231129	F	55	1,4077	17	1,029	1,011	1,100
Vífilstaðavatn	7/9/2023	ZJ23121	M	40	0,4606	14	1,036	1,009	0,984
Vífilstaðavatn	7/9/2023	ZJ23122	F	40	0,4902	14	1,012	0,935	1,061

Vífilstaðavatn	7/9/2023	ZJ23123	F	40	0,4998	13	0,971	1,072	1,050
Vífilstaðavatn	7/9/2023	ZJ23124	M	38	0,5302	12	1,185	1,040	1,060
Vífilstaðavatn	7/9/2023	ZJ23125	M	40	0,4537	10	0,969	0,899	0,988
Vífilstaðavatn	7/9/2023	ZJ23126	F	40	0,4720	13	1,078	1,012	1,195
Vífilstaðavatn	7/9/2023	ZJ23127	F	36	0,3002	12	1,007	1,030	1,038
Vífilstaðavatn	7/9/2023	ZJ23128	F	34	0,2569	11	1,009	1,051	0,972
Vífilstaðavatn	7/9/2023	ZJ23129	F	32	0,2163	NA	NA	NA	NA
Vífilstaðavatn	7/9/2023	ZJ231210	F	33	0,2880	11	1,136	1,075	1,112
Vífilstaðavatn	7/9/2023	ZJ231211	M	38	0,4419	14	1,008	1,109	1,050
Vífilstaðavatn	7/9/2023	ZJ231212	F	32	0,2375	11	1,116	1,033	1,004
Vífilstaðavatn	7/9/2023	ZJ231213	F	35	0,2931	17	1,055	1,073	1,035
Vífilstaðavatn	7/9/2023	ZJ231214	F	35	0,4208	14	1,123	1,325	1,168
Vífilstaðavatn	7/9/2023	ZJ231215	F	34	0,2363	NA	NA	NA	NA
Vífilstaðavatn	7/9/2023	ZJ231216	F	35	0,2466	14	1,019	1,114	1,007
Vífilstaðavatn	7/9/2023	ZJ231217	F	31	0,1888	15	0,933	0,975	1,057
Vífilstaðavatn	7/9/2023	ZJ231218	F	34	0,2340	8	1,052	1,020	0,977
Vífilstaðavatn	7/9/2023	ZJ231219	F	34	0,1933	11	NA	NA	NA
Vífilstaðavatn	7/9/2023	ZJ231220	F	34	0,2470	10	NA	NA	NA
Vífilstaðavatn	7/9/2023	ZJ231221	F	34	0,2300	18	1,217	0,985	0,993
Vífilstaðavatn	7/9/2023	ZJ231222	F	41	0,4530	13	1,051	0,961	1,000
Vífilstaðavatn	7/9/2023	ZJ231223	F	32	0,1655	13	1,028	1,176	1,474
Vífilstaðavatn	7/9/2023	ZJ231224	M	32	0,1900	10	1,010	0,954	0,985
Vífilstaðavatn	7/9/2023	ZJ231225	F	33	0,2011	12	1,453	1,481	1,033
Vífilstaðavatn	7/9/2023	ZJ231226	F	35	0,2220	10	1,126	1,095	1,020
Vífilstaðavatn	7/9/2023	ZJ231227	F	32	0,1890	14	1,011	1,345	0,985
Vífilstaðavatn	7/9/2023	ZJ231228	F	37	0,2944	15	0,999	1,018	0,996
Vífilstaðavatn	7/9/2023	ZJ231229	F	35	0,2366	15	0,997	0,971	0,793
Vífilstaðavatn	7/9/2023	ZJ231230	M	31	0,1600	13	1,261	0,967	0,835
Vífilstaðavatn	7/9/2023	ZJ23007	F	63	3,9455	13	1,042	1,050	1,036

