Investigation of DNA variation in genes of the immune system in wild populations of *Apodemus sylvaticus* in relation to infection by *Toxoplasma gondii* and helminth parasites.

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Table of Contents:

Tables of Figures:

Table of Tables:

Statement of Authorship:

I hereby certify that I am the sole owner of this thesis. The work contained in this thesis has not previously submitted for a degree or diploma at any other universities or institutions.

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List of Abbreviations:

TLR	Toll like receptors
AIDS	Acquired Immune Deficiency Syndrome
IFAT	Immunofluorescence antibody
LAT	Latex agglutinin Test
SAG	Surface Antigen of *T. gondii*
GRA	Dense Granule Protein
MLN	Mesenteric Lymph Nodes
HSE	*H. polygyrus* execratory secretory antigen
IFNγ	Interferon Gamma
IL-12	Interleukin 12
NBD	NOD-like receptors
NBD	Nucleotides Binding Domain
STAT-3	Signal Transducer and Activator of Transcription 3
BN	Norway mice
SNPs	Single Nucleotide Polymorphism
P2x7r	P2X Purinoceptor 7
BMDM	Bone Marrow Derived Macrophage
LPS	Lipopolysaccharides
LLRs	Leucine Rich Repeat
ssRNA	Single Strand RNA
MYD88	Myeloid Differentiation Primary Response 88
TRIF	TIR-Domain-Containing Adapter-Inducing Interferon-β
TIRAP	Toll-Interleukin 1 Receptor (TIR) Domain Containing Adaptor Protein
CCL2	Chemokine Ligand 2
NF-kappa B.	The Nuclear Factor Kappa B
HSV-1	Herpes Simplex Virus
SM	Smith Antibody
PAMPs	Pathogen Associated Molecular Pattern
FliC	Flagillin
PCR	Polymerases Chain Reaction
dNTP	Deoxyribonucleotide triphosphate
xTBE	Tris/Borate/EDTA Buffer
DC	Dendritic Cell
UNC93B1	Unc-93 homolog B1 Protein
TgPRF	*Toxoplasma gondii* Profilin
PYD	Pyrin Domain
CASP1	Caspase 1
IL-1B	Interleukin 1 Beta
CARD	Caspase Recruitment *Domain*
ASC linker	Apoptosis-Associated Speck-Like Protein Containing a CARD

Abstract:

The aims of this study are to investigate DNA sequence variation in immune genes from *Apodemus sylvaticus* in relation to parasite infection. The purpose is to investigate the hypothesis that immune gene variation can influence parasite infection. Evidence from other studies, such as infection of wild voles with *Borrelia* and cattle with bovine tuberculosis, has demonstrated that the DNA sequence (and therefore the protein sequence) of toll-like receptors (TLRs) is correlated with infection status. Recent laboratory studies on mice showed that the genes for TLR11 or TLR12 are essential for recognition of the parasite *Toxoplasma gondii*. Furthermore, the NLRP1b gene, a key component in regulating inflammation during infection, her been found in laboratory studies to be responsible for resistance of the mice against toxoplasmosis. Also, it is located at the *Toxo1* locus which has been shown to be a key host locus for controlling *Toxoplasma* parasite proliferation and recent studies on inbred mice confirm that NLRP1b is the main gene that is responsible for this control. But little is known of the role of these innate immune system genes in natural populations. The purpose of this study is to investigate gene diversity in relation to parasite infection in wild rodent populations.

A recent study in our laboratory resulted in 126 wild wood mice (*Apodemus sylvaticus*) being tested for *T. gondii* infection and other helminth parasites. This provides a topical opportunity to investigate DNA polymorphisms in NLRP1 and TLRs that could be associated with infection.

In a study which investigated polymorphisms in relation to TLR11 and TLR 12, several relations were obtained but only one of them is significant which is the relationship between *Syphacea* infection with H2 and non H2 haplotype

At the start of the project no sequence was available for the NLRP1b gene from *A. sylvaticus*. Using Clustal alignment of DNA sequences from *Mus musculus* and other rodents, several combinations of PCR primer pairs were designed and tested for the amplification of parts of this gene from *A. sylvaticus*. The amplification is complex due to the arrangement of exons and introns, but successful sequences have been obtained for Exon 3 part 2 and Exon 3 part 3 (approximately 450 base pairs) which covers the function region, called NACHT, from 80 mice. Polymorphisms were found at amino acid positions 15 and 22 of exon 3-2 and positions 11 and 36 of exon 3-3. Exon 3-2 amino acid polymorphism is either methionine or leucine at position 15 and glutamic acid or alanine at position 22. Exon 3-3 amino acid polymorphism is either histidine or leucine at position 16 and glutamic acid or lysine at position 36. The polymorphisms found in the NLRP1b gene were examined for any relationship to a broad

range of nematode parasites. One of the exon 3-2 haplotypes (H1/H2) shows a significant association with different nematodes and two haplotypes (H2/H3 and H4/H4) have a significant association with *Toxoplasma gondii* in respect for different helminth parasites. Furthermore, three of the exon 3-3 haplotypes (H1/H4, H4/H4 and H3/H3) show a significant association with different nematodes and one haplotype (H4/H4) has a significant association with *Toxoplasma gondii*. In addition, one haplotype of exon 3-3 which is H2/H2 showed a significant association with parasitic infection status (negative or multiple parasites). Also, the homozygosity and heterozygosity of the SNPs were investigated. For exon 3-2, the homozygosity of the first SNPs locus showed significant association with *Toxoplasma gondii*. For exon 3-3, the homozygosity of the first SNPs locus, and the second SNPs locus when analysed separately show significant association which *Toxoplasma gondii*. But, when analysed together, the heterozygosity of both SNPs locus showed significant association with *Toxoplasma gondii* in respect for different helminth parasites.

In this study, DNA sequence variation was found in the immune genes (TLR11, 12 and NLRP1b) natural populations of wood mice. There was an association between some haplotypes and parasite infection. This provides evidence in support of the hypothesis that variation in immune gene sequences can influence parasite infection. Future studies should be aimed at identifying the detailed interactions between parasites and host immune genes in natural populations of wild animals.

Chapter 1

Introduction

1. Introduction.

1.1 Adaptive Immune System.

The adaptive immune response is considered to be a specific mechanism of defence due to its ability to fight specific pathogens. An adaptive immune response requires more time than an innate immune response to be activated. However, it has the ability to fight the same antigen faster if it invades the body again (Alberts *et al.*, 2002). This is very important, because an innate immune response cannot clear all kinds of infection (Janeway *et al*., 2001). Also, an adaptive immune response works as a memory for pathogens, which helps in the clearance process in a case of acquiring the same infection repeatedly. The adaptive immune response is controlled by the T and B Lymphocytes. This kind of immune response has two main parts that play key roles in the defence mechanism. The first part is antibody production, while the second part is a cell-mediated response. The most important cell involved in antibody production is the B cell. The function of a cell-mediated response is to activate different cells, such as macrophages and natural killer cells. Also, a cell-mediated response is involved in the production of cytokines. An adaptive immune response is controlled by an innate immune response via an activation sequence (Schnare *et al*., 2001).

Antigens of foreign pathogens are presented to CD4 and CD 8 T lymphocytes by B cells, macrophages and dendritic cells (Jenkins *et al*., 2001). CD4 T lymphocytes are very important for antibody production by B lymphocytes (Johnson and Sayles, 2002). CD8 T lymphocytes' main function is to destroy parasites and other pathogens and give resistance to infection. It produces some cytokines such as interferon gamma to fight infections. The production of interferon gamma occurs by two processes, CD40/CD40L interaction and perforin-mediated cytolysis of infected host cells (Reichmann *et al*., 2000; Denkers *et al*., 1997). In vitro experiments show that CD4 and CD8 T lymphocyte depletion from previously infected mice leads to reactivation of the disease and severe symptoms (Gazzinelli *et al*., 1992).

1.2 Innate Immune System.

There are two types of immune defence in the mammalian body. The first defence is an innate immune response, while the second is an adaptive immune response. Both mechanisms have a main function, which is protection against any infection (Alberts *et al.*, 2002)An innate immune response is one that exists in the mammalian body from birth (Simon *et al.*, 2015). As such, it response is very important because it is the first defence mechanism to be triggered directly after infection, while an adaptive immune response requires 4–7 days (Janeway *et al*., 2001). The innate immune response is considered the first mechanism of defence against foreign antigens. It is known as a non-specific response due to its ability to be activated by different types of pathogens. The innate immune response consists of three levels that work together against foreign pathogens. The first level is an anatomic barrier, e.g. skin, tears, saliva, mucus and cilia in the intestinal and respiratory tracts. The second level is a humoral barrier, e.g. the complement system and Interleukin-1. The third level is a cellular barrier, e.g. neutrophils, macrophages, dendritic cells, natural killer cells and natural killer T cells. Each type of cell plays a very important role in the innate immune response. The innate immune response plays an important role in the control of *T. gondii* infection. Many studies have shown that T-lymphocyte-deficient animals have high levels of parasites (Lindberg and Frenkel, 1977). Following infection, three types of immune system cells are directed to the site of a parasite, these are monocytes, neutrophils and dendritic cells (Dunay *et al*., 2008; Bliss *et al*., 2000; Tait *et al*., 2010). After that, IL-12 is produced by the antigen presenting cells, which leads to the production of interferon gamma by T lymphocytes and natural killer cells. IFN-γ production is very important for protection against *Toxoplasma gondii* replication and infection (Gazzinelli, 1993; Hunter, 1994). In vitro studies show that an IL-12 or IFN-γ deficiency in laboratory mice infected with *T. gondii* offers no control over the parasite (Suzuki, 1988; Gazzinelli, 1994). *Toxoplasma gondii* must be recognized by the host in order to start the mechanism of an innate immune response. TLR receptors are responsible

for detection of parasites. Then, MyD88 sends a signal from the TLR receptors to initiate a response. MyD88 deficient mice show susceptibility to infection (Scanga, 2002). There are many types of toll-like receptors and four of them, TLRs 2, 4, 9 and 11, are involved in activation of the immune response against *T. gondii* infection (Yarovinsky *et al*., 2005; Jenkins *et al*., 2010). Many types of cells are involved in fighting *Toxoplasma gondii* infection. Four cells are responsible for production of IL-12, these are dendritic cells (Scanga *et al*., 2002), neutrophils which store IL-12 (Bliss *et al*., 2000), macrophages and inflammatory monocytes. Dendritic cells are the main source of IL-12 during *T. gondii* infection (Reis e Sousa *et al*., 1997). Neutrophils are important not only for IL-12 production but as an important mechanism for killing parasites. For example, the release of reactive chemical species, the formation of extracellular traps and the formation of an enzyme that is important for oxidative burst are neutrophil-dependent (Abi Abdallah *et al*., 2012; Chtanova *et al*., 2008; Konishi *et al*.,1992). Natural killer cells are innate producers of interferon gamma (Gazzinelli, 1993).

1.3 Immune Genes

1.3.1 Toll-like receptors.

The immune system has two main components, innate immunity and adaptive immunity. These two components have the ability to select any invading microbial pathogens. Microbial pathogens are recognized by the immune system as non-self and this leads to activation of the immune system to clear them. Toll-like receptors are a group of receptor proteins that play a major role in the innate immune response. They have the ability to detect certain pathogen molecules due to their pathogen-associated molecular pattern. This process is called pattern recognition (Christmas, 2010). A study in 1990 confirmed that toll-like receptors are very important in innate immunity since mutations in the toll gene led the fruit fly *Drosophila melanogaster* to become more susceptible to infection (Lemaitre *et al*., 1996). Toll-like

receptors are type 1 membrane proteins that consist of a trans-membrane domain and external and internal domains. The external domain has small structural domains within it (Leulier & Lemaitre, 2008).

Toll-like receptors were identified as the main receptors that start host immunity against fungal infection in *Drosophila* at the end of the 20th century (Lemaitre *et al.*, 1996). After that, TLR4 was discovered to have a function in the expression of a gene that plays a role in inflammatory response(Medzhitov *et al*., 1997). Also, TLR 4 mutation in some mouse strains makes them not respond to LPS. TLR 4 is considered to be the first discovered mammalian TLR. Later, many proteins that are structurally similar to TLR4 were discovered and called toll-like receptors (Rock *et al.*, 1998). The TLR family consists of approximately 13 TLRs in mammalian species (Takeda *et al*., 2003). Both humans and mice share the same TLRs from TLR1 to TLR9. TLR 10 is functional in humans but not in mice. TLR 11 is functional in mice but has a stop codon in humans which responds in the absence of TLR 11 (Zhang, 2004).

After discovering the role of TLRs, TLRs were first called the Toll/IL-1 receptor (TIR) domain due to the strong similarity between the cytoplasmic part of TLRs and the IL-1 receptor family. However, there is no structural similarity between the extracellular parts of TLRs and IL-1. The extracellular part of TLRs is leucine-rich repeats (LRRs), while the extracellular part of IL-1 has immunoglobulin. IL-1 receptors possess an immunoglobulin-like domain, whereas TLRs reside in the extracellular domain (Poltorak, 1998) In the beginning, the role of TLR 4 was established, and subsequently the role of each individual TLR was discovered (Figure 1.4).

Toll-like receptors are a type 1 membrane protein that consists of a trans-membrane domain and external and internal domains. The external domain has small structural domains within it (Figure 1.1)(Leulier & Lemaitre, 2008). Pathogen molecules bind to the external domain.

This binding activates the internal domain (cytoplasmic), which leads to the activation of different types of protein. Those proteins include MYD88, TRAM, TRIF and MAL/TIRAP (Johnston, 2007). MYD88, TRAM, TRIF and MAL/TIRAP are five adaptor proteins that are involved in the signalling pathway (O'Neill & Bowie, 2007)**.** The binding between TLRs and MyD88, Mal/TIRAP, TRAM and TRIF mediates the signalling of host-microbial interaction. For example, TLR4 use two proteins, Mal/TIRAP-MyD88 and TRAM-TRIF, to initiate the MyD88-dependent pathway (Figure 1.1). (Choi *et al*., 2010).

Also, other types of protein are activated inside the cell by this process, which leads to signal transduction events as a response to a pathogen (Leulier & Lemaitre, 2008). Binding of toll-like receptors to foreign microbes leads to the activation of an innate immune response (Table1.1) and the production of inflammatory cytokines, such as interleukin-1, tumour necrosis factor-α and interleukin-6. Subsequently, an adaptive immune response is activated (Johnston, 2007). The TLRs are a family of genes and their exact numbers vary from species to species.

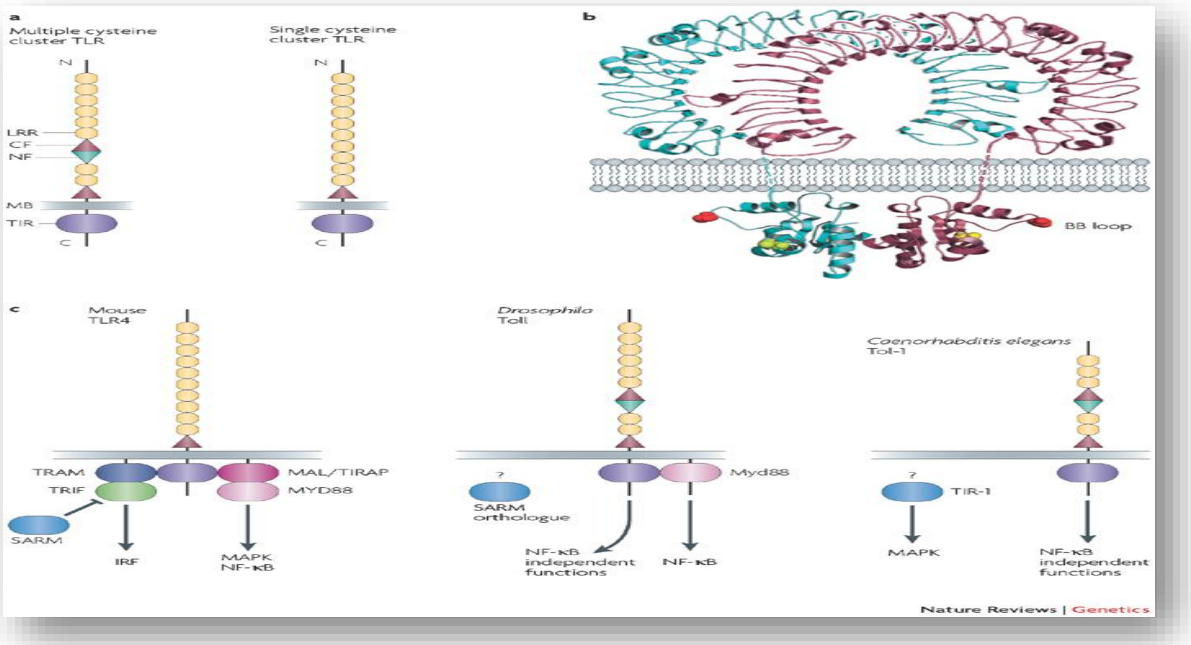

Figure 1.1. Toll-like receptor structure (Leulier & Lemaitre, 2008).

There are about 13 toll-like receptors in the mammalian immune system. The family of toll-like receptors consists of various numbers depending on the mammal. Humans have 10 toll-like receptors, TLR 1–10. Mice have 12 toll-like receptors, TLR1–9 and TLR11–13. Each toll-like receptor has a specific ligand, location and outcome (Figure 1.2) (Takeda & Akira, 2005). TLR 11 is expressed in mice and has a pseudogene in humans (Roach *et al*., 2005). TLR11 is a receptor that is located intracellularly and resides in the endoplasmic reticulum (Pifer *et al.*, 2011). TLR11 plays a key role in the detection of *Toxoplasma gondii* (Yarovinsky, 2005). *Toxoplasma gondii* profilin-like protein (TgPRF) has been identified as an activator of the innate immune response. This protein is detected by TLR 11 and thus leads to initiation of the production of cytokine interleukin-12 by dendritic cells (DC), which have a protective effect against Toxoplasma infection (Yarovinsky, 2005). The activation of dendritic cells against a *Toxoplasma gondii* infection is regulated by the UNC93B1 protein. UNC93B1 comprises 12 membrane-spanning endoplasmic reticulum-resident proteins directly coupled to TLR11 *in vivo*. Ineffective UNC93B1 protein evokes the secretion of IL-12 by the dendritic cells and critically increases the sensibility to infection due to constraining the Th1 response (Pifer *et al.*, 2011).

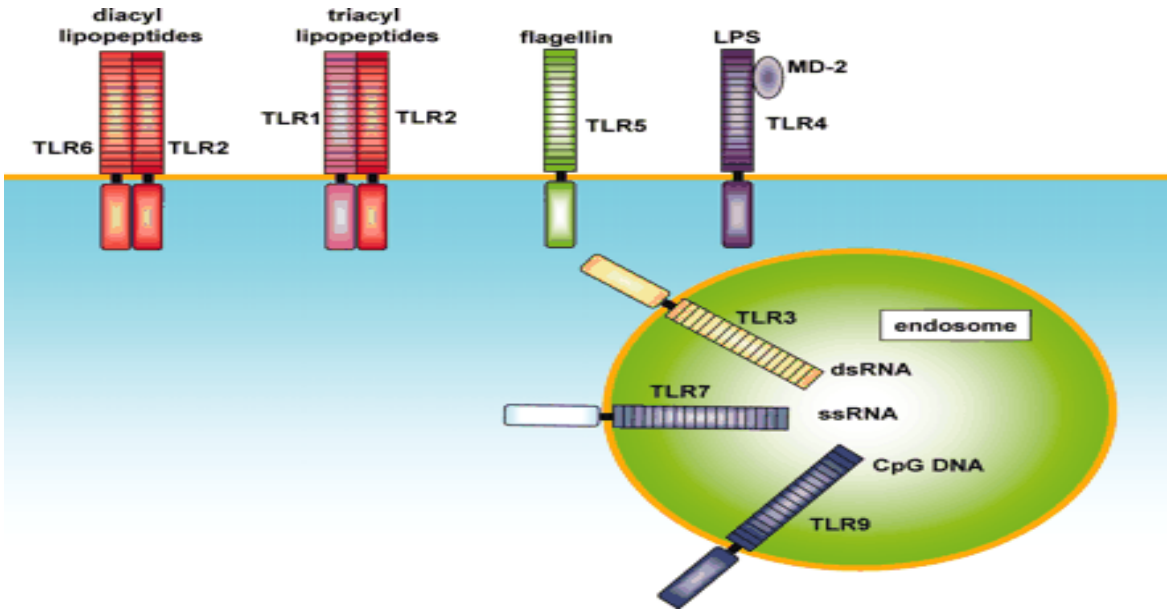

Figure 1.2. Specific legend of each member of the TLR family. Microbial lipopeptides are detected by TLR2, LPS is detected by TLR4, CpG DNA is recognized by TLR9, viral dsRNA is detected by TLR3, viral-derived ssRNA is recognized by TLR 7 and TLR 8, TLR5 recognizes flagellin (taken from Meylan et al., 2004).

Many studies have investigated TLR ligands. The first study found that non-methylated CpG DNA is a ligand for toll-like receptor 9, which is located in the endosome (Klinman, 2006). Another study on TLR 7 and TLR 8 found that single-strand RNA is their ligand and located this in the endosome, while a further study confirmed that double-strand RNA is a ligand for toll-like receptor 3 (Figure 1.2) (Pichlmair *et al*., 2011; Kato *et al*., 2008).

Table1.1. Some TLRs and their outcomes.

TLR	Outcome	Reference(s)
TLR 2	Th1 response and T cytotoxic	(Underhill *et al*., 1999) (Duthie *et al*., 2011)
TLR 3	Interferon production	(Kato *et al*., 2008)
TLR 4	Th1 response and T cytotoxic	(Duthie *et al*., 2011).
TLR 5	Th1 response and T cytotoxic	(Franchi *et al*., 2006).
TLR 7	Th1 response	(Hemmi *et al*., 2002)
TLR 8	Th1 response	(Philbin & Levy, 2007)
TLR 9	Th1 response and T cytotoxic	(Klinman, 2006)

Toll-like receptors play a very important role in pathogen detection and activation of an innate immune response in mammals and insects. In 2001, Medzhitov explained that toll-like receptors have the ability to detect specific microbial elements (Medzhitov, 2001).

A study carried out in China found that toll-like receptor 2 plays a crucial role in patient pulmonary tuberculosis susceptibility in some particular areas, such as Asia and Europe. It also found that toll-like receptor 1 increases the susceptibly to pulmonary tuberculosis in Africa and America. On the other hand, toll-like receptor 6 is responsible for decreased risk of the disease. That result was achieved via a meta-analysis (Zhang *et al*., 2013).

Toll-like receptor 1 plays an important role in the case of infection, e.g. *Yersinia enterocolitica*. It is responsible for cytokine CCL2 production. This kind of chemokine is very important in a *Yersinia enterocolitica* infection (Sugiura *et al*., 2013). Toll-like receptor 2 is very important in the case of acute immune complex-driven arthritis. A deficiency in TLR 2 causes severe symptoms and results in acute immune complex-driven arthritis. It also causes many other symptoms, such as joint, bone and cartilage diseases. The effect of TLR2 is expressed in managing FcγRson macrophages (Abdollahi-Roodsaz *et al*., 2013).

TLR 3 has the ability to detect the double-strand RNA of pathogens. The detection of dsRNA leads to the activation and production of different things such as NF-kappaB and type 1 interferon. An experiment that was done on mice confirmed that TLR3 is required to produce some kinds of cytokines (Alexopoulou *et al*., 2001). Central nervous system epithelial tissue has toll-like receptor 3. This receptor is very important and plays a vital role in herpes simplex virus1 (HSV-1) encephalitis in the central nervous system. On the other hand, it is not required in the defence against many pathogens (Zhang *et al*., 2007).

Plasmacytoid dendritic cells and B cells have the ability to detect many kinds of viruses, such as vesicular stomatitis virus and influenza virus. They have this ability because of toll-like receptor 7 that they express. TLR 7 catches viral single-strand RNA. This process results in the activation of co-stimulatory molecules and the production of cytokines. An experiment done on mice showed that mice deficient in TLR7 have a lower response to vesicular stomatitis virus (Lund *et al*., 2004).

Experiments on autoimmune antibody disorders have shown the involvement of toll-like receptors. For example, toll-like receptor 7 has a major impact on Systemic Lupus Erythematosus disease. Systemic Lupus Erythematosus is an autoimmune disease where the immune system attacks the body and causes damage. Scientists have observed some reductions in chromatin, Sm and rheumatoid factor autoantibody titres in autoimmune prone mice. These kind of mice lack the toll-like receptor adaptor protein myeloid differentiation adaptor protein (MyD88) (Lau *et al*., 2005).

Toll-like receptor 8 is also involved in Systemic Lupus Erythematosus. TLR 8 affects the production of interferon 1 after the activation of an innate immune response (Vollmer *et al*., 2005).

Toll-like receptors have the ability to detect bacterial DNA because it has un-methylated CpG di-nucleotides. TLR 9 is responsible for detecting un-methylated CpG di-nucleotides of bacteria when they enter the body. An experiment found that mice with no toll-like receptor 9

had no cell response in the presence of un-methylated CpG di-nucleotides. (Hemmi *et al*., 2002). Also, TLR9 has the ability to regulate natural interferon-producing cells (IPC) that are responsible for the detection of some viruses and the production of type 1 interferon and interleukin 12. For instance, TLR 9 plays an important role in the detection and clearance of murine cytomegalovirus (Krug *et al*., 2004).

The susceptibility to parasitic infection varies among individuals. This might be due to genetic diversity. Several mechanisms control host resistance, such as negative frequency-dependent selection, over dominance or temporal and spatial variation in the composition of the parasite community.

Genetic diversity in host resistance can be maintained by several non-mutually exclusive mechanisms (Hedrick, 2002;Apanius *et al.*, 1997;Woolhouse *et al.*, 2002) . Due to their intimate interactions with parasites, the genes involved in parasite recognition, rather than immune signaling or effector genes, are predicted to be the main targets of parasite-mediated directional selection (Tiffin & Moeller, 2006;Sackton *et al.*, 2007;Turner *et al.*, 2012)

Various approaches in biomedical research have been used to identify immune genes that have the fundamental function of pathogen defense. For example, gene knockout in mice to investigate the susceptibility of mice to some laboratory pathogens after impairing the function of certain genes (Li, Corraliza & Langhorne, 1999;Alexopoulou *et al.*, 2002;Cooke & Hill, 2001).

Whereas such an approach can provide important insights into parasite infection and host defense pathways, it is agnostic about the level of naturally occurring polymorphisms (laboratory mice are usually highly inbred) or the role of genes identified in coevolutionary processes in natural populations. Indeed, parasite-mediated selection can only act if there is variation in host defense traits. Thus, it remains generally unclear if candidate genes for

parasite resistance identified in a laboratory setting mediate variation in host resistance under ecologically meaningful conditions, especially in non-human vertebrates.

Also, there is accumulating evidence that environmental factors play a crucial role in shaping immune system functioning, and thus the ways in which hosts interact with their parasites(Abolins *et al.*, 2011;Boysen, Eide & Storset, 2011). Therefore, we may gain important insights into the evolution and function of the vertebrate immune system by studying patterns of infection in natural host-parasite systems (Pedersen & Babayan, 2011;Maizels & Nussey, 2013;Turner & Paterson, 2013) This could prove particularly fruitful for our understanding of defense strategies against zoonotic diseases, given the long co-evolutionary history of wildlife with disease-causing agents.

TLRs are a group of proteins that play an important role in pathogen recognition. They can detect a part of pathogens called pathogen-associated molecular patterns (PAMPs) either extracellularly or inside the endosome. After they interact with PAMPs, they send signals to start an immune response and antimicrobial action (West *et al.*, 2006).TLR 11 plays an important role in the detection of *Toxoplasma gondii* profilin (TPRF)(Yarovinsky, 2005). Recent studies confirm that TLR 11 also plays an important role in the detection of flagellin (FliC) in some bacteria such as *Salmonella* and *Typhimurium* (Mathur *et al.*, 2012).

TLR polymorphism is found to have a major effect in the susceptibility to different parasites in humans and some domestic animals (e.g. Brightbill, 1999;Texereau *et al.*, 2005;Garantziotis *et al.*, 2008) .

TLR12 is another of the TLR receptor proteins that plays an important role in the immune system. TLR 12 also detects profilin protein and induces an innate immune response reaction (Yarovinsky, 2005;Kucera *et al.*, 2010). Profilin is required for motility, host-cell invasion and virulence for the parasite (Plattner *et al.*, 2008). Two major roles have been identified for TLR12 against toxoplasma infection. The first role is the activation of TLR12 after exposure

to the TgPRF of *Toxoplasma gondii* that then activates plasmacytoid dendritic cells (PDCs) (Koblansky *et al.*, 2013), while the second function is to form heterodimers with TLR11 (Andrade *et al.*, 2013; Raetz *et al.*, 2013). Experiments on laboratory mice show a higher mortality rate among mice deficient in TLR12 than mice deficient in TLR11, when mice are in the acute phase of infection (Koblansky *et al*., 2013). TLR12 exists in the mouse genome but not in *the human* genome(Roach *et al*., 2005).

1.3.2 NLRP1 gene

NLRP1b is the scientific name of a gene in the NOD-like receptor (NLR) molecule family (Wagner, Proell, Kufer & Schwarzenbacher, 2009). This family is characterized by the presence of the NACHT and nucleotide binding domains (NBD) and Leucine rich repeats (Ting *et al.*, 2008). The main function of the NLRP1 gene is to synthesize key proteins in the nucleotide-binding domain that are leucine-rich and contain NLR proteins. This protein is involved in initial immune response activation to regulate inflammation by stimulating the grouping of inflammasomes that initiate an immune response against pathogens such as parasites and bacteria (Wagner, Proell, Kufer & Schwarzenbacher, 2009). Also, it plays an important role in apoptosis (Bruey *et al*., 2007; Fernandes-Alnemri *et al*., 2007; Franchi *et al*., 2006). Recent studies show that an increase in congenital toxoplasmosis is related to NLRRP1 polymorphism (Dinarello *et al*., 1998).

T. gondii is considered to be one of the most successful intracellular parasites that exist inside the host cell for a long period of time, due to many secretions by the parasite in the host cytosol. The function of those secretions is to cut the host cell signals, such as ROP16, ROP 5 and ROP 18, and dense granule proteins. ROP 16 stops host molecules such as JAK kinases, and phosphorylating STAT-3 and STAT-6. ROP 5 and 18 stop GTPases and inhibits their ability to attack the parasitophorous vacuole membrane. Dense granules interrupt some of the

host cell functions by altering gene expression (Ewald *et al*., 2014; Ong *et al*., 2010; Behnke *et al*., 2012; Fleckenstein *et al*., 2012; Bougdour *et al*., 2013).

Inflammasomes work as sensors for all microbial and foreign pathogens which have the ability to wipe out cell cytosol as a sign of infection. Those inflammasome belong to the NLRP and PYRIN/HIN families. This protein families contain domain which are responsible about cell death and their known function of signaling specially during the assembly of the inflammasome (Chu *et al.*, 2015). The direct interaction between those sensors and caspase1 and 11, via the linker ASC, leads to the accumulation of those sensors and subsequently the secretion of IL-1 cytokines, a pyroptotic cell death. IL-1 and pyroptotic cell death employs other types of cell to inhibit parasite replication (Martinon, 2002).

Mice and rats vary in their reactions to *Toxoplasma gondii* infection. The mice group is susceptible to infection. The rat group has the ability to fight infection and protect itself. For example, Lewis rats restrict infection while BN (Brown Norway) rats are more permissive. *Toxo1* locus is found to be a responsible for resistance of rats to Toxoplasmosis. *Toxo1* locus is a single 1.7-centimorgan region located at chromosome 10 (Cavailles *et al*., 2006). *Toxo 1* locus is thought to be controlled by the NLRP1 gene at chromosome 10. Also, the same gene controls the sensitivity to anthrax lethal factor susceptibility (Newman *et al*., 2010). Susceptibility to congenital toxoplasmosis was found to be related to single nucleotide polymorphisms (SNPs) in NLRP1 and the NLRP3 inflammasome activator *p2x7r* (Witola *et al*., 2011).

An experiment by Ewald and colleagues (2014) found that bone-marrow derived macrophage (BMDM) in response to a *T. gondii* infection does not require a lethal factor proteolysis site. This was discovered in 129 mice that had alleles of NLRP1b and this was enough for the activation of a macrophage response. Also, the N terminal activation mechanism, which is

known to date, was absent during a *T. gondii* infection. Therefore, NLRP1b is considered to be the main sensor of an innate immune response to *Toxoplasma gondii* (Ewald *et al*., 2014).

Another study was carried out by Gorfu and his team (2014) on inbred mice, they found that NLRP1 is the main gene responsible for the control of parasite proliferation and IL-1 and IL-18 processing. Also, *Toxoplasma gondii* is the main activator of the NLRP1 sensor in rat macrophage. They confirmed that knocking out the NLRP1 gene in sensitive macrophages resulted in the increased replication of parasites, and over-expression of the NLRP1 gene in sensitive macrophages made them resistant (Gorfu *et al*., 2014).

In China, polymorphisms of NLRP1 gene were proven to have influences on gene transcription which is a risk factor for rheumatoid arthritis (Sui *et al.*, 2012).

NLRP1 gene polymorphism was investigated and six SNPs in the NLRP1 region were discovered. However, none of the SNPs were associated with disease susceptibility or the ocular, neurological, and dermatological manifestations of Koyanagi-Harada Disease which is an auto immune disease(Horie *et al.*, 2011).

1.4 Justification for testing laboratory hypothesis of stuyding immune genes in the field mice.

TLRs have an essential role for pathogen detection (Tschirren *et al.*, 2013). Laboratory studies have suggested that TLR 11 and 12 have importance in detecting *Toxoplasma gondii* (Morger *et al.*, 2014). No association between TLR 11 and 12 haplotypes and genotypes with susceptibility and resistance to *Toxoplasma gondii* was found; however, the study found that TLR 11 and 12 were polymorphic despite the fact that TLR 11 and 12 are very important in recognition of *T.gondii* (Morger *et al.*, 2014). These mice were also infected with 6 species of helminth parasite which provided a topical opportunity to investigate TLR 11 and 12 polymorphisms in relation to these parasites.

Several studies investigated the role of NLRP1 gene role and revealed the importance of this gene in during parasitic infection and laboratory studies suggest that NLRP1 is activated during the *T.gondii* infection independent of the lethal factor (Gorfu *et al.,* 2014; Ewalds *et al.,* 2014; Cavailles *et al*., 2006; Martinon, 2002). The NLRP1b gene has not previously characterized in the *Apodemus sylvaticus*. Therefore, the availability of the 126 wood mice samples that were used previously provided a topical opportunity to investigate the NLRP1b gene and study the polymorphisms in relation to these parasites.

1.5 Parasites of Rodent

Rodents are animals that have the ability to transfer many kinds of parasites, bacteria and viruses to humans and other animals 9 (Table1.2). Rodents are considered to be a major pathogen carrier around the world. Therefore, many studies have been conducted to study the role of rodents in disease transmission.

Table 1.2. Examples of different pathogens that are transferred by rats in different parts of the world.

Pathogen	Rodent species	Country	Reference
Toxoplasma gondii	*R. norvegicus*	UK	Webster, 1994
Toxoplasma gondii	*R. norvegicus*	USA	Smith *et al*., 1992
Toxoplasma gondii	*R. rattus*	Netherlands	Kijlstra *et al*., 2008
Listeria spp.	*R. norvegicus*	UK	Webster *et al*., 1995
Cryptosporidium parvum	*R. norvegicus*	UK	Webster and MacDonald, 1995
Salmonella spp.	*M. musculus domesticus*	UK	Pocock *et al*., 2001
Salmonella enteritidis	Mice	UK	Davies and Wray, 1995

Rodents play a key role in the transfer of zoonotic parasites such as *Toxoplasma gondii*. Since cats are a definitive host of this parasite, rodents are considered to be the main source of transmission to cats. A study of 523 samples of wild rodents was conducted using different detection methods, such as an Indirect Fluorescent Antibody Test (IFAT) and a Latex Agglutination Test (LAT), 17% of them were seropositive (Dabritz *et al*., 2008). Also,

rodents play a major role in the transmission of *T. gondii* to pigs. RT PCR was used to determine the prevalence of the parasite in infected hearts and brain from rodents and shrews. The prevalence of *T. gondii* was as follows: 10.3% in *Rattus norvegicus*, 6.5% in *Mus musculus*, 14.3% in *Apodemus sylvaticus* and 13.6% in *Crocidura russula*. The sero-prevalence percentage amongst slaughtered pigs was 8–17%, and this decreased to 0–10% after they controlled rodents (Weigel *et al*., 1995; Kijlstra *et al*., 2008).

Toxoplasma gondii has an effect on rodent behaviour. It changes rodents' behaviour to increase transmission of the parasite. A study showed that *T. gondii* makes rodents more attracted to cat urine while normally they are averse to it (Berdoy *et al*., 2000). Interestingly, *T. gondii* can change rodent behaviour only for cat pheromones and not for other predator and non-predator animals (Lamberton *et al*., 2008; Vyas *et al*., 2007). A study conducted to investigate congenital transmission in mice showed that two species of mice, *Mus musculus* and *Apodemus sylvaticus*, delivered more than 80% of infected pups (Owen *et al*., 1998).

Congenital transmission in some species, such as BALB/c mice and *Rattus*, mostly occurs when the animal is infected during pregnancy, while a chronic infection is less likely to cause congenital transmission (Freyre *et al*., 2006). Mice showed 75% of vertical transmission in a natural population (Hide *et al*., 2009).

1.5.1 *Apodemus sylvaticus*

Apodemus sylvaticus, or the wood mouse, is the most common mouse in Great Britain. They are also called long-tail mice due to their long tails. The tail size is usually the same as the head and body combined. They are characterized by a grey belly and a brown fur head and can be distinguished from the very similar yellow-necked mice in lacking a yellow collar around their neck. The head and body size is usually around 81–103 mm, and the tail size 71–95 mm. Their weight is around 13–27 g. They are mostly nocturnal, but young males and females sometimes appear during the day. Their food includes fungi seeds, fruit, nuts,

seedlings, invertebrates and moss. They reproduce from March or April until October andthey live for 18–20 months.

Seasonal variation is known about *Apodemus sylvaticus*. Their number is higher in the winter andthen the number starts to decline in the spring until it reaches to a low stable number in the summer (Flowerdew, 1974). In arable farmland eco system in UK, the reproduction of the wood mice usually takes place in the winter. But there is a certain time in the winter which the reproduction is absent. Male *Apodemus sylvaticus* has a very fast growth during the sexual development maturity time which corresponds to the spring season. Both males and females gain extra weight during the breeding seasons. Some factors affect the juvenile mice survival such as food supply and adult density in the area. Endosperms of wheat and grain seed are considered as one of the most parts of wood mice food in autumn and early winter (Green, 1979).

Mice are considered to play a major role in *T. gondii* transmission, since they are potential prey for cats (Tenter *et al*., 2000). Both serological and PCR studies have been applied to check the prevalence of *T. gondii* in mice. Serological studies found low prevalence, but PCR studies found high prevalence (Franti *et al*., 1976; Jackson *et al*., 1986; Marshall *et al*., 2004; Zhang *et al*., 2004). Beverley explains that serological studies might not be effective for studying *T. gondii* in mice, especially in chronic infection cases (Beverley, 1959). A study carried out on *Apodemus sylvaticus* in the UK showed relatively high prevalence. A total of 206 DNA samples extracted from the brains of *Apodemus sylvaticus* were tested. The prevalence of *T. gondii* was 40.78% (Thomasson *et al*., 2011). The samples used in this study were collected from a low cat-density area (2 cats per square km.) based on this paper (Thomasson *et al*., 2011). Nonetheless, the prevalence of the parasite was very high despite the low number of cats. This might be explained by congenital transmission of the parasite from mother to foetus. This was confirmed by investigating an infection of pregnant mice, showing that congenital transmission was indeed occurring (Thomasson *et al*., 2011).

A study carried out by Bajnok investigated a host genotype and spatial location in relation to *T. gondii* infection. A total of 126 samples (*Apodemus sylvaticus*) were collected from four locations. PCR markers, SAG1, SAG2, SAG3 and GRA6, were used to detect *T. gondii* in infected tissue. They found 44 positive samples, giving an infection rate of 34.92%. Significant differences in *T. gondii* prevalence were found in different genotypically derived mouse populations, but not between geographically defined populations (Bajnok *et al*., 2015).

Apodemus sylvaticus mice are known to be infected by different parasites. For example

Nine species of helminths were discovered among 134 wood mice in southern UK which includes five different nematodes which are *Heligmosomoides polygyrus, Syphacea stroma, Pelodera strongyloides, Trichuris muris, Capillaria murissylvatici* and two cestodes; *Microsomacanthus crenata* and *Taenia taeniaeformis*. These mice were also infected by two trematodes which are *Corrigia vitta* and *Brachylaemus recurvum*. 91.8% of this population were infected by more than one helminth parasite (Behnke *et al.*, 1999). Furthermore, *Plagiorchis elegans* was studied by Boyce and a group of researchers at Malham Tarn. They found high prevalence (25%) of this trematode species and mouse age, as well as interestingly, mouse sex, appeared to associate with the *P. elegans* infections (Boyce *et al.*, 2014).

Also, wood mice can host bacteria known to be zoonotic. *Apodemus sylvaticus* was found to be reservoir for *Borrelia burgdorferi* in The Netherlands since 47 % of the tested mice were infected (De Boer *et al.*, 1993). Furthermore, *Borrelia lusitaniae* was reported among 196 wood mice in Portugal (de Carvalho *et al.*, 2010).

Viruses also transmitted by the wood mice. Serology and PCR analysis revealed that MHV-4 herpesvirus 4 is endemic in wood mice in the UK (Blasdell *et al.*, 2003).

1.6 Toxoplasmosis.

Toxoplasmosis is a disease caused by a unicellular parasite, *Toxoplasma gondii*. Millions of people have this parasite in their blood but only some of them show symptoms of the disease. This might be due to the efficiency of some people's immune system, which protects them from this disease. This disease, though, may cause serious symptoms in some cases, such as pregnant women and immune-deficient patients (Centers for Disease Control and Prevention, 2017).

1.6.1 *Toxoplasma gondii*

Toxoplasma gondii is an obligate intracellular parasite that belongs to the Phylum Apicomplexa. It has a complex life cycle with three stages: sporozoites, tachyzoites and bradyzoites (Dubey *et al*., 1998). *Toxoplasma gondii* was first discovered in 1908 by Nicolle and Manceaux in a rodent in the United States of America and *Ctenodactylus gondii* in Brazil (Ferguson *et al*. 2005; Ferguson, 2009). Since then, this parasite has been seen as a common infection in many warm-blooded animals and humans. Later, in 1920, scientists discovered a clinical effect of this parasite in children who were born with hydrocephalus, retinochoroiditis and encephalitis. In 1980 it was discovered that *Toxoplasma gondii* is a major cause of fatal encephalitis in AIDS patients (Luft & Remington, 1992).

1.6.2 Life Cycle

Toxoplasma gondii is an obligate intracellular parasite that has a complex life cycle (Figure1.3). This includes sexual and asexual cycles (Dubey, 2004). The sexual cycle only occurs in domestic and wild cats (Dubey *et al*., 1998; Dubey, 2009). The parasite can be transmitted by vertical or horizontal transmission (Hutchison, 1965; Frenkel, 1973). The life cycle starts after the ingestion of a cyst by a cat, which is a definitive host. Five sexual stages of the parasite are seen in the cat before the formation of gametocytes. Two to 15 days after infection, gametocytes are found in the small intestine with high numbers in the ileum. In the

small intestine, a microgamete fertilizes a macrogamete and forms a fertilized oocyst inside an enterocyst. Then, the enterocyst wall ruptures and the oocyst is released in cat faeces. The period between ingestion of the cyst and release of the oocyst is around 7 to 21 days (Dubey and Frenkel, 1972). The oocyst becomes sporulated after 1 to 5 days of shedding and divides into two sporocysts, each with 4 sporozoites. The sporocyst remains highly infectious for a long period of time (Dubey *et al*., 1998, Dubey, 2009). A sexual cycle also occurs in cats when bradyzoites penetrate the lamina propria and divide as tachyzoites. These tachyzoites spread out through the body of the cat and encyst. An intermediate host ingests the oocyst from contaminated food or water. Then, the parasites differentiate into tachyzoites that spread through the body and rapidly divide by endodyogeny. The tachyzoites invade macrophages and lymphocytes and stay free in plasma. This stage of infection is called the acute phase. Tachyzoites may transform into another stage, bradyzoites, and form tissue cysts. Tissue cysts are present in the muscles of the intermediate host and are highly infectious (Sibley, 2011, Dubey, 1997, Dubey *et al*., 1998; Dubey & Frenkel, 1976).

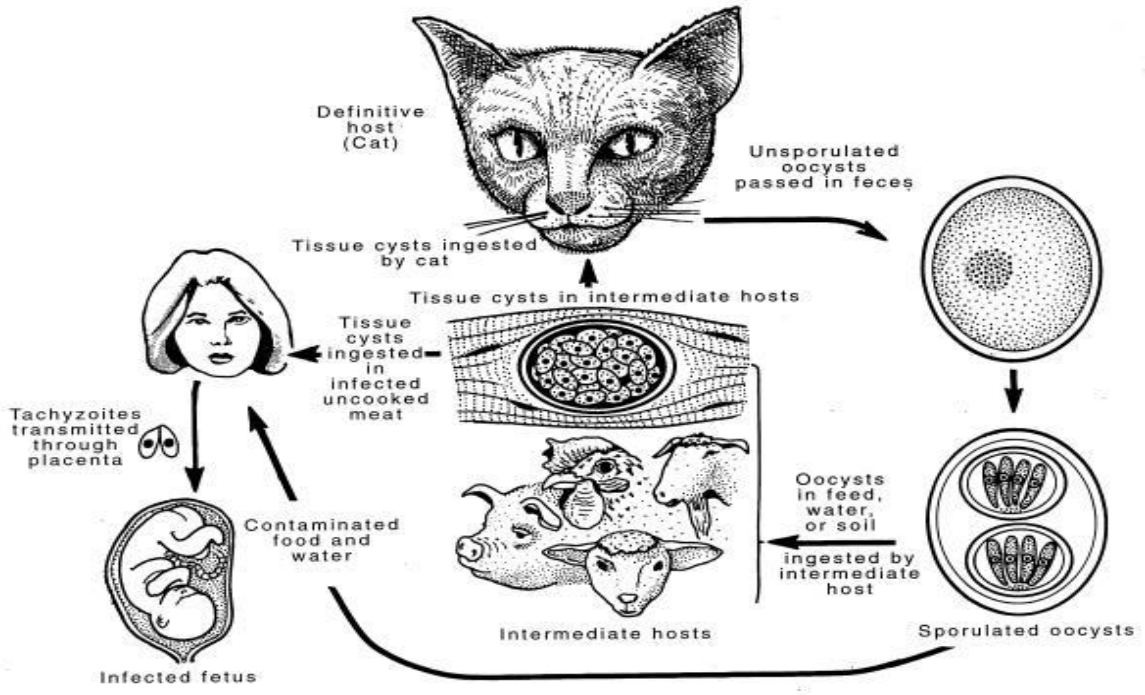

Figure 1.3. *Toxoplasma gondii* Life Cycle (taken from Dubey et al., 1998).

1.6.3 Transmission.

There are three forms of the infectious stages of *T. gondii*, i.e. tachyzoites, bradyzoites and sporozoites inside the oocyst (Figure1.4). Tachyzoites cause an acute infection while bradyzoites cause a chronic infection, and both of them reproduce asexually. The oocyst remains infectious in the environment and reproduces sexually inside the cat (Dubey *et al*., 1998; Sibley *et al*., 2009). There are different modes of transmission for *Toxoplasma gondii.* The first one is ingestion of a tissue cyst from contaminated food or water (Hill & Dubey, 2002). A cat can get a cyst if it ingests an infected rodent or an animal that has a cyst in its brain or tissues; and after that, a sexual cycle occurs in the cat to produce oocysts (Dubey *et al*., 1970). A human can get a cyst from eating undercooked meat, e.g. infected pork or beef (Dubey *et al*., 2005; Hill *et al*., 2010).

Cats shed up to 10 million oocysts per day. This might last for around two weeks after infection. Following shedding, an oocyst takes around five days to become infectious or may stay uninfectious for around a year in warm soil (Dubey *et al*., 1998). Humans and animals can easily be infected from ingesting fruit and vegetables contaminated with oocysts (Dubey,

2010; Pereira and Franco *et al*., 2010). Oocysts are found to cause more severe infections in humans than cysts (Dubey, 2010). Oocysts are highly resistant to environmental factors and can survive for up to six months in seawater (Lindsay & Dubey, 2009).

The second mode of transmission is congenital transmission from mother to child (Hill & Dubey, 2002). This happens when the mother has acute toxoplasmosis and tachyzoites circulate in her blood. The tachyzoites are able to cross the mother's placenta and cause a serious infection in the foetus (Jones *et al*., 2003; Montoya & Remington, 2008). The severity of the infection and the frequency of transmission depend on the stage of the pregnancy. Transmission occurs less during the first trimester (<20%) but high transmission (>80%) occurs during the late stages of pregnancy (Jones *et al*., 2003; Ortiz-Alegria *et al*., 2010). Transmission during the first trimester can cause a severe infection, with clinical symptoms such as mental retardation and abortion, while transmission in the last trimester causes less severe infections, which might be asymptomatic or present as chorioretinitis (Montoya and Liesenfeld, 2004). Studies show that the vertical transmission rate from mother to child in humans is about 9.9 % (Haq *et al*., 2016).Other studies carried out on sheep and rodents found that sheep have a 65% vertical transmission (Williams *et al*., 2005). while rate and rodents around 75% (Marshall *et al* 2004; Hide *et al*., 2009) when measured using PCR.

Also, tachyzoites may be transmitted during organ transplantation and blood transfusion. It is not clear which mode of transmission is more common than the other, this might depend on the culture and eating habits (Tenter *et al*., 2000).

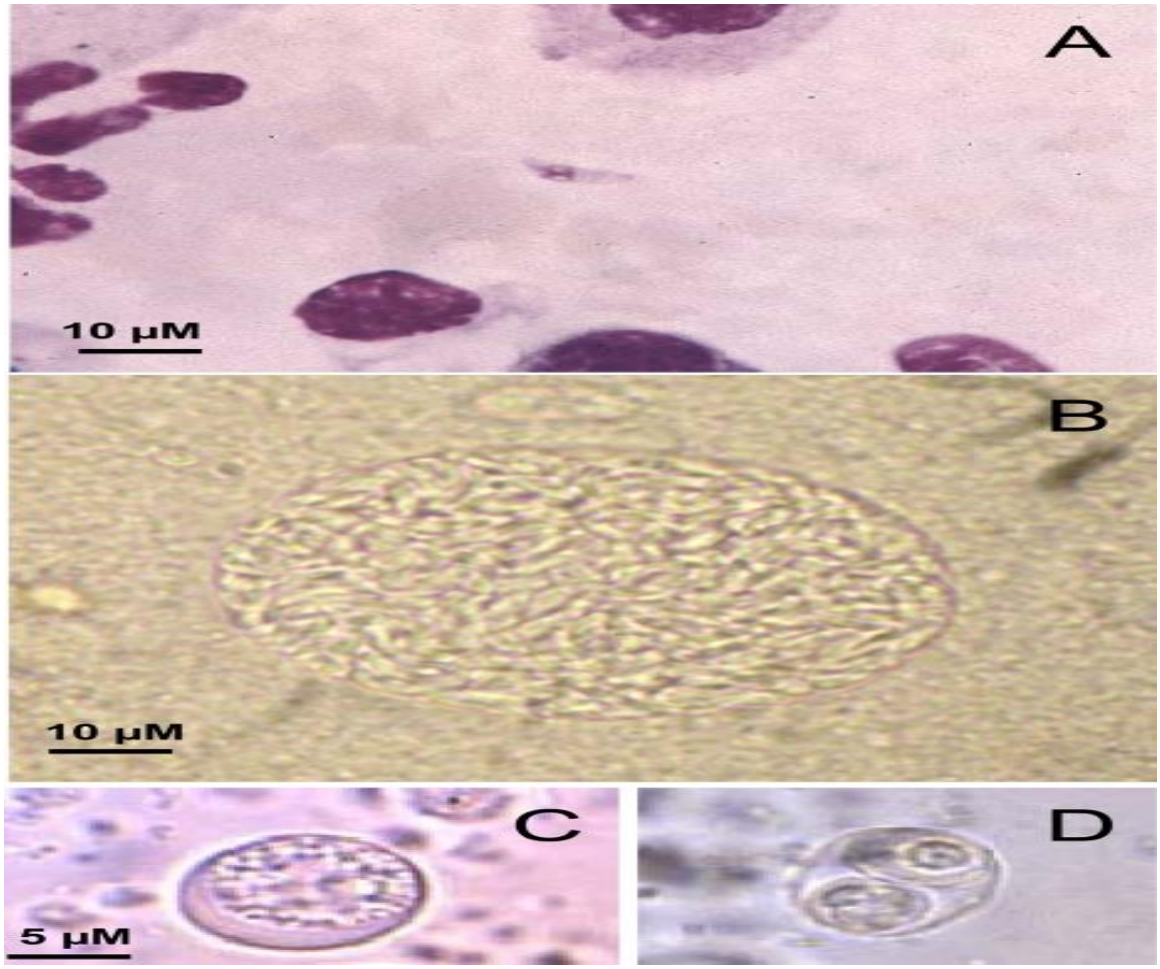

Figure 1.4. Different stages of *Toxoplasma gondii*. (A) tachyzoites, (B) cyst, (C) unsporulated and (D) sporulated oocyst (taken from Robert-Gangneux & Laure Dardé, 2012)

1.6.4 Disease

1.6.4.1 Asymptomatic Toxoplasmosis

Toxoplasma gondii infection is mostly asymptomatic in normal healthy people. The parasite may exist in a host body with no symptoms in their entire life. In some cases, the parasite is activated by factors such as AIDS or cancer and causes severe symptoms (Hoyen, 1990).

1.6.4.2 Symptomatic infection in immunocompetent host.

Despite the fact that *Toxoplasma gondii* infection is mostly asymptomatic in healthy people, a primary infection may cause muscle weakness fever and cervical or occipital lymphadenopathy for around 4 to 6 weeks (Ho-Yen, 2009).

1.6.4.3 Congenital Toxoplasmosis

Congenital transmission of *T. gondii* can lead to major problems in the foetus, such as hydrocephalus or microcephalus, cerebral calcification and retinochoroiditis (Table1.3), and it may result in abortion or stillbirth (Goldenberg and Thompson, 2003; Gibbs, 2002). During pregnancy, a newly infected mother can pass the infection to her foetus while previously infected women have strong immunity to the parasite which may prevent reinfection. The severity of the symptoms depends on the time of becoming infected. Having an infection during the first trimester of pregnancy is believed to be more serious, with harsher symptoms but less congenital transmission. Infection during the third trimester has high congenital transmission (Wong and Remington, 1994). A high percentage of newborn babies do not have any physical symptoms; however, they may have chorioretinitis, neurological damage or delayed growth as they get older (Zhou *et al*., 2011).

Table1.3. The prevalence of *T. gondii* infection in disabled children in China (Zhou *et al*., 2011).

Children's symptoms	***T. gondii* prevalence**
Hypophrenia	21.7%
Epilepsy	20.0%
Retinochoroiditis	26.1%
Cardiovascular defects	25.0%
Respiratory system defects	14.3%

1.6.4.4 Toxoplasmosis in Immunocompromised Patients

Toxoplasmosis can be very harmful in an immunocompromised patient due to the reactivation of a chronic infection. If the immune system is damaged by an immune disease, the parasite becomes active (Araujo, 2010) (Table1.4). Studies have found a high prevalence of the parasite among immunocompromised individuals in different countries.

