ASSOCIATION BETWEEN NINE IMMUNE SYSTEM GENE POLYMORPHISMS
AND TOLERANCE TO ALEUTIAN MINK DISEASE VIRUS INFECTION

by

Ronakkumar Desai

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ABSTRACT

Aleutian mink disease virus (AMDV) causes a major health concern for the mink industry worldwide. There is no vaccine or treatment for this disease and the only control measure is regular testing of mink for antibodies against AMDV and removal of seropositive animals. This strategy has not been effective in controlling the virus. Selecting mink that can tolerate AMDV and do not succumb to the disease would be useful for the mink industry. Allele frequency changes in 16 single nucleotide polymorphisms (SNPs) in nine immune system genes (IL-6, LEP, FcεR1G, TLR2, TLR3, TLR4, TLR7, TLR9 and TGF-β1) were determined in two black mink herds naturally exposed to AMDV and selected for tolerance and four control herds. There was evidence that TLR2, TLR4 and TLR7 genes were possibly under selection in one of the tolerant herds. SNPs in these genes may serve as putative molecular markers for tolerance to AMDV infection.

LIST OF ABBREVIATIONS USED

AD	Aleutian disease
AMDV	Aleutian mink disease virus
CIEP	Counter-immunoelectrophoresis
DNA	Deoxyribonucleic acid
Fab	Fragment antigen binding
Fc	Fragment crystallize
FcεR1G	Fc epsilon receptor subunit gamma
F_{IS}	Inbreeding coefficient
GH	Growth hormone
H_E	Expected heterozygosity
HIV	Human immunodeficiency virus
H_O	Observed heterozygosity
HWE	Hardy Weinberg Equilibrium
IAT	Iodine agglutination test
Ig	Immunoglobulin
IL-10	Interleukin-10
IL-6	Interleukin-6
KASP	Kompetitive allele specific PCR genotyping
LEP	Leptin
MALDI-TOF	Matrix associated laser desorption/ionization-time-of-flight
MAS	Marker assisted selection
mRNA	Messenger ribonucleic acid
NCBI	National center for biotechnology information
NS	Nova Scotia
PCR	Polymerase chain reaction
pi	Post infection/inoculation
PRL	Prolactin
QTL	Quantitative trait loci
RFLP	Restriction fragment length polymorphism
SNP	Single nucleotide polymorphism
TGF-β1	Transforming growth factor beta-1
TLR	Toll-like receptor
TLR2	Toll-like receptor-2
TLR3	Toll-like receptor-3
TLR4	Toll-like receptor-4
TLR7	Toll-like receptor-7
TLR9	Toll-like receptor-9
TNF-α	Tumor necrosis factor alpha
TYR	Tyrosinase

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CHAPTER 1. INTRODUCTION

Mink (*Neovison vison*) farming is a growing agricultural activity in Nova Scotia (NS), with a high financial return. More than 1,980,000 ranch-raised mink pelts were sold by NS ranchers in 2014 (Statistics Canada, 2014), making this province the largest producer of mink pelts in Canada. Aleutian mink disease virus (AMDV) is prevalent in NS (Farid *et al*., 2012) and in other mink producing countries (Knuuttila *et al.*, 2009; Themudo *et al.*, 2011). AMDV infection causes significant economic losses to the mink industry by reducing litter size, increasing adult and embryonic mortalities and reducing fur quality (Hansen and Lund, 1988; Farid and Ferns, 2011). Currently, there is no vaccine or treatment for this disease and the only control measure is regular testing of mink for antibodies against AMDV by counter immunoelectrophoresis (CIEP) (Cho and Ingram, 1972), followed by removal of seropositive animals. Although the test-and-removal strategy has been used in NS for over 30 years, it has not been effective in eradicating the virus from many ranches (Farid *et al*., 2012).

Genetic selection of mink that can tolerate AMDV is an alternative approach to the test-and-removal strategy, and could be useful for the mink industry. Classical selection is not practical as it involves deliberate infection of mink herds with the virus, leading to high mortalities and wastage of resources that most ranchers cannot financially endure. Marker assisted selection (MAS) is a possible option, but it requires molecular markers. When developing molecular markers, it is necessary to have groups of animals that differ in the trait of interest.

A rancher in NS has established a herd (MM herd) of tolerant mink by selecting animals on his infected ranch for over 20 years. Selection was based on animal health and on

iodine agglutination test (IAT) based serum gamma globulin levels. This unique mink herd is an excellent resource for developing molecular markers for mink which are tolerant to AMDV infection. Apart from the MM herd, another AMDV-infected herd (MBL) located in western NS was included in this study. This herd never made any attempts to eradicate the virus from the premises. Breeder animals on this ranch were selected based on their health status and reproductive performance. In addition, mink from four other herds across NS were included as controls in this study. Three of these herds have been practicing a test-and-kill strategy (NG, MJ, PE) to test their mink health status at least once per year by counter immuno-electrophoresis (CIEP); followed by eliminating the seropositive animals. The last herd (AC herd) contained mink originating from ranches which have been free of AMDV for several years and had been AMDV-free for many years until recently.

The overarching objective of this study was to identify markers for AMDV tolerance using a candidate gene approach. This approach was appropriate for the current study because high density SNP panels for mink have not been created and available linkage maps (Anistoroaei et al., 2007) are not adequately saturated enough for QTL mapping. The sequences and the positions of 16 SNPs of nine immune system genes, namely, IL-6, LEP, FcεR1G, TLR2, TLR3, TLR4, TLR7, TLR9 and TGF-β1 were available and hence, were selected as candidates for this study. Interestingly, 13 of the 16 SNP loci studied were polymorphic in every herd. The largest number of significant differences between pairs of herds within each SNP locus was observed for three polymorphic sites: TLR2-C1509T, TLR4-C1992T, and TLR7-T651C, highlighting the possible role of TLR genes in tolerance to AMDV infection.

This study identified changes in immune system genes as a result of selection for healthy animals. These changes might be associated with AMDV tolerance and could serve as putative molecular markers. However, additional studies are warranted to confirm and validate these findings.

CHAPTER 2. LITERATURE REVIEW

2.1 Immune responses of mink to AMDV infection

The AMDV is a non-enveloped, single stranded DNA virus belonging to the family of *Parvoviridae* (Bloom *et al*., 1980). This infectious agent causes AD, an immune mediated disease in mink (McGuire *et al*., 1971). After infection, AMDV replicates in different tissues and attains high viral titer around 10 days post inoculation and subsequently, the viral replication decreases (Porter *et al*., 1969; Alexandersen, *et al*., 1988). The virus is sequestered in lymphoid tissues and a low viral load persists in some mink for a long time after infection (Larsen and Porter, 1975; Hadlow *et al*., 1985).

The AMDV infected mink develop antibodies against structural (VP1 and VP2) and non-structural (NS1) virion proteins (Bloom *et al*., 1982; Porter *et al*., 1984). It has also been demonstrated that antibodies against AMDV DNA are produced after infection (Slugin *et al*., 1975; Hahn and Kenyon, 1980). However, these antibodies against AMDV are not detectible during the early periods after infection. Antibody titer attains peak levels at 6 to 8 weeks after infection and in some mink, the level starts to decline after 8 weeks, while it progressively increases in other mink until death (Bloom *et al*., 1975; Hadlow *et al*., 1985).

Blood serum is comprised of two major high molecular weight proteins: albumin and globulins. There are four components of globulins; namely alpha-1, alpha-2, beta and gamma. A major class of gamma globulins is immunoglobulins (antibodies) which are

produced by the plasma B cells (Goldsby *et al.*, 2003). Following is a typical pattern of serum proteins in healthy mink, determined by serum electrophoresis (Figure 1).
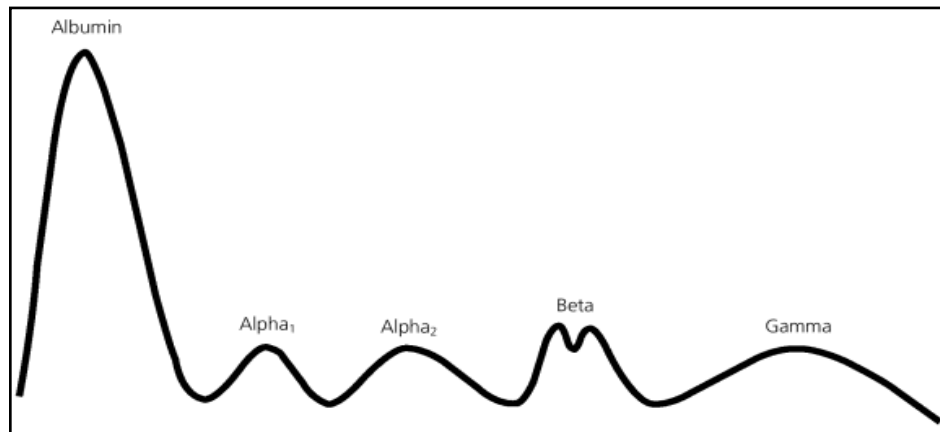
Figure 1. Serum protein profile of a healthy mink separated by serum electrophoresis (Henson et al., 1961)

The AMDV infection causes increased total serum proteins, increased gamma globulins (hypergammaglobulinemia) and decreased albumin (Henson *et al.*, 1961; Kenyon *et al.*, 1963). The abnormally high levels of gamma globulin cause albumin to decrease in order for the blood viscosity to remain stable, resulting in high gamma globulin to albumin ratio (Eastham, 1954). Infected mink produces variable levels of gamma globulin (Hadlow *et al.*, 1984) and increased serum gamma globulin levels play a critical role in the pathogenesis of the disease (Eklund *et al.*, 1968).

2.1.1 Differences among mink in developing AD symptoms

Differences in the severity of AD symptoms have been observed in both naturally (Kenyon *et al.*, 1963) and experimentally infected mink (Bloom *et al.*, 1975; Hadlow *et al.*, 1984). Development of AD in mink depends on both host and viral factors, and non-Aleutian type of mink fail to develop the disease when inoculated with low pathogenic

strains of the virus (Hadlow *et al*., 1983; Bloom *et al*., 1994). There is also a large variation among individual animals within each colour type in the onset of infection, occurrence of viremia, extent of viral replication in tissues, serum gamma globulin level, antibody production and development of AD symptoms, following exposure to AMDV (Bloom *et al*., 1975; Larsen and Porter, 1975; Hadlow *et al*., 1984, 1985).

Mink have been classified into three groups in response to infection with AMDV: progressive, persistent non-progressive and non-persistent non-progressive infection. In a progressive AMDV infection, high anti-AMDV antibody titers mark an increase in gamma globulin levels and immune complex mediated lesions are frequently seen, which eventually lead to death (Hadlow *et al*., 1983). Persistent non-progressive infection is characterized by permanent viral infection with low anti-AMDV antibody titers, low serum gamma globulin levels and absence of immune complex mediated lesions (Hadlow *et al*., 1983). In non-persistent and non-progressive infection, along with low anti-AMDV antibody titers, eventual virus clearance is observed (Larsen and Porter, 1975). The presence of differences in the severity of the disease symptoms, among individual animals within colour types, suggests the possibility of genetic selection for natural resistance.

2.1.2 Relationships between antibody titer, gamma globulin and AD symptoms

Hadlow *et al.,* (1985) reported that the occurrence of AD symptoms is often correlated with increased levels of serum anti-AMDV antibodies. On the contrary, animals with low antibody titer sometimes exhibit disease symptoms. For example, in naturally infected mink, a correlation was observed between serum gamma globulin levels and AD

symptoms, including lesions in kidney, liver and spleen (Kenyon *et al.*, 1963). Conversely, in the same experiment, some infected animals with abnormally high gamma globulin levels did not exhibit advanced tissue lesions, suggesting that hypergammaglobulinemia was not strongly related to the severity of the disease symptoms.

Hahn and Kenyon (1980) experimentally infected 10 pastel mink with the Connecticut strain of ADMV and compared them with 10 chronically infected pastel mink from an infected ranch and 10 AMDV-free mink (controls). Chronically infected mink had four times higher anti-DNA antibodies compared to controls (P=0.001). Significant and positive correlation was observed between the level of anti-DNA antibodies and gamma globulin, although three of the chronically infected mink had normal levels of anti-DNA antibodies. The results indicate large variation (up to 6 times) for anti-DNA antibodies in chronically infected mink, and that some of the gamma globulins were anti-AMDV DNA. Levels of immunoglobulins and anti-DNA antibodies were measured at the time of inoculation and on days 35 and 86 pi, in experimentally infected mink. The levels of immunoglobulins were normal but anti-DNA antibodies were higher compared to controls at the time of inoculation; suggesting a difference in the level of anti-DNA antibody between the control and experimental groups at this time. The level of immunoglobulins rises on days 35 and 86 pi, but levels of anti-DNA antibodies peaked at day 35 pi and then dropped by day 86 pi to the level at the time of inoculation. There was no correlation between anti-DNA antibodies and gamma globulin on day 86 pi.