Location	Fish_ID	SP 1	SP2	PS	Plates_number
ASKJA EAST	SG24001	0,98	1,043	1,082	4
ASKJA EAST	SG24002	0,917	1,068	1,102	7
ASKJA EAST	SG24003	0,929	1,008	1,054	6
ASKJA EAST	SG24004	1,04	1,08	1,348	12
ASKJA EAST	SG24005	1,739	1,869	2,852	5
ASKJA EAST	SG24006	0,895	1,029	1,058	4
ASKJA EAST	SG24007	0,897	1,088	NA	6
ASKJA EAST	SG24008	0,901	1,056	1,062	5
ASKJA EAST	SG24009	0,939	1,016	1,096	6
ASKJA EAST	SG24010	0,965	1,058	1,168	6
ASKJA EAST	SG24011	0,92	1,096	1,146	5
ASKJA EAST	SG24012	0,842	0,987	1,047	10
ASKJA EAST	SG24013	0,795	0,809	1,068	4
ASKJA EAST	SG24014	0,921	0,992	1,007	6
ASKJA EAST	SG24015	1,038	1,063	1,143	14
ASKJA EAST	SG24016	0,97	0,996	1,389	5
ASKJA EAST	SG24017	0,656	0,982	1,035	4
ASKJA EAST	SG24018	1,05	1,06	NA	4
ASKJA EAST	SG24019	0,961	1,031	1,088	6
ASKJA EAST	SG24020	1,126	1,319	1,355	7
ASKJA EAST	SG24021	0,942	1,02	1,087	4
ASKJA EAST	SG24022	0,902	1,016	1,039	7
ASKJA EAST	SG24023	1,001	1,022	1,067	4
ASKJA EAST	SG24024	0,874	1,047	1,054	5
ASKJA EAST	SG24025	0,927	1,011	1,081	6
ASKJA EAST	SG24026	0,84	1,011	1,12	5
ASKJA EAST	SG24027	0,906	0,945	1,002	5
ASKJA EAST	SG24028	0,751	0,927	0,931	4
ASKJA EAST	SG24029	1,082	1,114	1,326	4
ASKJA EAST	SG24030	1,01	1,056	1,084	6
ASKJA WEST	SG24111	0,926	1,044	1,155	16
ASKJA WEST	SG24112	0,933	1,048	1,114	7
ASKJA WEST	SG24113	0,897	0,996	1,017	11
ASKJA WEST	SG24114	0,841	0,993	1,028	13
ASKJA WEST	SG24115	0,853	0,947	0,997	3
ASKJA WEST	SG24116	0,927	1,004	1,069	5
ASKJA WEST	SG24117	1,227	1,268	1,423	6
ASKJA WEST	SG24118	0,917	0,941	1,145	4
ASKJA WEST	SG24119	0,851	0,893	1,043	6
ASKJA WEST	SG24120	0,914	1,029	1,039	5

ASKJA WEST	SG24121	0,865	0,922	1,064	9
ASKJA WEST	SG24122	0,891	1,017	1,143	5
ASKJA WEST	SG24123	1,003	1,048	1,083	4
ASKJA WEST	SG24124	0,825	1,024	1,038	5
ASKJA WEST	SG24125	0,833	0,988	1,216	4
ASKJA WEST	SG24126	0,814	1,078	1,093	3
ASKJA WEST	SG24127	0,896	0,903	1,015	15
ASKJA WEST	SG24128	0,954	1,035	1,058	4
ASKJA WEST	SG24129	0,965	1,057	1,14	12
ASKJA WEST	SG24130	1,001	1,087	1,29	4
ASKJA WEST	SG24131	0,862	0,988	1,001	6
ASKJA WEST	SG24132	0,882	1,098	1,123	5
ASKJA WEST	SG24133	1,085	1,129	1,134	4
ASKJA WEST	SG24134	0,844	1,008	1,188	6
ASKJA WEST	SG24135	0,929	1,096	1,144	7
ASKJA WEST	SG24136	0,834	0,97	1,411	4
ASKJA WEST	SG24137	0,857	0,939	1,136	12
ASKJA WEST	SG24138	1,026	1,084	1,109	7
ASKJA WEST	SG24139	1,059	1,085	1,088	4
ASKJA WEST	SG24140	0,835	0,927	1,038	4
ASKJA WEST	SG24141	0,97	1,014	1,015	6
Àstjörn	ZJ23101	0,816	1,07	1,078	4
Àstjörn	ZJ23102	0,895	0,945	1,015	4
Àstjörn	ZJ23103	0,91	1,127	1,143	4
Àstjörn	ZJ23104	0,862	0,994	1,106	4
Àstjörn	ZJ23105	1,021	1,112	1,485	3
Àstjörn	ZJ23106	0,958	1,046	1,181	6
Àstjörn	ZJ23107	0,903	0,952	0,97	4
Àstjörn	ZJ23108	0,891	1,04	1,088	5
Àstjörn	ZJ23109	1,03	1,493	1,43	4
Àstjörn	ZJ231010	NA	1,129	1,189	4
Àstjörn	ZJ231011	NA	NA	0,98	3
Àstjörn	ZJ231012	0,8	0,904	1,051	5
Àstjörn	ZJ231013	0,749	0,941	1,061	4
Àstjörn	ZJ231014	0,834	0,987	1,14	4
Àstjörn	ZJ231015	NA	0,948	0,988	4
Àstjörn	ZJ231016	0,723	0,981	1,004	3
Àstjörn	ZJ231017	NA	NA	NA	3
Àstjörn	ZJ231018	NA	0,88	0,964	4
Àstjörn	ZJ231019	NA	NA	NA	5
Àstjörn	ZJ231020	0,794	0,946	1,113	4
Àstjörn	ZJ231021	NA	NA	NA	4