Table 1.4. The prevalence of *T. gondii* in different cancer patients in China (Zhou *et al*., 2011)

Cancer type	**Prevalence of *T. gondii***
Rectal cancer	63.6%
Nasopharyngeal cancer	46.2%
Breast cancer	78.9%
Hepatic cancer	77.8%
Gastric cancer	61.1%
Leukaemia	15.1%
Refractory leukaemia	35.0%

There is a high prevalence (30%) of *T. gondii* parasite in immunocompromised patients in Egypt (Baiomy *et al*., 2010). Also, studies in Korea have found similar results to those in Egypt (Shin, 2009). Toxoplasmosis in immunocompromised patients mostly affects the central nervous system. Infection may lead to encephalitis, chorioretinitis, pneumonitis, acute respiratory failure and haemodynamic abnormalities (Ho-Yen, 2009; Luft and Remington, 1992). Another study reported that 26% of AIDS patients have *T. gondii* infection in China (Zhou *et al*., 2011).

1.6.4.5 Ocular Toxoplasmosis

This disease occurs after 30% of the middle layer of the eye becomes inflamed in individuals in the Western world (uveitis). Ocular lesions may be present in both acute and chronic phases of a toxoplasma infection. Seventy per cent of ocular scar formation is suggested as being due to congenital transmission. Congenital ocular toxoplasmosis may lead to several symptoms, such as malformation, with anophthalmus, chorioretinal, congenital cataract, optic neuritis, strabismus, amblyopia and congenital aniridia. The most common symptoms of ocular toxoplasmosis found in China were central exudative chorioretinitis and uveitis (Zhou *et al*., 2011). Ocular lesion results from contracting an infection after birth and are due to an immune response that leads to tissue destruction (Gaddi, 2007). *Toxoplasma gondii* is found to be present in about 38% of ophthalmology cases in China (Zhou *et al*., 2011).

1.6.4.6 Schizophrenia and Toxoplasmosis.

Many studies have investigated the presence of *T. gondii* in some psychosis patients. These studies suggest that *T. gondii* might be present as a major pathogen. Practically, *T. gondii* parasites are found to have an effect that leads to an increase in dopamine levels in the mouse brain, which plays an important role in some cases such as bipolar disorder and schizophrenia (Huber *et al*., 2007; Skallova, 2006). Schizophrenic patients in China are found to have 1% to 28.7% sero-prevalence of *T. gondii* infection (Xiao *et al*., 2010). Sixty-seven children with schizophrenia and aged from 6–17 years old were tested for *Toxoplasma*-IgG and 85.7% of them were positive (Zhou *et al*., 2011).

1.7 Helminths

In this study, a population of mice were used to investigate Toxoplasma infection in relation to immune gene variation. Additionally, data was available for other helminth parasites, including two trematodes and 4 nematodes and hence these parasites were also included in the study. The following text provides background information on these other parasites.

Helminth parasites are a group of elongated, flat or round invertebrate worms. Medically, they are divided into two groups. The first group is the platyhelminths, or flat worms, which includes flukes and tapeworms. The second group is the nematodes, or roundworms.

1.7.1 Trematodes

Trematodes comprise about 18,000 species of flat worm (Greiman *et al.*, 2013). Their life cycle is complicated with three groups of hosts involved in it. Intermediate hosts are normally gastropods (mollusks) but they can also be found in several animals such as vertebrates and arthropods, and these animals are second intermediate hosts. The definitive host is normally all vertebrates. Laboratory work on trematodes requires the cultivation of trematodes themselves, gastropods and all other hosts due to their complicated life cycle. Such work on

trematodes is very helpful for producing vaccines against trematode infections and in biochemical and diagnostic studies (Greani *et al*., 2014;Mwangi *et al*., 2014).

1.7.1.1 *Plagiorchis elegans*

Plagiorchis elegans is related to the plagiorchiid digeneans found across Europe and in some regions of North America (Rudolphi, 1802). As a trematode, it is has a huge range of definitive mammalian hosts and also some reptiles (Ndiaye *et al*, 2013).

According to natural and experimental studies of *Plagiorchis elegans* by Styczynska-Jurewicz (1962), the life cycle starts with *Lymnaea stagnalis* which act as an intermediate host in nature. The second intermediate host is a *Culicidae*, and additional experimental laboratory work has shown that a definitive host is the white mouse (Bock, 1984; Styczyńska-Jurewicz,1962).

1.7.1.2 *Brachylaemus recurvum*

There are very limited studies on *Brachylaemus recurvum* (Loxton *et al*., 2016;Behnke *et al*., 1999; Boyce *et al*., 2013). The related *Brachylaemus erinacei* was shown to have 2 % prevalence in a study of 40 hedgehogs in Iran (Naem *et al*., 2015)

1.7.2 Nematodes

The phylum Nematoda is one of the most diverse phylums with an estimate of more than 10 million species around the world (Lambshead, 1993), many of which infect humans and other animal. Across the globe, 2.9 billion people are infected with parasitic nematodes according to WHO (Ranganathan *et al*., 2009). Due to the number of infections among humans around the world, especially in tropical areas, nematodes are considered to be the most common infectious parasite of humans; 120 million are infected with filariasis, 700 million with hookworm, 700 million with trichuriasis and 1200 million with ascariasis (Lustigman *et al*., 2012).

1.7.2.1 *Heligmosomoides polygyrus*

H. polygyrus, previously called *Nematospiroides dubius*, is a nematode parasite from the family Trichstrongylidae that infects the intestines of mouse populations. A *H. polygyrus* infection can last for a long period of time in mice, which makes the parasite a good model for chronic helminthiases.

Experimental studies show that 24 hours after ingestion of the L3 larvae of the parasite by mice, the larvae migrate to the small intestine of the mouse, penetrate the submucosa and transform into two different forms; after moulting, it goes back to the lumen as an adult parasite. In the lumen, the parasite feeds on the tissue of the small intestine (Bansemir & Sukhdeo, 1994). Two processes happen in the small intestine, mating and producing eggs that are shed in faeces. After that, the eggs hatch and transform twice to become L3 larvae (Figure 1.5) (Reynolds *et al*., 2012).

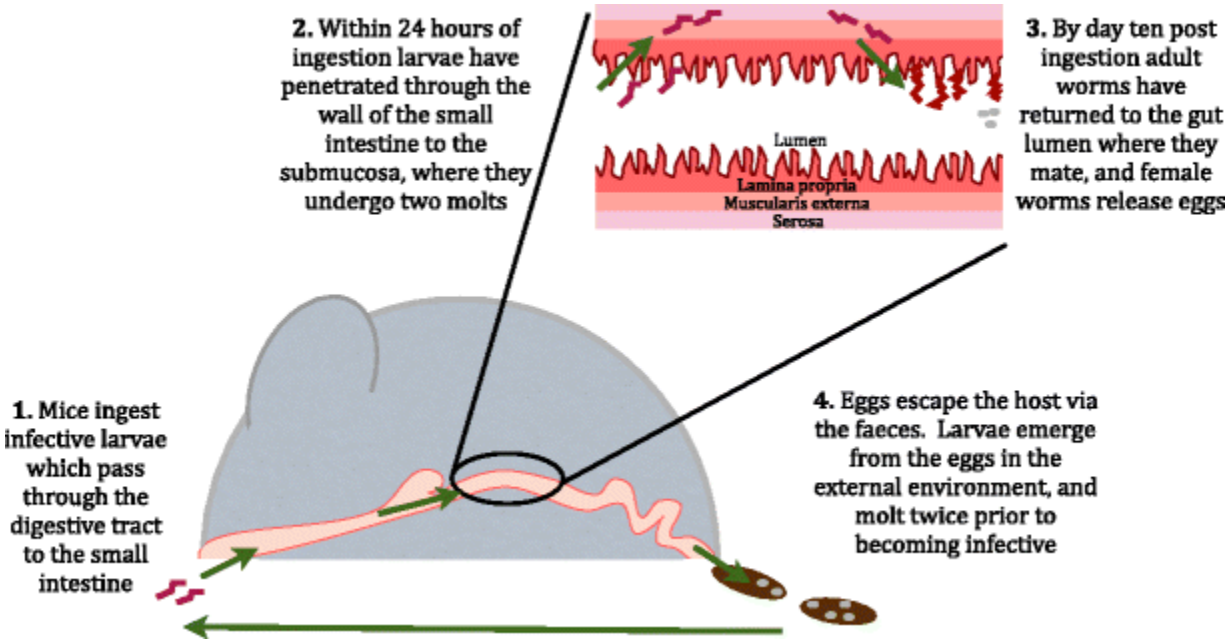

Figure 1.5 Life cycle of *Heligmosomoides polygyrus* in mice (taken from Reynolds et al., 2012).

Two ways can give an idea about the time *H. polygyrus* spends in a host. The first way is to count the number of eggs that are shed in the faeces of an infected animal. The second way is to count the number of adult parasites in the small intestine of an infected animal (Reynolds *et al*., 2012). Also, *H.polygyrus* excretory-secretory antigens (HES) can be measured after cultivation in the laboratory. After collection, the HSE can be identified (Hewitson *et al*., 2011). Thus is very helpful in vaccine production and immunological studies (Reynolds *et al*., 2012).

Laboratory mice show a variable immune response against *H. polygyrus* infection, from slow to rapid. For example, CBA, H3c and SL mouse strains show weak responses against infection. The Mesenteric Lymph (MLN) cell count is a very low number. Also, the mast cell number is low in the gut of mice (Lawrence & Pritchard, 1994) and there is low eosinophil levels in the white blood cells (Zhong & Dobson, 1996). A/J mice show a weak response and protective action is absent (Behnke *et al.*, 2003), whereas,C57BL/6, C57BL/10 and 129/J mice showed intermediate response. The immune response is slow but more rapid than in the previous group and there is low eosinophil levels in the blood circulation (Wakelin & Donachie, 1983). The third group, which includes DBA/2, BALB/c and NIH mice, has a fast response and lymphocyte, neutrophil and monocyte counts in white blood cells are higher than in the intermediate group (Ali *et al*., 1985). SJL and SWR mice also have a fast immune response. An antibody response is generated and fast HSE antigen recognition occurs and is measured using a western blot. Also, titres of a parasite-specific antibody are found in serum (Ben-Smith *et al.*, 1999).

1.7.2.2 *Syphacea stroma*

Syphacea species was first mentioned by Von Linstow in 1884 and he called the parasite *Oxyuris stroma.* This species was discovered in wood mice *(A.sylvaticus*) and was thought for a long time to be the same as *Syphacea obevtala,* which was widespread in mice and

humans. In 1915, Seurat suggested that *Oxyuris stroma* and *Syphacea obevtala* were the same species and subsequently, he referred to *O.stroma* as *S.obvetala*. No studies had investigated *S.obvetala* in *A.sylvaticus*. It is difficult to find any differences between these nematodes and Linstow concluded that the tail ends of both male parasites were the same. It is difficult to study the male tail ends of both parasites due to the rare appearance of the male. This seems to be due to the death of the male after fertilizing the female, which occurs before the female gets to full size. In1932, Morgan had a chance to study and investigate the males of both species from field mice. He found that some males had distinguishing characteristics which differentiate *S.obvetala* from *Mus musculus*. As the host was the same one that Linstow described, this species was therefore called *Syphacea stroma* (Morgan, 1932).

The length of the *S.stroma* female is about 3.45 to 3.95 mm, the width is about 0.05mm at the head and increases to 0.21mm in the vulva region, the width decreases to 0.08mm at the tail and the tail is very sharp. The length of the male is about 1.74 to 2.20 mm, the maximum width is about 0.15mm and the tail is coiled at the end (Morgan, 1932).

1.7.2.3 *Capillaria murissylvatici*

Capillaria murissylvatici is a nematode parasite that infects the small and large intestines of *Apodemus sylvaticus* (Lewis, 1967). *Capillaria murissylvatici* was reported among 134 *Apodemus sylvaticus* in the southern part of the UK collected in the September of four consecutive years, 1994–1997, along with other helminthic parasites such as *Heligmosomoides polygyrus*, *Syphacea stroma* and *Pelodera* (Behnke *et al.*, 1999).

1.7.2.4 *Pelodera strongyloides*

The nematode *Pelodera strongyloides* is a free-living parasite that lives its life in organic matter. The length of this parasite is about 1.0–1.5 mm. It has been reported in humans and in different animals, including dogs, horses cattle, sheep and guinea pigs (Yeruham & Perl, 2005). The infection leads to skin lesions that are generally polymorphic, with papules,

pustules, scaling and alopecia (Tanaka *et al.*, 2004). *Pelodera strongyloides* larvae invade the skin of mammals, whereupon they became third-stage larvae; however, but dermatitis rarely occurs. Organic soil is a natural habitat for *Pelodera strongyloides*. *Pelodera strongyloides* is a cause of damp straw bedding, erythema, alopecia, papulocrustous and skin lesions, and pruritus usually occurs due to direct contact with the habitat, which is organic soil (Saari & Nikander, 2006)

Pelodera strongyloides has been reported among bank voles (*Clethrionomys glareolus*) and wood mice (*Apodemus sylvaticus*) (Hominick & Aston, 1981).

In the south of the United Kingdom, *Pelodera strongyloides* was reported in wood mice, *Apodemus sylvaticus*, between 1994 and 1997, along with nine other helminthic parasites such as the nematodes, *Heligmosomoides polygyrus* and , *Syphacea stroma* and also, trematodes (Behnke *et al*., 1999).

1.8 Aim

The broad aim of this thesis is to investigate the relationship between the genetic variations in host immune genes within a natural population of animals in response to their infection with parasites. The overall hypothesis is that variation in host gene DNA sequences (and therefore the proteins they produce) influences the susceptibility of animals to infection by *Toxoplasma gondii* and helminth parasites.

Chapter 2

Materials and Methods

2. Materials and Methods

2.1. Sample Collection

Wood mice for the collection were trapped in the Malham Tarn woodland area in North Yorkshire using Longworth small mammal traps. This area is located in Northwest England at an altitude of 375m above sea level. Multiple studies were carried out in this area to investigate different parasites (Rogan *et al*., 2007; Behnke *et al*., 2009; Boyce *et al*.,2014). The collection was done by others as described previously (Boyce *et al*., 2013; Boyce *et al*., 2014;Bajnok *et al*., 2015). Ethical approval (College of Science and Technology Research Ethics Panel, University of Salford Reference, CST 12/36) was obtained for the collection by the previous authors, as described (Bajnok *et al*., 2015). The samples were obtained from four different sites (Tarn Wood 54°06′03.3″N, 002°09′44.9″W; Spigot Hill 54°05′72.9″N, 002°10′43.1″W; Ha Mire Plantation 54°05′64.5″N, 002°09′53.7″W; Tarn Fen 54°06′00.0″N, 002°10′43.4″W) between October 2009 and October 2011 within the border of Malham Tarn Nature Reserve in North Yorkshire, UK.

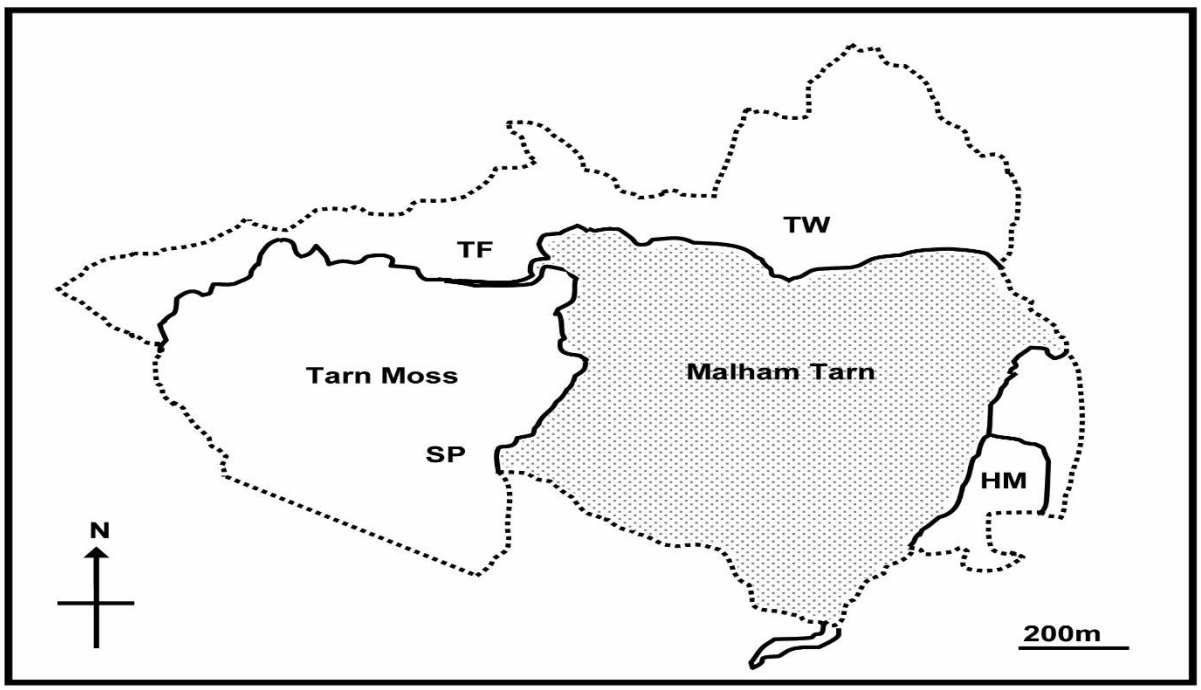

Figure 2.1. Map of Malham Tarn showing the 4 different collection locations: Ha Mire (HM), Tarn Wood (TW), Spiggot Hill (SP) and Tarn Fen (TF) (Taken from Boyce et al., 2014).

Permission was obtained from the National Trust (Boyce *et al.*, 2013;Boyce *et al*., 2012) and the Field Studies Council co-operated with the work as reflected in the co-authorship of some of their staff. In all cases, animal body weight and length was measured at the time of collection and age/maturity and sex status was determined. Body length was measured from the animal's nose to its anus. The mouse morphometric and infection data are summarised below (Tables 2.1 and 2.2).

Table 2.1. Summary of the capture details and morphometric data on the 80 wood mice studied in this thesis. Data derived from Boyce *et al*. (2014).

Number	Date	Location	Sex	length (cm)	weight (g)	age group
6	31.10.09	Ha Mire	M	8.5	19	Young adult
9	02.11.09	Ha Mire	M	7	15	Young adult
10	07.02.10	Tarn Wood	M	8.7	20	Adult
11	11.05.10	Tarn Wood	F	8.6	19	Young adult
12	12.05.10	Tarn Wood	M	8.5	24	Adult
15	01.07.10	Spiggot Hill	M	8.2	14	Young adult
18	02.07.10	Tarn Fen	M	7.4	15	Young adult
23	16.09.10	Spiggot Hill	F	7	14	Young adult
25	16.09.10	Spiggot Hill	M	8	20	Adult
26	16.09.10	Spiggot Hill	F	7	18	Young adult
27	16.09.10	Spiggot Hill	F	8	22	Adult
28	16.09.10	Spiggot Hill	M	7.6	21	Adult
31	16.09.10	Spiggot Hill	M	5.8	8	Juvenile
32	16.09.10	Spiggot Hill	F	7.3	17	Young adult
33	18.09.10	Tarn Wood	F	5.9	9	Juvenile
34	18.09.10	Tarn Wood	M	8.8	22	Adult
36	19.09.10	Tarn Fen	F	4.5	4	Juvenile
37	19.09.10	Tarn Fen	M	8.4	11	Juvenile
38	19.09.10	Tarn Fen	F	5.5	7	Juvenile
39	19.09.10	Tarn Fen	M	7.5	16	Young adult
40	19.09.10	Tarn Fen	F	7.5	13	Young adult
41	19.09.10	Tarn Fen	M	7.4	11	Juvenile
43	20.09.10	Ha Mire	F	7.5	15	Young adult
44	20.09.10	Ha Mire	M	8.5	24	Adult
45	20.09.10	Ha Mire	M	8.7	20	Adult
46	20.09.10	Ha Mire	M	8.2	20	Adult
47	20.09.10	Ha Mire	F	7	15	Young adult
48	20.09.10	Ha Mire	F	6.6	14	Young adult
53	20.09.10	Ha Mire	F	7.9	20	Adult
56	20.09.10	Ha Mire	M	7.2	19	Young adult
57	16.01.11	Ha Mire	M	7.8	19	Young adult
58	17.01.11	Tarn Wood	F	8.3	21	Adult
60	23.01.11	Tarn Fen	F	6.7	12	Juvenile
61	03.02.11	Spiggot Hill	M	7.6	20	Adult
64	05.05.11	Tarn Wood	F	7	26	Adult
66	05.05.11	Tarn Wood	M	7.3	23	Adult
70	29.06.12	Tarn Fen	M	7.2	19	Young adult
71	29.06.13	Tarn Fen	F	8.7	17	Young adult
72	28.06.11	Ha Mire	M	8.4	20	Adult
73	19.07.11	Tarn Wood	M	8.2	20	Adult
74	19.07.12	Tarn Wood	M	8	19	Young adult
75	19.07.13	Tarn Wood	M	8.3	15	Young adult

76	19.07.14	Tarn Wood	M	9.2	22	Adult
77	19.07.15	Tarn Wood	M	9.5	19	Young adult
78	19.07.16	Tarn Wood	M	7.5	15	Young adult
82	27.09.11	Tarn Wood	M	8.6	21	Adult
84	27.09.11	Tarn Wood	M	8.7	0	Adult
85	27.09.11	Tarn Wood	M	7.8	0	Young adult
86	27.09.11	Tarn Wood	M	8.8	0	Adult
87	30.10.11	Tarn Fen	F	7.8	12	Juvenile
88	30.10.11	Tarn Wood	F	7.2	17	Young adult
89	30.10.11	Tarn Wood	F	8.7	20	Adult
90	30.10.11	Tarn Wood	M	9.2	22	Adult
91	30.10.11	Tarn Wood	F	8	15	Young adult
92	30.10.11	Tarn Wood	F	8.1	16	Young adult
94	30.10.11	Tarn Wood	F	8.5	19	Young adult
95	30.10.11	Tarn Wood	M	7.4	20	Adult
96	30.10.11	Tarn Wood	M	7.9	14	Young adult
99	30.10.11	Tarn Wood	F	8.5	19	Young adult
100	30.10.11	Tarn Wood	M	7.9	16	Young adult
101	30.10.11	Tarn Wood	M	9	19	Young adult
102	30.10.11	Tarn Wood	M	8	18	Young adult
103	30.10.11	Tarn Wood	F	8	17	Young adult
104	30.10.11	Tarn Wood	M	8.5	17	Young adult
106	30.10.11	Tarn Wood	M	7	16	Young adult
108	30.10.11	Tarn Fen	F	8	15	Young adult
110	30.10.11	Tarn Wood	F	8	19	Young adult
112	30.10.11	Tarn Wood	M	8	13	Young adult
115	30.10.11	Tarn Wood	M	8	16	Young adult
116	30.10.11	Tarn Wood	M	7.2	18	Young adult
117	30.10.11	Ha Mire	M	7.3	12	Juvenile
118	30.10.11	Ha Mire	F	7.5	15	Young adult
119	30.10.11	Ha Mire	F	8.5	18	Young adult
120	30.10.11	Ha Mire	F	8	19	Young adult
121	30.10.11	Ha Mire	M	7.5	13	Young adult
122	30.10.11	Ha Mire	M	9	14	Young adult
123	30.10.11	Ha Mire	M	7.5	16	Young adult
124	30.10.11	Ha Mire	M	8.8	20	Adult
125	30.10.11	Ha Mire	F	7.2	12	Juvenile
126	30.10.11	Ha Mire	M	9.3	25	Adult

Table 2.2. Summary of the parasite infection status of the 80 mice studied in this thesis. Data derived from Boyce *et al*. (2014).

Number	*Toxoplasma*	*Plagiorchis*	*Heligmosomoides*	*Syphacea*	*Capillaria*	*Pelodera*	*Brachylaemus*
6	Pos	0	5	0	0	0	0
9		0	0	0	0	0	0
10		0	13	0	0	0	0
11		0	8	0	0	0	0
12		0	9	0	0	0	0
15		0	5	0	0	0	0
18		0	2	0	0	0	3
23	Pos	0	6	77	0	0	0
25		275	0	0	0	0	0
26		0	2	23	0	0	0
27		0	25	0	0	0	0
28	Pos	0	20	0	0	0	0
31	Pos	0	0	0	0	0	0
32	Pos	0	1	44	0	0	0
33		0	0	0	0	0	0
34		3	0	0	0	0	0
36		0	0	0	0	0	0
37		0	1	0	0	1	0
38		0	0	0	0	0	0
39		0	2	0	0	0	0
40		0	0	0	0	8	0
41		0	3	0	0	1	0
43	Pos	0	5	0	0	0	0
44	Pos	1	7	0	0	0	0
45		66	2	0	0	0	0
46		4	22	0	0	0	0
47		1	0	0	0	0	0
48		1	0	0	0	0	0
53	Pos	26	5	0	0	0	0
56		1	7	0	0	0	0
57		0	1	0	0	0	0
58		0	2	0	0	0	0
60		0	1	0	0	0	0
61		0	9	13	0	0	0
64		0	1	5	0	0	0
66		179	2	4	0	0	0
70		6	16	11	0	0	0
71	Pos	0	2	8	0	0	0
72	Pos	0	1	0	0	0	0
73		2	3	7	0	0	0
74		0	1	21	0	0	0

75		0	3	32	0	0	0
76		0	0	0	0	0	0
77		2	3	0	0	0	0
78	Pos	0	4	16	0	0	0
82		12	32	0	0	0	0
84	Pos	6	65	1	0	0	0
85	Pos	0	0	0	0	0	0
86	Pos	1	10	3	0	0	0
87	Pos	0	8	0	0	0	0
88	Pos	0	0	1	1	58	0
89	Pos	0	4	0	0	0	0
90		0	0	0	1	0	0
91		0	4	0	0	0	0
92		0	1	0	0	0	0
94		0	8	0	1	0	0
95		0	33	0	0	0	0
96		0	0	0	0	0	0
99	Pos	0	1	5	0	0	0
100		3	6	0	0	0	0
101		0	4	0	2	0	0
102	Pos	0	0	0	0	0	0
103	Pos	0	2	0	0	0	0
104		24	25	0	0	0	0
106	Pos	2	0	10	0	0	0
108	Pos	0	0	56	0	0	0
110	Pos	0	1	0	0	0	0
112	Pos	0	0	0	0	0	0
115	Pos	0	0	87	0	0	0
116	Pos	0	2	100	0	0	0
117	Pos	0	0	0	0	0	0
118	Pos	0	0	0	0	0	0
119	Pos	0	0	0	0	0	0
120	Pos	0	2	0	0	0	0
121	Pos	0	0	0	0	0	0
122		0	0	0	0	0	0
123	Pos	0	0	10	8	0	0
124	Pos	1	5	0	0	0	0
125	Pos	0	0	35	0	0	0
126		0	15	0	0	0	0
Total	34	19	53	22	5	4	1

2.2 DNA Extraction

Mus musculus and *Apodemus sylvaticus* mice had previously been used for various studies on parasitic infections (Marshall *et al*., 2004; Boyce *et al*., 2013, 2014; Morger *et al*., 2014; Bajnok *et al*., 2015). DNA was extracted from the mice using standard methods and stored at -20°C (Bajnok *et al*., 2015).

2.3 Primer Synthesis

Gene sequences for two variants of NLRP1b were retrieved from the GeneBank database at NCBI http://www.ncbi.nlm.nih.gov/) and Ensembl (http://www.ensembl.org/index.html)and their translations were checked. The first variant is labelled MEQSQPKKK (NP_001035786.1) and the second is MEESQYKQ (NP_001155886.1) and a ClustalW alignment was done using the alignment tool from EBI (http://www.ebi.ac.uk/Tools/msa/clustalo/) for both DNA variant sequences and both translations. After that, possible primers were designed, and ambiguity codes were added to recognize more than one base. Primer 3 (http://primer3.ut.ee/) is online software that is used for primer validation and checking all criteria that must be met for optimal performance. The primers were synthesized by Eurofins Genomics (Table 2.3).

Table 2.3. Sequences of NLRP1b primers.

Primer (forward)	Primer (reverse)	Name
5’ ATGGAASAATCTCAGYMCAAGMAG 3’	5’ RMTTSAAGKKWCYCTCTGGSTTGG 3’	EXON 2
5’ AWTTCCARAAGTAYAYATRAAWCA 3’	5’ACATAGCTCAGGATTCTGGTCTGC 3’	EXON 3-1
5’TRYRAGMAGCTGAGTCTGGCTGAG 3’	5’ GCAAAGAACTCCTGGAGACACAAG 3’	EXON 3-2
5’GAGGATGCCRTTGCCAYTTTCCTG 3’	5’ CTGTCAGYKYATRTCCYTGYTGTC 3’	EXON 3-3
5’ GCTGAGTCTGGCTGAGCTCATAGC 3’	5’ GAACTCCTGGAGACACAAGTGG 3’	EXON 3-2N
5’ CCTTCAAAAGCAGGCCAGC 3’	5’ GCAGAAAGTGACCACCATGAG 3’	EXON3-3N
5’ CCCACTGTGCGCTTTCTAT ’3	5’ AGTGACCACCATGAGCTTC’3	SEQ
5’ TGCTGCATCTGTTACCTCTTG’3	5’ GGGTCCCCTAAGATGATCCA’3	SEQ2
5’ GAGACTCTGGACCCCACAT’3	5’ GACTTCCCAATTCCAGCAG’3	EXON 3-1
5’ AGAGAGCCTGGAAGGAAGG’3	5’ AGCTCAGGATTCTGGTCTGC’3	EXON3-2
5’ TGACCTCACAGACCACA’3	5’ CCTGCTTTTGAAGGATACCG’3	EXON3-3

2.4. PCR

2.4.1. Mammalian Tubulin PCR

PCR amplification of the mouse tubulin gene was used as a control to confirm that DNA extractions were of suitable quality for subsequent assay. The cycling conditions and primers used for the tubulin PCR are shown in Tables 2.4 and 2.5.

Table 2.4. Mammalian tubulin PCR cycling conditions.

Cycle	Conditions	Number of Cycles
1	94°C for 5 minutes	1
2	94°C for 40 seconds 60°C for 40 seconds 72° *C* for 1 minute 30 seconds	40
3	72°C for 10 minutes	1

The first denaturation cycle was at 94°C for 5 minutes followed by a second cycle that included denaturation at 94°C for 40 seconds, then annealing at 72°C for 40 seconds and

extension at 72°C for 1 minute and 30 seconds. The last step was a final elongation at 72°C for 10 minutes.

Table 2.5. Sequences of the Tubulin primers.

Tubulin forward Primer	Tubulin reverse primer
5'CGTGAGTGCATCTCCATCCAT'3	5'GCCCTCACCCACATACCAGTG'3

The PCR master mix (Table 2.6) consisted of a 10X Bioline NH4 PCR buffer (no $MgCl_2$), $MgCl_2$, dNTPs mix (Bioline), forward and reverse tubulin primers and distilled water (Sigma), which made a total volume of 24µl. The final total volume in each PCR tube was 25 µl as 1 µl of mouse DNA was then added (1 µl of distilled water was used for a negative control purpose).

Table 2.6. PCR Master mix contents, concentrations and amount per sample for amplification of the mouse tubulin and NLRP1b genes.

Material	**Concentration**	**Amount**
Bioline NH_4 PCR buffer	-----	2.5 µl
$MgCl_2$	50 mM	1 µl
dNTPmix	25 mM each	0.25 µl
Forward primer	25 pm/µl	0.5 µl
Reverse primer	25 pm/µl	0.5 µl
Distilled water	–	18.75 µl
Taq DNA polymerase	5 unit/µl	0.5 µl

2.4.2. Polymerase Chain Reaction

A master mix was prepared comprising: 2.5 µl of 10x Bioline NH4 PCR buffer, 1 µl of 50 mM $MgCl_2$ (Bioline), 0.25 µl each of 25 mM dNTPs, 0.5 of 25pm/µl forward primer, 0.25 of 25pm/µl of our designed reverse primer, 18.75 µl of distilled water and 0.5 µl of 5unit/µl Taq DNA polymerase (Table 2.4). Standard PCR cycling conditions were as follows: DNA denaturation at 94°C for 10 minutes for one cycle, followed by 40 cycles of DNA denaturation at 94°C for 40 seconds; primer annealing was done at different temperatures

according to the type of primer and this was for 40 seconds with an extension at 72°C for 90 seconds. A final extension step at 72°C for 10 minutes was carried out.

Also, a Bioline MyTaq Mix was used at a certain times with some of the PCRs. It comprised a MyTaq DNA Polymerase buffer, dNTPs and $MgCl_2$ and required adding only water, primers and DNA as follows: 1 µl of 25 pm/µl forward primer, 1 µl of 25 pm/µl reverse primer, 1 µl of DNA templet, 12.5 µl of MyTaq Mix and 9.5 µl of water.

Table 2.7. Details of NLRP1b primers annealing temperature and expected fragment size.

Primer	Annealing temperature	Size (bp)
EXON2	57°C	142
EXON3Part1	59°C	–
EXON3Part2	52°C	770
EXON3Part3	52°C	610
EXON3Part2N	60°C	757
EXON3Part3 N	60°C	610
EXON3-1	59°C	215
EXON3-2	59°C	235
EXON3-3	59°C	235

2.4.3. PCR Optimization

Two factors were taken into account during PCR optimization; annealing temperature and $MgCl_2$ concentration. The optimization used may improve target segment quantity and specificity. The optimal annealing temperature was determined by setting up a temperature gradient across a thermocycler plate. For example, if the PCR plate has 12 columns, each column will have an increment of 1°C in temperature. This should help to determine the best primer annealing temperature. A $MgCl_2$ dilution series was used in some cases to enhance the recovery of PCR product.

2.5. Gel Electrophoresis

Agarose gel electrophoresis was used to check for the recovery of PCR products. A 1.5% gel concentration was used for gel electrophoresis and this was prepared by addition of 1.5 g of agarose powder (Bioline) to 100 ml of 1x TBE buffer (Bioline). The mixture was heated in a microwave for about 30 seconds to allow the agarose powder to dissolve. The solution was put onto a rotary shaker, and when it had cooled to approximately 50°C, 3 µl of concentrated gel red solution (Biotium) was added. The agarose solution was then poured into a gel casting tray that contained appropriate dams and a comb and it was allowed to solidify. Then, approximately 400 ml of the 1xTBE buffer was poured into the casting tank, the casting dams and comb were removed and the DNA (with 3 µl of the concentrated 6x loading buffer from Bioline) was loaded into the wells. The DNA was allowed to electrophorese for 1 hour at 100V and 100 mA. The DNA was then visualized by placing the gel in a UV transilluminator (SynGene). The gel was imaged and the photo saved.

2.6. DNA extraction from agarose gel.

A Bioline Isolated II Gel Kit was used to extract DNA from the agarose gel. Using UV eye protection, a clean scalpel was used to cut the required DNA fragment from the gel. Excess agarose was removed from the fragment and the weight of the slice was measured. 200µl of CB binding buffer was added to the slice in a clean tube and incubated in a 50°C water bath for 5–10 minutes with a vortex every 2 minutes to completely dissolve the gel slice. After that, an Isolated II PCR column was placed in a 2ml collection tube and the sample was loaded and centrifuged for 30 seconds at 11,000 rpm. The flow-through was discarded and 700 µl of CW washing buffer was added to the Isolated II PCR column, another centrifuge was done for the same time and speed. The flow-through from the previous step was discarded and another centrifuge was carried out to remove any ethanol from the washing buffer. The last step was to place the Isolated II PCR column in a 1.5ml tube with about 25 µl

of an elution buffer, followed by incubation at room temperature for 1 minute before a last centrifuge was done for 1 minute at 11,000 rpm.

2.7. PCR Product clean-up

A Bioline Isolated II PCR Kit was used in this stage. The sample was prepared by adding water to adjust the total volume to 50μl. Then, one volume of the sample was mixed with two volumes of CB binding buffer. The Isolated II PCR column was placed in a 2 ml collection tube and the sample loaded and centrifuged for 30 seconds at 11,000 rpm. The flow-through was discarded and 700 μl of CW washing buffer was added to the Isolated II PCR column, another centrifuge was done for the same time and speed. The previous step was repeated to remove and minimize any Chaotropic salt carry-over. The flow-through was discarded and another centrifuge carried out to remove any ethanol from the washing buffer. The Isolated II PCR column was placed in a 1.5 ml tube with about 25 μl of an elution buffer, followed by incubation at room temperature for 1 minute before a last centrifuge was done for 1 minute at 11,000 rpm.

2.8. DNA concentration measurement

A Nanodrop spectrophotometer (ThermoFisher Scientific) was used to measure the concentration of the recovered PCR product, since a specific concentration is required by Source Bioscience, the DNA sequencing company utilised throughout this study. An aliquot of 2–3 μl of deionized water was placed on the lower optical surface and the upper optical arm was closed to clean both optical surfaces. Then, the water was wiped away and 2–3 μl of buffer was added to the optical surface and the arm closed to blank the measurement. The type of sample was closed, double-strand DNA, and 1μl of the sample was loaded onto the lower optical surface and the upper arm closed to measure the concentration.

2.9. Sequencing

PCR products were prepared to be sent to Source Bioscience Lifesciences Company in order to sequence them. There are some requirements from the company to get a good quality sequence data. The forward and reverse primers must be 5 µl volumes and at a concentration of 3.2 pmol/µl. Samples must be a 5µl volume for each PCR product and the concentration must be 1ng/µl per 100bp. Also, it is preferable that primer lengths are between 18–23 base pairs, the primer annealing temperature between 55°C and 60°C, and that primers should have about 40% and 60% GC content.

The date were analysed using various software: FinchTV (http://officialsite.pp.ua/?p=2958497), ChromasPro, NCBI blast search (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch), clustal alignment (http://www.ebi.ac.uk/Tools/msa/clustalo/), protein translation Expasy (http://web.expasy.org/translate/) and ChromasPro (http://technelysium.com.au/wp/chromaspro/). FinchTV and ChromasPro were used for sequencing visualization and analysis. NCBI Blast searches was used to check the similarity to mouse references. Clustal alignment was used to align all sequences and find any differences between them. Expasy was used to translate all sequences into their proteins.

2.10. Statistics

Minitab 16 was used for statistical analyses of the data (licensed to the University of Salford). Also, GraphPad, online software, was used in parallel with Minitab (http://graphpad.com/quickcalcs/contingency1.cfm).

Chapter 3

Result: Investigation into the variation of Toll-like Receptor (TLR) genes in relation to helminth infections in woodmice (*Apodemus sylvaticus*)

3. Result: Investigation into the variation of Toll-like Receptor (TLR) genes in relation to helminth infections in wood mice (*Apodemus sylvaticus*)

3.1. Introduction

3.1.1. Toll-like Receptor gene variation in *Apodemus sylvaticus*

A number of researchers have shown that variations in toll-like receptors can have an influence on resistance or susceptibility to infectious agents in both laboratory studies and natural populations. A previous study was carried out in which a large collection of wild wood mice were tested with infections from both *T. gondii* (Bajnok *et al*., 2015) and a range of helminth and other parasites (Boyce *et al*., 2014). Individual mice in this population also had their genes for TLR11 and TLR12 sequenced as part of a study to investigate any specific relationship between TLRs gene polymorphism and *T. gondii* infection. A large quantity of other parasitological data on helminth infections was also obtained for these mice (Morger *et al*., 2014; Morger *et al*., 2014; Boyce *et al*., 2014). Since evidence suggests that TLR11 and TLR12 may interact with parasites other than *Toxoplasma gondii* the aim of this chapter is to investigate whether there is any association between variations in TLR11 and TLR 12 gene sequences and helminth infection.

According to the first quantitative study on the genetic diversity of TLR 11 and TLR12 in nature in *Apodemus sylvaticus*, an intermediate level of genetic diversity was observed in TLR 11 andresulting amino acid changes give four haplotypes (Morger *et al*., 2014). Two of these haplotypes are frequently present and two of them are very rare (Figure 3.1). TLR 12 shows a higher genetic diversity compared to TLR 11, with nine amino acid changes and nine haplotypes (Morger *et al*., 2014). Only one TLR12 haplotype is very frequent (Figure 3.1). These variations in TLR 11 and TLR 12 appear to have a minimal effect on the susceptibility or resistance of the studied population of *Apodemus sylvaticus* to *T. gondii* infection (Morger *et al*., 2014).

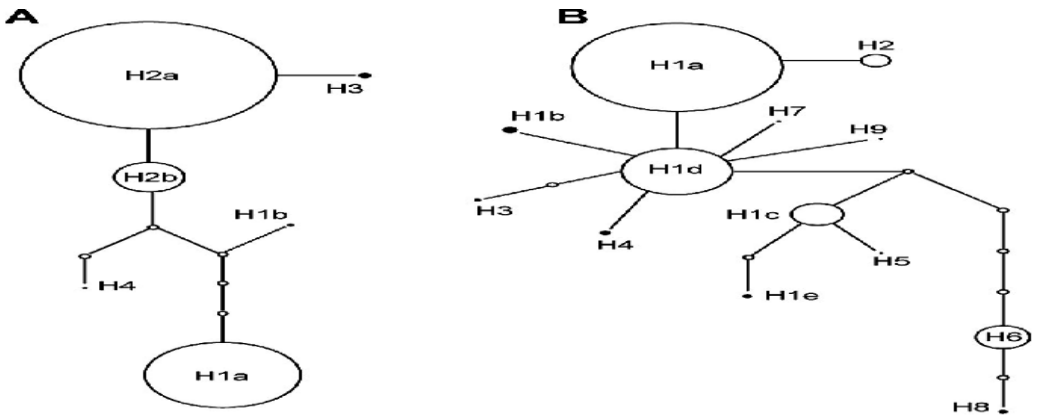

Figure 3.1. Genetic diversity of TLR 11 and TLR 12 in *Apodemus sylvaticus* (taken from Morger *et al*., 2014).

3.2. Study Rationale.

Little is known about the interactions between parasitic infections in natural populations of *Apodemus sylvaticus* and genetic variations in genes of the immune system. The availability of a population of wood mice well characterised for their parasite infections (Boyce, 2013; Boyce *et al*., 2014), and which had had their TLR11 and TLR12 genes sequenced (Morger *et al.*, 2014), provided an opportunity to explore this further.

As TLRs are an important mechanism for detecting parasites and triggering the innate immune system, a broad hypothesis might be that different variants of each TLR have different efficiencies of detection and therefore the ability to resist infection. Furthermore, heterozygous mice have two different variants and might have a greater ability to detect and deal with infection than homozygous mice. If these hypotheses are correct, one might expect certain variants to be associated with specific infections or with a lack of infection. Furthermore, heterozygous mice might be more likely to be less infected than their homozygous counterparts. Looking at this from another perspective, parasite infection could be acting as a selective force driving TLR variants or heterozygosity in wild populations, such as *Apodemus sylvaticus*. Additionally, the repertoire of TLRs could also have an influence on or be influenced by multiple or co-infections. The availability of this collection of mice and genetic data offers the opportunity to investigate these questions.

3.3. Objectives.

There are several questions that would be interesting to address regarding these infected samples and their genotypes:

1- Is there any relationship between the H1 haplotype of TLR11, negative, single and multiple parasitic infections and non-infected mice?
2- Is there any relationship between the H2 haplotype of TLR11, negative, single and multiple parasitic infections and non-infected mice?
3- Is there any relationship between the H1 haplotype of TLR12 and negative, single and multiple parasitic infections?
4- Do TLR11 homozygosity and heterozygosity have a relationship with each parasitic infection?
5- Do TLR12 homozygosity and heterozygosity have a relationship with each parasitic infection?
6- Is there any relation between the H1 haplotype of TLR11 and each parasitic infection?
7- Is there any relation between the H1 haplotype of TLR12 and each parasitic infection?
8- Is there any relation between the H2 haplotype of TLR11 and each parasitic infection?

3.4. Material and methods.

Wood mice sample collection and investigations were carried out by Boyce and her group (Boyce *et al*., 2014). TLR polymorphism and any association with *T. gondii* were carried out by Morger and his group (Morger *et al*., 2014). The prevalence data for *Toxoplasma gondii* were investigated by Bajnok and his group (Bajnok *et al.*, 2015)

3.4.1. Data Analysis

Graphpad online software was used to calculate associations using a Fisher's exact test and a two-tailed P value analysis. A two-tailed value of $P \leq 0.05$ was considered significant.

3.5. Results

3.5.1. Investigating the relation between negative, single and multiple parasitic infections and presence of the H1 genotype of TLR11

One hundred and twenty mice were examined for helminth parasitic infections and six species of helminths were found. The prevalence of infection with at least one parasite was 79.2% while the negative infection prevalence was 20.8%. The number of mice with multiple parasitic infections was slightly higher than those with a single parasitic infection; 49 (51.6%) and 46 (48.4%), respectively. The H2/H2 TLR 11 genotype comprised about 48% of the sample while H1 genotypes were 21.6%; the remaining samples were a mixture of H1/H2, H1/H3, H2/H3 and H2/H4 genotypes. The following tables show the relationship between negative, single and multiple parasite infected mice and non-infected mice, and according to the presence of the H1 and non-H1 genotypes of TLR11.

Table 3.1. Investigation of the relationship between negative, single and multiple parasitic infections and the presence of TLR11 haplotype 1

	H1 Genotype	Non H1 Genotype	Total
No infection	4	21	25
Single Parasite infection	6	40	46
Multiple Parasites infection	8	41	49
Total	18	102	120

The table above (Table 3.1) was constructed to investigate the association between parasitic infection statuses according to the H1 haplotype of TLR11. Six out of 46 mice were H1 and infected with a single parasite, and 8 out of 49 mice were H1 and infected with more than one parasite. The two-tailed P value was calculated for the previous table and was 0.77. According to the two-tailed P value, the association between single and multiple parasitic infections and the presence of the H1 haplotype of TLR11 is insignificant.

Also, it presents the data for negative and multiple infection mice based on the TLR 11 presence of haplotype1 1 to find out the relation between them. A total of 18 mice were H1, 4 of them were not infected and 8 were multiple parasites infected. The calculated two-tailed P value is > 0.05, which shows no significant relation between negative and multiple parasitic infections in the presence of H1 of TLR 11.

The association between negative and single parasitic infections in the presence of TLR11 H1 haplotype is investigated in Table 3.3. Four out of 25 mice were H1 and had no parasitic infection and 6 out of 46 mice were H1 and infected with a single parasite. No association between negative and single parasitic infection and the presence of haplotype H1 of TLR11 is found since the two-tailed P value is 0.73.

3.5.2. Investigating the relation between negative, single and multiple parasitic infections and the presence of H2 genotypes of TLR11.

The next table show the relation investigated between TLR11 H2 haplotypes and each parasitic infection: negative, single and multiple.

Table 3.2. Investigation of the relationship between negative, single and multiple parasitic infections and the presence of TLR11 haplotype 2.

	H2 Genotype	Non H2 Genotype	Total
No infection	15	10	25
Single Parasite infection	23	23	46
Multiple Parasites infection	20	29	49
Total	58	62	120

The table above (Table 3.2) was constructed to investigate the association between parasitic infection statuses according to the H2 haplotype of TLR11. Twenty-three out of 46 mice were H2 and infected with a single parasite, which makes 50% of them, and 20 out of 49 mice were H2 and infected with more than one parasite. The two-tailed P value was calculated for the previous table and is 0.43. According to the two-tailed P value, the

association between single and multiple parasitic infections and the presence of the H2 haplotype of TLR11 is insignificant.

Another relation from the previous table (Table 3.2) was investigated which is the relation between negative and multiple infection mice based on the TLR 11 presence of haplotype 2. A total of 58 mice were H2, 15 of them were not infected and 20 were multiple parasites infected. The two-tailed P value calculated is 0.14, which indicates no significant relation between negative and multiple parasitic infections in the presence of H2 of TLR 11.

The association between negative and single parasitic infection in the presence of TLR11 H2 haplotype is investigated in Table 3.2. Fifteen out of 25 mice were H2 and had no parasitic infection and 23 out of 46 mice were H2 and infected with a single parasite. No association was found between negative and single parasitic infection and the presence of the haplotype H2 of TLR11 since the two-tailed P value is 0.46.

3.5.3. Investigating the relation between negative, single and multiple parasitic infections and H1 genotypes of TLR12.

Sixty per cent of TLR 12 genotypes were H1 homozygotes and the other 40% were a mix of H1/H2–H1/H9. The next few tables show the interaction between infection status and the genotypes of TLR12.

Table 3.3. Investigation of the relationship between negative, single and multiple parasitic infection and the presence of TLR12 haplotype 1

	H1 Genotype	Non H1 Genotype	Total
No infection	15	10	25
Single Parasite infection	28	18	46
Multiple Parasites infection	29	20	49
Total	72	48	120

The table above (Table 3.3) was constructed to investigate the association between parasitic infection statuses according to the H1 haplotype of TLR12. Twenty-eight out of 46 mice were H1 and infected with a single parasite and 29 out of 49 mice were H1 and infected with

more than one parasite. The two-tailed P value calculated for the above table is> 0.05. According to this two-tailed P value, the association between single and multiple parasitic infections and the presence of the H1 haplotype of TLR12 is insignificant.

Also the table (Table 3.3) presents the data for negative and multiple infection mice based on the TLR 12 presence of haplotype 1 to determine the relation between them. A total of 72 mice were H1, 15 of them were not infected and 29 were multiple parasite infected. The two-tailed P value calculated is > 0.05, which indicates no significant relation between negative and multiple parasitic infections in the presence of H1 of TLR 12.

The association between negative and single parasitic infections in the presence of TLR12 H1 haplotype is investigated in Table 3.3. 15 out of 25 mice were H1 and had no parasitic infection and 28 out of 46 mice were H1 and infected with a single parasite. No association was found between negative and single parasitic infections and the presence of haplotype H1 of TLR12 since the two-tailed P value is > 0.05.

3.5.4. Investigating the relationship between each helminth infection and TLR11 and 12 homozygotes and heterozygotes

3.5.4.1. *Plagiorchis*

Plagiorchis infection has been discovered among *Apodemus sylvaticus* mice; 20.8% of wood mice were infected while 79.2% of wood mice were negative for *Plagiorchis* infection. The two tables below show the details for *Plagiorchis* infection and the TLR11 and 12 genotypes heterozygosity and homozygosity.

Table 3.4. Investigation of the relationship between *Plagiorchis* infection and the presence of TLR11 homozygous and heterozygous haplotypes.

	Homozygote	Heterozygote	Total
Plagiorchis	16	9	25
No *Plagiorchis*	60	35	95
Total	76	44	120

The table above (Table 3.4) was constructed to investigate the association between *Plagiorchis* infection and the genotypes' allele status (homozygote or heterozygote) for TLR

11. Sixteen mice infected with *Plagiorchis* were homozygous and 9 infected mice were heterozygous; 60 out of 95 mice were not infected with *Plagiorchis* and were homozygous for TLR11, and 35 of the negative ones were heterozygous. The two-tailed P value calculated for the above table is > 0.05, which means there is no association between the numbers of *Plagiorchis* infected mice and non-infected mice in the presence of TLR11 heterozygosity and homozygosity.

Table 3.5. Investigation of the relationship between *Plagiorchis* infection and the presence of the TLR12 homozygous and heterozygous haplotypes.

	Homozygote	Heterozygote	Total
Plagiorchis	16	9	25
No *Plagiorchis*	56	39	95
Total	72	48	120

Table 3.5 shows the association between *Plagiorchis* infection and the TLL12 allele status (homozygote or heterozygote) for TLR 12. Sixteen mice infected with *Plagiorchis* and were homozygous and 9 infected mice were heterozygous; 56 were not infected with *Plagiorchis* and were homozygous for TLR12, and 39 of the negative ones were heterozygous. The two-tailed P value calculated for the above table is 0.8190, which means there is no association between the numbers of *Plagiorchis* infected mice and non-infected mice in the presence of TLR12 heterozygosity and homozygosity.

3.5.4.2. *Heligmosomoides*

81 out of 120 samples were infected with *Heligmosomoides* and 39 mice were negative. The tables below show the relation between *Heligmosomoides* infection and the genotype status of TLR 11and 12, respectively.

Table 3.6. Investigation of the relationship between *Heligmosomoides* infection and the presence of the TLR11 homozygous and heterozygous haplotypes.

	Homozygote	Heterozygote	Total
Heligmosomoides	47	34	81
No *Heligmosomoides*	29	10	39
Total	76	44	120

The association between *Heligmosomoides* infection and allele status (homozygote or heterozygote) for TLR 11 is investigated in the table above (Table 3.6); 47 mice were homozygous and infected with *Heligmosomoides*, and 29 homozygous ones were negative; 34 of the heterozygous mice were infected and 10 were negative. The two-tailed P value calculated for the above table is 0.1, which means there is no significant association between the numbers of *Heligmosomoides* infected mice and non-infected mice in the presence of TLR11 heterozygosity and homozygosity.

Table 3.7. Investigation of the relationship between *Heligmosomoides* infection and the presence of the TLR12 homozygous and heterozygous haplotypes.

	Homozygote	Heterozygote	Total
Heligmosomoides	48	33	81
No *Heligmosomoides*	24	15	39
Total	72	48	120

Table 3.7 shows the association between *Heligmosomoides* infection and the allele status (homozygote or heterozygote) for TLR 12; 48 mice infected with *Heligmosomoides* were homozygous and 33 infected mice were heterozygous; 24 were not infected with *Heligmosomoides* and were homozygous for TLR12, and 15 of the negative ones were heterozygous. The two-tailed P value calculated for the above table is 0.56, which mean there is no association between the numbers of *Heligmosomoides* infected mice and non-infected mice in the presence of TLR12 heterozygosity and homozygosity.

3.5.4.3. *Syphacea*

27.5% of the mice were infected with *Syphacea* while 72.5% of mice were not infected; 51.5% of infected mice were TLR 11 homozygotes, while 48.5% were heterozygotes. Also, 67.8% of the negative samples were homozygous and the rest were heterozygous (Table 3.8). TLR12 homozygote genotype samples were about 63.6% while the heterozygote genotype samples were around 36.3% (Table 3.8).

Table 3.8. Investigation of the relationship between *Syphacea* infection and the presence of the TLR11 homozygous and heterozygous haplotypes.

	Homozygote	Heterozygote	Total
Syphacea	17	16	33
No *Syphacea*	59	28	87
Total	76	44	120

The association between *Syphacea* infection and the TLR11 allele status (homozygote or heterozygote) is shown in the table above (Table 3.8); 17 mice were homozygous and infected with *Syphacea*, 59 of the homozygotes were negative; 16 of the heterozygous mice were infected and 28 heterozygotes were negative. The two-tailed P value calculated for the above table is 0.14, which means there is no significant association between the numbers of *Syphacea* infected mice and non-infected mice in the presence of TLR11 heterozygosity and homozygosity.

Table 3.9. Investigation of the relationship between *Syphacea* infection and the presence of the TLR12 homozygous and heterozygous haplotypes.

	Homozygote	Heterozygote	Total
Syphacea	21	12	33
No *Syphacea*	51	36	87
Total	72	48	120

Table 3.9 shows the association between *Syphacea* infection and the TLR12 allele status (homozygote or heterozygote); 21 mice infected with *Syphacea* were homozygous and 12 were heterozygous; 51 were not infected with *Syphacea* and were homozygous for TLR12, and 36 of the negative ones were heterozygotes. The two-tailed P value calculated for the above table is 0.68, which means there is no association between the numbers of *Syphacea* infected mice and non-infected mice in the presence of TLR12 heterozygosity and homozygosity.

3.5.4.4. *Capillaria*

The number of mice infected by *Capillaria* was relatively low, as shown in Tables 3.10 and 3.11.

