FINE MAPPING OF TRYPANOSOMOSIS RESISTANCE LOCI IN MURINE BY HAPLOTYPE APPROACH

A Thesis submitted to the University of Nairobi, Faculty of Veterinary Medicine; Department of Animal Production in partial fulfillment for the Degree of Master of Science in Animal Science (Animal Breeding and Genetics option)

By

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DECLARATION

I, Kamau, Joseph Muiruri hereby declare that this thesis is my original work and has not been presented for award of a degree in any other university.

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This thesis has been submitted for examination with our approval as university supervisors.

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This thesis is dedicated to my loving parents Priscah Kamau and the late Joseph Kamau

My brothers and sisters for their never ending love, support and encouragement.

And to my daughter Michelle Muiruri and wife Emily Muiruri for their patience, love and care!

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ABSTRACT

Mapping of trypanotolerance QTL in two F2 resource populations generated by crossing BALB/c and A/J with C57BL/6J has previously been achieved by Kemp *et al.*, (1997). The subsequent fine mapping using the advanced intercross line (AIL) found the same to be true in F6 using same strains of mice (Iraqi *et al.*, 2000). To confirm and fine map the QTL, a genome-wide scan for quantitative trait loci (QTLs) affecting trypanotolerance in a population of 300 F2 progeny derived from a cross between 129/J and, C57BL/6J. Two trypanotolerance QTLs (*Tir1 and Tir3*) were detected at the genome-wide 5% or less level. *Tir1* and *Tir3*, located on Chromosomes (Chrs) 17and 1, respectively, had main effects on trypanotolerance. However, *Tir2* on Chr 5 was not detected at the same level and had LOD score bellow 2. Resistant (tolerant) alleles derived from C57BL/6J were responsible for the high trypanotolerance effect. These results indicated that the genetic control of trypanotolerance is complex and the identified QTLs may provide new insights into the pathogenesis of trypanotolerance in mice as well as in livestock and humans.

CHAPTER 1

INTRODUCTION

1.1 Trypanosomosis

Trypanosomosis is a disease of man, domestic livestock as well as wildlife. It is among the many diseases that affect human and animal health and by extension, the pace, direction and nature of livestock-based rural development in much of tropical Africa and South America. The causative organisms of the disease are a number of species of trypanosome, protozoan parasites, which are transmitted between the mammalian hosts, wild as well as domestic, by various species of tsetse flies, which feed only on vertebrate blood. About one-third of Africa is affected by trypanosomosis (WHO, 1986).

Trypanosomosis restricts the keeping of domestic animals, particularly cattle. In turn, this not only affects supplies of meat and milk but also limits the development of mixed arable and livestock farming (WHO, 1979; Murray and Gray, 1984).

The disease is caused by different species of the genus trypanosoma. They include *T. vivax, T. congolense, T. evansi, T. simiae, T. equiperdum, T. godfreyi* and *T. brucei* (Masake *et al.,* 1984). Different sub-species and strains exist and they cause different disease syndromes in different hosts (Myler, 1993). In man, trypanosomosis is caused by *Trypanosoma brucei rhodesiense (T.b. rhodesiense)* and *T.b. gambiense.* There are two forms of human Trypanosomosis, the acute and the chronic form. The acute form caused by *T.b. rhodesiense*, is characterized by severe headache and fever associated with waves of parasitemia. Normally there is severe toxaemia and death ensues in untreated patients. The acute form of the disease is a true zoonosis with the vector (Glossina *morsitans)*

cyclically transmitting the parasite between man and wildgame (Jordan, 1986). The chronic form, caused by *T.b. gambiense*, is characterized by intermittent mild fever, facial edema, enlarged lymph nodes and general emaciation. The vectors for *T.b. gambiense* sleeping sickness are *G. palpalis, G. fuscipes* and *G. tachinoides*.

Animal trypanosomosis is caused by several species of trypanosomes. The disease in cattle, commonly known as Nagana, is caused by *T. congolense, T. vivax* and *T.b. brucei. T. vivax* and *T. congolense* cause the greatest economic loss with respect to reduced meat and milk production (WHO, 1979, 1991). It is difficult to clinically distinguish most infections although acute haemorrhagic infection of *T. vivax* has a distinct syndrome (Jordan, 1986). Many African cattle breeds, although readily infected by *T.b brucei* do not show clinical signs of the disease unlike the exotic breeds which are highly susceptible (Godfrey, 1981). Other domestic animals are normally infected by trypanosomes other than *T. congolense, T. vivax* and *T.b. brucei.* These include *T. simiae* and *T. suis* which are predominantly the causative agents of porcine trypanosomosis (Jordan, 1986). *T. evansi* causes trypanosomosis in camels, horses and donkeys. The disease is commonly known as dourine in horse. Cats and dogs are known also to become infected once they consume an infected goat meat (Moloo *et al.*, 1973).

1.2 Background and Justification of the Project

Genome-wide search (scan) in two murine F_2 populations for quantitative trait loci (QTLs) that influence survival following *T. congolense* infection have been reported (Kemp *et al.*, 1997). Three loci *Tir1*, *Tir2*, and *Tir3*, were identified and mapped to mouse chromosomes (Chrs) 17, 5, and 1 respectively, with confidence intervals (CIs) in the range of 10-40 cM. The larger size of these CIs was to a large degree the consequence of limited number of recombination events in small chromosomal regions in F_2 populations (Kemp *et al.*, 1997).

Subsequent, fine mapping of the three QTL was carried-out by applying an advanced intercross line (AIL) approach (Clapcott, 1998, Iraqi *et al.*, 2000) and the advanced intercross in this case was generation 6 (G6). The reduction in CIs for the *Tir* loci ranged from 2.5 to more than ten-fold in G6 populations by comparison with CIs obtained previously in the equivalent F_2 generations.

However, high resolution mapping of the QTL to a small genomic confidence interval is required for allowing positional cloning of genes underlying the QTL. It is known that most inbred lines of mice derive from fairly small number of recent common ancestral outbred populations. The *consequence* is that the majority of ancestral functional polymorphisms carried by inbred strains should lie within haplotypes that are conserved among strains that share the same polymorphism. Thus, if it can be demonstrated that a number of mouse strains carry the same QTL allele, then, there is a possibility that the

causative mutation will lie within a shared haplotype. Identification of shared haplotypes within the QTL regions will therefore point to the most likely location of the causative gene/s. Therefore in this study, *haplotype mapping* approach will be utilized to achieve very fine localization of the QTL.

The 129/J mouse strain is known phenotypically to be susceptible to *T. congolense* infection (Morrison and Murray, 1979). However, that does not necessarily mean that it carries susceptible alleles at *Tir1*, *Tir2*, and *Tir3* loci.

1.3 Hypothesis

The following hypothesis will be tested;

i. 129/J-mouse strain carries the same susceptible alleles as A/J and BALB/c on chromosome 1, 2, 3, 5, 15 and 17

ii. These susceptible mice strains carry the same QTL allele within a shared haplotypes

1.4 Objectives

To fine map and analyse QTLs responsible for trypanotolerance

1.4.1 Specific objective

i. To confirm if 129/J mouse strain, also carries the susceptible alleles at the *Tir1*, *Tir2* and *Tir3* loci, which were previously mapped in A/J, and BALB/c strains

ii. To confirm whether the three QTLs identified on Chromosomes 2, 3 and 15 in F2 and F6 using FDR approach are present in F2 C57BL/6 x 129J inbred crosses.

iii. To identify any shared haplotypes through Single Nucleotide Polymorphisms (SNPs) genotyping in the three strains (above), which can lead to improved fine mapping of the quantitative trait loci.

CHAPTER 2

Literature review

2.1 Trypanosomosis and livestock production in sub-Saharan Africa

Livestock have had an intimate and formative association with human civilizations in the past and continue to fulfill key agricultural, economic, cultural and religious roles in different societies (Bradley *et al.*, 1996). The livestock sector accounts for over half of agricultural output in developed countries and more than a quarter in developing countries LID (1998). Livestock products include meat, milk, wool, hides and skins. Livestock also play a critical role in providing manure and draught power in mixed crop-and-livestock farming systems. The contribution of livestock to social values, rituals and beliefs of most communities in Africa is also of great importance (Rege and Gibson, 2002).