Àstjörn	ZJ231022	NA	NA	NA	3
Àstjörn	ZJ231023	0,964	1,021	1,146	4
Àstjörn	ZJ231024	NA	NA	NA	4
Àstjörn	ZJ231025	NA	NA	NA	3
Àstjörn	ZJ231026	0,98	1,007	1,311	4
Àstjörn	ZJ231027	NA	NA	NA	4
Àstjörn	ZJ231028	0,96	1,005	1,03	4
Àstjörn	ZJ231029	NA	NA	NA	3
Àstjörn	ZJ231030	NA	NA	NA	4
Kópavogslækur	ZJ23110	0,894	1,026	1,094	5
Kópavogslækur	ZJ23111	1,091	1,124	1,138	6
Kópavogslækur	ZJ23112	1,055	1,095	1,199	5
Kópavogslækur	ZJ23113	0,913	0,928	0,99	4
Kópavogslækur	ZJ23114	0,956	1,033	1,083	4
Kópavogslækur	ZJ23115	0,819	0,998	1,02	7
Kópavogslækur	ZJ23116	0,933	1,08	1,159	10
Kópavogslækur	ZJ23117	0,912	0,962	1,094	5
Kópavogslækur	ZJ23118	0,894	1,049	1,122	4
Kópavogslækur	ZJ23119	0,936	0,974	1,068	5
Kópavogslækur	ZJ231110	0,851	0,988	1,127	5
Kópavogslækur	ZJ231111	0,998	1,059	1,081	4
Kópavogslækur	ZJ231112	1,06	1,065	1,211	4
Kópavogslækur	ZJ231113	0,789	0,978	1,766	5
Kópavogslækur	ZJ231114	0,989	1,038	1,067	5
Kópavogslækur	ZJ231115	1,253	1,31	1,342	4
Kópavogslækur	ZJ231116	0,869	0,938	1,002	4
Kópavogslækur	ZJ231117	1,019	1,122	1,151	4
Kópavogslækur	ZJ231118	0,975	1,007	1,624	4
Kópavogslækur	ZJ231119	1,019	1,059	1,142	5
Kópavogslækur	ZJ231120	0,921	1,116	1,185	5
Kópavogslækur	ZJ231121	1,061	1,078	1,165	17
Kópavogslækur	ZJ231122	0,919	0,947	1,052	4
Kópavogslækur	ZJ231123	0,925	1,019	1,089	7
Kópavogslækur	ZJ231124	0,966	1,001	1,052	7
Kópavogslækur	ZJ231125	0,871	0,914	1,062	4
Kópavogslækur	ZJ231126	0,967	1,104	1,033	4
Kópavogslækur	ZJ231127	0,902	1,082	1,091	5
Kópavogslækur	ZJ231128	0,9	0,924	1,04	5
Kópavogslækur	ZJ231129	0,956	1,048	1,163	4
Vífilstaðavatn	ZJ23121	0,896	NA	1,096	2
Vífilstaðavatn	ZJ23122	NA	1,038	NA	2
Vífilstaðavatn	ZJ23123	0,898	0,912	NA	3

