Characterizing the immune response to *Flavobacterium psychrophilum*, and possible resistance/susceptibility conferring major histocompatibility alleles in *Oncorhynchus mykiss*

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

Oncorhynchus mykiss, commonly known as rainbow trout, is an economically important species. Rainbow trout can be found in freshwater habitats across Canada and is present in over 1000 lakes and streams in British Columbia alone. In addition to food production, the aquaculture industry of Canada maintains restocking programs so sport fishing does not deplete natural resources. Coldwater disease is an increasingly prevalent issue and has a deleterious impact on farmed populations in Canadian aquaculture. Mortalities can approach approximately 80% in infected populations, causing significant monetary losses. However, the immune response to *Flavobacterium psychrophilum,* the causative agent of coldwater disease in rainbow trout, has yet to be fully elucidated. This project assessed that response using several measures. Firstly, Major Histocompatibility (MH) genes play an important role in initiating the immune response. Specific alleles of these genes can be related to resistance or susceptibility to disease. Thus DNA of individual rainbow trout from four British Columbia populations was extracted and sequenced to identify allelic differences between subpopulations. Secondly, the immune response to the bacteria was characterized utilizing whole blood with two immunological assays, chemiluminescent detection of oxygen burst responses and enzyme-linked immunosorbent assay for assessing antibody production. There were significant differences not only in MH class II β variability of sub-populations of rainbow trout but also in the immune response between vaccinated and non-vaccinated rainbow trout.

Acknowledgements

First and foremost I would like to thank my supervisor Dr. Brian Dixon and my committee for their immense knowledge and guidance in pursuing this project. Without their guidance I would not be here today. I would also like to thank the countless graduate and undergraduate students who have been a part of the Dixon Dozen, you've taught me every molecular technique I know.

Last, but not least, I would like to thank my family for their continued love and support that has allowed me to complete my Masters degree. As well, I would like to specifically thank my aunts Janie and Melissa Hodgins for their immeasurable support and guidance throughout my degree, without you the past two years would have been infinitely more difficult.

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General Introduction

Oncorhynchus mykiss, commonly known as rainbow trout, is an economically important species of fish. This has allowed the aquaculture industry to become the fastest growing industry in Canada. In 2006 alone the industry produced approximately 912 million Canadian dollars in fish which is an increase over 1986, as reported by the United Nations, when it was valued at only 35 million dollars (Canadian Aquaculture Systems Inc., 2009). However, due to the high demand of fish species for food consumption throughout Canada, there is a growing concern with the potential decline of fish in their natural habitats. In order to sustain wild populations, the aquaculture industry runs restocking programs to maintain these populations. Within the aquaculture industry Bacterial Coldwater Disease (BCD) is prevalent and has a deleterious impact on farmed trout populations where mortalities can approach approximately 80% of infected populations, which can lead to substantial monetary losses (Freshwater Fisheries Society of British Columbia, 2009). This evidence stresses the necessity of understanding the immune system of fish more completely and developing tools to reduce the impact of coldwater disease on farmed populations.

The goal of this research was to characterize the important immune system molecules known as the Major Histocompatibility (MH) receptors in rainbow trout. This in turn will help develop a greater understanding of the immune response and survival of rainbow trout to BCD. In addition to characterizing the MH alleles, a greater understanding of the immune response effector mechanisms that respond to *Flavobacterium psychrophilum*, the causative agent of bacterial coldwater disease, is required. Together this work will allow for further development of

coldwater disease prevention protocols and management tools to improve the aquaculture industry.

Chapter 1: Major histocompatibility alleles and possible *Flavobacterium psychrophilum* resistance/susceptibility in rainbow trout (*Oncorhynchus mykiss*)

1.1 Introduction

1.1.1 General Overview of Vertebrate Immune Function

The development of an immune system is essential for the survival of living organisms. In vertebrates, immunity can be divided into two components; the innate immune response and the adaptive immune response. The innate immune response is the initial line of defense against infection which includes the physical barriers, and a cellular response. The adaptive immune response is capable of specific antigen recognition and is responsible for the secondary immune response (Dixon & Stet, 2001).

First and foremost, physical barriers maintain the first line of defense against invading pathogens which includes a mucosal layer, the dermal and epidermal layers. The mucosal layer is responsible for the secretion of anti-pathogenic factors such as proteases, lysins and agglutinins which act to destroy the pathogens (Dalmo *et al.*, 1997). Should the physical barriers be ineffective and the pathogen penetrates the epithelium the first cellular line of defense is the innate effector cells. For example, macrophages and neutrophils are responsible for the engulfment and subsequent degradation of the peptides. Cells of the innate immune system have a diverse array of functions. Some cells are phagocytic, allowing them to engulf and degrade pathogenic particles. Other cells produce and secrete cytokines and chemokines that can stimulate and help guide the migration of cells and further direct the immune response. A

fundamental role of the innate immune response is to initiate the adaptive response (Janeway *et al.,* 2001).

The adaptive response generally starts four days after infection and is capable of recognizing specific protein motifs of peptides which leads to a response that increases in both speed and magnitude with each successive exposure (Dixon & Stet, 2001). The main effector cells of the adaptive immune response are the lymphocytes, specifically B cells and T cells. While the developmental process of each is similar the thymic environment is required for development of T cells and the bone marrow environment is required for B cells (Anderson *et al.,* 1996). When B cells are activated, they are capable of differentiating into plasma cells which can secrete antibodies. Antibodies are proteins that are capable of binding epitopes on antigens. Upon activation T cells differentiate into either helper T cells or cytotoxic T cells. Helper T cells are capable of activating other cells of the adaptive immune response such as B cells and macrophages. Cytotoxic T cells are able to kill cells that have been infected upon activation. It is possible to distinguish between the two cell types by their cell surface molecules; while helper T cells contain $CD4^+$ molecules on their surface; cytotoxic T cells contain $CD8^+$ molecules on their cell surface (Janeway *et al.,* 2001). Both lymphocytes, however, are capable of producing an antigen-specific immune response due to the production T-cell receptors on their surface (Anderson *et al.,* 1996).

B cells contribute to the adaptive immune response by producing immunoglobulins; a population of B cells can produce antibodies that bind a large variety of epitopes. The antigen-specific immune response associated with T cells however is far more complex. The receptors (TCR) are made solely as membrane-bound proteins which serve to signal T cells for activation. Also, unlike antibodies, TCRs do not bind antigens directly; instead they require the Major

Histocompatibility glycoproteins to present the short antigen peptides to the TCRs (Janeway *et al.,* 2001).

1.1.2 Major Histocompatibility Receptors - Structure and Function

MH genes are an essential component of the adaptive immune response of vertebrates. They encode a set of membrane glycoproteins that play a crucial role in eliminating foreign pathogens by being responsible for antigen presentation to the T cell receptors. In higher vertebrates such as humans and mice the MH genes are referred to as a complex (MHC) as all the genes are located on the same chromosome. These genes encode the MHC class I, II and III molecules and being linked allows the genes to be inherited as a complex by subsequent generations (Janeway *et al.*, 2001). The organization of the teleost fish MH genes differs from that of higher vertebrates in that the MH genes classes I, II and III of teleost fish are not linked and therefore do not form a complex. Instead, the genes for both class I and class II can be found on different chromosomes (Bingulac-Popovic *et al*., 1997). Although the organization of the genes differs, the proteins themselves function in the same way (Dixon *et al.*, 1995).

MHC class I molecules can be divided into two families, including the class I classical and non-classical genes. The two families have similar structures, but varying functions. Classical molecules are the most highly polymorphic genes known to date, where polymorphism refers to genes with multiple alleles. The non-classical molecules, on the other hand, show only very limited polymorphism (O'Callaghan & Bell, 1998). In salmonid fishes, including rainbow trout, MH class I are also divided into classical and non-classical loci where class I molecules are highly polymorphic but only encoded by few loci. Non-classical loci are oligomorphic, meaning fewer changes than in polymorphic genes, but can be encoded by many loci (Stet *et al.*, 1996).

The structure of class I molecules appears to be fairly consistent among species with an adaptive immune response (Janeway *et. al.,* 2001). The basic structure consists of two polypeptide chains; the first having three extracellular domains termed α_1, α_2, and α_3 as well as transmembrane and cytoplasmic regions (class I heavy chain); and the second being the extracellular β_2-microglobulin peptide, as seen in Figure 1.1 and is responsible for stability of the protein (Dixon *et al.,* 1995). Due to the occlusions at the end of the peptide binding groove, MH class I molecules are restricted to binding peptides that are 8-12 amino acids in length. These peptides are capable of interacting with six binding pockets in the groove, two of which are used as anchors for the N- and C-terminus of the peptides (Bjorkman & Parham, 1990). Unique to MH class I molecules is the fact that they present endogenously derived peptides such as viral components to the cells of the immune system. This presentation elicits a cellular mediated immune response through the stimulation of CD8 cytotoxic T cells therefore leading to the death of the infected cell (Bjorkman & Parham, 1990).

The MH class I heavy chain genes have been shown to be extremely polymorphic in the α_1 and α_2 domains which may have been due to pathogen selection pressures throughout evolution. This polymorphism contributes to the protection of the population as a whole whereas individuals are protected from a diverse array of pathogens due to the expression of six alleles in most species (Dixon *et al.,* 1995). With evolution and species radiation a need to recognize new pathogens and parasites within different environment increased dramatically and with the aid of polymorphism this was possible (Miller *et al.,* 2002).

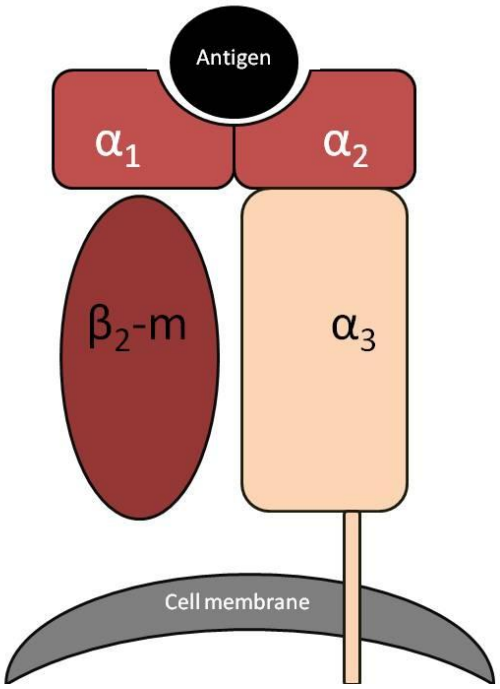


Figure 1.1: Structural diagram of MHC class I. The structure consists of two polypeptide chains the first having three extracellular domains termed α_1, α_2, and α_3 as well as transmembrane and cytoplasmic regions (class I heavy chain) and the second being the extracellular β_2-microglobulin peptide (Dixon *et al.*, 1995). The α_1 and α_2 domains make up the peptide binding region which presents endogenously derived peptides such as viral components to the cells of the immune system.

Much like class I, class II genes are responsible for encoding the cell surface proteins that bind and present foreign molecules to T cells. However, unlike class I, class II genes are only expressed in antigen-presenting cells and generally present peptides derived from exogenously derived peptides and stimulate CD4 helper T cells (Glimcher & Kara, 1992; Bjorkman & Parham, 1990).

The genes of MH class II code for a receptor that is made up of two protein chains: an α chain with two extracellular domains α_1 and α_2, and a β chain consisting of two extracellular domains β_1 and β_2, as seen in Figure 1.2 (Stet *et al.,* 1996). Both chains have been shown to contain a highly conserved transmembrane region and cytoplasmic domains. The two chains form a peptide binding region in the groove formed by the α_1 and β_1 subunits that can accommodate peptides larger than that of class I. Due to the lack of restrictions in the groove class II can bind peptides 12-24 amino acids in length (Stet *et al.,* 1996). This peptide binding region has been shown to be extremely polymorphic which, like class I, is due to the selection pressures brought on by a need to recognize a greater array of potential pathogens (Miller *et al.,* 2002). Evidence suggests that with greater sequence and allelic diversity the greater immunity against extracellular pathogens (Grimholt *et al.,* 1993).

1.1.3 Expression of MH Genes in Salmonids

Teleost fish are so diverse in numbers and species that they account for over half of the living vertebrates. While mammals and even cartilaginous fish have been shown to have their MH genes linked to form a complex this is not the case with teleosts including the salmonids where the genes can be found on different linkage groups (Grimholt *et al.,* 2002). It has been shown that the rainbow trout genome encodes one major expressed MH class I locus termed UBA with eight additional non-classical class I genes (Miller *et al.,* 2006). Rainbow trout

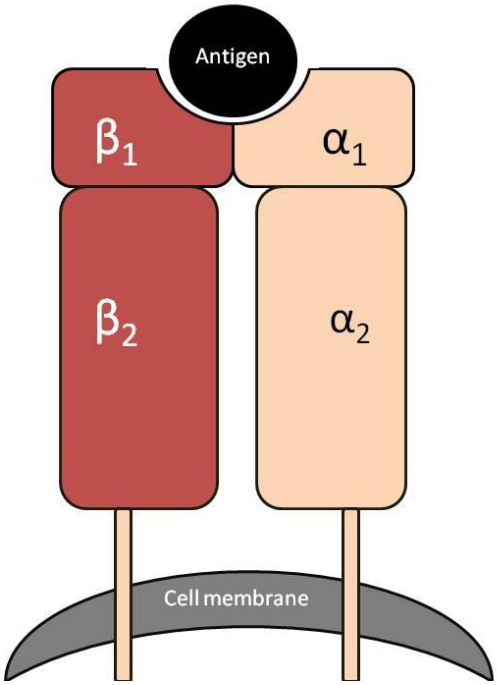


Figure 1.2: Structural diagram of MHC class II. The structure consists of two protein chains: an α chain with two extracellular domains α_1 and α_2, and a β chain consisting of two extracellular domains β_1 and β_2 containing a highly conserved transmembrane region and cytoplasmic domains (Stet *et al.*, 1996). The β_1 and α_1 domains make up the peptide binding region which presents exogenously derived peptides such as bacterial components to the cells of the immune system.

express one major MH class II α and one major β gene termed DAA and DAB respectively, which are located on the same chromosome but different linkage groups (Stet *et al.*, 2002). While research on non-classical class II genes is limited, a recent study was conducted with Atlantic salmon which showed two additional divergent class II alpha sequences, which was interpreted to be two additional loci. These additional loci showed a lack of expression and polymorphism and therefore was concluded that the genes were non-classical class II genes. This evidence shows a more complex molecule and expresses the possibility for additional species of teleosts to potentially contain multiple expressed loci (Harstad *et al.*, 2008).

1.1.4 Analytical Techniques

A common technique used when analyzing sequence diversity is the polymerase chain reaction (PCR). However, when conducting PCR for DNA sequence analysis in individuals with multiple copies of similar sequences, certain errors can arise within the elongation phase which could result in erroneous sequence diversity. This could be due to the incomplete extension of an amplimer in a single PCR cycle followed by the further extension of that amplimer bound to a different template during subsequent cycles to form a complete strand (Boriello & Krauter, 1990). Another mode in which this could occur is through the creation of heteroduplexes where two complete strands of DNA that were derived from different templates throughout the denaturation-annealing steps of the PCR form double-stranded DNA. These resulting errors in the sequences are referred to as artifacts (Boriello & Krauter, 1990).

In order to avoid the production of artifacts a technique called PCR+1 was developed. The technique utilizes forward and reverse primers that are made to contain a restriction enzyme site at their 5’ ends. The DNA is then asymmetrically initially amplified using a high concentration of the forward primer with the added restriction site and a low concentration of the

reverse primer lacking the restriction site. During the last cycle of the PCR a third primer, the reverse primer containing the restriction site, is added at a high concentration. This final cycle guarantees that only homoduplexes can be amplified and that they have both the forward and reverse primers containing the restriction site (L'Abbe *et al.*, 1991).