Sub-Saharan Africa occupies an area of 23 million square kilometers comprising 49 countries. It is one of the most rapidly growing human populations in the world. The human population in this region is currently about 673 million (2003 World population data sheet, www.prb.org) and it is expected to reach about 1300 million by the year 2025. This rapid population increase comprising both expanding rural populations and urban populations is expected to create even greater markets and demand for food including livestock and livestock products. Meeting this demand presents a significant challenge, and unless suitable measures are taken, an increasing shortfall in the supply of meat and milk products is expected.

The sub-humid and the non-forested parts of the humid zones of Sub-Saharan cover approximately nine million square kilometers and comprise more than 40% of sub-Saharan Africa. These areas are characterized by the presence of good-quality grazing land and plenty of water supplies, and therefore hold great potential for significant increases in livestock populations and livestock productivity. Yet the sub-humid zones of sub-Saharan, which constitute 22% of the land, account for only 20% of the ruminant population. On the other hand, the humid zones, which cover 19% of the land, account for only 6% of the ruminant population (d'Ieteren *et al.*, 1998).

Animal diseases, particularly those caused by parasites (such as rinderpest, trypanosomosis, helminthosis and theileriosis) are major impediment to livestock productivity in the humid and sub humid zones of Africa where the disease is endemic, limiting the full development of livestock and crop-based agriculture in these areas. Of the diseases, trypanosomosis is arguably the most important. Approximately 40% of sub-Saharan Africa constitutes the ecological niche of tsetse flies (*Glossina sp.*), the vector of human-sleeping sickness and African trypanosomosis caused by *trypanosoma* spp. In this area, which covers 49 African countries, African animal trypanosomosis has hindered livestock production to such an extent that only approximately 15% of the potential total number of cattle are kept. This represents an annual loss of 1.8 million tones in meat production alone (Hoste, 1987).

If control of trypanosomosis were achieved (FAO, 2002), introduction of livestock production into these areas would be possible leading to a major contribution to the

national incomes and improved living standards of the citizens. Assuming that ruminant stocking densities in areas currently free of trypanosomosis would also apply to areas freed from trypanosomosis, it has been projected that meat supply in these ecozones would increase by approximately 80%, and milk supply by 70%, of current production levels even at the current low rates of production (Jahnke *et al.*, 1988). Such increases would represent 16% and 14% of the current total ruminant meat and milk production in sub-Saharan Africa, respectively (Kristjanson *et al.*, 1999).

2.1 Trypanosomes of clinical importance

Various species of the genus *Trypanosoma* are important from a medical and veterinary point of view (Hoare, 1972). *T. brucei gambiense* and *T brucei rhodesiense* cause African sleeping sickness in humans, while *T. vivax, T. congolense, T. simiae, T. brucei brucei* and *T. suis* affect domestic animals. All these species are transmitted by tsetse flies. *T. cruzi*, which is an intracellular parasite that is transmitted via blood-sucking bugs (*Triatoma*), causes Chaga's disease in humans. *T. cruzi* is found in a number of animals other than humans, including dogs, cats and rodents.

2.2 Clinical manifestations and diagnosis of trypanosomes

Trypanosomosis is a complex disease, involving a wide range of effects. Clinical manifestations of the disease vary considerably depending on the species of infecting trypanosome and on the livestock host species. The pathogenicity of strains (demes) within a species also varies and so do the clinical signs. For instance, individual infections with various isolates of *T. congolense* show considerable variations in

pathogenicity for different mammalian species and for animals of the same species (Stephen, 1970).

Clinical manifestations of trypanosomosis may also be greatly influenced by a number of other factors such as the state of nutrition, the occurrence of intercurrent bacterial, viral and helminth infections and stress due to vaccination regiments, trekking, thirst, and temperature changes. The severity of the challenge, that is, the number of metacyclic trypanosomes introduced into an animal in any unit of time, is also an important factor in character of the resultant infection (Stephen, 1970).

Several types of *Trypanosoma* do exist (Godfrey, 1981). For example, *T. congolense* are primarily confined to the bloodstreams, although they can attach to erythrocytes or endothelial cells that line blood vessels walls. *T. congolense* infections are generally characterized by a moderate fluctuating parasitemia and a progressive acute anemia in susceptible breeds of cattle (Murray and Dexter, 1988). *T. vivax*, however, are able to invade tissues. Certain strains of *T. vivax*, (mainly isolated from Eastern Africa) cause acute disease characterized by high parasitemia, with massive hemorrhage. These isolates of hemorrhagic *T. vivax* can result in death within a few weeks of infection. Other strains of *T. vivax*, mainly isolated from West Africa, are far less pathogenic and produce an anemia that is less severe than observed in *T. congolense* infections.

In the field, diagnosis of trypanosomosis is often based on microscopic examination of wet blood films or thick/thin smear preparation on a slide stained with Giemsa.

Occasionally, examination of fluid aspirated from a superficial lymph node may reveal infection with *T. vivax* more readily than microscopic examination of blood smears. The three characteristics which are used to differentiate the two pathogenic species (*T. Vivax* and congolense) of trypanosomes affecting cattle are motility and morphology (Boyt, 1980).

In the laboratory, diagnosis of trypanosome infection can be performed by inoculation of susceptible laboratory animals such as mice, rats and guinea pigs with blood, lymph or edema fluids from an animal suspected to have an infection (Godfrey and Killick-Kendrick, 1961). This technique is more sensitive than direct microscopic examinations. However, some isolates of *T. vivax, T. simiae* and *T. congolense* do not grow in the laboratory rodents (Leeflang *et al.,* 1976; Gathuo *et al.,* 1987). For diagnosis of trypanosomes infection at low parasitemia, the trypanosomes in the host blood can be concentrated using an anion-exchange column such as diethyl aminoethyl (DEAE)-cellulose to separate the trypanosomes from the cellular elements in the blood (Lanham and Godfrey, 1970). Capillary tube centrifugation coupled with examination of the Buffy coat using phase contrast or dark background illumination has been used in diagnosis of trypanosomosis (Murray *et al.*, 1979a, b).

Immunodiagnostic techniques including indirect haemogglutination (Gill, 1964), the indirect immunoflourescent antibody test (IFAT; Katende *et al.*, 1987) and enzyme linked immunosorbent assays (ELISA) for antigen and antibody (Luckins *et al.*, 1979; Nantulya and Lundqvist, 1989) appear to be useful in determining the prevalence of the

antibody and antigen in epidemiological studies. Other techniques are card agglutination test for trypanosomosis "CATT" (Magnus *et al.*, 1978), enzyme electrophoresis (Godfrey and Kilgour, 1976) and blood incubation infectivity test "BIIT" (Rickman and Robson, 1972). DNA hybridization diagnostic techniques are most useful in identification of the trypanosomes which are difficult to isolate and identify (Patterson and Hyypia, 1985).

2.3 Control of trypanosomosis

Currently, there are three main approaches to the control of trypanosomosis. These are: combating the vector by control or eradication of tsetse fly populations; combating the disease in the mammalian host through chemotherapy and/or chemoprophylaxis and reducing the effects of the disease through the use of trypanotolerant cattle (Jordan, 1976).

2.3.1 Vector control

2.3.1.1 Non-chemical methods

Several non-chemical methods have been used to control tsetse flies, such as biotope modification (destruction of habitats, mass killing of reservoir hosts, etc), traps and targets with or without attractants, and biological control.

When there was no known trypanocide to control trypanosomosis in livestock, biotope modification and reservoir-host eradication (e.g., wildlife) were used to control vectors. Clearing of vegetation was based on partial or total cutting down of trees so as to "replace bush or woodland by treeless grassland." However, the ecological consequences were

disastrous. In Eastern and Southern Africa, between the 1920s and 1960s, about 1.3 million heads of wildlife were destroyed for the purpose of control method, but the detrimental effect of this method on the environment was tremendous (Jordan, 1986).

2.3.1.2 Chemical methods

Chemical methods of control are based on the use of insecticides. The main insecticides used are organochlorines such as DDT (first used during the Second World War against malaria and yellow fever vectors), dieldrin, endosulfan or synthetic pyrethroids such as deltamethrin, flumethrin or cypermethrin.