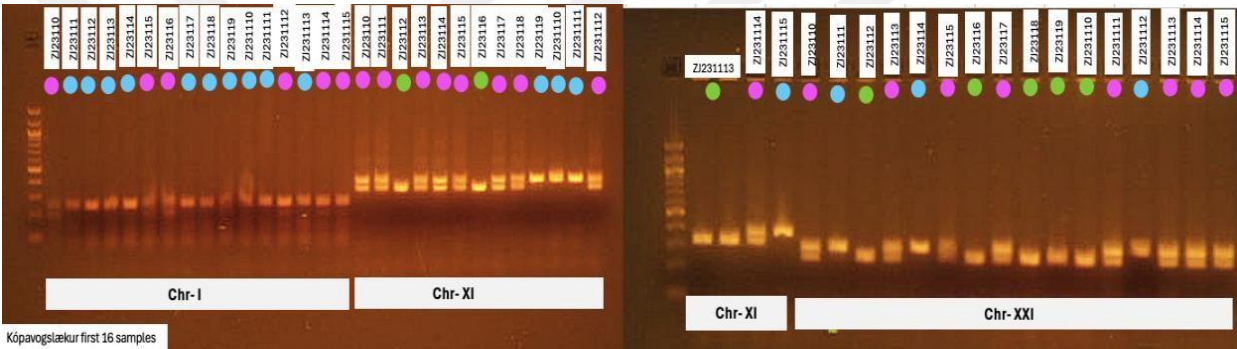
Vífilstaðavatn	ZJ23124	NA	0,986	NA	3
Vífilstaðavatn	ZJ23125	NA	NA	NA	3
Vífilstaðavatn	ZJ23126	0,923	1,144	NA	2
Vífilstaðavatn	ZJ23127	0,863	0,949	1,078	3
Vífilstaðavatn	ZJ23128	NA	NA	NA	2
Vífilstaðavatn	ZJ23129	NA	NA	NA	3
Vífilstaðavatn	ZJ231210	NA	NA	NA	3
Vífilstaðavatn	ZJ231211	0,919	NA	NA	2
Vífilstaðavatn	ZJ231212	NA	NA	NA	3
Vífilstaðavatn	ZJ231213	1,062	1,246	NA	3
Vífilstaðavatn	ZJ231214	0,965	0,996	NA	3
Vífilstaðavatn	ZJ231215	NA	NA	NA	3
Vífilstaðavatn	ZJ231216	0,98	NA	NA	4
Vífilstaðavatn	ZJ231217	NA	1,015	NA	3
Vífilstaðavatn	ZJ231218	0,956	1,026	NA	2
Vífilstaðavatn	ZJ231219	0,935	1,033	NA	3
Vífilstaðavatn	ZJ231220	0,948	0,956	NA	3
Vífilstaðavatn	ZJ231221	0,965	1,03	NA	4
Vífilstaðavatn	ZJ231222	0,926	1,021	NA	0
Vífilstaðavatn	ZJ231223	1,025	1,08	NA	2
Vífilstaðavatn	ZJ231224	0,891	0,988	NA	3
Vífilstaðavatn	ZJ231225	0,923	1,007	NA	2
Vífilstaðavatn	ZJ231226	0,922	0,998	NA	1
Vífilstaðavatn	ZJ231227	0,95	1,013	NA	3
Vífilstaðavatn	ZJ231228	0,927	1,052	NA	2
Vífilstaðavatn	ZJ231229	1,03	1,113	NA	3
Vífilstaðavatn	ZJ231230	0.963	1.091	NA	3
viðvassarvatn	ZJ23007	0.947	0.996	1.000	10

4.2 APPENDIX 3

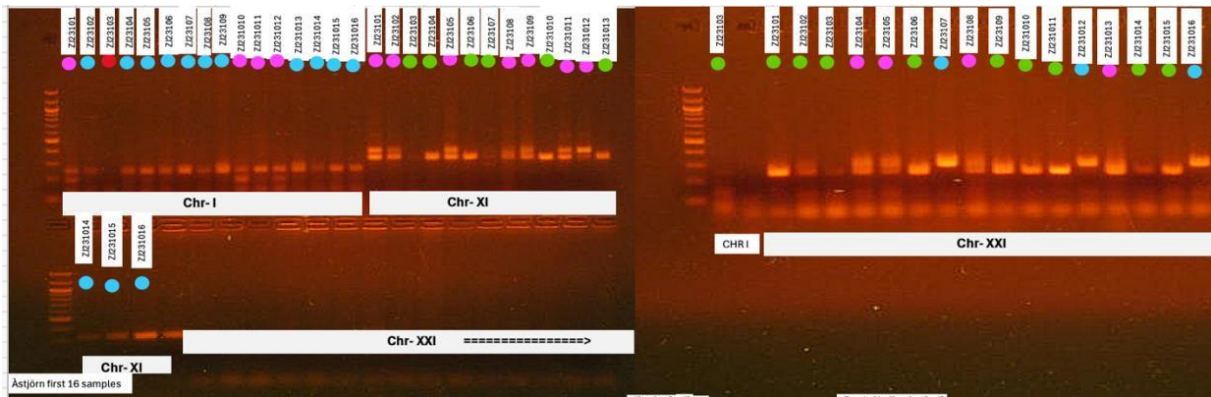
Tables of inversion chromosomes in each population And the gels of PCR results