1.1.5 Purpose of Study

The goal of this study was to characterize and analyze MH class II allele variability in multiple sub-populations of British Columbia stocked rainbow trout to determine which populations have a greater capacity to bind bacterial peptides. Evidence suggests that with greater sequence and allelic diversity there is a greater immunity against extracellular pathogens (Grimholt *et al.*, 1993). Preliminary research suggested that some sub-populations of rainbow trout may be more susceptible to disease, particularly *Flavobacterium*, than others (Freshwater Fisheries Society of British Columbia, 2009). For example, due to the susceptibility of the Blackwater rainbow trout to disease in hatcheries there would therefore be less sequence diversity within the population. Additionally, due to the larger geographic range of the Fraser Valley rainbow trout the allelic diversity within the population was thought to be higher than that of the others. By understanding the immune responses of salmonid fishes techniques can be implemented in order to decrease aquaculture costs and losses such as stocking lakes and rivers with strains resistant to disease.

1.2 Materials and Methods

1.2.1 Experimental Fish

A total of 800 rainbow trout were collected from the Freshwater Fisheries Society of British Columbia (FFSBC) comprising four sub-populations: 100 diploid and 100 all-female

Figure 1.3: Geographic locations of the four sub-populations of rainbow trout. The four subpopulations include Fraser Valley, Pennask Lake, Dragon and Tunkawa Lakes and Tzenzaicut Lake.

triploid Pennask rainbow trout from Pennask Lake, 100 diploid and 100 triploid Blackwater rainbow trout from Dragon Lake and Tunkawa Lake, 100 all-female diploid and 100 triploid Fraser Valley rainbow trout from Fraser Valley Lake and last, 100 diploid and 100 triploid Tzenzaicut rainbow trout from Tzenzaicut Lake (Figure 1.3).

1.2.2 Tissue Collection

Fish were euthanized with 0.1% tricaine methane sulphonate (MS-222) and immediately sampled. Blood was drawn from the caudal vein utilizing a 23 gauge 1 inch needle containing approximately 100 µL of heparin (20 units/mL) and dispensed into a 1.5 mL eppendorf tube. The blood was immediately centrifuged at 10,000 rpm for 5 min. The upper plasma layer was then transferred into a new 1.5mL eppendorf tube and frozen at -20°C. Following blood extraction, the entire caudal fin was clipped and stored in 95% ethanol for later DNA extraction. In addition to the fin clips, a 1 cm by 1 cm piece of muscle was also clipped and stored in 95% ethanol. One complete set of gills, including all four arches, were removed and placed in a 2 mL O-ringed screw cap tube containing 1mL of RNAlater, an RNA preserving solution (25 mM $C_6H_5NaO_7$, 10 mM of Ethylenediaminetetraacetic acid (EDTA) and 4 M ($(NH_4)_2SO_4$). Last, the spleen and head kidney were also removed and placed in RNAlater. All samples were then frozen at -20°C.

1.2.3 DNA Extraction

Genomic DNA was extracted from an approximately 1cm by 1cm piece of caudal fin from each fish sampled. The pieces were submerged in 1.5 mL of Tris-EDTA (TE) buffer (100 mM Tris, 1mM EDTA) for approximately 6 hrs then transferred into a fish extraction buffer (100 mM Tris, 10 mM EDTA, 240 mM NaCl, 1% SDS) with 300 µg/ml proteinase K overnight at 45°C. Following this the samples were then treated to two phenol (pH 6.6) extractions and one chloroform extraction. The DNA was then precipitated out of solution using sodium acetate and

washed with 70% ethanol. The pellets were then suspended in 100 µL of Milli Q water and the concentration of each was determined using a NanoDrop Spectrophotometer after which the DNA was frozen at -20°C.

1.2.4 Primer Design and PCR Conditions

Due to the variability of exon two of the MH class II β gene, the forward primer was developed to the leader peptide (exon one) which has been shown to be conserved throughout many species of salmonids (Shum *et al.,* 2001). The reverse primer was developed to a conserved region at the end of exon two (F: 5'-TGCCAATTGCCTTCTACATTTGCCTG-3' and R: 5'-TGGGGGCTCAACTGTCTTGTCCAGT-3'). The fragment was amplified using 50 ng/µl of DNA template and an initial denaturation of 95°C for 5 min, plus 35 amplification cycles of denaturation for 1 min at 95°C, primer annealing for 30 sec at 55°C and extension for 1.5 min 72°C. A final extension period at 72 °C for 10 min was also performed to ensure the completion of all amplified products. All PCR reactions were performed in a Master Cycler (Eppendorf). Once PCR conditions were optimized primers were developed with the *Hind*III restriction site to conduct PCR+1 (F: 5'-TTCAAGCTTTGCCAATTGCCTTCTACATTTGCCTG-3'and R: 5'-TTCAAGCTTTGGGGGCTCAACTGTCTTGTCCAGT-3'). The fragment was then amplified utilizing the PCR+1 technique which included an initial denaturation for 5 min at of 95°C, with two cycles of denaturation for 1 min at of 95°C, primer annealing for 30 sec at 50°C and extension for 1.5 min 72°C. Following the completion of the initial two cycles, there was 35 cycles of denaturation for 1 min at 95°C, primer annealing for 30 sec at 55°C and extension for 1.5 min at 72°C with a final extension of 72°C for 2 min. After completion of the short extension, the final primer was added and the final cycle was run at 95°C for 5 min, with one

cycle of 95°C of denaturation for 5 sec, 55°C of primer annealing for 30 sec and 72°C of extension for 10 min.

To determine whether the expected band size of 750 base pairs was achieved the PCR amplicons were electrophoresed on a 1% agarose gel electrophoresis. Samples were then extracted and purified directly from the gel using Qiaquick Gel Extraction Kit (Qiagen Sciences, Maryland).

1.2.5 Ligation and Transformation

Purified PCR products were ligated overnight at 4°C into pGEM-T Easy Vector system, which allows blue/white selection of recombinants due to the insertional inactivation of the α-peptide of β-galactosidase (Promega Corporation, Madison, WI). Ligation products contained 5 µL of ligation buffer, 1 µL of ligase and 1 µL pGEM-T Easy Vector with 3 µL of the purified PCR product. Ligation products were then transformed into XL-1 Blue MRF' chemically competent strains of *E. coli* bacteria by adding 50 µL of the competent cells to each ligation product. Tubes were then incubated on ice for 20 min, heat shocked for 45 sec in a 42°C water bath and placed in a 37°C water bath shaking at 225 rpm for 1 hr with 400 µL of SOC (0.5% yeast extract, 2% tryptone, 10 mM sodium chloride, 10 mM magnesium chloride, 2.5 mM potassium chloride, 10 mM magnesium sulphate and 20 mM glucose) for recovery. Following recovery, products were then plated on Luria Bertani (LB) media (1% tryptone, 0.5% yeast extract and 172 mM sodium chloride) supplemented with 100 µg/mL ampicillin plus 1 µM isopropyl-β-D-1-thiogalactopyranoside (IPTG) (Fermentas) and 100 µg/ml X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) (Fermentas). The plates were then incubated overnight at 37°C.

1.2.6 Plasmid Purification

White colonies were selected and each colony was inoculated in 3 mL of LB/amp broth and incubated in a 37°C water bath shaking at 225 rpm overnight. The following day, 1.5 mL of the culture was centrifuged at 10,000 rpm for 5 min and the tubes were placed on ice. The pellet was re-suspended in 100 µL of solution I (50 mM glucose, 25 mM Tris-HCl pH8 and 10 mM EDTA pH8) and mixed by inverting the tubes eight times. The cells were then lysed by the addition of 200 µL of solution II (0.2N sodium hydroxide and 1% sodium dodecyl sulfate) and after 5 min incubation on ice, 150 µL of solution III (3M potassium acetate and 11.5% acetic acid) was added and mixed by inverting eight times to neutralize the sample. Following the addition of solution III, the tubes were centrifuged at 15000 rpm for 5 min to clear the protein-SDS complexes. The supernatant was then transferred to a new 1.5 mL eppendorf tube containing 20 µL of 0.1% RNaseA solution (Sigma # R6513) to degrade intracellular RNA and incubated at 37°C for 30 min. Following the incubation, 300 µL of chilled isopropanol was added and the solution was mixed by inverting 15 times and placed at -20°C overnight. The samples were then centrifuged for 15 min at 15000 rpm, the supernatant discarded and the pellet was washed twice with 1 mL of 70% ethanol. Following the final wash the pellets were then vacuumed dry for 2 min in the DNA 110 SpeedVac (Thermo Scientific) and re-suspended in 20 µL of Milli Q water. Samples were then heated at 70°C for 10 min and the concentration was measured using a NanoDrop Spectrophotometer. If necessary the samples were then diluted to 1000 ηg/µL and stored at -20°C.

1.2.7 Digestion and Sequencing

The isolated plasmid samples were then digested to ensure the complete 750 base pair bands including the *Hind*III restriction sites produced through the PCR+1 procedure and that it

was successfully ligated into the vector. Each digestion product consisted of 16 µL of water, 2 µL of FastDigest green buffer, 1 µL of *Hind*III restriction enzyme (Fermentas) and 1 µL of the isolated plasmid DNA. The samples were then digested for 1 hr in a 37°C water bath and then run on a 1% agarose gel to determine the size of the insert. Samples with the expected band size containing the restriction site were then stored at -20°C.

The samples with the insert and containing the restriction site were then diluted to approximately 250 ηg/µL and 7 µL were sent to Sick Kids Hospital: The Centre for Applied Genomics sequencing facility for sequencing, as per facility instructions. Each sample was sequenced using the Sp6 primer which was provided by the sequencing facility.

1.2.8 Data analysis

Sequences were initially analyzed utilizing the software Bioedit: Biological Sequence Alignment Editor (Hall, 1999; http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Sequences were aligned using the ClustalW Multiple Alignment option in the editor and compared to cDNA alignments previously published on the National Centre for Biotechnology Information database (http://www.ncbi.nlm.nih.gov). Once each sequence was determined to be rainbow trout each allele was entered into the software Arlequin Ver. 3.5.1.2 – An Integrated Software Package for Population Genetics Data Analysis (Excoffier, 2010; http://cmpg.unibe.ch/software/arlequin35). The software enabled exact tests of population differentiation to test the distribution of alleles in the population samples (Raymond & Rousset, 1990) as well as calculate rough F-statistics.

1.3 Results

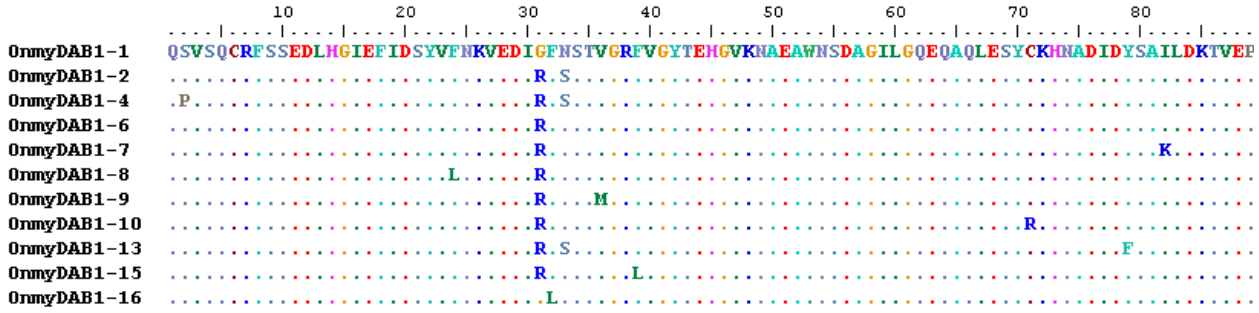
The DNA was extracted from twenty-five individuals from each of the four sub-populations of rainbow trout including the Pennask (PN), Fraser Valley (FV), Blackwater (BW)

and Tzenzaicut (TZEN) populations. A 750-bp fragment of the MH class II gene was successfully amplified, cloned and sequenced. For analysis, exon 2 was examined which encoded 90 amino acid residues. For each population, multiple clones from each individual were sequenced to determine all possible alleles in the populations (Figures 1.4-1.7). A great degree of confidence can be attributed to the legitimacy of the alleles due to the conservative parameters set in place for all populations. Each fish produced one to four alleles when sequenced which suggests alleles from up to two loci were amplified. Neighbour-joining analysis of the exon sequences revealed a clustering of alleles in fish with more than two alleles (Figure 1.8).

Rainbow trout have been shown to have one major expressed locus which suggests only two possible alleles, however; due to the presence of up to four alleles this data suggests the possibility of a second expressed locus. Using phylogenetic analysis, alleles that were different enough to form a new clade were identified as a second locus and termed DAB2. The remaining alleles were identified as the major expressed locus and termed DAB1. More sequences grouped into DAB1 than DAB2 due to primer bias within the samples where primers were designed for the amplification of DAB1 and not DAB2. As well, some individuals, when sequenced, contained alleles that fell only within the DAB1 locus while others contained alleles within both groups.

Figure 1.4: Unique amino acid sequences of the Fraser Valley rainbow trout population. Unique amino acid sequences based on MHC class II β gene full length exon 2 isolated from Fraser Valley rainbow trout genomic DNA, (a) DAB1 (b) DAB2

(a)



(b)

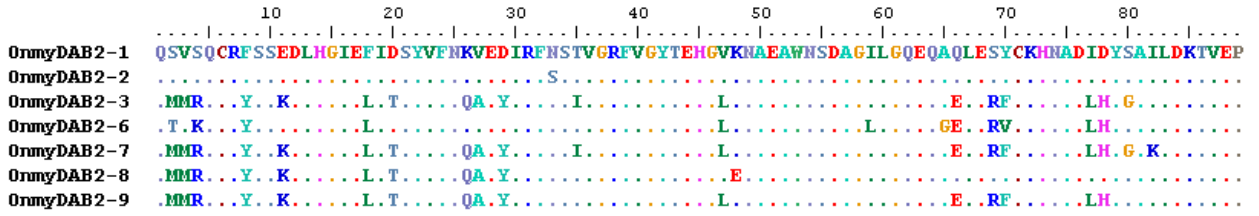


Figure 1.5: Unique amino acid sequences of the Pennask rainbow trout population. Unique amino acid sequences based on MHC class II β gene full length exon 2 isolated from Pennask rainbow trout genomic DNA, (a) DAB1 (b) DAB2

(a)

(b)

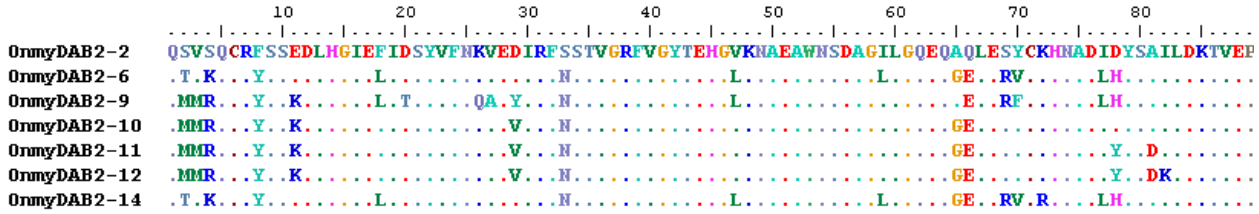
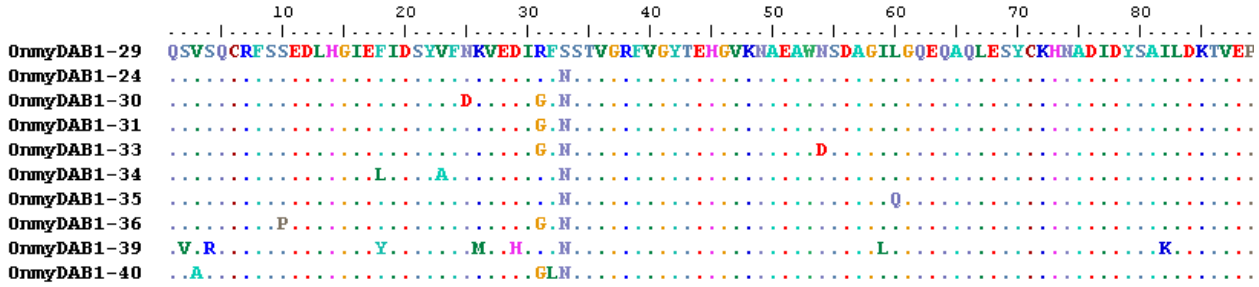