The above insecticides are used in formulations such as wettable powders, emulsifiable concentrates (which allow dispersion of droplets of less than 10mm in diameter), ultra-low-volume solutions or pour-on applications. The methods by which they are applied include ground spraying, aerial spraying (aircraft or helicopter) and pour-on application on animals.

The main aim is to destroy adult tsetse flies either emerging from the pupae (burrowed in the ground) or on their resting sites or when they feed on animals treated with pour-on applications. This method has been proven effective in eradicating the vector (Jordan, 1986) but the subsequent environmental side effects are not worth the success that is obtained.

2.3.2 Chemotherapy

Chemotherapy is the treatment of disease by the use of chemical drugs. Such drugs are *curative*. They disrupt or block one or more of the vital processes, which are essential to the invading micro-organism. Certain compounds have specific effects on some enzyme system or block essential metabolic pathways, but the exact way in which they work is often not known or only incompletely understood, and this is true of most of the *trypanocides* (chemotherapeutic drugs which kill trypanosomes or inhibit their development).

However, different trypanosomes have developed resistance to different trypanocidal drugs thus rendering the whole process ineffective (Leach and Roberts, 1981; Ainanshe *et al.*, 1992; Mugunieri G.L and Murilla G.A, 2003).

2.3.3 Trypanotolerant breeds

The alternative method is the use of trypanotolerant breed(s). Trypanotolerance, the ability of some livestock species and breeds to survive, reproduce and remain productive under trypanosome risk without the aid of trypanocidal drugs (Palling *et al.*, 1991a), was recognized and exploited by farmers long before research on trypanotolerance began. Trypanotolerance in cattle is well documented, particularly in N'Dama cattle, the most numerous trypanotolerant breeds, and in the West African shorthorn (Murray *et al.*, 1982; Soller and Beckmann, 1987). While significant differences in resistance to trypanosomes also occur among various zebu (Bos indicus) types (Njogu *et al.*, 1985), most *Bos indicus* cattle in tsetse fly-infested areas still require regular treatment or are found only on the

fringes of fly belts. However, exotic breeds cannot be maintained even in areas of low tsetse fly risk without intensive trypanocidal drug therapy and veterinary care. But there is a glimmer of hope –with the traditional African cattle breeds such as N'Dama that have evolved natural resistance to trypanosomosis and other diseases to which they have been exposed on evolutionary timescales, and are as productive as some of the best hybrid breeds (Paris, 1988).

N'Dama is the most numerous and most extensively studied trypanotolerant cattle. Trypanotolerant cattle are descendants of the earliest domesticated cattle breeds in Africa and have the longest history of selection by trypanosomosis, having been in Africa for between 5,000 to 7,000 years (Murray and Trail, 1984; Hanotte *et al.*, 2002). Consequently, they are able to withstand levels of trypanosome challenge which prove fatal to other cattle types (Palling *et al.*, 1991a, b). Trypanotolerance is attributed to a superior ability to control parasitemia and anemia following trypanosome infection. In addition to their resistance to trypanosomosis, trypanotolerant cattle, and the N'Dama in particular, have been reported to be resistant to other infectious diseases such as dermatophilosis (Stewart, 1937), heartwater, anaplasmosis and babesiosis (Oduye and Okumaiya, 1971). The success of introducing N'Dama cattle in the most humid parts of West and Central Africa is recognized to be equally attributed to their resistance to trypanosomosis and dermatophilosis (d'Ieteren *et al.*, 1998). N'Dama has lower tick burdens when compared to zebu cattle (Claxton and Leperre, 1991; *Mattioli et al.*, 1993; Mattioli and Casama, 1995; Mattioli, *et al.*, 1995) as well as lower prevalence of

strongyle infections (Mattioli, *et al.*, 1992). These genetic advantages contribute to their potential use in livestock development programs in the tropics.

The exploitation of trypanotolerant breeds is practiced as a major (if not the only) option for sustainable livestock production in 19 countries in the most humid parts of West and Central Africa. But, in spite of these successful attempts at encouraging their use, the farming of trypanotolerant livestock has not been widely adopted for various reasons. First, the resistant animals are few in number, accounting for only a third of the cattle in the tsetse-infested areas and are concentrated in West Africa. The low numbers of trypanotolerant animals that are available for stocking limits the rate at which such cattle could be introduced into other tsetse-infested areas. Secondly, it is perceived that trypanotolerant livestock do not combine disease resistance with other important economic traits desired by farmers, such as large body size (suitable for traction), high fertility and fecundity, high milk yield and early carcass maturity. In the absence of disease challenge, trypanotolerant cattle are thought to be less productive than exotic breeds (such as Boran and European breeds). However, under conditions of heavy trypanosome challenge, various studies have demonstrated their superior productivity advantage, compared to Boran cattle, which are preferred beef breed (Trail, *et al.*, 1985; Feron, *et al.*, 1988; Agyemang, *et al.*, 1994).

Using such animals to work the land and for milk and meat production, is an environmentally friendly and affordable approach to controlling the effects of trypanosomosis in Africa. Researchers are beginning to realize that Africa's unparalleled

genetic pool offers possibilities of exploiting disease resistant traits already present in cattle through mapping of quantitative trait (QTL) and selective breeding programs. Recently Hanotte, *et al.*, (2002) have reported the first results of mapping trypanotolerance QTL in N'Dama cattle breed. 10 major trypanotolerance QTLs were identified.

2.4 Trypanotolerance in mice

Mice are valuable animal model organisms. Their use is enhanced by the ready availability of many inbred lines, which use less space than the target livestock animals because of their smaller sizes. The mouse genome is already mapped, sequenced and characterized, and the production of large numbers of progeny in a short period of time, eases genetic experimentation. All commercially available mouse strains have accurate and detailed pedigrees and cost less than cattle to acquire and maintain. Because of the above advantages, mice are well suited to genome analysis (Moore and Nagle, 2000). The identification and characterization of resistance genes in mice could help elucidate basic mechanisms for resistance in livestock through comparative studies.

The mouse has become the premier mammalian system for the identification of the genetic basis of both mono- and oligogenic disorders, as well as the understanding of complex diseases with gene-gene and gene-environment interactions. The similarity between human and mouse genetic disease is sometimes striking, while in other cases the phenotypes are less similar. The ability to genetically map and then rapidly clone single gene disorders and the emerging technologies that will allow the economical

identification of the polygenes controlling quantitative traits further demonstrate the utility of the mouse as a model for gene discovery. Additionally, the ability to genetically manipulate the mouse through transgenesis and gene targeting allows for the testing of hypotheses regarding specific gene function and their roles in disease (West *et al.*, 2000). The utility of the mouse extends beyond being just a gene discovery tool to provide prevalidated targets. It can also be used for the development of animal models, and the testing of compounds in specifically constructed transgenic and knockout strains to further define the target and pathway of a therapeutic compound.

Considerable understanding of the relationship between susceptibility, parasite growth and the immune response has been provided by the work of Black *et al.*, 1983 (reviewed in 1985) on the mouse model. Mice vary in their susceptibility to *T. brucei* (Black *et al.*, 1983), to *T. congolense* (Morrison *et al.*, 1978) and to *T. vivax* (Mahan *et al.*, 1986). It has been found that the more resistant strains of mice such as the C57BL/6J are better able to control parasitemia and produce a superior antibody response.

However, there, would appear to be more important differences in the role played by the antibody responses in controlling *T. brucei, T. congolense* and *T. vivax* parasitemia. Antibody responses to *T. brucei* (Sendashoga and Black, 1982; Black *et al.*, 1983) and *T. congolense* (Mitchell and Pearson, 1986; Whitelaw *et al.*, 1983) parasitemia occur after most organisms have differentiated to non-dividing forms. In contrast, antibody control of parasitemia in C57BL/6J mice infected with *T. vivax* occurred during the exponential phase of parasite population increase in the blood (Mahan *et al.*, 1986). Thus, the

capacity to control *T. brucei* and *T. congolense* parasitemia appears to lie in the ability to regulate parasite growth followed by the induction of the immune response, while in *T. vivax* infections parasitemia appears to be directly dependent on the ability to mount an immune response.