Table 3.10. Investigation of the relationship between *Capillaria* infection and the presence of the TLR11 homozygous and heterozygous haplotypes.

	Homozygote	Heterozygote	Total
Capillaria	4	4	8
No *Capillaria*	72	40	112
Total	76	44	120

The association between *Capillaria* infection and the TLR11 allele status (homozygote or heterozygote) is shown in the table above (Table 3.10). Four mice were homozygous and infected with *Capillaria* and 72 of the homozygotes were negative. Four of the heterozygote mice were infected and 40 heterozygotes were negative. The two-tailed P value calculated for the above table is 0.42, which means there is no significant association between the numbers of *Capillaria* infected mice and non-infected mice in the presence of TLR11 heterozygosity and homozygosity.

Table 3.11. Investigation of the relationship between *Capillaria* infection and the presence of the TLR112 homozygous and heterozygous haplotypes.

	Homozygote	Heterozygote	Total
Capillaria	5	3	8
No *Capillaria*	67	45	112
Total	72	48	120

Table 3.11 shows the association between *Capillaria* infection and the TLR12 allele status (homozygote or heterozygote). Five mice infected with *Capillaria* were homozygous and 3 infected mice were heterozygotes; 67 were not infected with *Capillaria* and were homozygous for TLR12 and 45 of the negative ones were heterozygotes. The two-tailed P value calculated for the above table is > 0.05, which means there is no association between the numbers of *Capillaria* infected mice and non-infected mice in the presence of TLR12 heterozygosity and homozygosity.

3.5.4.5. *Pelodera*

Pelodera was the second least prevalent infection among the mice. Only 5 mice were positive while the other 115 were negative.

Table 3.12. Investigation of the relationship between *Pelodera* infection and the presence of the TLR11 homozygous and heterozygous haplotypes.

	Homozygote	Heterozygote	Total
Pelodera	1	4	5
No *Pelodera*	75	40	115
Total	76	44	120

The association between *Pelodera* infection and the TLR11 allele status (homozygote or heterozygote) is shown in the above table (Table 3.12). One mouse was homozygous and infected with *Pelodera* and 75 of the homozygotes were negative. Four of the heterozygous mice were infected and 40 were negative. The two-tailed P value calculated for the above table is 0.6, which means there is no significant association between the numbers of *Pelodera* infected mice and non-infected mice in the presence of TLR11 heterozygosity and homozygosity.

Table 3.13. Investigation of the relationship between *Pelodera* infection and the presence of the TLR12 homozygous and heterozygous haplotypes.

	Homozygote	Heterozygote	Total
Pelodera	4	1	5
No *Pelodera*	68	47	115
Total	72	48	120

Table 3.13 shows the association between *Pelodera* infection and the TLR12 allele status (homozygote or heterozygote). Four mice infected with *Pelodera* were homozygotes and one was a heterozygote; 68 mice were not infected with *Pelodera* and were homozygous for TLR12, 47 of the negative ones were heterozygotes. The two-tailed P value calculated for the above table is 0.65, which means there is no association between the numbers of *Pelodera* infected mice and non-infected mice in the presence of TLR12 heterozygosity and homozygosity.

3.5.4.6. *Brachylaemus*

The least prevalent among the six parasites was *Brachylaemus*. Only 3 mice were positive, as shown in Tables 3.14 and 3.15.

Table 3.14. Investigation of the relationship between *Brachylaemus* infection and the presence of the TLR11 homozygote and heterozygote haplotypes.

	Homozygote	Heterozygote	Total
Brachylaemus	2	1	3
No *Brachylaemus*	74	43	117
Total	76	44	120

The association between *Brachylaemus* infection and the TLR11 allele status (homozygote or heterozygote) is shown in the above table (Table 3.14). Two mice were homozygotes and infected with *Brachylaemus* and 74 of the homozygotes were negative. One of the heterozygote mice was infected and 43 were negative. The two-tailed P value calculated for the above table is > 0.05, which means there is no significant association between the numbers of *Brachylaemus* infected mice and non-infected mice in the presence of TLR11 heterozygosity and homozygosity.

Table 3.15. Investigation of the relationship between *Brachylaemus* infection and the presence of the TLR12 homozygous and heterozygous haplotypes.

	Homozygote	Heterozygote	Total
Brachylaemus	2	1	3
No *Brachylaemus*	68	47	117
Total	72	48	120

Table 3.15 shows the association between *Brachylaemus* infection and the TLR12 allele status (homozygote or heterozygote). Two mice infected with *Brachylaemus* were homozygotes and one was heterozygous; 68 mice not infected with *Brachylaemus* were homozygous for TLR12 and 47 of the negative ones were heterozygotes. The two-tailed P value calculated for the above table is > 0.05, which means there is no association between the numbers of infected mice and non-infected mice in the presence of TLR12 heterozygosity and homozygosity.

3.5.5. Investigating the relationship between each helminth infection in the presence of the H1 genotypes of TLR11 and 12

In this study, the H1 genotype of TLR 11 and TLR12 was investigated in relation to each helminth infection.

3.5.5.1. *Plagiorchis*

The tables below show the relation between *Plagiorchis* infection and the H1 genotypes of TLR 11 and TLR 12.

Table 3.16. Investigation of the relationship between *Plagiorchis* infection and the presence of TLR11 haplotype 1.

	H1	Non-H1	Total
Plagiorchis	5	20	25
No *Plagiorchis*	13	82	95
Total	18	102	120

The above table (Table 3.16) shows *Plagiorchis* infection was higher in the non-H1 genotype than the H1 genotype of TLR11; 80% and 20% respectively. Twenty positive mice were non-H1 while only 5 were H1 for TLR11. The two-tailed P value indicates that there is no relation between *Plagiorchis* infection and the H1 genotype of TLR 11 (P value = 0.53).

Table 3.17. Investigation of the relationship between *Plagiorchis* infection and the presence of TLR12 haplotype 1.

	H1	Non-H1	Total
Plagiorchis	16	9	25
No *Plagiorchis*	57	38	95
Total	73	47	120

Table 3.17 shows *Plagiorchis* infection was higher in mice of TLR12 H1 genotype. Sixteen positive mice were H1 while only 9 were non-H1 for TLR12. Negative *Plagiorchis* infected mice of the H1 genotype were 57 and non-H1 were 38. The two-tailed P value indicates that there is no relation between *Plagiorchis* infection and H1 and non-H1 genotypes for TLR 12 (P value = 0.82).

3.5.5.2. *Heligmosomoides*

The tables below show the relation between *Heligmosomoides* infection and TLR 11 and TLR 12 H1 genotypes.

Table 3.18. Investigation of the relationship between *Heligmosomoides* infection and the presence of TLR11 haplotype 1.

	H1	Non-H1	Total
Heligmosomoides	10	71	81
No *Heligmosomoides*	8	31	39
Total	18	102	120

The above table (Table 3.18) for TLR 11 showsthat only 10 H1 mice were infected with *Heligmosomoides* while the other 71 positive mice were non-H1. Also, negative H1 mice were 8 while non-H1 were 31. The P value outcome from the above table is 0.28 which means there is no significant relation between *Heligmosomoides* and the H1 haplotype of TLR11.

Table 3.19. Investigation of the relationship between *Heligmosomoides* infection and the presence of TLR12 haplotype 1.

	H1	Non-H1	Total
Heligmosomoides	49	32	81
No *Heligmosomoides*	24	15	39
Total	73	47	120

Table 3.19 shows the data for TLR 12 haplotype 1 and *Heligmosomoides* infection. The data shows that 49 H1 mice were infected with *Heligmosomoides* while the other 32 positive ones were non-H1. Also, negative H1 mice were 24 while non-H1 mice were 15. The P value outcome from the above table is > 0.05, which means there is no significant relation between *Heligmosomoides* and the H1 haplotype of TLR12.

3.5.5.3. *Syphacea*

The tables below show the relation between *Syphacea* infection and the H1 genotypes of TLR 11 and TLR 12.

Table 3.20. Investigation of the relationship between *Syphacea* infection and the presence of TLR11 haplotype 1.

	H1	Non-H1	Total
Syphacea	8	25	33
No *Syphacea*	10	77	87
Total	18	102	120

The table above shows that *Syphacea* was prevalent in 33 out of 120 mice. According to Table 3.20, for TLR 11 only 8 mice were H1 and infected with *Syphacea* and the other 25 infections were in non-H1 mice. Furthermore, there were 87 negative mice, including 10 H1 and 77-non H1 for TLR 11. The calculated two-tailed P value for TLR 11 mice is 0.09, which is not significant.

Table 3.21. Investigation of the relationship between *Syphacea* infection and the presence of TLR12 haplotype 1.

	H1	Non-H1	Total
Syphacea	22	11	33
No *Syphacea*	51	36	87
Total	73	47	120

Table 3.21 shows the data for *Syphacea* infection in relation to the H1 genotype of TLR12; 22 positive mice were H1 while 11 positive mice were non-H1. The negative mice were as follows: 51 H1 and 36 non-H1. The two-tailed P value calculated for TLR 12 mice is > 0.05, which is not significant.

3.5.5.4. *Capillaria*

The relation between *Capillaria* parasite infection and H1 for TLR11 and 12 is investigated in the next two tables 3.22 and 3.23.

Table 3.22. Investigation of the relationship between *Capillaria* infection and the presence of TLR11 haplotype 1.

	H1	Non-H1	Total
Capillaria	1	7	8
No *Capillaria*	17	95	112
Total	18	102	120

The above table (Table 3.22) for TLR11 shows that only one mouse with H1 genotype was *Capillaria* infected while 17 H1 genotype mice were negative. Also, 7 infected mice were

non-H1 while 95 negative mice were non-H1. No significant two-tailed P value was obtained from the above table as it is > 0.05.

Table 3.23. Investigation of the relationship between *Capillaria* infection and the presence of TLR12 haplotype 1.

	H1	Non-H1	Total
Capillaria	5	3	8
No *Capillaria*	68	44	112
Total	73	47	120

Table 3.23 shows that 5 infected mice and 68 non-infected mice had the TLR12 H1 genotype. Also, 3 infected and 44 non-infected mice had the non-H1 genotype. No significant two-tailed P value was obtained from the above table as it is > 0.05 for TLR12.

3.5.5.5. *Pelodera*

The relation between *Pelodera* infection and H1 for TLR11 and 12 is investigated in the next two tables 3.24 and 3.25.

Table 3.24. Investigation of the relationship between *Pelodera* infection and the presence of TLR11 haplotype 1.

	H1	Non-H1	Total
Pelodera	0	5	5
No *Pelodera*	18	97	115
Total	18	102	120

According to Table 3.24, which displays the data for TLR11, no infected mice were found with the H1 genotype and all 5 infected mice had the non-H1 genotype; 115 were not infected and among these mice 18 were H1 and 97 were non-H1. The TLR11 P value is equal to > 0.05, which means there is no association between the number of *Pelodera* infection and H1 genotype of TLR11.

Table 3.25. Investigation of the relationship between *Pelodera* infection and presence of TLR12 haplotype 1.

	H1	Non-H1	Total
Pelodera	4	1	5
No *Pelodera*	69	46	115
Total	73	47	120

Table 3.25 shows the data for TLR12. In contrast to the TLR 11 data, 4 positive mice were H1 and 1 mouse was non-H1. The number of H1 negative mice was higher than non-H1 mice at 69 and 46, respectively. The two-tailed P value calculated for the above table is 0.65, which means there is no association between the number of *Pelodera* infections and the H1 genotype of TLR12.

3.5.5.6. *Brachylaemus*

The relation between *Brachylaemus* infection and H1 for TLR11 and 12 is investigated in the next two tables 3.26 and 3.27.

Table 3.26. Investigation of the relationship between parasitic *Brachylaemus* infection and the presence of TLR11 haplotype 1.

	H1	Non-H1	Total
Brachylaemus	0	3	3
No *Brachylaemus*	18	99	117
Total	18	102	120

Brachylaemus infection prevalence was 3 out of 120 mice. According to Table 3.26 for TLR 11 data, no mice were H1 while the other 3 mice were non-H1. Furthermore, there were 117 negative mice, comprising 18 H1 and 99 non-H1. The calculated two-tailed P value for the above table is > 0.05, thus there is no significant association between *Brachylaemus* infection and the presence of the H1 genotype of TLR 11.

Table 3.27. Investigation of the relationship between parasitic *Brachylaemus* infection and the presence of TLR12 haplotype 1.

	H1	Non-H1	Total
Brachylaemus	2	1	3
No *Brachylaemus*	71	46	117
Total	73	47	120

Table 3.27 shows the data for *Brachylaemus* infection in relation to the H1 genotype. Two positive mice were H1 while only one positive mouse was non-H1. The negative mice were as follows: 71 H1 and 46 non-H1. The calculated two-tailed P value for the above table is > 0.05, thus there is no significant association between *Brachylaemus* infection and the presence of the H1 genotype of TLR12.

3.5.6. Investigating the relationship between each helminth infection in the presence of the H2 genotype of TLR11

3.5.6.1. *Plagiorchis.*

Twenty-five five mice were infected with *Plagiorchis* and 95 mice were *Plagiorchis* negative (Table 3.28).

Table 3.28. Investigation of the relationship between *Plagiorchis* infection and the presence of TLR11 haplotype 2.

	H2	Non-H2	Total
Plagiorchis	11	14	25
No *Plagiorchis*	47	48	95
Total	58	62	120

Table 3.28 shows the data for *Plagiorchis* parasite against the H2 and non-H2 genotypes of TLR11; 11 positive mice were H2 while 14 were non-H2. The negative mice numbered 95; 47 of them were H2, while 48 were non-H2. The two-tailed P value for the above table is 0.66, which is more than 0.05 and means there is no significant relation between *Plagiorchis* infection and the H2 TLR11genotype.

3.5.6.2. *Heligmosomoides*

Eighty-one out of 120 mice were infected with *Heligmosomoides*, while the other 39 were *Heligmosomoides* negative. *Heligmosomoides* parasite had the highest prevalence among the six other helminth parasites (Table 3.29).

Table 3.29. Investigation of the relationship between *Heligmosomoides* infection and the presence of TLR11 haplotype 2.

	H2	Non-H2	Total
Heligmosomoides	37	44	81
No *Heligmosomoides*	21	18	39
Total	58	62	120

Like *Plagiorchis* infection, the positive *Heligmosomoides* H2 infected mice were fewer than the non-H2 ones at 37 and 44, respectively. But there were more H2 negative mice than non-H2 infected mice at 21 and 18, respectively. There is no significant association between *Heligmosomoides* and the TLR11 H2 genotype, since the P value is 0.44.

3.5.6.3. *Syphacea*

Thirty-three mice out of 120 were infected with *Syphacea* while the other 81 were *Syphacea* negative (Table 3.30).

Table 3.30. Investigation of the relationship between *Syphacea* infection and the presence of TLR11 haplotype 2.

	H2	Non-H2	Total
Syphacea	9	24	33
No *Syphacea*	49	38	87
Total	58	68	120

Like the previous two tables, H2 infected mice with *Syphacea* were fewer than non-H2 infected ones. The H2 infected mice numbered 9 while non-H2 were 24; 49 out of 87 non-infected mice with *Syphacea* were H2 and 38 were non-H2 TLR11 genotype. The calculated two-tailed P value for both TLR mice variants is 0.007, i.e. < 0.05. Therefore, the P value shows a strong statistically significant association between *Syphacea* infection and the non

H2 genotype of TLR 11. Upon further analysis of the *Syphacea* infection, all data showed that the association was not significant with respect to the *Heligmosomoides* co-infection (P value = 0.28) (see appendix 1, Table1)

3.5.6.4. *Capillaria*

Eight mice out of 120 were infected with *Capillaria* while the other 12 were *Capillaria* negative (Table 3.31).

Table 3.31. Investigation of the relationship between *Capillaria* infection and the presence of TLR11 haplotype 2.

	H2	Non-H2	Total
Capillaria	2	6	8
No *Capillaria*	56	56	112
Total	58	62	120

As shown in the above table (Table3.31), the majority of *Capillaria* infected mice were non-H2 genotype and only two mice were H2; 112 mice were not infected, half of them were H2 and the other half were non-H2. There is no significant association between *Capillaria* infection and the TLR11 H2 genotype since the P value is 0.27.

3.5.6.5. *Pelodera*

Five mice out of 120 were infected with *Pelodera* while the other 115 were *Pelodera* negative (Table 3.32).

Table 3.32. Investigation of the relationship between *Pelodera* infection and the presence of TLR11 haplotype 2.

	H2	Non-H2	Total
Pelodera	1	4	5
No *Pelodera*	57	58	115
Total	58	62	120

Table 3.32 shows the data for the *Pelodera* parasite in relation to the H2 and non-H2 genotypes of TLR11. Only one positive mouse was H2 while 4 were non-H2. The negative mice numbered 115; 57 of them were H2 while 58 were non-H2 genotype (Table 3.32). The

two-tailed P value for the above table is 0.36 i.e. more than 0.05, which means there is no significant relation between *Pelodera* parasitic infection and the TLR11genotype.

3.5.6.6. *Brachylaemus.*

Three mice out of 120 were infected with *Brachylaemus* while the other 117 were *Brachylaemus* negative (Table 3.33).

Table 3.33. Investigation of the relationship between *Brachylaemus* infection and the presence of TLR11 haplotype 2.

	H2	Non-H2	Total
Brachylaemus	2	1	3
No *Brachylaemus*	56	61	117
Total	58	62	120

TLR11 data indicate that only 2 H2 mice were infected with *Brachylaemus*, while only 1 positive mouse was non-H1. Also, negative H2 mice numbered 8 while non-H2 were 31.

There is no significant association between *Brachylaemus* infection and the H2 genotype of TLR11 since the P value is 0.61.

The following tables (Tables 3.34, 3.35 and 3.36) summarise the statistical investigations carried out in this chapter with respect to any associations between TLR 11 and TLR 12 haplotypes and helminth infections. Note, the only significant link was found with the TLR 11 haplotype 2 and resistance towards *Syphacea* (Table 3.36).

Table 3.34. Summary of the statistical significance between TLR 11 (haplotypes H1 and H2), TLR12 (haplotype H1) and negative, single and multiple parasitic infections.

H1 haplotype of TLR11's relation to:	P value	Significance
Single and multiple parasitic infections	0.77	No
Negative and multiple parasitic infections	> 0.05	No
Negative and single parasitic infections	0.73	No
H2 haplotype of TLR11's relation to		
Single and multiple parasitic infections	0.43	No
Negative and multiple parasitic infections	0.14	No
Negative and single parasitic infections	0.46	No
H1 haplotype of TLR12's relation to		
Single and multiple parasitic infections	> 0.05	No
Negative and multiple parasitic infections	> 0.05	No
Negative and single parasitic infections	> 0.05	No

Table 3.35. Summary of the statistical significance between homozygosity and heterozygosity of TLRs 11 and 12 and infections with different parasites.

TLR11 homozygote or heterozygote relation to:	P value	Significance
Plagiorchis	> 0.05	No
Heligmosomoides	0.10	No
Syphacea	0.14	No
Capillaria	0.43	No
Pelodera	0.6	No
Brachylaemus	> 0.05	No
TLR12 homozygote or heterozygote relation to:		
Plagiorchis	0.82	No
Heligmosomoides	0.56	No
Syphacea	0.68	No
Capillaria	> 0.05	No
Pelodera	0.65	No
Brachylaemus	> 0.05	No

Table 3.36. Summary of the statistical significance between haplotypes of TLRs 11 and TLR 12 and different parasite infections.

TLR11 haplotype H1 against:	P value	Significance
Plagiorchis	0.53	No
Heligmosomoides	0.28	No
Syphacea	0.09	No
Capillaria	> 0.05	No
Pelodera	> 0.05	No
Brachylaemus	> 0.05	No
TLR12 haplotype H1 against:		
Plagiorchis	0.82	No
Heligmosomoides	> 0.05	No
Syphacea	> 0.05	No
Capillaria	> 0.05	No
Pelodera	0.65	No
Brachylaemus	> 0.05	No
TLR11 haplotype H2 against:		
Plagiorchis	0.66	No
Heligmosomoides	0.44	No
Syphacea	0.0073	Yes
Capillaria	0.27	No
Pelodera	0.36	No
Brachylaemus	0.61	No

3.6. Discussion

Little is known about the interactions between parasitic infections in natural populations of *Apodemus sylvaticus* and genetic variation in genes of the immune system. The purpose of this chapter is to study the association between the TLR11 and 12 haplotypes and six helminth parasites: *Plagiorchis, Heligmosomoides, Syphacea, Capillaria, Pelodera* and *Brachylaemus.* It is also to determine the effects of TLR heterozygosity and homozygosity on infection.

To our knowledge this is the first study that investigates the relations between TLR polymorphisms and the above helminth parasites. Only one published study was found that investigated the association between goat TLRs and infection with the nematode *Haemonchus contortus*. Indeed, polymorphisms of the goat TLR gene were found to be associated with *Haemonchus contortus* infection; a total of 31 SNPs were discovered and 9 of them showed statistically significant associations (Alim *et al.*, 2016).

Multiple studies have investigated TLR polymorphism in relation to different diseases. For example, a study that was carried out in China found that TLR 9 polymorphism is associated with knee diseases in humans (Zheng *et al.*, 2017). Also, another study showed that TLR9 polymorphisms are associated with altered IFN-γ levels in children with cerebral malaria (Sam-Agudu *et al.*, 2010). Furthermore, a study of TLR 2 polymorphisms and tuberculosis revealed an association between cattle tuberculosis and susceptibility/resistance of cattle (Bhaladhare *et al.*, 2016).

This current study presents evidence that the H2 genotype of TLR 12 in wood mice confers resistance against *Syphacea* infection, having a two-tailed P value of 0.0073. No other associations were discovered between the TLR11 and TLR12 genotypes and the helminth parasites investigated. It would be interesting to address further how the H2 haplotype apparently confers resistance to *Syphacea.* A comprehensive literature unfortunately revealed that no existing studies that might help explain the date presented within this chapter.

The prevalence of some parasites, e.g. *Pelodera and Brachylaemus,* was very low, which clearly would impact the quality of the data analysis. For example, an increase of 1 infected mice with certain parasites would make the relationship being analysed significant. Only five mice were infected with *Pelodera,* while only 3 mice were infected with *Brachylaemus.* This might be due to different factors, such as age, sex, season of sample collection and other factors that might affect the numbers of infected mice.

Apodemus sylvaticus, infected with helminth parasites, has been studied in two different areas, Aberystwyth and Skomer Island. Aberystwyth is a grassland area and Skomer Island is woodland. The prevalence of helminth parasites was higher in adults than juveniles. The composition of helminthic parasites and some seasonal variations in their prevalence in mice were discovered (Lewis, 2009). Both extrinsic factors, which include seasonal and site effects, and intrinsic factors, which include age and sex, interact with helminths prevalence and abundance (Langley and Fairley, 1982; Montgomery and Montgomery, 1989; Abu-Madi *et al.*, 2000). Seasonal variation (extrinsic) appears to be the factor most affecting variations in prevalence and abundance. This could be through indirect effects occurring in the reproductive cycles of animals and through direct effects such as seasonal differences in climate which affect the transmission and survival of each helminth stage(Abu-Madi *et al.*, 1998; Montgomery and Montgomery, 1990). Age is the most important intrinsic factor that affects the richness of helminths. This can be explained as young infected animals having fewer parasites and fewer species (Montgomery and Montgomery, 1989; Abu-Madi, Behnke, Lewis & Gilbert, 1998). Sex has very few effects on richness and abundance (Behnke *et al*., 1999; Ferrari *et al*., 2004). Synergistic and antagonistic relationships between parasites may affect the richness of helminth parasites (Behnke *et al.*, 2001). For example, some hosts can be exposed to two infective stages of different parasites (synergistic) at the same time (Lotz *et al*., 1995). On the other hand, some helminths make their hosts more or less susceptible to other species. Three mice with *Pelodera* were infected with different helminth parasites, and

other parasites might make the host more susceptible to infection. There were no mice infected only with *Pelodera*.

A hypothesis by Behnke (2005) suggests that the presence of the intestinal nematode *Heligmosomoides polygyrus* influences wood mice to have more than one helminth parasite. It was confirmed that wood mice in Portugal infected with *H. polygyrus* had a higher prevalence of other helminths, but other factors also had an influenced, such as the season (Behnke *et al*., 2009). In this data that is reported in this chapter, mice carrying *H. polygyrus* were more likely to be infected with other species, but only older mice. This confirms that *H. polygyrus* is a factor that increases the co-infection of wood mice with other helminth parasites (Behnke *et al*., 2009). The prevalence of *H. polygyrus* among mice in the current study was very high, and most of the mice were infected with more than one species of helminth.

Chapter 4

Determination of the Genomic arrangement and DNA Sequence of the NLRP1b Gene from *Apodemus sylvaticus* and its Relation to *Toxoplasma gondii* Infection

4.1 Introduction

The NLRP1b protein is found within inflammasomes and works as a sensor system to scan sterile host-cell cytosol for any foreign germs (Martinon *et al.*, 2002). Therefore, the NLRP1 gene plays a vital role in innate immune response activation.

The NLRP1 gene, like all the NLR family, has a central nucleotide binding domain and a short leucine-rich repeat domain (LRR). NLRP1 also has a domain called function to find FIIND (Martinon *et al*., 2009). The FIIND domain is similar to the ZU-5 and UPA domain, as found in non-NLR family proteins, such as PIDD and UNC5B (D'Osualdo *et al.*, 2011). The function of these domains is to go through different processes, mature and make the NLRP1 able to respond to stimuli (Finger *et al.*, 2012; Frew, Joag & Mogridge, 2012). Another two domains have also been found in the NLRP1 gene structure, the N-terminal pyrin domain (PYD) and the C-terminal caspase activating and recruitment domain (CARD). These two domains are related to the pyroptosis superfamily and are structurally close (Figure 4.1) (Chavarría-Smith & Vance, 2015) .

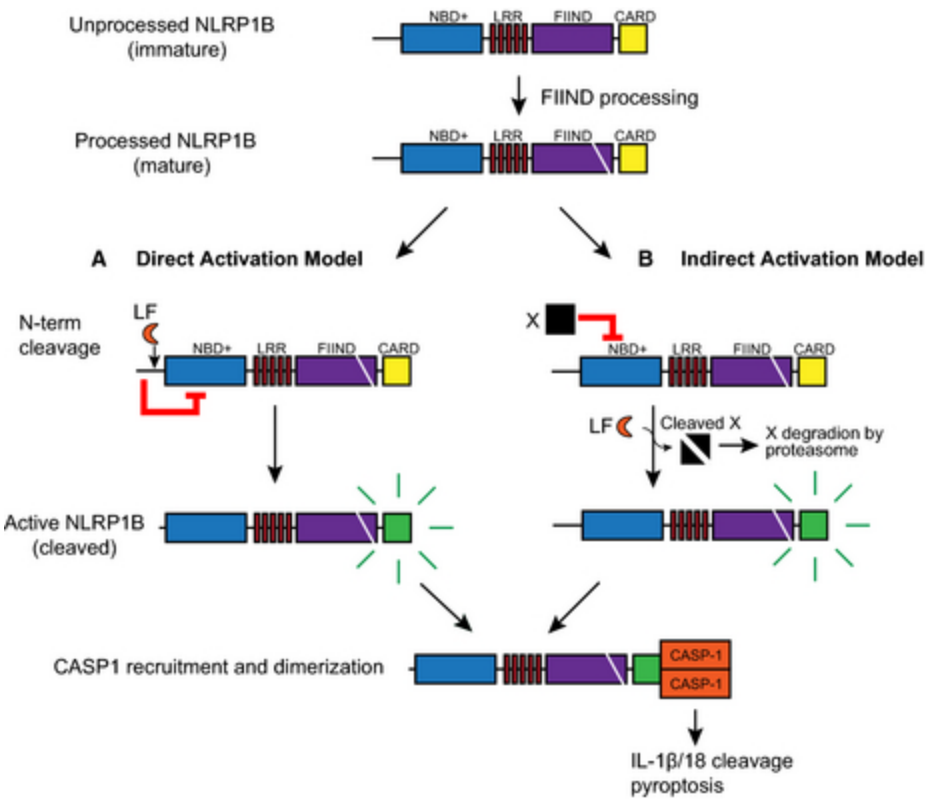

Figure 4.1. Domain structure and activation of NLRP1. The NLRP1 gene has different domains including a nucleotide binding domain (NBD) accompanied by helical domain 1, a leucine-rich repeat (LRR) domain, a function to find domain (FIIND), a caspase activating and recruitment domain (CARD) and a N-terminal pyrin domain (PYD) This figure shows how the anthrax factor activates the NLRP1b in mouse. First FIIND self-process starts for the NLRP1b to become mature and response for the anthrax lethal factor (LF). After that, direct or indirect activation takes place. Direct activation occurs when the N terminus is removed from the N terminal part that is cleaved by the lethal factor which leads to the activation of the NLRP1b. N terminal regulates the NLRP1b and keeps it off. This process subsequently leads to recruitment of the CARD domains which activate Caspase 1 (CASP1) and initiate the IL1B and IL-18 cleavage which end by programed cell death. The in directed activation initiate when unknown (X) factor which keep the NLRP1b off is cleaved by the lethal factor and affect the stabilization of the NLRP1b. This subsequently, break down the unknown factor by proteasome and lead to the activation which recruit the CASP1 and cleaves IL-1B and IL18 (Chavarría-Smith & Vance, 2015).

The NLRP1 gene is found in different mammals and takes on diverse forms. For instance, the mouse NLRP1b gene goes through two gene duplications which have resulted in three different paralogs, NLRP1A, NLRP1B and NLRP1C, in the mouse genome. Within the single paralog of NLRP1b, five allelic variants have been found and identified in different inbred mouse strains, with extensive protein coding differences among them (Boyden &

Dietrich, 2006). The NLRP1b gene is very diverse in mouse genomics and it has been found that the same gene codes only one paralog in humans. Therefore, the NLRP1 gene is considered to be one of the top 150 genes that goes through a process called signature selection, which is indicative of positive selection (George *et al.,* 2011)

Laboratory studies have found that NLRP1 gene activity causes *T. gondii* parasite resistance and susceptibility in rats (Cirelli *et al.,* 2014). Also, single nucleotide polymorphisms in the NLRP1 gene lead to susceptibility to congenital toxoplasmosis. Knocking down NLRP1 in human monocytes boosts *Toxoplasma* growth (Lees *et al*., 2010; Witola *et al.*, 2011). However, there are almost no studies of the role of the NLRP1b gene in wild animals. Since we had access to 126 samples of *Apodemus sylvaticus* that were used in previous studies and these had been tested for *Toxoplasma* infection (Morger *et al.*, 2014; Bajnok *et al*., 2015) it was of interest to study the NLRP1 gene locus in these animals.

4.2. Aim

The aims of this chapter are to sequence relevant fragments of the NLRP1b gene from the wood mouse, *Apodemus sylvaticus,* and investigate whether variations in the gene sequences in natural populations of wood mice are related to infection with the parasite *Toxoplasma gondii*.

4.3. Study Rationale

No information exists about the interactions between *Toxoplasma gondii* infections in natural populations of *Apodemus sylvaticus* and genetic variation in the NLRP1b gene. The availability of a population of wood mice well characterized for their parasite infections (Morger *et al*., 2014; Boyce *et al*., 2014) provided an opportunity to explore this further.

As NLRP1b is an important mechanism for detecting parasites and triggering the innate immune system, a broad hypothesis is that different variants of each NLRB1b strain might have a different efficiency of detection and therefore ability to resist infection. Furthermore, heterozygous mice, which have two variants, might have a greater ability to detect and deal with infection than homozygous mice. If these hypotheses are correct, one might expect certain variants to be associated with *T. gondii* infection or with a lack of infection. Furthermore, heterozygous mice might be more likely to have fewer infections than their homozygous counterparts. Looking at this from another perspective, parasite infection could be acting as a selective force driving NLRP1b variants or heterozygosity in wild populations, such as in *Apodemus sylvaticus*. The availability of this collection of wild mice with known parasite infections offers an opportunity to investigate these questions.

4.4. Objectives

1. Produce a map of the NLRP1b gene based on sequence information from *Mus musculus*.

2. Design primers for specific exons which could be used to amplify relevant sections of the homologous gene from *Apodemus sylvaticus*.

3. PCR amplification of specific regions of the NLRP1b from *Apodemus sylvaticus*.

4. PCR amplification of specific sections of the NLRP1b gene from a population of wood mice and determine sequence polymorphisms.

5. Analysis of DNA sequence variants across the wood mouse population and investigation of the relationship of NLRP1b gene variation with *Toxoplasma* infection.

4.5. Results

4.5.1. Produce a map of the NLRP1b gene from *Mus musculus*

In order to design different sets of primers to be able to amplify the NLRP1b gene from *Apodemus sylvaticus,* it was first necessary to search for sequences from a closely related species, *Mus musculus,* to produce a map of the mouse NLRP1b gene. This was done by searching the NCBI gene database using the search terms "*Mus*" and "NLRP1b". Several entries were found indicating that there were variants of the gene, or at least combinations of exons. The DNA sequence of the first of these (variant 1) is shown in Figure 4.2.

Nucleotide Sequence of NLRP1b variant 1 – MEQSQPKKK (NP_001035786.1)
Nucleotide Sequence (3525 nt):

ATGGAACAATCTCAGCCCAAGAAGAAAAGTCGCACAAAGGTGGCTCAGCATGAGGGTCAACTAAATTTGA
ACCCCACCTTCAAAACAAGGAAACGTAAAGAGGTGGAGCTGATGAAGCGCAGACCCAAGCCAGAGGGACA
CTTGAAGCTAGGGACAATTCCAAAAGTACACATAAAACAAAAAGGAGAGACTCTGGACCCCACATGGTCA
AGGAAAAGAAAGAATCTGGTCCAAAAGTTAACAAACCTACTACTCTTTGAAAAATTGTGCTCAAGAGGCT
CAGAAAACTTGATCAGGAAAAGCTGGTATTCATGTGAAGAAGAAGAAAGAGGACATATGATTGAGATCCA
AGACTTATTTGGCCCAAACAGAGGTACCCATAAAAAGCCTCAATTAGTCATAATAGAGGGTGCTGCTGGA
ATTGGGAAGTCAACACTGGCCAGGCAGGTGAAGAGAGCCTGGAAGGAAGGCCAGCTCTACAGGAATCACT
TCCAGCATGTCTTCTTCCTCAGTTGCAGGGAGCTGGCCCAGTATGAGCAGCTGAGTCTGGCTGAGCTCAT
AGCACAAGGCCAGGAAGTGCCCACCGTTCCCATCAGGCAGATCCTGTCTCACCCTAAGGAGCTGCTCTTC
ATCCTGGATGGCATAGATGAGCCAGCATGGGTCTTGGCAGACCAGAATCCTGAGCTATGTCTGCACTGGA
GTCAAAGACAGCCTGTGCACACTCTGCTGGGCAGTTTACTAGGGAAATCCATCCTTCCTGGGGCTTCCTT
CTTGCTCACAGCTCGGACCACAGCTCTACAGAAGATCATTCCTTATGTGGGGCAGCCACGTCGGGTGGAA
GTCCTGGGGTTCTCCAAGTTTGAACGGGAGGTCTATTTCCGCAAATATTTTGTGAAGGAGAGCGATGCAA
TTGCTGCTTTTAGATTGGTGGTATCTAACCCAGTGCTCCTGACCCTGTGTGAGGTTCCCTGGGTGTGCTG
GTTGGTCTGCACTTGCCTGAAAAAGCAGATGGAGCAGGGTGGAGAGCTCTCCCTGACCTCACAGACCACC
ACAGCCCTCTGCCTGAAATACCTTTCCCTGACAATCCCAGGGCAGCACATGAGGACCCAGCTCAGAGCCC
TCTGCTCACTGGCTGCTGAGGGGATCTGCCAAAGAAGAACCCTGTTCAGTGAAAGCGACCTCTGTAAGCA
AGGGTTAGCAGAGGATGCCATTGCCACTTTCCTGAAGATCGGTATCCTTCAAAAGCAGGCCAGCTCTCTG
AGCTACAGCTTTGCCCACTTGTGTCTCCAGGAGTTCTTTGCAGTCATGTCTTACATCTTGGAGGATAGTG
ATGAAAGATGTGATGGTATGGAATTCAAAAGAACTGTGGAAACACTGATAGAAGTATATGGAAGACATAC
TCTGTGTGAGGAACCCACTGTGCACTTTCTATTTGGTCTTGTGAATGAACAGGGAATGAGAGAAATGAAA
AAGATCTTTGACTGCAAACTGCCTTTGGGGACAGAGTTGAAGATGCTAAAGAGCACCCTAGGGAACCCAA
CCTATCAACATCATCTGGGTTTGCTCCACTGTCTGTATGAAAGTCAGGAAGAGGTGCTCCTGACATATGT
GTTATGTAATCTTCACTTAACAGGGCCTGACAAAAATTACATGGAAGCCACAGTGTCACAGACAAATGTG
AAGCACCTGGTGATACAGACAGATATGGAGCTCATGGTGGTCACTTTCTGCATTCAGTTCTGTTGTCACG
TGAGGAGTCTTCGGGTGAACATGAAAGGACAGCAGGGACATAAGCTGACAGTTGCCAGTATGGTTCTGTA
CAGGTGGACCCCAATCACTAATGCCAGTTGGAAGATTCTTTTCTACAATCTTAAATTCAACAGTAATCTG
GAGGGGTTGGACCTCAGTGGAAATCCACTGAGTTACTCTGCAGTGCAATATCTCTGTGATGCCATGATAT
ACCCTGGCTGCCAACTAAAGACTTTGTGGCTTGTTGAATGCGGCCTCACACCCACATACTGCTCACTCCT
GGCCTCAGTGCTCAGTGCCTGCTCCAGCCTGAGAGAGCTAGACCTGCAGCTAAATGACCTGTGTGACGAT
GGTGTAAGGATGCTGTGTGAGGGGCTCAGGAATCGTGCCTGCAACCTCAGAATCCTGCGGCTGGACCTGT
ACTCTCTCAGTGCCCAGGTGATTACAGAGCTCAGGACACTGGAGGAAAATAATCTGAAGCTGCACATCTC
CAGCATCTGGATGCCACAAATGATGGTCCCCACTGAGAACATGGATGAAGAAGATATCCTGACCTCATTC
AAGCAGCAGAGACAACAGTCAGGAGCCAATCCCATGGAAATTCTGGGGACTGAAGAAGACTTCTGGGGCC
CTATAGGACCTGTGGCTACTGAGGTGGTTTACAGAGAAAGGAACCTGTACCGAGTTCAATTGCCCATGGC
TGGTTCCTACCACTGTCCCAGCACAAGACTCCACTTTGTAGTGACAAGGGCAGTGACAATAGAGATCGAA
TTCTGTGCCTGGAGCCAATTCCTGGACAAGACTCCCTTGCAGCAGAGTCACATGGTGGTTGGACCTCTGT
TTGACATCAAGGCTGAGCAAGGAGCTGTGACTGCAGTGTACCTCCCTCATTTTGTGTCCCTCAAAGACAC
AAAGGCAAGCACATTTGACTTCAAGGTGGCCCACTTTCAAGAACATGGGATGGTTCTAGAAACGCCAGAT
AGGGTGAAGCCTGGATACACAGTACTGAAAAACCCAAGCTTCTCCCCAATGGGAGTTGTACTGAGAATAA
TCCCTGCTGCCCGGCACTTCATCCCCATTACTTCCATCACACTGATCTACTATCGAGTCAATCAGGAAGA
AGTCACCCTTCACCTCTACCTAGTCCCTAATGACTGCACCATACAGAAGGCCATAGATGATGAAGAAATG
AAGTTTCAGTTTGTGAGAATAAACAAGCCACCCCCAGTAGACAATCTTTTCATTGGCTCCCGCTACATTG
TGTCTGGTTCTGAGAACCTGGAAATCACCCCAAAGGAACTGGAGCTGTGCTACAGGAGCAGCAAGGAATT
CCAACTCTTCTCTGAGATATACGTTGGAAACATGGGTTCAGAGATTAAGCTGCAAATCAAAAACAAAAAG
CACATGAAACTCATATGGGAAGCCTTGCTGAAACCAGGAGACCTCAGACCTGCTCTGCCCAGGATTGCCC
AAGCCCTCAAAGATGCCCCTTCCTTGCTGCACTTCATGGACCAGCATCGGGAGCAGCTGGTAGCCCGAGT
GACATCAGTGGACCCTCTCTTGGACAAGCTGCATGGTCTGGTGCTGAATGAAGAATCCTATGAGGCAGTG
CGGGCTGAGAACACAAACCAAGATAAGATGAGGAAGCTCTTCAACCTCAGCCGGTCCTGGAGTCGAGCCT
GCAAAGACCTATTCTACCAAGCTCTGAAGGAGACCCATCCTCACCTGGTCATGGACCTCTTAGAAAAGTC
AGGTGGGGTCTCTTTGGGATCCTGA

Figure 4.2. DNA sequence of Variant 1 of the NLRP1b gene from *Mus musculus*. The blue regions indicate alternating exons and the black regions are other exons.

Variant 1 was found to be 3,525 nucleotides in length. It has a total of 15 exons. Figure 4.3 shows the protein translation for variant 1.

Translation (1174 aa) of Nucleotide Sequence of NLRP1b variant 1 – MEQSQPKKK
MEQSQPKKKSRTKVAQHEGQLNLNPTFKTRKRKEVELMKRRPKPEGHLKLGTIPKVHIKQKGETLDPTWS
RKRKNLVQKLTNLLLFEKLCSRGSENLIRKSWYSCEEEERGHMIEIQDLFGPNRGTHKKPQLVIIEGAAG
IGKSTLARQVKRAWKEGQLYRNHFQHVFFLSCRELAQYEQLSLAELIAQGQEVPTVPIRQILSHPKELLF
ILDGIDEPAWVLADQNPELCLHWSQRQPVHTLLGSLLGKSILPGASFLLTARTTALQKIIPYVGQPRRVE
VLGFSKFEREVYFRKYFVKESDAIAAFRLVVSNPVLLTLCEVPWVCWLVCTCLKKQMEQGGELSLTSQTT
TALCLKYLSLTIPGQHMRTQLRALCSLAAEGICQRRTLFSESDLCKQGLAEDAIATFLKIGILQKQASSL
SYSFAHLCLQEFFAVMSYILEDSDERCDGMEFKRTVETLIEVYGRHTLCEEPTVHFLFGLVNEQGMREMK
KIFDCKLPLGTELKMLKSTLGNPTYQHHLGLLHCLYESQEEVLLTYVLCNLHLTGPDKNYMEATVSQTNV
KHLVIQTDMELMVVTFCIQFCCHVRSLRVNMKGQQGHKLTVASMVLYRWTPITNASWKILFYNLKFNSNL
EGLDLSGNPLSYSAVQYLCDAMIYPGCQLKTLWLVECGLTPTYCSLLASVLSACSSLRELDLQLNDLCDD
GVRMLCEGLRNRACNLRILRLDLYSLSAQVITELRTLEENNLKLHISSIWMPQMMVPTENMDEEDILTSF
KQQRQQSGANPMEILGTEEDFWGPIGPVATEVVYRERNLYRVQLPMAGSYHCPSTRLHFVVTRAVTIEIE
FCAWSQFLDKTPLQQSHMVVGPLFDIKAEQGAVTAVYLPHFVSLKDTKASTFDFKVAHFQEHGMVLETPD
RVKPGYTVLKNPSFSPMGVVLRIIPAARHFIPITSITLIYYRVNQEEVTLHLYLVPNDCTIQKAIDDEEM
KFQFVRINKPPPVDNLFIGSRYIVSGSENLEITPKELELCYRSSKEFQLFSEIYVGNMGSEIKLQIKNKK
HMKLIWEALLKPGDLRPALPRIAQALKDAPSLLHFMDQHREQLVARVTSVDPLLDKLHGLVLNEESYEAV
RAENTNQDKMRKLFNLSRSWSRACKDLFYQALKETHPHLVMDLLEKSGGVSLGS

Figure 4.3. Protein sequence of Variant 1 of the NLRP1b gene from *Mus musculus*. The blue regions indicate amino acids from alternating exons. Red highlighting indicates amino acids encoded across a splice junction. The black regions indicate amino acids from other exons.

Variant 1 protein translation consists of 1,174 amino acids. The blue labelled amino acids in Figure 4.3 show the amino acids encoded by alternating exons. The second variant is shown as a DNA sequence in Figure 4.4.

Nucleotide Sequence of NLRP1b variant 2 – MEESQYKQ （NP_001155886.1)
Nucleotide Sequence (3534 nt):

ATGGAAGAATCTCAGTACAAGCAGGAACATAACAAAAAGGTAGCTCAGGATGAAGGTCAAGAGGACAAGG
ACACCATCTTCGAAACAATAGAAGCTATAGAGGCAAAGCTGATGGAGCTCAAAACCAACCCAGAGAGTAC
CTTCAATTATGGAATATTTCCAGAAGTATATATGAATCAGGGAGAAGAGATACTTTACCCAGCATGGTCA
TTGAAAGAGGAGAATTTGTTTCAAACTTTCAAAAGTCTACGACTGTTTCAAAAATTGTGCCCAAGAGGCT
CAGGGAACTTGGTCAAGAAAAGCTGGTATCCATGTGTACCAGAAGAAGGAGGACATATAATTAATATCCA
AGACTTATTTGGCCCAAATATAGGTACTCAGAAAGAGCCTCAATTAGTCATAATAGAAGGGGCTGCTGGG
ATTGGGAAGTCAACACTGGCCAGGCAGGTGAAGAGAGCCTGGATGGAAGGCGAGCTCTACAGGGATCACT
TCCAACATGTCTTCTTCTTCAGCTGCAGAGAGCTGGCCCAGTGCAAGAAGCTGAGTCTGGCTGAGCTCAT
AACACAAGGCCAGGATGTGCCCACAGCTCCCATAAATCAGATCCTGTCTCACCCTGAGAAGCTGCTCTTC
ATCCTGGATGGCATAGATGAGCCAGCATGGGTCTTGGCAGACCAGAATCCTGAGCTATGTCTGTACTGGA
GTCAAACACAGCCTGTGCACACACTACTGGGAAGTTTACTAGGGAAATCCATCCTTCCTGAGGCTTCCTT
CTTGCTCACAACTCGCACCACAGCTCTACAGAAGTTCATTCCTTCTTTGCCACAGTCATGTCAGGTGGAA
GTCCTAGGATTCAGTGATTTTGAACAGGAGATCTATATCTACAAATATTTTGCAAAGCAAATATTTGGAA
TTAAAGCTCTTATGATGGTTGAGTCTAACCCAGTTCTCCTGACCCTGTGTGAGGTTCCCTGGGTGTGCTG
GTTGGTCTGCAATTGCCTGAAAAAGCAGATGGAGCAGGGTGGAGATGTCTCCCTGACCTCACAGACAACC
ACAGCCATCTGCCTAAAATACATTTCCCTAACAATCCCAGTGCATCATATGAGGACCCAGCTCAGAGCCC
TCTGCTCATTGGCTGCTGAAGGCATCTGGAAAAGAAGGACCCTGTTCAGTGAAAGCGACCTCTGTAAGCA
AGGGTTAGACGAGGATGCCGTTGCCATTTTCCTGAAGACTGGTGTCCTTCAAAAGCAGGCCAGCTCTCTG
AGCTATAGCTTTGCCCACTTGTGTCTCCAGGAGTTCTTTGCATCAATGTCTTGCATCTTGGAGGATAGTG
AGGAACGACATGGTGATATGGAAATGGACAGAATTGTGGAAACACTGGTAGAACGGTATGGAAGACAAAA
CCTGTTTGAGGCACCCACTGTGCGCTTTCTCTTTGGTCTTTTGAGTAAAGAGGGATTGAAAGAAATGGAA
AAGCTCTTTTCCTGCAGCCTTCCTGGGAAGACAAAGTTGAAACTACTATGGCACATCCTTGGGAAATCCC
AACCCCATCAACCACCTTGCCTGGGTTTGCTCCACTGTCTATATGAAAATCAGGATATGAAGCTCCTGAC
ACATGTGATGCATGATCTTCAAGGAACAATAGTGCCTGACACAGATGACATTACACACACAGTGTTGCAG
ACAAATGTGAAGCACCTGGTGGTACGGACAGACATGGAGCTCATGGTGGTCACTTTCTGCATTCAGTTCT
GCTCTCACATGAGGAGTCTACAGCTGAATATGGAAGGACAACAAGGATATGCACTGACAGCCCCAAGGAT
GGTTCTGTACAGGTGGACCCCAATCACTAATGCCAGTTGGAAGATTCTTTTCTACAATCTTAAATTCAAC
AGTAATCTGGAGGGGTTGGACCTCAGTGGAAATCCACTGAGTTACTCTGCAGTGCAATATCTCTGTGATG
CCATGATATACCCTGGCTGCCAACTAAAGACTTTGTGGCTTGTTGAATGCGGCCTCACACCCACATACTG
CTCACTCCTGGCCTCAGTGCTCAGTGCCTGCTCCAGCCTGAGAGAGCTAGACCTGCAGCTAAATGACCTG
TGTGACGATGGTGTAAGGATGCTGTGTGAGGGGCTCAGGAATCGTGCCTGCAACCTCAGAATCCTGCGGC
TGGACCTGTACTCTCTCAGTGCCCAGGTGATTACAGAGCTCAGGACACTGGAGGAAAATAATCTGAAGCT
GCACATCTCCAGCATCTGGATGCCACAAATGATGGTCCCCACTGAGAACATGGATGAAGAAGATATCCTG
ACCTCATTCAAGCAGCAGAGACAACAGTCAGGAGCCAATCCCATGGAAATTCTGGGGACTGAAGAAGACT
TCTGGGGCCCTATAGGACCTGTGGCTACTGAGGTGGTTTACAGAGAAAGGAACCTGTACCGAGTTCAATT
GCCCATGGCTGGTTCCTACCACTGTCCCAGCACAAGACTCCACTTTGTAGTGACAAGGGCAGTGACAATA
GAGATCGAATTCTGTGCCTGGAGCCAATTCCTGGACAAGACTCCCTTGCAGCAGAGTCACATGGTGGTTG
GACCTCTGTTTGACATCAAGGCTGAGCAAGGAGCTGTGACTGCAGTGTACCTCCCTCATTTTGTGTCCCT
CAAAGACACAAAGGCAAGCACATTTGACTTCAAGGTGGCCCACTTTCAAGAACATGGGATGGTTCTAGAA
ACGCCAGATAGGGTGAAGCCTGGATACACAGTACTGAAAAACCCAAGCTTCTCCCCAATGGGAGTTGTAC
TGAGAATAATCCCTGCTGCCCGGCACTTCATCCCCATTACTTCCATCACACTGATCTACTATCGAGTCAA
TCAGGAAGAAGTCACCCTTCACCTCTACCTAGTCCCTAATGACTGCACCATACAGAAGGCCATAGATGAT
GAAGAAATGAAGTTTCAGTTTGTGAGAATAAACAAGCCACCCCCAGTAGACAATCTTTTCATTGGCTCCC
GCTACATTGTGTCTGGTTCTGAGAACCTGGAAATCACCCCAAAGGAACTGGAGCTGTGCTACAGGAGCAG
CAAGGAATTCCAACTCTTCTCTGAGATATACGTTGGAAACATGGGTTCAGAGATTAAGCTGCAAATCAAA
AACAAAAAGCACATGAAACTCATATGGGAAGCCTTGCTGAAACCAGGAGACCTCAGACCTGCTCTGCCCA
GGATTGCCCAAGCCCTCAAAGATGCCCCTTCCTTGCTGCACTTCATGGACCAGCATCGGGAGCAGCTGGT
AGCCCGAGTGACATCAGTGGACCCTCTCTTGGACAAGCTGCATGGTCTGGTGCTGAATGAAGAATCCTAT
GAGGCAGTGCGGGCTGAGAACACAAACCAAGATAAGATGAGGAAGCTCTTCAACCTCAGCCGGTCCTGGA
GTCGAGCCTGCAAAGACCTATTCTACCAAGCTCTGAAGGAGACCCATCCTCACCTGGTCATGGACCTCTT
AGAAAAGTCAGGTGGGGTCTCTTTGGGATCCTGA

Figure 4.4. DNA sequence of Variant 2 of the NLRP1b gene from *Mus musculus*. The blue regions indicate alternating exons.

Variant 2 was found to be 3,525 nucleotides in length. It has a total of 13 exons. Figure 4.5 shows the protein translation for variant 2.

Translation (1177 aa) of Nucleotide Sequence of NLRP1b variant 2 – MEESQYKQ

MEESQYKQEHNKKVAQDEGQEDKDTIFETIEAIEAKLMELKTNPESTFNYGIFPEVYMNQGEEILYPAWS
LKEENLFQTFKSLRLFQKLCPRGSGNLVKKSWYPCVPEEGGHIINIQDLFGPNIGTQKEPQLVIIEGAAG
IGKSTLARQVKRAWMEGELYRDHFQHVFFFSCRELAQCKKLSLAELITQGQDVPTAPINQILSHPEKLLF
ILDGIDEPAWVLADQNPELCLYWSQTQPVHTLLGSLLGKSILPEASFLLTTRTTALQKFIPSLPQSCQVE
VLGFSDFEQEIYIYKYFAKQIFGIKALMMVESNPVLLTLCEVPWVCWLVCNCLKKQMEQGGDVSLTSQTT
TAICLKYISLTIPVHHMRTQLRALCSLAAEGIWKRRTLFSESDLCKQGLDEDAVAIFLKTGVLQKQASSL
SYSFAHLCLQEFFASMSCILEDSEERHGDMEMDRIVETLVERYGRQNLFEAPTVRFLFGLLSKEGLKEME
KLFSCSLPGKTKLKLLWHILGKSQPHQPPCLGLLHCLYENQDMKLLTHVMHDLQGTIVPDTDDITHTVLQ
TNVKHLVVRTDMELMVVTFCIQFCSHMRSLQLNMEGQQGYALTAPRMVLYRWTPITNASWKILFYNLKFN
SNLEGLDLSGNPLSYSAVQYLCDAMIYPGCQLKTLWLVECGLTPTYCSLLASVLSACSSLRELDLQLNDL
CDDGVRMLCEGLRNRACNLRILRLDLYSLSAQVITELRTLEENNLKLHISSIWMPQMMVPTENMDEEDIL
TSFKQQRQQSGANPMEILGTEEDFWGPIGPVATEVVYRERNLYRVQLPMAGSYHCPSTRLHFVVTRAVTI
EIEFCAWSQFLDKTPLQQSHMVVGPLFDIKAEQGAVTAVYLPHFVSLKDTKASTFDFKVAHFQEHGMVLE
TPDRVKPGYTVLKNPSFSPMGVVLRIIPAARHFIPITSITLIYYRVNQEEVTLHLYLVPNDCTIQKAIDD
EEMKFQFVRINKPPPVDNLFIGSRYIVSGSENLEITPKELELCYRSSKEFQLFSEIYVGNMGSEIKLQIK
NKKHMKLIWEALLKPGDLRPALPRIAQALKDAPSLLHFMDQHREQLVARVTSVDPLLDKLHGLVLNEESY
EAVRAENTNQDKMRKLFNLSRSWSRACKDLFYQALKETHPHLVMDLLEKSGGVSLGS

Figure 4.5. Protein sequence of variant 2 of the NLRP1b gene from *Mus musculus*. The black and blue regions indicate alternating exons. Red highlighting indicates amino acids encoded across a splice junction.