In another experiment, Hahn and Hahn (1983) collected serum samples from 6 months old mink naturally infected with AMDV, twice in a two-week interval, and demonstrated significant increases in the levels of total gamma globulin and anti-AMDV DNA antibodies, but there was no significant increase in anti-AMDV antibody titer measured by CIEP during that period, suggesting a strong correlation (r=0.90) between gamma globulin and anti-DNA antibodies. The above experiments suggest that the levels of gamma globulins at later stages of infection are mainly due to anti-AMDV DNA antibodies.

2.2 Methods for controlling AMDV

Efforts to make a vaccine for AMDV have so far failed as vaccine constructs resulted in the enhancement of tissue lesions (Porter *et al*., 1972) and increased mortalities for the vaccinated mink (Aasted *et al*., 1998). Treatment of AMDV infected mink with immunosuppressive agents, namely levamisole and cyclophosphamide, has not been successful in preventing development of disease symptoms (Kenyon, 1978).

A common method employed by ranchers is to test for serum antibodies against the viral capsid proteins by using CIEP (Cho and Ingram 1972) and eliminating the seropositive mink. This test-and-removal strategy has been successful in reducing the rate of AMDV infection, but has not been effective in the complete elimination of the virus in NS (Farid *et al*., 2012) and in other mink producing regions (Themudo *et al*., 2011). Another test-and-removal strategy proposed for AMDV eradication is the use of PCR (Stahl *et al*., 2010). However, viral replication will decrease dramatically in some mink a few months after infection (Larsen and Porter, 1975; Alexandersen *et al*., 1988) and the virus may not

be detectable in blood or other body fluids (Farid *et al.*, 2014). Because of unsuccessful attempts in eradicating AMDV by the test-and-removal strategy, many ranchers in Nova Scotia (NS) and elsewhere are interested in selecting mink for increased tolerance to AD symptoms.

2.3 Methods of selecting mink for tolerating AD symptoms

Because some animals do not develop AD symptoms and remain healthy after exposure to AMDV, selecting healthy animals with satisfactory reproductive performance and fur quality traits in an infected environment, is an obvious approach to fight against AMDV. This method is not economically feasible for the ranchers as it might result in high mortality and reduced reproductive performance for several years before a tolerant herd is established. Additionally, all healthy animals on an infected ranch may not be genetically tolerant because the exact time of infection is unknown and all animals are not uniformly exposed to the virus. For these reasons, ranchers do not solely rely on the health of their animals and often use one of a few available laboratory tests for the identification of tolerant mink.

2.3.1 Anti-AMDV antibody methods

Anti-AMDV antibody titer has been measured by CIEP test in serially diluted plasma (Bloom *et al.*, 1975; Hadlow et al., 1985) and can be used to identify animals with low levels of anti-AMDV antibodies, which may be able to tolerate the virus. This method is not practical on commercial ranches because of the high cost of testing many serially diluted samples. Quantitative ELISA is another potential laboratory test to measure anti-

AMDV antibody titer in blood (Knuuttila *et al*., 2009; Chen *et al*., 2015; Farid and Segervall, 2014), but its merit in the selection of tolerant animals has not been investigated (Farid and Rupasinghe, 2016). Methods based on anti-AMDV antibody titer may not be reliable selection tools as the level of anti-AMDV antibodies in the serum is not constant after an infection (Hadlow *et al*., 1984). Both ELISA and CIEP tests are based on VP2 protein of the virus (Dam-Tuxen *et al*., 2014) and are not capable of measuring anti-AMDV DNA antibodies so they might not be accurate methods for selection of animals that can tolerate the infection.

2.3.2 Gamma globulin based methods

Serum electrophoresis is a technique where serum is placed on a cellulose acetate paper or agarose gel and electric current is applied to separate the serum proteins, based on their size and electric charge (Hahn and Hahn, 1983). The gel is then scanned to quantify the amount of albumin and each type of globulins and a high gamma globulin to albumin ratio is a measure of animal sickness. This laboratory test is commonly used in research to quantify mink serum proteins (Porter *et al*., 1969; Hadlow *et al*., 1984), but has not been used commercially because of its high cost.

It has been known for many years that high gamma globulin/albumin ratio causes clumping of the serum proteins when mixed with iodine. A mixture of iodine and potassium iodide in distilled water was used to detect sub-clinically sick animals. This test was called iodine agglutination test or IAT (Mallen *et al*., 1950). Henson *et al*., (1962) used IAT as a simple field procedure to detect mink infected with AMDV. Plasma or serum samples with high levels of gamma globulin/albumin ratio form agglutinated

particles which are visible as dark brown clumps. The clumps are formed when serum gamma globulin is higher than 2 g in 100 mL of serum (Gorham *et al.*, 1965). Serum from healthy mink appears as a clear brown drop (Henson *et al.*, 1962). Because IAT is easy to perform and is inexpensive, it has been used on several ranches in North America and the Netherlands, but little has been published on its merit as a selection tool for tolerant animals. The only report is from a ranch in NS where IAT has been used for over 20 years to identify healthy mink on an AMDV infected ranch with a considerable success (Farid, 2010).

Animals in their early stages of AMDV infection do not show high levels of gamma globulin or high gamma globulin to albumin ratios and thus cannot be detected by any of these methods. Positive IAT results occur three weeks after infection (Gorham *et al.*, 1965). Additionally, IAT is a non-specific test for AMDV infection because it detects mink that have high gamma globulin to albumin ratios as a result of infection with any other pathogens (Gorham *et al.*, 1976). Although IAT is not accurate for virus eradication in test-and-removal strategies, it could be useful in selection for tolerance. Since the time of infection is not known and the kinetics of gamma globulin titer varies among mink (Kenyon *et al.*, 1963; Porter *et al.*, 1969), it can be speculated that low levels of gamma globulin or low gamma globulin to albumin ratios do not necessarily indicate a tolerant animal. During AMDV infection, the levels of antibody and gamma globulin in serum are not constant in each animal, and the time of infection and viral dose are not known in the field. Hence, the serological tests are not dependable for the identification and selection of animals that can tolerate the infection.

2.4 Marker Assisted Selection (MAS)

For a complex trait, such as disease resistance, classical selection becomes difficult as direct exposure to infectious agents leads to high mortalities; which are economically damaging for farmers. Additionally, collecting accurate records on disease incidence is difficult as not all animals are uniformly exposed to infectious agents at the same time and at the same rate. There are problems associated with serum gamma globulin and anti-AMDV antibody levels, as discussed above. Consequently, phenotypic and serological data are not accurate, which necessitates the development of molecular markers to improve disease resistance in farm animals. The MAS is a selection tool which is based on using molecular markers rather than assessing the phenotype of animals for a particular trait (Foolad and Sharma, 2005). In a review, Gibson and Bishop (2005) suggested that MAS is particularly useful for traits which are difficult, expensive and/or time consuming to record. The MAS also facilitate early selection for traits which are expressed at maturity, as well as those which are expressed in only one sex.

2.4.1 Strategies for molecular marker development

In a review, Andersson (2001) summarized three major strategies for developing molecular markers, namely candidate genes, high density SNP panels and QTL mapping. The use of next generation sequencing has become popular in recent years (Zhang *et al*., 2011). Marker development studies also require specifically bred resource populations with groups of animals that differ in the trait of interest and in which the specific trait is segregating.

2.4.1.1 Candidate gene

Tabor *et al*. (2002) reported that in candidate gene studies, researchers begin with the selection of suitable candidate gene(s) on the basis of any prior evidence which indicates a critical role of those gene(s) with traits of interest in the same or related species. Candidate genes for disease resistance are those that play a role in the immune system (Tabor *et al*., 2002). The resource population is established from disease-affected animals and is compared to controls, which are a disease free group. The frequency of SNP is compared in the affected and non-affected individuals in the resource population. This approach has been successful in identifying genetic variants that have a significant effect on resistance or susceptibility to diseases in livestock, including mastitis in cattle, where the chemokine receptor allele was found to have significant association with subclinical mastitis in Holsteins (Youngerman *et al*., 2004). Scrapie in sheep (Belt *et al*., 1995) and goats (Corbière *et al*., 2013) are another example where prion protein alleles were found to be highly correlated with the disease incidence and have been extensively used in many countries for selecting resistant sheep and goats. Kuhnlein *et al*., (1997) used non-inbred strains of White Leghorns that were selected for resistance to challenge by Marek's disease virus and also selected for egg production. Using RFLP, Kuhnlein *et al*., (1997) compared the frequency of growth hormone alleles between the selected and unselected control strains, demonstrating that the frequency of one growth hormone allele was higher in the resistance selected Leghorn strain than in the control strain. A similar study in bovines showed differences in the frequency of Toll like receptor 1 (TLR1) alleles between bovine tuberculosis infected and non-infected animals (Sun *et al*., 2012).

These studies reflect that allelic variations in candidate genes might have an implication in disease resistance or susceptibility in livestock.

2.4.1.1.1 Selection of candidates

Selection of genes is the first step in candidate gene approach. Function-dependent, comparative-genomics and combined approaches are a few of the proposed strategies for a candidate gene method (reviewed by Zhu and Zhao, 2007). Identification of candidate genes with known functional significance in signalling, immunity, metabolic and/or developmental pathways involved in the trait of interest is known as function-dependent strategy. Gene expression profiles, knock-out animal and cell culture methods provide useful information for selection of functional candidates in livestock (Schwerin *et al.,* 2003). Comparative-genomics method is a cross-species approach to identify putative candidates (reviewed by Zhu and Zhao, 2007).

In a comparative genomics approach, knowledge is derived from the available information-rich species, including mouse, to identify putative candidate genes in agricultural species. Studies have revealed conservation of genetic elements between humans, mustelids (mink), and bovines (cow) (Hameister *et al*., 1997; Chowdhary *et al*., 1996). However, genetic architecture for the trait of interest, particularly complex traits, can be different in related species, which poses a limitation for this strategy. Currently, combinations of the above strategies to identify candidates for complex traits is a popular and successful approach (de Dorlodot *et al.,* 2007). Furthermore, to recognise the most potential candidate genes from large lists, computationally efficient "gene prioritization tools" are available such as ToppGene (https://toppgene.cchmc.org/) and TOM

(http://www.micrel.deis.unibo.it/~tom/), which saves time and resources invested in candidate gene approach (Tranchevent *et al.,* 2011).

2.4.1.1.2 Selection of SNPs for genotyping

Selection of SNPs is a challenging yet crucial step in a candidate gene study. It is important to select a limited number of SNPs and perform genotyping in a cost-effective manner. The location and nature of the polymorphism are critical for prioritizing genetic variants. Non-synonymous SNPs which either change the amino acid or result in the formation of pre-mature stop codons are usually assigned highest importance for genotyping (Tabor *et al*., 2002). Also, SNPs located in the non-coding sequences, although they do not change gene function, can influence gene expression (Lozano and Levine, 1991) by altering splice sites for mRNA splicing or by affecting regulation of transcription rates. For example, Sander *et al*. (2005) demonstrated bovine prion protein gene promoter (PRNP) polymorphisms modulated PRNP gene expression by influencing the binding of transcription factors on promoter site. They performed promoter reporter gene assays *in vitro* and measured mRNA levels by qRT-PCR in different tissues of calves to establish this association between prion protein gene alleles and transcription rate (Sander *et al*., 2005). Another consideration for selection of SNPs is linkage disequilibrium (LD). Alleles of SNPs in strong LD tend to be inherited together and the variants are highly correlated serving as proxies for each other (reviewed by Hirschhorn and Daly, 2005), so only single SNP can be selected out of the several SNPs in LD.

2.4.1.2 Whole genome scan

Doerge (2002) reviewed QTL mapping, which is a method to identify regions of the genome containing a gene, or genes, associated with variations in the quantitative trait of interest. For QTL mapping, a suitable population must be developed in which the trait of interest is segregating, such as crosses between inbred lines or diverse breeds (Edfors-Lilja *et al*., 1998; Désautés *et al*., 2002). A saturated linkage and physical map of the species is also required, which will be used to select microsatellite markers for genotyping of the individuals of the mapping population. The QTL mapping is not currently suitable for detecting markers associated with AD tolerance in mink, because neither a proper mapping population nor a saturated linkage map of the mink exists. The existing linkage map (Anistoroaei *et al*., 2007) contains markers, but is not saturated enough for QTL mapping. The use of high-density microarrays for the identification of association between SNP and phenotypic traits, including disease resistance, has greatly increased in recent years (Davies *et al*., 2009). Currently, high density SNP panels are not available for mink. On the basis of these limitations, the candidate gene approach is the most practical strategy at the present time.