pulationname	Population abbreviation	Nb-Fish	Nb-males	Nb-Females	Year	Chr1 M	Chr1 H	Chr1 F	Chr1 ND	Chr11 M	Chr11 H	Chr11 F	Chr11 ND	Chr21 M	Chr21 H	Chr21 F	Chr21 ND
Askja East	SG240	30	5	25	2024	1	3	26	0	10	10	6	4	12	8	6	4
Askja West	SG241	30	18	13	2024	3	6	11	10	6	4	8	12	6	8	3	12
Àstjörn	ZJ2310	30	15	15	2023	0	9	21	1	16	10	3	1	23	4	3	0
Kópavogslækur	ZJ2311	30	9	21	2023	4	12	14	0	5	14	10	1	7	15	7	1
Vífilstaðavatn	ZJ2312	31	6	25	2023	1	8	22	0	20	0	1	10	31	0	0	0

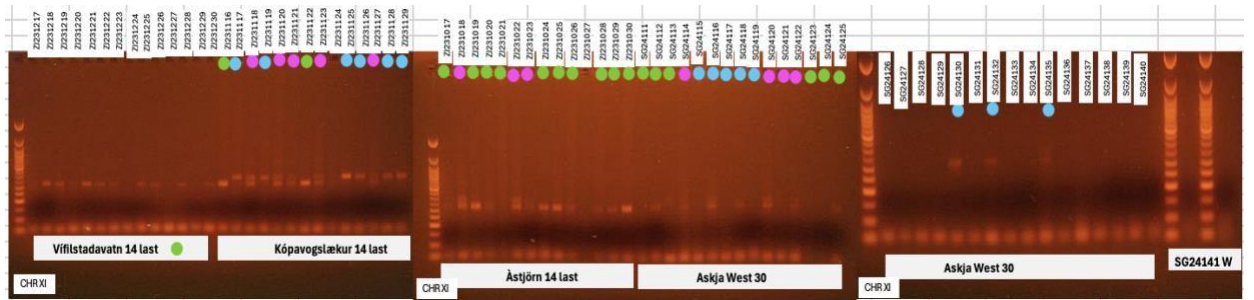
Kópavogslækur first 16 samples



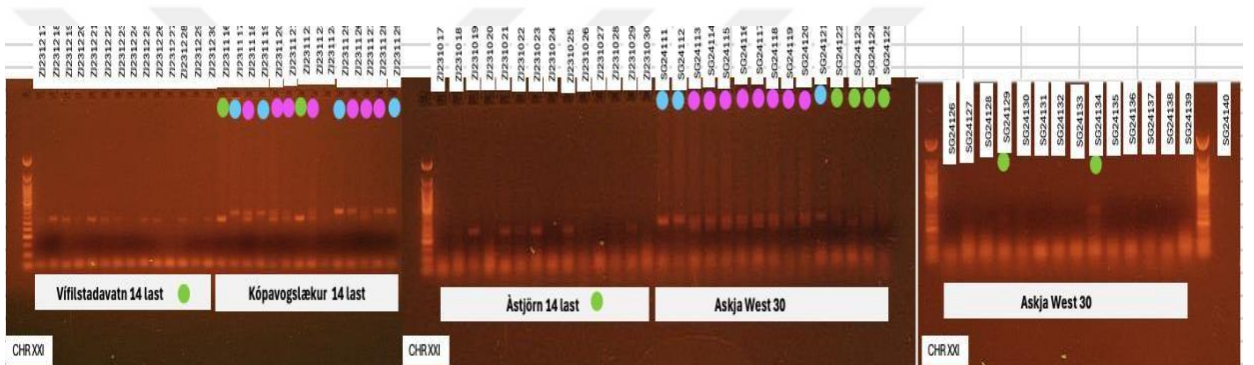
Àstjörn first 16 samples



Inversion Chromosome XI for last 14 samples of Kópavogslækur+ Vífilstadavatn+ Àstjörn and all samples of Askja West.



Inversion Chromosome XXI for last 14 samples of Kópavogslækur+ Vífilstadavatn+ Àstjörn and all samples of Askja West.