Variant 2 protein translation consists of 1,177 amino acids. There are three nucleotides difference between variant one and variant two which subsequently affect the number of the amino acids that transcript form each variant. Also there is some variation between both variants which is shown in next figure (Figure 4.6).

```
V1      MEQSQPKKKSRTKVAQHEGQLNLNPTFKTRKRKEVELMKRRPKPEGHLKLGTIPKVHIKQ        60
V2      MEESQYKQEHNKKVAQDEGQEDKDTIFETIEAIEAKLMELKTNPESTFNYGIFPEVYMNQ        60
        **:** *:: ..****.*** : :  *:* :  *.:**: : :**. :: * :*:*:::*

V1      KGETLDPTWSRKRKNLVQKLTNLLLFEKLCSRGSENLIRKSWYSCEEEERGHMIEIQDLF        120
V2      GEEILYPAWSLKEENLFQTFKSLRLFQKLCPRGSGNLVKKSWYPCVPEEGGHIINIQDLF        120
          * * *:** *.:**.*.:..* **:*** *** **::**** *  ** **:*:*****

V1      GPNRGTHKKPQLVIIEGAAGIGKSTLARQVKRAWKEGQLYRNHFQHVFFLSCRELAQYEQ        180
V2      GPNIGTQKEPQLVIIEGAAGIGKSTLARQVKRAWMEGELYRDHFQHVFFFSCRELAQCKK        180
        *** **:*:************************* **:***:*******:******* ::

V1      LSLAELIAQGQEVPTVPIRQILSHPKELLFILDGIDEPAWVLADQNPELCLHWSQRQPVH        240
V2      LSLAELITQGQDVPTAPINQILSHPEKLLFILDGIDEPAWVLADQNPELCLYWSQTQPVH        240
        *******:***:***.**.******::************************:*** ****

V1      TLLGSLLGKSILPGASFLLTARTTALQKIIPYVGQPRRVEVLGFSKFEREVYFRKYFVKE        300
V2      TLLGSLLGKSILPEASFLLTTRTTALQKFIPSLPQSCQVEVLGFSDFEQEIYIYKYFAKQ        300
        ************* ******:*******:** : *  :*******.**:*:*: ***.*:

V1      SDAIAAFRLVVSNPVLLTLCEVPWVCWLVCTCLKKQMEQGGELSLTSQTTTALCLKYLSL        360
V2      IFGIKALMMVESNPVLLTLCEVPWVCWLVCNCLKKQMEQGGDVSLTSQTTTAICLKYISL        360
          .* *: :* ******************.**********::*********:****:**

V1      TIPGQHMRTQLRALCSLAAEGICQRRTLFSESDLCKQGLAEDAIATFLKIGILQKQASSL        420
V2      TIPVHHMRTQLRALCSLAAEGIWKRRTLFSESDLCKQGLDEDAVAIFLKTGVLQKQASSL        420
        *** :***************** :*************** ***:* *** *:********

V1      SYSFAHLCLQEFFAVMSYILEDSDERCDGMEFKRTVETLIEVYGRHTLCEEPTVHFLFGL        480
V2      SYSFAHLCLQEFFASMSCILEDSEERHGDMEMDRIVETLVERYGRQNLFEAPTVRFLFGL        480
        ************** ** *****:** ..**:.* ****:* ***:.* * ***:*****

V1      VNEQGMREMKKIFDCKLPLGTELKMLKSTLGN--PTYQHHLGLLHCLYESQEEVLLTYVL        538
V2      LSKEGLKEMEKLFSCSLPGKTKLKLLWHILGKSQPHQPPCLGLLHCLYENQDMKLLTHVM        540
        :.::*::**:*:*.*.**  *:**:*   **:  *     *********.*:  ***:*:

V1      CNLHL-TGPDKNYMEATVSQTNVKHLVIQTDMELMVVTFCIQFCCHVRSLRVNMKGQQGH        597
V2      HDLQGTIVPDTDDITHTVLQTNVKHLVVRTDMELMVVTFCIQFCSHMRSLQLNMEGQQGY        600
         :*:    **.: :  ** ********::***************.*:***::**:****:

V1      KLTVASMVLYRWTPITNASWKILFYNLKFNSNLEGLDLSGNPLSYSAVQYLCDAMIYPGC        657
V2      ALTAPRMVLYRWTPITNASWKILFYNLKFNSNLEGLDLSGNPLSYSAVQYLCDAMIYPGC        660
         **.  ************************************************

V1      QLKTLWLVECGLTPTYCSLLASVLSACSSLRELDLQLNDLCDDGVRMLCEGLRNRACNLR        717
V2      QLKTLWLVECGLTPTYCSLLASVLSACSSLRELDLQLNDLCDDGVRMLCEGLRNRACNLR        720
        ************************************************************

V1      ILRLDLYSLSAQVITELRTLEENNLKLHISSIWMPQMMVPTENMDEEDILTSFKQQRQQS        777
V2      ILRLDLYSLSAQVITELRTLEENNLKLHISSIWMPQMMVPTENMDEEDILTSFKQQRQQS        780
        ************************************************************

V1      GANPMEILGTEEDFWGPIGPVATEVVYRERNLYRVQLPMAGSYHCPSTRLHFVVTRAVTI        837
V2      GANPMEILGTEEDFWGPIGPVATEVVYRERNLYRVQLPMAGSYHCPSTRLHFVVTRAVTI        840
        ************************************************************

V1      EIEFCAWSQFLDKTPLQQSHMVVGPLFDIKAEQGAVTAVYLPHFVSLKDTKASTFDFKVA        897
V2      EIEFCAWSQFLDKTPLQQSHMVVGPLFDIKAEQGAVTAVYLPHFVSLKDTKASTFDFKVA        900
        ************************************************************



V1      HFQEHGMVLETPDRVKPGYTVLKNPSFSPMGVVLRIIPAARHFIPITSITLIYYRVNQEE        957
V2      HFQEHGMVLETPDRVKPGYTVLKNPSFSPMGVVLRIIPAARHFIPITSITLIYYRVNQEE        960
        ************************************************************
```

```
V1      VTLHLYLVPNDCTIQKAIDDEEMKFQFVRINKPPPVDNLFIGSRYIVSGSENLEITPKEL        1017
V2      VTLHLYLVPNDCTIQKAIDDEEMKFQFVRINKPPPVDNLFIGSRYIVSGSENLEITPKEL        1020
        ************************************************************

V1      ELCYRSSKEFQLFSEIYVGNMGSEIKLQIKNKKHMKLIWEALLKPGDLRPALPRIAQALK        1077
V2      ELCYRSSKEFQLFSEIYVGNMGSEIKLQIKNKKHMKLIWEALLKPGDLRPALPRIAQALK        1080
        ************************************************************

V1      DAPSLLHFMDQHREQLVARVTSVDPLLDKLHGLVLNEESYEAVRAENTNQDKMRKLFNLS        1137
V2      DAPSLLHFMDQHREQLVARVTSVDPLLDKLHGLVLNEESYEAVRAENTNQDKMRKLFNLS        1140
        ************************************************************

V1      RSWSRACKDLFYQALKETHPHLVMDLLEKSGGVSLGS      1174
V2      RSWSRACKDLFYQALKETHPHLVMDLLEKSGGVSLGS      1177
        ***********************************
```

Figure 4.6. Clustal alignment between NLRP1b protein variants 1 and 2 showing the differences between them.

Variants 1 and 2 of NLRP1b have some differences between them. As shown in Figure 4.6, many amino acids differ between the two variants and also, variant 1 is missing two amino acids located at positions 512 and 513, which makes the total length of the protein sequence 1,174 amino acids.

In conclusion, this sequence forms a starting point for primer design. Figure 4.7 shows a genomic map for NLRP1b variants 1 and 2 from *Mus musculus* (mouse).

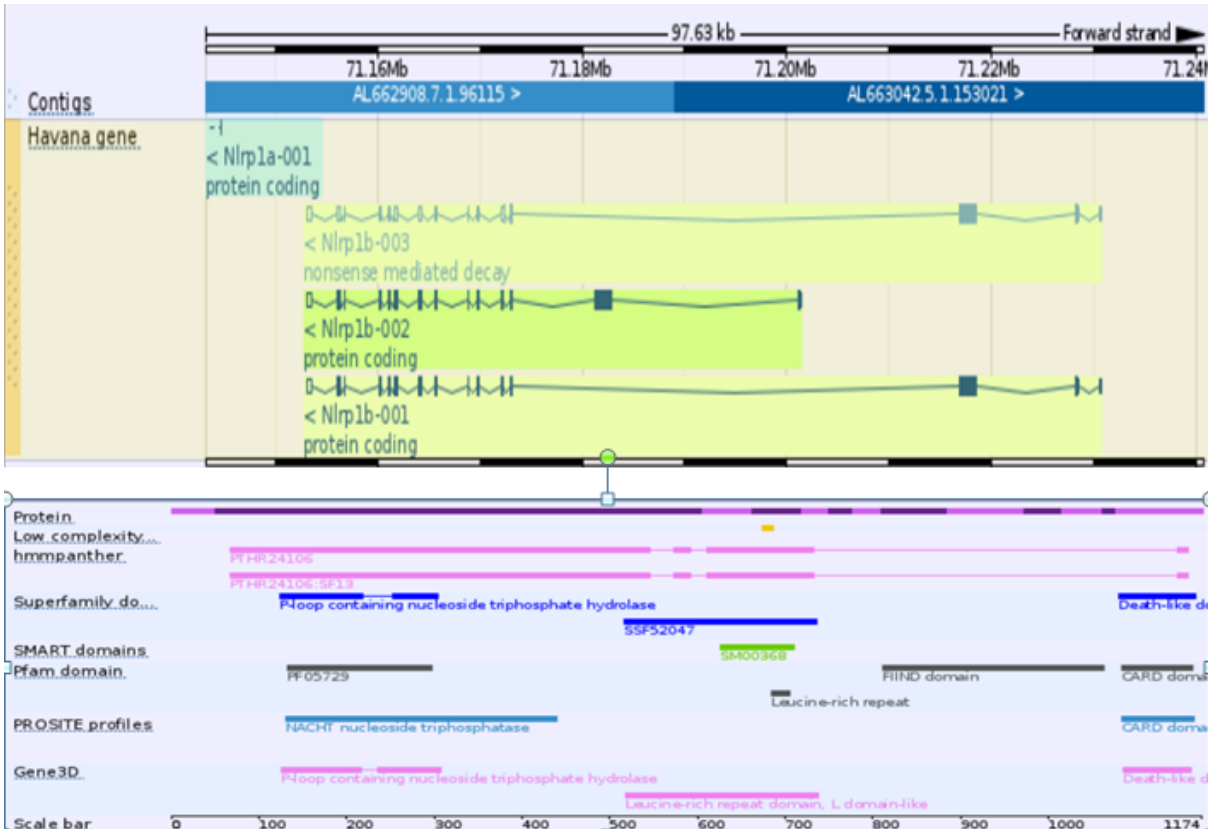

Figure 4.7. Map of NLRP1b gene variants 1 and 2 that shows exons and introns. The dark blue regions are the exons while the light blue ones are the introns. Also, it shows the location of the protein domains.

NLRP1b consists of 15 exons and 14 introns. The tables below (Tables 4.1 and 4.2) show the

domains of each variant, their locations in the variants and their accession numbers.

Table 4.1 Start and end of each protein domain of NLRP1b variant 1

Start	End	Protein domain	Accession
1084	1164	CARD domain	PF00619
1084	1167	CARD domain	PS50209
1080	1168	Death-like domain	SSF47986
1085	1163	Death-like domain	1.10.533.10
812	1064	FIIND domain	PF13553
684	706	Leucine-rich repeat	PF13516
519	738	Leucine-rich repeat domain, L domain-like	3.80.10.10
131	440	NACHT nucleoside triphosphatase	PS50837
124	218	P-loop containing nucleoside triphosphate hydrolase	SSF52540
126	141	P-loop containing nucleoside triphosphate hydrolase	3.40.50.300
142	216	P-loop containing nucleoside triphosphate hydrolase	3.40.50.300
236	308	P-loop containing nucleoside triphosphate hydrolase	3.40.50.300
253	304	P-loop containing nucleoside triphosphate hydrolase	SSF52540

Table 4.2 Start and end of each protein domain of NLRP1b variant 2

Start	End	Protein domain	Accession
1087	1167	CARD domain	PF00619
1087	1170	CARD domain	PS50209
1083	1171	Death-like domain	SSF47986
1088	1166	Death-like domain	1.10.533.10
815	1067	FIIND domain	PF13553
687	709	Leucine-rich repeat	PF13516
521	741	Leucine-rich repeat domain, L domain-like	3.80.10.10
131	440	NACHT nucleoside triphosphatase	PS50837
30	46	P-loop containing nucleoside triphosphate hydrolase	3.40.50.300
126	218	P-loop containing nucleoside triphosphate hydrolase	SSF52540
130	301	P-loop containing nucleoside triphosphate hydrolase	3.40.50.300
253	292	P-loop containing nucleoside triphosphate hydrolase	SSF52540

4.5.2 Designing primers for specific regions of *Apodemus sylvaticus* and amplification of sections of the NLRP1b gene from *Mus musculus* and *Apodemus sylvaticus*.

4.5.2.1 Initial primer design and amplification of sections of the NLRP1b gene.

After finding two variants of the NLRP1b gene of *Mus musculus* (laboratory mice), DNA alignments were carried out using ClustalW, as described in Section 2.8, to check the similarities between the two variants (Figure 4.8). These two variants contain the NACHT domain, leucine rich repeats and PYD domains. Four primers were initially designed to cover exon 2 and exon 3 part-one, exon 3 part 2 and exon 3 part 3 (Table 4.3). The area shaded yellow in Figure 4.8 indicates the forward and reverse primer sequence of exon 2, while the dark green shaded area indicates the forward and reverse primer sequence of exon 3 part one. The fuchsia shaded area represents the forward and reverse primer sequence of exon 3 part 2 and the aqua shaded area represents the forward and reverse primer sequence of exon 3 part 3 (Figure 4.8). These primers were chosen from certain positions for a number of reasons. For example, some positions were more stable than others and contained high G and C content. Also, the difference between the melting temperatures of the forward and reverse primers is very low, which will be helpful during the PCR reaction. Another reason is to find some position that has variability between variants one and two.

DNA Clustal Alignments

```
                        Exon 2 Forward
nlrp1bvar1      ATGGAACAATCTCAGCCCAAGAAGAAAAGTCGCACAAAGGTGGCTCAGCATGAGGGTCAA 60
nlrp1bvar2      ATGGAAGAATCTCAGTACAAGCAGGAACATAACAAAAAGGTAGCTCAGGATGAAGGTCAA 60
                ****** ******** .****.**.**..*..**.******.****** ****.******

nlrp1bvar1      CTAAATTTGAACCCCACCTTCAAAACAAGGAAACGTAAAGAGGTGGAGCTGATGAAGCGC 120
nlrp1bvar2      GAGGACAAGGACACCATCTTCGAAACAATAGAAGCTATAGAGGCAAAGCTGATGGAGCTC 120
                 :..* ::*.**.*** ****.****** ..**  **:***** ..********.*** *

                        Exon2 reverse      Start of Exon3 -Exon spanning AAs
nlrp1bvar1      AGACCCAAGCCAGAGGGACACTTGAAGCTAGGGACAATTCCAAAAGTACACATAAAACAA 180
nlrp1bvar2      AAAACCAACCCAGAGAGTACCTTCAATTATGGAATATTTCCAGAAGTATATATGAATCAG 180
                *.*.**** ******.*:..*** **  ::**.* *:*****.***** * **.**:**.
                                                    Exon 3 Part 1 Forward

nlrp1bvar1      AAAGGAGAGACTCTGGACCCCACATGGTCAAGGAAAAGAAAGAATCTGGTCCAAAAGTTA 240
nlrp1bvar2      GGAGAAGAGATACTTTACCCAGCATGGTCATTGAAAGAGGAGAATTTGTTTCAAACTTTC 240
                ..**.***** :**  ****..********: ****....***** ** * ****. **.

nlrp1bvar1      ACAAACCTACTACTCTTTGAAAAATTGTGCTCAAGAGGCTCAGAAAACTTGATCAGGAAA 300
nlrp1bvar2      AAAAGTCTACGACTGTTTCAAAAATTGTGCCCAAGAGGCTCAGGGAACTTGGTCAAGAAA 300
                *.**. **** *** *** *********** ************..******.***.****

nlrp1bvar1      AGCTGGTATTCATGTGAAGAAGAAGAAAGAGGACATATGATTGAGATCCAAGACTTATTT 360
nlrp1bvar2      AGCTGGTATCCATGTGTACCAGAAGAAGGAGGACATATAATTAATATCCAAGACTTATTT 360
                ********* ******:* .*******.**********.***.* ***************

nlrp1bvar1      GGCCCAAACAGAGGTACCCATAAAAAGCCTCAATTAGTCATAATAGAGGGTGCTGCTGGA      420
nlrp1bvar2      GGCCCAAATATAGGTACTCAGAAAGAGCCTCAATTAGTCATAATAGAAGGGGCTGCTGGG      420
                ******** * ****** ** ***.**********************.** ********.

nlrp1bvar1      ATTGGGAAGTCAACACTGGCCAGGCAGGTGAAGAGAGCCTGGAAGGAAGGCCAGCTCTAC      480
nlrp1bvar2      ATTGGGAAGTCAACACTGGCCAGGCAGGTGAAGAGAGCCTGGATGGAAGGCGAGCTCTAC      480
                ********************************************:******* ********

                                                                Exon 3 Part 2 Forward
nlrp1bvar1      AGGAATCACTTCCAGCATGTCTTCTTCCTCAGTTGCAGGGAGCTGGCCCAGTATGAGCAG      540
nlrp1bvar2      AGGGATCACTTCCAACATGTCTTCTTCTTCAGCTGCAGAGAGCTGGCCCAGTGCAAGAAG      540
                ***.**********.************ **** *****.*************. .**.**
                                                    Exon 3-2N F

nlrp1bvar1      CTGAGTCTGGCTGAGCTCATAGCACAAGGCCAGGAAGTGCCCACCGTTCCCATCAGGCAG      600
nlrp1bvar2      CTGAGTCTGGCTGAGCTCATAACACAAGGCCAGGATGTGCCCACAGCTCCCATAAATCAG      600
                **********************.*************:********.* ******.*. ***


nlrp1bvar1      ATCCTGTCTCACCCTAAGGAGCTGCTCTTCATCCTGGATGGCATAGATGAGCCAGCATGG      660
nlrp1bvar2      ATCCTGTCTCACCCTGAGAAGCTGCTCTTCATCCTGGATGGCATAGATGAGCCAGCATGG      660
                ***************.**.****************************************

                        Exon 3 Part 1 Reverse
nlrp1bvar1      GTCTTGGCAGACCAGAATCCTGAGCTATGTCTGCACTGGAGTCAAAGACAGCCTGTGCAC      720
nlrp1bvar2      GTCTTGGCAGACCAGAATCCTGAGCTATGTCTGTACTGGAGTCAAACACAGCCTGTGCAC      720
                ******************************** ************ *************

nlrp1bvar1      ACTCTGCTGGGCAGTTTACTAGGGAAATCCATCCTTCCTGGGGCTTCCTTCTTGCTCACA      780
nlrp1bvar2      ACACTACTGGGAAGTTTACTAGGGAAATCCATCCTTCCTGAGGCTTCCTTCTTGCTCACA      780
                **:**.*****.****************************.******************

nlrp1bvar1      GCTCGGACCACAGCTCTACAGAAGATCATTCCTTATGTGGGGCAGCCACGTCGGGTGGAA      840
nlrp1bvar2      ACTCGCACCACAGCTCTACAGAAGTTCATTCCTTCTTTGCCACAGTCATGTCAGGTGGAA      840
                .**** ******************:*********.* **  .*** ** ***.*******

nlrp1bvar1      GTCCTGGGGTTCTCCAAGTTTGAACGGGAGGTCTATTTCCGCAAATATTTTGTGAAGGAG      900
nlrp1bvar2      GTCCTAGGATTCAGTGATTTTGAACAGGAGATCTATATCTACAAATATTTTGCAAAGCAA      900
                *****.**.***:  .* *******.****.*****:** .*********** .*** *.

nlrp1bvar1      AGCGATGCAATTGCTGCTTTTAGATTGGTGGTATCTAACCCAGTGCTCCTGACCCTGTGT      960
nlrp1bvar2      ATATTTGGAATTAAAGCTCTTATGATGGTTGAGTCTAACCCAGTTCTCCTGACCCTGTGT      960
                * . :** ****..:*** *** .:**** *:.*********** ***************

nlrp1bvar1      GAGGTTCCCTGGGTGTGCTGGTTGGTCTGCACTTGCCTGAAAAAGCAGATGGAGCAGGGT     1020
nlrp1bvar2      GAGGTTCCCTGGGTGTGCTGGTTGGTCTGCAATTGCCTGAAAAAGCAGATGGAGCAGGGT     1020
                ******************************.****************************

nlrp1bvar1      GGAGAGCTCTCCCTGACCTCACAGACCACCACAGCCCTCTGCCTGAAATACCTTTCCCTG     1080
nlrp1bvar2      GGAGATGTCTCCCTGACCTCACAGACAACCACAGCCATCTGCCTAAAATACATTTCCCTA     1080
                *****  ******************.*********.*******.******.*******.
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nlrp1bvar1      ACAATCCCAGGGCAGCACATGAGGACCCAGCTCAGAGCCCTCTGCTCACTGGCTGCTGAG      1140
nlrp1bvar2      ACAATCCCAGTGCATCATATGAGGACCCAGCTCAGAGCCCTCTGCTCATTGGCTGCTGAA      1140
                ********** *** ** ****************************** **********.



nlrp1bvar1      GGGATCTGCCAAAGAAGAACCCTGTTCAGTGAAAGCGACCTCTGTAAGCAAGGGTTAGCA      1200
nlrp1bvar2      GGCATCTGGAAAAGAAGGACCCTGTTCAGTGAAAGCGACCTCTGTAAGCAAGGGTTAGAC      1200
                ** ***** .*******.****************************************..

                    Exon3 part 3 forward                    Exon3-3N forward
nlrp1bvar1      GAGGATGCCATTGCCACTTTCCTGAAGATCGGTATCCTTCAAAAGCAGGCCAGCTCTCTG      1260
nlrp1bvar2      GAGGATGCCGTTGCCATTTTCCTGAAGACTGGTGTCCTTCAAAAGCAGGCCAGCTCTCTG      1260
                *********.****** ***********  ***.**************************

                                  Exon 3 part 2 reverse
nlrp1bvar1      AGCTACAGCTTTGCCCACTTGTGTCTCCAGGAGTTCTTTGCAGTCATGTCTTACATCTTG      1320
nlrp1bvar2      AGCTATAGCTTTGCCCACTTGTGTCTCCAGGAGTTCTTTGCATCAATGTCTTGCATCTTG      1320
                ***** ********************************  .*******.*******

nlrp1bvar1      GAGGATAGTGATGAAAGATGTGATGGTATGGAATTCAAAAGAACTGTGGAAACACTGATA      1380
nlrp1bvar2      GAGGATAGTGAGGAACGACATGGTGATATGGAAATGGACAGAATTGTGGAAACACTGGTA      1380
                *********** ***.** .**.**.*******:* .*.**** *************.**

nlrp1bvar1      GAAGTATATGGAAGACATACTCTGTGTGAGGAACCCACTGTGCACTTTCTATTTGGTCTT      1440
nlrp1bvar2      GAACGGTATGGAAGACAAAACCTGTTTGAGGCACCCACTGTGCGCTTTCTCTTTGGTCTT      1440
                ***  .***********:*. **** ***** .***********.*******.*********

nlrp1bvar1      GTGAATGAACAGGGAATGAGAGAAATGAAAAAGATCTTTGACTGCAAACTGCCTTTGGGG      1500
nlrp1bvar2      TTGAGTAAAGAGGGATTGAAAGAAATGGAAAAGCTCTTTTCCTGCAGCCTTCCTGGGAAG      1500
                 ***.*.** *****:***.*******.*****.***** .*****..** ***  *..*

nlrp1bvar1      ACAGAGTTGAAGATGCTAAAGAGCACCCTAGGGAAC--CCAAC-CTATC---AACATCAT      1554
nlrp1bvar2      ACAAAGTTGAAACTACTATGGCACATCCTTGGGAAATCCCAACCCCATCAACCACCTTGC      1560
                ***.*******..*.***:.*..** ***:*****.  ***** * ***   .**.* .

nlrp1bvar1      CTGGGTTTGCTCCACTGTCTGTATGAAAGTCAGGAAGAGGTGCTCCTGACATATGTGTTA      1614
nlrp1bvar2      CTGGGTTTGCTCCACTGTCTATATGAAAATCAGGATATGAAGCTCCTGACACATGTGATG      1620
                ********************.*******.******:.:*.:********** *****:*.

nlrp1bvar1      TGTAATCTTCACTTA---ACAGGGCCTGACAAAAATTACATGGAAGCCACAGTGTCACAG      1671
nlrp1bvar2      CATGATCTTCAAGGAACAATAGTGCCTGACACAGATGACATTACACACACAGTGTTGCAG      1680
                 .*.*******.  *   * ** ********.*.** **** ..* .******** .***

                                                                Exon 3-3N Reverse
nlrp1bvar1      ACAAATGTGAAGCACCTGGTGATACAGACAGATATGGAGCTCATGGTGGTCACTTTCTGC      1731
nlrp1bvar2      ACAAATGTGAAGCACCTGGTGGTACGGACAGACATGGAGCTCATGGTGGTCACTTTCTGC      1740
                ********************.***.****** **************************

nlrp1bvar1      ATTCAGTTCTGTTGTCACGTGAGGAGTCTTCGGGTGAACATGAAAGGACAGCAGGGACAT      1791
nlrp1bvar2      ATTCAGTTCTGCTCTCACATGAGGAGTCTACAGCTGAATATGGAAGGACAACAAGGATAT      1800
                *********** * ****.**********:*.* **** ***.*******.**.*** **

                      Exon 3 Part 3 Reverse          End of Exon 3
nlrp1bvar1      AAGCTGACAGTTGCCAGTATGGTTCTGTACAGGTGGACCCCAATCACTAATGCCAGTTGG      1851
nlrp1bvar2      GCACTGACAGCCCCAAGGATGGTTCTGTACAGGTGGACCCCAATCACTAATGCCAGTTGG      1860
                ...*******   *.** ****************************************

nlrp1bvar1      AAGATTCTTTTCTACAATCTTAAATTCAACAGTAATCTGGAGGGGTTGGACCTCAGTGGA      1911
nlrp1bvar2      AAGATTCTTTTCTACAATCTTAAATTCAACAGTAATCTGGAGGGGTTGGACCTCAGTGGA      1920
                ************************************************************

nlrp1bvar1      AATCCACTGAGTTACTCTGCAGTGCAATATCTCTGTGATGCCATGATATACCCTGGCTGC      1971
nlrp1bvar2      AATCCACTGAGTTACTCTGCAGTGCAATATCTCTGTGATGCCATGATATACCCTGGCTGC      1980
                ************************************************************

nlrp1bvar1      CAACTAAAGACTTTGTGGCTTGTTGAATGCGGCCTCACACCCACATACTGCTCACTCCTG      2031
nlrp1bvar2      CAACTAAAGACTTTGTGGCTTGTTGAATGCGGCCTCACACCCACATACTGCTCACTCCTG      2040
                ************************************************************

nlrp1bvar1      GCCTCAGTGCTCAGTGCCTGCTCCAGCCTGAGAGAGCTAGACCTGCAGCTAAATGACCTG      2091
nlrp1bvar2      GCCTCAGTGCTCAGTGCCTGCTCCAGCCTGAGAGAGCTAGACCTGCAGCTAAATGACCTG      2100
                ************************************************************

nlrp1bvar1      TGTGACGATGGTGTAAGGATGCTGTGTGAGGGGCTCAGGAATCGTGCCTGCAACCTCAGA      2151
nlrp1bvar2      TGTGACGATGGTGTAAGGATGCTGTGTGAGGGGCTCAGGAATCGTGCCTGCAACCTCAGA      2160
                ************************************************************

nlrp1bvar1      ATCCTGCGGCTGGACCTGTACTCTCTCAGTGCCCAGGTGATTACAGAGCTCAGGACACTG      2211
nlrp1bvar2      ATCCTGCGGCTGGACCTGTACTCTCTCAGTGCCCAGGTGATTACAGAGCTCAGGACACTG      2220
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                ************************************************************

nlrp1bvar1      GAGGAAAATAATCTGAAGCTGCACATCTCCAGCATCTGGATGCCACAAATGATGGTCCCC      2271
nlrp1bvar2      GAGGAAAATAATCTGAAGCTGCACATCTCCAGCATCTGGATGCCACAAATGATGGTCCCC      2280
                ************************************************************

nlrp1bvar1      ACTGAGAACATGGATGAAGAAGATATCCTGACCTCATTCAAGCAGCAGAGACAACAGTCA      2331
nlrp1bvar2      ACTGAGAACATGGATGAAGAAGATATCCTGACCTCATTCAAGCAGCAGAGACAACAGTCA      2340
                ************************************************************

nlrp1bvar1      GGAGCCAATCCCATGGAAATTCTGGGGACTGAAGAAGACTTCTGGGGCCCTATAGGACCT      2391
nlrp1bvar2      GGAGCCAATCCCATGGAAATTCTGGGGACTGAAGAAGACTTCTGGGGCCCTATAGGACCT      2400
                ************************************************************

nlrp1bvar1      GTGGCTACTGAGGTGGTTTACAGAGAAAGGAACCTGTACCGAGTTCAATTGCCCATGGCT      2451
nlrp1bvar2      GTGGCTACTGAGGTGGTTTACAGAGAAAGGAACCTGTACCGAGTTCAATTGCCCATGGCT      2460
                ************************************************************

nlrp1bvar1      GGTTCCTACCACTGTCCCAGCACAAGACTCCACTTTGTAGTGACAAGGGCAGTGACAATA      2511
nlrp1bvar2      GGTTCCTACCACTGTCCCAGCACAAGACTCCACTTTGTAGTGACAAGGGCAGTGACAATA      2520
                ************************************************************

nlrp1bvar1      GAGATCGAATTCTGTGCCTGGAGCCAATTCCTGGACAAGACTCCCTTGCAGCAGAGTCAC      2571
nlrp1bvar2      GAGATCGAATTCTGTGCCTGGAGCCAATTCCTGGACAAGACTCCCTTGCAGCAGAGTCAC      2580
                ************************************************************

nlrp1bvar1      ATGGTGGTTGGACCTCTGTTTGACATCAAGGCTGAGCAAGGAGCTGTGACTGCAGTGTAC      2631
nlrp1bvar2      ATGGTGGTTGGACCTCTGTTTGACATCAAGGCTGAGCAAGGAGCTGTGACTGCAGTGTAC      2640
                ************************************************************

nlrp1bvar1      CTCCCTCATTTTGTGTCCCTCAAAGACACAAAGGCAAGCACATTTGACTTCAAGGTGGCC      2691
nlrp1bvar2      CTCCCTCATTTTGTGTCCCTCAAAGACACAAAGGCAAGCACATTTGACTTCAAGGTGGCC      2700
                ************************************************************

nlrp1bvar1      CACTTTCAAGAACATGGGATGGTTCTAGAAACGCCAGATAGGGTGAAGCCTGGATACACA      2751
nlrp1bvar2      CACTTTCAAGAACATGGGATGGTTCTAGAAACGCCAGATAGGGTGAAGCCTGGATACACA      2760
                ************************************************************

nlrp1bvar1      GTACTGAAAAACCCAAGCTTCTCCCCAATGGGAGTTGTACTGAGAATAATCCCTGCTGCC      2811
nlrp1bvar2      GTACTGAAAAACCCAAGCTTCTCCCCAATGGGAGTTGTACTGAGAATAATCCCTGCTGCC      2820
                ************************************************************

nlrp1bvar1      CGGCACTTCATCCCCATTACTTCCATCACACTGATCTACTATCGAGTCAATCAGGAAGAA      2871
nlrp1bvar2      CGGCACTTCATCCCCATTACTTCCATCACACTGATCTACTATCGAGTCAATCAGGAAGAA      2880
                ************************************************************

nlrp1bvar1      GTCACCCTTCACCTCTACCTAGTCCCTAATGACTGCACCATACAGAAGGCCATAGATGAT      2931
nlrp1bvar2      GTCACCCTTCACCTCTACCTAGTCCCTAATGACTGCACCATACAGAAGGCCATAGATGAT      2940
                ************************************************************

nlrp1bvar1      GAAGAAATGAAGTTTCAGTTTGTGAGAATAAACAAGCCACCCCCAGTAGACAATCTTTTC      2991
nlrp1bvar2      GAAGAAATGAAGTTTCAGTTTGTGAGAATAAACAAGCCACCCCCAGTAGACAATCTTTTC      3000
                ************************************************************


nlrp1bvar1      ATTGGCTCCCGCTACATTGTGTCTGGTTCTGAGAACCTGGAAATCACCCCAAAGGAACTG      3051
nlrp1bvar2      ATTGGCTCCCGCTACATTGTGTCTGGTTCTGAGAACCTGGAAATCACCCCAAAGGAACTG      3060
                ************************************************************

nlrp1bvar1      GAGCTGTGCTACAGGAGCAGCAAGGAATTCCAACTCTTCTCTGAGATATACGTTGGAAAC      3111
nlrp1bvar2      GAGCTGTGCTACAGGAGCAGCAAGGAATTCCAACTCTTCTCTGAGATATACGTTGGAAAC      3120
                ************************************************************

nlrp1bvar1      ATGGGTTCAGAGATTAAGCTGCAAATCAAAAACAAAAAGCACATGAAACTCATATGGGAA      3171
nlrp1bvar2      ATGGGTTCAGAGATTAAGCTGCAAATCAAAAACAAAAAGCACATGAAACTCATATGGGAA      3180
                ************************************************************

nlrp1bvar1      GCCTTGCTGAAACCAGGAGACCTCAGACCTGCTCTGCCCAGGATTGCCCAAGCCCTCAAA      3231
nlrp1bvar2      GCCTTGCTGAAACCAGGAGACCTCAGACCTGCTCTGCCCAGGATTGCCCAAGCCCTCAAA      3240
                ************************************************************

nlrp1bvar1      GATGCCCCTTCCTTGCTGCACTTCATGGACCAGCATCGGGAGCAGCTGGTAGCCCGAGTG      3291
nlrp1bvar2      GATGCCCCTTCCTTGCTGCACTTCATGGACCAGCATCGGGAGCAGCTGGTAGCCCGAGTG      3300
                ************************************************************

nlrp1bvar1      ACATCAGTGGACCCTCTCTTGGACAAGCTGCATGGTCTGGTGCTGAATGAAGAATCCTAT      3351
nlrp1bvar2      ACATCAGTGGACCCTCTCTTGGACAAGCTGCATGGTCTGGTGCTGAATGAAGAATCCTAT      3360
                ************************************************************

nlrp1bvar1      GAGGCAGTGCGGGCTGAGAACACAAACCAAGATAAGATGAGGAAGCTCTTCAACCTCAGC      3411
nlrp1bvar2      GAGGCAGTGCGGGCTGAGAACACAAACCAAGATAAGATGAGGAAGCTCTTCAACCTCAGC      3420
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nlrp1bvar1      CGGTCCTGGAGTCGAGCCTGCAAAGACCTATTCTACCAAGCTCTGAAGGAGACCCATCCT      3471
nlrp1bvar2      CGGTCCTGGAGTCGAGCCTGCAAAGACCTATTCTACCAAGCTCTGAAGGAGACCCATCCT      3480
                ************************************************************

nlrp1bvar1      CACCTGGTCATGGACCTCTTAGAAAAGTCAGGTGGGGTCTCTTTGGGATCCTGA 3525
nlrp1bvar2      CACCTGGTCATGGACCTCTTAGAAAAGTCAGGTGGGGTCTCTTTGGGATCCTGA 3534
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Figure 4.8. Clustal alignment of variants 1 and variant 2 of the NLRP1b gene from *Mus musculus*. The red region indicates the start of exon 3, while the grey region indicates its end. The aqua shaded area is exon 3-3 forward and reverse. The dark purple shaded area is exon 3-3N forward and reverse. The green shaded area is exon 3-2N forward and reverse, while the bright purple shaded area is exon 3-2 forward and reverse.

The two variants were aligned to find the similarities between them and design the primers. There were strong similarities between them and specific regions were chosen to design the primers, as shown in Table 4.3. One primer was designed for exon 2 and 3 primers were designed for exon 3.

Table 4.3. Possible initial primer sequences for amplification of fragments of the mouse NLRP1b gene and primers after adding ambiguity letters to improve recognition.

Possible primer	**After adding ambiguity letters**

Exon 2 Forward ATGGAACAATCTCAGCCCAAGAAG ATGGAAGAATCTCAGTACAAGCAG	5’ ATGGAASAATCTCAGYMCAAGMAG 3’
Exon2 Reverse CCAAGCCAGAGGGACACTTGAAGC CCAACCCAGAGAGTACCTTCAATT	5’ RMTTSAAGKKWCYCTCTGGSTTGG 3’
Exon 3 part 1 Forward AATTCCAAAAGTACACATAAAACA ATTTCCAGAAGTATATATGAATCA	5' AWTTCCARAAGTAYAYATRAAWCA 3.'
Exon 3 part 1 Reverse GCAGACCAGAATCCTGAGCTATGT GCAGACCAGAATCCTGAGCTATGT	5' ACATAGCTCAGGATTCTGGTCTGC 3.'
Exon 3 part 2 Forward TATGAGCAGCTGAGTCTGGCTGAG T GCAAGAAGCTGAGTCTGGCTGAG	5' TRYRAGMAGCTGAGTCTGGCTGAG 3.'
Ex 3 Part 2 Reverse CTTGTGTCTCCAGGAGTTCTTTGC CTTGTGTCTCCAGGAGTTCTTTGC	5’ GCAAAGAACTCCTGGAGACACAAG 3’
Exon 3 part 3 Forward GAGGATGCCATTGCCACTTTCCTG GAGGATGCCGTTGCCATTTTCCTG	5' GAGGATGCCRTTGCCAYTTTCCTG 3
Exon 3 Part 3 Reverse GACAGCAGGGACATAAGCTGACAG GACAACAAGGATATGCACTGACAG	5’ CTGTCAGYKYATRTCCYTGYTGTC 3’

The above table shows both possible primers from the retrieved sequences (Table 4.3). Since we had two variants and were not sure of the exact sequences, ambiguity letters were added to improve the likelihood of success. Those are bases where ambiguities are reported using the IUPAC set of characters (other than ATCGU). For example, (R) Purine represents (A or G), (Y) Pyrimidine accepts (C or T/U) and (K) Keto accepts (G or T/U). The letters shaded yellow are the differences between the two variant primer sequences. Oligonucleotide sequences were sent to Eurofins MGW Operon to carry out a synthesis of the primers. The table below shows some details of the characteristics of the primers (Table 4.4).

Table 4.4. Forward and reverse initial primer details

Primers	Temp	Molecular Weight
MUSNLRP1B EXON 2 FOR 5' ATGGAASAATCTCAGYMCAAGMAG 3'	60.2°C	7391
MUSNLRP1B EXON 2 REV 5' RMTTSAAGKKWCYCTCTGGSTTGG 3'	63.6°C	7382
MUSNLRP1B EXON 3 Part1 FOR 5' AWTTCCARAAGTAYAYATRAAWCA 3'	52.5°C	7336
MUSNLRP1B EXON 3Part1 REV 5'ACATAGCTCAGGATTCTGGTCTGC 3'	62.7°C	7343
MUSNLRP1B EXON 3Part2 FOR 5'TRYRAGMAGCTGAGTCTGGCTGAG 3'	64.4°C	7453
MUSNLRP1B EXON 3Part2 REV 5' GCAAAGAACTCCTGGAGACACAAG 3'	62.7°C	7388
MUSNLRP1B EXON 3Part3 FOR 5'GAGGATGCCRTTGCCAYTTTCCTG 3'	64.4°C	7335
MUSNLRP1B EXON 3Part3 REV 5' CTGTCAGYKYATRTCCYTGYTGTC 3'	62.7°C	7281

The primers shown in Table 4.4 were the first attempt to amplify the exon 2 and exon 3 regions of the NLRP1b gene of *Apodemus sylvaticus.*

After that, mammalian specific beta tubulin PCR was carried out to check the quality of the *Mus musculus* DNA. The expected band size for the PCR product was 1000 bp. A 1.5% gel was used to identify the expected band (Figure 4.9).

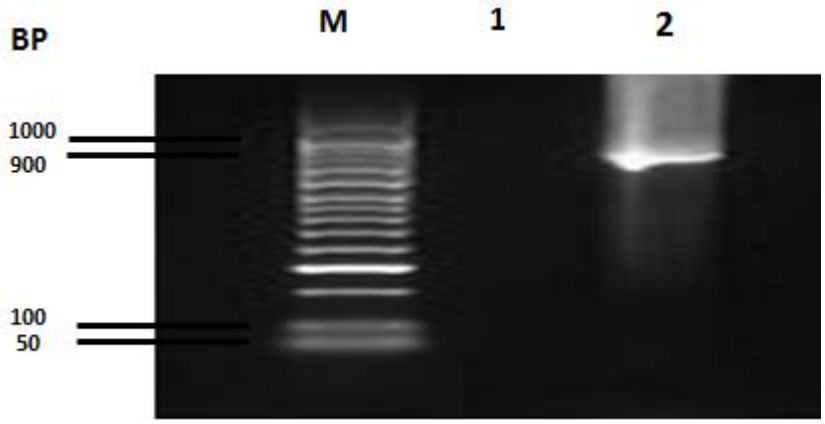

Figure 4.9. Representative agarose gel (1.5%) image showing PCR amplification of the *Mus musculus* beta tubulin fragment. M, 50bp hyperladder; 1, negative control (water); 2, *Mus musculus* targeted DNA (1000bp).

After that, a further mammalian specific beta tubulin PCR was carried out to check the quality of the *Apodemus sylvaticus* DNA. The expected band size of the PCR product was around 1000 bp. A 1.5% gel was used to identify the expected band (Figure 4.10).

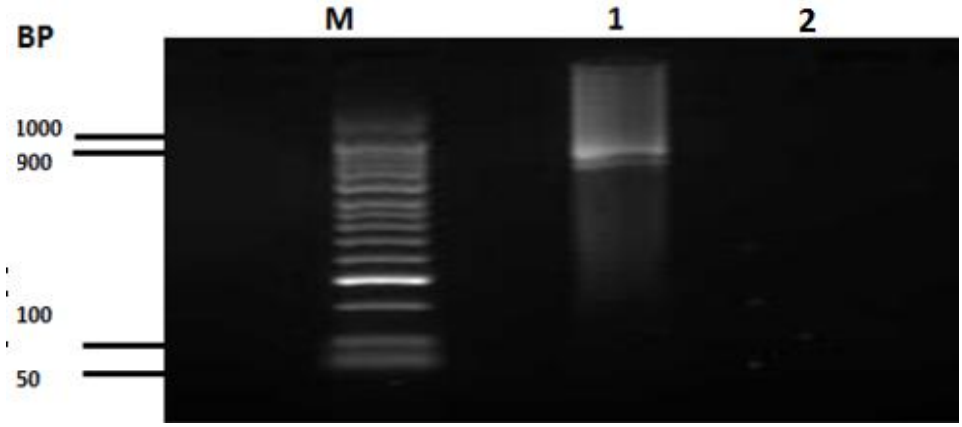

Figure 4.10. Representative agarose gel (1.5%) image showing PCR amplification of the *Apodemus sylvaticus* beta tubulin fragment. M, 50bp hyperladder; 1, *Apodemus sylvaticus* targeted fragment (1000bp); 2, negative control (water).

The quality of the extracted DNA was suitable to start the experiment.

After checking the quality of DNA, all designed primers were tested on *Mus musculus* DNA samples. Figure 4.11 shows a 1.5% electrophoresis for 3 different PCR products. *Mus musculus* DNA was used with exon 2, exon 3 part 2 and exon 3 part 3 primers. The expected

band size for exon two is 142 base pairs, for exon three part two 770 base pairs, and for exon 3 part 3 610 base pairs (Figure 11).

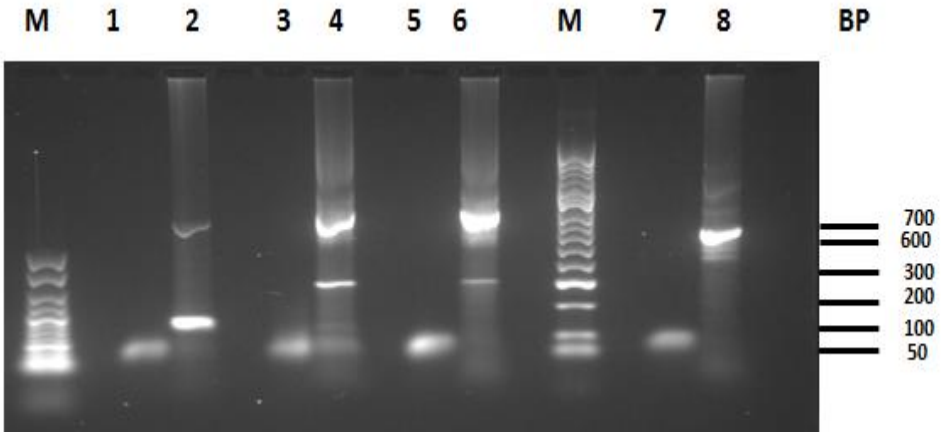

Figure 4.11. Representative agarose gel (1.5%) image showing PCR amplification of *Mus musculus* NLRP1b fragments using exon 2, 3-3 and 3-2 primers, 50bp hyperladder; 1, negative control (water); 2, *Mus musculus* targeted fragment of exon 2 (142 bp); 3, negative control (water); 4, *Mus musculus* targeted fragment of exon 3-2 (770 bp); 5, negative control (water); 6, repeated *Mus musculus* targeted fragment of exon 3-2 (770 bp); M, 50bp hyperladder; 7, negative control (water); 8, *Mus musculus* targeted fragment of exon 3-3 (610 bp).

The next step was to apply these primers to the *Apodemus sylvaticus* DNA. Two control samples were available, *Apodemus sylvaticus* samples, numbers 301 and 303. Figure 4.12 shows gel electrophoresis for the PCR product of *Apodemus sylvaticus* DNA control (303) using exon 3-2 and exon 3 part 3 primers.

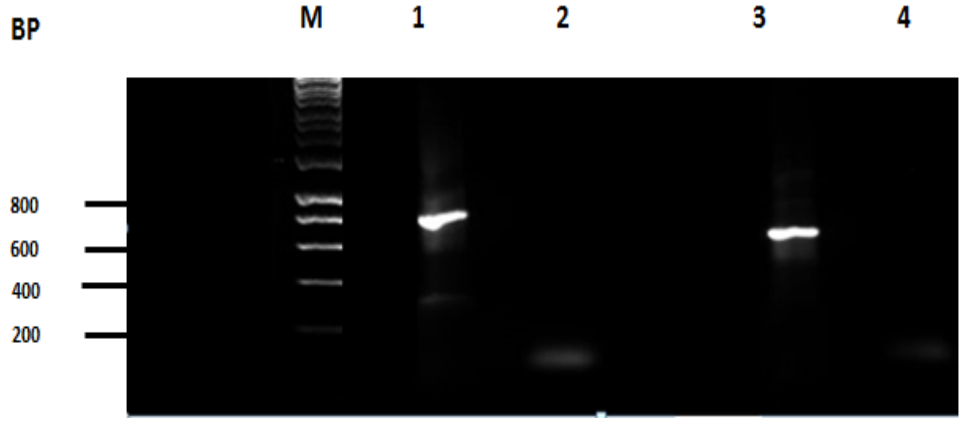

Figure 4.12. Representative agarose gel (1.5%) image showing PCR amplification of *Apodemus sylvaticus* NLRP1b fragments using exon 3-3 and 3-2 primers. M, 50bp hyperladder; 1 *Apodemus sylvaticus* targeted fragment of exon 3-2 (770 bp); 2, negative control (water); 3, *Apodemus sylvaticus* targeted fragment of exon 3-3(610) bp; 4, negative control (water).

The exon 2 primer was also tested with *Apodemus sylvaticus* DNA after successfully amplifying regions of exon 3-2 and 3-3. Figure 4.13 shows gel electrophoresis for the PCR product of *Apodemus sylvaticus* DNA (303) using exon 2 primer.

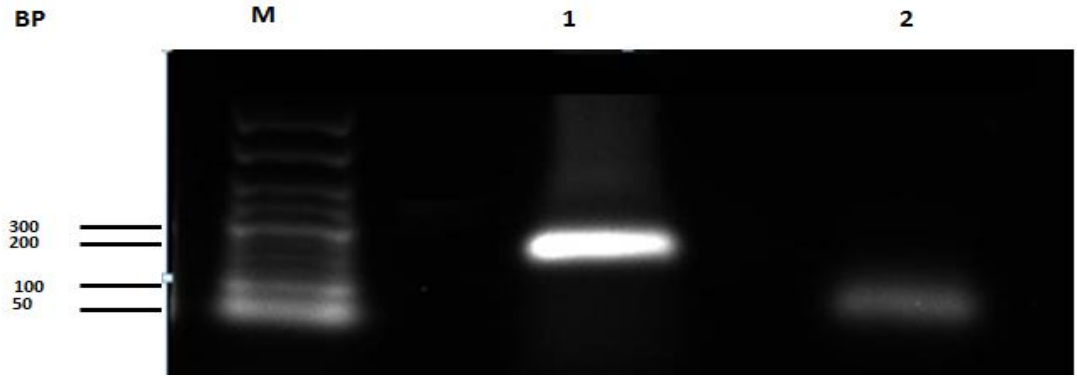

Figure 4.13. Representative agarose gel (1.5%) image showing PCR amplification of the *Apodemus sylvaticus* NLRP1b gene using exon 2 primer. M, 50bp hyperladder; 1, *Apodemus sylvaticus* targeted fragment of exon 2 (142 bp); 2, negative control (water).

Exon 3-1 primer was unsuccessful for amplification of *Mus musculus*. Also, it was not successful with *Apodemus sylvaticus* (controls 301and 303) (data not shown).

Later, amplification was carried out on four *Apodemus sylvaticus* samples, 3, 12, 30 and 78, using exon 3-3 primer. These samples were picked at random from the sample set we had. The next figure shows a gel electrophoresis for four samples of *Apodemus sylvaticus* using exon 3-3 primer (Figure 4.14).

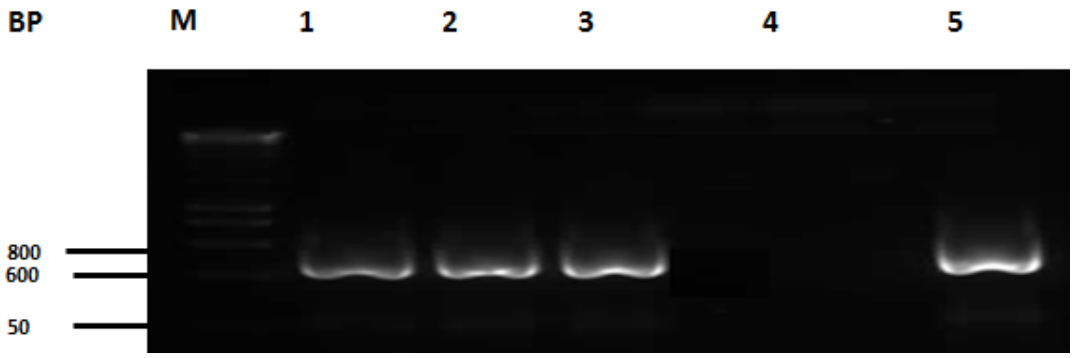

Figure 4.14. Representative agarose gel (1.5%) image showing PCR amplification of the *Apodemus sylvaticus* NLRP1b gene using exon 3-3 primer. M, 50bp hyperladder; 1, *Apodemus sylvaticus* targeted fragment of exon 3-3 (610 bp) of sample 3; 2, *Apodemus sylvaticus* targeted fragment of exon 3-3 (610 bp) of sample 12; *3, Apodemus sylvaticus* targeted fragment of exon 3-3 (610 bp) of sample 30; 4, negative control (water); 5, *Apodemus sylvaticus* targeted fragment of exon 3-3 (610 bp) of sample78.

Some regions of exon 2, exon 3 part 2 and exon 3 part 3 were successfully amplified from *Apodemus sylvaticus.* Unfortunately, after applying the primers to four samples, for some reason, the primers failed to work with the control and the other sample. Many attempts were made but no successful amplification was achieved. A different procedure was considered before the second primer design took place, such as reordering new stock of the same primer which would solve primer degradation, should that have occurred for any reason. Also, repeating the PCR reaction, temperature gradient and magnesium gradient PCR was done three times. Another attempt was made using a different PCR machine which would solve any problems that might arise due to machine failure. Such failure might be due to using ambiguity letters which may be picked up as not belonging to any specific region of the DNA template.

As the DNA sequences had to be analysed to produce a consensus sequence, several steps were taken. An example is shown for exon 3 part 3 of the first attempt at amplifying the exon 3-3 region from *Apodemus sylvaticus*. A similar analysis was carried out on all other sequences.

The first attempted products of amplified control samples of *Apodemus sylvaticus* using exon 3 part 3 primer were sent for sequencing and the sequences came back in a raw form that had to be processed. These sequences were analysed to check for any SNPs but it was difficult to show that with low-quality sequences. After four attempts on positive PCR products of Exon 3-3, the sequences came back much improved due to protocol optimization. An analysis was carried out for all forward sequences to check for heterozygotes and SNPs and to compare them with the reference sequences of *Mus musculus*. Sequence analysis was carried out on each sample, as follows. First, a raw forward sequence was retrieved (Figure 4.15).

Raw sequence sample 3 – forward sequence

NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNTCTGAGNNNNNNNNNNNGTCCATTTGTNNNN
NNCNNGAGTTCTTTGCAGCCATGTCCTACATTTTGGAGGATAGTGAGGAAAGATATGGTAATAAG
GACAATGAAAGAATTGTGGAAACACTGATTGAACATTATGGAAAACAAAACCTGTTTGAGGCACC
CACTGTGCGCTTTTTGTTTGGCCTTTTGAGTAAAAAGGGATGGAAAAAAATGAAAAAGTTCTTTTA
CTGCAACTTTTCTAGGAAGACAAAGTTGAAGCTGCTATGGCACATCCTAGCAAAAACCCAACCCC
ATCAACCACCTTGTCTGGGTTTGCTCTACTGTCTGTATGAAAATCAGGAAAAGGAACTCCTGACAC
ACGTGATGCATNATTTTCAAGGAACAATAGTGCCTGGCCCAAATGATAAGGCACACACAGTGTTA
CAGACAAACATGAATCACCTGGTGATACAGACAGACATGGATCTCATGGTGGTTACTTTCTGCATT
AGGTTCTGCTCTCATGTAAAGAGTCTTCAGGTGAATATGGAAAGACAACAAGGACATNNNCCTGA
CAGANNNNNNNNANCNNNTANCNNCNNNGNANTNNCGNACNGNNNNNNNNNNNNGNNNNNCN
NNNNNNNNNNNNAANNNTTTNTNNNTNNNNANGANTANANTTNANTNGAA

Figure 4.15. Raw sequence of sample 3. The yellow shaded area shows an unreliable sequence.

Then, the raw sequence for the reverse was retrieved (Figure 4.16). The yellow shaded (N) letters represent unreliable sequences.

Raw sequence – reverse sequence

NNNNNNNNNNNNNNATNNNNNNNNNTGACAGCAGACCTAATGCAGAAAGTAACCACCATGAGA
TCCATGTCTGTCTGTATCACCAGGTGATTCATGTTTGTCTGGAACACTGTGTGTGCCTTATCATCTG
GGCCAGGCACTATTGTTCCTTGAAAATTATGCATCACGTGTGTCAGGAGCTCCTTTTCCTGATTTTC
ATACAGACAGTAGAGCAAACCCAGACAAGGTGGTTGATGGGGTTGGGTTTTTGCTAGGATGTGCC
ATAGCAGCTTCAACTTTGTCTTCCTAGAAAAGTTGGAGTAAAAGTACTTTTTCACTTTTTTCCATCC
CTTTTTACTCAAAAGGCCAAACAAAAAGCGCACAGTGGGTGCCTCAAACAGGTTTTGTTTTCCATA
ATGTTCTATCAGTGTTTCCACAATTCTTTCATTGTCCTTATTACCATGTCTTTCCTCACTATCCTCCA
ANATGTAGGATATGGCTGCNAAGAACTCCTGGAGATACAAATGGACAAAGCTGTAGCTCAAAGAG
CTGGGCTNATNTTGAAGGACACCCATCTTCAGGAAAATGGCAACGNNCATCCTCA

Figure 4.16. Reversed raw sequence of sample 3. The yellow shaded area shows an unreliable sequence.

Then, unreliable bases were corrected against forward sequences to make each sequence more reliable (Figure 4.17). This was done by deleting all unknown nucleotides on the 3 and 5 ends of the sequences. They were also checked manually against chromatograms to improve accuracy.

GAGTTCTTTGCAGCCATGTCCTACATTTTGGAGGATAGTGAGGAAAGATATGGTAATAAGGACAAT
GAAAGAATTGTGGAAACACTGATTGAACATTATGGAAAACAAAACCTGTTTGAGGCACCCACTGT
GCGCTTTTTGTTTGGCCTTTTGAGTAAAAAGGGATGGAAAAAAATGAAAAAGTTCTTTTACTGCAA
CTTTTCTAGGAAGACAAAGTTGAAGCTGCTATGGCACATCCTAGCAAAAACCCAACCCCATCAACC
ACCTTGTCTGGGTTTGCTCTACTGTCTGTATGAAAATCAGGAAAAGGAACTCCTGACACACGTGAT
GCATNATTTTCAAGGAACAATAGTGCCTGGCCCAAATGATAAGGCACACACAGTGTTACAGACAA
ACATGAATCACCTGGTGATACAGACAGACATGGATCTCATGGTGGTTACTTTCTGCATTAGGTTCT
GCTCTCATGTAAAGAGTCTTCAGGTGAATATGGAAAGACAACAAGGACAT

Figure 4.17. Sample 3 forward sequences after correcting unreliable sequences.

After that, unreliable bases were corrected against reverse sequences in the same way as forward sequences in order to make each sequence more reliable, and the reverse complement was retrieved (Figure 4.18).

GAGTTCTTTGCAGCCATGTCCTACATTTTGGAGGATAGTGAGGAAAGATATGGTAATAAGGACAAT
GAAAGAATTGTGGAAACACTGATTGAACATTATGGAAAACAAAACCTGTTTGAGGCACCCACTGT
GCGCTTTTTGTTTGGCCTTTTGAGTAAAAAGGGATGGAAAAAAATGAAAAAGTTCTTTTACTGCAA
CTTTTCTAGGAAGACAAAGTTGAAGCTGCTATGGCACATCCTAGCAAAAACCCAACCCCATCAACC
ACCTTGTCTGGGTTTGCTCTACTGTCTGTATGAAAATCAGGAAAAGGAACTCCTGACACACGTGAT
GCATNATTTTCAAGGAACAATAGTGCCTGGCCCAAATGATAAGGCACACACAGTGTTACAGACAA
ACATGAATCACCTGGTGATACAGACAGACATGGATCTCATGGTGGTTACTTTCTGCATTAGGTTCT
GCTCTCATGTAAAGAGTCTTCAGGTGAATATGGAAAGACAACAAGGACAT

Figure 4.18. Sample 3 reverse sequence after correcting for unreliable sequences.

Later, Clustal alignment between forward sequences and reverse complement sequences was performed to check their similarity. The similarity between them was high (Figure 4.19). However, bases that still did not correspond were rechecked against chromatograms to reach a consensus sequences.

Clustal alignment

Clustal 2.1 Multiple sequence alignment.