Another aspect of the inability of susceptible mice to control parasitemia results from an impaired capacity of parasite-induced antibody-containing cells to secrete immunoglobulin. Such cells regained the ability to secrete antibody within 24 hours after trypanosome elimination by treatment with trypanocidal drug (Black *et al.*, 1986; Mahan *et al.*, 1986), suggesting that the block in antibody secretion was maintained by living parasites or short-lived components of degenerating parasites. Table1 vividly shows different responses to Trypanosomosis on inbred mouse strains (Morrison *et al.*, 1978).

TABLE 1: Survival of inbred strains of mice infected with *T. congolense*

Mouse strain	% deaths (days) post infection							Mean. survival
	15	20	40	60	80	120	140	time (days)
A/J	67.8	85.7	100					15.8
SWR/J	42.0	88.0	100					16.9
129/J	36.6	58.5	90.2	100				22.6
BALB/CJ	15.5	20.0	28.8	64.4	100			49.5
DBA/1J	0	0	62.2	100				36.3
C3H/HeJ	0	0	12.5	50.0	100			59.0
AKR/A	0	0	7.9	13.6	45.4	97.7	100	81.7
C57B1/6J	0	0	2.8	5.5	13.9	61.1	94.4	110.2

Source: Morrison *et al.*, (1978)

Performing a series of crosses with these strains may reveal the identity of the genes underlying the phenotypic differences. These inbred lines of mice show marked difference in survival time following infection with *trypanosoma congolense*. C57BL/6J mouse strain shows a higher degree of resistance to *T. congolense* than A/J or BALB/c mouse strains (Morrison *et al.*, 1978: Jennings *et al.*, 1978). Understanding the genetics and pathophysiology behind trypanotolerance would be a great step towards understanding diseases both in human and domestic animals.

2.5 Breeding of genetically disease resistance livestock

The potential value of the trypanotolerance trait for livestock production in tsetse-infested areas has made it the subject of considerable study since it was first described (d'Ieteran, *et al.*, 1998). The degree of trypanotolerance varies both between and within trypanotolerant breeds. For breeding strategies, the analysis of phenotypic variation and uncovering the contribution of genetic factors is very important. Greater exploitation of

genetic resistance coupled with improving production potential of trypanotolerant livestock would provide a robust and low input control method with considerable advantages over other options in terms of sustainability, cost efficacy and environmental considerations.

Exploitation of such genes would be greatly facilitated by marker-assisted selection (MAS) for trypanotolerance, an approach which requires identification of genetic markers closely linked to trypanotolerance. Such markers would subsequently be used as a selection tools for within-breed improvement of trypanotolerance and for cross-breeding through marker-assisted introgression (MAI) programs aimed at integrating disease resistance with productivity traits that are important to farmers in tsetse-infested parts of the tropics.

2.6 QTL mapping

Identification of genes underlying complex traits has been aided by quantitative trait locus (QTL) mapping approaches. The discovery of genes underlying multigenic diseases and traits is one of the most important challenges currently facing geneticists (Wayne and McIntyre 2002; Kriste *et al.*, 2002). A QTL is a chromosomal region that contains a gene or genes that influence a quantitative trait. The idea of QTL mapping coined by Thoday (1961) simply relates genetic markers scattered throughout the genome of an organism of interest to a given trait. Segregation of these markers is used to detect and estimate the effects of linked QTLs (Lander *et al.*, 1987). In this case information from phenotypes

and marker genotypes is utilized to pinpoint chromosomal areas that explain a substantial amount of genetic variation.

The premise for QTL mapping is that chromosomal markers will tend to segregate with loci associated with the trait of interest. The first step towards detecting genes affecting quantitative traits is the creation of a genetic (or linkage) map which describes the relative positions of polymorphic genetic markers across the genome. Genetic maps provide the representation of chromosome on which the markers and QTL reside, and therefore supply the structure in which to search for QTL. For QTL mapping within individual populations, the population's structures include daughter, granddaughter and full-sib family designs (Weller *et al.*, 1990; Knott and Halley, 1992; Forrest and Feingold, 2000). For QTL mapping of crosses between populations, these include F2 and backcross (BC) populations between inbred or near-inbred lines (Soller *et al.*, 1976) and between lines that share the same segregating marker alleles, but are supposedly at or close to fixation for alternative alleles at the QTL that affect the trait of interest (Beckmann and Soller, 1988).

Earlier work by Kemp *et al.* (1997), Iraqi *et al.* 2000 and Hanotte *et al.* (2002) has shown that, there is clear evidence that trypanotolerance is partly under genetic control, and heritable (Murray *et al.*, 1984); continuously distributed and is also influenced by environmental factors (Wissocq *et al.*, 1993; Agyemang *et al.*, 1994, Trail *et al.*, 1994). Traits which show continuous variation in a population are referred to as complex traits or quantitative traits. Continuous phenotypic variation reflects the influence of many

genes (Falconer and MacKay, 1996) each with a small effect, affecting the quantitative trait and a limited number of loci with a large effect (Shrimpton and Robertson, 1988). These genes or loci can provide insights into questions about the genetic architecture of complex traits, such as the number of loci potentially affecting the trait, the distribution of gene effects and the underlying patterns of gene action, including additivity, dominance, sex-specificity, epistasis and pleiotropy. QTLs are identified via statistical procedures that integrate genotypic and phenotypic data. They are assigned to chromosome locations based on the position of markers on a linkage map and are located to regions of the genome at specified levels of statistical probability following linkage analysis.

2.7 Microsatellite Markers

Microsatellite markers consist of di-, tri-, or tetra-nucleotides repeated up to 40 times. Oligonucleotides are synthesized to nucleotide sequences that flank these repeats and the sequences can be used in PCR, to amplify loci from genomic DNA (Holmes *et al.*, 1993; Faber and Medrano, 2003). Microsatellites are now being used as genetic markers for linkage analysis as they can be highly polymorphic and usually behave as single locus markers (Weber, 1990). Previously microsatellites were used for genetic mapping and have also been used extensively for linkage analysis to find out association with economic traits that includes disease susceptibility/resistance genes.

2.8 Polymerase Chain Reaction

PCR is an *in vitro* method for the enzymatic synthesis of specific DNA sequences, using oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA (Mullis and Faloona, 1987). A repetitive series of cycles involving template denaturation, primer annealing and the extension of the annealed primers by the DNA polymerase results to exponential accumulation of a specific fragment whose termini are defined by the 5′ end of the primers.

During the denaturation, usually at 95°C the double strand melts and opens to single stranded DNA, all enzymatic reactions stop. At annealing temperature, which is dependent on the specific pair of primers; hydrogen bonds are constantly formed and broken between the single stranded primer and the single stranded template. Extension occurs at 72°C. DNA fragments matching both primers are amplified exponentially, rather than linearly as at each cycle the number of fragments doubles. 72°C is the ideal (optimum) working temperature for the polymerase.

Since the primer extension products synthesized approximately double at each cycle, a relatively small number of cycles are needed to obtain enough DNA, typically between 25 and 35 cycles, for allele size separation on polyacrylamide or capillary gels.

2.11 Value of comparative mapping

Availability of DNA sequences from various animal species has made it possible for one to compare the similarities or differences of one species DNA sequence or genes present. Bioinformatics has been used as a tool to establish similarities or differences where conservation of gene order in mammals is considerable (Montgomery *et al.*, 1993; Basset *et al.*, 1995).

Establishing a gene order in one species is made easier by application of bioinformatics tool. Domestic livestock are being intensively studied in order to map traits of economic value. Cattle, sheep and pig are the major targets.

Comparative mapping has been applied in various species where species genome is complete. For example, mouse Genome Informatics (MGI) is a comprehensive repository for information on the biology and genetics of the laboratory mouse. MGI contains homology information for mouse, human and over 60 other mammalian species. The completion of human, mouse and now the bovine genome sequences provides a valuable resource for decoding other mammalian genomes. With a match to either of the genome sequences, the approximate location of a query sequences can be predicted to the species of interest (Liu *et al.*, 2004)

2.12 Progress in QTL mapping affecting Trypanotolerance

Trypanosomosis has been studied in some detail in mice and cattle, however the systemic and non-specific nature of the infection has made it difficult to establish which particular cell or tissue types are responsible for disease control of trypanosomosis infection. Although the parasite can be maintained in culture, there is no satisfactory *in vitro* model of the response to trypanosomosis infection.