Figure 1.6: Unique amino acid sequences of the Blackwater rainbow trout population. Unique amino acid sequences based on MHC class II β gene full length exon 2 isolated from Blackwater rainbow trout genomic DNA, (a) DAB1 (b) DAB2

(a)



(b)

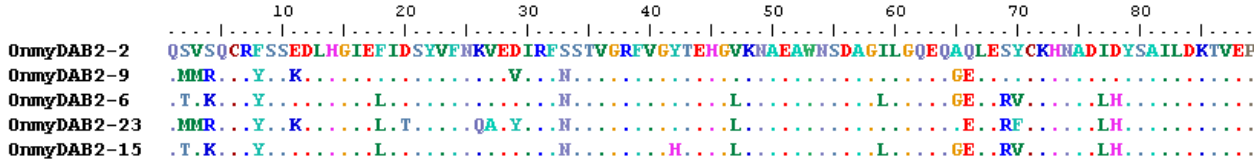
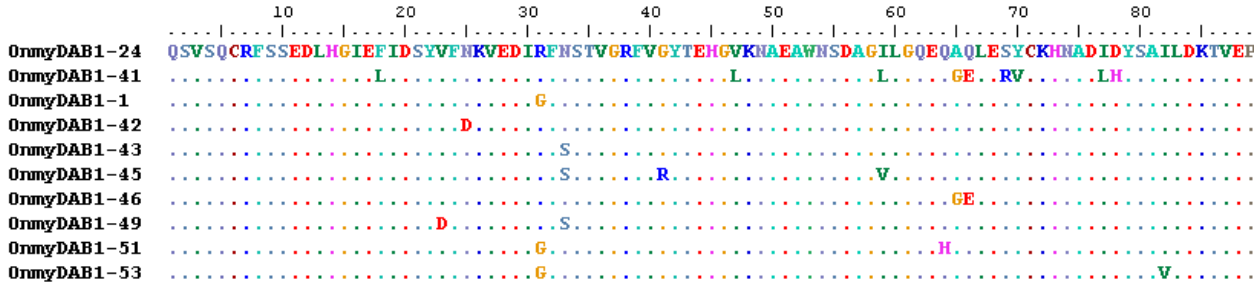
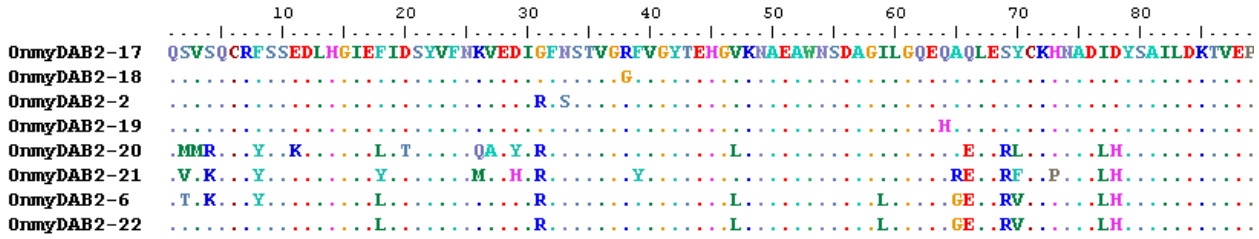


Figure 1.7: Unique amino acid sequences of the Tzenzaicut rainbow trout population. Unique amino acid sequences based on MHC class II β gene full length exon 2 isolated from Tzenzaicut rainbow trout genomic DNA, (a) DAB1 (b) DAB2

(a)



(b)



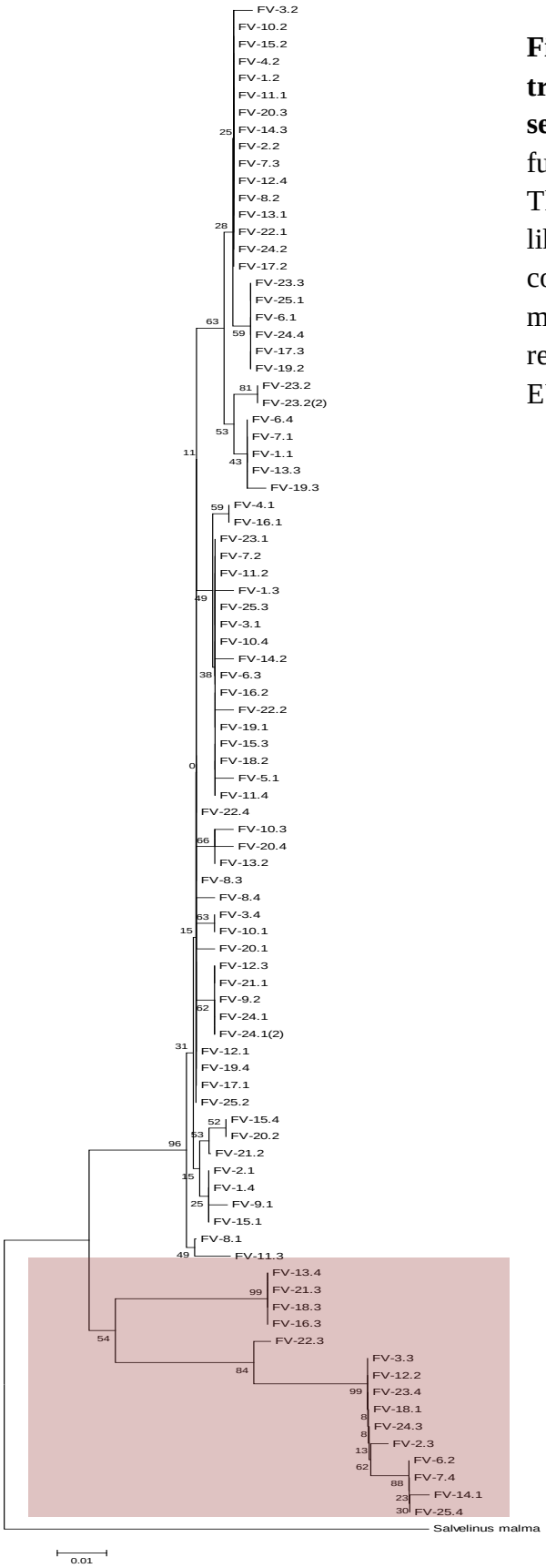


Figure 1.8: Complete neighbour-joining tree of full length rainbow trout exon sequences. Initial nucleotide sequences of the full length exon sequences of MH class II β. The pink box indicates sequences that are likely from a second locus. The tree was constructed using the Jukes and Cantor model. Bootstrap values were based on 1000 replicates with *Salvelinus malma* (Accession EU159601.1) as the outgroup.

Each population produced a varying number of alleles for the DAB1 and DAB2 loci (Tables 1.1 and 1.2). Within a population of 25, FV contained 11 unique alleles, based on nucleotide sequences, which is more than PN with 5 unique, BW with 10 unique, and TZEN with 10 unique alleles at the DAB1 locus. At the DAB2 locus, FV contained 7 unique alleles which is more than PN with 7 unique, BW with 5 unique, and TZEN with 8 unique alleles. While no complete sequences were found to be shared with other species, some alleles were found to be shared between populations of *O. mykiss* (Appendix). The level of nucleotide diversity differed between the four populations of rainbow trout (Tables 1.3 and 1.4). The within species diversity ranged from 0.011 to 0.006 and the base composition between sequences remained constant.

In the phylogenetic analysis of the rainbow trout MH class II β nucleotide sequences (neighbour-joining tree in Appendix) each population did not group separately, however, at the DAB1 locus FV and TZEN tended to group together. This indicates FV and TZEN to be more closely related. To determine the level of variance between the four populations rough F-statistics (F_{ST}) were determined for each locus (Tables 1.5-1.6). The F_{ST} was calculated with the assumption that there was only one locus per individual. Due to there being more than two alleles for some fish at the DAB1 locus, and it being unknown which allele is at which locus in these cases, the F_{ST} is just a rough estimate. Pairwise F_{ST} results for the DAB1 locus showed that there was a significant difference between all populations ($p < 0.0001$) except between FV and TZEN. The DAB2 locus showed a significant difference between only FV and BW ($p < 0.0001$) and BW and TZEN ($p < 0.0001$) (Tables 1.7 and 1.8).

The levels of synonymous (d_S) and non-synonymous (d_N) substitutions per population of rainbow trout sequences are shown in Tables 1.9 and 1.10 for each of the DAB1 and DAB2 loci.

For each population at the DAB1 locus the d_S was always shown to be greater than the d_N and overall the d_S/d_N ranged from 0.2820 to 0.6232. However, the DAB2 locus shows a greater difference in the d_N and d_S substitutions where the d_N/d_S ratio overall is 2.56.

Table 1.1: Summary of rainbow trout MH class II β alleles found in the four sub-populations at DAB1. The table represents the number of total alleles found for each population (n=25) at the DAB1 locus as well as the unique and shared numbers between each of the populations at the amino acid level. Asterisk (*) indicates that the same alleles were shared multiple times within the population. Numbers based on unique amino acid sequences.

Population (n=25)	Number of alleles	Number of unique alleles	Shared alleles between populations			
			Fraser Valley	Pennask	Blackwater	Tzenzaicut
Fraser Valley	23	11		3*	3*	2*
Pennask	9	5	3*		4*	1*
Blackwater	15	10	3*	4*		1*
Tzenzaicut	16	10	2*	1*	1*	

Table 1.2: Summary of rainbow trout MH class II β alleles found in the four sub-populations at DAB2. The table represents the number of total alleles found for each population (n=25) at the DAB2 locus as well as the unique and shared numbers between each of the populations at the amino acid level. Asterisk (*) indicates that the same alleles were shared multiple times within the population. Numbers based on unique amino acid sequences.

Population (n=25)	Number of alleles	Number of unique alleles	Shared alleles between populations			
			Fraser Valley	Pennask	Blackwater	Tzenzaicut
Fraser Valley	9	7		3*	3*	2*
Pennask	8	7	3*		4*	1*
Blackwater	6	5	3*	4*		1*
Tzenzaicut	8	8	2*	1*	1*	

Table 1.3: Summary of the composition of MH class II β alleles found in the four sub-populations. Table indicates the nucleotide diversity at the DAB1 locus in the four sub-populations of rainbow trout. Numbers based on all nucleotide sequences found within the population.

Populations	N	Diversity	Base Composition			
			C	T	A	G
Fraser Valley	23	0.0111	19.21%	24.11%	28.26%	28.43%
Pennask	9	0.0067	19.02%	24.34%	28.33%	28.30%
Blackwater	15	0.0129	19.04%	24.22%	28.29%	28.45%
Tzenzaicut	16	0.0091	19.11%	24.21%	28.16%	28.52%

Table 1.4: Summary of the composition of MH class II β alleles found in the four sub-populations. Table indicates the nucleotide diversity at the DAB2 locus in the four sub-populations of rainbow trout. Numbers based on all nucleotide sequences found within the population.

Populations	N	Diversity	Base Composition			
			C	T	A	G
Fraser Valley	9	0.0385	19.30%	24.16%	28.36%	28.18%
Pennask	8	0. 0453	18.96%	24.19%	28.57%	28.28%
Blackwater	6	0.0462	19.35%	24.06%	28.59%	28.401%
Tzenzaicut	8	0.0348	19.14%	24.27%	28.34%	28.25%

Table 1.5: Summary of the F_{ST} values calculated for the MH class II β alleles found in the four sub-populations at DAB1. Table indicates the F_{ST} values between populations at the DAB1 locus. F_{ST} values indicates the total variance accounted for by differences between populations where values closer to 1 show greater genetic diversity and values closer to 0 show a fixation of alleles. Numbers based on all nucleotide sequences found within the population.

	Fraser Valley	Pennask	Blackwater	Tzenzaicut
Fraser Valley	0.0000			
Pennask	0.1507	0.0000		
Blackwater	0.0970	0.0849	0.0000	
Tzenzaicut	0.02269	0.25243	0.12229	0.0000

Table 1.6: Summary of the F_{ST} values calculated for the MH class II β alleles found in the four sub-populations at DAB2. Table indicates the F_{ST} values between populations at the DAB2 locus. F_{ST} values indicates the total variance accounted for by differences between populations where values closer to 1 show greater genetic diversity and values closer to 0 show a fixation of alleles. Numbers based on all nucleotide sequences found within the population.

	Fraser Valley	Pennask	Blackwater	Tzenzaicut
Fraser Valley	0.0000			
Pennask	0.0258	0.0000		
Blackwater	0.1499	0.0351	0.0000	
Tzenzaicut	0.0069	0.0459	0.1785	0.0000

Table 1.7: Summary of the F_{ST} p-values calculated for the MH class II β alleles found in the four sub-populations at DAB1. Table indicates the F_{ST} p-values of each population at the DAB1 locus which shows whether the populations were significantly different. Numbers based on all nucleotide sequences found within the population.

	Fraser Valley	Pennask	Blackwater	Tzenzaicut
Fraser Valley	*			
Pennask	0.0001	*		
Blackwater	0.0001	0.0001	*	
Tzenzaicut	0.05405	0.0001	0.0001	*

Table 1.8: Summary of the F_{ST} p-values calculated for the MH class II β alleles found in the four sub-populations at DAB2. Table indicates the F_{ST} p-values of each population at the DAB2 locus which shows whether the populations were significantly different. Numbers based on all nucleotide sequences found within the population.

	Fraser Valley	Pennask	Blackwater	Tzenzaicut
Fraser Valley	*			
Pennask	0.1802	*		
Blackwater	0.0001	0.1261	*	
Tzenzaicut	0.2613	0.0901	0.0001	*

Table 1.9: Summary of the d_N/d_S ratios calculated for the MH class II β alleles found in the four sub-populations at DAB1. The number of non-synonymous substitutions (d_N) to the number of synonymous substitutions (d_S) for each population of rainbow trout which is used as an indicator of selective pressure acting on a protein-coding gene.

	Nonsynonymous (dN)	Synonymous (dS)	dN/dS
Fraser Valley	0.0086	0.0305	0.2820
Pennask	0.0129	0.0207	0.6232
Blackwater	0.0146	0.0317	0.4606
Tzenzaicut	0.0156	0.0266	0.5865
Overall	0.0128	0.0295	0.4350

Table 1.10: Summary of the d_N/d_S ratios calculated for the MH class II β alleles found in the four sub-populations at DAB2. The number of non-synonymous substitutions (d_N) to the number of synonymous substitutions (d_S) for each population of rainbow trout which is used as an indicator of selective pressure acting on a protein-coding gene.

	Nonsynonymous	Synonymous	dN/dS
Fraser Valley	0.0618	0.0216	2.8611
Pennask	0.0597	0.0249	2.3976
Blackwater	0.0554	0.0242	2.2893
Tzenzaicut	0.0623	0.0176	3.5398
Overall	0.0599	0.0234	2.5637

1.4 Discussion

1.4.1 MH class II β diversity among rainbow trout populations

Due to the extreme polymorphism of MH genes, rainbow trout are capable of efficiently recognizing a vast array of pathogenic peptides. This is thought to be due to the selection pressures throughout evolution (Miller *et al.,* 2002). A population's chance of survival is directly proportional to the sequence and allelic diversity of MHC genes it carries. With greater diversity comes a greater immunity against pathogens and therefore a greater chance of survival (Grimholt *et al.,* 1993).