```
SAM3F           ------------------------------------------------------------
SAM3RC          CGTTGCCATTTTCCTGAAGATGGGTGTCCTTCAANATNAGCCCAGCTCTTTGAGCTACAG 60

SAM3F           ----------------------GAGTTCTTTGCAGCCATGTCCTACATTTTGGAGGATAG 38
SAM3RC          CTTTGTCCATTTGTATCTCCAGGAGTTCTTNGCAGCCATATCCTACATNTTGGAGGATAG 120
                                      ******** ******** ******** ************
SAM3F           TGAGGAAAGATATGGTAATAAGGACAATGAAAGAATTGTGGAAACACTGATTGAACATTA 98
SAM3RC          TGAGGAAAGACATGGTAATAAGGACAATGAAAGAATTGTGGAAACACTGATAGAACATTA 180
                ********** ****************************************** ********

SAM3F           TGGAAAACAAAACCTGTTTGAGGCACCCACTGTGCGCTTTTTGTTTGGCCTTTTGAGTAA 158
SAM3RC          TGGAAAACAAAACCTGTTTGAGGCACCCACTGTGCGCTTTTTGTTTGGCCTTTTGAGTAA 240
                ************************************************************
SAM3F           AAAGGGATGGAAAAAAATGAAAAAGTTCTTTTACTGCAACTTTTCTAGGAAGACAAAGTT 218
SAM3RC          AAAGGGATGGAAAAAAGTGAAAAAGTACTTTTACTCCAACTTTTCTAGGAAGACAAAGTT 300
                *************** ********* ******** ************************
SAM3F           GAAGCTGCTATGGCACATCCTAGCAAAAACCCAACCCCATCAACCACCTTGTCTGGGTTT 278
SAM3RC          GAAGCTGCTATGGCACATCCTAGCAAAAACCCAACCCCATCAACCACCTTGTCTGGGTTT 360
                ************************************************************
SAM3F           GCTCTACTGTCTGTATGAAAATCAGGAAAAGGAACTCCTGACACACGTGATGCATNATTT 338
SAM3RC          GCTCTACTGTCTGTATGAAAATCAGGAAAAGGAGCTCCTGACACACGTGATGCATAATTT 420
                ******************************** ******************** ****
SAM3F           TCAAGGAACAATAGTGCCTGGCCCAAATGATAAGGCACACACAGTGTTACAGACAAACAT 398
SAM3RC          TCAAGGAACAATAGTGCCTGGCCCAGATGATAAGGCACACACAGTGTTCCAGACAAACAT 480
                ************************ ********************** ************
SAM3F           GAATCACCTGGTGATACAGACAGACATGGATCTCATGGTGGTTACTTTCTGCATTAGGTT 458
SAM3RC          GAATCACCTGGTGATACAGACAGACATGGATCTCATGGTGGTTACTTTCTGCATTAGGT- 539
                ************************************************************
SAM3F           CTGCTCTCATGTAAAGAGTCTTCAGGTGAATATGGAAAGACAACAAGGACAT 510
SAM3RC          CTGCTGTCA---------------------------------------------- 548
                ***** ***
```

Figure 4.19. Clustal alignment of sample 3 exon 3-3 forward and reverse complement sequences.

Mouse reference DNA sequences were obtained from NCBI and aligned with both forward and reverse sequences to examine their similarity (Figure 4.20). To help with resolution, the *Apodemus* sequences were investigated for their similarities to mouse DNA sequences.

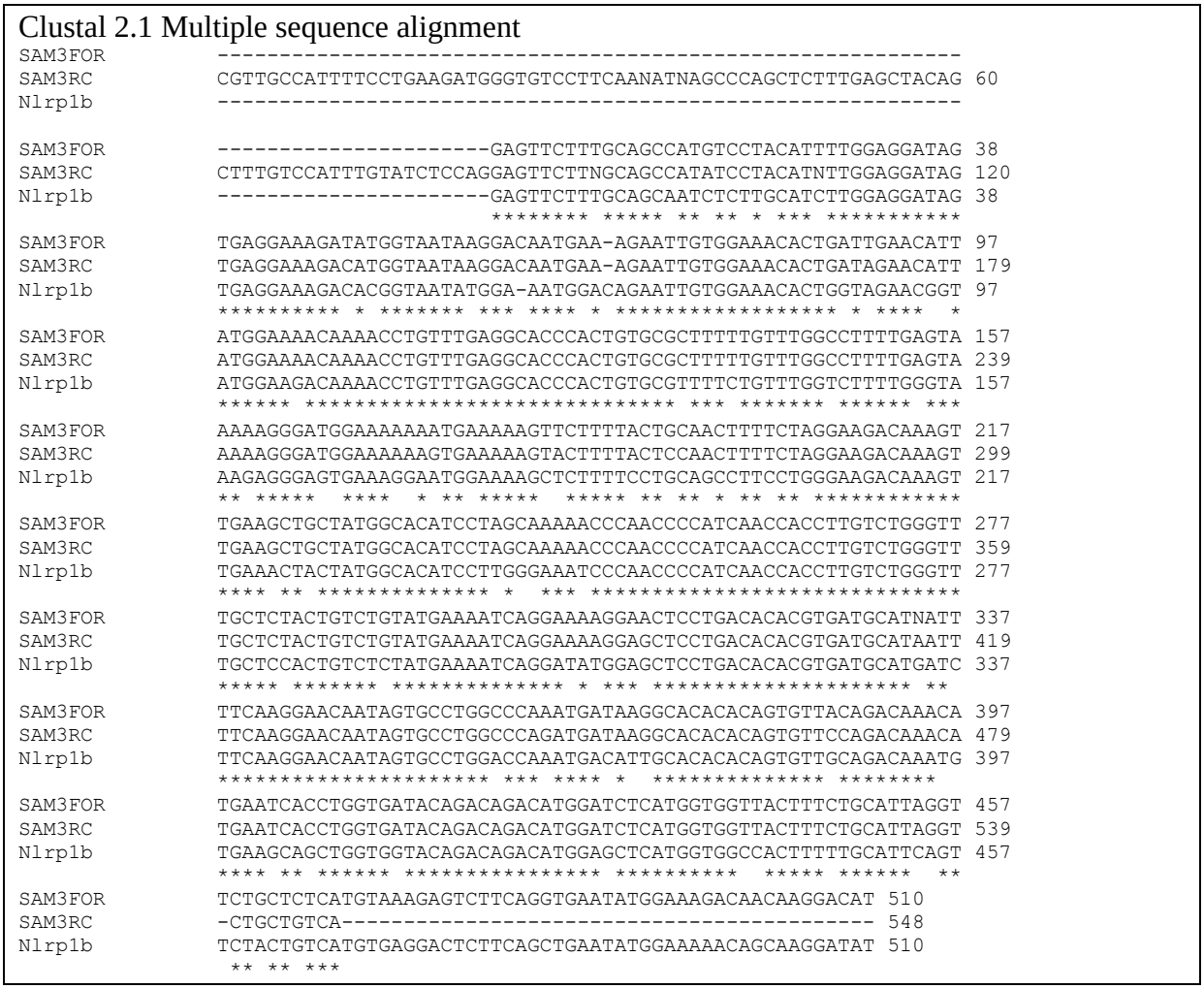

Figure 4.20. Clustal alignment of sample 3 exon 3-3 forward and reverse complement sequences with a mouse reference sequence (NLRP1b)

These sequence manipulations were carried out on four representative *Apodemus* DNA samples (3, 12, 30 and 78) and consensus sequences were obtained. A Clustal alignment was done for all consensus sequences of the four samples to compare the heterozygote nucleotide of each one in relation to the other sample (Figure 4.21).

Figure 4.21. Clustal alignment for forward sequences of all four samples. Each shaded nucleotide means that it is heterologous. Green shaded nucleotides may be G or A. Red shaded nucleotides may be C or T. Purple shaded nucleotides may be T or A.

The primer suddenly failed to work. Several procedures were followed and attempts made to get the primer to work and all of them failed, as explained previously. Therefore, new sets of primers (exon 3-2N, exon 3-3N) were designed and the products of PCR with the new

primers were sent for sequencing. Protein translation was not carried out due to the number of samples, four samples not being enough to move forward and continue our work.

4.5.2.2 Second attempt at primer design and amplification of sections of the NLRP1b gene and analysis of the data

Some difficulties were encountered with the previously designed primers; therefore, two new sets of primers were designed for exon 3 part 2 and exon 3 part 3. Later, two other sets were designed when a problem was encountered with the sequence of the new first set. The new first set were from variants one and two (Exon 3-2N, Exon3-3N) and the other two were from the sequence that came back from the sequencing company to be used for sequencing purposes only (SEQ1, SEQ2). SEQ 1 and SEQ 2 were designed from the sequencing product of the exon 3-2N primers (Tables 4.5, 4.6). The green shaded nucleotides represent forward and reverse primers of exon 3-2N while the purple shaded nucleotides show forward and reverse primers of exon 3-3N (Figure 4.8).

Table 4.5. Forward and reverse exon 3-2N and exon 3-3N primer details

Primer		Temperature	MW
Exon 3-2N Forward	5’ GCTGAGTCTGGCTGAGCTCATAGC 3’	66.1°C	7385
Exon 3-2N Reverse	5’ GAACTCCTGGAGACACAAGTGG 3’	62.1°C	6793
Exon 3-3N Forward	5’ CCTTCAAAAGCAGGCCAGC 3’	58.8°C	5767
Exon 3-3N Reverse	5’ GCAGAAAGTGACCACCATGAG 3’	59.8°C	6473

The primers shown in Table 4.3 were the second attempt at primers designed for exon 3 part2.

Table 4.6. Forward and reverse SEQ and SEQ2 primer details

Primer		Temperature	MW
SEQ F	5’ CCCACTGTGCGCTTTCTAT ’3	56.7°C	5706
SEQ R	5’ AGTGACCACCATGAGCTTC’3	56.7°C	5773
SEQ2 F	5’ TGCTGCATCTGTTACCTCTTG’3	57.9°C	6354
SEQ2 R	5’ GGGTCCCCTAAGATGATCCA’3	59.4°C	6102

SEQ and SEQ 2 primers were designed to be used for sequencing from the product of exon 3-2N. The purpose of these primers was to clarify an issue encountered with some of the sequences of exon 3-2N (Figures 4.22, 4.23).

Figure 4.22. SEQ forward and reverse locations in the sequence of the exon 3-2 product.

Figure 4.22 shows one of the sequences of the previous product of *Apodemus sylvaticus*, which was amplified using exon 3-2N primer. This sequence was used to design and determine the location of SEQ primer in sequence. The SEQ primer was used for sequence purposes only.

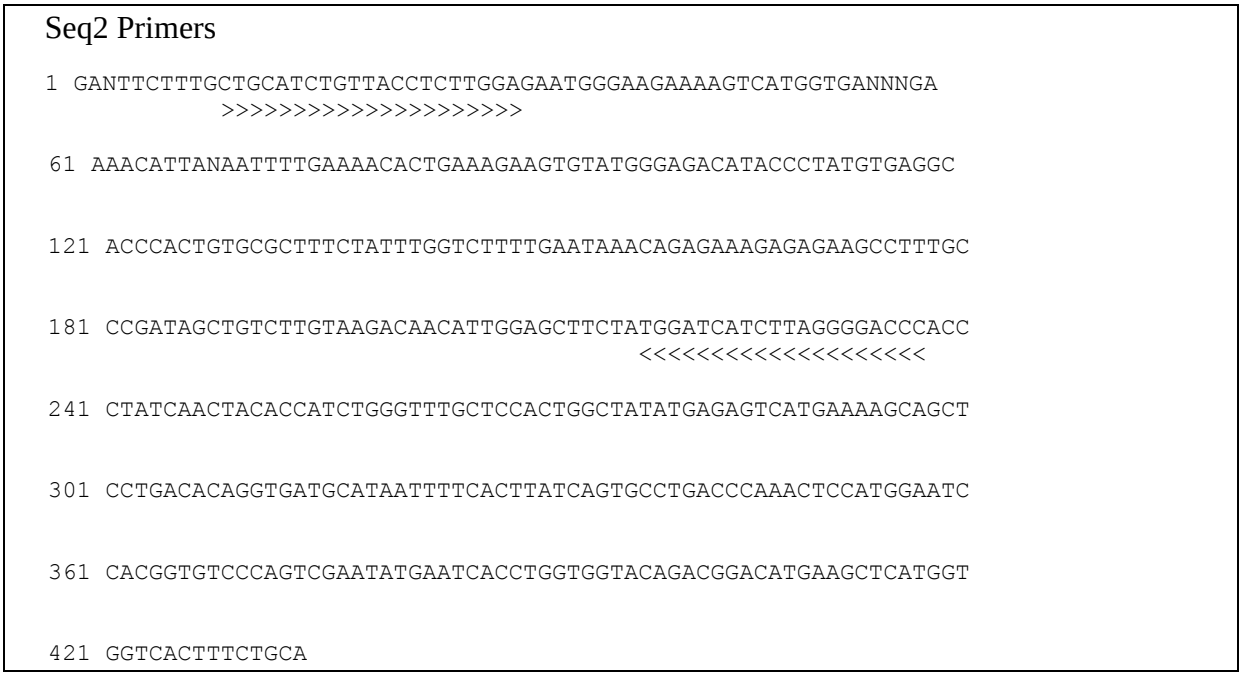

Figure 4.23. SEQ2 primers forward and reverse locations in the sequence of the exon 3-2N product.

Figure 4.23 shows the same sequence that was used to design the SEQ primer and also used to design SEQ 2 primers at a different location.

The failure of the first set of primers to amplify the NLRP1b gene from *Apodemus* led us to design two new primer sets called primer 3-2 N and primer 3-3N. The expected product size of the exon 3-2N primers was 757 bp, and for exon 3-3N 445bp. Furthermore, the primers were tested on *Mus musculus* DNA first and then PCR was carried out using exon 3-3N primers with *Apodemus sylvaticus* DNA samples 301, 303 and 111. Successful amplification was achieved for the *Apodemus* DNA samples (Figure 4.24).

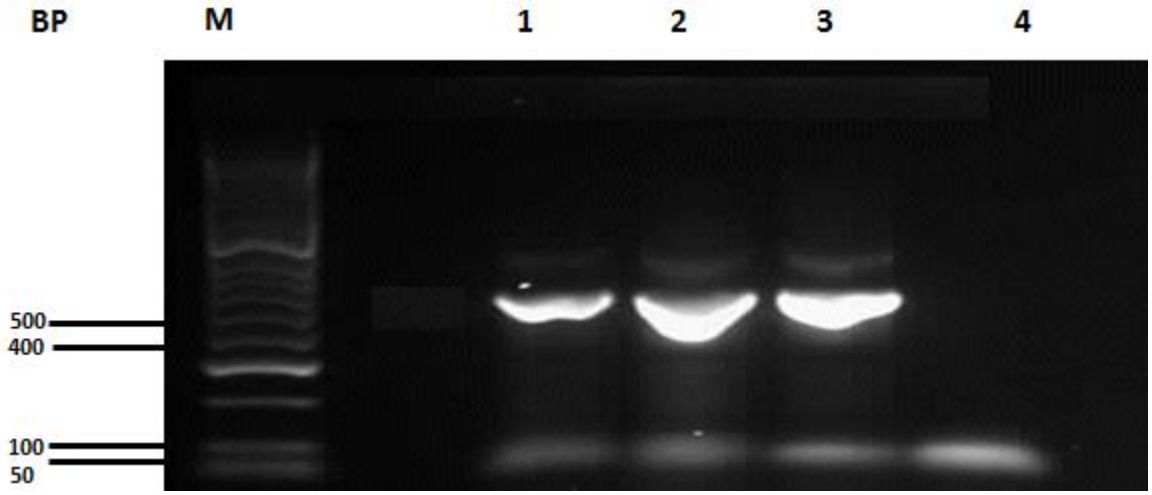

Figure 4.24. Representative agarose gel (1.5%) image showing PCR amplification of *Apodemus sylvaticus* NLRP1b fragment using primers 3-3N. M, 50bp hyperladder; 1, *Apodemus sylvaticus* targeted fragment of sample 301 (445bp); 2, *Apodemus sylvaticus* targeted fragment of sample 303 (445bp); 3, *Apodemus sylvaticus* targeted fragment of sample 111 (445bp); 4, negative control (water).

After that, exon 3-2N primers were tested on both *Mus musculus and Apodemus sylvaticus* DNA and gel electrophoresis was carried out to identify the expected band size (Figure 4.25). Gel electrophoresis showed successful amplification for the exon 3-2 region using exon 3-2N primers from *Apodemus sylvaticus*.

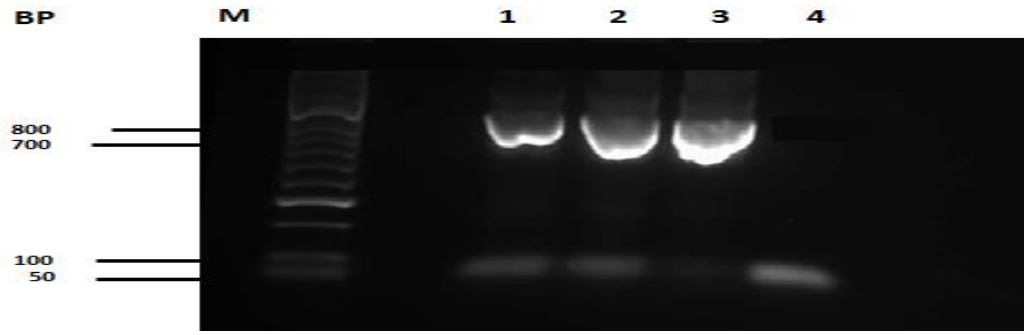

Figure 4.25. Representative agarose gel (1.5%) image showing PCR amplification of *Apodemus sylvaticus* NLRP1b fragment using primers 3-2N. M, 50bp hyperladder; 1, *Apodemus sylvaticus* targeted fragment of sample 301 (757bp); 2, *Apodemus sylvaticus* targeted fragment of sample 303 (757bp); 3, *Apodemus sylvaticus* targeted fragment of sample 111 (757bp); 4, negative control (water).

The previous products of exon 3-2N and exon 3-3N were sent for sequencing. Exon3-2N sequences came back with half good and half with overlapped peaks as shown in Figure 4.26. Therefore, only the first 330 bp was analysed.

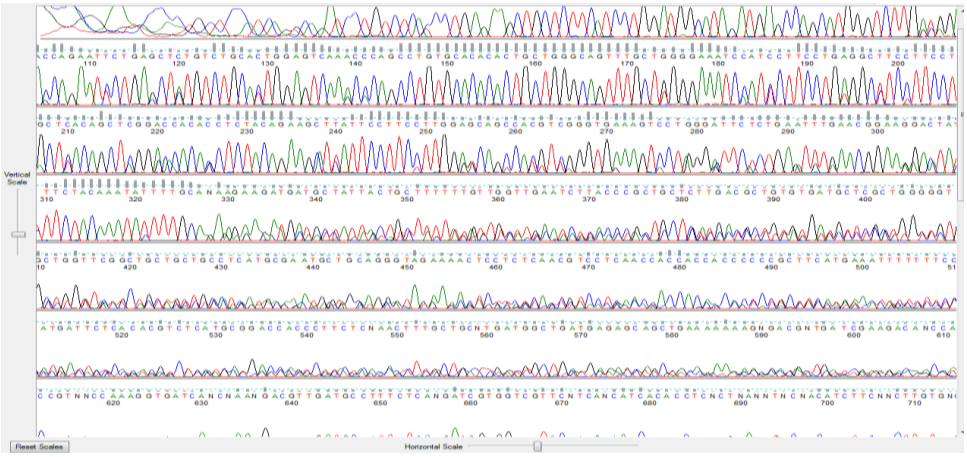

Figure 4.26. Sequence of the PCR product of exon 3-2N.

Figure 4.27 shows half of a good sequence and the other half with overlapped peaks. All the samples that were sent for sequencing and came back with the same overlapped peaks started at the same location, i.e. after 330 bases. To investigate this issue further the data were analysed in two stages. First, 330 bases were identified using an NCBI search. Then, each possibility from the overlapped peaks was analysed separately. Figure 4.27 showing blast searches for the first 330 bp of the sequences shows 92% similarity with NLRP1b gene of *Mus musculus* and 100 % coverage (Figure 4.27).

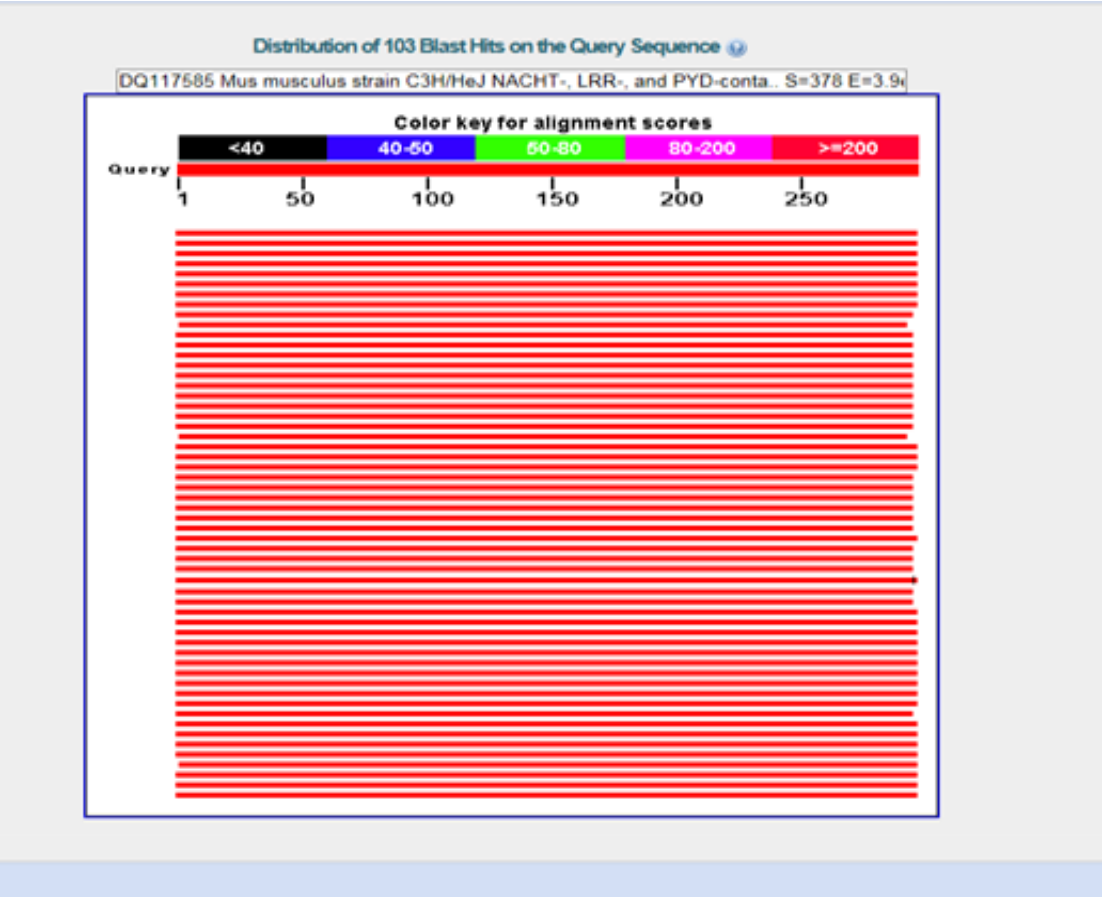

Figure 4.27. BlastN search of the first 330 bp of exon 3-2 shows highest similarity (92%; Blast score = 378; E-value $3e^{-90}$) with *Mus musculus* NLR1P1b protein (Accession number: DQ117585).

Furthermore, all samples were aligned using Clustal alignment and the same four SNPs were found at the same locations (Figure 4.28).

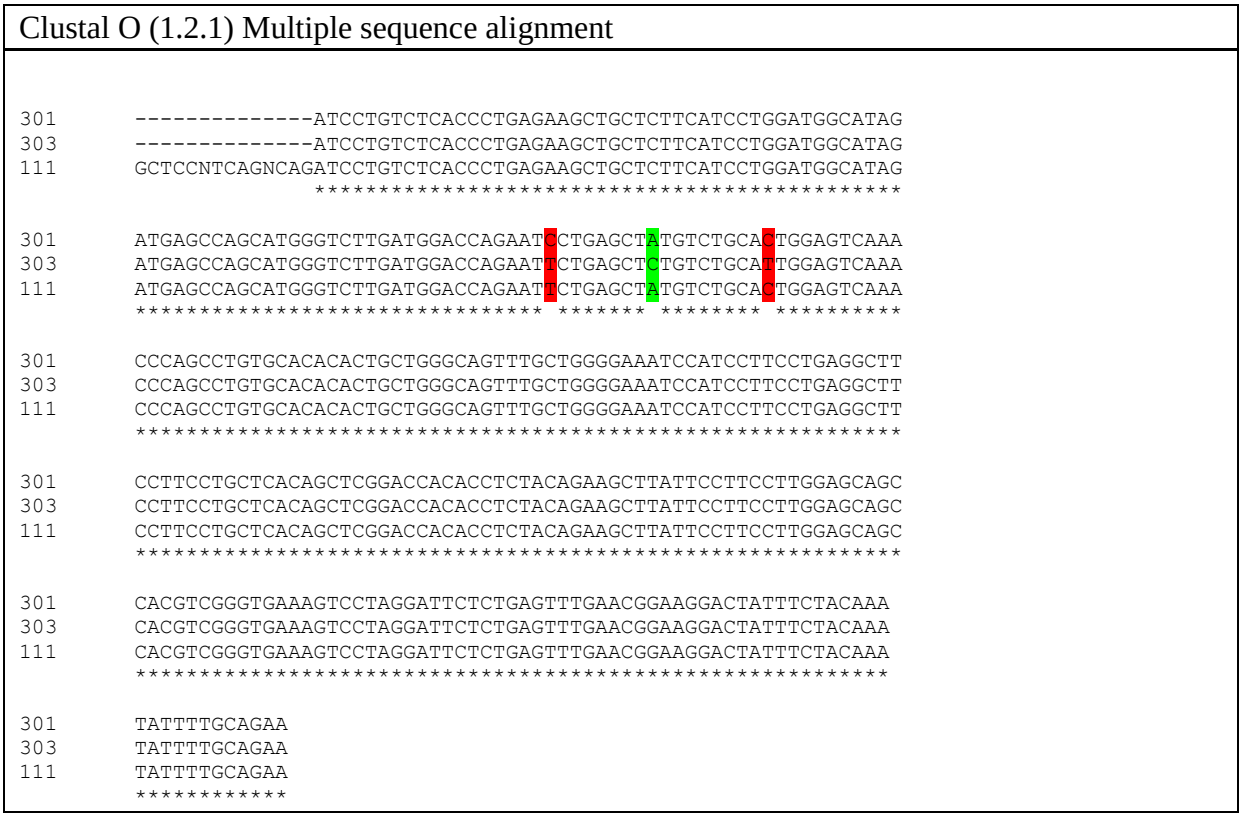

Figure 4.28. Clustal alignment of sequences 301, 303, 111. The red shaded nucleotides may be C or T. The dark green shaded nucleotides may be C or A.

No protein translation was carried out due to the confusion over the half of the sequence that had overlapped peaks. SEQ1 and SEQ2 primers were used for sequencing the product of exon 3-2N primers, but the sequence showed no similarity or match with the NLRP1b gene fragment of *Mus musculus*. The possibility of overlapped peaks did not match any *Mus musculus* genes and therefore, we decided to design further new sets of primers.

Exon 3-3 sequences were not good enough to be analysed. They had overlapping peaks all around the sequence; therefore, they were difficult to investigate. SEQ 1 and SEQ 2 primers were only used for sequencing the previous product, but unfortunately, they did not work well and no sequence was significant.

4.5.2.3 Successful attempt at primer design and amplification of sections of the NLRP1b gene and data analysis

The failure of previous primers was due to bad sequence products which occurred with two primers. The first exon 3-2 primer showed a large band in the gel electrophoresis, but the sequence was half overlapping peaks and half clear peaks. All possibilities of an overlapped two sequence were investigated but none of them were the correct sequence. The other half of the sequence was of good quality but we could not rely on it because the primer was not picking a specific sequence in the first part of the sequence. The second primer from exon 3-3 N product was not clear after sequencing. That might have been due to the specificity of the primers which might have been picking something else. Also, it might be due to designing the primers from different species that may have differences in their DNA. Therefore, after the previous two attempts to amplify some regions of the NLRP1b gene from *Apodemus sylvaticus*, three further primers were designed for exon 3 part 1, exon 3 part 2 and exon 3-1, as shown in Table 4.7.

Table 4.7. Forward and reverse exon 3-1, exon 3-2 and exon 7 primer details

Primer	Temperature	Molecular weight
Exon 3-2 forward AGAGAGCCTGGAAGGAAGG	58.8	5976
Exon 3-2 reverse AGCTCAGGATTCTGGTCTGC	59.4	6124
Exon 3-1 forward GAGACTCTGGACCCCACAT	58.8	5758
Exon 3-1 reverse GACTTCCCAATTCCAGCAG	56.7	5733
Exon 3-3 forward TGACCTCACAGACCACA	59.4	6000
Exon 3-3 reverse CCTGCTTTTGAAGGATACCG	57.3	6108

The third set of primers were applied to *Apodemus sylvaticus* and gel electrophoresis showed very clear bands in both exon 3-2 and exon 3-3 regions. After that, the PCR products were cleaned up as described in Section 2.6 and sent for sequencing. The sequences came back

very clean and analyses were conducted. The same steps as described in Section 4.4.2.1 were taken to find consensus sequences of both exon 3-2 and exon 3-3.

In conclusion, three attempts at primer design were carried out from variant one and variant two sequences. The first set comprises four primers: exon 2, exon 3-1, exon 3-2 and exon 3-3. The second set comprises exon 3-2N, exon 3-3N, SEQ and SEQ2. Also, a third set of primers was designed later after the failure of the previous two attempts, which were primers for exon 3 parts 1, 2 and 3.

The third set of primers was tested on both *Mus musculus* and *Apodemus sylvaticus* DNA. Initially, a temperature gradient was used with all primers, as described Section 2.3.3, to find the best annealing temperature (Figure 4.29).

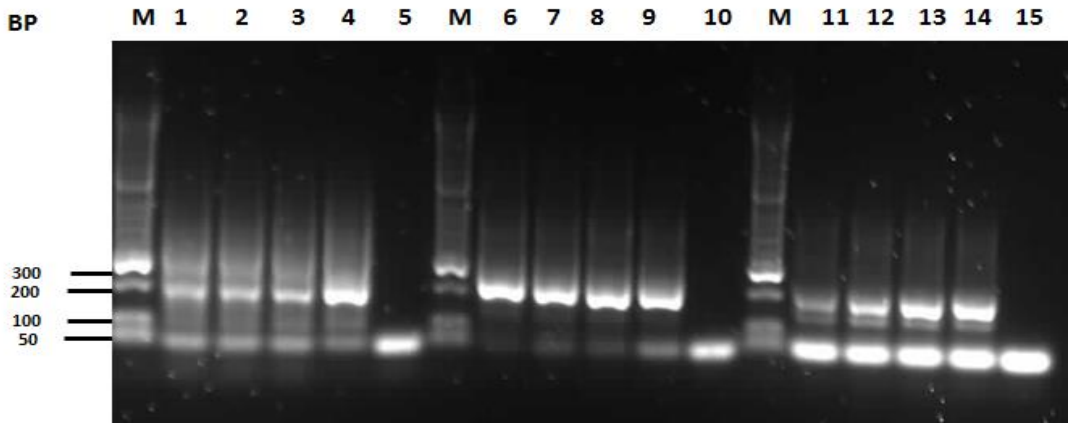

Figure 4.29. Representative agarose gel (1.5%) image showing temperature gradient PCR amplification of *Apodemus sylvaticus* NLRP1b fragments using exon 3-3, 3-2 and exon 3-1 primers. M, 50bp hyperladder; 1, *Apodemus sylvaticus* targeted exon 3-3 fragment of 235bp with annealing temperature of 56 °C; 2, *Apodemus sylvaticus* targeted exon 3-3 fragment of 235bp with annealing temperature of 57°C; 3, *Apodemus sylvaticus* targeted exon 3-3 fragment of 235bp with annealing temperature of 58°C; 4, *Apodemus sylvaticus* targeted exon 3-3 fragment of 235bp with annealing temperature of 59°C; 5, Negative control (water); M, 50bp hyperladder; *6, Apodemus sylvaticus* targeted exon 3-2 fragment of 235bp with annealing temperature of 56°C; 7, *Apodemus sylvaticus* targeted exon 3-2 fragment of 235bp with annealing temperature of 57°C; 8 *Apodemus sylvaticus* targeted exon 3-2 fragment of 235bp with annealing temperature of 58°C; 9, *Apodemus sylvaticus* targeted exon 3-2 fragment of 235bp with annealing temperature of 59°C; 10, Negative control (water); M, 50bp hyperladder; 11, *Apodemus sylvaticus* targeted exon 3-1 fragment of 215bp with annealing temperature of 56°C; 12, *Apodemus sylvaticus* targeted exon 3-1 fragment of 215bp with annealing temperature of 57°C; 13, *Apodemus sylvaticus* targeted exon 3-1 fragment of 215bp with annealing temperature of 58°C; 14, *Apodemus sylvaticus* targeted exon 3-1 fragment of 215bp with annealing temperature of 59 °C; 15, Negative control (water).

Then, the primers were tested at 59°C and the result was perfect, all primers showed successful amplification (Figure 4.30).

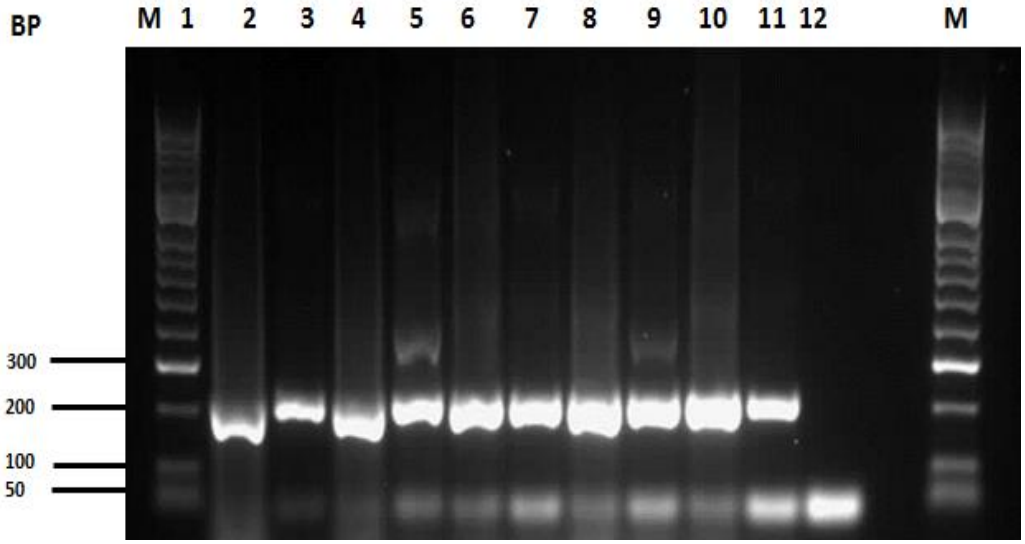

Figure 4.30. Representative agarose gel (1.5%) image showing PCR amplification of *Apodemus sylvaticus* NLRP1b fragments using primer 3-2. M, 50bp hyperladder; 1, *Apodemus sylvaticus* targeted fragment of sample 102 (235); 1, *Apodemus sylvaticus* targeted fragment of sample 102 (235); 2, *Apodemus sylvaticus* targeted fragment of sample 102 (235); 3, *Apodemus sylvaticus* targeted fragment of sample 102 (235); 4, *Apodemus sylvaticus* targeted fragment of sample 102 (235); 5, *Apodemus sylvaticus* targeted fragment of sample 102 (235); 6, *Apodemus sylvaticus* targeted fragment of sample 102 (235); 7, *Apodemus sylvaticus* targeted fragment of sample 102 (235); 8, *Apodemus sylvaticus* targeted fragment of sample 102 (235); 9, *Apodemus sylvaticus* targeted fragment of sample 102 (235);10, *Apodemus sylvaticus* targeted fragment of sample 102 (235); 11, negative control (water); 12, M 50bp hyperladder.

After that, exon 3-3 primers were also tested at 59ºC and showed successful amplification (Figure 4.31).

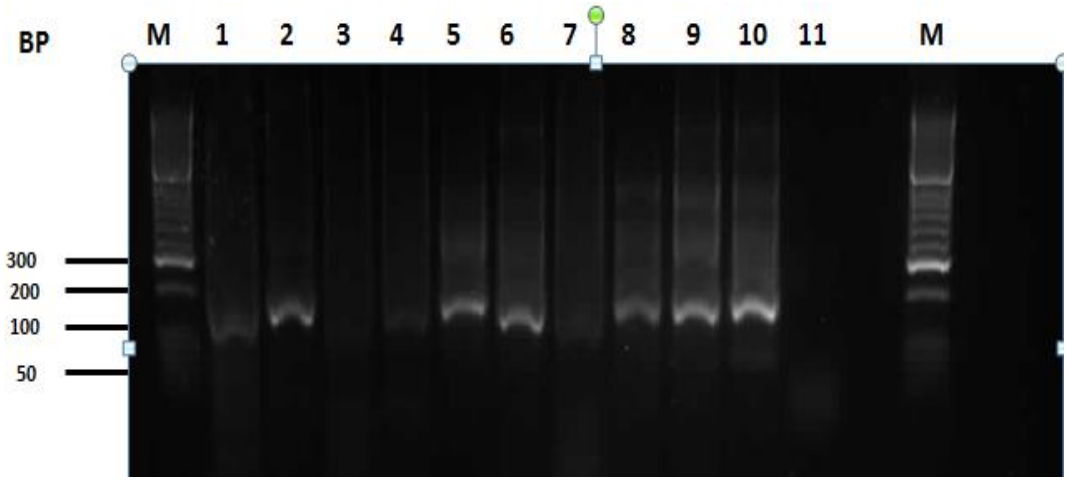

Figure 4.31. Representative agarose gel (1.5%) image showing PCR amplification of *Apodemus sylvaticus* NLRP1b fragments using primers 3-3. M, 50bp hyperladder; 1, *Apodemus sylvaticus* targeted fragment of sample 102 (235); 1, *Apodemus sylvaticus* targeted fragment of sample 102 (235); 2, *Apodemus sylvaticus* targeted fragment of sample 102 (235); 3, *Apodemus sylvaticus* targeted fragment of sample 102 (235); 4, *Apodemus sylvaticus* targeted fragment of sample 102 (235); 5, *Apodemus sylvaticus* targeted fragment of sample 102 (235); 6, *Apodemus sylvaticus* targeted fragment of sample 102 (235); 7, *Apodemus sylvaticus* targeted fragment of sample 102 (235); 8, *Apodemus sylvaticus* targeted fragment of sample 102 (235); 9, *Apodemus sylvaticus* targeted fragment of sample 102 (235);10, *Apodemus sylvaticus* targeted fragment of sample 102 (235); 11, negative control (water); 12, M 50bp hyperladder.

After testing both primers, exon 3-2 and 3-3, exon 3-1 primer was tested and the targeted band size was successfully determined (Figure 4.32).

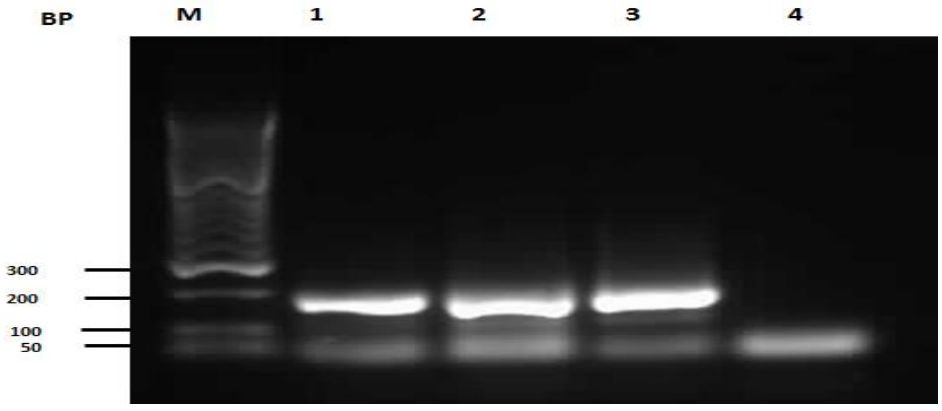

Figure 4.32. Representative agarose gel (1.5%) image showing PCR amplification of *Apodemus sylvaticus* NLRP1b fragments using primer 3-1. M, 50bp hyperladder; 1, *Apodemus sylvaticus* targeted fragment of sample 86 (220bp); 2, *Apodemus sylvaticus* targeted fragment of sample 102 (220bp); 3, *Apodemus sylvaticus* targeted fragment of sample 103 (220bp); 4, negative control (water)

In conclusion, different PCRs were carried out to determine the optimal conditions for each primer. First, *Apodemus sylvaticus* DNA was successfully amplified using two different sets of primers. The first set included exon 2, exon 3-2 and exon 3-3, while the second set included exon 3-2N and exon 3-3N. The last set of primers comprised exon 3 primers parts 1, 2 and 3. All the primers used are summarized in the table below (Table 4.8).

Table 4.8. Summary of primers that had been designed and used to amplify fragments of the mouse NLRP1b gene.

Exon	Primer	*Mus Musculus*	*Apodemus sylvaticus*
2	E2	✓	✓
3-1	E3-1	x	x
3-2	E3-2	✓	✓
3-3	E3-3	✓	✓
3-2N	E3-2N	✓	✓
3-3	E3-3N	✓	✓
3-3	SEQ	N/A	(sequencing only)
3-3	SEQ2	N/A	(sequencing only)
3-2	3-2	✓	✓
3-3	3-2	✓	✓
3-1	3-1	✓	✓

4.5.2.3.1 Analysis of exon 3-2

The first step of the analysis was to arrive at a consensus sequence for exon 3-2 as described in Section 4.4.2.1. Figure 4.33 shows the consensus sequence of exon 3-2.

```
GATGTTTTCTTCTTCAGTTGCAGAGAGTTGGCCCAGTACAAGCAGATGAGTCTGGCTGAGCT
CATAGAACAAGGCCAGGAAGTACCCACAGCTCCCATCAGTCAGATCCTGTCTCATCCTGAGA
AGCTGCTCTTCATCCTGGATGGCATAGATGAGCCAGCATGGGTCTTGGCAGACCAGAATCCT
GAGCTC
```

Figure 4.33. Consensus sequence of exon 3-2. The red shaded nucleotides show the SNP locations.

Two SNP locations were found in all 80 samples, bases 46 and 68 (marked in red in Figure 4.33), and were investigated by carrying out a protein translation (Figure 4.34).

```
GATGTTTTCTTCTTCAGTTGCAGAGAGTTGGCCCAGTACAAGCAGATGAGTCTGGCTGAG
 D  V  F  F  F  S  C  R  E  L  A  Q  Y  K  Q  M  S  L  A  E
CTCATAGAACAAGGCCAGGAAGTACCCACAGCTCCCATCAGTCAGATCCTGTCTCATCCT
 L  I  E  Q  G  Q  E  V  P  T  A  P  I  S  Q  I  L  S  H  P
GAGAAGCTGCTCTTCATCCTGGATGGCATAGATGAGCCAGCATGGGTCTTGGCAGACCAG
 E  K  L  L  F  I  L  D  G  I  D  E  P  A  W  V  L  A  D  Q
AATCCTGAGCTC
N  P  E  L
```

Figure 4.34. Protein translation of exon 3-2 sequence.

According to the two SNPs, four haplotypes were discovered, haplotypes 1, 2, 3 and 4 (Figure 4.35).

```
Haplotype 1
GATGTTTTCTTCTTCAGTTGCAGAGAGTTGGCCCAGTACAAGCAGCTGAGTCTGGCTGAGCT
CATAGCACAAGGCCAGGAAGTACCCACAGCTCCCATCAGTCAGATCCTGTCTCATCCTGAGA
AGCTGCTCTTCATCCTGGATGGCATAGATGAGCCAGCATGGGTCTTGGCAGACCAGAATCCT
GAGCTC
Haplotype 2
GATGTTTTCTTCTTCAGTTGCAGAGAGTTGGCCCAGTACAAGCAGCTGAGTCTGGCTGAGCT
CATAGAACAAGGCCAGGAAGTACCCACAGCTCCCATCAGTCAGATCCTGTCTCATCCTGAGA
AGCTGCTCTTCATCCTGGATGGCATAGATGAGCCAGCATGGGTCTTGGCAGACCAGAATCCT
GAGCTC
Haplotype 3

GATGTTTTCTTCTTCAGTTGCAGAGAGTTGGCCCAGTACAAGCAGATGAGTCTGGCTGAGCT
CATAGAACAAGGCCAGGAAGTACCCACAGCTCCCATCAGTCAGATCCTGTCTCATCCTGAGA
AGCTGCTCTTCATCCTGGATGGCATAGATGAGCCAGCATGGGTCTTGGCAGACCAGAATCCT
GAGCTC
Haplotype 4
GATGTTTTCTTCTTCAGTTGCAGAGAGTTGGCCCAGTACAAGCAGATGAGTCTGGCTGAGCT
CATAGCACAAGGCCAGGAAGTACCCACAGCTCCCATCAGTCAGATCCTGTCTCATCCTGAGA
AGCTGCTCTTCATCCTGGATGGCATAGATGAGCCAGCATGGGTCTTGGCAGACCAGAATCCT
GAGCTC
```

Figure 4.35. Four different haplotypes of the exon 3-2 region of NLRP1b. The red shaded nucleotides show the SNP locations.

As we had four different haplotypes, a protein translation was carried out for each one, as described in Section 2.8, to find any differences in protein sequence (Figure 4.36).

Haplotype 1

```
GATGTTTTCTTCTTCAGTTGCAGAGAGTTGGCCCAGTACAAGCAGCTGAGTCTGGCTGAG
 D  V  F  F  F  S  C  R  E  L  A  Q  Y  K  Q  L  S  L  A  E
CTCATAGCACAAGGCCAGGAAGTACCCACAGCTCCCATCAGTCAGATCCTGTCTCATCCT
 L  I  A  Q  G  Q  E  V  P  T  A  P  I  S  Q  I  L  S  H  P
GAGAAGCTGCTCTTCATCCTGGATGGCATAGATGAGCCAGCATGGGTCTTGGCAGACCAG
 E  K  L  L  F  I  L  D  G  I  D  E  P  A  W  V  L  A  D  Q
AATCCTGAGCTC
 N  P  E  L
```

Haplotype 2

```
GATGTTTTCTTCTTCAGTTGCAGAGAGTTGGCCCAGTACAAGCAGCTGAGTCTGGCTGAG
 D  V  F  F  F  S  C  R  E  L  A  Q  Y  K  Q  L  S  L  A  E
CTCATAGAACAAGGCCAGGAAGTACCCACAGCTCCCATCAGTCAGATCCTGTCTCATCCT
 L  I  E  Q  G  Q  E  V  P  T  A  P  I  S  Q  I  L  S  H  P
GAGAAGCTGCTCTTCATCCTGGATGGCATAGATGAGCCAGCATGGGTCTTGGCAGACCAG
 E  K  L  L  F  I  L  D  G  I  D  E  P  A  W  V  L  A  D  Q
AATCCTGAGCTC
 N  P  E  L
```

Haplotype 3

```
GATGTTTTCTTCTTCAGTTGCAGAGAGTTGGCCCAGTACAAGCAGATGAGTCTGGCTGAG
 D  V  F  F  F  S  C  R  E  L  A  Q  Y  K  Q  M  S  L  A  E
CTCATAGAACAAGGCCAGGAAGTACCCACAGCTCCCATCAGTCAGATCCTGTCTCATCCT
 L  I  E  Q  G  Q  E  V  P  T  A  P  I  S  Q  I  L  S  H  P
GAGAAGCTGCTCTTCATCCTGGATGGCATAGATGAGCCAGCATGGGTCTTGGCAGACCAG
 E  K  L  L  F  I  L  D  G  I  D  E  P  A  W  V  L  A  D  Q
AATCCTGAGCTC
 N  P  E  L
```

Haplotype 4

```
GATGTTTTCTTCTTCAGTTGCAGAGAGTTGGCCCAGTACAAGCAGATGAGTCTGGCTGAG
 D  V  F  F  F  S  C  R  E  L  A  Q  Y  K  Q  M  S  L  A  E
CTCATAGCACAAGGCCAGGAAGTACCCACAGCTCCCATCAGTCAGATCCTGTCTCATCCT
 L  I  A  Q  G  Q  E  V  P  T  A  P  I  S  Q  I  L  S  H  P
GAGAAGCTGCTCTTCATCCTGGATGGCATAGATGAGCCAGCATGGGTCTTGGCAGACCAG
 E  K  L  L  F  I  L  D  G  I  D  E  P  A  W  V  L  A  D  Q
AATCCTGAGCTC
 N  P  E  L
```

Figure 4.36. Protein translations of the four different haplotypes of exon 3-2.

Later, a clustal alignment was carried out, as described in Section 2.8, for all four protein haplotypes to check for any differences between them (Figure 4.37).