While most breeds of cattle are highly susceptible to trypanosomosis, it has been known for many years that certain breeds show a remarkable resistance to infection. This has been termed 'trypanotolerance' because the host tolerates the presence of parasites while apparently controlling levels of parasitemia and, critically, without showing the severe anemia and production loss characteristic of infection in susceptible hosts (Kemp and Teale, 1998).

Following observation in breeds of cattle in response to trypanosomosis, a unique F_2 resource population derived from trypanotolerant N'Dama bulls crossed with susceptible Boran cows was developed at ILRI for mapping the quantitative trait loci (QTL) controlling trypanotolerance (Kemp and Teale, 1991; Kennedy., 1992). Analysis revealed 9 QTL (Hanotte *et al.*, 2002).

Tolerance to trypanosomosis is not only found in breeds of cattle, but it is also well known that there is a range of response to trypanosome infection in inbred strains of mice (Morrison *et al.*, 1978: Kemp and Teale, 1998). Research on a mouse model at ILRI has

lead to mapping (Kemp *et al.*, 1997) and subsequently fine mapping (Iraqi *et al.*, 2000) of QTL in mice that account for most of the variance in survival time following trypanosome challenge. The major resistance QTL - *Tir1* - has been located to a 95% confidence interval of 0.9cM in two independent crosses (Iraqi *et al.*, 2000)

Marker assisted introgression (MAI) in a mouse model has fixed combinations of the resistant alleles of the murine QTL onto susceptible backgrounds, phenotyping these mice has in every case, confirmed the location and effect of the major QTL (Koudande *et al.*, 2005). Recent developments in both human and murine genome sequencing, and more recently the bovine genome has now provided access to a virtually complete set of full-length non-redundant clones that span the mouse *Tir* loci.

Comparative mapping has been completed between three of the cattle QTL and the three trypanotolerance QTL located in mice (Kanga, 2002). To date one very small region of homology (0.2 cM) has been located between the cattle QTL on Bta 7 and the mouse QTL on Mmu 17, which provides good prospects for eventually identifying the common gene involved (Teale *et al.*, 1999).

2.13 Single nucleotide polymorphisms (SNP)

The most common type of variation in the human genome is the Single nucleotide polymorphism (SNP), where a single base differs between individuals. The combination of QTL mapping and SNP analysis is an effective approach for identifying candidate genes (Moreno-Vazquez *et al.*, 2003).

SNPs occur about once every 1000 base pairs in the genome, making up the bulk of the 3 million variations found in the genome, and the frequency of a particular polymorphism tends to remain stable in the population, importantly, physically linked SNPs are co-inherited as a series of alleles in pattern known as a haplotype.

Unlike the other, rarer kinds of variations, many SNPs occur in genes and in the surrounding regions of the genome that control their expression. The effect of a single SNP on a gene may not be that common as such – perhaps influencing the activity of the encoded protein in *a subtle* way – but even subtle effects can result in susceptibility to common diseases (Wiltshire *et al.*, 2003). The nature and organization of polymorphisms or differences between genomes of individuals are of great interest, because these variations can be associated with or even underlie phenotypic traits under study. Simple sequence polymorphisms (SSLPs, dinucleotide repeats) show similar distributions for QTL analysis as well as positional cloning of monogenic loci.

CHAPTER 3

MATERIALS AND METHODS

3.1 General methods

The procedures presented in the general methods i.e. Preparation of samples for loading and extraction of high molecular DNA, were used as standard unless otherwise stated.

3.2 The trypanosome

The trypanosome used for this study was *T. congolense* clone 1L1180. It was stored in liquid nitrogen. When the clone was required for use it was propagated in A/J mice and prepared for challenge.

3.3 Mice

Two different strains of mice were used. These are 129/J and C57BL/6J strains. The 129/J mouse strain is known and previously was confirmed by our group at ILRI (unpublished results) to be susceptible to *T. congolense* infection. C57BL/6 is known to be relatively resistant to *T. congolense* infection. The two mouse strains are inbred lines and thus they are entirely homozygous. 129/J mouse strain was obtained from Jackson Laboratory and maintained at ILRI small animal for five years before use in this experiment, while C57BL/6J was obtained from Harlan UK (Bicester, Oxon, UK). The mice were fed on the same diet.

3.4 Experimental design

120 mice of F_1 129/J x C57BL/6J generated by reciprocal crossing of 20 pairs (10 male and 10 females) of each 129/J mouse strain with 20 pairs (10 males and 10 females) of

C57BL/6J strain, Table 1 shows how reciprocal crossing was done. The 60 breeding pairs of F_1 were intercrossed in order to generate 300- F_2 129/J x C57BL/6J resource populations. These resource populations as well as 40 mice of each parental line were challenged with *T. congolense* at the age of 12 weeks. The data of the control animals was used for quality control of the infection as well as for determining the phenotypic variance between the parental strains.

Table1: Reciprocal crossing used in this experiment

Groups	Sire of Sire	Dam of Sire	Dam of Dam	Sire of Dam	Animals
A	129/J	C57BL/6	C57BL/6	129/J	71
B	C57BL/6	129/J	129/J	C57BL/6	67
C	129/J	C57BL/6	129/J	C57BL/6	72
D	C57BL/6	129/J	C57BL/6	129/J	72

3.5 Trypanosomosis challenge

The F_2 129/J x C57BL/6J resource population together with the control parental mouse strain mice were challenged at 12 weeks of age by intraperitoneal inoculation of 10^4 blood stream forms of *T. congolense* clone IL1180 (Masake *et al*, 1983 ; Nantulya *et al*, 1984). In the following 14 days, blood sample were collected daily from the tail tip of all challenged mice and examined for evidence of infection by parasitemia observation by microscope.

3.6 Phenotyping

Phenotypic data was defined as survival time in days following the day of challenge (day 0). The first group to succumb were taken as the most susceptible (S), while the last one

to succumb to infection were presumed to be resistant(R), and those ones in between were taken as the intermediate group (I). The mouse that was not parasitemic was excluded from further analysis.

3.7 Genomic DNA extraction

Following the challenge and monitoring, high-molecular-weight genomic DNA from the phenotypic extremes of F_2 129/J x C57BL/6J and the parental strain mice was prepared from tails by a urea lysate method. The method used is as follows:

Chop 1-1.5 cm of mouse tail in tinny pieces into a 2ml Eppendorf tube.

Add 0.8ml of 8M Urea pH7.6, mix gently

Incubate at 37°c overnight in a water bath

Pipette out the 0.8ml lysate into a new tube using a large bore pipette tip

Add an equal volume of Phenol: Chlorform: Isoamyl alcohol (25:24:1) into the lysate

Mix gently on wing rollers for 30 min

Spin in microfuge 12000rpm for 2 min at RT

Recover the clear supernatant into a new Eppendorf tube

Repeat step 5-8

Add an equal volume of Chlorform only and repeat step 6-8

Add $1/10^{th}$ volume 3M Sodium Acetate pH 5.2, followed by 2 volumes of ice-cold absolute Ethanol

Mix by gentle inversion over 1 min and leave to precipitate at RT for 1 hour

Aspirate the supernatant gently and discard

Add 1 ml of 70% Ethanol to the pellet, mix gently for 10 min on wing roller

Spin in microfuge at 12000rpm 2 min at RT and discard the supernatant

Dry the pellet under vacuum for ~5 min. DO NOT OVER DRY

Resuspend the pellet in 200µl T.E. with RNAase dissolved

3.8 Simple sequence length polymorphism (SSLP) loci studied

Thirty six (39) autosomal microsatellite loci were used. They included markers in the six chromosomes, namely chromosomes 1, 2, 3, 5, 15, and 17. The appendix **2** gives the name of loci studied, the MGD position as it is in the mouse genome informatics and their primer sequences.

3.9 Polymerase chain reaction (PCR) amplification

PCR amplification was done using a 10ng DNA template in a 10µl PCR reaction volume. The 10µl reaction volume contained 1µl template DNA, 5µl of ABgene and 0.2µl (40pmol/µl) of both forward and reverse primers scaled up to 10µl by adding double distilled water to final volume.