Population name	Population abbreviation	Year	FISH_ID	Chr 1 genotypes	Chr 11 genotype	Chr 21 genotype
ASKJA EAST	SG240	2024-04-05	SG24001	H	M	F
ASKJA EAST	SG240	2024-04-05	SG24002	F	M	M
ASKJA EAST	SG240	2024-04-05	SG24003	F	F	H
ASKJA EAST	SG240	2024-04-05	SG24004	F	H	M
ASKJA EAST	SG240	2024-04-05	SG24005	F	H	M
ASKJA EAST	SG240	2024-04-05	SG24006	F	ND	ND
ASKJA EAST	SG240	2024-04-05	SG24007	F	H	F
ASKJA EAST	SG240	2024-04-05	SG24008	F	H	M
ASKJA EAST	SG240	2024-04-05	SG24009	F	M	H
ASKJA EAST	SG240	2024-04-05	SG24010	F	H	H
ASKJA EAST	SG240	2024-04-05	SG24011	F	ND	ND
ASKJA EAST	SG240	2024-04-05	SG24012	F	M	F
ASKJA EAST	SG240	2024-04-05	SG24013	H	M	M
ASKJA EAST	SG240	2024-04-05	SG24014	F	M	M
ASKJA EAST	SG240	2024-04-05	SG24015	F	H	M
ASKJA EAST	SG240	2024-04-05	SG24016	F	F	F

ASKJA EAST	SG240	2024-04-05	SG24017	F	ND	ND
ASKJA EAST	SG240	2024-04-05	SG24018	F	F	M
ASKJA EAST	SG240	2024-04-05	SG24019	F	H	H
ASKJA EAST	SG240	2024-04-05	SG24020	F	H	M
ASKJA EAST	SG240	2024-04-05	SG24021	F	M	H
ASKJA EAST	SG240	2024-04-05	SG24022	F	H	M
ASKJA EAST	SG240	2024-04-05	SG24023	F	M	F
ASKJA EAST	SG240	2024-04-05	SG24024	F	M	M
ASKJA EAST	SG240	2024-04-05	SG24025	F	ND	ND
ASKJA EAST	SG240	2024-04-05	SG24026	F	M	F
ASKJA EAST	SG240	2024-04-05	SG24027	F	F	M
ASKJA EAST	SG240	2024-04-05	SG24028	F	H	H
ASKJA EAST	SG240	2024-04-05	SG24029	H	F	H
ASKJA EAST	SG240	2024-04-05	SG24030	M	F	H
ASKJA WEST	SG241	2024-04-05	SG24111	H	M	F
ASKJA WEST	SG241	2024-04-05	SG24112	H	M	F
ASKJA WEST	SG241	2024-04-05	SG24113	F	M	H
ASKJA WEST	SG241	2024-04-05	SG24114	F	H	H
ASKJA WEST	SG241	2024-04-05	SG24115	F	F	H
ASKJA WEST	SG241	2024-04-05	SG24116	H	F	H
ASKJA WEST	SG241	2024-04-05	SG24117	M	F	H
ASKJA WEST	SG241	2024-04-05	SG24118	H	F	H
ASKJA WEST	SG241	2024-04-05	SG24119	M	F	H
ASKJA WEST	SG241	2024-04-05	SG24120	M	H	H
ASKJA WEST	SG241	2024-04-05	SG24121	F	H	F
ASKJA WEST	SG241	2024-04-05	SG24122	F	H	M
ASKJA WEST	SG241	2024-04-05	SG24123	F	M	M
ASKJA WEST	SG241	2024-04-05	SG24124	ND	M	M
ASKJA WEST	SG241	2024-04-05	SG24125	F	M	M
ASKJA WEST	SG241	2024-04-05	SG24126	ND	ND	ND
ASKJA WEST	SG241	2024-04-05	SG24127	ND	ND	ND
ASKJA WEST	SG241	2024-04-05	SG24128	ND	ND	ND
ASKJA WEST	SG241	2024-04-05	SG24129	ND	ND	M
ASKJA WEST	SG241	2024-04-05	SG24130	H	F	ND
ASKJA WEST	SG241	2024-04-05	SG24131	F	ND	ND
ASKJA WEST	SG241	2024-04-05	SG24132	F	F	ND
ASKJA WEST	SG241	2024-04-05	SG24133	F	ND	ND
ASKJA WEST	SG241	2024-04-05	SG24134	ND	ND	M
ASKJA WEST	SG241	2024-04-05	SG24135	F	F	ND