```
H4      DVFFFSCRELAQYKQMSLAELIAQGQEVPTAPISQILSHPEKLLFILDGIDEPAWVLADQ
H3      DVFFFSCRELAQYKQMSLAELIEQGQEVPTAPISQILSHPEKLLFILDGIDEPAWVLADQ
H1      DVFFFSCRELAQYKQLSLAELIAQGQEVPTAPISQILSHPEKLLFILDGIDEPAWVLADQ
H2      DVFFFSCRELAQYKQLSLAELIEQGQEVPTAPISQILSHPEKLLFILDGIDEPAWVLADQ
        ***************:****** ************************************

H4      NPEL
H3      NPEL
H1      NPEL
H2      NPEL
        ****
```

Figure 4.37. Clustal alignment of the four different protein haplotypes

The next table (Table 4.9) shows a summary of the four haplotypes and their nucleotide SNP locations at both DNA and protein levels. It also shows the effect of each SNP that affects protein translation.

Table 4.9. Summary of SNP positions and amino acid changes.

Haplotype	SNPS	Amino acid variation	Mutation
H1	Position 46/C Position 68/C	Position 16/L Position 23/A	Missense Missense
H2	Position 46/C Position 68/A	Position 16/L Position 23/E	Missense Missense
H3	Position 46/A Position 68/A	Position 16/M Position 23/E	Missense Missense
H4	Position 46/A Position 68/C	Position 16/M Position 23/A	Missense Missense

As shown in Table 4.9, haplotype 1 has leucine at position 16 and alanine at position 23. Haplotype 2 has leucine at position 16 and glutamic acid at position 23. Haplotype 3 has methionine at position 16 and glutamic acid at position 23. Haplotype 4 has methionine at position 16 and alanine at position 23.

According to the protein haplotypes, the 80 mouse samples were each divided into different groups of haplotypes (Table 4.10).

Table 4.10. Table of each mouse ID and its NLRP1b exon 3-2 haplotype.

Haplotype	Mouse ID	Haplotype	Mouse ID	Haplotype	Mouse ID	Haplotype	Mouse ID
H1/H1	6	H1/H1	40	H4/H4	74	H1/H1	101
H2/H3	9	H2/H2	41	H4/H4	75	H2/H3	102
H1/H1	10	H2/H2	43	H1/H3	76	H1/H2	103
H2/H2	11	H2/H2	44	H1/H2	77	H2/H2	104
H2/H2	12	H2/H2	45	H3/H2	78	H1/H1	106
H2/H3	15	H4/H4	46	H2/H2	82	H2/H3	108
H1/H2	18	H4/H4	47	H1/H1	84	H2/H3	110
H2/H3	23	H4/H4	48	H1/H1	85	H2/H2	112
H2/H3	25	H2/H3	53	H1/H3	86	H2/H3	115
H2/H3	26	H1/H2	56	H1/H2	87	H1/H1	116
H1/H2	27	H1/H3	57	H3/H3	88	H2/H2	117
H2/H3	28	H1/H2	58	H1/H2	89	H2/H3	118
H1/H3	31	H1/H2	60	H1/H2	90	H2/H2	119
H1/H3	32	H3/H2	61	H1/H2	91	H2/H2	120
H1/H2	33	H2/H2	64	H4/H4	92	H2/H3	121
H1/H2	35	H1/H2	66	H1/H2	94	H2/H3	122
H4/H4	36	H2/H2	70	H1/H2	95	H1/H1	123
H4/H4	37	H2/H2	71	H1/H3	96	H1/H2	124
H1/H1	38	H1/H2	72	H2/H3	99	H2/H2	125
H1/H2	39	H4/H4	73	H1/H2	100	H2/H2	126

The above table includes the 80 mice used in our experiment. It also shows each mouse ID number and its specific haplotype out of the nine haplotypes discovered.

Out of the 80 samples that were investigated, ten mice were H1/H1 haplotypes, 20 were H1/H2, 17 were H2/H2, two were H3/H2, six were H1/H3,15 were H2/H3, only one was H3/H3 and nine were H4/H4 (Table 4.10 & Figure 4.38).

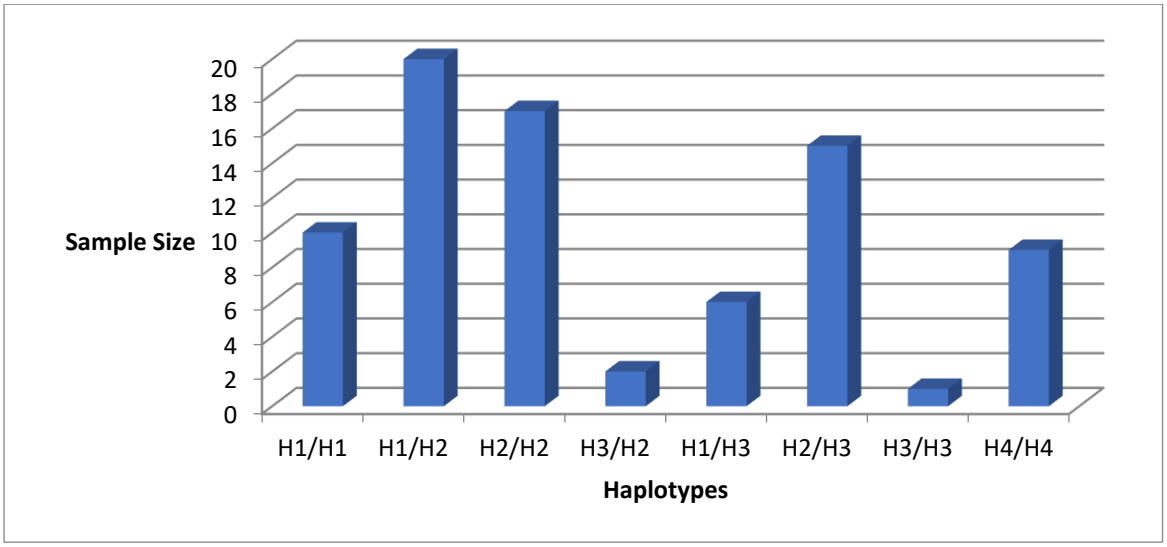

Figure 4.38 Association between the haplotypes of exon 3-2 and the numbers of the samples.

Next, the number of *Toxoplasma gondii* infected mice and the specific haplotype of each infected and non-infected mouse was investigated (Figure 4.39).

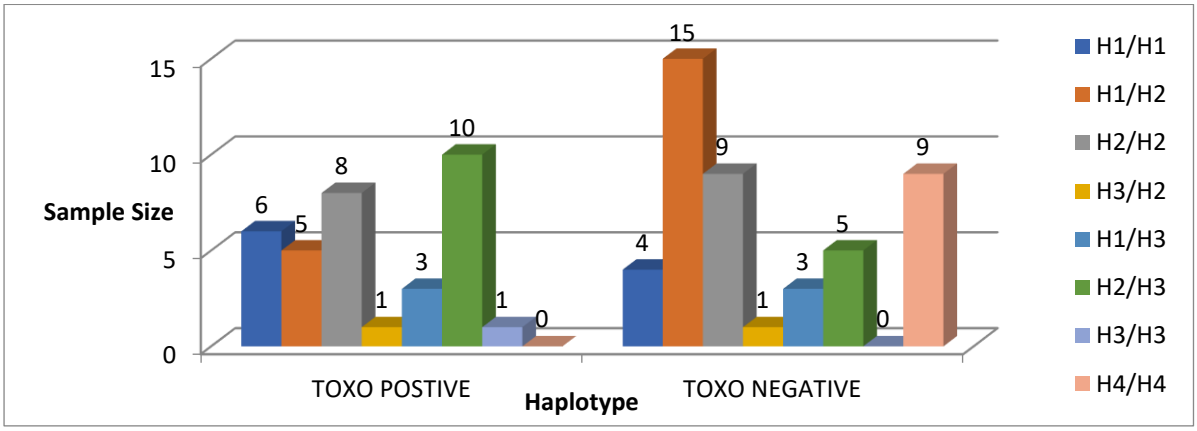

Figure 4.39. *Toxoplasma* infected mice and their haplotypes.

In total, 34 out of 80 mice *were Toxoplasma* positive and 46 *Toxoplasma* negative: 6 out of 10 H1/H1 mice were *Toxoplasma* positive, 4 were negative; 5 out of 20 H1/H2 were infected, 15 were not infected; 8 out of 17 H2/H2 were *Toxoplasma* positive, 9 of them were negative; 1out of 2 H3/H2 mice was *Toxoplasma* positive, 1 was negative; 3 out of 6 H1/H3 were infected, 3 were not infected; 10 out of 15 H2/H3 were *Toxoplasma* positive, 5 were

Toxoplasma negative; only one mouse was H3/H3 and was *Toxoplasma* positive; and finally, none of the 9 H4/H4 mice were *Toxoplasma* positive (Figure 4.42).

Then, the relation between haplotype and susceptibility or resistance to *Toxoplasma* infection was investigated. Only two haplotypes were discovered to have a significant relation with *Toxoplasma* infection, H2/H3 and H4/H4 (Figures 4.40, 4.41).

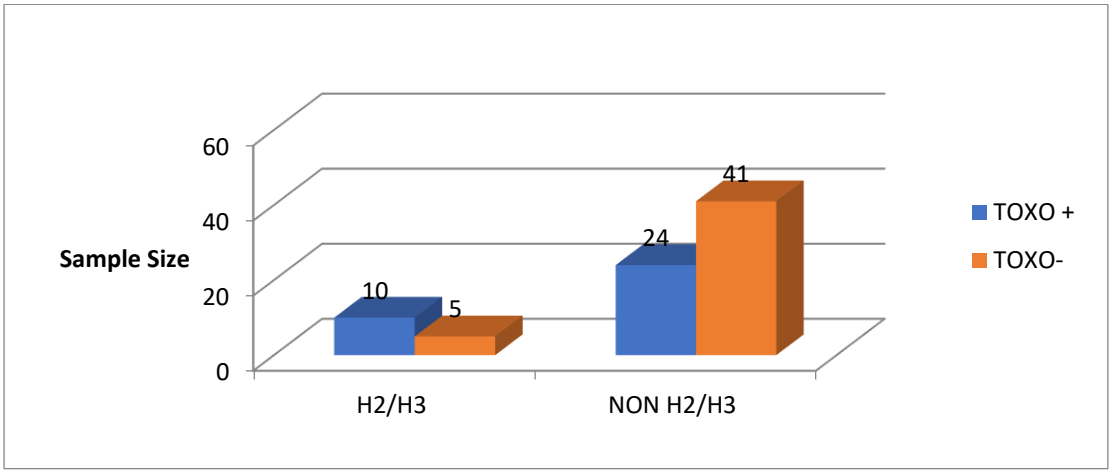

Figure 4.40. Relation between H2/H3 haplotype and *Toxoplasma* positive and negative mice.

An exact Fisher's test was carried out to find the two-tailed P value, which is equal to 0.045, which means a significant relation between H2/H3 haplotypes and *Toxoplasma gondii* infection with respect to different helminth parasites as mice with this haplotype appeared more susceptible to infection.

Upon further investigation of *Toxoplasma* infected mice, the data showed that the association was not significant with respect to the single *Toxoplasma gondii* infection (P value = 0.67). (see appendix 2, Table1)

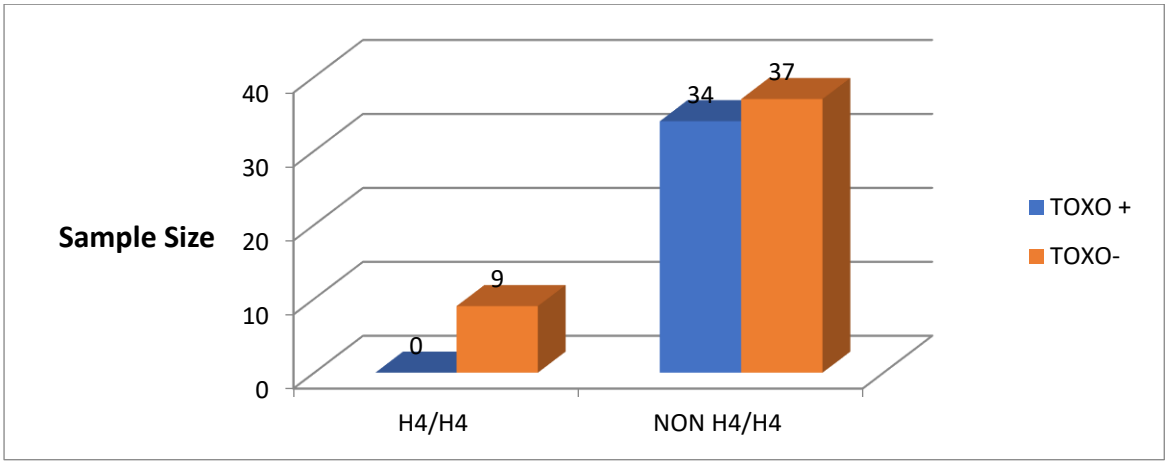

Figure 4.41. Relation between H4/H4 haplotype and *Toxoplasma* positive and negative mice.

A significant association was discovered between H4/H4 haplotypes and *Toxoplasma* infection with respect to different helminth parasites as this haplotype appeared to confer resistance to infection. A two-tailed P value was calculated, which is 0.0086. No significant relation was discovered between any other haplotype and *Toxoplasma gondii* infection.

Upon further investigation of *Toxoplasma* infected mice, the data showed that the association was not significant between H4/H4 with respect to the single *Toxoplasma gondii* infection (P value > 0.05) (see appendix 2, Table1)

The next table summarizes each haplotype of infected and non-infected mice, their calculated two-tailed P values and the significance of those P values (Table 4.11).

Table 4.11. Calculated two-tailed P value for each haplotype of exon 3-2.

Haplotypes	Two-tailed P value	Significance
H1/H1	0.31	No
H1/H2	0.075	No
H2/H2	0.78	No
H3/H2	> 0.05	No
H1/H3	0.69	No
H2/H3	0.045	Yes
H3/H3	0.42	No
H4/H4	0.0086	Yes

After that, the relation between homozygosity, heterozygosity and *Toxoplasma* positive and negative mice was investigated at the first SNP location alone (Figure 4.42), the second SNP location alone (Figure 4.3) and both SNP locations together (Figure 4.44).

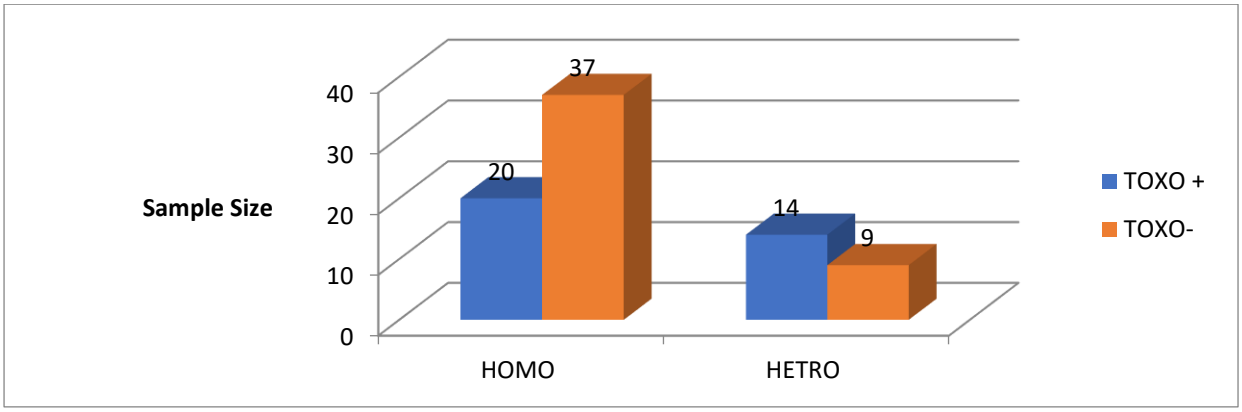

Figure 4.42. Association between homozygosity and heterozygosity at the 16 amino acid SNP location and *Toxoplasma* infection status.

With respect to different helminth parasites a total of 34 mice were infected with *Toxoplasma gondii*, 20 were homozygote and 14 were heterozygote. 37 homozygote mice were discovered among 46 *Toxoplasma gondii* negative mice, the remaining mice were heterozygote. A significant association was discovered between homozygosity and

heterozygosity at the 16 amino acid SNP location since homozygous mice appeared to show resistance towards *Toxoplasma* infection (two-tailed P value = 0.0465). Upon further investigation of *Toxoplasma* infected mice, the data showed that the association was not significant between homozygosity and heterozygosity at the first SNP locus with respect to the single *Toxoplasma gondii* infection (P value =0.7).

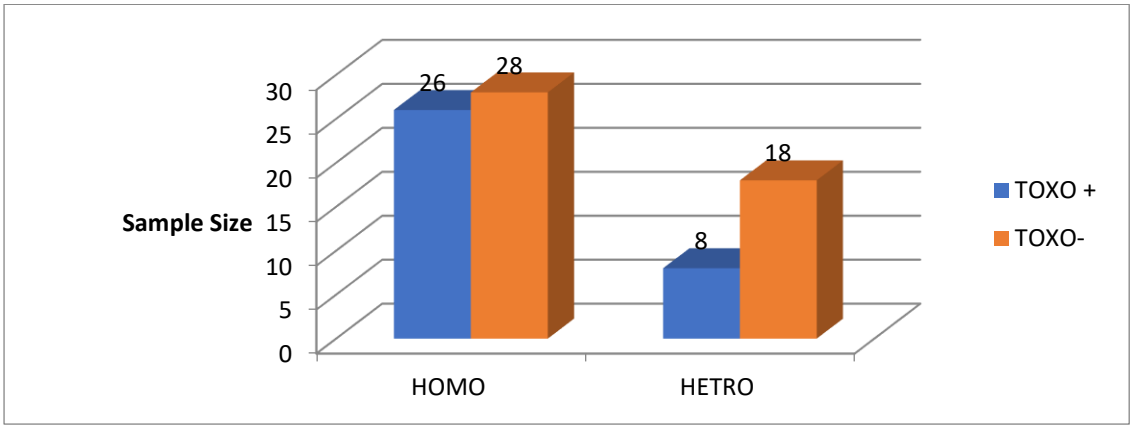

Figure 4.43. Association between homozygosity and heterozygosity at 23 amino acid SNP location and *Toxoplasma* infection status.

No significant association was discovered between homozygosity and heterozygosity at the 23 amino acid SNP location and *Toxoplasma* infection with respect to different helminth parasites after calculating the two-tailed P value, which is 0.16.

Upon further investigation of *Toxoplasma* infected mice, the data showed that the association was not significant between homozygosity and heterozygosity of the second SNP locus with respect to the single *Toxoplasma gondii* infection (P value =0.6).

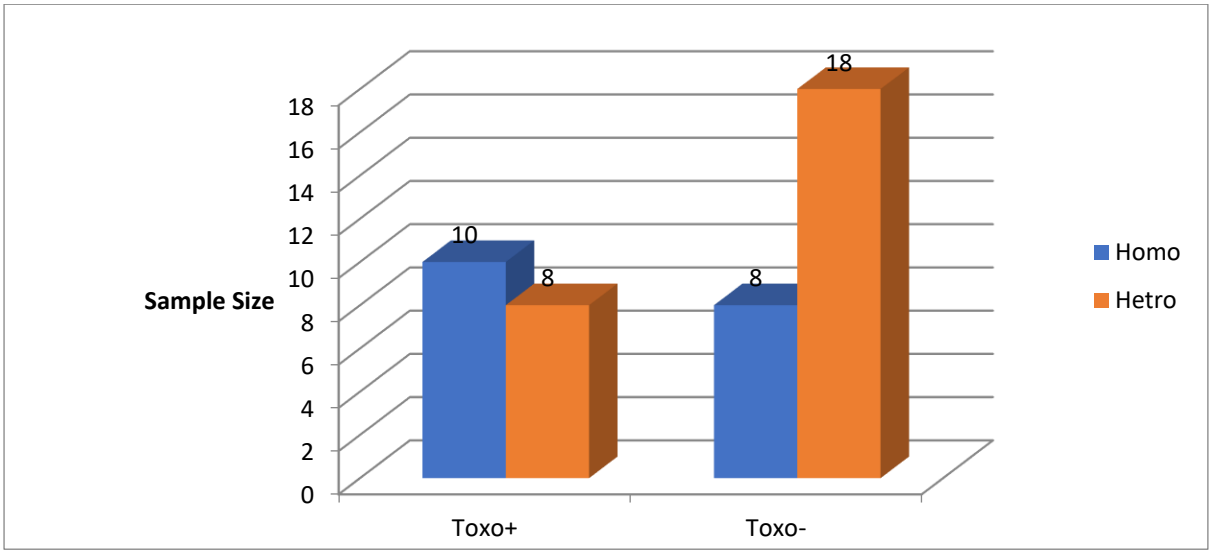

Figure 4.44. Association between homozygosity and heterozygosity at both amino acid SNP locations (16 & 23) and *Toxoplasma* infection status.

With respect to different helminth parasites the two-tailed P value for the previous figure is 0.13, which indicates that there is no significant association homozygosity and heterozygosity at both amino acid SNP locations taken together and *Toxoplasma* infection. Upon further investigation of *Toxoplasma* infected mice, the data showed that the association was not significant between homozygosity and heterozygosity at both SNP loci with respect to the single *Toxoplasma gondii* infection (P value > 0.05).

4.5.2.3.2 Analysis of exon 3-3

After finishing the analysis of exon 3-2, exon 3-3 was analysed in same way as exon 3-2. A consensus sequence for exon 3-3 was determined after taking the same steps as those described in Section 4.4.2.1 .Figure 4.45 shows the consensus sequence of exon 3-3.

AAATACCTTTCCCTGACAATCCCAGGTCAGCACATGGGGACCCAGCTCAGAGCC
CTCTGCTCACTGGCTGCTGAGGGGATCTGCCAAAGAAGGACTCTATTCAGTAAAA
GCGACCTCCGTAAGCAAGGGTTAGCAGACGTTGCCATTGACACCTTCGTGAAGA
TCGGTATCCTTCAAAAGCAGG

Figure 4.45. Consensus sequence of exon 3-3, the red shaded nucleotides show the SNP locations.

Like exon 3-2, two SNPs were discovered in exon 3-3, located at bases 32 and 106, and four haplotypes were determined from the sequences (Figure 4.46).

HAPLOTYPE1
AAATACCTTTCCCTGACAATCCCAGGTCAGCACATGGGGACCCAGCTCAGAGCC
CTCTGCTCACTGGCTGCTGAGGGGATCTGCCAAAGAAGGACTCTATTCAGTAAAA
GCGACCTCCGTAAGCAAGGGTTAGCAGACGTTGCCATTGACACCTTCGTGAAGA
TCGGTATCCTTCAAAAGCAGG
HAPLOTYPE2
AAATACCTTTCCCTGACAATCCCAGGTCAGCACATGGGGACCCAGCTCAGAGCC
CTCTGCTCACTGGCTGCTGAGGGGATCTGCCAAAGAAGGACTCTATTCAGTGAAA
GCGACCTCCGTAAGCAAGGGTTAGCAGACGTTGCCATTGACACCTTCGTGAAGA
TCGGTATCCTTCAAAAGCAGG
HAPLOTYPE3
AAATACCTTTCCCTGACAATCCCAGGTCAGCTCATGGGGACCCAGCTCAGAGCCC
TCTGCTCACTGGCTGCTGAGGGGATCTGCCAAAGAAGGACTCTATTCAGTAAAA
GCGACCTCCGTAAGCAAGGGTTAGCAGACGTTGCCATTGACACCTTCGTGAAGA
TCGGTATCCTTCAAAAGCAGG
HAPLOTYPE4
AAATACCTTTCCCTGACAATCCCAGGTCAGCTCATGGGGACCCAGCTCAGAGCCC
TCTGCTCACTGGCTGCTGAGGGGATCTGCCAAAGAAGGACTCTATTCAGTGAAA
GCGACCTCCGTAAGCAAGGGTTAGCAGACGTTGCCATTGACACCTTCGTGAAGA
TCGGTATCCTTCAAAAGCAGG

Figure 4.46. Four different haplotypes of exon 3-3, the red shaded nucleotides show the SNP locations.

A protein translation was carried out for each haplotype (Figure 4.47).

HAPLOTYPE1

```
AAATACCTTTCCCTGACAATCCCAGGTCAGCACATGGGGACCCAGCTCAGAGCCCTCTGC
 K  Y  L  S  L  T  I  P  G  Q  H  M  G  T  Q  L  R  A  L  C
TCACTGGCTGCTGAGGGGATCTGCCAAAGAAGGACTCTATTCAGTAAAAGCGACCTCCGT
 S  L  A  A  E  G  I  C  Q  R  R  T  L  F  S  K  S  D  L  R
AAGCAAGGGTTAGCAGACGTTGCCATTGACACCTTCGTGAAGATCGGTATCCTTCAAAAG
 K  Q  G  L  A  D  V  A  I  D  T  F  V  K  I  G  I  L  Q  K
CAGG
 Q
```

HAPLOTYPE2

```
AAATACCTTTCCCTGACAATCCCAGGTCAGCACATGGGGACCCAGCTCAGAGCCCTCTGC
 K  Y  L  S  L  T  I  P  G  Q  H  M  G  T  Q  L  R  A  L  C
TCACTGGCTGCTGAGGGGATCTGCCAAAGAAGGACTCTATTCAGTAAAAGCGACCTCCGT
 S  L  A  A  E  G  I  C  Q  R  R  T  L  F  S  K  S  D  L  R
AAGCAAGGGTTAGCAGACGTTGCCATTGACACCTTCGTGAAGATCGGTATCCTTCAAAAG
 K  Q  G  L  A  D  V  A  I  D  T  F  V  K  I  G  I  L  Q  K
CAGG
 Q
```

HAPLOTYPE3

```
AAATACCTTTCCCTGACAATCCCAGGTCAGCTCATGGGGACCCAGCTCAGAGCCCTCTGC
 K  Y  L  S  L  T  I  P  G  Q  L  M  G  T  Q  L  R  A  L  C
TCACTGGCTGCTGAGGGGATCTGCCAAAGAAGGACTCTATTCAGTGAAAGCGACCTCCGT
 S  L  A  A  E  G  I  C  Q  R  R  T  L  F  S  E  S  D  L  R
AAGCAAGGGTTAGCAGACGTTGCCATTGACACCTTCGTGAAGATCGGTATCCTTCAAAAG
 K  Q  G  L  A  D  V  A  I  D  T  F  V  K  I  G  I  L  Q  K
CAGG
 Q
```

HAPLOTYPE4

```
AAATACCTTTCCCTGACAATCCCAGGTCAGCTCATGGGGACCCAGCTCAGAGCCCTCTGC
 K  Y  L  S  L  T  I  P  G  Q  L  M  G  T  Q  L  R  A  L  C
TCACTGGCTGCTGAGGGGATCTGCCAAAGAAGGACTCTATTCAGTGAAAGCGACCTCCGT
 S  L  A  A  E  G  I  C  Q  R  R  T  L  F  S  E  S  D  L  R
AAGCAAGGGTTAGCAGACGTTGCCATTGACACCTTCGTGAAGATCGGTATCCTTCAAAAG
 K  Q  G  L  A  D  V  A  I  D  T  F  V  K  I  G  I  L  Q  K
CAGG
 Q
```

Figure 4.47. Four different haplotypes of exon 3-3.

Protein translation was done for each haplotype and clustal alignment for all the protein sequences to show changes at the protein level (Figure 4.48).

```
H4      KYLSLTIPGQLMGTQLRALCSLAAEGICQRRTLFSESDLRKQGLADVAIDTFVKIGILQK
H3      KYLSLTIPGQLMGTQLRALCSLAAEGICQRRTLFSKSDLRKQGLADVAIDTFVKIGILQK
H1      KYLSLTIPGQHMGTQLRALCSLAAEGICQRRTLFSKSDLRKQGLADVAIDTFVKIGILQK
H2      KYLSLTIPGQHMGTQLRALCSLAAEGICQRRTLFSESDLRKQGLADVAIDTFVKIGILQK
        ********** ************************:************************