Fluorescent phosphoramidites dyes were used to label the above primers namely, Fam (blue), Vic (green), Ned (yellow) and Pet (red) These dyes emit fluorescence when excited by a laser, and facilitate detection and quantification of the resulting fluorescent DNA fragments by the ABI prism 3730 DNA analyzer. The PCR product was diluted (1:40) with triple distilled de-ionized water. 1 ml aliquot of the diluent was mixed with 9 ml of the LIZ standard/formamide (1:100) by gentle pipetting. Fragment analysis was

then done using automated capillary ABI 3730 DNA sequencers and analysed using the GENEMAPPER™ software.

3.9 Genotyping

A selective genotyping approach was used in this experiment. This is the genotyping of only individuals, which are extreme for the quantitative trait (i.e. from the high and the low tails of the trait distribution) for detection of linkage between marker and QTL (Korol *et al.*, 1996). In this way the number of individuals that need to be genotyped is reduced (Darvasi and Soller, 1992; Ronin *et al.*, 2003; Darvasi, 1997). Depending on the design, selective genotyping can be based on phenotypic observations or estimated breeding values (Soller *et al.*, 1976; Weller *et al.*, 1990; Chatziplis and Haley, 2000 Bovenhuis and Spelman, 2000; Darvasi 1997).

DNA extracted was amplified through PCR using specific primers according to the suppliers' recommendation. Informative DNA markers between C57BL/6J and 129/J mouse strains located within the six previously mapped (trypanotolerance) QTL intervals were used in genotyping. Screening using a number of microsatellite DNA markers was carried on each QTL to determine the informative markers. Genotyping was initially restricted to mice representing the phenotypic extremes (10-20% either side). Thus, the first to succumb to infection, the last mice to succumb and together with the control were genotyped.

3.10 DNA fragment analysis (Size-Calling) and Genescan™ analysis

3rd-order least square method was used. The least square method uses regression analysis to build a best-fit size calling curve. This curve compensates for any fragments that may run anomalously. Consequently, this method results in the least amount of deviation for all the fragments, including the size standards and the samples.

The Genescan™ analysis software version 3.1.2 was used to size the resultant DNA fragments following electrophoresis. The software operates with the Perkin Elmer ABI Prism™ 3730 DNA sequencer to automatically size and quantify DNA fragments by automated fluorescence detection, allowing more accurate and faster analysis than traditional methods.

3.11 Linkage analysis and QTL mapping

Genotype frequencies in resistant and susceptible groups of mice were checked against Hard-Weinberg equilibrium (HWE) (Deng *et al.*, 2000, 2003; Deng and Chen, 2000). Where HWE states that, in a large population with random mating and no selection, mutation, or migration, the gene frequencies and the genotype are constant from generation to generation; in this study, there was selective genotyping and the law was not obeyed.

Multipoint analysis was performed with MAPMAKER/EXP version 3.0 (Lincoln *et al.*, 1992a), and map distances were calculated with the Haldane function. QTL interval mapping analysis were performed with the maximum likelihood (ML) approach of

MAPMAKER/QTL version 1.1 (Lincoln *et al.*, 1992b) and with the least Square (LS) approach of QTL express (Seaton *et al.*, 2002). Mapmaker assumes the existence of a QTL and estimates its effect on the phenotype in individuals carrying the three possible genotypes at the QTL. The significant LOD score in Mapmaker/QTL was defined as the interval above the two LOD scores; however in QTL express permutation test was run 1000 times randomly across the data and significant F value (LOD score) was defined by the software. QTL express was used with marker orders and distances from the sequenced mouse genome (Waterston *et al.*, 2002). The markers that were used were first tested for linkage analysis as described by Lincoln and Lander (1992). The QTL position and significance was confirmed by maximum likelihood estimation method using Mapmaker/QTL programs by incorporating marker order (Lander *et al.*, 1987).

3.12 Single Nucleotide Polymorphism (SNP) haplotype analysis

The murine trypanotolerance QTL have been mapped in two separate crosses; A/J x C57BL/6J and BALB/c x C57BL/6J. The susceptible A/J and BALB/c strains are known to share a common ancestral population, making it highly likely that they share the same mutation at the QTL. The recent *in silico* mapping of QTL loci by regressing phenotype on marker haplotype across inbred mouse lines (Grupe *et al.*, 1901) shows that most mutations underlying QTL in mouse strains have a common ancestor. It is therefore possible to fine-map QTL by identifying regions of shared haplotype in inbred lines of known QTL status (Grupe *et al.*, 1901). We therefore undertook rapid F_2 QTL mapping of susceptible 129/J to determine its QTL allele status. This was possible with small numbers of markers and mice because *de novo* mapping was not required. Genes within

such regions for which susceptible strains share confirmed haplotypes which becomes strong positional candidates.

In a separate study C3H/J mouse strain was shown to carry the susceptible alleles of trypanotolerance on chromosomes 1 and 17 (Amwayi, 2006). The mapped QTL region in five different mouse strains (i.e. 129/J, BALB/c, A/J, C3H/J and C57BL/6J) was aligned to the sequenced mouse genome to identify the shared single nucleotide polymorphism (SNP) for the purpose of fine mapping and possible candidate gene(s) identification.

The mouse SNP database http://mousesnp.roche.com/cgi-bin/msnp_public.pl and the Jackson Laboratory database http://aretha.jax.org/pub-cgi/phenome/mpdcgi?rtn-docs/home was screened for SNPs within each QTL for total SNPs available, and then filtered to keep only the SNPs for 129J, A/J, BALB/c, C3H and C57BL/6 as indicated in appendix 3.

CHAPTER 4

RESULTS

4.1 Phenotyping

The survival time of the F2 129J x C57BL/6 population and the parental lines are presented in Figure 1. Fifty percent (50%) of the two parental strains 129/J and the C57BL/6J population died by day 140 post *T. congolense* infection. The A/J strain, which is known to be most susceptible to trypanosomosis, had all died by day 100 post challenge. The mean survival times were 60, 79 and 82 for A/J, 129/J and C57BL/6J, respectively. The F2 population had a higher survival time of 140 days comparing with the parental lines. The F2 group showed higher survival rate than the resistant parental C57BL/6 mouse strain. A high proportion of the susceptible group (F2) were found to be more resistant than the parental 129/J mouse strain, while A/J mouse - the most susceptible of all the mice strains had the lowest survival mean of 60 days.

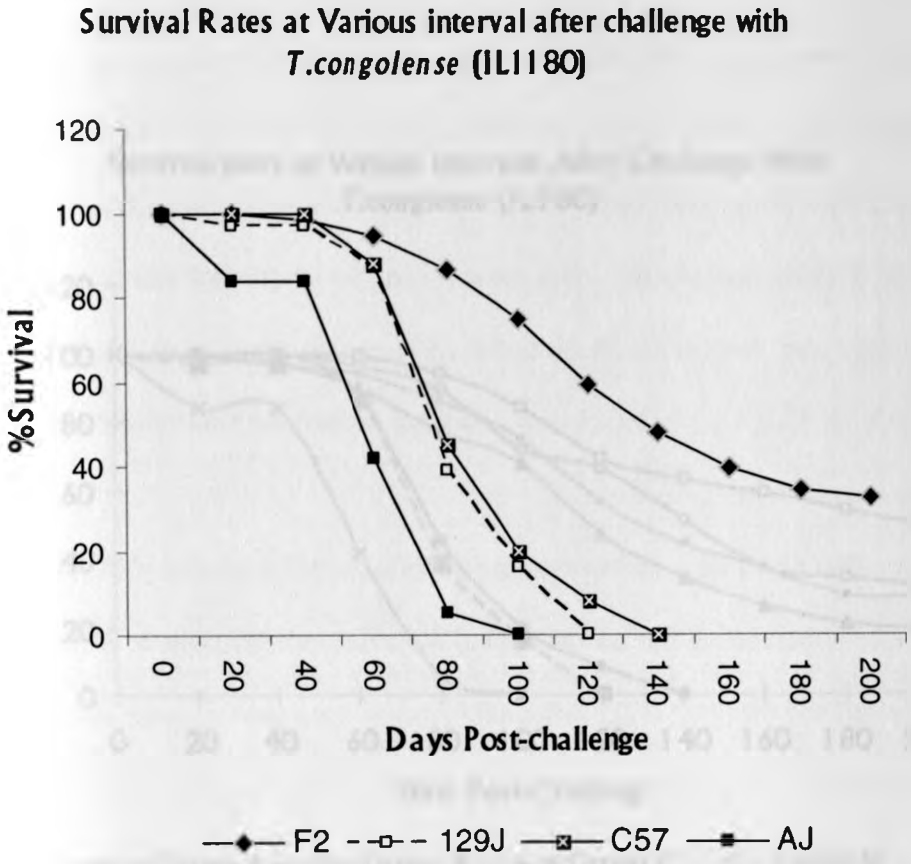

Figure 1: Survival rates (%) at various intervals after challenge with *T. congolense* (IL1180).