In order to evaluate the polymorphic content of the MH class II β gene, exon 2 sequences were compared from four sub-populations of rainbow trout. It was expected that up to two alleles would be found per individual; however, this was not the case, as many as one to four alleles were found in each individual which suggests two loci were amplified. Steps were taken to make certain no erroneous sequences were amplified such as the PCR+1 procedure which ensured great degree of confidence in the possibility of two different loci. As well, due to the conservative estimates on allele differences within populations no extraneous alleles can be found in this data. When intron and exon sequences were analyzed several alleles were shown to be different enough to form a new clade in a neighbour-joining phylogenetic tree, supporting the possibility of a second locus. This accounted for some, but not all, of the extra alleles. The intron sequences also contained low levels of nucleotide diversity throughout the four populations which indicates that the loci are little differentiated. It is likely that, due to the lack of sequence diversity, the primers could not distinguish between loci and therefore amplified both copies of the class II locus. However, studies conducted on *Xenopus* by Courtet *et al.,* (2001) discovered that there was the reduction of the MHC genes to a disomic inheritance pattern in a tetraploid

species. These results suggest there are three optimally expressed loci for class II. To efficiently recognize a vast array of pathogens the peptide binding region of the MH alleles has been shown to be extremely polymorphic this which can be attributed to the selection pressures throughout evolution (Miller *et al.*, 2002). With greater sequence and allelic diversity there is a greater chance of survival (Grimholt *et al.*, 1993).

Natural selection maintains high levels of polymorphism and heterozygosity in MH genes of vertebrate organisms, including rainbow trout. Rainbow trout, not being native to BC, migrated north approximately 200 million years ago requiring increased variability in their MH genes. This variability promotes long evolutionary perseverance of populations and strongly differentiated allelic lineages. Due to the strong genetic differentiation at the MH class II β locus between each population besides FV and TZEN, it can be assumed that gene flow among various rainbow trout populations originating from four different lake systems, even those in close geographical proximity, is restricted enough to allow for strong adaptations at loci subject to natural selection (Miller *et al.*, 2001). In addition to the polymorphism, the FV population exhibited a higher number of alleles in comparison to the other three populations which suggests a selective advantage for FV. Populations with more potential binding sites suggest a greater chance of binding peptides from all potential pathogens such as *F. psychrophilum.* This leads to a heterozygous advantage and survival of the population. When a neighbour-joining phylogenetic tree was created (Appendix) all populations were interspersed amongst each other which suggest a common pool of ancestral alleles.

To determine the level of variance between the four populations the F_{ST} values were determined for each. Pairwise F_{ST} values calculated showed no significant differences between FV and TZEN which suggests that some MH class II β alleles which is especially interesting in

that Tzenzaicut Lake and Fraser Valley are the furthest apart geographically than the other two lakes. These findings suggest each population were under the same selective pressures or lesser selection pressures as compared with the others. This could also indicate the two populations diverged long after BW and PN so although they all share the same ancestral pools of alleles, FV and TZEN have a more recent common ancestor than BW and PN. This data suggests a difference in pathogen recognition and presentation capabilities between the significantly different populations and therefore a different in ability to recognize bacteria, such as *F. psychrophilum*, effectively. This could potentially lead to a particular resistance or susceptibility of these rainbow trout populations to infection by *F. psychrophilum.*

A closer look at the at the polymorphism of the DAB1 and DAB2 loci of the MH class II β alleles in rainbow trout show an excess of synonymous mutations over non-synonymous in DAB1 however; the opposite was found at the DAB2 locus. The increase of non-synonymous relative to synonymous substitutions found in codons of DAB2 indicates that selection could be maintaining variability within the populations which is responsible for the allelic diversity. However, positive selection cannot be completely identified. It was expected that more non-synonymous mutations would have been found in the DAB1 locus as well which would have increased the number of potential peptide binding sites within a population but this was not the case. This could mean that selection pressures were not as great for the fish of these populations.

1.4.2 Selection pressures among populations

Polymorphism is a common characteristic between the MH alleles of many species including rainbow trout. These glycoproteins play a crucial role in the identification and presentation of foreign peptides to the adaptive immune system (Janeway *et al.*, 2001). The level

of diversity and polymorphism in each population can be driven by pathogen fluctuations in various environments. Environments that contain high levels of pathogens are more likely to experience relatively higher selection pressures for maintaining polymorphism in their MH genes as compared to environments with lower rates of infectious disease (Trowsdale *et al.,* 1989). However, if a population is consistently exposed to one particular pathogen, allele variability targeted to the pathogen will be selected for thus decreasing polymorphism within a population. Within species, geographic location in the populations of rainbow trout as well as species and virulence of the pathogen will also create differences in the selection pressures exerted on populations (Miller *et al.,* 2001). For example, rainbow trout populations that share habitats with infectious bacteria, such as *F. psychrophilum,* are more likely to be selectively pressured to maintain higher levels of polymorphism and motifs to recognize *F. psychrophilum* at the class II locus.

The MH allelic diversity data of this study demonstrates significant differences in the selective forces experienced with the rainbow trout β alleles in multiple sub-populations, and therefore lakes. Specifically, the allelic diversity between FV and PN, FV and BW as well as PN and TZEN suggest varying capabilities of antigen recognition. This data shows potential genetic differences in susceptibility to specific disease among rainbow trout populations with Fraser Valley and Tzenzaicut populations having potentially the best chances against invading pathogens, such as *F. psychrophilum,* and Blackwater and Pennask potentially being most susceptible.

1.4.3 Future Research

The results of this study represent the vast array of genetic variation of the MH class II β alleles of four sub-populations of rainbow trout. Although there is much variation it is difficult to say whether or not the differentiated peptide binding regions are beneficial to populations of rainbow trout in their respective lakes. There is simply not enough information on the pathological histories of the Fraser Valley, Pennask, Dragon, Tunkawa and Tzenzaicut lakes and the involvement of the MH in disease and parasite susceptibility, to elucidate the exact selective forces on the MH genes. In the future, characterizing specific pathogens to specific variations in MH polymorphism could be beneficial to breeding rainbow trout in hatcheries. In addition to increased information of the pathological histories in specific habitats, there has been confusion surrounding possible numbers of β_2 loci in rainbow trout. To determine the number of loci within a population, Southern blots can be conducted. Once exact loci numbers are determined expression analysis can be conducted through RT-PCR to determine which loci is expressed in rainbow trout.

Chapter 2: Characterizing the immune response to *Flavobacterium psychrophilum* in rainbow trout (*Oncorhynchus mykiss*).

2.1.1 Introduction

Since the late 1940s the aquaculture industry has been afflicted with a bacterium called *Flavobacterium psychrophilum* which has resulted in the substantial loss of rainbow trout fry in hatcheries across Europe (Daalsgard, 1993; Bernardet *et al.,* 1988). *Flavobacterium psychrophilum* is a flexible, rod-shaped, Gram-negative bacterium, which is capable of slow motility (Bernardet & Kerouault, 1989). It is the causative agent of Bacterial Coldwater Disease (BCD) and Rainbow Trout Fry Syndrome (RTFS) and is now recognized as a pathogen occurring worldwide in freshwater aquaculture (Wiklund & Dalsgaard, 2001; Nematollahi *et al*., 2003).

The bacteria mainly affect salmonid fishes, namely rainbow trout and coho salmon. It has also been known to affect non-salmonid fish, and causes systemic disease in cyprinids and eels however it is predominantly an issue for the commercial salmonid industry of fish farming (Lehman *et al.,* 1991). It has been suggested that even though disease transmission between rainbow trout is extremely effective, *F. psychrophilum* can also utilize multiple fish species in natural habitats to act as carriers from which they can infect the more susceptible salmonid species thus making disease eradication increasingly more important (Lehman *et al.,* 1991).

2.1.2 Clinical Presentation and Histopathology

While the molecular pathogenesis of *F. psychrophilum* is not well known in comparison to other bacteria such as *E. coli,* the virulence of the disease has been studied. Most isolates of *F. psychrophilum* are proteolytic and are able to degrade casein, gelatin, elastin and some strains

have the capacity to degrade collagen and fibrinogen (Bernadet *et al.*, 1996; Holt, 1987). This direct damage by digestion of the extracellular tissues of the fish leads to increased ports of entry for the bacteria (Dalsgaard & Madsen, 2000).

Other clinical and histopathological observations as well as mortality rates differ depending on species. In rainbow trout, *F. psychrophilum* causes RTFS specifically which, when the bacterium is introduced into a population can lead to up to 80% of mortalities (Chua, 1991). In populations of fry, approximately 0.2-2 grams, the disease is marked by anemia which is indicated by pale gills, kidney, intestines and liver. In addition to anemia, the most prominent histopathological change occurs in the spleen. Affected fish had enlarged spleens with extensive necrosis. This was indicated by the presence of phagosomes and residual bodies in splenic reticuloendothelial cells. This evidence indicated widespread lysosomal activity within the phagocytes (Nematollahi *et al.*, 2003). The connective and muscular tissue of infected fry have been shown to be targeted by *F. psychrophilum* specifically in cases of BCD. The bacteria produce proteolytic enzymes, which target these tissues as well as the cartilage and bone of the cranium, gills, bony fins and ribs leading to mass degradation of the tissues (Lumsden *et al.*, 1996). Increased tissue degradation and damage leads to increased ports of entry for the bacteria's invasion into the body. Clinically, the fry appear lethargic and swim close to or at the surface of the water and go off feed (Rangdale, 1995).

2.1.3 Transmission

In addition to the causation of disease with *F. psychrophilum* the virulence is also determined by the port of entry and transmission of the bacteria. Although port of entry has yet to be positively identified, evidence shows the bacteria to reside in and around the gills, operculum, skin mucus and connective tissue of the skin which suggests that the bacteria is part

of the normal flora of rainbow trout (Holt *et al*., 1993). However, Liu *et al*., (2001) showed that in naturally infected rainbow trout *F. psychrophilum* infiltrates the lumen as well as the mucosa of the stomach which suggests the gastro-intestinal tract as port of entry. In addition to port of entry, two types of transmission have also been studied including vertical (mother to offspring) and horizontal transmission (the spread of infection between organisms). Evidence of vertical transmission in rainbow trout with *F. psychrophilum* was identified by Brown and colleagues in 1997 where they were able to isolate the bacteria from the contents of newly fertilized, surface-disinfected eggs. For horizontal transmission, water is the medium in which viable *F. psychrophilum* moves which is by far the most intrusive to the habitat. The bacteria are usually spread through contact of pathogen-carrier fish, bacteria-shedding diseased and dead fish (Starliper, 2010). The bacteria can alter its morphology and utilize nutrients in the fish's habitat to cope with the varying conditions such as starvation. This allows *F. psychrophilum* to survive in water for months infecting all salmonids inhabiting the area and suggests horizontal transmission is correlated with high bacterial numbers and increased disease (Brown *et al*., 1997).

2.1.4 Immune response and Analysis Techniques

The immune response to *F. psychrophilum* has yet to be fully elucidated, however it has been shown that once infected, the rainbow trout spleen becomes the site of phagocytic and lysosomal activity within the phagocytes which shows the activity of the innate response (Rangdale, 1995). However, *F. psychrophilum* is contained within a capsule which has been shown to be anti-phagocytic. In addition to the spleen, the production of reactive oxygen species was seen in the phagocytes of rainbow trout head kidney (Lammens *et al.,* 2000). As well, preliminary evidence demonstrates the ability of rainbow trout to produce a specific antibody

response to *F. psychrophilum* however the extent to which it confers protection is mostly unknown (LaFrentz *et al.,* 2002). Therefore, a more complete characterization of the immune response is needed.

2.1.4.1 Chemiluminescence

To characterize the immune response of rainbow trout to *F. psychrophilum* it is necessary to determine both the innate and adaptive responses. One of the earliest initiatives of the inflammatory response is the migration of phagocytes from the blood to the location of bacterial invasion or injury. This leads to an accumulation of phagocytes, specifically monocytes, in the circulating blood and mature macrophages in the tissues of all teleosts (Griffin, 1984). The phagocytes in both the blood and tissues perform multiple bactericidal activities including respiratory burst (Griffin, 1984). Respiratory burst is a process in which phagocytic cells begin to convert oxygen into the reactive oxygen species (ROS) superoxide and hydrogen peroxide, which act as microbicidal oxidants capable of inflicting lethal injury on bacteria (Baboir, 1984). One assay that can be utilized to measure respiratory burst is chemiluminescence.

Zymosan-enhanced chemiluminescence (CL) is a technique widely used to measure respiratory burst. The production of ROS generates electronically excited states, which, on relaxation to the ground state, emit photons. The photon intensity can then be measured using a luminometer and the response can be chemically enhanced using luminol and zymosan. Zymosan, a yeast cell wall preparation, which contains glucose and mannose polymers, can be used to induce oxygen radical production. These carbohydrate polymers are bound by complement as well as many other proteins including adhesion proteins (Gilbertson *et al.,* 2003; Marnila *et al.,* 1995). When the assay is conducted using whole blood, the oxygen radical production is measured from circulating neutrophils and monocytes (however neutrophils are

more common in blood), which are an essential component of the innate immune response (Gilbertson *et al.,* 2003).

2.1.4.2 Enzyme-Linked Immunosorbent Assay

The enzyme-linked immunosorbent assays (ELISA) is one of the most widely used serological techniques due to its ability to detect extremely low amounts of antibody in serum samples. There are two different types of commonly used ELISA techniques, a direct and in indirect ELISA. A direct ELISA uses monoclonal antibodies to detect the presence of a particular antigen in a sample whereas an indirect ELISA is used to detect the presence of a specific antibody in a serum sample. In an indirect ELISA, microtiter plates which have been previously coated with a known antigen are incubated with the test serum. The assay allows for the detection of the antibodies that bind to the antigen through chemiluminescence. In this way the indirect ELISA can be used to quantify the antibodies in the serum to bacteria, specifically *F. psychrophilum*, which will in turn represent the level of the adaptive immune response (Hornbeck, 1991).

2.1.5 Purpose of Study

The goal of this study was to characterize and analyze the immune response between vaccinated and non-vaccinated rainbow trout to the bacteria *F. psychrophilum.* Preliminary research has shown conflicting evidence of the effects of vaccine on mortality rates. Various immunological methods can be used to estimate individuals' immunocompetence including chemiluminescence and ELISAs. It has been suggested that these two methodologies effectively analyze the principle components of the immune response; the innate and adaptive response (Vainikka *et al.,* 2009). Therefore, the objectives of this study were to characterize the innate

and adaptive immunity of rainbow trout following vaccination with two preparations of formalin-killed *F. psychrophilum* and subsequent experimental infection.

2.2 Materials and Methods

2.2.1 Exposure Trial

Four hundred and fifty juvenile Fraser Valley triploid rainbow trout (FVRB 3n) were housed at the University of Guelph Hagen Aqualab. The fish were obtained from the FFSBC having already been vaccinated. Fish were vaccinated five months prior to infection with one of two formalin-killed vaccines that were produced by growing the vaccine strain FFSBC-92 at 8°C and 18°C (Microtek International Inc.). Vaccine strain of *F. psychrophilum* FFSBC-92 was previously isolated from a clinically infected rainbow trout at the Vancouver Island Trout Hatchery. Each fish was vaccinated by immersion in a static bath of 1 L vaccine to 9 L of water for 1 minute with supplemental oxygen. Procedure was repeated for each fish approximately 8-10 times and was completed by Indervesh Dahiya. The infection trials commenced at the University of Guelph.

The 450 FVRB 3n were split into ten groups of 45 fish into tanks with a volume of approximately 148 L. The fish were designated into one of four tanks for vaccine strain FFSBC-92 at 8°C, one of four tanks for vaccine strain FFSBC-92 at 18°C and one of two tanks for rainbow trout that were not previously vaccinated. Forty fish were sampled prior to infection for a time point 0 hrs. Fish were then infected by injecting $1x10^8$ cfu/mL of *F. psychrophilum* into the peritoneal cavity of all fish. After infection forty fish were sampled at each time point, four fish per tank, including 24 hrs, 4, 7, 21 and 28 days.