For this study, the resource populations are unique tolerant herds of mink developed by two ranchers in NS. One rancher (MM) has selected animals on his infected ranch based on their health and serum gamma globulin level, using IAT for over 20 years (Farid, 2010). The other rancher (MBL) selected his animals based on physical health with no laboratory test for many years. These herds are excellent resources for developing molecular markers for mink which are tolerant to AMDV infection.

2.4.2 Candidate genes for immune response to AMDV infection in mink

The candidate genes selected for this study are IL-6 (Donkor, 2007), LEP (Arju, 2010), FcεR1G (NCBI acc# KC986377), TGF-β1, TLR2, TLR3, TLR4, TLR7 and TLR9 because they are the only immune system genes for which SNPs have been identified in mink. These genes were selected because of their critical roles in immune responses during infection, and have also been reported in other species. There is no SNP available for mink in dbSNP (http://www.ncbi.nlm.nih.gov/snp). There is only one SNP identified in the promoter region of TNF-α gene to date (Yudin *et al*., 2012) and there are no discriminative enzymes for this SNP. A limited number of SNPs in other genes, which are not related to the immune system, have been reported in mink. The distributions of SNPs in different segments (promoter, exon, introns, 3'-UTR) of all the genes of the mink that had been reported at the start of the present study (2013), are reported in Table 1. The number of SNPs investigated in mink populations in general, and in black mink in particular, is very low indeed. In addition to the published SNPs, potential SNPs in six immune system genes (TLR2, TLR3, TLR4, TLR7, TLR9, TGF-β1) were identified by comparing sequences of transcriptomes of 12 black mink generated by next generation sequencing.

Most of the reported SNPs in Table 1 are intronic, which do not directly change amino acids but could influence gene expression (Lozano and Levine, 1991) by altering splice sites for mRNA splicing, or by affecting transcript stability or regulation of transcription. Additionally, studies have established a significant association between intronic SNPs and diseases including autoimmune hepatitis (Vogel *et al*., 2002).

Table 1. Published SNPs in mink genome by 2013.

| Gene | Colour type* | # mink | SNPs | | | | Total | References ** |
			Promoter	Intron	Exon	3'-UTR		
IL-6	B, P, Br, W	20	0	7	1	0	8	1
LEP	B, P, S, Br, W	20	-	6	0	11	17	2
FcεR1G	B, P, S, Br, W	-	-	5	-	-	5	3
TNF-α	Not specified	-	1	-	-	-	1	4
GH	S, W, P, B	-	-	7	5	-	12	5
PRL	B, Br, P, W	4	-	3	0	-	3	6
TYR	M, B	-	-	-	1	-	1	7

*B-Black, P-Pastel, S-Sapphire, Br-Brown, W-Wild, M-Marbled
**References (1: Donkor, 2007; 2: Arju, 2010; 3: NCBI acc# KC986377; 4: Yudin *et al*., 2012; 5: Skorupski and Kmieć, 2012; 6: Vardy, 2002; 7: Benkel *et al*., 2009)

2.4.2.1 Interleukin-6 (IL-6)

IL-6 is an appropriate gene to be studied in the mink population as this multifunctional cytokine contributes to a wide range of biological functions. IL-6 is a pleiotropic cytokine that regulates immune responses, inflammatory events, hematopoiesis and acute phase response. It stimulates the differentiation of B cells into antibody secreting plasma cells and also leads to activation of the thymus and peripheral T-cells (Lotz *et al*., 1988). In a review, Bloom *et al*. (1994) showed that higher levels of IL-6 mRNA were detected at 10 and 60 days after AMDV infection, indicating a progressive increase in IL-6 level as the disease developed. Several SNPs of the IL-6 gene in the introns and exons of humans and animals have been identified. Barrett *et al*. (2003) demonstrated that a C(-174)G polymorphism in IL-6 gene was associated with viral clearance in hepatitis C infected patients. He reported that CC genotype was significantly associated with viral clearance and caused low IL-6 production. These experimental evidences support the role of IL-6

as a candidate gene for investigation during AMDV infection. In addition, the entire coding region of the IL-6 gene has been sequenced in mink (Genebank accession: EF620932.1), and eight SNPs have been identified for black mink (Donkor, 2007).

2.4.2.2 Leptin

LEP, a product of the obese gene, promotes the phagocytic function of monocytes and macrophages (Mancuso *et al*., 2004). LEP also activates human B cells to secrete cytokines, such as IL-6, IL-10, and TNFα (Agrawal *et al*., 2011). A study has suggested that a significant association exists between LEP gene promoter polymorphism and anemia in HIV infected individuals (Vanasse *et al*., 2011). A Study by Abdel and Rashed (2011) reported that a G(2548)A polymorphism in the LEP gene could be a predictor for increased risk of psoriasis, and might also serve as a useful marker for psoriasis-related risk in humans. Significant difference in the frequency of LEP gene allele G(2548)A was identified between clinically diagnosed psoriasis patients and controls. Additionally, measurements of plasma leptin levels were significantly higher in the same psoriasis patients compared to controls.

Protection from autoimmunity in LEP deficient mice has been observed in experimentally induced glomerulonephritis. This is an immune complex-mediated inflammatory disease, where LEP is required for the induction and maintenance of immune-mediated glomerulonephritis (Tarzi *et al*., 2004). Above studies have demonstrated that hyperleptinaemia and polymorphism in LEP gene are associated with many immune-mediated inflammatory disorders, such as psoriasis and experimentally induced glomerulonephritis.

Recent literature suggests that LEP is an appropriate gene to be investigated as a marker for mink response to AMDV infection (Arju, 2010). Role of the LEP gene in host defence mechanisms with activating effects on monocytes, macrophages, neutrophils, natural killer cells, lymphocytes and B cells has been reviewed (Lam and Lu, 2007). Therefore, LEP gene is a potential candidate gene for investigation, to speculate on the role that this gene may play in the control of immunity in mink.

2.4.2.3 Fc fragment of IgE high affinity Receptor I for Gamma polypeptide (FcεRIG)

During digestion, the enzyme papain breaks the immunoglobulin (Ig) molecule in the hinge region, resulting in the formation of Fab (Fragment antigen binding) and Fc (Fragment crystallisable) (Goldsby, 2003). The Fab is the antigen binding region of the Ig molecule and Fc is the remainder region of the Ig molecule which interacts with the receptors (Goldsby, 2003). In a review, Fridman (1991) showed that the Fc portion of Ig molecules interacts with the cell surface receptors, called Fc receptors, and FcγR, FcεR, FcαR and FcμR bind to IgG, IgE, IgA and IgM, respectively.

There are two types of receptors for IgE: high affinity FcεRI receptor and low affinity FcεRII receptor (Kinet, 1989). In a review, Bournazos *et al*., (2009) suggested that FcεR1 is a multimeric structure comprised of an alpha subunit, a beta subunit and a gamma subunit which are encoded by FcεR1A (Fc fragment of IgE high affinity Receptor I for alpha polypeptide), MS4A2 (Membrane Spanning 4-domains, Subfamily A, Member 2) and FcεR1G (Fc fragment of IgE high affinity Receptor I for Gamma polypeptide) genes respectively. FcγR has the identical gamma chain as FcεRI and it has been demonstrated

that targeted disruption of the gamma chain resulted in suppression of immune responses in mice (Takai *et al*., 1994). FcεRIG plays an important role in the stimulation of signal transduction pathways necessary for mast cell degranulation (Sakurai *et al*., 2004) and mediates necessary signals which up regulates the mast cell activation and survival and hence, it is a key gene involved in immune responses. The A(237)G polymorphism in FcεRIG gene has been reported to be associated with aspirin intolerant asthma in humans, indicating that this polymorphism could be a risk factor contributing for susceptibility towards asthma (Palikhe *et al*., 2008).

2.4.2.4 Toll like receptors

Toll like receptors (TLRs) are an important class of receptors that recognize pathogens and are involved in initiating both innate and adaptive immune responses (Takeda *et al*., 2003). The TLRs are also a type of pattern recognition receptors (PPRs) (Akira *et al*., 2006; Medzhitov, 2001), as they recognise evolutionary conserved molecular patterns associated with pathogens. The TLRs were discovered based on their noticeable homologies to the membrane toll protein in *Drosophila melanogaster*, which is critical for antifungal response and embryogenesis in *Drosophila* (Hashimoto *et al*., 1988).

There are different types of TLRs (TLR1, 2, 3, 4, 5, 6, 7, 8 and 9), which recognize specific microbial molecules (Takeda *et al*., 2003; Akira *et al*., 2006). TLRs are mainly expressed on the cell surface; however, several TLRs recognize the viral nucleic acids are located in the intracellular compartments. Binding of a pathogen to a TLR, causes dimerization and a conformational changes which facilitates the recruitment of intracellular signalling molecules. Upon engagement of these molecules to the TLRs, a

sequential signalling cascade is activated which, eventually initiates the inflammatory cytokines and type 1 interferon (IFN) and induces an antiviral state in the infected cells (Akira and Takeda, 2004; Akira *et al*., 2006).

2.4.2.4.1 Toll like receptor 2 (TLR2)

Of all the mammalian TLRs, TLR 2 is able to recognize the widest range of pathogens including fungi, gram-positive bacteria, gram-negative bacteria and viruses (Lien *et al*., 1999; Compton *et al*., 2003) The expression of TLR2 is restricted to certain cell types that mediate acute immune responses, with the highest expression in the hematopoietic-derived cells (Herman *et al*., 2014). Additionally, TLR2 is also expressed on the T and B immune- specific cells which are involved in cell-mediated immunity. In gene knock-out models, TLR2-deficient mice showed reduced survival rates after intravenous challenge with *Staphylococcus aureus* as compared with the wild type controls (Takeuchi *et al*., 2000). TLR2-knockout mice after mycobacterial aerosol challenge failed to exhibit an effective immune response (Drennan *et al*., 2004). Similar results were observed in TLR2-knockout mice models infected intravenously with *Candida albicans. (*Villamón *et al*., 2004). These findings suggest sufficient evidence that TLR2 is crucial to initiate host immune responses against a wide range of pathogens. A few of the nonsynonymous SNPs of the TLR2 gene have been associated with increased susceptibility to bacterial infection, including Arg677Trp polymorphism which had a significantly higher frequency among the tuberculosis patients compared to the control group (Ben-Ali *et al*., 2004). Additionally, some synonymous variants in TLR2, including C597T, have been shown to be associated with TB meningitis and leprosy (Bochud *et al*., 2007; Thuong *et*

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al., 2007). Genetic and functional studies in animal models support the importance of TLR2 in immune responses against infection.

2.4.2.4.2 Toll like receptor 3 (TLR3)

The TLR3 has been found to recognise viral dsRNA (Alexopoulou *et al*., 2001). A study by Tabeta *et al.* (2004) reported that TLR3-knockout mice models are more susceptible to murine cytomegalovirus (MCMV) infections compared to the wild-type controls (Tabeta *et al*., 2004). Also, increased susceptibility to west nile viral infection has been observed in TLR3-knockout mice (Daffis *et al*., 2008). This susceptibility is the result of reduced productions of interferons and natural killer cells. Stimulation of the dsRNA-dependent protein kinase signalling pathway and activation of the interferon regulatory factor3 (IRF3) contribute towards the ability of TLR3 to trigger the production of interferons (Doyle *et al*., 2002; Horng *et al*., 2001). A few studies in humans patients have also demonstrated that genetic polymorphisms in TLR3 are associated with susceptibility to chronic hepatitis C virus (HCV) and hepetitis B virus (HBV) viral infections in humans (Medhi *et al*., 2011; Al-Qahtani *et al*., 2012). However, current understanding of the genetic variants of TLR3 in establishment of host immune responses against viruses is sparse and more population-based association studies are needed to confirm the findings.