The F2 population was further divided into four different groups as indicated in Figure 2. These groups were defined based on reciprocal crossing of the original founders in producing F1 and subsequent F2 populations as shown in Table1. In these groups, group B had the highest mean survival time of ~ 188.7days, while group C had the lowest of ~ 132.6days. Groups A and D had mean survival time of ~ 150 and 140 days respectively as indicated on Figure 2.

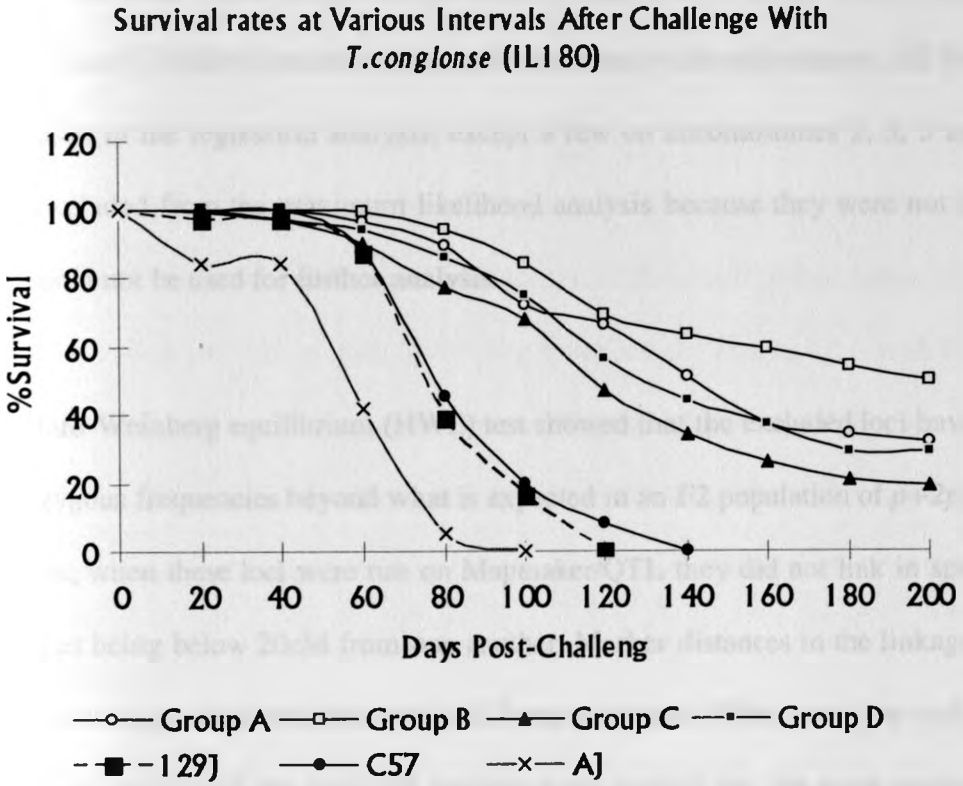

Figure 2: Survival rates (%) of various breeding groups at various intervals after challenge with *T. congolense* (IL1180).

4.1 Genotyping

A total of 39 microsatellite loci (Appendix 1) selected in chromosomes 1, 2, 3, 5, 15 and 17 from the MGI within the previously mapped regions were screened for polymorphism in 129/J and C57BL/6J mouse strains and were found to be informative. All the markers were used in the regression analysis, except a few on chromosomes 2, 3, 5 and 17 that were excluded from the maximum likelihood analysis because they were not linked and thus could not be used for further analysis.

The Hard-Weinberg equilibrium (HWE) test showed that the excluded loci having excess homozygous frequencies beyond what is expected in an F2 population of $p+2pq+q=1$. In addition, when these loci were run on Mapmaker/QTL they did not link in spite of their distances being below 20cM from one another. Marker distances in the linkage mapping were on average about four times the MGI map distances. When genotype and the allelic frequency analysis of the excluded markers were carried out, the same markers having excess homozygousity were picked out to be problematic. Likewise the marker order and distances were found to be inconsistent between the MGI and ENSEMBL v36.

4.2 Linkage analysis

Initially, analysis of the data to locate QTL was done using Least squares interval mapping (Haley and Knott, 1992) with threshold for statistical significance determined by permutation test for each chromosome separately and confidence interval for QTL location were obtained by bootstrapping (Vischer *et al.*, 1996). This data consisted of 138 genotyped mice. Maximum likelihood interval mapping methods was later used, which

gave equally informative and similar QTL results. The QTL analysis revealed presence of loci that influence survival of mice under trypanosome challenge on chromosome 1 and 17 (Figures 3a and b), which was consistent with earlier reports by Kemp *et al.*, (1997). The Bootstrapping experimental specific threshold for QTL express was used to estimate the Confidence Interval. The threshold obtained with least square method was comparable to the two LOD score level of significance assigned to the mapmaker results. The statistical test for the QTL showed evidence of *Tir1* and *Tir3* on previously mapped region as reported by Kemp *et al.* (1997). *Tir*3 was resolved into a QTL with LOD scores of 3.026 (Figure 3a) *and* was located between 70 and 90cM on the MGI scale within 95% CI of 20cM. *Tir*1 was resolved into a QTL with LOD scores 2.58 (Figure 3b). This QTL was located between 20 and 35cM on MGI scale within 95% CI of 15cM (Table 2).