2.2.2 Blood Collection

Fish were euthanized with 0.1% benzocaine and immediately sampled. Blood was drawn utilizing a 23 gauge 1 inch needle containing approximately 100 µL of heparin (20 units/mL) and dispensed into a 1.5 mL eppendorf tube. The blood was allowed to clot at 4°C over night and subsequently centrifuged at 13,000 rpm for 5 min. The upper serum layer was then transferred into a new 1.5mL eppendorf tube and stored at 4°C.

2.2.3 Chemiluminescence Reagent Preparation

Zymosan A (Sigma #Z4250, Oakville Ontario) was prepared by boiling 100 mg of zymosan A in 5 mL of Hanks balanced salt solution (HBSS) for 30 min. The sample was then centrifuged at 3000 rpm for 10 min, the supernatant was removed and the process was repeated. Following the second wash the zymosan pellet was re-suspended in 5 mL of HBSS to a final concentration of 20 mg/mL and was then stored at 4°C. A 100 mM stock of luminol (5-amino-2,3-dihydro-1,4-pthalazinedione, Sigma #A8511 Oakville) was prepared by dissolving 17.7 mg of luminol in 1 mL of cell culture grade dimethyl sulfoxide (DMSO, Sigma #D2650, Oakville Ontario). A working stock of 1 mM luminol was prepared by diluting the 100 mM stock in HBSS.

2.2.4 Respiratory Burst

The respiratory burst activity of circulating blood neutrophils and monocytes was measured using zymosan-induced chemiluminescence (CL) in individual rainbow trout. Triplicate 10 µL samples of whole blood from individual rainbow trout were added to each well of white 96-well plates (Fisher Scientific #3917, Ottawa, Ontario). Following addition of the whole blood, 110 µL of HBSS, 60 µL of 1 mM luminol and 20 µL of zymosan A were added to each well and mixed thoroughly. The plate was then placed in a POLARStar Omega microplate

(BMG Labtech) reader to determine the CL from each well. The measurements were performed at room temperature and the relative light units (RLU) produced was measured every 2 min for 120 min to obtain kinetic curves for each sample.

2.2.5 Bacterial Culture for Antigen Production

For ELISA antigen production *F. psychrophilum* was obtained from the University of Guelph. The cells were grown in 300 mL of cytophaga broth to early stationary phase at 15°C. Cells were then inactivated using 0.5% formalin and incubated at 15°C for 24 h. The following day the bacterial preparation was washed with PBS twice and stored at 4°C.

To better ensure antibody detection the process of bead beating was utilized to release the proteins from the cells of the *F. psychrophilum* culture. To do this, 2 mL screw cap tubes were filled with 0.8 mL zirconia/silica (0.1 mm) beads and 1 mL of the *F. psychrophilum* culture. The tubes were then placed in a BioSpec mini-beadbeater and agitated at full speed for 45 sec followed by 1 min incubation on ice. The procedure was repeated five times and centrifuged at 10,000 rpm for 15 min and the supernatant recovered to sterile 1.5 mL eppendorf tubes. The agitated *F. psychrophilum* was then diluted by 30% with coating buffer (35 mM $NaHCO_3$, 15 mM Na_2CO_3, and 3 mM NaN_3, pH 9.6) and stored at 4°C.

2.2.6 Enzyme-linked Immunosorbent Assay

Enzyme-linked immunosorbent assays (ELISA) were conducted on all serum samples to identify anti- *F. psychrophilum* antibodies. The wells of NUNC 96-well plates were coated with 100 µL of the diluted antigen as well as positive and negative controls and incubated at 4°C overnight. The positive control consisted of 10 µg/ml keyhole limpet hemocyanin (KLH) diluted to 1/1000 with coating buffer. The negative control consisted of 100 µL of coating buffer to

indicate non-specific binding of serum. The following day the plates were washed three times with TBS-T (0.14 M NaCl, 2.7 mM KCl, 25 mM Tris, 0.5% Tween 20, pH 8) and blocked with 300 µl of 5% skim milk powder dissolved in TBS-T for 3 hrs at room temperature. Wells were then washed three times with TBS-T prior to the addition of 100 µl of the trout serum samples, plated in triplicate, which had been diluted 1/10 in the 5% skim milk and incubated for 2 hrs at room temperature. After three washes with TBS-T, anti-rainbow trout IgM monoclonal antibody (Aquatic Diagostics Ltd., Stirling, Scotland) was diluted to 1:100 and 100 µl was added to each well and incubated for 1hr at room temperature. Following three more washes 100 µL of a 1:1000 dilution of conjugate (anti-mouse IgG-HRP diluted with 1% Bovine Serum Albumin (BSA) dissolved in TBS-T) was added to each well and incubated at room temperature for 1 hr. The plate was then washed three final times with TBS-T before the addition of 100 µL of Sigma Fast p-nitrophenyl phosphate (p-NPP) tablet set (Sigma- Aldrich, ON, Canada). The plate was then covered and incubated in complete darkness for 30 min. The reaction was stopped by the addition of 50 µL of 0.03M NaOH and the absorbance was read at 405 ηm using the Versamax microplate reader (Molecular Devices, CA, USA).

2.3 Results

2.3.1 Chemiluminescence

Respiratory burst responses of rainbow trout circulating blood neutrophils and monocytes to stimulation with zymosan are illustrated in Figure 2.1. Chemiluminescence responses began immediately after stimulation and the peak reactivity occurred approximately one hour after stimulation (Figure 2.1). Comparing the three treatments at 24 hrs post-infection, an analysis of variance demonstrated a significant effect between the vaccinated and non-vaccinated groups (*p*

< 0.01). However, between the vaccine strains FFSBC-92 grown at 8°C and 18°C there was no significant difference in the rate of respiratory burst although the RLU produced were still significantly ($p < 0.05$) above background levels produced in the absence of whole blood.

When comparing time points post-infection (Figure 2.2), an analysis of variance demonstrated a significant effect ($p < 0.01$) between the respiratory burst of vaccinated versus non-vaccinated fish at 24 hrs as compared to days 4 and 7. This decrease in response by day 7 could be attributed to there being a decline in the number of neutrophils over time or just a decrease in the effective response of the cells after 7 days. However, there was no significant difference between the two vaccinated groups at any of the three time points (Figure 2.2). As well, there was a significant decrease in response from 24 hrs to Day 7 ($p < 0.01$). The overall trend of the respiratory burst activity decreased over time which is expected of an innate immune response which decreases in time as the adaptive response increases.

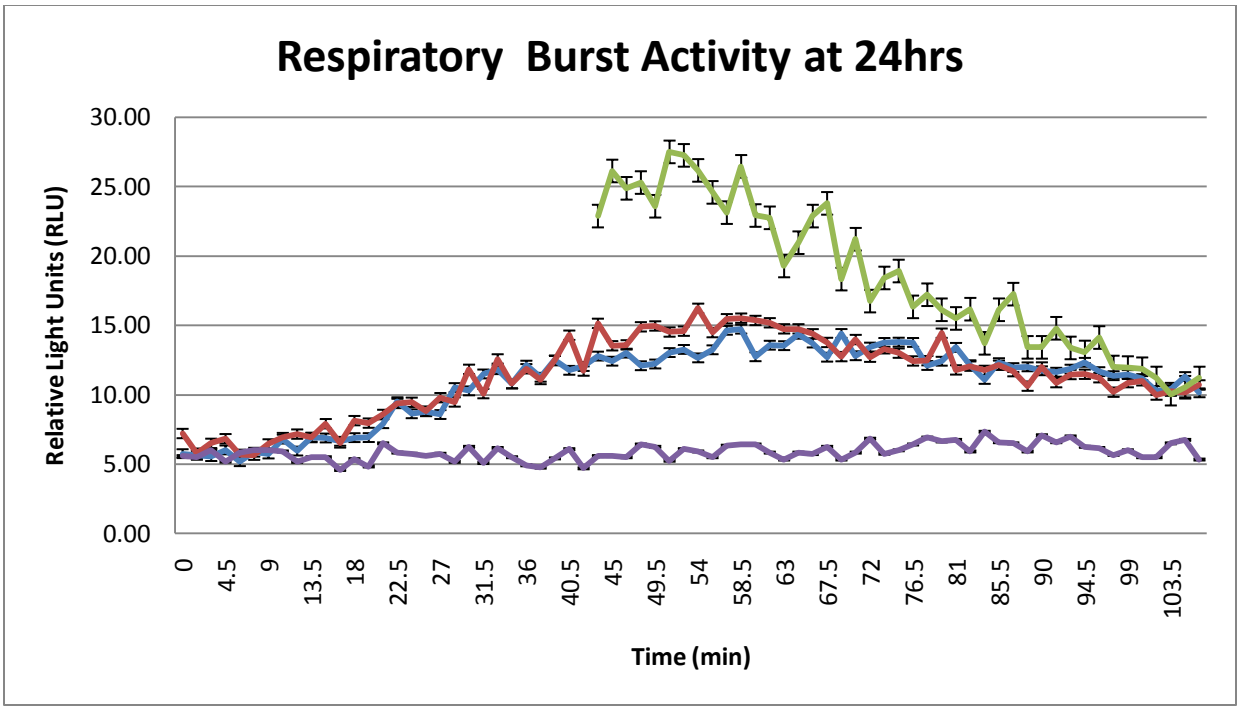


Figure 2.1: Respiratory burst activity at 24 hrs.
Respiratory burst responses of rainbow trout circulating blood neutrophils stimulated with Zymosan A 24-hours post *Flavobacterium psychrophilum* infection. Fish were vaccinated five months prior to infection with one of two formalin-killed vaccines that were produced by growing the vaccine strain FFSBC-92 at 8°C and 18°C. The green line represents the average respiratory burst activity of the non-vaccinated fish. The red line represents the vaccine strain FFSBC-92 grown at 8°C. The blue line represents the vaccine strain FFSBC-92 grown at 8°C. The purple line represents the assay control. Data shown is mean and standard error of 40 fish total, 16 for each vaccinated group and 8 for the non-vaccinated, with a significant difference ($p < 0.01$) between vaccinated and non-vaccinated fish.

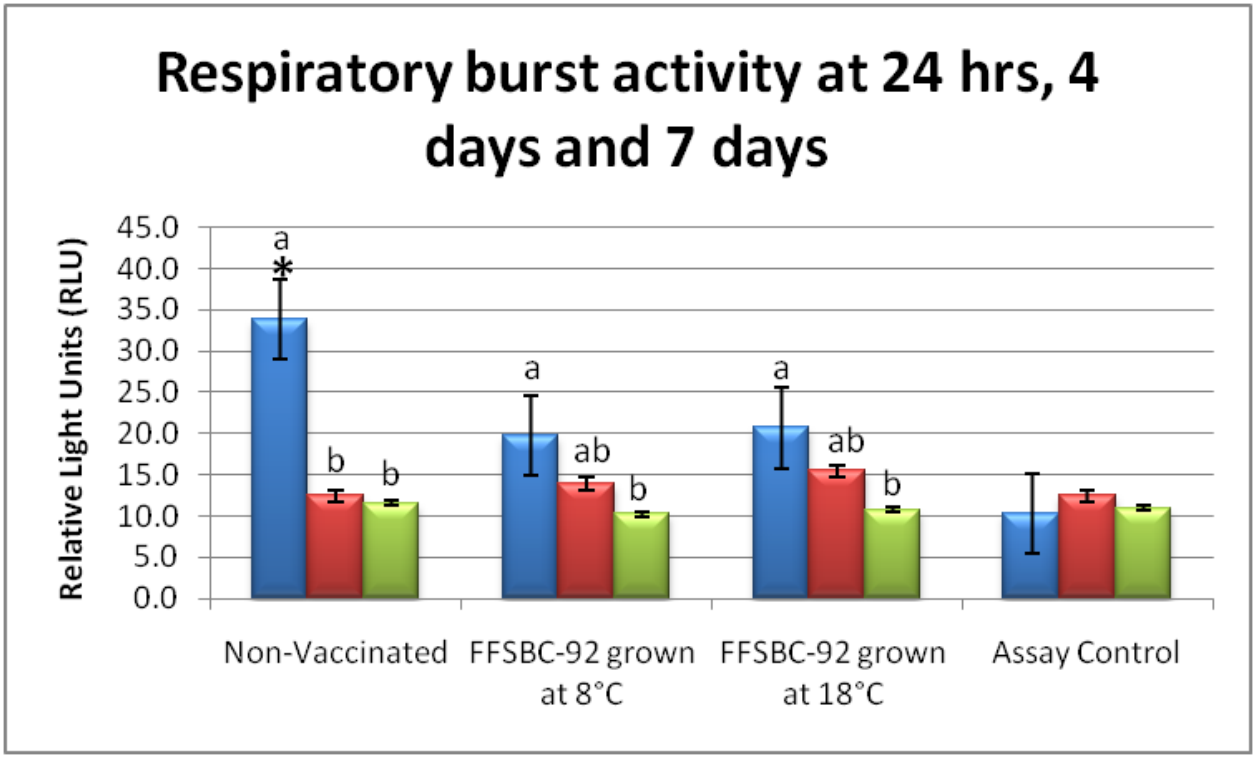


Figure 2.2: Respiratory burst activity at 24 hrs, 4 days and 7 days.
Respiratory burst responses of rainbow trout circulating blood neutrophils stimulated with zymosan A 24 hrs, 4 days and 7 days post *Flavobacterium psychrophilum* infection. Fish were vaccinated with one of two formalin-killed vaccines that were produced by growing the vaccine strain FFSBC-92 at 8°C and 18°C. The blue column represents the peak of respiratory burst activity at 24 hrs. The red column represents the peak of respiratory burst activity at 4 days and the green column represents the peak of respiratory burst activity at 7 days. Data shown is means and standard error of 120 fish, 16 for each vaccinated group and 8 for the non-vaccinated in each time-point, with a significant difference (*$p < 0.01$) between vaccinated and non-vaccinated fish at 24 hrs as compared to days 4 and 7. Days 4 and 7 produced no significant difference between vaccinated and non-vaccinated fish. Post-hoc tests confirmed significance between 24 hrs and Day 7 within each group. Groups with the same letter are not significantly different ($p < 0.01$).

2.3.1 ELISA

The antibody response of vaccinated versus non-vaccinated fish were measured in this experiment over a 28 day time period. Antibody responses of rainbow trout against *F. psychrophilum* are illustrated in Figure 2.3. Antibody responses began within the first 24 hrs where an analysis of variance demonstrated a significant increase from Day 0 ($p < 0.01$) in all three treatments including the vaccinated and non-vaccinated groups. Antibody response continued to increase significantly overall ($p < .01$) until the trial was terminated at Day 28.

Comparing the three treatments within each time-point, an analysis of variance concluded there was no significant difference between the non-vaccinated group and the groups that had been previously vaccinated with the vaccine strains FFSBC-92 grown at 8°C and 18°C. Overall it was determined that the exposure to the pathogen did induce a protective response in all fish. However, the trend of antibody response from Day 0 to Day 28 show that, while not being significant, the two vaccinated groups exhibited a lower antibody response than the non-vaccinated group but appeared to be reaching the same levels at approximately Day 28.