2.4.2.4.3 Toll like receptor 4 (TLR4)

TLR4 is needed to initiate host innate immune responses against lipopolysaccharide (LPS) which is a component of the gram-negative bacterial envelope (Lu *et al*., 2008). The critical role of TLR4 in lipopolysaccharide (LPS) responsiveness is confirmed by the

observation that disruption of TLR4 in mice models resulted in a LPS resistant phenotype (Bihl *et al*., 2001; Hoshino *et al*., 1999). Recognition of LPS by TLR4 facilitates the recruitment of the adaptor protein myeloid differentiation primary response gene 88 (MyD88) to the outer membrane and triggers the nuclear translocation of nuclear factor kappa light chain enhancer of activated B cells (NF-κB) transcription factors (Zhang and Ghosh, 2001). The role of NF-κB signalling has been implicated in a wide range of autoimmune and inflammatory disorders (Roman-Blas and Jimenez, 2006; Neurath *et al*., 1998).

Two genetic polymorphisms of TLR4, including D299G and T399I have been studied extensively in genetic association. Both these non-synonymous SNPs were found to be correlated with systemic inflammatory hyporesponsiveness after inhalation of LPS (Michel *et al*., 2003). Another study demonstrated that these two polymorphisms conferred increased resistance against pulmonary infection by *Legionella pneumophila* (Hawn *et al*., 2005). Studies have also found an association of SNP D299G with atherosclerosis and myocardial infarction (Kiechl *et al*., 2002; Edfeldt *et al*., 2004). On the contrary, few studies have reported no associations of these polymorphisms with cardiovascular diseases (Koch *et al*., 2006; Paulus *et al*., 2007). This could be explained by small sample sizes, different experimental stimulatory conditions (inhaled vs intravenous), and type and dosage of LPS. Therefore, validation studies are required to confirm the variable findings.

2.4.2.4.4 Toll like receptor 7 (TLR7)

Of the mammalian TLRs, TLR7 recognizes ssRNA viruses (Lund *et al.*, 2004). Recognition of ssRNA viruses by TLR7 triggers the activation of costimulatory molecules and stimulates the release of cytokines mounting an effective innate immune response. Studies have found an association of an intronic C(4170)G polymorphism with systemic lupus erythematosus (SLE) (Kawasaki *et al.*, 2011; Shen *et al.*, 2010). Type I interferon and interleukin-10 levels were also elevated in these SLE patients. Hence, it can be postulated that activation of the proinflammatory cytokine network is a mechanism by which TLR7 mounts an innate immune response. A study by Nazmi *et al.* (2014) revealed significant differences in susceptibility to Japanese encephalitis virus (JEV) infection between TLR7-knockout mice and the wild-type controls. As a major component of the TLR7 response, the production of type-1 interferon contributes towards a beneficial effect in systemic infection. Overall, the studies imply that genetic polymorphisms of TLR7 are important to elicit an immune response against specific viruses and since, TLR7 is located on the X-chromosome a gender-specific effect is commonly observed (Shen *et al.*, 2010).

2.4.2.4.5 Toll like receptor 9 (TLR9)

TLR9 has been shown to recognize the unmethylated bacterial and viral CpG (cytosine-phosphate-guanine) motifs (Dalpke *et al.*, 2006). Studies have also implicated the role of TLR9 in host immune responses against fungal infections (Bellocchio *et al.*, 2004; Ramirez-Ortiz *et al.*, 2008). Surprisingly, the TLR9-knockout mice showed increased survival rates and reduced inflammation after challenge with fungal pathogen *Candida*

albicans and *Aspergillus fumigatus* compared to wild-type controls. Another study by Bochud *et al.* (2007) identified an intronic SNP and a synonymous SNP in TLR9 to be associated with increased progression in HIV (Bochud *et al.*, 2007). Additionally, genetic variants of TLR9 have been shown to be associated with SLE and coronary heart diseases (Lazarus *et al.*, 2003; Noguchi *et al.*, 2004). However, majority of these findings were of marginal significance and failed to replicate in different populations (Hur *et al.*, 2005; Ng *et al.*, 2005). Another study also investigated the role of TLR9 in regulating host immune responses against *Mycobacterium tuberculosis* (Bafica *et al.*, 2005). The study documented that in the TLR2/9 double knockout mice, enhanced susceptibility to the bacterial infection and reduced cytokine production were observed when subjected to aerosol challenge with the pathogen. These studies support the conclusion that TLR9 is important for innate immune responses.

2.4.2.5 Transforming growth factor-beta1 (TGF-β1)

TGF-β is a multifunctional cytokine which regulates multiple biological processes for growth, differentiation, inflammation and host repair (Sporn *et al.*, 1986; Li *et al.*, 2006). This pleiotropic peptide is a key modulator in regulating host immune responses (Li *et al.*, 2006). TGF-β also controls inflammatory responses by regulating proliferation, maturation, and survival of key immune cells, including lymphocytes, granulocytes, natural killer cells and dendritic cells (Letterio and Roberts, 1998). Three TGF-β isoforms (TGF-β1, TGF-β2, and TGF-β3) have been identified in mammals which are expressed in specific tissues (Govinden and Bhoola, 2003). Among these, TGF-β1 is largely expressed in the immune system and often upregulated in tumour cells. A study by Grainger *et al.*

(1999) reported that the concentration of plasma TGF-β1 is primarily under genetic control.

Targeted disruption of TGF-β1 gene in mice caused excessive inflammatory response with infiltration of lymphocytes in major organs and tissue necrosis and eventually, these mice succumbed to early death (Kulkarni *et al*., 1993; Shull *et al*., 1992). It has also been reported that 60% of TGF-β1 knockout mice die *in utero* (Kulkarni *et al*., 1995). Studies have demonstrated significant correlation of plasma TGF-β1 levels with several diseases, including Systemic Lupus Erythematosus (SLE) (Metawie *et al*., 2015), advanced atherosclerosis (Grainger *et al*., 1995) and inflammatory diseases (Liberek *et al*., 2013). Studies have also documented the role of TGF-β1 polymorphisms in the disease occurrence and susceptibility to multiple diseases including cancer (Pasche *et al*., 1999), myocardial infarction (Yokota *et al*., 2000) and autoimmune diseases (Yamada *et al*., 2008). Overall, these studies highlight the importance of this cytokine in immune-mediated inflammatory responses and hence, is crucial for establishment of host innate immune responses.

2.5 Conclusion

In farm animals, production and functional traits, including body weight, reproductive performance and disease resistance, are controlled by multiple genes. Each gene makes a contribution, in a direct or indirect manner, towards regulation of biological pathways which affect the trait. Identification of polymorphisms in candidate genes that are responsible for a major proportion of variation in the trait could be used as putative molecular markers for MAS. All available SNPs in mink associated with immune

response to infection, as well as new SNPs in six immune system genes, were selected for analysis in this study.

CHAPTER 3. OBJECTIVE AND HYPOTHESIS

The objective of this study was to determine the changes that have taken place at sixteen SNP loci as a result of selection for tolerance in two mink herds (MBL and MM) naturally exposed to the AMDV.

I hypothesized that allele frequencies at the loci which were under selection for tolerance were significantly different between these two mink herds and the other four control herds, particularly the AC herd.

CHAPTER 4. MATERIALS AND METHODS

4.1 Source of animals

Mink from six ranches in NS were used in this study (Table 2). Information on the selected ranches is presented below.

4.1.1 MM ranch

This mink ranch is located in Cape Breton Island in eastern NS. It was established in 1944, but all the animals were discarded in 1990. Approximately 700 to 800 black breeder females and 180 black males were purchased in 1990. The ranch was closed to outside stock between 1995 and 2005, but a group of pregnant females were purchased from an AMDV-free ranch in 2006 and were gradually mixed with the existing animals. IAT has been performed in December every year without interruption for more than 20 years, and all IAT positive mink have been discarded from the herd. In addition to IAT results, selection criteria were primarily based on animal health, litter size and fur quality traits. CIEP has not been used as a selection tool on this herd (Farid, 2010). In 2000, 2004 and 2005, samples of mink were tested by CIEP. The proportions of CIEP positive mink in this ranch were 84.7% in 2000, 84.5% in 2004 and 80.2% in 2005 (Farid *et al.*, 2012). In 2000, 773 mink were tested by IAT and CIEP and the proportion of IAT positive mink was 5.3% and the proportion of CIEP positive mink was 84.7%, suggesting that the large percentage of mink were infected by AMDV (Farid, 2010). The low proportion of IAT positive mink suggests that most of the mink were healthy because IAT detects only mink that have serum gamma globulin higher than 2 gm in 100 mL of serum (Henson *et*

al., 1962). These data suggest that long-term selection for healthy animals which were IAT negative on this ranch favoured mink that can tolerate AMDV without succumbing to the disease.

4.1.2 Canadian Centre for Fur Animal Research (AC)

This is the mink ranch of Dalhousie University, Faculty of Agriculture, located in central NS, and has been AMDV-free until recently. The stock of this ranch came from different mink ranches in NS, and thus had a broad genetic base. This ranch has been maintained with 400 to 500 breeding females each year, and additional stock from other ranches has been used for breeding. The breeding program was to choose the best fur quality mink available, the females were mated twice when possible to the same male and inbreeding had been avoided. This herd was used as a control group to evaluate the changes that have taken place on other ranches.

4.1.3 MBL ranch

This AMDV-infected ranch is located in western NS and is one of the oldest mink ranches in the province which is still in operation. The ranch always had AMDV and no attempt has ever been made to eradicate the virus from the premises. Selection was originally based on health and high fur quality traits, which were mostly from small litters. Later, attention was paid to litter size, and replacements were selected from litters of three or larger. Consequently, average litter size improved over the years. Starting in 2012, attention was paid to fur quality traits once again. Selection of breeder animals has

always been based on health status, with no IAT or CIEP test. Mink with white guard hair fibres were culled.

4.1.4 Ranches that practiced test-and-cull strategy

Samples from three other ranches, one in eastern NS and two in the western part of the province were also used. These ranches have followed a strict test-and-cull strategy, namely testing their animals at least once per year by CIEP and eliminating all seropositive animals as soon as the test results became known. These herds were used to separate random genetic drift from actual effect of selection for tolerance to AMDV infection. A total of 749 samples were available for this study (Table 2).

Table 2. Total number of mink samples available from each ranch, sampling year, and selection method.

Ranch	Number of samples	Sampling year	Selection method	Location
MM	369	2004, 2005, 2008, 2009	IAT, health, production	East
AC	233	2007, 2009	Production	Central
MJ	36	2005, 2006, 2007	CIEP and production	West
PE	45	2003	CIEP and production	West
NG	54	2003, 2004	CIEP and production	East
MBL	12	2005, 2006, 2007	Health and production	West

4.2 Laboratory analysis

4.2.1 DNA preparation

Genomic DNA was extracted using the high-salt procedure of Miller et al. (1988) after certain modifications. Approximately 30 to 50 mg of frozen tissue (liver, spleen or kidney) was homogenized with 50 µL of TESDS buffer (50 mM Tris-HCl pH-8.0, 20 mM EDTA pH-8.0, 2% SDS) in a microfuge tube. Another 350 µL of TESDS buffer was added to homogenate and mixed, 75 µL of a 14 mg/mL Proteinase K was added and homogenate was incubated at 55°C overnight in a hybridization oven at 4 rpm. After incubation, homogenate was cooled on ice for 5 min, 4 µL of RNase (10 µg/µL) was added to the mixture and incubated at 37°C for 30 min in a shaking water bath. The tube was chilled on ice for 5 min and 200 µL of saturated NaCl was added to the mixture. The tube was shaken vigorously several times and kept at room temperature for 10 min. The tube was centrifuged at 14000 rpm (15996*g) for 10 min (Eppendorf 5415C) and 400 µL of supernatant was transferred to another tube. For precipitation of DNA, 2.5 volume (1000 µL) of cold 100% ethanol was added, tube was inverted 10 to 15 times and kept at room temperature for 20 min. DNA was pelleted by centrifugation at 16,000 g (Eppendorf 5415C) for 10 min. Supernatant was discarded and the DNA pellet was air dried, DNA pellet was washed with 1000 µL of 70% ethanol in a chemistry mixer (Fisher Scientific) for 30 min to overnight. The tube was centrifuged at 14000 rpm (15996*g) (Eppendorf 5415C) for 10 min, DNA pellet was air dried to remove traces of ethanol and re-suspended in 1X TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0).

The DNA quality was assessed by gel electrophoresis (Agarose I, Amresco, Solon, OH, USA) and the concentration was measured by a spectrophotometer (ND-1000, NanoDrop Technologies Inc.®, Wilmington, DE, USA) in triplicate. Working solutions (75 ng/µL) were prepared using 1X TE buffer and the stock DNA was stored at -80 °C.