H4      Q
H3      Q
H1      Q
H2      Q
        *
```

Figure 4.48. Clustal alignment of four protein haplotypes of exon 3-3.

The next table shows a summary of the four haplotypes and their nucleotide SNP locations at both DNA and protein levels. Also, it shows the effect of each SNP that affects protein translation (Table 4.12).

Table 4.12. Summary of SNP positions and amino acid changes.

Haplotype	SNP	Amino acid variation	Mutation
H1	Position 32/A Position 106/A	Position 11/H Position 36/K	Missense Missense
H2	Position 32/A Position 106/G	Position 11/H Position 36/E	Missense Missense
H3	Position 32/T Position 106/A	Position 11/L Position 36/K	Missense Missense
H4	Position 32/T Position 106/G	Position 11/L Position 36/E	Missense Missense

As shown in Table 4.12 haplotype 1 has histidine at position 11 and lysine at position 36. Haplotype 2 has histidine at position 11 and glutamic acid at position 36. Haplotype 3 has leucine at position 11 and lysine at position 36. Haplotype 4 has leucine at position 11 and glutamic acid at position 36.

According to the protein haplotypes, the 80 samples were divided into different groups (Table 4.13).

Table 4.13. Table of each mouse and its NLRP1b exon 3-3 haplotype.

Haplotypes	Mouse ID	Haplotypes	Mouse ID	Haplotypes	Mouse ID	Haplotypes	Mouse ID
H1/H1	6	H2/H2	40	H1/H4	74	H1/H4	101
H1/H1	9	H3/H2	41	H1/H4	75	H3/H4	102
H1/H1	10	H3/H2	43	H1/H4	76	H3/H4	103
H1/H1	11	H3/H2	44	H1/H4	77	H4/H4	104
H1/H1	12	H3/H2	45	H1/H4	78	H4/H4	106
H1/H1	15	H3/H2	46	H1/H4	82	H4/H4	108
H1/H2	18	H4/H2	47	H1/H4	84	H4/H4	110
H1/H2	23	H3/H3	48	H1/H4	85	H4/H4	112
H1/H2	25	H3/H3	53	H1/H4	86	H4/H4	115
H1/H2	26	H3/H3	56	H1/H4	87	H4/H4	116
H2/H2	27	H1/H4	57	H1/H4	88	H4/H4	117
H2/H2	28	H1/H4	58	H1/H4	89	H4/H4	118
H2/H2	31	H1/H4	60	H1/H4	90	H4/H4	119
H2/H2	32	H1/H4	61	H1/H4	91	H4/H4	120
H2/H2	33	H1/H4	64	H1/H4	92	H4/H4	121
H2/H2	34	H1/H4	66	H1/H4	94	H4/H4	122
H2/H2	36	H1/H4	70	H1/H4	95	H4/H4	123
H2/H2	37	H1/H4	71	H1/H4	96	H4/H4	124
H2/H2	38	H1/H4	72	H1/H4	99	H4/H4	125
H2/H2	39	H1/H4	73	H1/H4	100	H4/H4	126

The above table includes the 80 mice used in our experiment. It also shows each mouse ID number and its specific haplotype out of the nine haplotypes that were discovered.

Out of the 80 samples investigated, six mice were H1/H1 haplotype, four mice were H1/H2, 31 were H1/H3, five were H3/H2, 3 were H3/H3, 2 were H3/H4, only one was H4/H2, 17 were H4/H4 and 11 were H2/H2 (Figure 4.49 and Table 4.13).

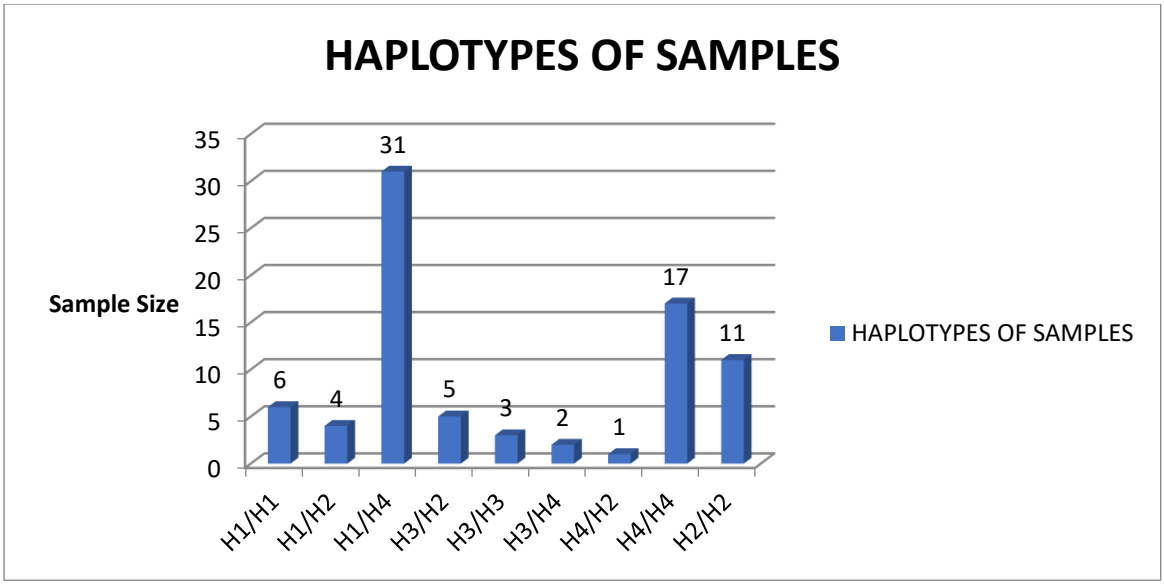

Figure 4.49 Association between the haplotypes of exon 3-3 and the numbers of samples.

In total, 34 out of 80 mice were *Toxoplasma gondii* positive and 46 were *Toxoplasma gondii* negative; only 1 out of 6 H1/H1 mice were *Toxoplasma gondii* positive, 5 were negative; 1 out of 4 H1/H2 were infected, 3 were not infected; 10 out of 31 H1/H4 mice were *Toxoplasma* positive, 21 of them were negative; 3 out of 11 H2/H2 mice were *Toxoplasma* positive infection, 8 were negative; 2 of the H3/H2 haplotypes was infected, 3 were negative; 1 H3/H3 mice were infected, 2 were negative. Only 2 mice of H3/H2 found and they were infected by *Toxoplasma gondii*. Only one H4/H2 mouse was found and negatively infected; 14 out of 17 H4/H4 mice were *Toxoplasma* positive, 3 were *Toxoplasma* negative (Figure 4.50).

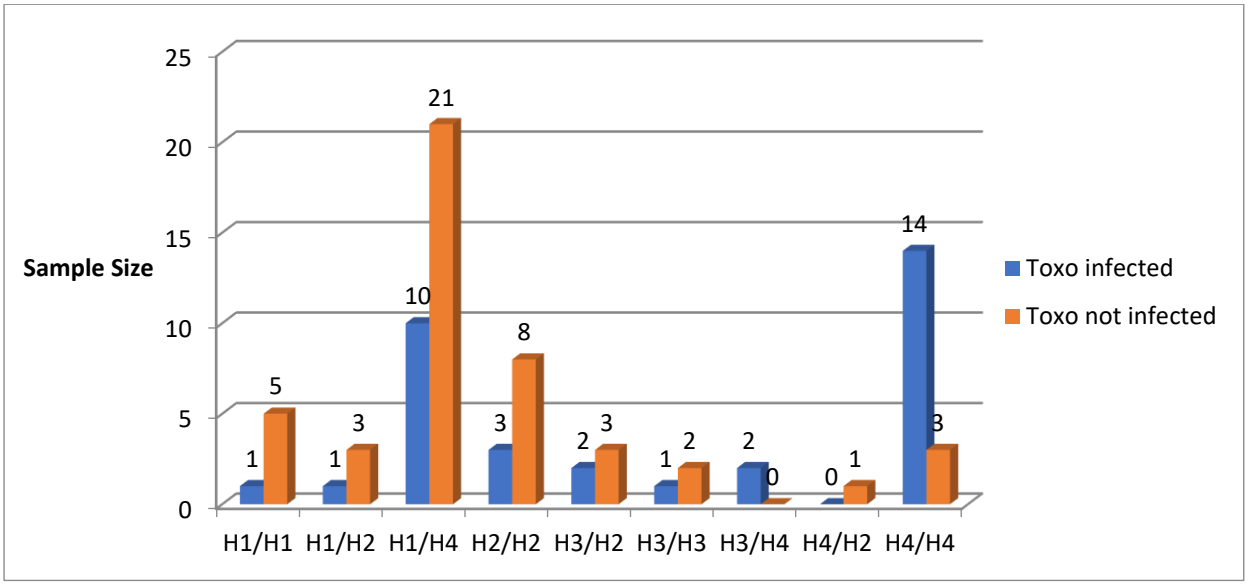

Figure 4.50. Relation between *Toxoplasma* infected mice and their haplotypes.

Then, the relation between the haplotypes and their susceptibility or resistance to *Toxoplasma* infection was investigated. Only one haplotype was discovered to have a significance relation with *Toxoplasma* infection in respect to different helminth parasites, H4/H4 (Figures 4.51)

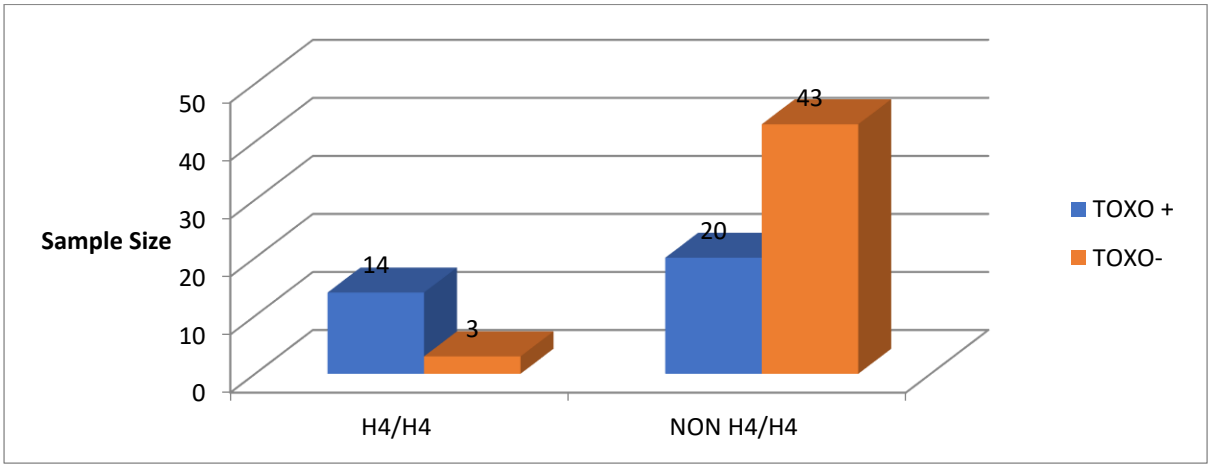

Figure 4.51. Relation between H4/H4 haplotypes and *Toxoplasma* positive and negative mice.

A significant association between H4/H4 haplotypes and susceptibility to *Toxoplasma* infection was discovered in respect to different helminth parasites after calculating the two-tailed P value for the previous chart, which is 0.0002. Upon further investigation of

Toxoplasma infected mice, the data showed that the association was not significant between H4/H4 haplotypes with respect to the single *Toxoplasma gondii* infection (P value > 0.2).

No significant relation was discovered between any other haplotypes and *Toxoplasma gondii* infection, as summarized in the table below (Table 4.14).

Table 4.14. Calculated two-tailed P values for each haplotype of exon 3-3.

Haplotypes	Two-tailed P value	Significance
H1/H1	0.23	No
H1/H2	0.63	No
H1/H4	0.17	No
H2/H2	0.34	No
H3/H2	> 0.05	No
H3/H3	> 0.05	No
H3/H4	0.18	No
H4/H2	> 0.05	No
H4/H4	0.00002	Yes

After that, the relationship between homozygosity and heterozygosity and *Toxoplasma* infection was investigated at the first SNP location alone (Figure 4.52), the second SNP location alone (Figure 4.53) and both SNP locations together (Figure 4.54).

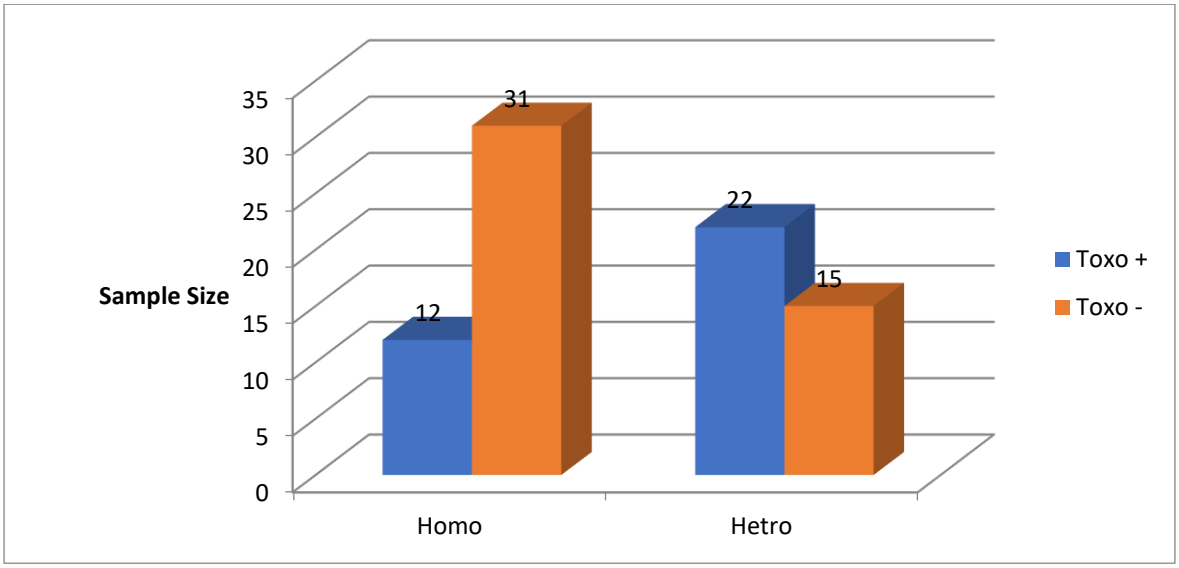

Figure 4.52. Association between homozygosity and heterozygosity at the 11 amino acid SNP location in exon 3-3 and *Toxoplasma* infection status.

A total of 34 mice were infected with *Toxoplasma gondii*, 12 of them were homozygote and 22 were heterozygote; 31 homozygote mice were discovered out of 46 *Toxoplasma gondii* negative mice, the remaining mice were heterozygote. With respect to different helminth parasites, a significant association was discovered between homozygosity at the 11 amino acid SNP location and resistance to *Toxoplasma* infection after calculating the two-tailed P value, which is 0.0064. Upon further investigation of *Toxoplasma* infected mice, the data showed that the association was not significant between homozygosity and heterozygosity at the first SNP locus with respect to the single *Toxoplasma gondii* infection (P value >0.05).

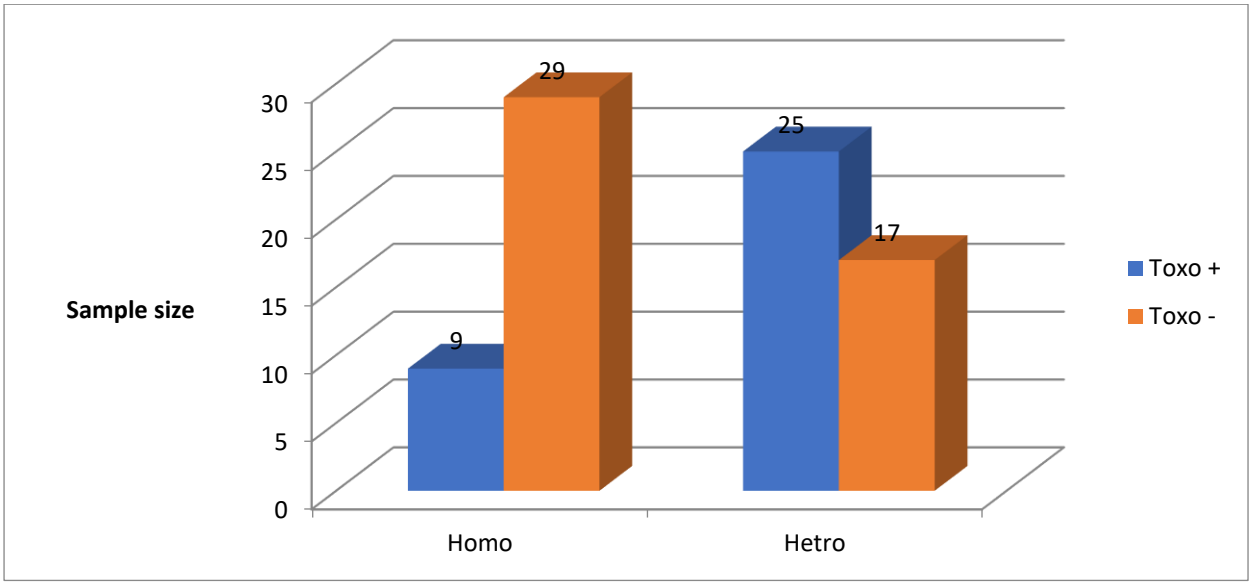

Figure 4.53. Association between homozygosity and heterozygosity at the 36 amino acid SNP position in exon 3-3 and *Toxoplasma* infection status.

A significant association was discovered between homozygosity at the 36 amino acid SNP position and resistance to *Toxoplasma* infection with respect to different helminth parasites after calculating the two-tailed P value, which is 0.0015. Upon further investigation of *Toxoplasma* infected mice, the data showed that the association was not significant between homozygosity and heterozygosity at the second SNP locus with respect to the single *Toxoplasma gondii* infection (P value >0.05).

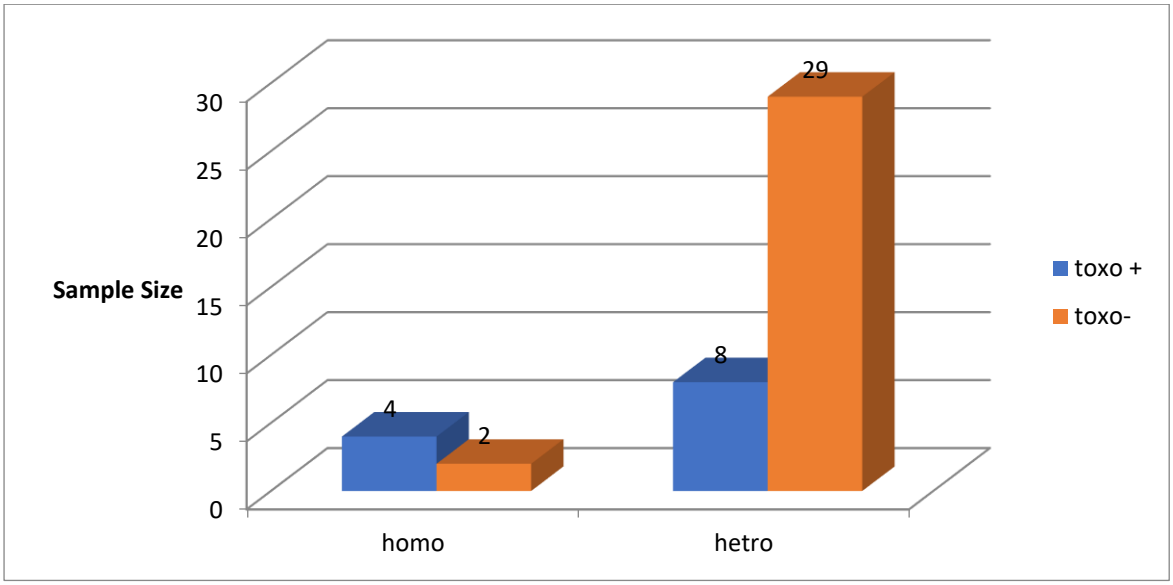

Figure 4.54. Association between homozygosity and heterozygosity at both the 11 and 36 amino acid SNP positions and *Toxoplasma* infection status.

With respect to different helminth parasites, a significant association was discovered between heterozygosity at both the 11 and 36 SNP amino acid positions and resistance to *Toxoplasma* infection after calculating the two-tailed P value, which is 0.042. Upon further investigation of *Toxoplasma* infected mice, the data showed that the association was not significant between homozygosity and heterozygosity at the first SNP locus with respect to the single *Toxoplasma gondii* infection (P value >0.05).

4.6 Discussion

4.6.1 Producing a map of NLRp1b gene from *Mus Musculus*

The first idea in this chapter was to produce a map for the NLRP1b gene for *Apodemus sylvaticus* and find out its variants. Although some parts of the *A. sylvaticus* genome were known there was no sequence in the database for the NLRP1b gene from *A. sylvaticus* at the time this work was being initiated. A search was carried out for *Mus musculus* NLRP1b sequence variants and two variants were found. The first was MEQSQPKKK (NP_001035786), which has around 3,525 nucleotides, while the second variant was MEESQYKQ (NP_001155886), which consists of 3,534 nucleotides. Clustal alignment was done for both DNA variant sequences, this showed a very high similarity between the two variants, which led to designing good PCR primers.

Initially, it was difficult to design NLRP1b PCR primers for *Apodemus sylvaticus* from *Mus musculus*; therefore, ambiguity nucleotides were used to improve the likelihood of success since we had two variants of the NLRP1b gene. Four primer sets were initially designed for exon 2, exon 3 part 1, exon 3 part 2 and exon 3 part 3. The primers for exon 3-2, exon 3-3 and exon 2 worked, but the primer for exon 3-1 failed. Some difficulties were encountered with the initial set of primers. Many attempts were made to make the primers work again, but none was successful. For example, changing the temperature gradient and magnesium gradient and ordering new stock of the same primers were done, but all these failed. Therefore, a decision was made to design a new primer. These issues might be due to using ambiguity letters, which could affect primer specificity.

Therefore, a second attempt at primer design was made. Two primer sets were synthesized for exon 3 part 2 and exon 3 part 3, which were called E3-2N and E3-3 N. Both primers were applied to *Mus musculus* and *Apodemus sylvaticus* and the expected fragment was amplified successfully. After sending the PCR product for sequencing, the sequence of exon 3-2 came

back with overlapped peaks from the begging to 330 base and good sequence from 331 to the end and the sequence of exon 3-3 came back with very poor quality. Several repeat attempts at PCR and sequencing were made, but the results were the same. The decision was made to stop working on exon 3-3 after several attempts at optimization failed and to investigate exon 3-2 instead. The sequence of exon 3-2 is divided into two parts. The first part has an overlapped sequences issue from base 1 to base 330, the second part is from base 331 to base 757. The first part had overlapped peaks and these peaks occur in all the samples. Therefore, two primers were designed from the sequence of exon 3-2 for sequencing purposes only, SEQ and SEQ2. Unfortunately, SEQ 1 and SEQ 2 primers did not solve the problem and the result of the sequencing did not match the required fragment of the NLRP1b gene. The reason for that over lapped peaks might be due amplification of two copies of the gene which might be found in the genome. Also, it might be due to picking nonspecific region of the gene or amplification of two related genes in the genome.

Later after the first two attempts were carried out, scaffold NLRP1b sequences were found in the NCBI database. This sequence was used with all the information that had been collected to design the third set of primers. This draft genome provided some help in mapping the NLRP1b in *Apodemus sylvaticus* and aligning the sequenced sample to check for any similarity. However, it did not give an explanation for why the previous two sets of primer did not work since the primers were aligned with draft genome.

After the previous two attempts, a third set of primers was designed. This was the most successful attempt to design primers. Three primers were designed for exon 3-1, 3-2 and 3-3. The second and third primers worked very well and gave excellent results. Exon 3-1 primers gave moderate results but not good enough to continue with further investigation. Exon 3-2 and 3-3 gave very good results and a further investigation was carried out.

There are no published studies on the NLRP1b gene in *Apodemus sylvaticus*, nor are there studies where people have investigated inflammasome variation in natural populations of

mice. Some studies investigated the association between the Anthrax lethal factor and the NLRP1b (Hellmich *et al*., 2012; Chavarría-Smith & Vance, 2013). Also, one other study that was carried out by Kuo-Chieh investigated the activation of NLRP1b by cytosolic ATP (Liao & Mogridge, 2013). Indeed, there are limited studies on the NLRP1b in any organism.

After successfully amplifying the exon 3-2 region from *Apodemus sylvaticus*, two SNPs were found, as shown in Table 4.7. These two SNPs changed the amino acid. The exon 3-2 fragments were variable with four haplotypes, which are described from 80 individual mice. Eight haplotypes were detected among the 80 mice. *Toxoplasma gondii* infection was investigated in the Malham Tarn mice (Boyce, 2013; Boyce *et al*., 2014), which was a perfect opportunity for us to investigate the relationship between parasite infection and variations in the NLRP1b gene haplotypes. Also, it helped us to address the question of whether *T. gondii* only, infection correlates with any of the NLRP1b haplotypes or whether different helminth parasites are an important component of any association. It was observed that only two haplotypes showed a significant relation with *T. gondii* infection and these were both linked with different helminth parasites. The H2/H3 haplotype has high frequency of *T. gondii* infection, while H4/H4 haplotype has 0% *T. gondii* infection; however, these relationships were not significant for *Toxoplasma gondii* infection only. Unexpectedly, homozygosity at the first SNP locus has lower frequency of *T. gondii* infection than the heterozygote with respect to different helminth parasites. Further investigation showed that the association was not significant with only *Toxoplasma* infected mice.

Furthermore, two SNPs were discovered in the exon 3-3 region. According to those SNPs, four haplotypes are described among the 80 mice (Table 4.11). Four haplotypes were generated from the variability of exon 3-3 fragments and these generated nine haplotypes among the 80 mice. It was observed that only one haplotype showed a significant relation with *T. gondii* infection in respect to different helminth parasites which is H4/H4. The H4/H4

haplotype statistically has high frequency of *T. gondii* infection. Unexpectedly, heterozygosity at the first and second location separately had a high frequency of toxoplasmosis while 2 SNPs at the 2 loci together had a low frequency of infection. These SNPs were found to locate to the NACHT domain. All the previous relationships were investigated in relation to *Toxoplasma* only and they were found not to be significant.

The nucleotide Binding domain consists of the NACHT domain and NAD domain which is NACHT associated domain (Macdonald *et al.*, 2013) (Figure 4.1). The NACHT domain plays an important role in binding to ribonucleotides which controls the self-oligomerization and inflammasome assembly (Latz, 2010). The NACHT domain also associates with some other domains such as the LRR domain which has the function of direct binding to procaspase-1 through intermediate adaptor proteins (Faustin *et al.*, 2007). Polymorphisms in the Nod-like receptor gene family such as NLRP and NLRP3 has been identified in different hosts but not the *Apodemus sylvaticus* since the genome is not available. Mutations have been discovered among the NACHT domain and nucleotide binding domain in humans. For example, NLRP3 missense mutations (R260W, D303N, and L353P) in the nucleotide binding domain has a relationship with some diseases such as Muckle–Wells syndrome as well as chronic infantile neurological cutaneous and articular (CINCA) syndrome. These known polymorphisms have a significant relationship with different diseases due to alteration of the inflammasome. However, the mechanism of alteration and the outcomes of these mutations are not well understood (Macdonald *et al.*, 2013).

These studies (Macdonald *et al.*, 2013: Faustin *et al.*, 2007; Latz, 2010) confirmed the role of the NACHT domain in inflammasome assembly and showed the important effects the polymorphism of the NACHT domain in relation to some disease. This also has been shown in this chapter as some haplotypes had a high prevalence of *Toxoplasma gondii* while others

had a very low prevalence and this might be due to some of the polymorphisms described within the NACHT domain of the wood mouse NLRP1b gene.

Furthermore, the homozygosity and heterozygosity of each SNP locus were investigated for both exons. For exon 3-2 SNPs, only homozygosity at the first SNP was statistically significant for association with negative *Toxoplasma gondii* infection while the second SNP was not significant and both SNPs together showed no significant association. For exon 3-3, SNPs at the first, second and both loci together showed a statistically significant association. The homozygosity at the first and second loci had a significant association with negative *T. gondii* infection while heterozygosity at both loci together has a significant association with negative infection. The findings with regard to homozygosity were somewhat surprising and difficult to explain and may reflect a limitation of the statistical analyses.

4.7 Summary

This chapter shows the difficulty of amplifying the NLRP1b gene from *Apodemus sylvaticus* for a number of reasons; not least that there was no *Apodemus sylvaticus* NLRP1b DNA sequence in the gene bank database at the time the project was initiated. Three different primers were used to design primers to amplify different regions of the gene. NLRP1b gene has four parts, exon 2, exon 3 part1, exon 3 part 2 and exon 3 part 3. Two parts of the gene were amplified successfully but the remaining parts were difficult. Two years after this work was initiated, an unpublished draft of the *Apodemus sylvaticus* genome (Scaffolds) was released which assisted in the primer design. Also, this draft was used to align the outcome sequence of exon 3-2 and exon 3-3 which gives more confidence in dealing with sequences. There was a high (95%) similarity between outcome sequence of both exons and some region on the *Apodemus sylvaticus* draft genome which were aligned separately. The 5% difference might be due to the genome which is currently only in draft form (ie. unpublished); however,

it is more likely to be due to the genetic variability of the NLRP1b gene in the Malham mice compared to the Wirral mice used for genome analysis.

A significant relationship was discovered between the NLRP1b variations of exon 3-2 and exon 3-3 and *Toxoplasma gondii* infection in the presence of helminths.

Chapter 5

Investigation into the variation in NLRP1b gene sequences in relation to *Toxoplama gondii* co-infection and helminth parasites in the wood mouse (*Apodemus sylvaticus*)

5. Investigation into the variation in NLRP1b genes in relation to *Toxoplasma gondii* and helminth infections in the wood mouse (*Apodemus sylvaticus*)

5.1. Introduction

5.1.1. NLRP1b gene

NLRP1b inflammasomes are immune sensors related to the NLR family. Their main function is to make a platform which activates the immune system caspase proteases, mostly caspase 1, but the molecular mechanism which leads to the activation of NLRs is unclear. NLRP1 is similar to all the NLR family which have NBD and LLR domains; however, the organization of the NLRP1 domain is different. First, the protein of NLRP1 contains a C-terminal Caspase Activation and Recruitment Domain (CARD). CARD is found to be N terminal in all other NLRs. Second, NLRP1 has a FIND domain which is located between LRRs and CARD (Chavarría-Smith & Vance, 2013).

NLRP1B was found to have the ability to detect the protease secreted by *Bacillus anthracis,* which is an anthrax lethal factor. Anthrax lethal factor cleaves the NLRP1b, which is enough and important for activation of the NLRP1b sensor (Chavarría-Smith & Vance, 2013).

Certain inbred mouse and rat strains induce pyroptosis in macrophages due to the lethal anthrax toxin. Other macrophages from inbred strains resist the lethal toxin (Hellmich *et al*., 2012). In rats, the presence of an LF cleavage site in the inflammasome sensor Nlrp1 determines the sensitivity of macrophages to toxin-induced cell death (Hellmich *et al*., 2012). This leads to activation of caspase 1 and, subsequently, cell death. Meanwhile, macrophages that resist the lethal toxin express NLRP1 protein, but this does not have a site for lethal factor cleavage. It has been reported that mouse NLRP1b is cleaved by a lethal factor (Hellmich *et al*., 2012). In contrast to rats, Balb/cJ and NOD/LtJ macrophages susceptibility and resistance have no relation to the capability of NLRP1b cleavage by a lethal factor because both NLRP and NLRP1b are cleaved. Two lethal factor location sites of cleavage

were determined in the 38 and 44 residue of NLRP1b in mice (Hellmich *et al*., 2012). Finally, the ability of NOD/LtJ macrophages to resist the lethal toxin and their inability to express or activate NLRP1b protein is more likely due to polymorphism rather than lethal factor cleavage sites (Hellmich *et al*., 2012).

The NLRP1b sensor plays a key role in *Toxoplasma gondii* infection. Studies carried out *in vitro* have shown that NLRP1b knocks down human monocyte cells, decreasing pyroptosis and the secretion of cytokines (Witola *et al*., 2011). It is not clear if NLRP1 senses *T. gondii* by N-terminal cleavage the same as anthrax lethal toxin, or by signals interacting with the LRR domain (Jorgensen & Miao, 2015).

A hypothesis of how an interaction between the intracellular inflammasome and extracellular parasites could occur is likely to focus upon the production of excretory-secretory fractions (ES) by the helminths since these molecules change the cellular physiology and influence the immune cells (Whits & Artavanis-Tsakonas, 2012). Indeed, ES molecules modulate the immune system and may affect the TLR signalling pathways. The TLR pathways do active inflammasome responses such as NLRP3 which activates signalling and production of pro-inflammatory cytokines (Ritter *et al.*, 2010).

5.2 Aim

The aim of this chapter is to investigate the relation of the NLRP1b gene polymorphism against *T .gondii* co-infections and find out the effect of homozygosity and heterozygosity of the alleles with respect to helminth parasites. Also, to investigate the relationship between each polymorphism of each exons of the NLRP1b gene with respect to each helminth parasite.

5.3. Study Rationale

Little is known about the NLRP1b gene and there is very limited information about its role during infection. Also, there are no studies that describe the gene sequence of the NLRP1b gene or its polymorphism. The interaction between the NLRP1b gene and *T. gondii* was studied in the previous chapter although there is no published study that investigated that interaction. Furthermore, no studies have described the interaction between the NLRP1b gene and helminth parasites. The availability of a population of wood mice well characterised for their parasite infections, which include *T. gondii* and six other helminth parasites (Boyce 2013; Boyce *et al*. 2014), provided an opportunity to explore this further. As NLRP1b is an important mechanism for detecting parasites and triggering the innate immune system, a broad hypothesis might be that different variants of NLRB1b might have different detection efficiencies and therefore abilities to resist infection. Furthermore, heterozygous mice have two different variants that might have a greater ability to detect and deal with infection than homozygous mice. If these hypotheses are correct, one might expect certain variants to be associated with infection or with a lack of infection. Furthermore, heterozygous mice might be more likely to be less infected than their homozygous counterparts. Looking at this from another perspective, parasite infection could be acting as a selective force driving NLRP1b variants or heterozygosity in wild populations, such as in *Apodemus sylvaticus*. Additionally, the availability of this collection of mice and genetic data offers the opportunity to investigate these questions.

5.4. Objectives.

There are several questions that it would be interesting to address regarding these infected samples and their genotypes:

1- Is there any relationship between the haplotype of exon 3-2 of the NLRP1b gene and negative, single and multiple parasitic infections and non-infected mice?
2- Is there any relationship between the haplotype of exon 3-2 of the NLRP1b gene and each helminth infection?
3- Is there any relationship between the haplotype of exon 3-3 of the NLRP1b gene and negative, single and multiple parasitic infections and non-infected mice?
4- Is there any relationship between the haplotype of exon 3-3 of the NLRP1b gene and each helminth infection?

5.5. Material and methods

5.5.1. Sample Collection

The sample collection location was described previously in section 2.1.

5.5.2. Data Analysis

The data analysis was carried out as described in section 3.4.1.

5.6 Results

5.6.1. Investigating the relationship between the presence of each haplotype of exon 3-2 of the NLRP1b gene and negative, single and multiple parasitic infections and non-infected mice

Exon 3-2 fragments were amplified and sequenced for 80 *Apodemus sylvaticus* mice. Different haplotypes were discovered. These mice were infected with *T.gondii* and six other helminth parasites; 26 mice were infected with a single parasite, 47 were infected with multiple parasites and only seven were not infected (Figure 5.1).

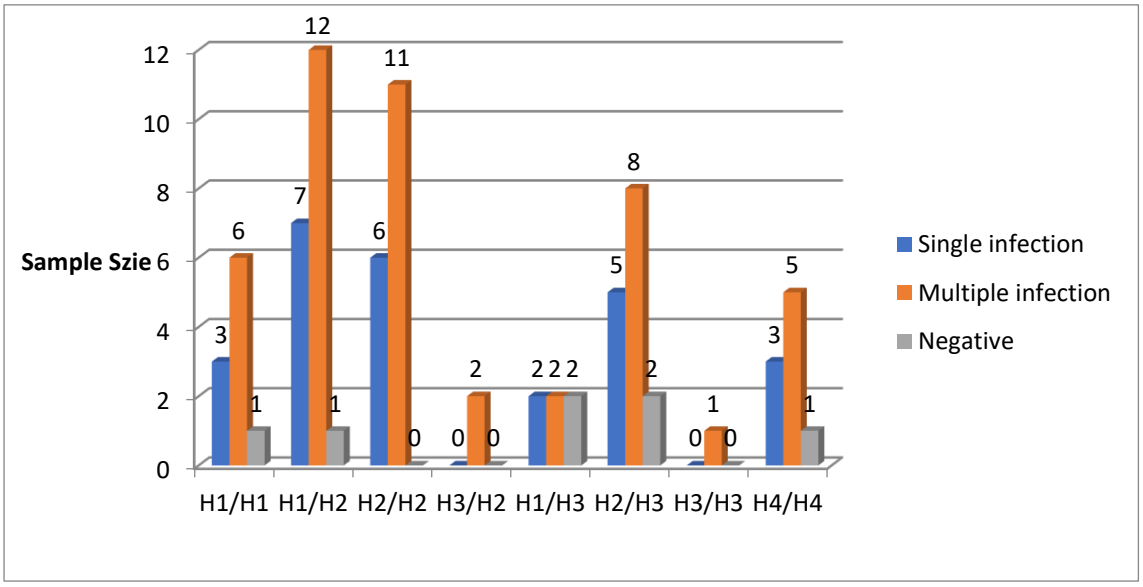

Figure 5.1. Haplotypes of exon 3-2 from 80 *Apodemus sylvaticus* mice and numerical infection status of each haplotype (negative, single or multiple infections).

The following tables show the relationship between negative, single and multiple parasite infected mice and non-infected mice, and according to the presence of the eight different haplotypes of exon 3-2 of the NLRP1b gene.

5.6.1.1. H1/H1 haplotype

Table 5.1. Investigation of the relationship between negative, single and multiple parasitic infections and the presence of exon 3-2 haplotype H1/H1 of the NLRP1b gene.

	H1/H1Genotype	Non H1/H1 Genotype	Total
No infection	1	6	7
Single Parasite infection	3	23	26
Multiple Parasites infection	6	41	47
Total	10	70	80

The table above (Table 5.1) was constructed to investigate the association between parasitic infection statuses according to the H1/H1 haplotype of exon 3-2 of the NLRP1b gene. Out of 73 mice, three H1/H1 mice had a single parasitic infection while the other six had multiple parasitic infections. The remaining 64 mice were non H1/H1, and 23 out of the 64 had a single parasitic infection while 42 had multiple parasitic infections. The two-tailed P value calculated for the above table was > 0.05. According to this value, the association between single and multiple parasitic infections and the presence of the H1/H1 haplotype of exon 3-2 of the NLRP1b gene is insignificant.

A total of seven mice were H1/H1, only one was not infected while six were multiple parasite infected; 47 mice were non-H1/H1, six mice were negative, and 41 mice had multiple infections. The two-tailed P value is > 0.05, which shows no significant relation between negative and multiple parasitic infections in the presence of H1/H1 of exon 3-2 of the NLRP1b gene.

The association between negative and single parasitic infections in the presence of the NLRP1b H1/H1 haplotype is investigated in (Table 5.1). Only one H1/H1 mouse had no parasitic infection while the other three mice had a single infection. Six non-H1/H1 mice showed no infection while 23 were infected by a single parasite species. No association between negative and single parasitic infections and the presence of haplotype H1/H1 of exon 3-2 of the NLRP1b gene is found since the two-tailed P value is > 0.05.

5.6.1.2. H1/H2 haplotype.

Table 5.2. Investigation of the relationship between negative, single and multiple parasitic infections and the presence of exon 3-2 haplotype H1/H2 of the NLRP1b gene.

	H1/H2Genotype	Non H1/H2 Genotype	Total
No infection	1	6	7
Single Parasite infection	7	19	26
Multiple Parasites infection	12	35	47
Total	20	60	80

The table above (Table 5.2) was constructed to investigate the association between parasitic infection statuses according to the H1/H2 haplotype of exon 3-2 of the NLRP1b gene. Out of 73 mice, seven H1/H2 mice had a single parasitic infection while the other 17 had multiple parasitic infections. The remaining 54 mice were non-H1/H2; 19 out of the 54 had a single parasitic infection while 35 had multiple parasitic infections. The two-tailed P value calculated for the above table is > 0.05. According to this value, the association between single and multiple parasitic infections and the presence of the H1/H2 haplotype of exon 3-2 of the NLRP1b gene is insignificant.

It is also presents the data for negative and multiple infection mice based on exon 3-2 of NLRP1b and the presence of haplotype H1/H2 to find out the relation between them; 13 mice were H1/H2, only one was not infected while 12 were multiple parasite infected; 41 mice were non-H1/H2, six were negative and 35 had multiple infections. The calculated two-tailed P value is > 0.05, which shows no significant relation between negative and multiple parasitic infections in the presence of H1/H2 of exon 3-2 of the NLRP1b gene.

The association between negative and single parasitic infections in the presence of NLRP1b H1/H2 haplotype is investigated in Table 5.2. Only one H1/H2 mouse had no parasitic infection while the other seven had a single infection. Six non-H1/H2 mice showed no

infection while 19 mice were infected by a single parasite species. No association between negative and single parasitic infection and the presence of haplotype H1/H2 of exon 3-2 of the NLRP1b gene is found since the two-tailed P value is 0.65.

5.6.1.3.H2/H2 haplotype.

Table 5.3. Investigation of the relationship between negative, single and multiple parasitic infections and the presence of exon 3-2 haplotype H2/H2 of the NLRP1b gene.

	H2/H2Genotype	Non H2/H2 Genotype	Total
No infection	0	7	7
Single Parasite infection	6	20	26
Multiple Parasites infection	11	36	47
Total	17	63	80

The table above (Table 5.3) was constructed to investigate the association between parasitic infection statuses according to the H2/H2 haplotype of exon 3-2 of the NLRP1b gene. Out of 73 mice, six H2/H2 had a single parasitic infection while the other 11 had multiple parasitic infections. The remaining 56 mice were non-H2/H2; 20 out of the 54 had a single parasitic infection while 35 had multiple parasitic infections. The two-tailed P value calculated for the above is > 0.05. According to this value, the association between single and multiple parasitic infections and the presence of the H2/H2 haplotype of exon 3-2 of the NLRP1b gene is insignificant.

The table above (Table 5.3) presents the data for negative and multiple infection mice based on exon 3-2 of NLRP1b and the presence of the haplotype H2/H2 to find out the relation between them. Eleven mice were H2/H2 and all of them had multiple parasites; 43 mice were non-H2/H2, seven were negative and 36 had multiple infections. The calculated two-tailed P value is 0.32, which shows no significant relation between negative and multiple parasitic infections in the presence of H2/H2 of exon 3-2 of the NLRP1b gene.

The association between negative and single parasitic infections in the presence of NLRP1b H2/H2 haplotype is investigated in Table 5.3. No mice of the H2/H2 genotype had any parasitic infection while the other six had a single infection. Seven non-H2/H2 mice showed no infection while 20 were infected by a single parasite species. No association between negative and single parasitic infection and the presence of haplotype H2/H2 of exon 3-2 of the NLRP1b gene is found since the two-tailed P value is 0.3.

5.6.1.4. H3/H2 haplotype.

Table 5.4. Investigation of the relationship between negative, single and multiple parasitic infections and the presence of exon 3-2 haplotype H3/H2 of the NLRP1b gene.

	H3/H2Genotype	Non H3/H2 Genotype	Total
No infection	0	7	7
Single Parasite infection	0	26	26
Multiple Parasites infection	2	45	47
Total	2	78	80

The table above (Table 5.4) was constructed to investigate the association between parasitic infection statuses according to the H3/H2 haplotype of exon 3-2 of the NLRP1b gene. Out of 73 mice, two were H3/H2 and these had multiple parasitic infections. The remaining 71 mice were non-H3/H2; 26 had a single parasitic infection while 45 had multiple parasitic infections. The two-tailed P value calculated for the above table is 0.53. According to this value, the association between single and multiple parasitic infections and the presence of the H4/H4 haplotype of exon 3-2 of the NLRP1b gene is insignificant.

The table above (Table 5.4) also presents the data for negative and multiple infection mice based on exon 3-2 of NLRP1b and the presence of the haplotype H3/H2 to find out the relation between them. Two mice were H3/H2 and both had multiple parasites; 52 mice were non-H3/H2, 7 were negative and 45 had multiple infections. The calculated two-tailed P value is

> 0.05, which shows no significant relation between negative and multiple parasitic infections in the presence of H3/H2 of exon 3-2 of the NLRP1b gene

The association between negative and single parasitic infections in the presence of the NLRP1b H3/H2 haplotype is investigated in Table 5.4. None of the H3/H2 mice had no infection or a single parasitic infection. The calculated two-tailed P value is > 0.05, which shows no significant relation between negative and multiple parasitic infections in the presence of H3/H2 of exon 3-2 of the NLRP1b gene.

5.6.1.5. H1/H3 haplotype.

Table 5.5. Investigation of the relationship between negative, single and multiple parasitic infections and the presence of exon 3-2 haplotype H1/H3 of the NLRP1b gene.

	H1/H3Genotype	Non H1/H3 Genotype	Total
No infection	2	5	7
Single Parasite infection	2	24	26
Multiple Parasites infection	2	45	47
Total	6	74	80

The table above (Table 5.5) was constructed to investigate the association between parasitic infection statuses according to the H1/H3 haplotype of exon 3-2 of the NLRP1b gene. Out of 73 mice, four were H1/H3. Half were single infected, and the other half were multiple infected. The remaining 69 mice were non-H1/H3; 24 had a single parasitic infection while 45 had multiple parasitic infections. The two-tailed P value calculated for the above table is 0.61. According to this value, the association between single and multiple parasitic infections and the presence of the H1/H3 haplotype of exon 3-2 of the NLRP1b gene is insignificant.

The table above (Table 5.5) presents the data for negative and multiple infection mice based on exon 3-2 of NLRP1b and the presence of haplotype H1/H3 to find out the relation between them. Two mice were H1/H3, the same as in the above table; half were single infected and

the other half were multiple infected; 50 mice were non-H1/H3, five were negative and 45 had multiple infections. The calculated two-tailed P value is 0.07, which shows no significant relation between negative and multiple parasitic infections in the presence of H1/H3 of exon 3-2 of the NLRP1b gene.

The association between negative and single parasitic infections in the presence of the NLRP1b H1/H3 haplotype is investigated in Table 5.5. Two mice of the H1/H3 genotype were negative and the other two were single infected. The calculated two-tailed P value is 0.19, which shows no significant relation between negative and multiple parasitic infections in the presence of H1/H3 of exon 3-2 of the NLRP1b gene.

5.6.1.6. H2/H3 haplotype.

Table 5. 6. Investigation of the relationship between negative, single and multiple parasitic infections and the presence of exon 3-2 H2/H3 of the NLRP1b gene haplotype.

	H2/H3Genotype	Non H2/H3 Genotype	Total
No infection	2	5	7
Single Parasite infection	5	21	26
Multiple Parasites infection	8	39	47
Total	15	65	80

The table above (Table 5.6) was constructed to investigate the association between parasitic infection statuses according to the H2/H3 haplotype of exon 3-2 of the NLRP1b gene. Out of 73 mice, 13 were H2/H3; five of them were infected by a single parasite while eight were infected by multiple parasites. The remaining 60 mice were non-H2/H3; 21 had a single parasitic infection while 39 had multiple parasitic infections. The two-tailed P value calculated for the above table is > 0.05. According to this value, the association between single and multiple parasitic infections and the presence of the H2/H3 haplotype of exon 3-2 of the NLRP1b gene is insignificant.

Also, the table above (Table 5.6) presents the data for negative and multiple infection mice based on exon 3-2 of NLRP1b and the presence of the haplotype H2/H3 to find out the relation between them. Ten mice were H2/H3; two had no infection while eight had multiple infections; 44 mice were non-H2/H3, 5 were negative and 39 had multiple infections. The calculated two-tailed P value is 0.6, which shows no significant relation between negative and multiple parasitic infections in the presence of H2/H3 of exon 3-2 of the NLRP1b gene.

The association between negative and single parasitic infections in the presence of the NLRP1b H1/H2 haplotype is investigated in Table 5.6. Only two H2/H3 mice had no parasitic infection while the other five had a single infection. Five non-H2/H3 mice showed no infection while 26 were infected by a single parasite species. No association between negative and single parasitic infection and the presence of haplotype H2/H3 of exon 3-2 of the NLRP1b gene is found, since the two-tailed P value is 0.62.

5.6.1.7. H3/H3 haplotype

Table 5.7. Investigation of the relationship between negative, single and multiple parasitic infections and the presence of exon 3-2 haplotype H3/H3 of the NLRP1b gene.

	H3/H3Genotype	Non H3/H3 Genotype	Total
No infection	0	7	7
Single Parasite infection	0	26	26
Multiple Parasites infection	1	46	47
Total	1	79	80

The table above (Table 5.7) was constructed to investigate the association between parasitic infection statuses according to the H3/H3 haplotype of exon 3-2 of the NLRP1b gene. Out of 73 mice, no H3/H3 had no infection and only one mouse had multiple parasitic infections. The remaining 72 mice were non-H3/H3; 26 had a single parasitic infection while 46 had multiple parasitic infections. The two-tailed P value calculated for the above table is > 0.05.

According to this value, the association between single and multiple parasitic infections and the presence of the H3/H3 haplotype of exon 3-2 of the NLRP1b gene is insignificant.

Also, Table 5.7 presents the data for negative and multiple infection mice based on exon 3-2 of NLRP1b and the presence of the haplotype H3/H3 to find out the relation between them. No H3/H3 mice infected by no parasite and only one infected had multiple parasites; 53 mice were non-H1/H3, seven were negative and 46 had multiple infections. The calculated two-tailed P value is > 0.05, which shows no significant relation between negative and multiple parasitic infections in the presence of H3/H3 of exon 3-2 of the NLRP1b gene.

The association between negative and single parasitic infections in the presence of the NLRP1b H3/H3 haplotype is investigated in Table 5.7. None of the H3/H3 mice had no infection or a single parasitic infection. The calculated two-tailed P value is >0.05, which shows no significant relation between negative and multiple parasitic infections in the presence of H3/H3 of exon 3-2 of the NLRP1b gene.

5.6.1.8. H4/H4 haplotype.

Table 5.8. Investigation of the relationship between negative, single and multiple parasitic infections and the presence of exon 3-2 haplotype H4/H4 of the NLRP1b gene.

	H4/H4Genotype	Non H4/H4 Genotype	Total
No infection	1	6	7
Single Parasite infection	3	23	26
Multiple Parasites infection	5	42	47
Total	9	71	80

The table above (Table 5.8) was constructed to investigate the association between parasitic infection statuses according to the H4/H4 haplotype of exon 3-2 of the NLRP1b gene. Out of 73 mice, eight were H1/H1; three of them had a single parasitic infection while the other five had multiple parasitic infections. The remaining 65 mice were non-H4/H4; 23 out of the 65 had a single parasitic infection while 42 had multiple parasitic infections. The two-tailed P

value calculated for the above table is > 0.05. According to this value, the association between single and multiple parasitic infections and the presence of the H4/H4 haplotype of exon 3-2 of the NLRP1b gene is insignificant.

Also, the table presents the data for negative and multiple infection mice based on exon 3-2 of NLRP1b and the presence of the haplotype H4/H4 to find out the relation between them. Six mice were H4/H4; only one was not infected while six were multiple parasites infected; 48 mice were non-H4/H4, six were negative and 41 had multiple infections. The two-tailed P value calculated is > 0.05, which shows no significant relation between negative and multiple parasitic infections in the presence of H4/H4 of exon 3-2 of the NLRP1b gene.

The association between negative and single parasitic infections in the presence of the NLRP1b H4/H4 haplotype is investigated in Table 5.8. Only one H4/H4 mouse had no parasitic infection while the other three had a single infection. Six non-H4/H4 mice showed no infection while 23 were infected by a single parasite species. No association between negative and single parasitic infection and the presence of the haplotype H4/H4 of exon 3-2 of the NLRP1b gene is found, since the two-tailed P value is > 0.05.

The table below (Table 5.9) presents the two-tailed P values calculated for the relation investigated between each haplotype of exon 3-2 of the NLRP1b gene and parasite infection status (single, negative and multiple infections). According to the results, no significant associations were discovered.

Table 5.9. Summary of the P values of the relation investigated between the parasitic infection status and each haplotype of exon 3-2 of NLRP1b.

	Single and multiple infections	Negative and multiple infections	Negative and single infections
H1/H1	> 0.05	> 0.05	> 0.05
H1/H2	> 0.05	> 0.05	0.65
H2/H2	> 0.05	0.32	0.30
H3/H2	0.53	> 0.05	> 0.05
H1/H3	0.61	0.08	0.19
H2/H3	> 0.05	0.60	0.62
H3/H3	> 0.05	> 0.05	> 0.05
H4/H4	> 0.05	> 0.05	> 0.05

5.6.2. Investigating the relationship between the presence of each haplotype of exon 3-2 of the NLRP1b gene and each helminth parasite infection

5.6.2.1. *Plagiorchi.*

The association between *Plagiorchis* infection and each haplotype of exon 3-2 of NLRP1b gene was investigated. No significant association was found between *Plagiorchis* infection and the presence of H3/H2 of exon 2 of the NLRP1b gene (see Appendix 1, Tables 1–8). The next table is a summary of the P values of all the haplotypes of exon 3-2 of NLRP1b which determine the relation with *Plagiorchis* infection (Table 5.10).

Table 5.10. Summary of P values of all haplotypes of exon 3-2 of the NLRP1b gene and *Plagiorchis* infection.

Haplotype	P value	Relation with *Plagiorchis*
H1/H1	> 0.05	Insignificant
H1/H2	> 0.05	Insignificant
H2/H2	0.53	Insignificant
H3/H2	> 0.05	Insignificant
H1/H3	> 0.05	Insignificant
H2/H3	0.5	Insignificant
H3/H3	> 0.05	Insignificant
H4/H4	0.2	Insignificant

The two-tailed P values of the table above were calculated from the data in Tables 1– 8 (see Appendix 1).

5.6.2.2. *Heligmosomoides*

One almost significant association was discovered between *Heligmosomoides* infection and the H1/H2 genotype of exon 3-2 of NLRP1b (Table 5.11).

Table 5.11. Investigation of the relationship between *Heligmosomoides* infection and the presence of exon 3-2 haplotype H1/H2 of the NLRP1b gene.

	H1/H2 Genotype	Non-H1/H2 Genotype	Total
Heligmosomoides	17	36	53
No *Heligmosomoides*	3	24	27
Total	20	60	80

Out of 20 H1/H2 mice: 17 were infected *Heligmosomoides* and only three were negative. The two-tailed P value calculated for the table above is 0.056, which means the relationship is not quite significant.

The remaining associations between *Heligmosomoides* and other haplotypes are not significant (see Appendix 1, Tables 9–15). The next table summarizes the two-tailed P values calculated to determine their relations.

Table 5.12. Summary of P values of all haplotypes of exon 3-2 of the NLRP1b gene and *Heligmosomoides* infection.

Haplotype	P value	Relation with *Heligmosomoides*
H1/H1	0.29	Insignificant
H1/H2	0.05	Almost significant
H2/H2	0.39	Insignificant
H3/H2	0.55	Insignificant
H1/H3	0.4	Insignificant
H2/H3	0.13	Insignificant
H3/H3	0.34	Insignificant
H4/H4	> 0.05	Insignificant

5.6.2.3. *Syphacea*

One significant association and one almost significant relationship were discovered between *Syphacea* infection and H1/H2 (Table 5.13) and H3/H2 (Table 5.14), respectively.

Table 5.13. Investigation of the relationship between *Syphacea* infection and the presence of exon 3-2 haplotype H1/H2 of the NLRP1b gene.

	H1/H2 Genotype	Non-H1/H2 Genotype	Total
Syphacea	1	21	22
No *Syphacea*	19	39	58
Total	20	60	80

The table above (Table 5.13) investigates the association between *Syphacea* infection and H1/H2 haplotypes of exon 3-2 of the NLRP1b gene. The majority of H1/H2 mice were not infected by *Syphacea*; 19 mice were negative, only one was infected. The two-tailed P value

calculated for the table above is 0.0089, which indicates a significant association between *Syphacea* resistance to infection and the H1/H2 haplotype of exon 3-2 of the NLRP1b gene.

Table 5.14. Investigation of the relationship between *Syphacea* infection and the presence of exon 3-2 haplotype H3/H2 of the NLRP1b gene.

	H3/H2 Genotype	Non-H3/H2 Genotype	Total
Syphacea.	2	20	22
No *Syphacea*	0	58	58
Total	2	78	80

Only two H3/H2 genotype mice were discovered and they were infected by *Syphacea*. The two-tailed P value calculated for the table above is 0.073, so an almost significant association is determined. The next table summarizes the two-tailed P values calculated for all haplotypes (see Appendix 1, Tables 16–21).

Table 5.15. Summary of P values of all haplotypes of exon 3-2 of the NLRP1b gene and *Syphacea* infection.

Haplotype	P value	Relation with *Syphacea*
H1/H1	0.45	Insignificant
H1/H2	0.0089	Significant
H2/H2	0.77	Insignificant
H3/H2	0.07	Insignificant
H1/H3	0.66	Insignificant
H2/H3	0.75	Insignificant
H3/H3	0.27	Insignificant
H4/H4	0.7	Insignificant

The table above shows the P values calculated for all the haplotypes of exon 3-2 in relation to *Syphacea* infection. Only one haplotype shows a significant association with *Syphacea*, the rest are not significant.

5.6.2.4. *Capillaria*

Table 5.16. Investigation of the relationship between *Capillaria* infection and the presence of exon 3-2 haplotype H3/H3 of the NLRP1b gene.

	H3/H3 Genotype	Non-H3/H3 Genotype	Total
Capillaria	**1**	**4**	**5**
No *Capillaria*	**0**	**75**	**75**
Total	**1**	**79**	**80**

Only one almost significant relation is discovered between H3/H3 haplotypes and *Capillaria* infection (Table 5.16). Only one mouse out of 80 was H3/H3 and was infected with *Capillaria,* the remaining infected mice were mixed haplotypes. The two-tailed P value calculated is 0.062. No other significant or almost significant association between *Capillaria* infection and any exon 3-2 of NLRP1b is discovered after calculating two-tailed P values (see Appendix 1, Tables 22–28) as summarised below in Table 5.17.

Table 5.17. Summary of P values of all haplotypes of exon 3-2 of the NLRP1b gene and *Capillaria* infection.

Haplotype	P value	Relation with *Capillaria*
H1/H1	0.11	Insignificant
H1/H2	0.59	Insignificant
H2/H2	0.58	Insignificant
H3/H2	0.32	Insignificant
H1/H3	> 0.05	Insignificant
H2/H3	0.58	Insignificant
H3/H3	0.062	Almost significant
H4/H4	> 0.05	Insignificant

5.6.2.5. *Pelodera*

Table 5.18. Investigation of the relationship between *Pelodera* infection and the presence of exon 3-2 haplotype H3/H3 of the NLRP1b gene.

	H3/H3 Genotype	Non-H3/H3 Genotype	Total
Pelodera	**1**	**3**	**4**
No *Pelodera*	**0**	**76**	**76**
Total	**1**	**79**	**80**

The same H3/H3 mouse that was infected by *Pelodera* was also infected by *Pelodera*. It is one of four mice that were infected by *Pelodera*. The two-tailed P value calculated for *Pelodera* infection from the table above (Table 5.18) shows an almost significant relation with H3/H3 haplotypes. No significant association was discovered with other haplotypes of exon 3-2 of NLRP1b from the P values calculated and (see Appendix 1, Tables 29–35) and summarized in Table 5.19, below.

Table 5.19. Summary of P values of all haplotypes of exon 3-2 of the NLRP1b gene and *Pelodera* infection.

Haplotype	P value	Relation with *Pelodera*
H1/H1	0.42	Insignificant
H1/H2	0.57	Insignificant
H2/H2	> 0.05	Insignificant
H3/H2	0.59	Insignificant
H1/H3	> 0.05	Insignificant
H2/H3	> 0.05	Insignificant
H3/H3	0.05	Almost significant
H4/H4	0.38	Insignificant

5.6.2.6. *Brachylaemus*

No significant association between *Brachylaemus* infection and any exon 3-2 of NLRP1b is discovered after calculating two-tailed P values from haplotype tables (see Appendix 1, Tables 35–43) and summarised in Table 5.20, below.

Table 5.20. Summary of P values of all haplotypes of exon 3-2 of the NLRP1b gene and *Brachylaemus* infection.

Haplotype	P value	Relation with *Brachylaemus*
H1/H1	> 0.05	Insignificant
H1/H2	0.25	Insignificant
H2/H2	> 0.05	Insignificant
H3/H2	> 0.05	Insignificant
H1/H3	> 0.05	Insignificant
H2/H3	> 0.05	Insignificant
H3/H3	> 0.05	Insignificant
H4/H4	> 0.05	Insignificant

4.5.3. Investigating the relationship between the presence of each haplotype of exon 3-3 of the NLRP1b gene and negative, single and multiple parasitic infections and non-infected mice

After successfully amplifying and sequencing the exon 3-3 fragments from 80 *Apodemus sylvaticus* mice, the association between the haplotypes discovered and parasitic infection status (negative, single and multiple parasitic infections) was investigated (Figure 5.2).

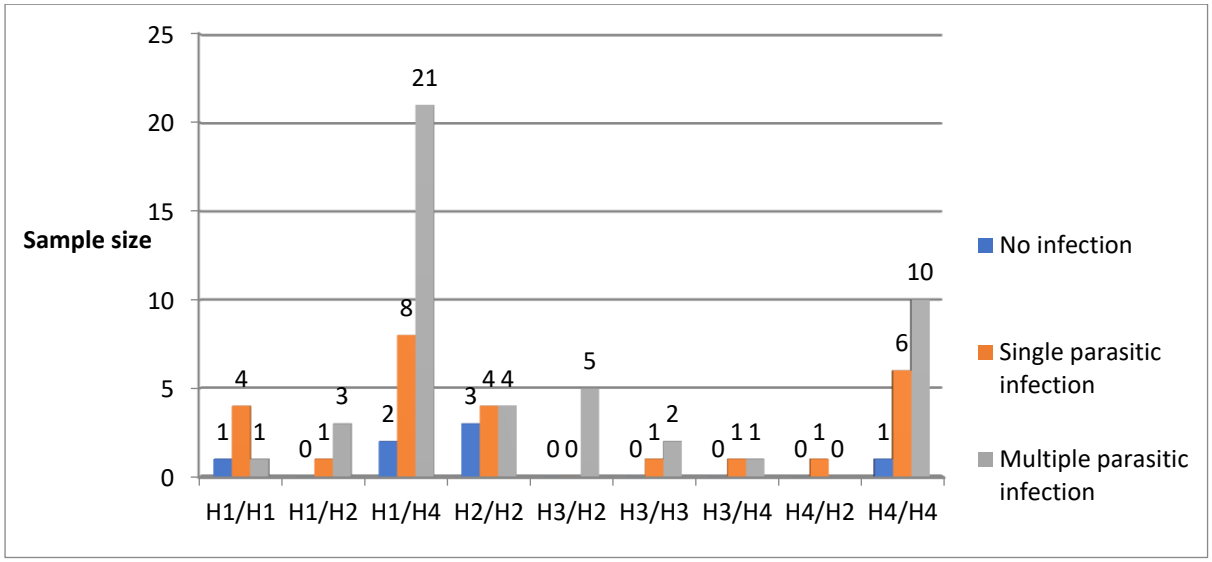

Figure 5.2. Haplotypes of exon 3-3 of the NLRP1b gene from 80 *Apodemus sylvaticus* mice and the infection status of each haplotype (negative, single or multiple infections).

According to the above figure, the mice haplotype with the most multiple parasite infections is H1/H4, whereas the mice haplotype with the least multiple parasitic infections is H4/H2. Also, the H4/H2 mice haplotype has the most single parasitic infections while no H3/H2 haplotype mice had any single parasitic infection. Furthermore, some mice of certain haplotypes had no parasitic infection, such as H1/H1 and H1/H4.

The following tables show the relationship between negative, single and multiple parasites of infected mice and non-infected mice according to the presence of the nine different haplotypes of exon 3-3 of the NLRP1b gene.

5.6.3.1. H1/H1 haplotype

Table 5.21. Investigation of the relationship between negative, single and multiple parasitic infections and the presence of exon 3-3 haplotype H1/H1of the NLRP1b gene.

	H1/H1Genotype	Non H1/H1 Genotype	Total
No infection	1	6	7
Single Parasite infection	4	22	26
Multiple Parasites infection	1	46	47
Total	6	74	80

The table above (Table 5.21) was constructed to investigate the association between parasitic infection statuses according to the H1/H1 haplotype of exon 3-3 of the NLRP1b gene. Twenty-six mice were infected by a single parasite and only four were H1/H1, the rest were other haplotypes. Forty-seven mice were infected by more than one parasite; only one mouse out of the 47 was H1/H1, the remaining 46 were other haplotypes. The two-tailed P value calculated for the above table is 0.0512. According to this value, the association between single and multiple parasitic infections and the presence of the H1 haplotype of NLRP1b is almost significant.

Table 5.21presents the data for negative and multiple infection mice based on exon 3-3 of the NLRP1b gene and presence of the haplotype H1/H1 to determine the relation between them. A total of seven mice were negative; one was H1/H1, the other six mice were non-H1/H1. Forty-seven of the mice were multiple parasites infected; only one of them was H1/H1, 46 were non-H1/H1. The two-tailed P value calculated is 0.24, which indicates no significant relation between negative and multiple parasitic infections in the presence of H1/H1 of exon 3-3 of NLRP1b.

The association between negative and single parasitic infections in the presence of NLRP1b H1/H1 haplotype is investigated in Table 5.21. Seven mice were negative; one mouse was H1/H1, and others were non-H1/H1. Twenty-six mice were infected by a single parasite; four were H1/H1, 22 mice were non-H1/H1. No association was found between negative and single parasitic infections and the presence of haplotype H1/H1 of the NLRP1b gene since the two-tailed P value is > 0.05.

5.6.3.2. H1/H2 haplotype

Table 5.22. Investigation of the relationship between negative, single and multiple parasitic infections and the presence of exon 3-3 haplotype H1/H2 of the NLRP1b gene.

	H1/H2Genotype	Non H1/H2Genotype	Total
No infection	0	7	7
Single Parasite infection	1	25	26
Multiple Parasites infection	3	44	47
Total	4	76	80

The table above (Table 5.22) was constructed to investigate the association between parasitic infection statuses according to the H1/H2 haplotype of exon 3-3 of the NLRP1b gene. Twenty-six mice were infected by a single parasite; only one mouse was H1/H2, the rest were non-H1/H2. Forty-seven mice were infected by more than one parasite; three out of the 47 mice were H1/H2, the remaining 44 were non-H1/H2. The two-tailed P value calculated for the above table is > 0.05. According to this value, the association between single and multiple parasitic infections and the presence of the H1/H2 haplotype of NLRP1b is insignificant.

Also, Table 5.22 presents the data for negative and multiple infection mice based on exon 3-3 of the NLRP1b gene and the presence of the haplotype H1/H2 to determine the relation between them. A total of seven mice were negative, all seven were non-H1/H2. Forty-seven of the mice were multiple parasites infected; three of them were H1/H2, 44 were non-H1/H2. The two-tailed P value calculated is > 0.05, which indicates no significant relation between negative and multiple parasitic infections in the presence of H1/H2 of exon 3-3 of NLRP1b.

The association between negative and single parasitic infections in the presence of NLRP1b H1/H2 haplotype is investigated in Table 5.22, above. Seven mice were negative; all seven were non-H1/H2. Twenty-six mice were infected by a single parasite; only one mouse was H1/H2, 25 were non-H1/H2. No association was found between negative and single parasitic infections and the presence of haplotype H1/H2 of the NLRP1b gene since the two-tailed P value calculated is > 0.05.

5.6.3.3. H1/H4 haplotype

Table 5.23 Investigation of the relationship between single and multiple parasitic infections and the presence of exon 3-3 haplotype H1/H4 of the NLRP1b gene.

	H1/H4Genotype	Non H1/H4 Genotype	Total
No infection	2	5	7
Single Parasite infection	8	18	26
Multiple Parasites infection	21	26	47
Total	31	49	80

The table above (Table 5.23) was constructed to investigate the association between parasitic infection statuses according to the H1/H4 haplotype of exon 3-3 of the NLRP1b gene. Twenty-six mice were infected by a single parasite; only eight mice were H1/H4, 18 were non-H1/H4. Forty-seven mice were infected by more than one parasite; 21 out of the 47 were H1/H4, the reaming 26 were non-H1/H4. The two-tailed P value calculated for the above table is 0.32. According to this value, the association between single and multiple parasitic infections and the presence of the H1/H4 haplotype of NLRP1b is insignificant.

Furthermore, table5.23 presents the data for negative and multiple infection mice based on exon 3-3 of the NLRP1b gene and the presence of the haplotype H1/H4 to determine the relation between them. A total of seven mice were negative; two mice were H1/H4, the other five were non-H1/H4. Forty-seven of the mice were multiple parasites infected; 21 of them were H1/H4, 26 were non-H1/H4. The two-tailed P value calculated is 0.68, which indicates no significant relation between negative and multiple parasitic infections in the presence of H1/H4 of exon 3-3 of NLRP1b.

The association between negative and single parasitic infections in the presence of the NLRP1b H1/H4 haplotype is investigated in the table above (Table 5.23). Seven mice were negative; one mouse was H1/H4, the others were non-H1/H4. Twenty-six mice were infected by a single parasite; two mice were H1/H4, 25 mice were non-H1/H4. No association was

found between negative and single parasitic infections and the presence of haplotype H1/H4 of the NLRP1b gene since the two-tailed P value is > 0.05.

5.6.3.4. H2/H2 haplotype

Table 5.24. Investigation of the relationship between negative, single and multiple parasitic infections and the presence of exon 3-3 haplotype H2/H2 of the NLRP1b gene.

	H2/H2Genotype	Non H2/H2 Genotype	Total
No infection	3	4	7
Single Parasite infection	4	22	26
Multiple Parasites infection	4	43	47
Total	11	69	80

The table above (Table 5.24) was constructed to investigate the association between parasitic infection statuses according to the H2/H2 haplotype of exon 3-3 of the NLRP1b gene. Twenty-six mice were infected by a single parasite; only four mice were H2/H2, the rest were non-H2/H2. Forty-seven mice were infected by more than one parasite; four out of the 47 were H2/H2, the remaining 43 were non-H2/H2. The two-tailed P value calculated for the above table is 0.44. According to this value, the association between single and multiple parasitic infections and the presence of the H2/H2 haplotype of NLRP1b is insignificant.

Also, table 5.24 presents the data for negative and multiple infection mice based on exon 3-3 of the NLRP1b gene and the presence of the haplotype H2/H2 to determine the relation between them. A total of seven mice were negative; three mice were H2/H2, the other four were non-H2/H2. Forty-seven of the mice were multiple parasitic infected; four of them were H2/H2, 43 were non-H2/H2. The two-tailed P value calculated is 0.039, which indicates a significant relation between negative and multiple parasitic infections in the presence of H2/H2 of exon 3-3 of NLRP1b.

The association between negative and single parasitic infections in the presence of the NLRP1b H2/H2 haplotype is investigated in the table above (Table 5.24). Seven mice were

negative; three mice were H2/H2, the others were non-H2/H2. Twenty-six mice were infected by a single parasite; only four mice were H2/H2, 22 were non-H2/H2. No association was found between negative and single parasitic infections and the presence of haplotype H2/H2 of the NLRP1b gene since the two-tailed P value is 0.14.

5.6.3.5. H3/H2 haplotype

Table 5.25. Investigation of the relationship between negative, single and multiple parasitic infections and the presence of exon 3-3 haplotype H3/H2 of the NLRP1b gene.

	H3/H2Genotype	Non H3/H2Genotype	Total
No infection	0	7	7
Single Parasite infection	0	26	26
Multiple Parasites infection	5	42	47
Total	5	75	80

The table above (Table 5.25) was constructed to investigate the association between parasitic infection statuses according to the H3/H2 haplotype of exon 3-3 of the NLRP1b gene. Twenty-six mice were infected by a single parasite and all of them were non-H3/H2. Forty-seven mice were infected by more than one parasite; five out of the 47 mice were H3/H2, the remaining 42 were non-H3/H2. The two-tailed P value calculated for the above table is 0.1533. According to this value, the association between single and multiple parasitic infections and the presence of the H3/H2 haplotype of NLRP1b is insignificant.

Furthermore, the table presents the data for negative and multiple infection mice based on exon 3-3 of the NLRP1b gene and the presence of haplotype H3/H2 to determine the relation between them. A total of seven mice were negative, all were non-H3/H2. Forty-seven of the mice were multiple parasites infected; five of them were H3/H2, 42 were non-H3/H2. The two-tailed P value calculated is > 0.05, which indicates no significant relation between negative and multiple parasitic infections in the presence of H3/H2 of exon 3-3 of NLRP1b.

The association between negative and single parasitic infections in the presence of the NLRP1b H3/H2 haplotype is investigated in the table above (Table 5.25). Seven mice were negative; all the H3/H2 mice had multiple parasitic infections. No association was found between negative and single parasitic infections and the presence of haplotype H3/H3 of the NLRP1b gene since the two-tailed P value is > 0.05.

5.6.3.6. H3/H3 haplotype.

Table 5.26. Investigation of the relationship between negative, single and multiple parasitic infections and the presence of exon 3-3 haplotype H3/H3 of the NLRP1b gene.

	H3/H3Genotype	Non H3/H3 Genotype	Total
No infection	0	7	7
Single Parasite infection	1	25	26
Multiple Parasites infection	2	45	47
Total	3	77	80

The table above (Table 5.26) was constructed to investigate the association between parasitic infection statuses according to the H3/H3 haplotype of exon 3-3 of the NLRP1b gene. Twenty-six mice were infected by a single parasite; only one mouse was H3/H3, the rest were non-H3/H3. Forty-seven mice were infected by more than one parasite; two out of the 47 mice were H3/H3, and remaining 45 were non-H3/H3. The two-tailed P value calculated for the above table is > 0.05. According to this value, the association between single and multiple parasitic infections and the presence of the H3/H3 haplotype of NLRP1b is insignificant.

Also, the table presents the data for negative and multiple infection mice based on exon 3-3 of the NLRP1b gene and the presence of the haplotype H3/H3 to determine the relation between them. A total of seven mice were negative, all seven were haplotype non-H3/H3. Forty-seven of the mice were multiple parasite infected; two of them were H3/H3, 45 were non-H3/H3. The two-tailed P value calculated is > 0.05, which indicates no significant relation between negative and multiple parasitic infections in the presence of H3/H3 of exon 3-3 of NLRP1b.

The association between negative and single parasitic infections in the presence of the NLRP1b H3/H3 haplotype is investigated in the table above (Table 5.26). Seven mice were negative and 26 mice had a single parasitic infection. Only one mouse was H3/H3 haplotype and it had a single parasite. No association was found between negative and single parasitic infections and the presence of haplotype H3/H3 of the NLRP1b gene since the two-tailed P value is > 0.05.

5.6.3.7. H3/H4 haplotype.

Table 5.27. Investigation of the relationship between negative, single and multiple parasitic infections and the presence of exon 3-3 haplotype H3/H4 of the NLRP1b gene.

	H3/H4Genotype	Non H3/H4Genotype	Total
No infection	0	7	7
Single Parasite infection	1	25	26
Multiple Parasites infection	1	46	47
Total	2	78	80

The table above (Table 5.27) was constructed to investigate the association between parasitic infection statuses according to the H3/H4 haplotype of exon 3-3 of the NLRP1b gene. Twenty-six mice were infected by a single parasite; only one mouse was H3/H2, the rest were non-H3/H4. Forty-seven mice were infected by more than one parasite; one mouse out of the 47 was H3/H4, the remaining 43 were non-H3/H4. The two-tailed P value calculated for the above table is > 0.05. According to this value, the association between single and multiple parasitic infections and the presence of the H3/H4 haplotype of NLRP1b is insignificant.

Moreover, the table presents the data for negative and multiple infection mice based on exon 3-3 of the NLRP1b gene and the presence of the haplotype H3/H4 to determine the relation between them. A total of seven mice were negative; there was only one mouse of H3/H4 haplotype that was infected by multiple parasites. The two-tailed P value calculated is > 0.05,