ASKJA WEST	SG241	2024-04-05	SG24136	ND	ND	ND
ASKJA WEST	SG241	2024-04-05	SG24137	H	ND	ND
ASKJA WEST	SG241	2024-04-05	SG24138	ND	ND	ND
ASKJA WEST	SG241	2024-04-05	SG24139	ND	ND	ND
ASKJA WEST	SG241	2024-04-05	SG24140	ND	ND	ND
ASKJA WEST	SG241	2024-04-05	SG24141	ND	ND	ND
ÀSTJÖRN	ZJ2310	2023-09-07	ZJ23101	H	H	M
ÀSTJÖRN	ZJ2310	2023-09-07	ZJ23102	F	H	M
ÀSTJÖRN	ZJ2310	2023-09-07	ZJ23103	M	M	M
ÀSTJÖRN	ZJ2310	2023-09-07	ZJ23104	F	M	H
ÀSTJÖRN	ZJ2310	2023-09-07	ZJ23105	F	H	H
ÀSTJÖRN	ZJ2310	2023-09-07	ZJ23106	F	M	M
ÀSTJÖRN	ZJ2310	2023-09-07	ZJ23107	F	M	F
ÀSTJÖRN	ZJ2310	2023-09-07	ZJ23108	F	H	H
ÀSTJÖRN	ZJ2310	2023-09-07	ZJ23109	F	H	M
ÀSTJÖRN	ZJ2310	2023-09-07	ZJ231010	H	M	M
ÀSTJÖRN	ZJ2310	2023-09-07	ZJ231011	H	H	M
ÀSTJÖRN	ZJ2310	2023-09-07	ZJ231012	H	H	F
ÀSTJÖRN	ZJ2310	2023-09-07	ZJ231013	F	M	H
ÀSTJÖRN	ZJ2310	2023-09-07	ZJ231014	F	F	M
ÀSTJÖRN	ZJ2310	2023-09-07	ZJ231015	F	F	M
ÀSTJÖRN	ZJ2310	2023-09-07	ZJ231016	F	F	F
ÀSTJÖRN	ZJ2310	2023-09-07	ZJ231017	F	M	M
ÀSTJÖRN	ZJ2310	2023-09-07	ZJ231018	F	H	M
ÀSTJÖRN	ZJ2310	2023-09-07	ZJ231019	H	M	M
ÀSTJÖRN	ZJ2310	2023-09-07	ZJ231020	F	M	M
ÀSTJÖRN	ZJ2310	2023-09-07	ZJ231021	F	M	M
ÀSTJÖRN	ZJ2310	2023-09-07	ZJ231022	H	H	M
ÀSTJÖRN	ZJ2310	2023-09-07	ZJ231023	F	H	M
ÀSTJÖRN	ZJ2310	2023-09-07	ZJ231024	F	M	M
ÀSTJÖRN	ZJ2310	2023-09-07	ZJ231025	F	M	M
ÀSTJÖRN	ZJ2310	2023-09-07	ZJ231026	F	M	M
ÀSTJÖRN	ZJ2310	2023-09-07	ZJ231027	F	ND	M
ÀSTJÖRN	ZJ2310	2023-09-07	ZJ231028	H	M	M
ÀSTJÖRN	ZJ2310	2023-09-07	ZJ231029	H	M	M
ÀSTJÖRN	ZJ2310	2023-09-07	ZJ231030	H	M	M
KÓPAVOGSLÆKUR	ZJ2311	2023-09-07	ZJ23110	H	H	H
KÓPAVOGSLÆKUR	ZJ2311	2023-09-07	ZJ23111	F	H	F
KÓPAVOGSLÆKUR	ZJ2311	2023-09-07	ZJ23112	F	M	M