4.2.2 Primer preparation and PCR optimization (IL6, LEP and FCεR1G genes)

The sequences and positions of SNPs of IL-6 gene (Donkor, 2007), LEP gene (Arju, 2010), FcεR1G gene (NCBI acc# KC986377), in mink were available. The primers were previously designed and tested for IL-6 (Donkor, 2007), LEP (Arju 2010) and FcεR1G gene (Farid, unpublished). Primer sets were synthesized by a commercial company (Sigma-Genosys, Oakville, ON) and were provided as a lyophilized powder. The information on molecular weight and amount of each primer set (provided by vendor) were used to make stock solutions (10X, 0.5 mM) in Tris-HCl (pH 7.4). Stock solutions were diluted with filtered dH_2O to make working solution (0.01 mM). Two phase dilution procedure was used because primer sequences are not stable at low concentrations and two phase dilutions improved shelf life of the primers. All aliquots of the stock and working primer solutions were stored at -80°C.

Optimum PCR conditions for each primer set were determined with various annealing temperatures (48 to 60°C) and $MgCl_2$ concentrations (1.5 and 3.0 mM) using gradient thermal cycler (Bio-Rad C1000). DNA of three animals were mixed for optimization with PCR reaction volume of 15 µL per tube. After initial optimization, a narrower range of temperature and $MgCl_2$ concentrations were used to find the best PCR amplification conditions. PCR products, along with a size marker, were tested on 2.0% agarose gels to

determine the amplification success, i.e., the presence of a single distinct band of correct size. The gel was stained with ethidium bromide (EtBr) and visualized using a gel documentation system (Bio-Rad, Molecular Imager®).

4.2.3 DNA amplification by PCR

Primer sets that amplified and produced a single distinct band on agarose gels were used to amplify DNA samples. PCR amplifications were carried out in 15 µL total reaction volumes containing 0.1% Tween 20, 1X PCR buffer, 0.2 mM each dNTPs, 400 nM each primer (forward and reverse), 1.5 mM $MgCl_2$, 0.8 unit of *Taq* DNA polymerase (Invitrogen, Burlington, ON) and 1.5 µL (approximately 115 ng) of genomic DNA. Amplification of PCR products longer than 1500 bp was carried out using TaKaRa *Taq* DNA polymerase (TaKaRa Bio USA, Madison, WI, USA). Thermal cycler conditions were 95°C denaturation for 5 min, primer specific annealing temperature for 60 s., extension at 72°C for 60 s., followed by 30 cycles of 94°C denaturation, primer specific annealing temperature, 72°C extension each for 60 s., and final extension for 6 min at 72°C. The annealing temperature of primers, size of amplicons and the SNPs to be tested are depicted in Table 3.

Table 3. Gene, SNP location, primer sequence, annealing temperature and amplicon size that were tested by RFLP-PCR.

Gene	SNP	Primer sequence (5'-3')	Annealing temperature (°C)	Amplicon size (bp)
IL-6	$T^{3039}C$,	F- CCACTGGTCTTCAGGAGTTTCA	60.1	1377
	$G^{3604}A$	R- TGGGGGTGGTCACTTCA		
LEP	$C^{306}T$,	F- TTTGTGGACCCTTGTGCCGATTC	59.0	1857
	$C^{1568}T$	R- GGGGTGCTGCCAAGATTGC		
FCεR1G	$C^{1364}G$,	F- GGCTGCCGTTCATCCTTGGTAAA	61.1	1039
	$T^{1920}C$	R- CAGGGTGAGGACGATACCGTAC		

4.2.4 Genotyping by RFLP-PCR

Proper restriction enzymes which discriminate between different genotypes have been previously identified (Donkor, 2007; Arju, 2010, Farid, Personal Comm.). RFLP-PCR is a well-established methodology for SNP genotyping and it was optimized for genotyping mink herds for each SNP. For optimization, proper quantity of each enzyme and electrophoresis conditions (agarose %, run-time and voltage) were tested. The enzyme that required additional treatment (1% SDS) and failed to differentiate between genotype was not used further. The enzyme recognition site and fragments generated by digestion are depicted in Table 4. Depending on the fragment size, digested fragments were separated on 1.5 to 3.0 % 3:1 agarose gels (Mandel Scientific, Guelph, ON) at 80 to 100 volts for 2 to 3 hours. An undigested PCR product, along with a digested product of a heterozygous individual, were used as internal markers. The gel was stained with ethidium bromide (EtBr) and visualized using a gel documentation system (Bio-Rad, Molecular Imager®). Samples with ambiguous results were tested again.

Table 4. Enzyme recognition sequences and fragment sizes generated by digesting with restriction enzyme.

Gene	SNP	Enzyme	Recognition site (§)	Cutting site (¥)	Fragment size (bp)	
					Pattern 1	Pattern 2
LEP	C306T	HphI	(5'-GGTGA(N)₈↕) (3'-CCACT(N)₇↕)	(5'-G**G**TGA(N)₈↕) (3'-C**C**ACT(N)₇↕)	**1552,135,** 116, 54	**1687,** 116,54
	C1568T	BsaHI	GR↕CGYC	GG↕**C**GTC	**972**, 591, **294**	**1266**, 591
IL-6	T3039C	BslI	CCNNNNN↕NNGG	C**C**GAATG↕GAGG	**722**,259, 160, 149, 87	**493**, 259, **229**, 160, 149, 87
	G3604A	BsiHKAI	GWGCW↕C	**G**GCA↕C	**911**,466	**740**, 466,**171**
FCεR1G	C1364G	BstUI	CG↕CG	CG↕**C**G	**1039,** 695, 344	695, 344
	T1920C	StuI	AGG↕CCT	AGG↕CC**T**	692, **206,** 141	692, **347**

§- N=either of the four nucleotides, R= A or G, Y=C or T, W=A or T
¥ - SNPs are bolded and underlined

4.2.5 Genotyping by KASP

Kompetitive Allele Specific PCR genotyping (KASP) is an allele specific, fluorescent endpoint genotyping technology (Robinson and Holme, 2011). It is a cost effective and a relatively simple genotyping technology. KASP was used for genotyping of SNPs which had no discriminatory restriction enzymes IL-6 (C2429T, C218T), LEP (G1540A), and those required additional treatment FcεR1G (C1793T). Exonic SNPs in TLR (2, 3, 4, 7, 9) and TGF-β1 genes were identified by next generation sequencing of the spleen mRNA of black mink.

KASP genotyping system contains three components, (i) KASP assay mix containing two allele specific labelled forward primers (one for each SNP allele) with a common reverse primer, (ii) KASP master mix containing Fluorescence resonance energy transfer (FRET)

cassette in optimized PCR buffer with *Taq* DNA polymerase and (iii) test DNA. Bi-allelic scoring of SNPs is performed through the competitive binding of the allele-specific forward primers. Candidate SNPs and their flanking sequences (50bp) for IL-6 (C2429T, C218T), LEP (G1540A), FcεR1G (C1793T), TLR2, TLR3, TLR4, TLR7, TLR9 and TGF-β1 were provided to LGC genomics based on their submission template. (http://www.lgcgroup.com/services/genotyping/setting-up-project/sequence-submission/#.Vrgsz1mQGyw). The DNA samples were sent in 96-well PCR plates at minimum concentration of 5ng/µL per sample on dry ice, and other guidelines provided by LGC genomics (http://www.lgcgroup.com/services/genotyping/setting-up-project/preparing-and-sending-your-samples/#.Vrgs1VmQGyw).

4.3 Statistical analysis

Allele frequency, observed and expected heterozygosity, F_{IS}, tests for homogeneity of allele frequency distributions in different herds and estimates of Nei's genetic distance were performed using the GenAlEx 6.5 computer software (Peakall and Smouse, 2012). Departures from HWE were assessed by chi-squared goodness of fit test (Emigh, 1980) using GenAlEx 6.5. Chi-squared goodness of fit test is based on the null-hypothesis that there is no difference between the observed and the expected genotype frequency values hence, the herd is in Hardy-Weinberg equilibrium (HWE). The Bonferroni correction (=α/(number of comparisons)) was used when multiple comparisons were made (Miller, 1981).

CHAPTER 5. RESULTS

5.1 Genetic variability within herds

5.1.1 Allele frequency distribution

Allele frequency distributions of the 16 loci from nine candidate genes in the six herds are shown in Table 5. All the 16 loci were polymorphic in every herd, except for three loci in eight loci-herd subclasses, which were monomorphic: IL6-C218T in the NG and MBL herds, IL6-C2429T in the MM, NG, NJ and MBL herds, and TLR4-C1992T in the MJ and PE herds. The IL6-C218T and IL6-C2429T loci had low levels of genetic variability in all herds and the frequency of the major alleles was higher than 0.913 in this gene. The TLR9-C315T and TGF-β1-C747T had high levels of genetic variability and the frequency of the major alleles were lower than 0.667 in all herds. The frequency of the TLR2-C1509T was 0.375 in the MBL herd, but was greater than 0.575 in other herds. The frequency of the TLR4-C1992T was 0.75 in the MBL herd, but was greater than 0.915 in other herds (Table 5). Allele frequencies over all herds ranged between 0.545 for TGF-β1- C747T and 0.989 for IL6-C2429T.

Table 5. Frequency of the major alleles of the 16 SNPs in six herds.

Gene	SNP	Allele	MM	AC	MJ	PE	NG	MBL	Overall
IL6	$C^{218}T$	C	0.964	0.913	0.944	0.989	1.0	1.0	0.968
	$C^{2429}T$	C	1.0	0.978	1.0	0.955	1.0	1.0	0.989
	$T^{3039}C$	T	0.783	0.739	0.861	0.878	0.898	0.750	0.818
	$G^{3604}A$	G	0.782	0.744	0.861	0.844	0.880	0.750	0.810
LEP	$T^{306}C$	T	0.574	0.574	0.667	0.489	0.585	0.875	0.627
	$G^{1540}A$	G	0.785	0.683	0.557	0.750	0.673	0.667	0.686
	$C^{1568}T$	C	0.594	0.674	0.847	0.700	0.769	0.458	0.674
FcεR1G	$T^{1364}C$	C	0.925	0.915	0.903	0.872	0.731	0.583	0.822
	$C^{1793}T$	C	0.655	0.742	0.778	0.636	0.745	0.583	0.690
	$T^{1920}C$	T	0.633	0.529	0.625	0.716	0.602	0.917	0.670
TLR2	$C^{1509}T$	C	0.808	0.813	0.847	0.773	0.575	0.375	0.699
TLR3	$C^{858}T$	C	0.658	0.670	0.736	0.693	0.798	0.708	0.711
TLR4	$C^{1992}T$	T	0.993	0.915	1.0	1.0	0.981	0.750	0.940
TLR7	$T^{651}C$	T	0.553	0.726	0.778	0.884	0.764	0.917	0.770
TLR9	$C^{315}T$	T	0.532	0.531	0.583	0.465	0.587	0.667	0.561
TGF-β1	$C^{747}T$	T	0.572	0.544	0.556	0.545	0.387	0.667	0.545

5.1.2 Heterozygosity and Inbreeding Coefficient (F_{IS})

Estimates of unbiased expected heterozygosity (H_E) of each herd at every locus, averaged over all loci for each herd and for each locus over all herds are shown in Table 6. The corresponding estimates of observed heterozygosity (H_O) are shown in Table 7. Averages of H_E and H_O over all herds and loci were 0.33 and 0.31, respectively. H_E of individual loci within herds ranged from 0.00 in the eight monomorphic herd-SNP subclasses (locus

IL6-C218T in NG and MBL herds, locus IL6-C2429T in MM, MJ, NG and MBL herds and locus TLR4-C1992T in MJ and PE herds) to 0.50 (locus LEP-T306C in PE herd). The estimates of H_E for loci TLR9-C315T and TGF-β1-C747T were greater than 0.44 in all herds, whereas the estimates for IL6-C218T and IL6-C2429T were smaller than 0.15 in all herds, implying that the magnitude of H_E in some loci was independent of the herd. The estimates of H_E of each locus over all herds ranged from 0.02 (IL6-C2429T) to 0.48 (TLR9-C315T and TGF-β1-C747T). The estimates of H_E of each locus over all herds were greater than 0.4 in half of the loci, and were smaller than 0.1 in only three loci, suggesting a large level of heterozygosity among different loci. In contrast, the estimates of H_E of each herd over all loci varied within a narrow range (from 0.30 in MJ to 0.36 in AC).