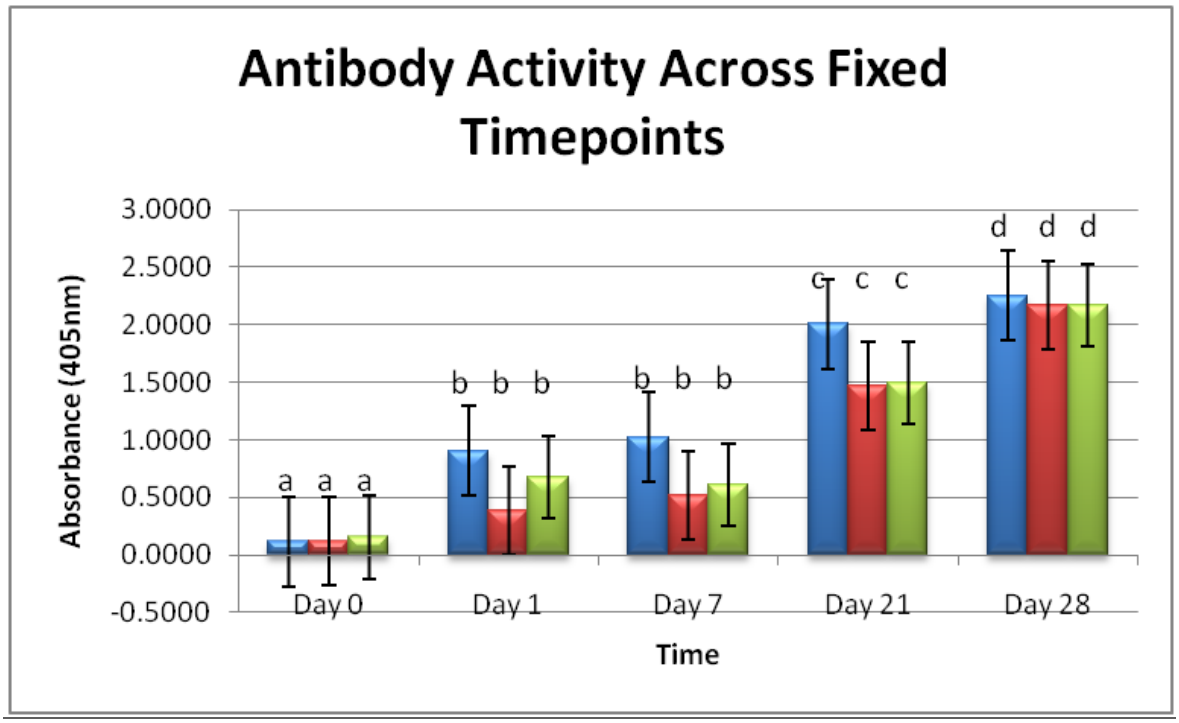


Figure 2.3: Antibody activity at fixed time-points

Antibody responses of infected rainbow trout at t=0, 1, 7, 21 and 28 days post *Flavobacterium psychrophilum* infection. Fish were vaccinated five months prior to infection with one of two formalin-killed vaccines that were produced by growing the vaccine strain FFSBC-92 at 8°C and 18°C. The blue column represents the antibody response of fish that were not vaccinated. The red column represents the antibody response for fish vaccinated with the strain FFSBC-92 grown at 8°C and the green column represents the antibody response for fish vaccinated with the strain FFSBC-92 grown at 18°C. Data shown is means and standard error of 200 fish, 16 for each vaccinated group and 8 for the non-vaccinated in each time-point, with a significant difference ($p < 0.01$) between all days except between Day 1 and Day 7. Groups with the same letter are not significantly different ($p < 0.01$).

2.5 Discussion

2.5.1 Efficacy of rainbow trout immune response against *F. psychrophilum*

The ability of the immune system to stage an effective immune response is essential for the well-being of all organisms. In rainbow trout, as well as other vertebrates, phagocytes play a crucial role throughout the innate immune response. Fish that were exposed to *F. psychrophilum* showed an increased level of respiratory burst over the assay control within the first 24 hrs which shows the immediate response of innate immunity. However, mortality rates between the two vaccinated groups and the non-vaccinated did not differ significantly.

In addition to the overall abundance of neutrophils in infected rainbow trout, the results from this study show that rainbow trout whole blood indicated significantly increased neutrophil numbers and/or activity in fish that were not vaccinated versus those that were. This increase in neutrophil number and activity implies an increase in the production of free radicals which implies an increase in bactericidal activity. With this increase there is a potential increase in the phagocytosis of potential pathogens. However, the lesser bactericidal activity in vaccinated fish could be due to the organisms having been exposed to *F. psychrophilum* five months prior to infection with the vaccine. It could be that the suppression of the innate immune response in groups vaccinated could be due to cell-signaling of the adaptive immune response having already been exposed to the pathogen.

Although there is still much debate of a memory response in teleosts it has been suggested that immunological memory requires approximately three months to develop in rainbow trout (Ackerman *et al.,* 2011). Due to the decrease in circulating blood neutrophil activity in fish that were previously vaccinated it can be suggested that immunological memory

was at play. Previous studies have shown high complement activity in fish infected with *F. psychrophilum* (Wiklund & Dalsgaard, 2002). It can be suggested that immunological memory triggered proteins of the complement system in addition to neutrophils whereby complement activity out competed neutrophil activity. Complement activity has been suggested as the most important non-specific immune mechanisms in the defense against invading pathogens, especially in fish (Yano, 1996).

Vaccines for the aquaculture industries have been licensed and proven effective for some fish pathogens, such as *Vibrio* species, and are available for the potential prevention of disease. The infection trial of this study showed an increase in the antibody levels in rainbow trout however, the values did not differ between vaccinated and non-vaccinated groups. This could be due to the preparation of the vaccine itself. There are two basic types of vaccines used in the aquaculture industry including recombinant and autogenous vaccines. With the recombinant protein vaccines, previous studies have identified the antigenic components of *F. psychrophilum* with molecular masses ranging from 22 to 100 kDa (Crump *et al.,* 2001). Vaccines that have been shown to be effective in the past are directed to the outer membrane fraction of the bacterium. The findings concluded that the enhanced immunogenicity of the vaccine may be due to the presence of outer membrane proteins in addition to the lipopolysaccharides on the cell walls (Rahman *et al.,* 2002). Additional findings concluded vaccines comprising high molecular weight antigens induced almost full protective immunity in rainbow trout (LaFrentz *et al.,* 2004). However, the vaccine produced in this study was an autogenous vaccine and therefore the vaccine was based on the whole cells recovered from a previously infected hatchery. Past studies have identified an extracellular polysaccharide capsule surrounding the bacteria which have been shown to not bind to MH and therefore not presented to T cells (Madetoja *et al*., 2006). This

could have resulted in a lower or less specific protection and implies a subunit vaccine could be more effective than a whole cell vaccine.

Even though significant numbers of circulating blood neutrophils and antibody activity was recorded, rainbow trout within the infection trial did not survive infection with *F. psychrophilum*. This could be attributed to too high a dose used throughout the infection trial. It also suggests that the innate and adaptive immune response of rainbow trout to the bacteria simply was not enough to combat the invading pathogen for either the non-vaccinated or the two vaccinated groups. For the virulent *F. psychrophilum* it is unclear whether resistance to the bacterial can be considered part of adaptive immunity. The majority of the mortalities within the infection trial occurred within 20 days of infection which was insufficient time for an effective adaptive response of rainbow trout. As well, while antibody activity is often used to determine the efficacy of the adaptive immune response, it might not be directly related to the protective value of a vaccine or even correlated with the survival of the rainbow trout. Thuvander and colleagues (1993) demonstrated that the outcome of an infection trial could potentially be independent of the agglutinating antibody titer in the serum of the infected fish. It could perhaps be the specificity of the antibodies in the serum instead of the sheer quantity that is produced that could be the determining factor (Olivier *et al.,* 1985). Additionally, the fish used in this study were triploid fish where certain cellular differences have been described. For example, not only are leukocyte populations decreased in triploids, but it has also been shown that in species containing more than six alleles for the MH gene, for example the *Xenopus* species, there is a possibility that they delete more than the standard 95% of T-cells, which would in turn make them more susceptible to pathogens (Benfey, 1999, Courtet *et al.,* 2001). These results suggest

that neutrophil and anybody response must act in unison with other factors of the immune system in order to effectively combat invading pathogens.

2.5.2 Future Studies

The results of this study show rainbow trout to have a quick immune response with respiratory burst and antibody response occurring within 24 hours of infection however, the vaccine was proven to be ineffective. This ineffectiveness could mean that although there was a quick antibody response, the response was not sufficient enough to combat the pathogen. Future studies need to look at how high an initial antibody response is required for less mortalities. Studies also need to be conducted on the immune response of diploid fish to get a full picture of immunity in hatchery populations. Additional studies are needed to determine the potential possibility of protective immunological memory following infection. Other immune cells, such as complement proteins, could potentially be affecting fish having already been vaccinated.

In addition to characterizing immunological memory in rainbow trout, additional studies are needed to assess the possible involvement of specific proteins of the FFSBC vaccine strains in the pathogenesis of *F. psychrophilum*. By identifying specific pathogenic subunits of the bacteria a more effective vaccine against *F. psychrophilum* can be developed, using the antigenic peptides characterized as possible immunity targets.

General Conclusion

In response to the high demand of consumers as well as the need to counteract the depletion of natural fish stocks, the production of fish, especially rainbow trout, in the aquaculture industry has evolved dramatically. In addition to fish farms, restocking programs are available to maintain natural populations of rainbow trout. However, disease caused by bacteria, for example *F. psychrophilum*, within the industry has caused devastating effects and has stressed the importance of characterizing the immune response to disease caused by *F. psychrophilum* (Freshwater Fisheries Society of British Columbia, 2009).

Rainbow trout major histocompatibility gene allelic diversity and polymorphisms contributed to the overall differences between populations. The greatest amino acid diversity in rainbow trout alleles were found in the Fraser Valley population however, the number of non-synonymous mutations were the lowest in this population. Since the Fraser Valley population was not significantly different than the Tzenzaicut population this suggests they share a more recent common ancestor with that population than with Pennask and Blackwater. Tzenzaicut also contained the second highest number of unique alleles within the population with a higher level of non-synonymous mutations than the Fraser Valley population which means the most unique alleles at the protein level. This indicates a greater ability of this population to bind a greater diversity of peptides, which implies a greater chance of binding *F. psychrophilum*. Therefore it is believed that the Tzenzaicut population has the best chance of survival through an outbreak. Using the same logic, it can be assumed that due to the lower levels of allelic diversity the Pennask and Blackwater populations would have lower survival in such an outbreak. However, the possibility exists that any one of these populations contains the allele necessary to fight *F. psychrophilum* best.

Overall, the results of this study have suggested the possibility of a second expressed locus for MH class II β (termed DAB2) which is contrary to most literary sources. However, the expression of a second locus could mean the difference between susceptibility and resistance to *F. psychrophilum* infection. The bacteria is known to be particularly virulent due to its cell-surface antigens and the exoenzymes produced by *F. psychrophilum* (Nematollahi, 2003). The possibility exists that the MH receptors which respond to the bacteria are the less expressed DAB2 which could be the reason why susceptibility to infection is so high in rainbow trout. Therefore strides should be taken to determine if specific populations of rainbow trout at the FFSBC have higher levels of DAB2 locus expression and whether or not increased DAB2 expression is correlated with increased resistance.

In addition to the possible interaction effects of *F. psychrophilum* on the DAB1 and 2 loci, the results of this study have indicated that neutrophil and antibody responses of rainbow trout were not enough to effectively combat invading pathogens and that vaccination did not increase either response, indeed vaccination seems to have reduced the neutrophils response. This could have been due to the usage of whole cell bacteria instead of utilizing immunogenic subunits of *F. psychrophilum.* It could also indicate a need for the FFSBC to look at the use of adjuvants or nutritional factors with the vaccine in order boost immunity since neither has been investigated with this infection trial. For example, studies have shown an increase in Vitamin E in fish feed has led to greater resistance against disease in fish (Puangkaew *et al.,* 2004). The immune response to *F. psychrophilum* may include other factors that were not measured in this study such as complement activation, and should be examined.

In conclusion, while many factors may influence resistance or susceptibility to disease in rainbow trout strides have been made with this study which has paved the way for many new

possibilities. This study has allowed not only a greater understanding of respiratory burst and antibody response to an autogenous vaccination but has shown the key differences between the peptide binding regions of multiple sub-populations of rainbow trout. These results will provide the Freshwater Fisheries Society of British Columbia and science as a whole, with a greater understanding of disease within fish stocks and will prevent the devastating effects of disease in the future.

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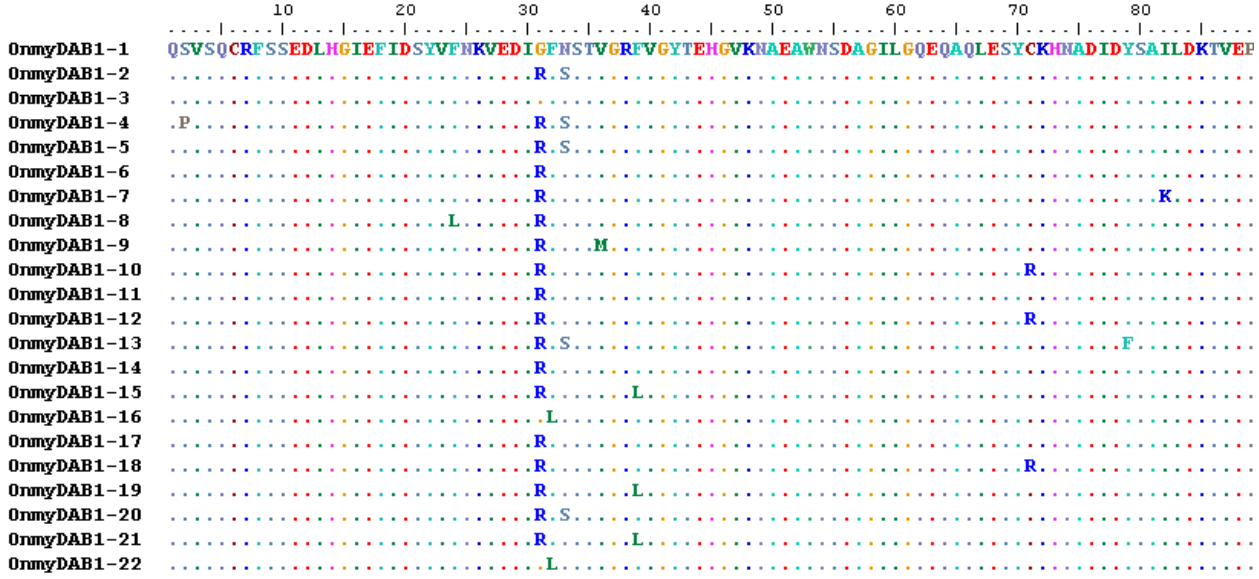
Appendix

Figure 1.4: Amino acid sequences of the Fraser Valley rainbow trout population. Amino acid sequences based on unique nucleotide sequences of the MHC class II β gene full length intron and exon sequences isolated from Fraser Valley rainbow trout genomic DNA, (a) DAB1 (b) DAB2

(a)



(b)

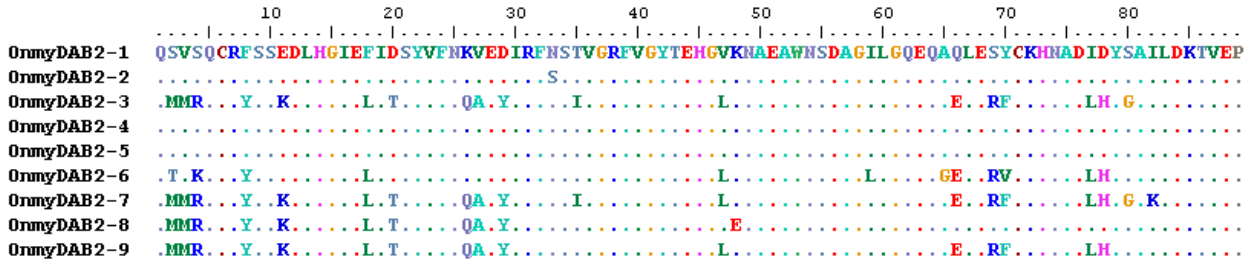
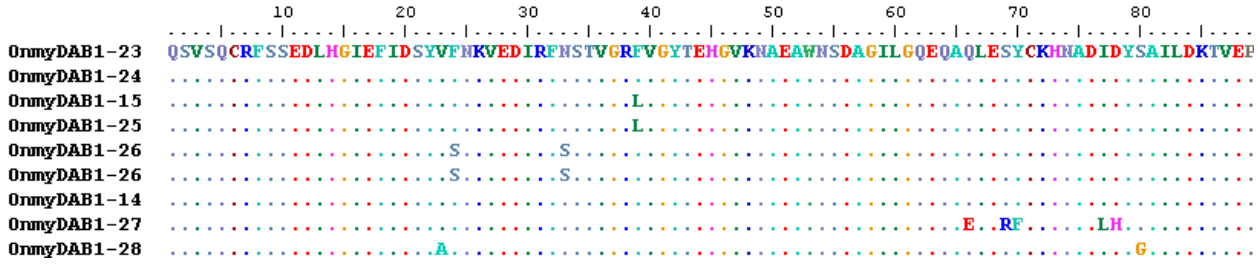