KÓPAVOGSLÆKUR	ZJ2311	2023-09-07	ZJ23113	F	H	H
KÓPAVOGSLÆKUR	ZJ2311	2023-09-07	ZJ23114	F	H	F
KÓPAVOGSLÆKUR	ZJ2311	2023-09-07	ZJ23115	H	H	H
KÓPAVOGSLÆKUR	ZJ2311	2023-09-07	ZJ23116	H	M	M
KÓPAVOGSLÆKUR	ZJ2311	2023-09-07	ZJ23117	F	H	H
KÓPAVOGSLÆKUR	ZJ2311	2023-09-07	ZJ23118	F	H	M
KÓPAVOGSLÆKUR	ZJ2311	2023-09-07	ZJ23119	F	F	M
KÓPAVOGSLÆKUR	ZJ2311	2023-09-07	ZJ231110	F	F	M
KÓPAVOGSLÆKUR	ZJ2311	2023-09-07	ZJ231111	F	F	H
KÓPAVOGSLÆKUR	ZJ2311	2023-09-07	ZJ231112	H	H	F
KÓPAVOGSLÆKUR	ZJ2311	2023-09-07	ZJ231113	F	M	H
KÓPAVOGSLÆKUR	ZJ2311	2023-09-07	ZJ231114	H	H	H
KÓPAVOGSLÆKUR	ZJ2311	2023-09-07	ZJ231115	H	F	H
KÓPAVOGSLÆKUR	ZJ2311	2023-09-07	ZJ231116	F	M	M
KÓPAVOGSLÆKUR	ZJ2311	2023-09-07	ZJ231117	F	F	F
KÓPAVOGSLÆKUR	ZJ2311	2023-09-07	ZJ231118	H	H	H
KÓPAVOGSLÆKUR	ZJ2311	2023-09-07	ZJ231119	F	F	F
KÓPAVOGSLÆKUR	ZJ2311	2023-09-07	ZJ231120	H	H	H
KÓPAVOGSLÆKUR	ZJ2311	2023-09-07	ZJ231121	F	H	H
KÓPAVOGSLÆKUR	ZJ2311	2023-09-07	ZJ231122	H	M	M
KÓPAVOGSLÆKUR	ZJ2311	2023-09-07	ZJ231123	H	H	H
KÓPAVOGSLÆKUR	ZJ2311	2023-09-07	ZJ231124	H	ND	ND
KÓPAVOGSLÆKUR	ZJ2311	2023-09-07	ZJ231125	H	F	F
KÓPAVOGSLÆKUR	ZJ2311	2023-09-07	ZJ231126	M	F	H
KÓPAVOGSLÆKUR	ZJ2311	2023-09-07	ZJ231127	M	H	H
KÓPAVOGSLÆKUR	ZJ2311	2023-09-07	ZJ231128	M	F	H
KÓPAVOGSLÆKUR	ZJ2311	2023-09-07	ZJ231129	M	F	F
VÍFILSTADAVATN	ZJ2312	2023-09-07	ZJ23121	F	M	M
VÍFILSTADAVATN	ZJ2312	2023-09-07	ZJ23122	F	M	M
VÍFILSTADAVATN	ZJ2312	2023-09-07	ZJ23123	F	M	M
VÍFILSTADAVATN	ZJ2312	2023-09-07	ZJ23124	H	M	M
VÍFILSTADAVATN	ZJ2312	2023-09-07	ZJ23125	H	M	M
VÍFILSTADAVATN	ZJ2312	2023-09-07	ZJ23126	H	M	M
VÍFILSTADAVATN	ZJ2312	2023-09-07	ZJ23127	F	M	M
VÍFILSTADAVATN	ZJ2312	2023-09-07	ZJ23128	F	M	M
VÍFILSTADAVATN	ZJ2312	2023-09-07	ZJ23129	H	M	M
VÍFILSTADAVATN	ZJ2312	2023-09-07	ZJ231210	H	M	M
VÍFILSTADAVATN	ZJ2312	2023-09-07	ZJ231211	F	M	M
VÍFILSTADAVATN	ZJ2312	2023-09-07	ZJ231212	H	M	M

VÍFILSTADAVATN	ZJ2312	2023-09-07	ZJ231213	H	M	M
VÍFILSTADAVATN	ZJ2312	2023-09-07	ZJ231214	H	M	M
VÍFILSTADAVATN	ZJ2312	2023-09-07	ZJ231215	F	M	M
VÍFILSTADAVATN	ZJ2312	2023-09-07	ZJ231216	F	M	M
VÍFILSTADAVATN	ZJ2312	2023-09-07	ZJ231217	F	M	M
VÍFILSTADAVATN	ZJ2312	2023-09-07	ZJ231218	F	M	M
VÍFILSTADAVATN	ZJ2312	2023-09-07	ZJ231219	F	M	M
VÍFILSTADAVATN	ZJ2312	2023-09-07	ZJ231220	F	M	M
VÍFILSTADAVATN	ZJ2312	2023-09-07	ZJ231221	F	M	M
VÍFILSTADAVATN	ZJ2312	2023-09-07	ZJ231222	F	M	M
VÍFILSTADAVATN	ZJ2312	2023-09-07	ZJ231223	F	M	M
VÍFILSTADAVATN	ZJ2312	2023-09-07	ZJ231224	F	M	M
VÍFILSTADAVATN	ZJ2312	2023-09-07	ZJ231225	F	M	M
VÍFILSTADAVATN	ZJ2312	2023-09-07	ZJ231226	F	M	M
VÍFILSTADAVATN	ZJ2312	2023-09-07	ZJ231227	F	M	M
VÍFILSTADAVATN	ZJ2312	2023-09-07	ZJ231228	F	M	M
VÍFILSTADAVATN	ZJ2312	2023-09-07	ZJ231229	F	M	M
VÍFILSTADAVATN	ZJ2312	2023-09-07	ZJ231230	F	M	M
VÍFILSTADAVATN	ZJ23007	2023-09-07	ZJ23007	M	F	M