Estimates of H_O of individual loci within herds ranged from 0.00 at the eight monomorphic herd-SNP subclasses to 0.59 (locus TGF-β1-C747T in PE herd) (Table 7). Comparable with the estimates of H_E, the H_O values for the locus TGF-β1-C747T were greater than 0.50 in all herds, and those for the locus TLR9-C315T were greater than 0.44 in all herds, whereas the estimates for IL6-C218T and IL6-C2429T were smaller than 0.14 in all herds. The H_O of each locus, using pooled data of all herds, ranged from 0.01 (IL6-C2429T) to 0.51 (TGF-β1-C747T), again showing a large degree of variability among loci. Six of the estimates over all herds were greater than 0.4. The estimates of H_O of each herd over all loci ranged from 0.29 (MJ) to 0.34 (AC), showing a rather small degree of variability among herds.

The estimates of H_E were larger than H_O in 47.7% (42 of 88) of the herd-SNP subclasses, whereas H_E was equal or smaller than H_O in 13.6% (12 of 88) and 37.5% (33 of 88), respectively (Table 8). The estimates of H_E for each locus over all herds was equal to H_O for one locus (IL6-C218T), but were smaller than H_O for six loci (FcεR1G-T1364C, FcεR1G-C1793T, TLR3-C858T, TLR4-C1992T, TLR9-C315T and TGF-β1-C747T) (Table 8). The greatest H_O /H_E of the loci over all herds (2.0) was observed for the IL6-C2429T locus, followed by the TLR7-T651C (1.78). The estimates of H_E for each herd over all loci was equal to H_O for the MBL herd, but were greater than H_O for the other five herds.

F_{IS} estimates of each herd at every locus, for each herd overall loci, and for each locus over all herds are shown in Table 9. F_{IS} of individual loci within herds ranged from -0.29 (locus TLR4-C1992T in MBL herd) to 1.0 (locus TLR7-T651C in MBL herd). Estimates of F_{IS} were positive in 61.3% (54 of 88) of SNP-herd subclasses, and the estimates for TLR7-T651C were positive for all six herds. Estimates of F_{IS} over all herds were positive for 10 of the 16 loci, and was the greatest for the TLR7-T651C locus (0.431). F_{IS} estimates were positive for all herds when data were pooled over all loci, and were close to each other (0.030 for MJ to 0.074 for MBL).

Table 6. Estimates of expected heterozygosity (H_E) within each herd–locus subclass, for each locus over all herds and for each herd over all loci.

Gene	SNP	MM	AC	MJ	PE	NG	MBL	Overall
IL6	$C^{218}T$	0.06	0.15	0.10	0.02	0.00	0.00	0.05
	$C^{2429}T$	0.00	0.04	0.00	0.08	0.00	0.00	0.02
	$T^{3039}C$	0.34	0.38	0.23	0.21	0.18	0.37	0.28
	$G^{3604}A$	0.34	0.38	0.23	0.26	0.21	0.37	0.30
LEP	$T^{306}C$	0.48	0.48	0.44	0.50	0.48	0.21	0.43
	$G^{1540}A$	0.33	0.43	0.49	0.37	0.44	0.44	0.42
	$C^{1568}T$	0.48	0.44	0.25	0.42	0.35	0.49	0.40
FcεR1G	$T^{1364}C$	0.13	0.15	0.17	0.22	0.39	0.48	0.26
	$C^{1793}T$	0.45	0.38	0.34	0.46	0.38	0.48	0.41
	$T^{1920}C$	0.46	0.49	0.46	0.40	0.47	0.15	0.41
TLR2	$C^{1509}T$	0.31	0.30	0.25	0.35	0.48	0.46	0.36
TLR3	$C^{858}T$	0.45	0.44	0.38	0.42	0.32	0.41	0.40
TLR4	$C^{1992}T$	0.01	0.15	0.00	0.00	0.03	0.37	0.09
TLR7	$T^{651}C$	0.49	0.39	0.34	0.20	0.36	0.15	0.32
TLR9	$C^{315}T$	0.49	0.49	0.48	0.49	0.48	0.44	0.48
TGF-β1	$C^{747}T$	0.49	0.49	0.49	0.49	0.47	0.44	0.48
Overall loci		0.34	0.36	0.30	0.31	0.32	0.33	0.33

Table 7. Estimates of observed heterozygosity (H_O) within each herd–locus subclass, for each locus over all herds and for each herd over all loci.

Gene	SNP	MM	AC	MJ	PE	NG	MBL	Overall
IL6	$C^{218}T$	0.06	0.14	0.11	0.02	0.00	0.00	0.05
	$C^{2429}T$	0.00	0.04	0.00	0.04	0.00	0.00	0.01
	$T^{3039}C$	0.32	0.35	0.27	0.15	0.20	0.33	0.27
	$G^{3604}A$	0.32	0.35	0.27	0.17	0.20	0.33	0.28
LEP	$T^{306}C$	0.48	0.36	0.33	0.44	0.49	0.25	0.39
	$G^{1540}A$	0.33	0.40	0.42	0.31	0.42	0.50	0.40
	$C^{1568}T$	0.46	0.46	0.25	0.42	0.31	0.41	0.38
FcεR1G	$T^{1364}C$	0.13	0.16	0.19	0.25	0.38	0.50	0.27
	$C^{1793}T$	0.43	0.37	0.44	0.40	0.39	0.50	0.42
	$T^{1920}C$	0.48	0.46	0.47	0.38	0.42	0.16	0.40
TLR2	$C^{1509}T$	0.30	0.30	0.19	0.45	0.28	0.41	0.32
TLR3	$C^{858}T$	0.44	0.46	0.36	0.47	0.40	0.41	0.42
TLR4	$C^{1992}T$	0.01	0.17	0.00	0.00	0.03	0.50	0.12
TLR7	$T^{651}C$	0.26	0.22	0.27	0.14	0.20	0.00	0.18
TLR9	$C^{315}T$	0.46	0.50	0.44	0.55	0.51	0.50	0.49
TGF-β1	$C^{747}T$	0.48	0.52	0.50	0.59	0.50	0.50	0.51
Overall loci		0.32	0.34	0.29	0.30	0.30	0.33	0.31

Table 8. Ratios of expected over observed heterozygosity within each herd–locus subclass, for each locus over all herds and for each herd over all loci.

Gene	SNP	MM	AC	MJ	PE	NG	MBL	Overall
IL6	$C^{218}T$	1.00	1.07	0.91	1.00	ϕ	ϕ	1.00
	$C^{2429}T$	0.00	1.00	ϕ	2.00	ϕ	ϕ	2.00
	$T^{3039}C$	1.06	1.09	0.85	1.40	0.90	1.12	1.04
	$G^{3604}A$	1.06	1.09	0.85	1.53	1.05	1.12	1.07
LEP	$T^{306}C$	1.00	1.33	1.33	1.14	0.98	0.84	1.10
	$G^{1540}A$	1.00	1.08	1.17	1.19	1.05	0.88	1.05
	$C^{1568}T$	1.04	0.96	1.00	1.00	1.13	1.20	1.05
FcεR1G	$T^{1364}C$	1.00	0.94	0.89	0.88	1.03	0.96	0.96
	$C^{1793}T$	1.05	1.03	0.77	1.15	0.97	0.96	0.98
	$T^{1920}C$	0.96	1.07	0.98	1.05	1.12	0.94	1.03
TLR2	$C^{1509}T$	1.03	1.00	1.32	0.78	1.71	1.12	1.13
TLR3	$C^{858}T$	1.02	0.96	1.06	0.89	0.80	1.00	0.95
TLR4	$C^{1992}T$	1.00	0.88	ϕ	ϕ	1.00	0.74	0.75
TLR7	$T^{651}C$	1.88	1.77	1.26	1.43	1.80	ϕ	1.78
TLR9	$C^{315}T$	1.07	0.98	1.09	0.89	0.94	0.88	0.98
TGF-β1	$C^{747}T$	1.02	0.94	0.98	0.83	0.94	0.88	0.94
Overall loci		1.06	1.06	1.03	1.03	1.07	1.00	1.04

ϕ - cannot be calculated

Table 9. F_{IS} estimates for SNPs in different locus-herd subclasses, for each locus over all herds and for each herd over all loci.

Gene	SNP	MM	AC	MJ	PE	NG	MBL	Overall
IL6	$C^{218}T$	0.044	0.072	-0.045	0.000	φ	φ	0.021
	$C^{2429}T$	φ	-0.020	φ	0.485	φ	φ	0.312
	$T^{3039}C$	0.059	0.074	-0.147	0.285	-0.104	0.154	0.051
	$G^{3604}A$	0.040	0.064	-0.147	0.333	0.047	0.154	0.073
LEP	$T^{306}C$	0.007	0.250	0.263	0.122	-0.0007	-0.100	0.097
	$G^{1540}A$	0.004	0.079	0.146	0.163	0.048	-0.082	0.047
	$C^{1568}T$	0.044	-0.052	0.048	0.006	0.124	0.203	0.051
FcεR1G	$T^{1364}C$	-0.0008	-0.033	-0.094	-0.134	0.019	0.015	-0.043
	$C^{1793}T$	0.036	0.017	-0.273	0.127	-0.034	0.015	-0.022
	$T^{1920}C$	-0.051	0.075	0.007	0.062	0.120	-0.048	0.027
TLR2	$C^{1509}T$	0.033	0.001	0.262	-0.284	0.429	0.154	0.105
TLR3	$C^{858}T$	0.023	-0.055	0.084	-0.110	-0.244	0.035	-0.051
TLR4	$C^{1992}T$	-0.006	-0.091	φ	φ	-0.009	-0.294	-0.241
TLR7	$T^{651}C$	0.469	0.433	0.209	0.332	0.432	1.000	0.431
TLR9	$C^{315}T$	0.078	-0.015	0.099	-0.110	-0.060	-0.082	-0.027
TGF-β1	$C^{747}T$	0.008	-0.0451	0.001	-0.180	-0.064	-0.082	-0.073
Overall loci		0.052	0.047	0.030	0.073	0.050	0.074	0.047

φ - cannot be calculated because loci were monomorphic

Table 10. Probability for departure of allele frequencies from Hardy-Weinberg equilibrium for 16 SNPs in different herds.

Gene	SNP	MM	AC	MJ	PE	NG	MBL	Overall
IL6	$C^{218}T$	0.372	0.390	1.000	φ	φ	φ	0.696
	$C^{2429}T$	φ	1.000	φ	0.067	φ	φ	0.250
	$T^{3039}C$	0.282	0.326	1.000	0.108	1.000	1.000	0.684
	$G^{3604}A$	0.453	0.384	1.000	0.051	0.563	1.000	0.565
LEP	$T^{306}C$	0.915	<0.000*	0.142	0.550	1.000	1.000	0.030
	$G^{1540}A$	1.000	0.285	0.496	0.415	0.758	1.000	0.904
	$C^{1568}T$	0.447	0.458	1.000	1.000	0.442	0.593	0.924
FcεR1G	$T^{1364}C$	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	$C^{1793}T$	0.560	0.863	0.159	0.514	1.000	1.000	0.891
	$T^{1920}C$	0.369	0.285	1.000	0.718	0.405	1.000	0.859
TLR2	$C^{1509}T$	0.609	1.000	0.162	0.086	0.002	1.000	0.040
TLR3	$C^{858}T$	0.725	0.455	0.674	0.721	0.099	1.000	0.763
TLR4	$C^{1992}T$	1.000	0.382	φ	φ	1.000	0.529	0.921
TLR7	$T^{651}C$	<0.000*	<0.000*	0.323	0.078	0.003	0.043	<0.000
TLR9	$C^{315}T$	0.140	0.894	0.731	0.546	0.776	1.000	0.889
TGF-β1	$C^{747}T$	0.914	0.508	1.000	0.359	0.773	1.000	0.981

φ - Cannot be tested
*- Significant after Bonferroni corrections (P<0.0005)

5.1.3 Hardy-Weinberg Equilibrium (HWE)

Genotype frequencies did not conform to HWE (P<0.05/88=0.0005) in three of the 88 herd-SNP subclasses: TLR7-T651C locus in MM and AC herds and for LEP-T306C in the AC herd (Table 10). The overall genotype frequencies deviated from HWE only for one of the 16 loci (TLR7-T651C). The test could not be performed for nine herd-SNP subclasses that were either monomorphic or all animals were homozygous except for one or two heterozygous individual (Table 10).