Figure 1.5: Amino acid sequences of the Pennask rainbow trout population. Amino acid sequences based on unique nucleotide sequences of the MHC class II β gene full length intron and exon isolated from Pennask rainbow trout genomic DNA, (a) DAB1 (b) DAB2

(a)



(b)

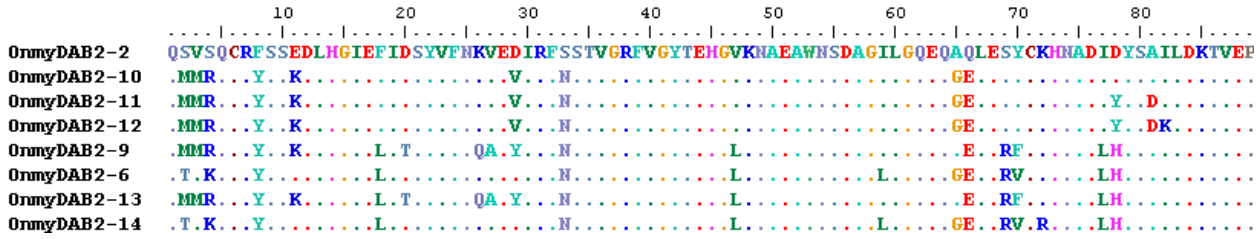


Figure 1.6: Amino acid sequences of the Blackwater rainbow trout population. Amino acid sequences based on unique nucleotide sequences of the MHC class II β gene full length intron and exon isolated from Blackwater rainbow trout genomic DNA, (a) DAB1 (b) DAB2

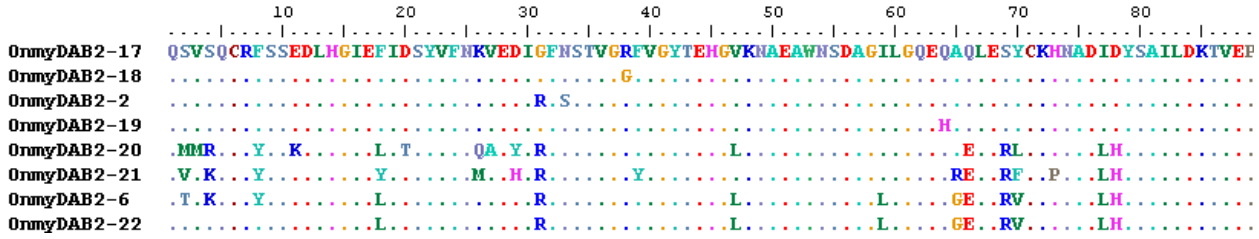
(a)

(b)

Figure 1.7: Amino acid sequences of the Tzenzaicut rainbow trout population. Amino acid sequences based on unique nucleotide sequences of the MHC class II β gene full length intron and exon isolated from Tzenzaicut rainbow trout genomic DNA, (a) DAB1 (b) DAB2

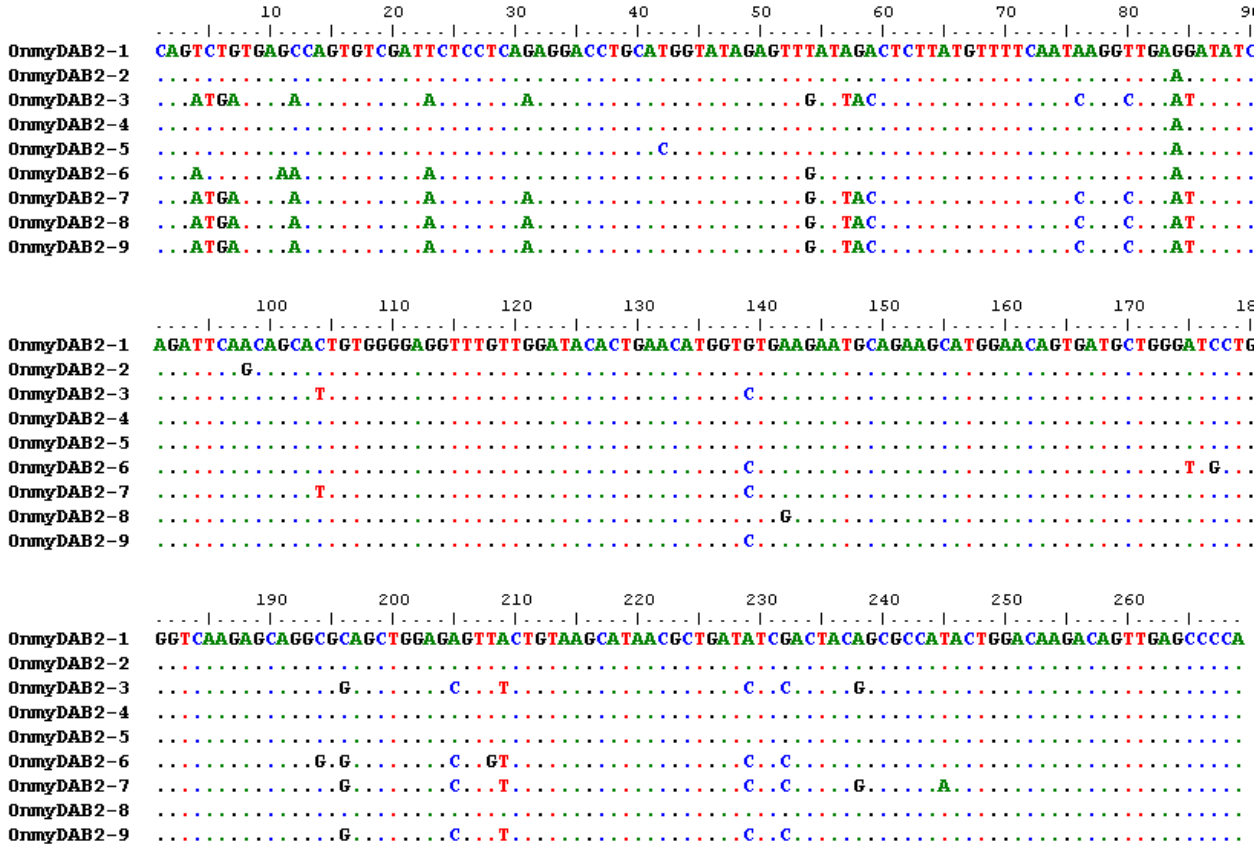
(a)

(b)

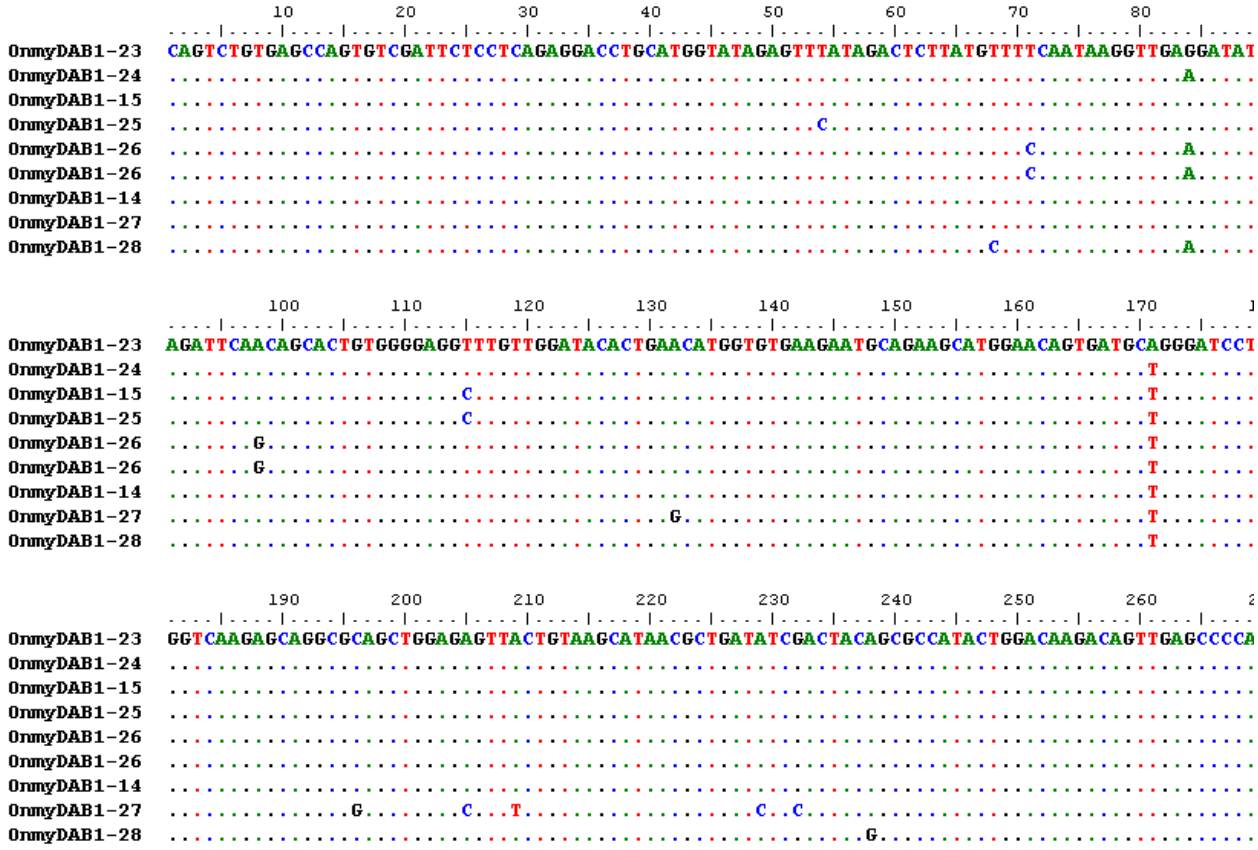


Nucleotide sequences of the Fraser Valley rainbow trout population. Nucleotide sequences of the MH class II β exon 2 DAB1 gene. Isolated from FV rainbow trout genomic DNA.

Nucleotide sequences of the Fraser Valley rainbow trout population. Nucleotide sequences of the MH class II β exon 2 DAB2 gene. Isolated from FV rainbow trout genomic DNA.



Nucleotide sequences of the Pennask rainbow trout population. Nucleotide sequences of the MH class II β exon 2 DAB1 gene. Isolated from PN rainbow trout genomic DNA.



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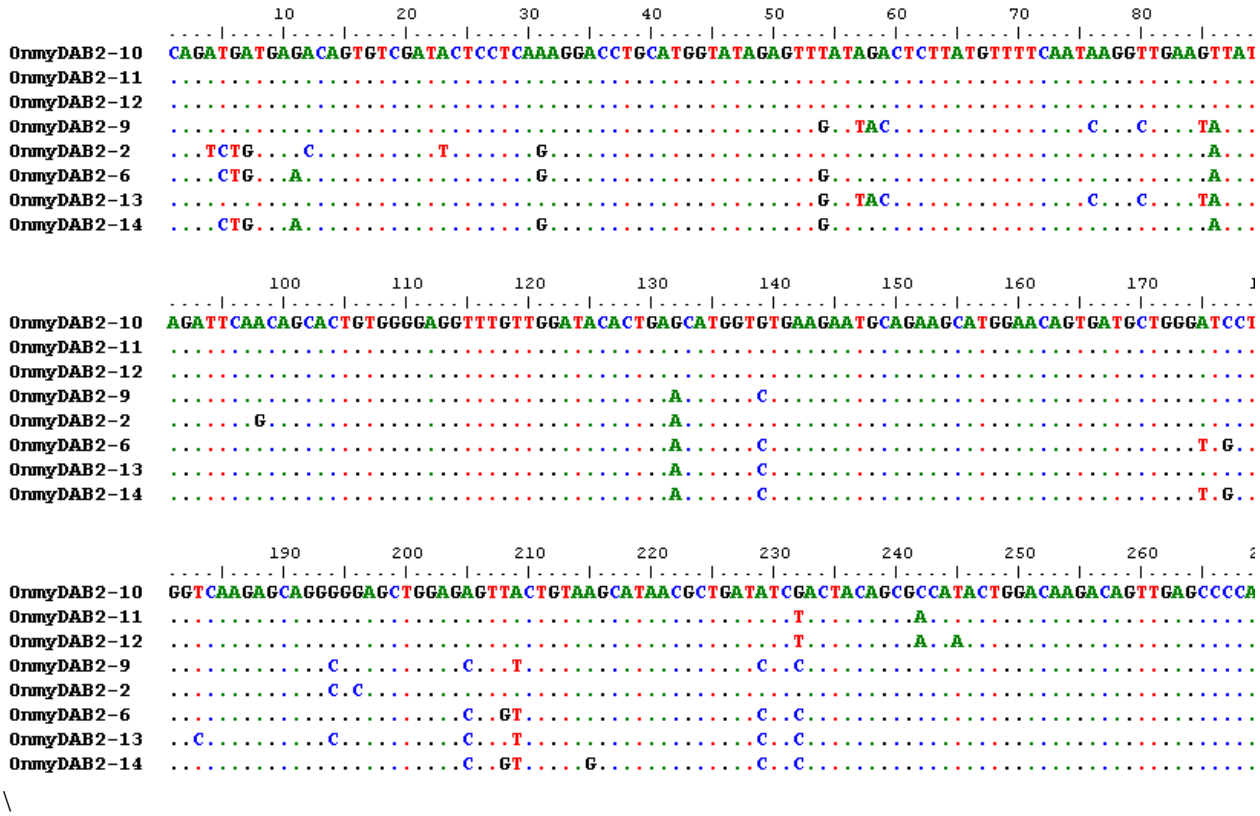
Nucleotide sequences of the Pennask rainbow trout population. Nucleotide sequences of the MH class II β exon 2 DAB2 gene. Isolated from PN rainbow trout genomic DNA.