which indicates no significant relation between negative and multiple parasitic infections in the presence of H3/H4 of exon 3-3 of NLRP1b.

The association between negative and single parasitic infections in the presence of the NLRP1b H3/H4 haplotype is investigated in the table above (Table 5.27). Seven mice were negative; there was only one mouse of H3/H4 that was infected by a single parasite. No association was found between negative and single parasitic infections and the presence of haplotype H3/H4 of the NLRP1b gene since the two-tailed P value is > 0.05.

5.6.3.8. H4/H2haplotype.

Table 5.28. Investigation of the relationship between negative, single and multiple parasitic infections and the presence of exon 3-3 haplotype H4/H2 of the NLRP1b gene.

	H4/H2Genotype	Non H4/H2 Genotype	Total
No infection	0	7	7
Single Parasite infection	1	25	26
Multiple Parasites infection	0	47	47
Total	1	79	80

The table above (Table 5.28) was constructed to investigate the association between parasitic infection statuses according to the H4/H2 haplotype of exon 3-3 of the NLRP1b gene. Twenty-six mice were infected by a single parasite; only one mouse was H4/H2, the rest mice were non-H4/H2. Forty-seven mice were infected by more than one parasite; none of them were H4/H2. The two-tailed P value calculated for the above table is 0.36. According to this value, the association between single and multiple parasitic infections and the presence of the H4/H2 haplotype of NLRP1b is insignificant.

Furthermore, Table 5.28 presents the data for negative and multiple infection mice based on exon 3-3 of the NLRP1b gene and the presence of the haplotype H4/H2 to determine the relation between them. No H4/H2 genotype was found to have multiple parasitic infections or no infection. The two-tailed P value calculated is 0.62, which indicates no significant relation

between negative and multiple parasitic infections in the presence of H4/H2 of exon 3-3 of NLRP1b.

The association between negative and single parasitic infections in the presence of the NLRP1b H4/H2 haplotype is investigated in Table 5.28, above. Seven mice were negative; all seven were non-H4/H2. Twenty-six mice were infected by a single parasite; only one mouse was H4/H2, 25 were non-H4/H2. No association was found between negative and single parasitic infections and the presence of haplotype H4/H2 of the NLRP1b gene since the two-tailed P value is > 0.05.

5.6.3.9. H4/H4 haplotype.

Table 5.29. Investigation of the relationship between negative, single and multiple parasitic infections and the presence of exon 3-3 H4/H4 of the NLRP1b gene haplotype.

	H4/H4Genotype	Non H4/H4 Genotype	Total
No infection	1	6	7
Single Parasite infection	1	25	26
Multiple Parasites infection	10	37	47
Total	12	68	80

The table above (Table 5.29) was constructed to investigate the association between parasitic infection statuses according to the H4/H4 haplotype of exon 3-3 of the NLRP1b gene. Twenty-six mice were infected by a single parasite; six were H4/H4, the rest were non-H4/H4. Forty-seven mice were infected by more than one parasite; 10 out of the 47 were H4/H4, the remaining 43 were non-H4/H4. The two-tailed P value calculated for the above table is > 0.05. According to this value, the association between single and multiple parasitic infections and the presence of the H4/H4 haplotype of NLRP1b is insignificant.

Moreover, the table presents the data for negative and multiple infection mice based on exon 3-3 of the NLRP1b gene and the presence of haplotype H4/H4 to determine the relation between them. A total of seven mice were negative, one mouse among them was non-H4/H4. Forty-seven of the mice were multiple parasites infected; ten of them were H4/H4, 37 were

non-H4/H4. The two-tailed P value calculated is > 0.05, which indicates no significant relation between negative and multiple parasitic infections in the presence of H4/H4 of exon 3-3 of NLRP1b.

The association between negative and single parasitic infections in the presence of the NLRP1b H4/H4 haplotype is investigated in Table 5.29, above. Seven mice were negative; one mouse was H4/H4, the others were non-H4/H4. Twenty-six mice were infected by a single parasite; six mice were H4/H4, 20 mice were non-H4/H4. No association was found between negative and single parasitic infections and the presence of haplotype H4/H4 of the NLRP1b gene since the two-tailed P value is > 0.05.

5.6.4. Investigating the relationship between the presence of each haplotype of exon 3-3 of the NLRP1b gene and each helminth parasite infection

5.6.4.1. *Plagiorchis*

Nine haplotypes were discovered in exon 3-3 of 80 *Apodemus sylvaticus* mice. One haplotype showed a significant relationship with *Plagiorchis* and two haplotypes showed an almost significant association.

Table 5.30. Investigation of the relationship between *Plagiorchis* infection and the presence of exon 3-3 haplotype H3/H3 of the NLRP1b gene.

	H3/H3 Genotype	Non-H3/H3 Genotype	Total
Plagiorchis	3	16	19
No *Plagiorchis*	0	61	61
Total	3	77	80

The table above (Table 5.30) shows 11 mice of H3/H3 genotypes that were discovered, none of them were infected by *Plagiorchis.* The two-tailed P value calculated for the previous table is 0.012, which indicates a significant association between the H3/H3 haplotype and susceptibility to *Plagiorchis* infection.

Table 5.31. Investigation of the relationship between *Plagiorchis* infection and the presence of exon 3-3 haplotype H3/H2 of the NLRP1b gene.

	H3/H2 Genotype	Non-H3/H2 Genotype	Total
Plagiorchis	3	16	19
No *Plagiorchis*	2	59	61
Total	5	75	80

The above table (Table 5.31) was constructed to determine the association between *Plagiorchis* infection and the H3/H2 haplotype of exon 3-3. Only three out of 19 mice infected by *Plagiorchis* were H3/H2. An almost significant association was found after calculating the two-tailed P value, which is 0.0841.

Table 5.32. Investigation of the relationship between *Plagiorchis* infection and the presence of exon 3-3 haplotype H2/H2 of the NLRP1b gene.

	H2/H2 Genotype	Non-H2/H2 Genotype	Total
Plagiorchis	0	19	19
No *Plagiorchis*	11	50	61
Total	11	69	80

The above table (Table 5.32) shows no H2/H2 mice were infected by *Plagiorchis.* Another almost significant association was discovered from the above table (Table 5.32) asthe two-tailed P value calculated is 0.058. The remaining haplotypes showed no significant relationships with *Plagiorchis* as the P value was calculated for each haplotype (see Appendix 1, Tables 44–49) and these are summarized in Table 5.33, below.

Table 5.33. Summary of P values of all haplotypes of exon 3-3 of the NLRP1b gene and *Plagiorchis* infection.

Haplotype	P value	Relation with *Plagiorchis*
H1/H1	0.33	Insignificant
H1/H2	> 0.05	Insignificant
H1/H4	0.79	Insignificant
H2/H2	0.058	Almost significant
H3/H2	0.084	Insignificant
H3/H3	0.012	Significant
H3/H4	> 0.05	Insignificant
H4/H2	0.24	Insignificant
H4/H4	0.75	Insignificant

5.6.4.2. *Heligmosomoides*

Two significant associations were obtained between *Heligmosomoides* infection and haplotypes H1/H4 and H4/H4 of exon 3-3 of the NLRP1b gene.

Table 5.34. Investigation of the relationship between *Heligmosomoides* infection and the presence of exon 3-3 haplotype H1/H4 of the NLRP1b gene.

	H1/H4 Genotype	Non-H1/H4 Genotype	Total
Heligmosomoides	26	27	53
No *Heligmosomoides*	5	22	27
Total	31	49	80

Table 5.34 shows that 26 mice of the H1/H4 haplotypes were infected by *Heligmosomoides.* That is more than 75 per cent of H1/H4 mice. The two-tailed P value calculated for the previous table is 0.0086, which means a significant association between susceptibility to *Heligmosomoides* infection and H1/H4 haplotype of the NLRP1b gene.

5.35. Investigation of the relationship between *Heligmosomoides* infections and the presence of exon 3-3 haplotype H4/H4 of the NLRP1b gene.

	H4/H4 Genotype	Non-H4/H4 Genotype	Total
Heligmosomoides	6	47	53
No *Heligmosomoides*	11	16	27
Total	17	63	80

A total of 17 H4/H4 mice were infected by *Heligmosomoides*. Six mice were H4/H4 genotype and 47 mice were mixed haplotypes, as shown in Table 5.35. The H4/H4 haplotypes account for 17 mice and less than the half of them were infected by *Heligmosomoides*. The two-tailed P value calculated for the above table is 0.0038. The relationship between *Heligmosomoides* and the H4/H4 haplotype of exon 3-3 is significant and relatively protective. For a summary of the other haplotypes P values see Table 5.36, below (see also Appendix 1, Tables 50–56).

Table 5.36. Summary of P values of all haplotypes of exon 3-3 of the NLRP1b gene and *Heligmosomoides* infection.

Haplotype	P value	Relation with *Heligmosomoides*
H1/H1	0.66	Insignificant
H1/H2	> 0.05	Insignificant
H1/H4	0.0086	Significant
H2/H2	0.17	Insignificant
H3/H2	0.16	Insignificant
H3/H3	> 0.05	Insignificant
H3/H4	> 0.050	Insignificant
H4/H2	0.34	Insignificant
H4/H4	0.0038	Significant

5.6.4.3. *Syphacea*

Table 5.37. Investigation of the relationship between *Syphacea* infection and the presence of exon 3-3 haplotype H1/H4 of the NLRP1b gene.

	H1/H4 Genotype	Non-H1/H4 Genotype	Total
Syphacea	13	9	22
No *Syphacea*	18	40	58
Total	31	49	80

A total of 22 mice were infected with *Syphacea*. Thirteen mice were H1/H4 genotype; nine mice were mixed haplotypes, as shown in Table 5.37. The H1/H4 haplotypes consist of 31 mice; only 13 of them were infected with *Syphacea*. The two-tailed P value calculated for the previous table is 0.038. The relationship between *Syphacea* and the H1/H4 haplotype of exon 3-3 is significant and shows susceptibility to infection. A summary of the other haplotypes P values is shown in Table 5.38, below (see also Appendix 1, Tables 57–64).

Table 5.38. Summary of P values of all haplotypes of exon 3-3 of the NLRP1b gene and *Syphacea* infection.

Haplotype	P value	Relation with *Syphacea*
H1/H1	0.18	Insignificant
H1/H2	0.3	Insignificant
H1/H4	0.038	Significant
H2/H2	0.27	Insignificant
H3/H2	0.31	Insignificant
H3/H3	0.56	Insignificant
H3/H4	> 0.05	Insignificant
H4/H2	> 0.05	Insignificant
H4/H4	0.54	Insignificant

5.6.4.4. *Capillaria*

Table 5.39. Investigation of the relationship between *Capillaria* infection and the presence of exon 3-3 haplotype H1/H4 of the NLRP1b gene.

	H1/H4 Genotype	Non-H1/H4 Genotype	Total
Capillaria	4	1	5
No *Capillaria*	27	48	75
Total	31	49	80

A total of five mice were infected with *Capillaria*. Four mice were H1/H4 genotype, as shown in Table 5.39. The H1/H4 haplotypes consist of 31 mice, only four of them were infected with *Capillaria*. The two-tailed P value calculated for the previous table is 0.07. The relationship between *Capillaria* and the H1/H4 haplotype of exon 3-3 is almost significant. A summary of the other haplotypes P values is shown in Table 5.40, below (see also Appendix 1, Tables 65–72).

Table 5.40. Summary of P values of all haplotypes of exon 3-3 of the NLRP1b gene and *Capillaria* infection.

Haplotype	P value	Relation with *Capillaria*
H1/H1	> 0.05	Insignificant
H1/H2	> 0.05	Insignificant
H1/H4	0.07	Insignificant
H2/H2	> 0.05	Insignificant
H3/H2	> 0.05	Insignificant
H3/H3	> 0.05	Insignificant
H3/H4	> 0.05	Insignificant
H4/H2	> 0.05	Insignificant
H4/H4	> 0.05	Insignificant

5.6.4.5. *Pelodera*

Table 5.41. Investigation of the relationship between *Pelodera* infection and the presence of exon 3-3 haplotype H2/H2 of the NLRP1b gene.

	H2/H2 Genotype	Non-H2/H2 Genotype	Total
Pelodera	2	2	4
No *Pelodera*	9	67	76
Total	11	69	80

Four mice were infected with *Pelodera*. Two mice were H2/H2 genotype, as shown in Table 5.41, above. The two-tailed P value calculated for the above table is 0.09. The relationship between *Pelodera* and the H2/H2 haplotype of exon 3-3 is almost significant. A summary of the other haplotypes P values is shown in Table 5.42, below (see also Appendix 1, Tables 73–80).

Table 5.42. Summary of P values of all haplotypes of exon 3-3 of the NLRP1b gene and *Pelodera* infection.

Haplotype	P value	Relation with *Pelodera*
H1/H1	> 0.05	Insignificant
H1/H2	> 0.05	Insignificant
H1/H4	> 0.05	Insignificant
H2/H2	0.09	Insignificant
H3/H2	0.23	Insignificant
H3/H3	> 0.05	Insignificant
H3/H4	> 0.05	Insignificant
H4/H2	> 0.05	Insignificant
H4/H4	0.57	Insignificant

5.6.4.6. *Brachylaemus*

Table 5.43. Investigation of the relationship between *Brachylaemus* infection and the presence of exon 3-3 of the NLRP1b gene haplotype H1/H2.

	H1/H2 Genotype	Non-H1/H2 Genotype	Total
Brachylaemus	1	0	1
No *Brachylaemus*	3	76	79
Total	4	76	80

Only one mouse was found to be infected by *Brachylaemus*, it had a H1/H2 genotype, as shown in Table 5.43. The two-tailed P value calculated for the previous table is 0.0500. The relationship between *Brachylaemus* and the H1/H2 haplotype of exon 3-3 is almost significant. A summary of the other haplotypes P values is shown in Table 5.44, below (see also Appendix 1, Tables 80–88).

Table 5.44. Summary of P values of all haplotypes of exon 3-3 of the NLRP1b gene and *Brachylaemus* infection.

Haplotype	P value	Relation with *Brachylaemus*
H1/H1	> 0.05	Insignificant
H1/H2	0.05	Almost significant
H1/H4	> 0.05	Insignificant
H2/H2	> 0.05	Insignificant
H3/H2	> 0.05	Insignificant
H3/H3	> 0.05	Insignificant
H3/H4	> 0.05	Insignificant
H4/H2	> 0.05	Insignificant
H4/H4	> 0.05	Insignificant

5.6.4.7 Summary of the significant relationships shown between the NLRP1b gene variants and parasite infections

Table 4.45. Summary of significant associations of exon 3-2 and 3-3. Red shaded haplotypes are showing resistance and yellow shaded haplotypes are showing susceptibility to the infection.

	Exon3-2
Infection	Haplotypes of exon 3-2
Syphacea	H1/H2
	Exon3-3
Infection	Haplotypes
Negative/Multiple	H2/H2
Plagiorchis	H3/H3
Heligmosomoides	H1/H4---- H4/H4
Syphacea	H1/H4

5.7 Discussion

NLRP1b is an inflammasome that binds to pathogens and initiates an immune response by starting caspase-1 processing. NLRP1B contains multiple domains, these include NACHT, a leucine-rich repeat (LRR), a domain which is called “function to find domain” (FIIND), and a carboxy-terminal caspase recruitment domain (CARD). The NACHT domain is considered to be a nucleotide-binding domain which works via self-association with NLRP1B. The LRR domain is involved in ligand recognition and in intramolecular interactions that mediate autoinhibition. The FIIND domain helps in the assembly of inflammasomes. The CARD domain of NLRP1B interacts directly with the CARD domain of procaspase-1 (Neiman-Zenevich *et al*., 2014).

One of the obstacles with the NLRP1b gene is the limited information about the gene and its regulation during infection. No studies in the wood mice have described its gene sequence and polymorphism. Inter actions between *T. gondii* and some other helminth parasites and the NLRP1b gene have not been evaluated and examined yet.

NLRP1b plays an important role in detecting parasites and triggering the innate immune system. One of the hypotheses of this study is that the different variants of each NLRP1b

vary in their role in the detection of parasitic infections and therefore the ability to resist infection.

No significant associations were found between any of the haplotypes of exon 3-2 and parasite infection status (negative, single and multiple parasites). On the other hand, some significant association were discovered between some haplotypes of exon 3-3 of NLRP1b and parasite infection status (negative, single and multiple parasites). Only one significant and one almost significant association were discovered between parasite infection status and the haplotypes of exon 3-3. A significant association was found between the H2/H2 haplotype and mice that are not infected and multiple parasite infected mice. An almost significant relationship was between the H1/H1 haplotypes and single and multiple parasite infected mice.

Only one significant association was discovered between among all the haplotypes of exon 3-2 of the NLRP1b gene and different helminth parasites which is the H2/H2 haplotype and *Syphacea* infection. Four other almost significant associations were discovered between the H3/H3 haplotype with *Capillaria* and *Pelodera* infections, H3/H2 with *Syphacea* and H1/H2 with *Heligmosomoides.*

Furthermore, four different significant associations were discovered between three haplotypes of the NLRP1b of exon 3-3 and different helminth parasites. These significant associations were H1/H4 with *Heligmosomoides* and *Syphacea, Plagiorchis* with H3/H3 and *Heligmosomoides* with H4/H4. Also, five almost significant associations were determined, which are H1/H2 with *Brachylaemus,* H1/H4 with *Capillaria,* H2/H2 with *Plagiorchis* and *Pelodera* and H3/H2 with *Plagiorchis.*

An interesting question is how would extracellular helminth parasites interact with the inter-cellular inflammasome. One possible answer to this question is based on understanding of the excretory-secretory system of helminth parasites. The helminth parasites have special secretions which is called excretory-secretory fraction (ES). The excretory-secretory fraction

can be defined as components that are secreted by most of the helminth parasites within the host which contain glycoproteins, proteins, glycolipids, and polysaccharides and have a huge effect in the cellular physiology and influence in the immune cells (Whits & Artavanis-Tsakonas, 2012).

For example, normally, MHC class II is presented in high level on the DCs. MHC II is considered as one of the most powerful component for detection of exogenous antigens through detection of strangulation from pathogens. After that, different processes occur such as maturation, pro inflammatory cytokines production and subsequent activation of different immune cells and macrophage localization at the infection site. On the other hand, the case can be different during helminth infection as the ES component affects the antigen presenting cells such as the dendritic cells and all the previous process and maturation, pro-inflammatory cytokines and activation of different immune cell do not occur. In conclusion, modulation of the immune system is essential for helminth parasite survival (White & Artavanis-Tsakonas, 2012). Surprisingly, the ES molecules that modulate the immune system TLRs and subsequently down regulate the TLR pathways do active inflammasome responses such as NLRP3 which activates signalling and production of pro-inflammatory cytokines. Poor information is available on the activation of NLRP inflammasome by helminth parasite. However, it is thought that overlapping mechanisms, including the presence of oxygen radicals and high extracellular potassium may lead to formation of membrane pores which in turn may result in recognition of the ES molecules by the NLRP inflammasome (Ritter *et al.*, 2010).

One of the limitations of this study is the size of the sample. We used 80 *Apodemus sylvaticus* as the sample. If we assume that we have double the sample size, i.e. 160, the relations of the haplotypes with some helminth parasites that were not quite significant would be highly significant. For example, the relationship between *Capillaria* infection and the presence of

exon 3-3 of the NLRP1b gene haplotype H1/H4 is not quite significant, as shown in Table 5.39. However, it would be if the same numbers were doubled (P=0.014).

Another limitation is the number of mice infected by some parasites such as *Pelodera* and *Brachylaemus.* Only four mice were infected by *Pelodera* and only one mouse was infected by *Brachylaemus.* Thus, it makes it difficult to find a significant association.

Some tentative associations have been found but more research is needed to establish whether these represent real biological associations.

To our knowledge this is the first study that investigates the relationships between NLRP1b gene polymorphisms and *T. gondii* co-infection (helminth parasites) that we investigated.

Chapter 6

Discussion and Summary

6. Discussion and Summary

The main objective of this thesis was amplifying the NLRP1b gene from a population of *Apodemus sylvaticus* sampled from the Malham Tarn woodland area in North Yorkshire (Boyce *et al*., 2014). This collection of mice has been tested for the presence of the parasite *Toxoplasma gondii* (Bajnok *et al* 2015). The aim was to investigate polymorphisms within the NLRP1b gene in relation to *Toxoplasma gondii* infection and infection with six helminth parasites: *Plagiorchis, Heligmosomoides, Syphacea, Capillaria*, *Pelodera* and *Brachylaemus*. A further aim was to investigate of the relationship between TLR11 and TLR 12 variants, which were also known for this *Apodemus* collection (Morger *et al*., 2014), with respect to helminth parasite infection.

Several significant associations were discovered between NLRP1b gene polymorphisms and parasitic infection as shown in Table 6.1.

Table 6.1. Summary of all significant associations between *Apodemus sylvaticus* TLR and NLRP1b gene variants and parasite infections.

Infection	NLRP1b Exon 3-2	NLRP1b Exon 3-3	TLR11 H1/H2	TLR12 H1
Single /Multiple	----------	----------	----------	----------
Negative/Multiple	----------	H2/H2	----------	----------
Negative/Single	----------	----------	----------	----------
Plagiorchis	----------	H3/H3	----------	----------
Heligmosomoides	----------	H1/H4 H4/H4	----------	----------
Syphacea	H1/H2	H1/H4	H2	----------
Capillaria	----------	----------	----------	----------
Pelodera	----------	----------	----------	----------
Brachylaemus	----------	----------	----------	----------
Toxoplasma gondii In respect to different helminth	H2/H3 H4/H4 First locus homozygosity	H4/H4 First and second locus homozygosity Heterozygosity of both locus together		

The NLRP1b gene encodes a protein that is a major component of inflammasome which act as a sensor for parasitic and other infections. Inflammasomes have been an interesting subject for researchers around the world. Multiple studies have investigated the association between the inflammasome and different non-infectious diseases, partial infectious diseases and

infectious diseases (see later). Specifically, the genes that encode the inflammasome, including NLRP1b, are located a genomic region known as *Toxo1* based on work that demonstrates that this genomic region is required for detection and resistance to *Toxoplasma gondii* (Cavailles *et al*., 2006). However, the majority of this work, especially that on the NLRP1b gene, has been carried out in laboratory animal systems and there is very little work on natural populations of animals in the wild. The context of this thesis is therefore to approach these studies from a field biology investigation using natural population of wood mice. Since this study commenced, a number of studies have been published which need to be reviewed in the light of the results found in this thesis.

A study by Guglindolo and his group (2017), evaluated the role of activation of the inflammasome in the SOD1 rat brain, a model of the Amyotrophic lateral sclerosis (ALS). The study demonstrated that inflammasome activation can lead to activation of caspase1 resulting in the NLRP3 protein level to increase. This increase in the NLRP3 level leads to an increase in IL18, IL-1β and IFNγ levels. The study concluded that the activation of the inflammasome in the brain of the SOD1 rat plays an important role in ALS (Gugliandolo, Giacoppo, Bramanti & Mazzon, 2017). Moreover, another study investigated the role of the inflammasome in acute microbial pneumonia. When the inflammasome is insufficiently controlled, the activation of the inflammasome in the lung will result in extensive inflammation and excessive tissue damage (Kumar *et al.*, 2017). Another study examined the role of the inflammasome in the skin wound healing and they found that the NLRP3 pathway activation plays an important role in wound repair and that Adenosine triphosphate (ATP) might be an important factor that could be used as an effective treatment in accelerating wound healing (Ito, Kanbe, Sakai & Seishima, 2017). In addition, the study by Chen and his group (2017), demonstrated that infection with Chikungunya virus will lead to increase level of the NLRP3 and this high level of NLRP3 is associated with the peak of inflammatory symptoms in human and mice. The inhibition of NLRP3 activation will lead to a reduced

inflammatory pathology associated with this virus. The study concluded that the NLRP3 is crucial factor for the virus- induced inflammation and the inhibition of NLRP3 is one of the useful treatment strategies against this virus (Chen *et al*., 2017). Another study done by Wang and his colleagues (2017), investigated the possible association between seizure and the inflammasome genetic polymorphisms in the NLRP1, NLRP3, and P2X7R genes. The study found that the frequency of the rs878329 (G>C) NLRP1b genotype was significantly lower in the seizure patients compared to controls. In addition, they found that there was a significant difference of the rs878329 (G>C) genotype among male patients and no significant differences among females. The study concluded that NLRP1 rs878329 (G>C) genotype might have an important role in the susceptibility for seizures and disease (Wang *et al*, 2017). These studies support the notion explored in this thesis that polymorphisms in inflammasome genes may influence disease susceptibility.

All these previous current studies and more studies available show how crucial and influential is the inflammasome. It is very important to investigate the role of the inflammasome in relation to parasitic infection due to their effect in controlling different diseases as shown above.

A number of studies have identified the *Toxo* 1 locus located on chromosome 10 of laboratory rodents and it appears to be important in controlling the *Toxoplasma* infection (Cavailles *et al*., 2014).

A study that investigated the *Toxo* 1 locus showed that some rats are resistant to *Toxoplasma gondii* due to the presence of *Toxo* 1 locus which of which one component is the NLRP1 gene. NLRP1 was shown to have a correlation with the production of IL-1β/IL-18, macrophage sensitivity to pyroptosis and prevention of *Toxoplasma gondii* proliferation. These findings reveal that the rat NLRP1 gene located in the macrophage is activated by *Toxoplasma* (Cirelli *et al*., 2014). A study by (Gorfu *et al*., 2014) showed the importance of the domains of the NLRP1 gene in mice, such as the nucleotide-binding domain and leucine-

rich repeat containing a pyrin domain (NLRP), during acute toxoplasmosis. They confirmed that mice deficient of NLRP1 showed increased parasitaemia and high mortality rate due to cytokine effects that are interrupted in mice deficient of NLRP1 (Gorfu *et al* .,2014).

Another study showed that NLRP1b in mice and rats is activated by *T. gondii* and this response is helping the mice and rats to resist the infection. This protective response accomplished by activation of caspase1 and 11 the ASC adaptor and the NLRP1b which leads to secretion of Interleukin 1β (IL-1β). NLRP1b is activated by the anthrax lethal factor although mice infected with *Toxoplasma gondii* showed an activation of NLRP1b independent of the lethal factor (Ewald, Chavarria-Smith & Boothroyd 2014). These studies all point to the importance of investigating polymorphisms in this gene in natural populations. Epidemiological studies show that the genetic arrangement of the host and the parasite both control the phenotypic expression in response to *T. gondii* (Mack *et al* 1999; Sibley, Mordue, *et al*., 2002). It is likely that the NLRP1b gene is involved in this. The NLRP1b gene leads to killing of the parasite by triggering pathogen intracellular sensing, pyroptosis (programmed-cell death), involving caspase-1 activation and cleavage of IL-1β. *In vivo*, studies showed that the ability of macrophages to restrict *T. gondii* growth and a *T. gondii* induced death of intracellular parasites and its host macrophages rely upon the *Toxo* 1 locus dependent intracellular resistance. Moreover, the *Toxo* 1 dependent response of the macrophage in infected rats might trigger two pathways which result in controlling the *Toxoplasma* infection. One pathway which seems important and the best candidate to mediate the parasite induced cell deaths is the NOD-like receptor NLRP1a/ Caspase-1 pathway (Cavailles *et al*., 2014).

Some rat species showed resistance for *T. gondii* infection such as LEW rat and other species showed susceptibility such as BN rat (Sergent *et al*., 2005). LEW rat resistance is a dominant trait and macrophages play an important role to limit the parasitaemia in laboratory studies. A locus in the rat genome was discovered and investigated after discovering the variation

between the LEW rat and BN rat in respect to *T. gondii* infection and called the *Toxo 1* locus. This locus is responsible for the resistance to toxoplasmosis in the LEW rat. Firstly, it was discovered that the *Toxo1* locus is located at chromosome 10 and limited to 7.6 mega base (Cavailles *et al* 2006).

Recent studies that investigated *Toxo 1* locus confirmed that NLRP1 gene is one of the main genes that is located at the *Toxo 1* locus and it is has a relationship with congenital toxoplasmosis (Witola *et al*., 2011)

Later, a study investigated and analysed the haplotype of chromosome 10 in LEW and BN rat using genetic method to established the group of the gene that locate at the *Toxo 1* locus and their arrangement. Also, they studied the effect of the locus on the resistant of LEW mice. They discovered that *Toxo 1* locus is highly conserved region and it is about 891 KB in the Lew rat. The sequence of locus of 90 LEW and BN rats revealed a mutation in the region which is located at the end of the locus around the NLRP1 gene. This mutation is responsible for the resistance of the rat by controlling cell death of the macrophage of the host and by killing the parasites. Further investigation proved that a conserved region located at *Toxo1* locus is the reason that inbred mice are resistant to *T. gondii*. 373 SNPs were discovered among the Toxo1 locus. Also 21 mutations which are either insertions or deletions were discovered among the refractory rats in the coding and non-coding sequence. Some mutations are at the end of the locus. The coding sequences contained about 23 SNPs and 16 of them were missense. One deletion mutation was also discovered. NLRP1 contained 2 SNPs (Cavailles *et al*., 2014). Based on these studies mentioned above; the *Toxo l* locus which contains the NLRP1 gene has an important role in controlling the *Toxoplasma gondii* infection.

There is very limited number of studies on the NLRP1b gene. No previous studies have investigated the relation between the NLRP1b gene and *T. gondii* infection in natural populations. Furthermore, there are no previous studies that consider helminth parasitic

infection in relation to the NLRP1b gene. Some published studies discuss the relationship between the NLRP1b gene and the anthrax lethal factor which was thought previously as the main activator of the NLRP1b gene. Subsequently, the NLRP1b gene was discovered to be activated by *Toxoplasma gondii* independent of the lethal factor (Chavarría-Smith & Vance., 2013; Neiman-Zenevich, Liao & Jeremy Mogridge., 2014; Hellmich *et al*.,2012; Terra *et al*., 2010; Averette *et al*.,2010).

After initiating the study, it was very difficult to amplify the NLRP1b gene from *A. sylvaticus* due to different reasons including lacking the genomic database of the NLRP1b gene and the lack of previous studies that investigated the NLRP1b gene in wild mice. All the previous studies were carried out in laboratory mice which might be different to the wild mice population. This resulted in some difficulties with design of NLRP1b specific primers for *A. sylvaticus*. Successful amplification allowed us to determine multiple associations between the NLRP1b exon variation and each specific parasite. Also, the relation of both exons of NLRP1b gene were investigated in respect to negative, single parasitic infection and co-infection.

Several attempts to design specific primers design *Mus musculus* were carried out and the last attempts provided successful amplification of two parts of exon 3, are part 2 and part 3.

Sequence analysis of both exons and protein translations revealed different SNPs in both parts of the exon. These polymorphisms represented in 8 haplotypes of exon 3 part 2 and 9 haplotypes of exon 3-3. These polymorphisms support the hypothesis that NLRP1b gene is polymorphic.

The relationship between the haplotypes and *Toxoplasma gondii* infection were investigated and revealed an interesting result. Some haplotypes showed an association with high frequency of the infection and some of them showed association with low frequency. Exon 3-2 showed as significant association between at the first SNPs location whereas exon 3-3 showed a significant relation at the first SNP, second SNP and both SNPs together. Mice

heterozygous at the exon 3-2 SNP (first location) showed a higher frequency of *T. gondii* infection. Interestingly, the first and second SNPs of exon 3-3 showed a higher frequency of *T. gondii* infection in heterozygous mice. However, it was a different case when both SNPs were taken in consideration since heterozygous mice then showed a lower frequency of *T. gondii* infection.

This work with the NLRP1b gene highlights a potential association between the gene and *T. gondii* infection in wood mice. It does not confirm that the NLRP1b gene is responsible for susceptibility or resistance to toxoplasmosis; it just expresses an association between them. Some other factors might interfere with the work; for example, another part of the NLRP1b gene, seasonal variation, genetic variation, and vertical transmission.

Other immune genes have an important role in immunity such as TLRs. Several studies have investigated the role of TLRs in the innate immune response. A study by Tschirren and her group (2013) investigated the *Myodes glareolus* TLR2 SNPs and checked the relationship of these SNPs upon infection by *Borrelia afzelii.* They discovered 15 haplotypes for TLR12 and these haplotypes represented in 10 amino acid haplotypes. These haplotypes differentiated to 3 cluster groups. They found a particular group of haplotypes that have low frequency of *Borrelia* infection and they suggest that TLR2 is subject to a positive selection mutation and the resulting polymorphism of TLRs could influence resistance (Tschirren *et al* 2013).

The study by Bajnok and colleagues (2015) investigated TLR 11 and TLR 12 polymorphism in relation to *Toxoplasma gondii* infection in the wood mice. They discovered that TLR 11 and TLR12 were polymorphic and contain different haplotypes. TLR 11 had 4 haplotypes and TLR 12 had 9 haplotypes. Also, no significant associations were found between the TLR11 and 12 and *T. gondii* infection (Bajnok *et al*., 2015).

The other parts of this thesis provide an understanding of the TLRs 11 and 12 immune gene relationship with the six helminth parasites that was described earlier. This work is an

extended analysis of the relation of TLR polymorphism work with *Toxoplasma gondii* that was done by Bajnok *et al*., 2015).

The hypothesis is that different TLRs variants have different efficiencies of detection and therefore the ability to resist infection. Our result show no association between TLR 12 variations and any resistance or susceptibility to any of the six parasitic infections. H2 genotypes of TLR 11 showed an association with the *Syphacea* infection and no other relationship with any of the remaining five helminth parasites.

Another hypothesis is that heterozygous mice that have two different variants might have a greater ability to detect and deal with infection than homozygous mice. Our result shows no relation between heterozygous mice, homozygous mice and any helminth infection and this means there are no differences between heterozygous mice and homozygous mice in dealing with the infections of the helminth parasites. Furthermore, the hypothesis of the repertoire of TLRs could also have an influence on, or be influenced by multiple or co-infections was not established because no association between the TLR 11 and 12 and single infection and the co infection was described.

Other parts of the TLRs and NLRP1b gene might have a stronger association with *T. gondii* and helminth parasites. Also, some other factors could be affecting the study such as seasonal variation, congenital transmission of *Toxoplasma gondii*, site variation exposure and hence differential exposure to the infection. Seasonal and site variation could have an influence on prevalence and abundance of the infection. A study by Abu-Madi, Behnke, Lewis & Gilbert investigated the effect of the season and site on infection in South England. *Capillaria murissylvatici* were was seasonal and site specific and *Syphacea stroma* showed no variation (Abu-Madi, Behnke, Lewis & Gilbert, 2000).

Exposure to the infection is really important in the case of *Toxoplasma*. Some mice might not be infected due to not being exposed to the cyst in the natural environment. Also, some mice might be infected due to congenital transmission and not natural exposure.

Indeed, this is the first report to investigate the NLRP1b gene in a group of *Apodemus sylvaticus* mice and investigate the effect of different variants on *Toxoplasma* and helminth infections. Also, it is the first report to study the association between TLR 11 and 12 of wild mice and different helminth infections.

6.1 Future Direction.

This study is recommended to be extended in several ways that would result in very interesting result.

Increase that the number of mouse samples which would interestingly affect the associations between the haplotypes of NLRP1b gene and *T. gondii* and the 6 other different parasites. Also, extend the analysis of these samples to cover TLR 11 and 12 haplotypes.

The large sample size will be very helpful in different ways which includes more confidence with the result, increased precision of the result and increasing our sample size can also give us greater ability to detect the significant association between the TLR and NLRP1b gene with different parasites.

Effort in this should focus on exon 3 part 2 and exon 3 part 3 of NLRP1b gene of *A. sylvaticus* and it would be interesting to design different primers for different region of the NLRP1b gene and investigate more exons to determine the full extent of polymorphisms. This also might increase the ability of detecting the most functional part of the gene and study it effects in relation to different parasites.

Using the draft genome could help with sequencing of the remaining exons on NLRP1b gene and other immune genes by facilitating primer design and saving much time and resources.

Investigation and studying the relationships between the immune genes with respect to other infections in the Malham wood mice such as blood parasites and bacteria to discover further potentially interesting relationships with immune gene variants. Indeed, as these are currently unknown, this may confound the current data interpretations.

Using different statistical analysis approaches would be beneficial since in this study, the data was analysed using pair wise combinations (2*2 contingency tables) and instead, use of multivariant modelling should improve the data analyses and robustness of conclusions.

Phylogenetic analysis of the NLRP1b gene will be very help full in giving an idea about how variant is the gene among *A.sylvaticus*. The phylogenetic analysis will be really helpful in studying the relation among the group of the mice that we have. Also, it will show the polymorphisms within the family and within different families. This will be through analysis of the phylogenetic interference of the inherited trait such as the DNA sequence.

Any NLRP1b gene confirmed polymorphism would be investigated and analysed using genetic study in model organisms. For instance, expressing the NLRP1b gene from *A. sylvaticus* in another NLRP1b mutant mouse and then investigating parasite infections.

Also, establishment of some techniques such as cell culture for *A. sylvaticus* since this would be important to study the binding of the NLRP1b gene and TLRs 11 and 12 with different specific parasites.

Also, study the association between the other exons of the NLRP1b gene with *T. gondii* and the other six helminth parasites. Other exons of the gene might be highly variable and might have a major function and effects on the susceptibility and resistance to the parasitic infection. By studying the other whole exons, a clearer understanding will be known about the NLRP1b gene in *A. sylvaticus* and the function of the whole gene.

TLR 11 and 12 were investigated in this study however, it is important to involve other *A. sylvaticus* TLRs that may have an important key role in the innate immune response to parasitic infection such as TLR 2 and TLR 4 which was studied in other animals. The outcomes of some TLRs are different as shown in Table 1.4. Some of them have a Th1 response and others have an interferon production response. By studying other TLRs, a

clearer observation will give an indication of the relation between the TLR responses and *Toxoplasma gondii* and helminth parasite infections.

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Appendix 1:

Table 1. 2*2 contingency table showing the relationship between *Plagiorchis* infections and the presence of exon 3-2 of NLRP1b gene haplotype H1/H1.

	H1/H1 Genotype	Non-H1/H1 Genotype	Total
Plagiorchis	2	17	19
No *Plagiorchis*	8	53	61
Total	10	70	80

Table 2. Table 1: 2*2 contingency table showing the relationship between *Plagiorchis* infections and the presence of exon 3-2 of NLRP1b gene haplotype H1/H2.

	H1/H2 Genotype	Non-H2/H2 Genotype	Total
Plagiorchis	5	14	19
No *Plagiorchis*	15	46	61
Total	20	60	80

Table 3. 2*2 contingency table showing the relationship between *Plagiorchis* infections and the presence of exon 3-2 of NLRP1b gene haplotype H2/H2.

	H2/H2 Genotype	Non-H2/H2 Genotype	Total
Plagiorchis	5	14	19
No *Plagiorchis*	12	49	61
Total	17	63	80

Table 4. 2*2 contingency table showing the relationship between *Plagiorchis* infections and the presence of exon 3-2 of NLRP1b gene haplotype H3/H2.

	H3/H2 Genotype	Non-H3/H2 Genotype	Total
Plagiorchis	0	19	19
No *Plagiorchis*	21	40	61
Total	21	59	80

Table 5. 2*2 contingency table showing the relationship between *Plagiorchis* infections and the presence of exon 3-2 of NLRP1b gene haplotype H1/H3.

	H1/H3 Genotype	Non-H1/H3 Genotype	Total
Plagiorchis	1	18	19
No *Plagiorchis*	5	56	61
Total	6	74	80

Table 6. 2*2 contingency table showing the relationship between *Plagiorchis* infections and the presence of exon 3-2 of NLRP1b gene haplotype H2/H3.

	H2/H3 Genotype	Non-H2/H3 Genotype	Total
Plagiorchis	0	19	19
No *Plagiorchis*	2	59	61
Total	2	78	80

Table7. 2*2 contingency table showing the relationship between *Plagiorchis* infections and the presence of exon 3-2 of NLRP1b gene haplotype H3/H3.

	H3/H3 Genotype	Non-H3/H3 Genotype	Total
Plagiorchis	0	19	19
No *Plagiorchis*	1	60	61
Total	1	79	80

Table 8. 2*2 contingency table showing the relationship between *Plagiorchis* infections and the presence of exon 3-2 of NLRP1b gene haplotype H4/H4.

	H4/H4 Genotype	Non-H4/H4 Genotype	Total
Plagiorchis	4	15	19
No *Plagiorchis*	5	56	61
Total	9	71	80

Table 9. 2*2 contingency table showing the relationship between *Heligmosomoides* infections and the presence of exon 3-2 of NLRP1b gene haplotype H1/H1.

	H1/H1 Genotype	Non- H1/H1 Genotype	Total
Heligmosomoides	5	48	53
No *Heligmosomoides*	5	22	27
Total	10	70	80

Table 10. 2*2 contingency table showing the relationship between *Heligmosomoides* infections and the presence of exon 3-2 of NLRP1b gene haplotype H2/H2.

	H2/H2 Genotype	Non-H2/H2 Genotype	Total
Heligmosomoides	13	40	53
No *Heligmosomoides*	4	23	27
Total	17	63	80

Table 11. 2*2 contingency table showing the relationship between *Heligmosomoides* infections and the presence of exon 3-2 of NLRP1b gene haplotype H3/H2.

	H3/H2 Genotype	Non-H3/H2 Genotype	Total
Heligmosomoides	2	51	53
No *Heligmosomoides*	0	27	27
Total	2	78	80

Table 12. 2*2 contingency table showing the relationship between *Heligmosomoides* infections and the presence of exon 3-2 of NLRP1b gene haplotype H3/H2.

	H1/H3 Genotype	Non-H3/H2 Genotype	Total
Heligmosomoides	3	50	53
No *Heligmosomoides*	3	24	27
Total	6	74	80

Table 13. 2*2 contingency table showing the relationship between *Heligmosomoides* infections and the presence of exon 3-2 of NLRP1b gene haplotype H2/H3.

	H2/H3Genotype	Non-H2/H3 Genotype	Total
Heligmosomoides	7	46	53
No *Heligmosomoides*	8	19	27
Total	15	65	80

Table 14. 2*2 contingency table showing the relationship between *Heligmosomoides* infections and the presence of exon 3-2 of NLRP1b gene haplotype H3/H3.

	H3/H3 Genotype	Non-H3/H3 Genotype	Total
Heligmosomoides	0	53	53
No *Heligmosomoides*	1	26	27
Total	1	79	80

Table15. 2*2 contingency table showing the relationship between *Heligmosomoides* infections and the presence of exon 3-2 of NLRP1b gene haplotype H4/H4.

	H4/H4 Genotype	Non-H4/H4 Genotype	Total
Heligmosomoides	6	47	53
No *Heligmosomoides*	3	24	27
Total	9	71	80

Table 16. 2*2 contingency table showing the relationship between Syphacea infections and the presence of exon 3-2 of NLRP1b gene haplotype H1/H1.

	H1/H1 Genotype	Non- H1/H1 Genotype	Total
Syphacea	4	18	22
No *Syphacea*	6	52	58
Total	10	70	80

Table 17. 2*2 contingency table showing the relationship between Syphacea infections and the presence of exon 3-2 of NLRP1b gene haplotype H1/H2.

	H2/H2 Genotype	Non-H2/H2 Genotype	Total
Syphacea	4	18	22
No *Syphacea*	13	45	58
Total	17	63	80

Table 18. 2*2 contingency table showing the relationship between Syphacea infections and the presence of exon 3-2 of NLRP1b gene haplotype H3/H2.

	H1/H3 Genotype	Non-H1/H2 Genotype	Total
Syphacea	2	20	22
No *Syphacea*	4	54	58
Total	6	74	80

Table 19. 2*2 contingency table showing the relationship between Syphacea infections and the presence of exon 3-2 of NLRP1b gene haplotype H2/H3.

	H2/H3Genotype	Non-H2/H3 Genotype	Total
Syphacea	5	17	22
No *Syphacea*	10	48	58
Total	15	65	80

Table 20. 2*2 contingency table showing the relationship between Syphacea infections and the presence of exon 3-2 of NLRP1b gene haplotype H3/H3.

	H3/H3 Genotype	Non-H3/H3 Genotype	Total
Syphacea	1	21	22
No *Syphacea*	0	58	58
Total	1	79	80

Table 21. 2*2 contingency table showing the relationship between Syphacea infections and the presence of exon 3-2 of NLRP1b gene haplotype H4/H4.

	H4/H4 Genotype	Non-H4/H4 Genotype	Total
Syphacea	3	19	22
No *Syphacea*	6	52	58
Total	9	71	80

Table 22. 2*2 contingency table showing the relationship between *Capillaria* infections and the presence of exon 3-2 of NLRP1b gene haplotype H1/H1.

	H1/H1 Genotype	Non- H1/H1 Genotype	Total
Capillaria	2	3	5
No *Capillaria*	8	67	75
Total	10	70	80

Table 23. 2*2 contingency table showing the relationship between *Capillaria* infections and the presence of exon 3-2 of NLRP1b gene haplotype H1/H2.

	H1/H2 Genotype	Non-H1/H2 Genotype	Total
Capillaria	2	3	5
No *Capillaria*	18	57	75
Total	20	60	80

Table 24. 2*2 contingency table showing the relationship between *Capillaria* infections and the presence of exon 3-2 of NLRP1b gene haplotype H2/H2.

	H2/H2 Genotype	Non-H2/H2 Genotype	Total
Capillaria	0	5	5
No *Capillaria*	17	58	75
Total	17	63	80

Table 25. 2*2 contingency table showing the relationship between *Capillaria* infections and the presence of exon 3-2 of NLRP1b gene haplotype H3/H2.

	H3/H2 Genotype	Non-H2/H2 Genotype	Total
Capillaria	0	5	5
No *Capillaria*	21	54	75
Total	21	59	80

Table 26. 2*2 contingency table showing the relationship between *Capillaria* infections and the presence of exon 3-2 of NLRP1b gene haplotype H1/H3.

	H1/H3 Genotype	Non-H3/H2 Genotype	Total
Capillaria	0	5	5
No *Capillaria*	6	69	75
Total	6	74	80

Table 27. 2*2 contingency table showing the relationship between *Capillaria* infections and the presence of exon 3-2 of NLRP1b gene haplotype H2/H3.

	H2/H3Genotype	Non-H2/H3 Genotype	Total
Capillaria	0	5	5
No *Capillaria*	15	60	75
Total	15	65	80

Table 28. 2*2 contingency table showing the relationship between *Capillaria* infections and the presence of exon 3-2 of NLRP1b gene haplotype H4/H4.

	H4/H4 Genotype	Non-H4/H4 Genotype	Total
Capillaria	0	5	5
No *Capillaria*	9	66	75
Total	9	71	80

Table 29. 2*2 contingency table showing the relationship between *Pelodera* infections and the presence of exon 3-2 of NLRP1b gene haplotype H1/H1.

	H1/H1 Genotype	Non- H1/H1 Genotype	Total
Pelodera	1	3	4
No *Pelodera*	9	67	76
Total	10	70	80

Table 30. Table 32. 2*2 contingency table showing the relationship between *Pelodera* infections and the presence of exon 3-2 of NLRP1b gene haplotype H1/H2.

	H1/H2 Genotype	Non-H1/H2 Genotype	Total
Pelodera	0	4	4
No *Pelodera*	20	56	76
Total	20	60	80

Table 31. 2*2 contingency table showing the relationship between *Pelodera* infections and the presence of exon 3-2 of NLRP1b gene haplotype H2/H2.

	H2/H2 Genotype	Non-H2/H2 Genotype	Total
Pelodera	1	3	4
No *Pelodera*	16	60	76
Total	17	63	80

Table 32. 2*2 contingency table showing the relationship between *Pelodera* infections and the presence of exon 3-2 of NLRP1b gene haplotype H1/H3.

	H1/H3Genotype	Non-H2/H3 Genotype	Total
Pelodera	0	4	4
No *Pelodera*	6	70	76
Total	6	74	80

Table 33. 2*2 contingency table showing the relationship between *Pelodera* infections and the presence of exon 3-2 of NLRP1b gene haplotype H2/H3.

	H2/H3 Genotype	Non-H3/H3 Genotype	Total
Pelodera	0	4	4
No *Pelodera*	15	61	76
Total	15	65	80

Table 34. 2*2 contingency table showing the relationship between *Pelodera* infections and the presence of exon 3-2 of NLRP1b gene haplotype H3/H3.

	H3/H3 Genotype	Non-H3/H3 Genotype	Total
Pelodera	1	3	4
No *Pelodera*	0	76	76
Total	1	79	80

Table 35. 2*2 contingency table showing the relationship between *Pelodera* infections and the presence of exon 3-2 of NLRP1b gene haplotype H4/H4.

	H4/H4 Genotype	Non-H4/H4 Genotype	Total
Pelodera	1	3	4
No *Pelodera*	8	68	76
Total	9	71	80

Table 36. 2*2 contingency table showing the relationship between *Brachylaemus* infections and the presence of exon 3-2 of NLRP1b gene haplotype H1/H1.

	H1/H1 Genotype	Non- H1/H1 Genotype	Total
Brachylaemus	0	1	1
No *Brachylaemus*	10	69	79
Total	10	70	80

Table 37. 2*2 contingency table showing the relationship between *Brachylaemus* infections and the presence of exon 3-2 of NLRP1b gene haplotype H1/H2.

	H1/H2 Genotype	Non-H1/H2 Genotype	Total
Brachylaemus	1	0	1
No *Brachylaemus*	19	60	79
Total	20	60	80

Table 38. 2*2 contingency table showing the relationship between *Brachylaemus* infections and the presence of exon 3-2 of NLRP1b gene haplotype H2/H2.

	H2/H2 Genotype	Non-H2/H2 Genotype	Total
Brachylaemus	0	1	1
No *Brachylaemus*	17	62	79
Total	17	63	80

Table 39. 2*2 contingency table showing the relationship between *Brachylaemus* infections and the presence of exon 3-2 of NLRP1b gene haplotype H3/H2.

	H3/H2 Genotype	Non-H3/H2 Genotype	Total
Brachylaemus	0	1	1
No *Brachylaemus*	21	58	79
Total	21	59	80

Table 40. 2*2 contingency table showing the relationship between *Brachylaemus* infections and the presence of exon 3-2 of NLRP1b gene haplotype H1/H3.

	H1/H3 Genotype	Non-H3/H2 Genotype	Total
Brachylaemus	0	19	1
No *Brachylaemus*	6	40	79
Total	6	59	80

Table 41. 2*2 contingency table showing the relationship between *Brachylaemus* infections and the presence of exon 3-2 of NLRP1b gene haplotype H2/H3.

	H2/H3Genotype	Non-H2/H3 Genotype	Total
Brachylaemus	0	1	1
No *Brachylaemus*	15	64	79
Total	15	65	80

Table 42. 2*2 contingency table showing the relationship between *Brachylaemus* infections and the presence of exon 3-2 of NLRP1b gene haplotype H3/H3.

	H3/H3 Genotype	Non-H3/H3 Genotype	Total
Brachylaemus	0	1	1
No *Brachylaemus*	1	78	79
Total	1	79	80

Table 43. 2*2 contingency table showing the relationship between *Brachylaemus* infections and the presence of exon 3-2 of NLRP1b gene haplotype H4/H4.

	H4/H4 Genotype	Non-H4/H4 Genotype	Total
Brachylaemus	0	1	1
No *Brachylaemus*	9	70	79
Total	9	71	80

Table 44. 2*2 contingency table showing the relationship between *Plagiorchis* infections and the presence of exon 3-3 of NLRP1b gene haplotype H1/H1.

	H1/H1 Genotype	Non-H1/H1 Genotype	Total
Plagiorchis	0	19	19
No *Plagiorchis*	6	55	61
Total	6	74	80

Table 45. 2*2 contingency table showing the relationship between *Plagiorchis* infections and the presence of exon 3-3 of NLRP1b gene haplotype H1/H2.

	H1/H2 Genotype	Non-H1/H2 Genotype	Total
Plagiorchis	1	18	19
No *Plagiorchis*	3	58	61
Total	4	76	80

Table 46. 2*2 contingency table showing the relationship between *Plagiorchis* infections and the presence of exon 3-3 of NLRP1b gene haplotype H1/H4.

	H1/H4 Genotype	Non-H1/H4 Genotype	Total
Plagiorchis	8	11	19
No *Plagiorchis*	23	38	61
Total	31	49	80

Table 47. 2*2 contingency table showing the relationship between *Plagiorchis* infections and the presence of exon 3-3 of NLRP1b gene haplotype H3/H4.

	H3/H4 Genotype	Non-H3/H4 Genotype	Total
Plagiorchis	0	19	19
No *Plagiorchis*	2	59	61
Total	2	78	80

Table 48. 2*2 contingency table showing the relationship between *Plagiorchis* infections and the presence of exon 3-3 of NLRP1b gene haplotype H4/H2.

	H4/H2 Genotype	Non-H4/H2 Genotype	Total
Plagiorchis	1	18	19
No *Plagiorchis*	0	61	61
Total	1	79	80

Table 49. 2*2 contingency table showing the relationship between *Plagiorchis* infections and the presence of exon 3-3 of NLRP1b gene haplotype H4/H4.

	H4/H4 Genotype	Non-H4/H4 Genotype	Total
Plagiorchis	3	16	19
No *Plagiorchis*	14	47	61
Total	17	63	80

Table 50. 2*2 contingency table showing the relationship between *Heligmosomoides* infections and the presence of exon 3-3 of NLRP1b gene haplotype H1/H1.

	H1/H1 Genotype	Non-H1/H1 Genotype	Total
Heligmosomoides	5	48	53
No *Heligmosomoides*	1	26	27
Total	6	74	80

Table 51. 2*2 contingency table showing the relationship between *Heligmosomoides* infections and the presence of exon 3-3 of NLRP1b gene haplotype H1/H2.

	H1/H2 Genotype	Non-H1/H2 Genotype	Total
Heligmosomoides	3	50	53
No *Heligmosomoides*	1	26	27
Total	4	76	80

Table 52. 2*2 contingency table showing the relationship between *Heligmosomoides* infections and the presence of exon 3-3 of NLRP1b gene haplotype H2/H2.

	H2/H2 Genotype	Non-H2/H2 Genotype	Total
Heligmosomoides	5	48	53
No *Heligmosomoides*	6	21	27
Total	11	69	80

Table 53. 2*2 contingency table showing the relationship between *Heligmosomoides* infections and the presence of exon 3-3 of NLRP1b gene haplotype H3/H2.

	H3/H2 Genotype	Non-H3/H2 Genotype	Total
Heligmosomoides	5	48	53
No *Heligmosomoides*	0	27	27
Total	5	75	80

Table 54. 2*2 contingency table showing the relationship between *Heligmosomoides* infections and the presence of exon 3-3 of NLRP1b gene haplotype H3/H3.

	H3/H3 Genotype	Non-H3/H3 Genotype	Total
Heligmosomoides	2	51	53
No *Heligmosomoides*	1	26	27
Total	3	77	80

Table 55. 2*2 contingency table showing the relationship between *Heligmosomoides* infections and the presence of exon 3-3 of NLRP1b gene haplotype H3/H4.

	H3/H4 Genotype	Non-H3/H4 Genotype	Total
Heligmosomoides	1	52	53
No *Heligmosomoides*	1	26	27
Total	2	78	80

Table 56. 2*2 contingency table showing the relationship between *Heligmosomoides* infections and the presence of exon 3-3 of NLRP1b gene haplotype H4/H2.

	H4/H2Genotype	Non-H4/H2 Genotype	Total
Heligmosomoides	0	53	53
No *Heligmosomoides*	1	26	27
Total	1	79	80

Table 57. 2*2 contingency table showing the relationship between *Syphacea* infections and the presence of exon 3-3 of NLRP1b gene haplotype H1/H1.

	H1/H1 Genotype	Non-H1/H1 Genotype	Total
Syphacea	0	22	22
No *Syphacea*	6	52	58
Total	6	74	80

Table 58. 2*2 contingency table showing the relationship between *Syphacea* infections and the presence of exon 3-3 of NLRP1b gene haplotype H1/H2.

	H1/H2 Genotype	Non-H1/H2 Genotype	Total
Syphacea	2	20	22
No *Syphacea*	2	56	58
Total	4	76	80

Table 59. 2*2 contingency table showing the relationship between *Syphacea* infections and the presence of exon 3-3 of NLRP1b gene haplotype H2/H2.

	H2/H2 Genotype	Non-H2/H2 Genotype	Total
Syphacea	1	21	22
No *Syphacea*	10	48	58
Total	11	69	80

Table 60. 2*2 contingency table showing the relationship between *Syphacea* infections and the presence of exon 3-3 of NLRP1b gene haplotype H3/H2.

	H3/H2 Genotype	Non-H3/H2 Genotype	Total
Syphacea	0	22	22
No *Syphacea*	5	53	58
Total	5	75	80

Table 61. 2*2 contingency table showing the relationship between *Syphacea* infections and the presence of exon 3-3 of NLRP1b gene haplotype H3/H3.

	H3/H3 Genotype	Non-H3/H3 Genotype	Total
Syphacea	0	22	22
No *Syphacea*	3	55	58
Total	3	77	80

Table 62. 2*2 contingency table showing the relationship between *Syphacea* infections and the presence of exon 3-3 of NLRP1b gene haplotype H3/H4.

	H3/H4 Genotype	Non-H3/H4 Genotype	Total
Syphacea	0	22	22
No *Syphacea*	2	56	58
Total	2	78	80

Table 63. 2*2 contingency table showing the relationship between *Syphacea* infections and the presence of exon 3-3 of NLRP1b gene haplotype H4/H2.

	H4/H2 Genotype	Non-H4/H2 Genotype	Total
Syphacea	0	22	22
No *Syphacea*	1	57	58
Total	1	79	80

Table 64. 2*2 contingency table showing the relationship between *Syphacea* infections and the presence of exon 3-3 of NLRP1b gene haplotype H4/H4.

	H4/H4 Genotype	Non-H4/H4 Genotype	Total
Syphacea	6	16	22
No *Syphacea*	11	47	58
Total	17	63	80

Table 65. 2*2 contingency table showing the relationship between *Capillaria* infections and the presence of exon 3-3 of NLRP1b gene haplotype H1/H1.

	H1/H1 Genotype	Non-H1/H1 Genotype	Total
Capillaria	0	5	5
No *Capillaria*	6	69	75
Total	6	74	80

Table 66. 2*2 contingency table showing the relationship between *Capillaria* infections and the presence of exon 3-3 of NLRP1b gene haplotype H1/H2.

	H1/H2 Genotype	Non-H1/H2 Genotype	Total
Capillaria	0	5	5
No *Capillaria*	4	71	75
Total	4	76	80

Table 67. 2*2 contingency table showing the relationship between *Capillaria* infections and the presence of exon 3-3 of NLRP1b gene haplotype H2/H2.

	H2/H2 Genotype	Non-H2/H2 Genotype	Total
Capillaria	0	5	5
No *Capillaria*	11	64	75
Total	11	69	80

Table 68. 2*2 contingency table showing the relationship between *Capillaria* infections and the presence of exon 3-3 of NLRP1b gene haplotype H3/H2.

	H3/H2 Genotype	Non-H3/H2 Genotype	Total
Capillaria	0	5	5
No *Capillaria*	5	70	75
Total	5	75	80

Table 69. 2*2 contingency table showing the relationship between *Capillaria* infections and the presence of exon 3-3 of NLRP1b gene haplotype H3/H3.

	H3/H3 Genotype	Non-H3/H3 Genotype	Total
Capillaria	0	5	5
No *Capillaria*	3	72	75
Total	3	77	80

Table 70. 2*2 contingency table showing the relationship between *Capillaria* infections and the presence of exon 3-3 of NLRP1b gene haplotype H3/H4.

	H3/H4 Genotype	Non-H3/H4 Genotype	Total
Capillaria	0	5	5
No *Capillaria*	2	73	75
Total	2	78	80

Table 71. 2*2 contingency table showing the relationship between *Capillaria* infections and the presence of exon 3-3 of NLRP1b gene haplotype H4/H2.

	H4/H2 Genotype	Non-H4/H2 Genotype	Total
Capillaria	0	5	5
No *Capillaria*	1	74	75
Total	1	79	80

Table 72. 2*2 contingency table showing the relationship between *Capillaria* infections and the presence of exon 3-3 of NLRP1b gene haplotype H4/H4.

	H4/H4 Genotype	Non-H4/H4 Genotype	Total
Capillaria	0	4	4
No *Capillaria*	17	59	76
Total	17	63	80

Table 73. 2*2 contingency table showing the relationship between *Pelodera* infections and the presence of exon 3-3 of NLRP1b gene haplotype H1/H1.

	H1/H1 Genotype	Non-H1/H1 Genotype	Total
Pelodera	0	4	4
No *Pelodera*	6	70	76
Total	6	74	80

Table 74. 2*2 contingency table showing the relationship between *Pelodera* infections and the presence of exon 3-3 of NLRP1b gene haplotype H1/H2.

	H1/H2 Genotype	Non-H1/H2 Genotype	Total
Pelodera	0	4	4
No *Pelodera*	4	72	76
Total	4	76	80

Table 75. 2*2 contingency table showing the relationship between *Pelodera* infections and the presence of exon 3-3 of NLRP1b gene haplotype H1/H4.

	H1/H4 Genotype	Non-H1/H4 Genotype	Total
Pelodera	1	3	4
No *Pelodera*	30	46	76
Total	31	49	80

Table 76. 2*2 contingency table showing the relationship between *Pelodera* infections and the presence of exon 3-3 of NLRP1b gene haplotype H3/H2.

	H3/H2 Genotype	Non-H3/H2 Genotype	Total
Pelodera	1	3	4
No *Pelodera*	4	72	76
Total	5	75	80

Table 77. 2*2 contingency table showing the relationship between *Pelodera* infections and the presence of exon 3-3 of NLRP1b gene haplotype H3/H3.

	H3/H3 Genotype	Non-H3/H3 Genotype	Total
Pelodera	0	4	4
No *Pelodera*	3	71	74
Total	3	75	78

Table 78. 2*2 contingency table showing the relationship between *Pelodera* infections and the presence of exon 3-3 of NLRP1b gene haplotype H3/H4.

	H3/H4 Genotype	Non-H3/H4 Genotype	Total
Pelodera	0	4	4
No *Pelodera*	2	74	76
Total	2	78	80

Table 79. 2*2 contingency table showing the relationship between *Pelodera* infections and the presence of exon 3-3 of NLRP1b gene haplotype H4/H2.

	H4/H2 Genotype	Non-H4/H2 Genotype	Total
Pelodera	0	4	4
No *Pelodera*	1	75	75
Total	1	79	80

Table 80. 2*2 contingency table showing the relationship between *Pelodera* infections and the presence of exon 3-3 of NLRP1b gene haplotype H4/H4.

	H4/H4 Genotype	Non-H4/H4 Genotype	Total
Pelodera	4	15	19
No *Pelodera*	5	56	61
Total	9	71	80

Table 81. 2*2 contingency table showing the relationship between *Brachylaemus* infections and the presence of exon 3-3 of NLRP1b gene haplotype H1/H1.

	H1/H1 Genotype	Non-H1/H1 Genotype	Total
Brachylaemus	0	1	1
No *Brachylaemus*	6	73	79
Total	6	74	80

Table 82. 2*2 contingency table showing the relationship between *Brachylaemus* infections and the presence of exon 3-3 of NLRP1b gene haplotype H1/H4.

	H1/H4 Genotype	Non-H1/H4 Genotype	Total
Brachylaemus	0	1	1
No *Brachylaemus*	31	48	79
Total	31	49	80

Table 83. 2*2 contingency table showing the relationship between *Brachylaemus* infections and the presence of exon 3-3 of NLRP1b gene haplotype H2/H2.

	H2/H2 Genotype	Non-H2/H2 Genotype	Total
Brachylaemus	0	1	1
No *Brachylaemus*	11	68	79
Total	11	69	80

Table 84. 2*2 contingency table showing the relationship between *Brachylaemus* infections and the presence of exon 3-3 of NLRP1b gene haplotype H3/H2.

	H3/H2 Genotype	Non-H3/H2 Genotype	Total
Brachylaemus	0	1	1
No *Brachylaemus*	5	74	79
Total	5	75	80

Table 85. 2*2 contingency table showing the relationship between *Brachylaemus* infections and the presence of exon 3-3 of NLRP1b gene haplotype H3/H3.

	H3/H3 Genotype	Non-H3/H3 Genotype	Total
Brachylaemus	0	1	1
No *Brachylaemus*	3	76	79
Total	3	77	80

Table 86. 2*2 contingency table showing the relationship between *Brachylaemus* infections and the presence of exon 3-3 of NLRP1b gene haplotype H3/H4.

	H3/H4 Genotype	Non-H3/H4 Genotype	Total
Brachylaemus	0	1	1
No *Brachylaemus*	2	77	79
Total	2	78	80

Table 87. 2*2 contingency table showing the relationship between *Brachylaemus* infections and the presence of exon 3-3 of NLRP1b gene haplotype H4/H2.

	H4/H2 Genotype	Non-H4/H2 Genotype	Total
Brachylaemus	0	1	1
No *Brachylaemus*	1	78	79
Total	1	79	80

Table 88. Investigation of the relationship between *Brachylaemus* infections and the presence of exon 3-3 of NLRP1b gene haplotype H4/H4.

	H4/H4 Genotype	Non-H4/H4 Genotype	Total
Brachylaemus	0	1	1
No *Brachylaemus*	17	62	79
Total	17	63	80