5.2 Genetic variability among herds

5.2.1 Differences among herds for allele frequency distribution

Of the 232 pairwise comparisons among the six herds for allele frequencies, 83 (35.8%) were significant at P<0.05 (Tables 11 and 12). Applying the Bonferroni corrections for multiple comparisons (α=0.05/232=0.0002), the number of significant differences reduced to 35 (15.1%). The largest number of significant differences between pairs of herds within each locus was observed for the TLR2-C1509T, whereas none of the herds were different from each other for allele frequencies at the IL6-G3604A, LEP-T306C, TLR3-C858T and TLR9-C315T loci. The MM and AC herds had the largest number of loci with significant allele frequency differences (seven), followed by MM and NG herds with five significant differences. No significant allele frequency difference for any of the 16 loci was observed between five herd pairs, including MJ and AC, and between PE with AC, MJ and NJ.

Pairwise comparisons between herds within each locus showed largest differences (ranging between 0.40 and 0.47) between MBL (0.375) and MM (0.808), AC (0.813), MJ (0.847) and PE (0.773) herds at the TLR2-C1509 locus. Other large allele frequency differences (ranging between 0.30 to 0.39) were between MBL (0.875) and PE (0.489), MM (0.574) and AC (0.574) herds at the locus IL6-T306T, between MBL (0.458) and NJ (0.769) and MJ (0.847) herds at the locus LEP-C1569T, between MBL herd (0.583) and MJ (0.903), AC (0.915) and MM (0.925) herds at the LEP-T1364C locus, between MBL (0.917) and AC (0.529) and NG (0.602) herds at the FcεR1G-T1920C locus, and between MM (0.553) and MBL (0.917) and PE (0.884) herds at the TLR7-T651C locus. The results show that all comparisons with large differences included the MBL herd, either having the largest or the smallest frequencies within each locus. Differences between all herds for allele frequencies, although significant in some cases, were smaller than 0.2 for IL6-C218T, IL6-C2429T, IL6-G3064A, FcεR1G-C1793T and TLR3-C858T loci.

5.2.2 Nei's genetic distance

Estimates of the Nei's genetic distances among the pairs of herds using SNP data are shown in Table 13. Genetic distances ranged from 0.008 (between AC and MM) to 0.074 (between MBL and MJ). The MBL herd had the largest genetic distance with all other herds (0.055 to 0.074).

Table 11. Pairwise comparison of allele frequency distributions of SNPs of IL-6, leptin and TGF-β1 among different herds.

Pair	IL-6				LEP			TGF-β1
	$C^{218}T$	$C^{2429}T$	$T^{3039}C$	$G^{3604}A$	$T^{306}C$	$G^{1540}A$	$C^{1568}T$	$C^{747}T$
MM & AC	0.000*	0.000*	0.072	0.138	1.000	0.000*	0.004	0.368
MM & MJ	0.345	φ	0.127	0.129	0.123	0.000*	0.000*	0.807
MM & PE	0.349	0.000*	0.041	0.221	0.134	0.500	0.063	0.644
MM & NG	0.059	φ	0.004	0.020	0.915	0.012	0.000*	0.000*
MM & MBL	1.000	φ	0.619	0.800	0.002	0.208	0.211	0.406
AC & MJ	0.492	0.374	0.026	0.041	0.164	0.046	0.001	0.898
AC & PE	0.014	0.254	0.004	0.046	0.163	0.250	0.714	1.000
AC & NG	0.000*	0.217	0.000*	0.002	0.914	0.905	0.066	0.004
AC & MBL	0.249	1.000	1.000	1.000	0.004	0.825	0.043	0.290
MJ & NG	0.027	φ	0.488	0.818	0.345	0.151	0.245	0.034
MJ & PE	0.172	0.126	0.816	0.823	0.024	0.017	0.038	1.000
MJ & MBL	0.567	φ	0.218	0.211	0.070	0.478	0.000*	0.471
PE & NG	0.457	0.041	0.650	0.533	0.207	0.265	0.325	0.030
PE & MBL	1.000	0.576	0.191	0.362	0.001	0.437	0.030	0.362
NG & MBL	φ	φ	0.079	0.112	0.009	1.000	0.004	0.019

φ - Both herd were monomorphic
*- Significant after Bonferroni corrections (P<0.0002)

Table 12. Pairwise comparison of allele frequency distributions of SNPs of FcεR1G, TLR2, TLR3, TLR4, TLR7 and TLR9 genes in different herds.

Pair	FcεR1G			TLR2	TLR3	TLR4	TLR7	TLR9
	$T^{1364}C$	$C^{1793}T$	$T^{1920}C$	$C^{1509}T$	$C^{858}T$	$C^{1992}T$	$T^{651}C$	$C^{315}T$
MM & AC	0.581	0.000*	0.000*	0.881	0.702	0.000*	0.000*	1.000
MM & MJ	0.489	0.035	0.898	0.527	0.197	1.000	0.000*	0.453
MM & PE	0.121	0.723	0.164	0.474	0.545	1.000	0.000*	0.265
MM & NG	0.000*	0.079	0.530	0.000*	0.004	0.224	0.000*	0.338
MM & MBL	0.000*	0.513	0.003	0.000*	0.670	0.000*	0.000*	0.216
AC & MJ	0.658	0.569	0.164	0.620	0.277	0.005	0.396	0.440
AC & PE	0.208	0.047	0.001	0.389	0.725	0.002	0.001	0.296
AC & NG	0.000*	1.000	0.192	0.000*	0.014	0.011	0.467	0.344
AC & MBL	0.000*	0.091	0.000*	0.000*	0.824	0.016	0.055	0.206
MJ & PE	0.609	0.063	0.240	0.312	0.599	φ	0.086	0.146
MJ & NG	0.004	0.724	0.876	0.000*	0.357	0.517	0.859	1.000
MJ & MBL	0.001	0.110	0.008	0.000*	0.796	0.000*	0.225	0.634
PE & NG	0.027	0.113	0.095	0.004	0.098	0.503	0.040	0.110
PE & MBL	0.005	0.640	0.056	0.000*	1.000	0.000*	1.000	0.106
NG & MBL	0.215	0.136	0.003	0.114	0.409	0.000*	0.159	0.498

φ - Both herd were monomorphic

*- Significant after Bonferroni corrections (P<0.0002)

Table 13. Nei's genetic distance for the six herds using 16 SNPs.

	MM	AC	MJ	PE	NG	MBL
MM	0.000	-	-	-	-	-
AC	0.008	0.000	-	-	-	-
MJ	0.019	0.010	0.000	-	-	-
PE	0.015	0.013	0.014	0.000	-	-
NG	0.025	0.020	0.015	0.016	0.000	-
MBL	0.072	0.071	0.074	0.061	0.055	0.000

CHAPTER 6. DISCUSSION

The 16 SNPs in nine candidate genes tested in this study were the only immune system genes for which SNPs had been previously identified in mink (IL6, LEP, FcεR1G) (Donkor, 2007; Arju, 2010; NCBI acc# KC986377) or were identified by next generation sequencing of the spleen mRNA of black mink (TLR2, 3, 4, 7, 9 and TGF-β1). Critical roles of these genes have been implicated in immune responses to infection in other species (Barrett *et al*., 2003; Bloom *et al.,* 1994; Abdel and Rashed, 2011; Palikhe *et al*., 2008; Ben-Ali *et al*., 2004; Medhi *et al*., 2011; Edfeldt *et al*., 2004; Kawasaki *et al*., 2011; Noguchi *et al*., 2004; Yamada *et al*., 2008). It was thus logical to assume that they might have been involved in mink immune response to AMDV infection.

Donkor (2007) sequenced twenty unrelated mink, four from each of five color type (wild, black, brown, pastel, sapphire) and showed 8 SNPs in 4678 bp (one SNP per each 585 bp) of which only one was exonic in the IL-6 gene. Using the same twenty mink as above, 17 SNPs were detected in 4738 bp of the LEP gene (one SNP per each 279 bp) and all were in the introns or the 3'UTR (Arju, 2010). Vardy (2002) found three intronic SNPs in 9,903 bp sequence of the mink prolactin gene (one SNP per each 3301 bp) by sequencing of one mink from each of the four color types (wild, black, brown, pastel). This low SNP density made it difficult to find a larger number of SNPs, particularly exonic SNPs, to perform this study. Sequencing of the mink entire genome by next generation sequencing can identify a large number of SNPs. SNPs in the five TLR and the TGF-β1 genes, which were identified in mRNA of three sets of four full-sib black mink by next generation sequencing, were all polymorphic in this study. This highlights the utility of next generation sequencing as a practical method of finding large numbers

of exonic SNPs. The intronic SNPs tested in this study may be linked to other alleles or genes with large effects on animal response to infection.

Previously designed RFLP-PCR tests (Donkor, 2007; Arju, 2010) were used to analyze six of the loci in the IL6, LEP and FcεR1G genes. Although RFLP is easy to perform, it is not a high throughput method and is a time-consuming genotyping technology. KASP was used for genotyping of SNPs which had either no discriminatory restriction enzyme (LEP-G1540A, IL6-C218T, IL6-C2429T), those which required additional treatments after enzyme digestion (FcεR1G-C1793T), as well as for the newly discovered SNPs in the six genes (TLR2, TLR3, TLR4, TLR7, TLR9 and TGF-β1). KASP is a low-cost and relatively simple genotyping technology (Robinson and Holme, 2011; Holdsworth and Mazourek, 2015; Tsai *et al*., 2015). The only limitation of this method is that the sequence of 50 bps flanking the SNP must be known, which may not always be possible when SNPs are obtained from mRNA sequencing or they are located close to exon-intron junctions.

6.1 Genetic variability within herds

The level of polymorphisms at the SNP loci, estimates of F_{IS}, H_O and H_E were the measures of genetic variability within herds. These parameters can shed light on the breeding structure of the mink herds, genetic consequences of keeping mink in captivity and the mating systems employed by mink ranchers. H_O is a measure of total number of heterozygous individuals in a population at a locus and H_E is the proportion of heterozygosity expected at a locus in a population under random mating. F_{IS} is the measure of the extent of inbreeding in a population, which is related to heterozygosity

and is defined as $(H_E-H_O)/H_E = 1-(H_O/H_E)$ (Nei, 1977). Positive F_{IS} values represent individuals which are more related to each other than the average in a population (Wright, 1922). A high degree of inbreeding within a herd may result in a decrease in animal performance, increased mortality and increased incidence of genetic disorders (Regan *et al.*, 1935; Gentile and Testoni, 2006), and thus needs to be corrected by introducing new breeding individuals into the herd.

Despite the fact that the black mink population in NS is rather small, 13 of the 16 loci studied were polymorphic in every herd, and 91.7% (88 of the 96) of the locus-herd subclasses were polymorphic (Table 5). In a previous study, frequencies of the major alleles at the LEP-T306C, LEP-G1540A and LEP-C1568T loci in a sample of 20 black mink from across NS were 0.70, 0.625 and 0.650, respectively (Arju, 2010), which are comparable with the estimates in the current study (0.627 to 0.686). Interestingly, the A allele of the LEP-G1540A locus was not detected in 20 wild mink samples collected in NS (Arju, 2010), suggesting that the LEP gene has possibly been under selection in farmed mink. The T alleles of the IL6-C218T and IL6-C2429T loci had very low frequencies in all herds, and were monomorphic in six of the herd-locus subclasses. In a previous study (Donkor, 2007), the T allele of the IL6-C218T locus had a low frequency in black mink and the IL6-C2429T locus was monomorphic. The IL6-C2429T locus was tested in the current study because it was an exonic SNP causing a substitution of a histidine to a tyrosine, and measuring its variability in a larger sample seemed logical. Allele frequencies at IL6-T3039C and IL6-G3604A were 0.76 and 0.78, respectively, in a sample of 20 black mink from across NS (Donkor, 2007), which are comparable with 0.82 and 0.81 in the current study.

The results of allele frequency distributions and the finding that estimates of H_E were greater than 0.30 in 65.6% of the locus-herd subclasses (0.30 to 0.50) and the H_O estimates were greater than 0.30 in 59.4% of the subclasses (0.30 to 0.59), indicate the presence of a moderate degree of genetic variability within herds. In addition, the estimates of H_E of the herds over all loci ranged between 0.30 (MJ) to 0.36 (AC), and the H_O varied between 0.29 (MJ) and 0.34 (AC), confirming the presence of moderate degrees of genetic variability in all herds. The F_{IS} values in locus-herd subclasses were mostly small, although positive in many cases, and the estimates overall loci were all smaller than 0.08, suggesting a low level of inbreeding.