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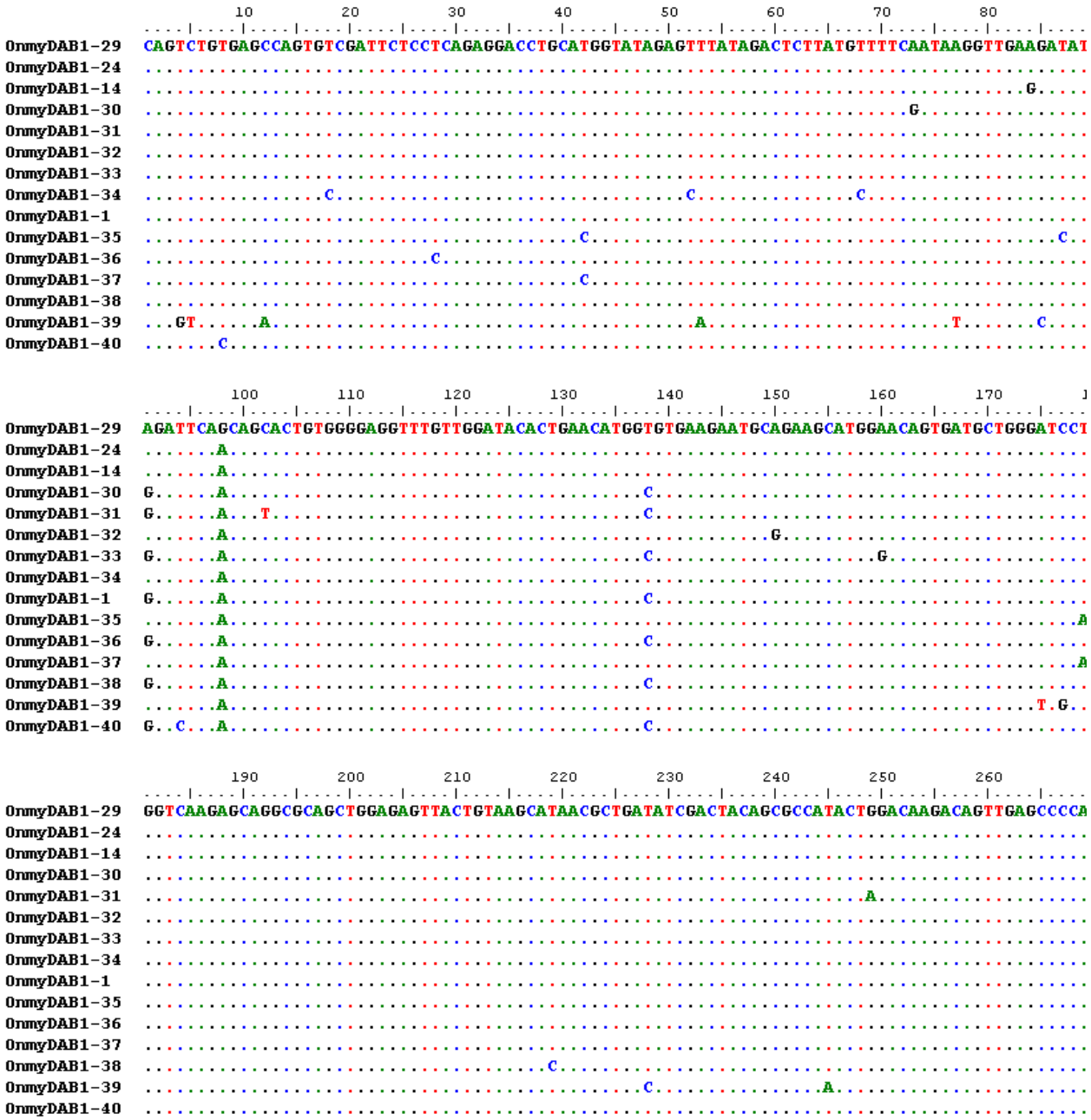
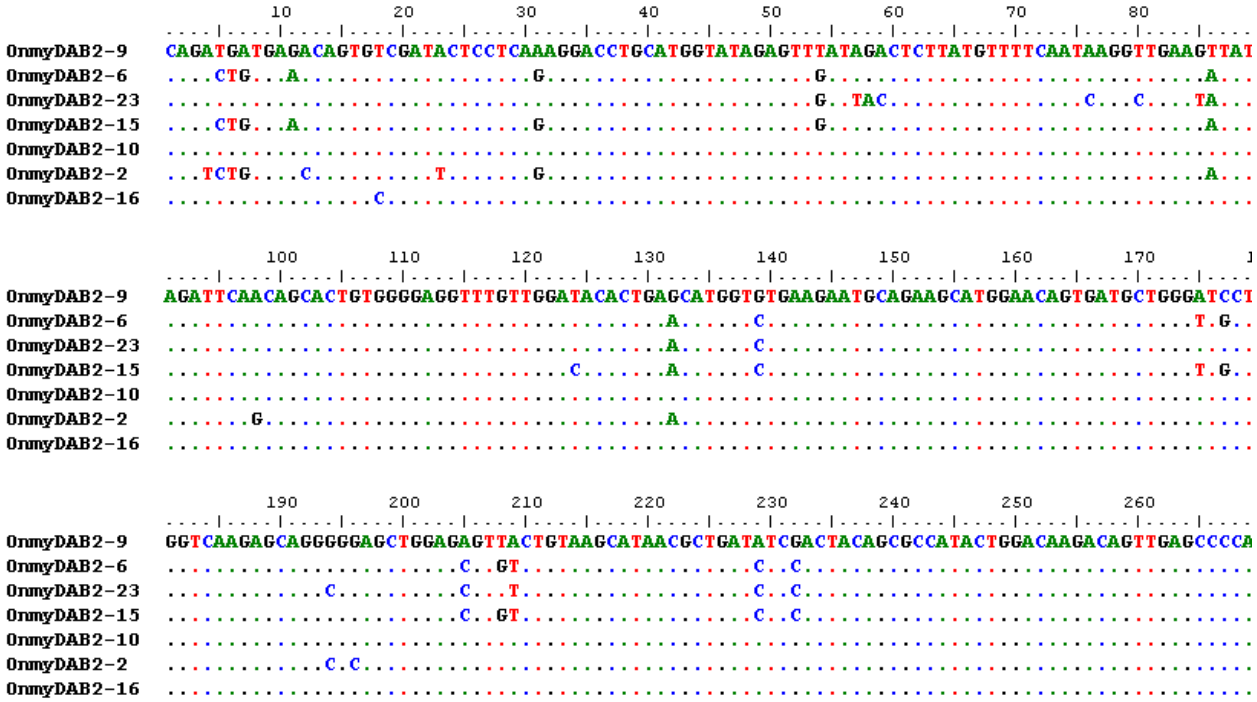
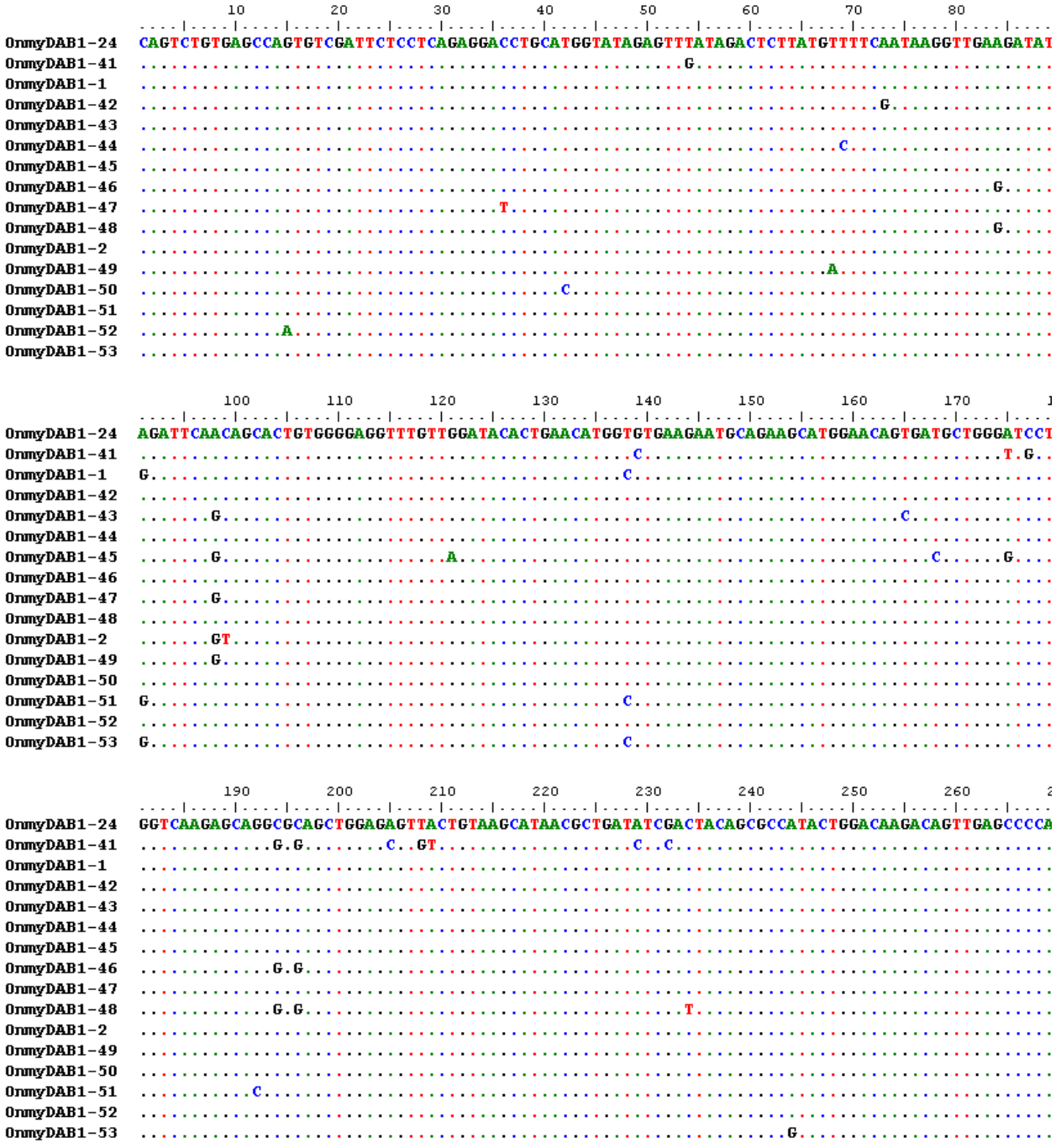
Nucleotide sequences of the Blackwater rainbow trout population. Nucleotide sequences of the MH class II β exon 2 DAB1 gene. Isolated from BW rainbow trout genomic DNA.



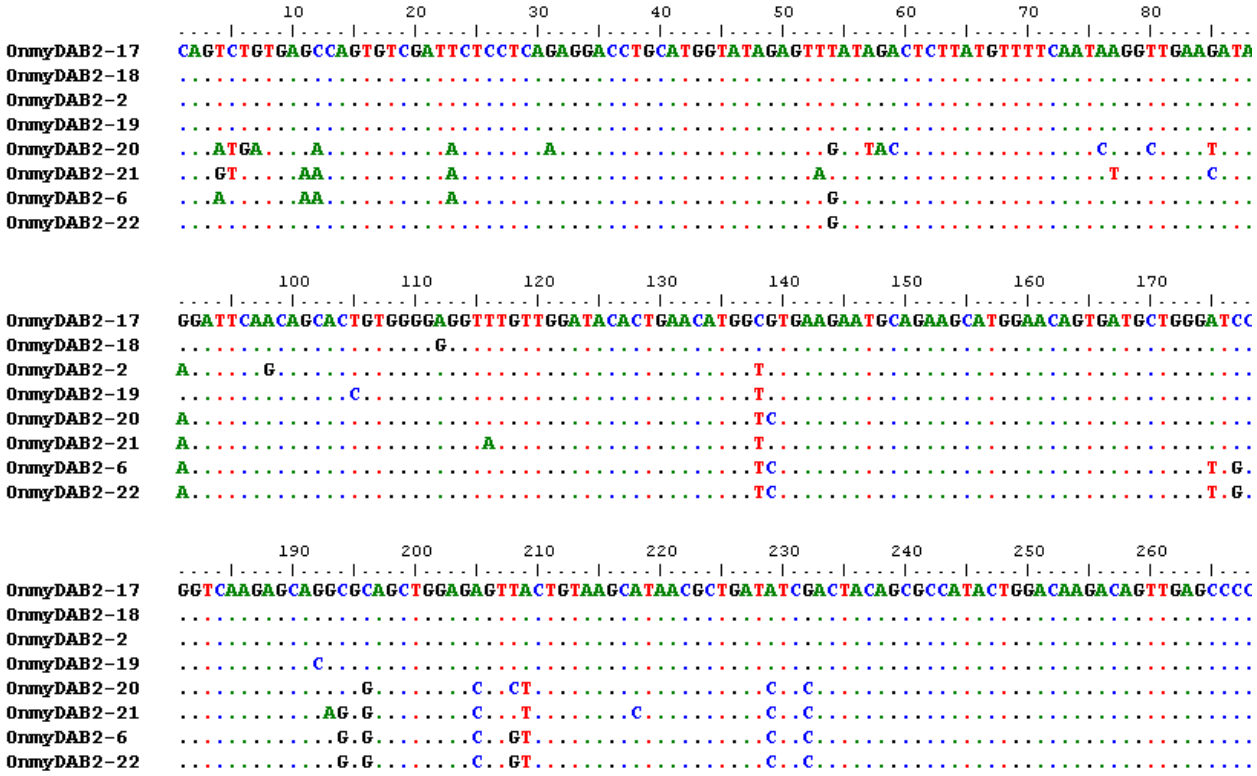
Nucleotide sequences of the Blackwater rainbow trout population. Nucleotide sequences of the MH class II β exon 2 DAB2 gene. Isolated from BW rainbow trout genomic DNA.

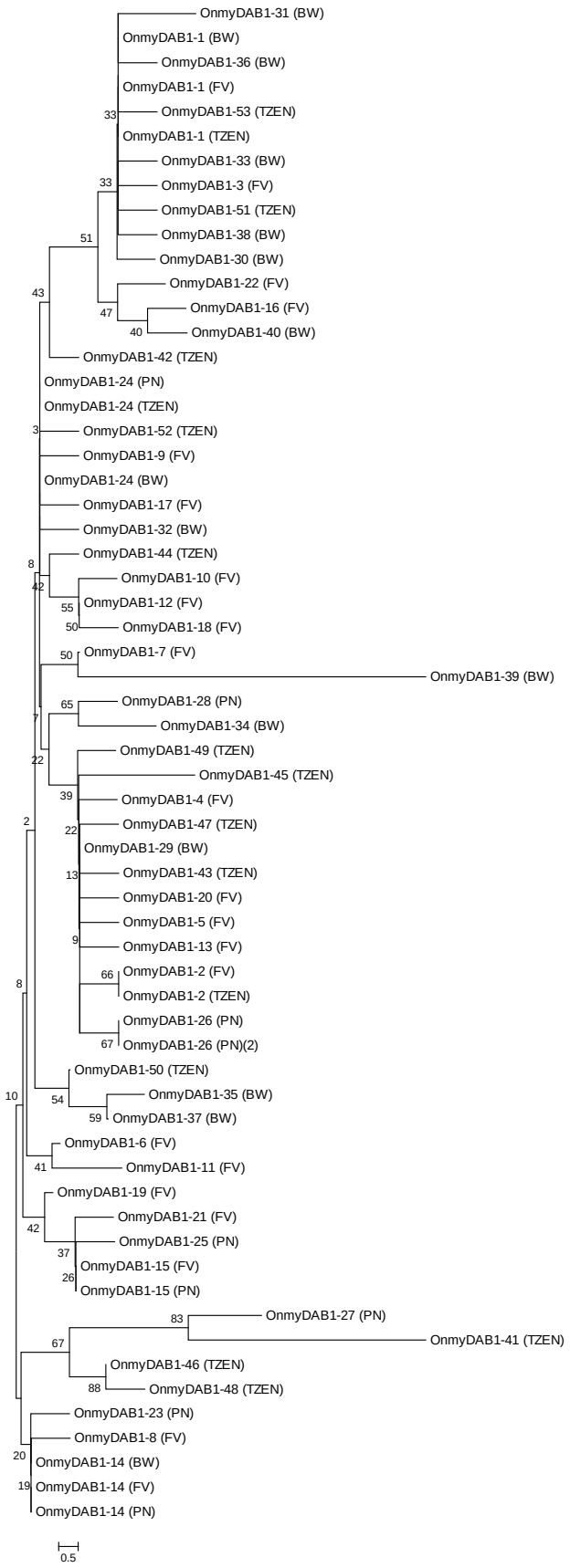


Nucleotide sequences of the Tzenzaicut rainbow trout population. Nucleotide sequences of the MH class II β exon 2 DAB1 gene. Isolated from TZEN rainbow trout genomic DNA.



Nucleotide sequences of the Tzenzaicut rainbow trout population. Nucleotide sequences of the MH class II β exon 2 DAB2 gene. Isolated from TZEN rainbow trout genomic DNA.





Complete neighbour-joining tree of all four populations' full length rainbow trout MH class II β exon 2 sequences. The nucleotide sequences of the 62 full length exon 2 sequences of MH class II β. The tree was constructed using the Jukes and Cantor model. Bootstrap values were based on 1000 replicates.