Moderate level of genetic variability can be attributed to the fact that the ranched mink originated from different subspecies of wild mink (Dunstone, 1993), and perhaps there has been a continuous gene flow among different ranches in the province. Most ranchers often purchased breeding stock from a few AMDV-free ranches in the province and mixed them with their stock, thus increasing the level of genetic variability within their herds. Gene flow between ranches in NS with those in other Canadian provinces and the USA has been limited because of the presence of Aleutian disease. However, some mink were imported from the USA by a few ranches in the past (Personal Comm.). Although ranchers avoid mating between parent-offspring, half- and full-sibs, mating between more distant relatives were unavoidable because only one generation of pedigree (the mother) is often available. Additionally, mink ranchers often breed each female with two different males to increase fertility, causing ambiguity of parentage.

Differences in allele frequencies, estimates of H_O, H_E and F_{IS} among loci over all herds were greater than those estimates for the herds over all loci, suggesting that variations in these parameters were mainly the characteristics of the loci rather than the feature of the herds. For instance, monomorphic loci were present only at the IL6-C218T, IL6-C2429T and TLR4-C1992T, and the frequencies of the alleles at these loci were greater than 0.9 in all herd-loci subclasses, as well as the estimates at these loci over all herds, except at the TLR4-C1992T locus in the MBL herd. Estimates of H_O and H_E were all low at these subclasses and over all loci. Similarly, estimates of H_O and H_E were greater than 0.4 at loci TLR9-C315T and TGF-β1-C747T in all herds. These findings may suggest that such loci were under selection for adaptation to captivity and/or production traits, such as fur quality, reproductive performance and/or immune response to infection, which are the traits where captive mink were favoured for over 100 years.

6.1.1 Hardy-Weinberg Equilibrium

The HWE principle states that within large populations, the allele and genotype frequencies remain constant from generation to generation unless mutation, migration, non-random mating (inbreeding), selection or genetic drift are introduced to misbalance the equilibrium (Benarie, 1981). The herd-SNP subclasses in this study did not show significant departures from HWE at any loci, except three (LEP-T306C in AC herd, TLR7-T651C in MM and AC herds). In this study, two of the three SNP-herd subclasses which significantly deviated from HWE were at the TLR7-T651C locus (MM and AC herds) suggesting that the HWE deviation was related to both the locus and the herd.

Arju (2010) reported that of the 40 SNP-herd subclasses in the LEP gene, involving wild mink and four captive color types, five subclasses deviated from HWE, four of them were in pastel and one was in brown mink. The five subclasses involved five different SNPs, and it was hence, concluded that deviation from HWE was the characteristic of the herd and not the locus. Another study identified significant deviations from HWE for 405 of the 8000 SNPs in five Ethiopian cattle populations (Edea *et al*., 2012). Further, they suggested that selection pressure and/or population stratification contributed to this deviation from HWE.

Excess homozygosity (positive F_{IS}) was the reason for deviation from HWE in every case. F_{IS} values were greater than 0.4 in two of the three SNP-herd subclasses which deviated from HWE, and LEP-T306C in AC herd was the only exception (F_{IS}=0.25). The H_E/H_O ratios were also large for cases where deviations from HWE were significant. The presence of excess homozygosity when a herd deviated from HWE was also reported in mink (Arju, 2010). Significant deviation from HWE, large F_{IS} estimates and large H_E/H_O ratios could be due to several factors:

Firstly, when the sample size is small, it is possible that the individuals in the samples are not representative of the allele counts in the herds. In order to sample as many alleles as possible, individuals should be as unrelated to each other as much as possible. Attempts were made to use individuals which were unrelated for at least one generation on MM and AC ranches but the animals might have been related to each other in previous generations. The sample sizes of the MM and AC ranches were large (369 and 233, respectively) whereas the MBL herd was represented by only 12 mink, but no clear trend was observed for the magnitude of F_{IS} and deviation from HWE and sample sizes.

Therefore, it is unlikely that sampling error played a significant role in the deviation of these herds from HWE. Furthermore, all loci in a herd would be deviated from HWE if sampling error was the major cause and not a certain locus in most herds, as was observed for TLR7-T651C in this study.

Secondly, some mink ranchers create several lines within their herds or breed the prolific males to the prolific females (positive assortative mating) to improve performance of their stock. Animals used in positive assortative mating may be related to each other, thus increasing inbreeding, excess homozygosity, positive F_{IS} and deviation from HWE. Positive assortative mating and inbreeding do not, however, fully explain the observation that only certain loci deviated from HWE.

Thirdly, genes that have been directly under artificial selection, those which modulate fitness, and those which were linked with such genes, would have high homozygosity at such loci. If this assumption was true, the TLR7 locus, and to some extent the LEP genes, have been under selection. Absence of the A allele at the LEP-G1540A locus in wild mink samples collected in NS (Arju, 2010), could be a supportive evidence for this hypothesis. Arju (2010) compared allele frequencies of 10 SNPs in the LEP gene among wild mink and four captive color types (black, brown, pastel, sapphire). The study concluded that the genomic region that harbours the LEP gene has been under selection in farmed mink as a result of the role that leptin plays on growth rate (Jiang and Gibson, 1999; Chen *et al*., 2004), reproductive performance (Zhang *et al*., 1994; Chen *et al*., 2004), and immune response of mink to the higher pathogen load on mink ranches. Genes involved in the recognition of pathogens, including TLRs, have been shown to be subjected to positive selection in mammals (Wlasiuk and Nachman, 2010) and birds

(Huang *et al*., 2011). Hence, it is reasonable to hypothesize that the genomic region carrying the TLR7 gene has been under selection pressure in farmed mink as TLR7 plays a crucial role in initiating immune responses against viruses (Lund *et al*., 2004). Selection seems to be the most admissible explanation, supported by positive F_{IS} values in all the loci that significantly deviated from HWE, and by the significantly larger H_E than H_O (Table 6 and 7) in almost every case where HWE was distorted.

Finally, the deviation from HWE may be the result of genotyping error (Salanti *et al.,* 2005). It is plausible that the genotyping method used in this study was not able to distinguish between heterozygous and homozygous individuals in these loci, i.e. KASP for TLR7-T651C locus and RFLP-PCR for the LEP-T306C.

A total of 88 x^2 tests were performed on the data, and thus the overall α (probability of rejecting a true hypothesis) was larger than 0.05. To keep the overall α at 0.05, the Bonferroni correction of p =α/n =.05/88= 0.00057 (Miller 1981) was considered significant. Statistical artefact was not thus a factor in significant departure of three loci-herd subclasses from HWE.

6.2 Genetic variability among population

The main goal of this study was to test whether long term selection for tolerance to AMDV infection using animal health status and reproductive success (MBL herd) or a combination of animal health, reproductive success and iodine agglutination test (MM herd) had changed allele frequency of 16 SNP loci in nine immune system genes. Three AMDV-infected herds which have been practicing test-and-kill strategy (NG, MJ, PE) and one farm which contained animals originated from farms which have been free of

AMDV for several years (AC herd) were used as the controls. The degree of differentiation among herds was estimated by the number of significant differences in allele frequency distributions among herds and estimates of the Nei's genetic distance. Genetic distance measures the degree of divergence between populations and consolidates a large number of data points among many populations and loci (232 comparisons in this study). This makes it advantageous over pairwise allele frequency differences. It is thus a very useful measure in uncovering the degree of differentiation of the herds. The smaller array of genetic distances which replace a large number of pairwise allele frequency differences, however, results in the loss of some information.

The Nei's genetic distance (D) is a measure of average number of nucleotide substitutions that have accumulated since any two populations separated from a common ancestral population (Nei, 1972). It has a linear relationship with time of divergence (t), i.e. $D=2\alpha t$, where α is the rate of nucleotide substitution per locus per generation. The number of generations that the herds in the current study were separated from an ancestral population is not known, and estimation of the time of divergence was not the objective of this study. Genetic distance estimates varied between zero and one, and although the values do not have a unit, their relative magnitude are the measure of the degree of divergence. There are some assumptions associated with the Nei's genetic distance (Nei, 1972). (i) mutations occur according to the infinite allele model, i.e., mutations result in a completely new allele and the probability of back mutation to the original allele would be negligible, and that the effect of mutant alleles ranges from neutral to deleterious (Kimura and Crow, 1964). This assumption does hold true in this study because an allele may mutate to one already existing in the population. (ii) it is

assumed that all loci have the same rate of neutral mutation, (iii) mutations within a locus occur independent of each other, (iv) the number of allelic substitutions per locus follows a Poisson distribution, (v) genetic variability initially in the population is at equilibrium between mutation and genetic drift, and (vi) the effective population size remains constant for each population. Although Nei's genetic distance is widely used in population genetics research (Belliveau et al., 1999), it is difficult to prove that the above assumptions hold true, as is the case in the current study. Yet, Nei's genetic distance is an acceptable measure of population differentiation. In addition, genetic distance increases drastically if a population goes through a tight bottleneck, i.e. a very small number of breeding individuals (2 to 10) for several generations (Chakrabotry and Nei, 1977). If population size increases over a long period of time, there will be a decrease in the rate of nucleotide substitution by drift and the estimate of genetic distance will be lower than the true time of divergence (Nei, 1976). The Nei's genetic distance is, however, quite robust to founder effect and change in population size (Nei 1975, 1976).

The largest degree of differentiation was observed between MBL and all other herds, shown by the greatest estimates of genetic distances (0.055 to 0.074), which were more than twice as high as those for the other herds (lower than 0.025). The large genetic distances were supported by the presence of one to four significant (after Bonferroni correction) allele frequency differences with the other herds. Interestingly, at least half of the significant pairwise comparisons of allele frequencies in the FcεR1G-T1364C, FcεR1G-T1920C, TLR2-C1509T and TLR4-C1992T loci involved MBL herd. These genes play important roles in immune response to infection, and the results imply that these genes need to be further studied for confirmation. The MBL herd has been infected

with AMDV for over 40 years and breeder animals have been selected based on their health status and reproductive performance, making it a unique mink herd in NS. This farm is located in Digby county, the centre of mink farming in NS, and has likely been infected with a large array of AMDV isolates circulating in this region. In contrast, the MM farm is located on the island of Cape Breton, where there are a few mink farms, and the MM herd was likely infected with one or a few AMDV isolates. It is thus possible that selection pressure on the MBL herd was greater than that on the MM herd. This herd, however, was represented by a small number of samples (twelve), which could be the reason for the large genetic distances. Despite several attempts, the farmer did not agree to supply additional samples.

Similarities among other mink herds for allele frequency and genetic distance was due to several factors; firstly, the Jetblack allele, which makes mink fur very dark and was discovered in Digby County, NS (Mullen, 1991). It has been incorporated into all black mink herds in the province resulting in all black mink in NS to be related to a single litter in the past. Secondly, most mink ranchers purchase breeding stock from a limited number of suppliers in NS and the USA who have mink with high quality pelt and are AMDV free. Thirdly, mink farmers in NS have heavily selected their animals for fur quality and litter size for many years. Strong selection pressure for a few traits applied to mink on different farms would likely result in the frequency of desirable alleles affecting those traits, as well as the frequency of alleles at linked loci, to change in the same direction. Genes that modulate fur quality traits, which have high heritability, are expected be particularly under selection pressure. There is one report on the estimate of genetic distances among four black mink herds in NS where microsatellite markers were used

(Belliveau et al., 1999), showing somewhat larger estimates of genetic distance (0.074 to 0.132).

The results of pairwise comparison of the allele frequencies highlighted some interesting points. First, the largest number of significant allele frequency differences (seven) was observed between the AC and MM herds, followed by those between MM and NG (five). The reason that these differences were not manifested in genetic distance estimates and is not clear, but suggests selection for tolerance at the MM herd had some effect on allele frequencies at several loci. Second, while some loci did not show any evidence of being under selection as no difference was observed amongst herds (IL6-G3604A, LEP-T306C, TLR3-C858T, and TLR9-C315T), the TLR2-C1509T showed seven significant pairwise differences, and TLR4-C1992T and TLR7-T651C, each showed five significant difference. All these alleles involved comparisons with either MM, MBL or both herds, which have been under selection for tolerance. Interestingly, allele frequencies of the TLR7-T651C were significantly different between the MM and all other herds, and no other comparison was significant. These findings suggest that TLR2, TLR4 and particularly TLR7 are possibly involved in tolerance to AMDV infection.

CHAPTER 7. CONCLUSION

Sixteen alleles of nine immune system genes were investigated in two herds selected for tolerance to AMDV and four control herds. The level of genetic variability in all herds was moderate, suggesting that intense selection for fur quality traits and reproductive success had not depleted genetic variability. There was evidence that TLR2, TLR4 and TLR7 genes were possibly under selection in tolerant farms. Future research should focus on these genes to see whether these genes modulate response of mink to infection by AMDV.

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