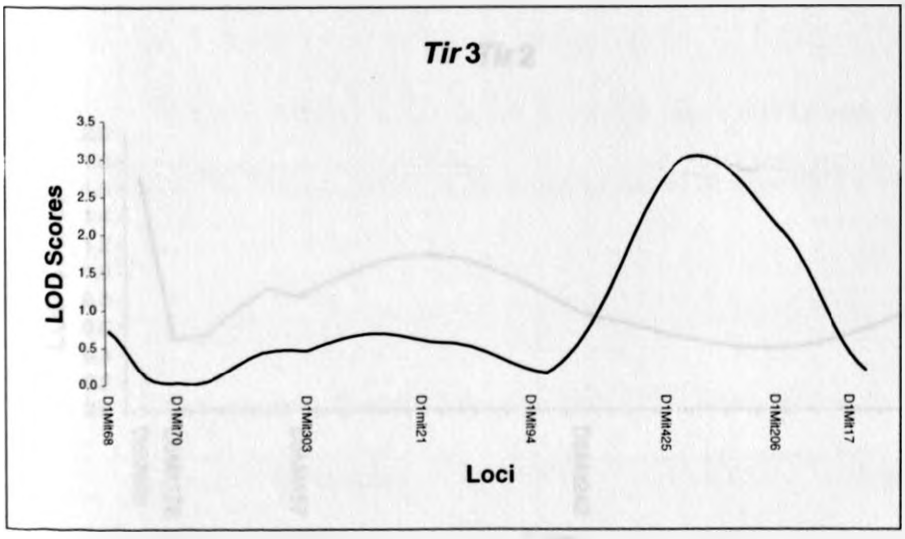

Figure 3a: Chr 1 (*Tir3*) QTL mapping using 129/J x C57BL/6J cross

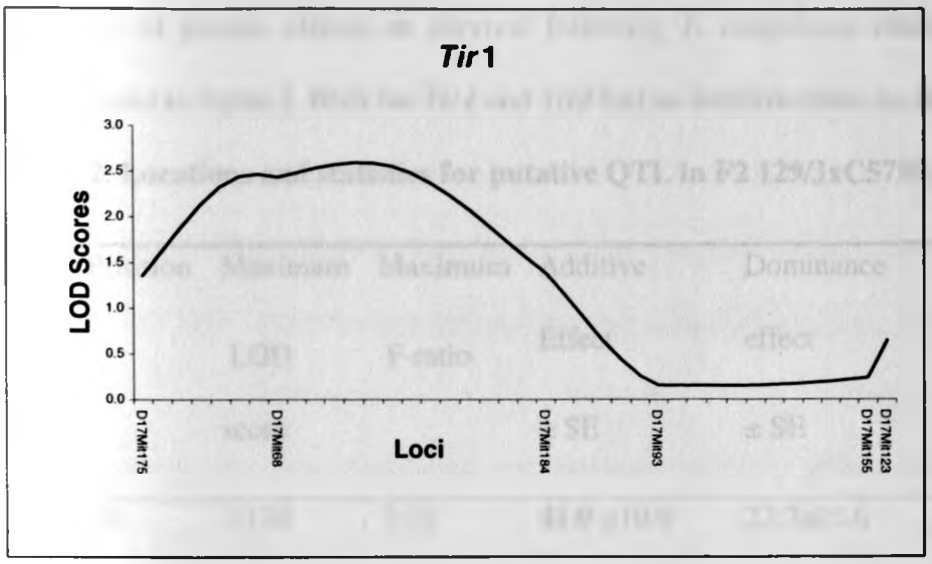

Figure 3b: Chr 17 (*Tir1*) QTL mapping using 129/J x C57BL/6J cross

Chromosome 5 (*Tir2*) did not show any significant QTL (Figure 3c). The LOD score was below the threshold of 2, though this QTL had been confirmed to exist in previous work done by Kemp *et al.* (1997) on A/J, BALB/c x C57BL/6 mouse strains.

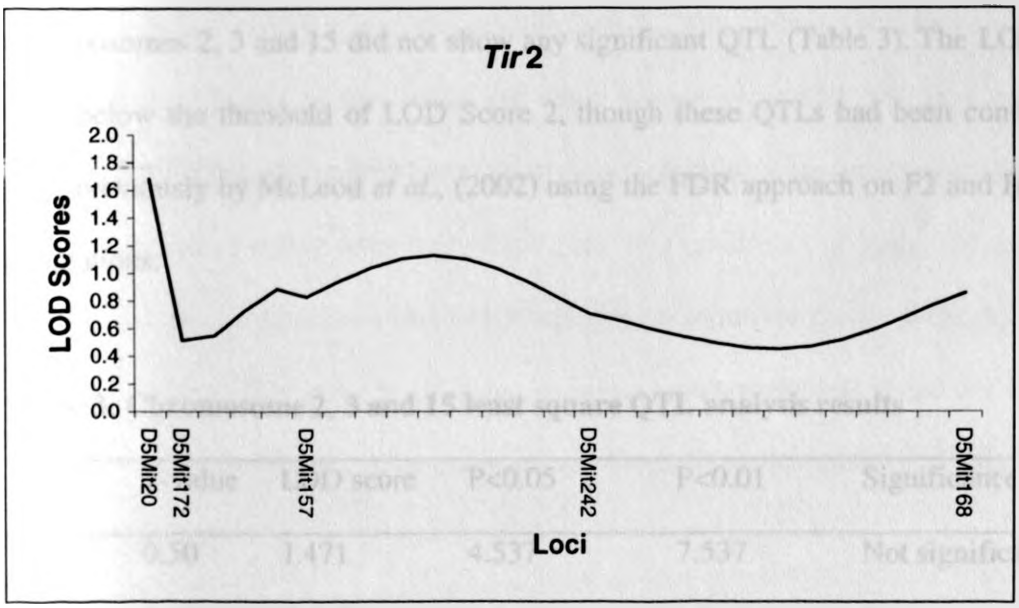

Figure 3c: Chr 5 (*Tir2*) QTL mapping using 129/J x C57BL/6J cross

Estimates of genetic effects on survival following *T. congolense* challenge in F2 are summarized in Table 2. Both the *Tir1* and *Tir3* had an additive effect on the phenotype.

Table 2. Locations and statistics for putative QTL in F2 129/JxC57BL/6J cross.

Chr	Position	Maximum LOD score	Maximum F-ratio	Additive Effect ± SE	Dominance effect ± SE	SSLP Flanking markers
1	78	3.138	7.64	**41.9** ±10.9	22.7±15.6	D1Mit425-D1Mit206
17	15	2.661	6.42	38.9 ±12.1	-6.32±18.7	D17Mit68-D17Mit184

Chromosomes 2, 3 and 15 did not show any significant QTL (Table 3). The LOD scores were below the threshold of LOD Score 2, though these QTLs had been confirmed to exist previously by McLeod *et al.*, (2002) using the FDR approach on F2 and F6 Mouse populations.

Table 3: Chromosome 2, 3 and 15 least square QTL analysis results

Chr	F-value	LOD score	P<0.05	P<0.01	Significance
2	0.50	1.471	4.537	7.537	Not significant
3	3.67	1.553	5.696	7.858	Not significant
15	0.86	0.371	4.888	7.110	Not significant

4.3 SNP /Haplotype analysis

Markers flanking each QTL were identified by the linkage analysis and subsequently mapped on the mouse genome sequence database. These markers were used to identify the reference SNP positions within the assembled genome sequences for database query. The mouse SNP database http://mousesnp.roche.com/cgi-bin/msnp_public.pl and the Oxford Laboratory database http://aretha.jax.org/pub-cgi/phenome/mpdcgi?rtn=docs/home was screened for SNPs within each QTL for total SNPs available, and then filtered to keep only the ones for 129/J, A/J, BALB/c, C3H/J and C57BL/6J as indicated in Appendix V.

DNA markers flanking *Tir*1 and *Tir*3 QTL were selected relative to LOD scores of 2.588 and 3.026 respectively. The regions focused ranged from 22-56Mb and 93-193Mb (ENSEMBL v36) in *Tir*1and *Tir*3 respectively. Additional data filtering of SNPs was done to increase confidence in the positive results. The filtering was by removing the SNPs which appeared in one strain and DNA sequences or SNP was not available in other strain. With additional filtering, the region focused was narrowed to smaller mosaic (blocks) regions, ranging from a few base pairs to a maximum of 5Mb. The region was categorized on chromosome 1 and 17 by haplotype approach as shown in the Appendix 3. It was possible to fine map the QTL region into a smaller genomic or mosaic haplotype blocks ranging from a few base pairs in one block to a maximum of 6 mega base in another block.

4.4 Possible candidate genes

Candidate fine mapped region for *Tir1* ranged from 32.48Mb-32.60Mb corresponding to previously fine mapped region using AIL in F6, while the F2 ranged from 32.48Mb-38.92Mb with many small mosaic regions. In *Tir3* the candidate fine mapped region, *Tir3a* ranged from 94.18Mb-96.20Mb, *Tir3b* from 128.42Mb-128.43Mb and *Tir3c* which had the largest region ranged from 171.139Mb-176.483Mb. Using the combined approach (QTL mapping and haplotype fine mapping) in this study, possible candidate genes were identified within the fine mapped (SNP) region. A total of 18 possible candidate genes were identified in *Tir3*. Among the candidate genes on chromosome 1 (*Tir3a*) are Sialidase 4 (EC 3.2.1.18) (Neuraminidase 4), and Programmed cell death protein 1 precursor (Protein PD-1); in *Tir3b* dipeptidylpeptidase 10 and C-X-C chemokine receptor type 4 (CXC-R4) were identified, while in *Tir3c* Apolipoprotein A-II precursor (Apo-AII), High affinity immunoglobulin epsilon receptor gamma-subunit precursor, PG regulatory-like protein, G-protein-activated inward rectifier potassium channel 3 (GIRK3), and a group of different Interferon-activatable proteins were identified.

A total of 24 genes were identified in *Tir1*, among the possible candidate genes included Trefoil factor 2 precursor, KIAA1-like protein, Death domain-associated protein 6, H-2 class II histocompatibility antigen-A beta chain precursor, Complement C4 precursor, Cytochrome P450 XXIA1.

However, among the possible candidate genes identified in chr17 Death domain-associated protein 6 (Daxx), Estradiol 17 beta-dehydrogenase 8, histocompatibility 2, O region alpha locus, Antigen peptide transporter 2 (APT2), and H-2 class II histocompatibility antigen-A beta chain precursor are within haplotype mapped in F6 population.

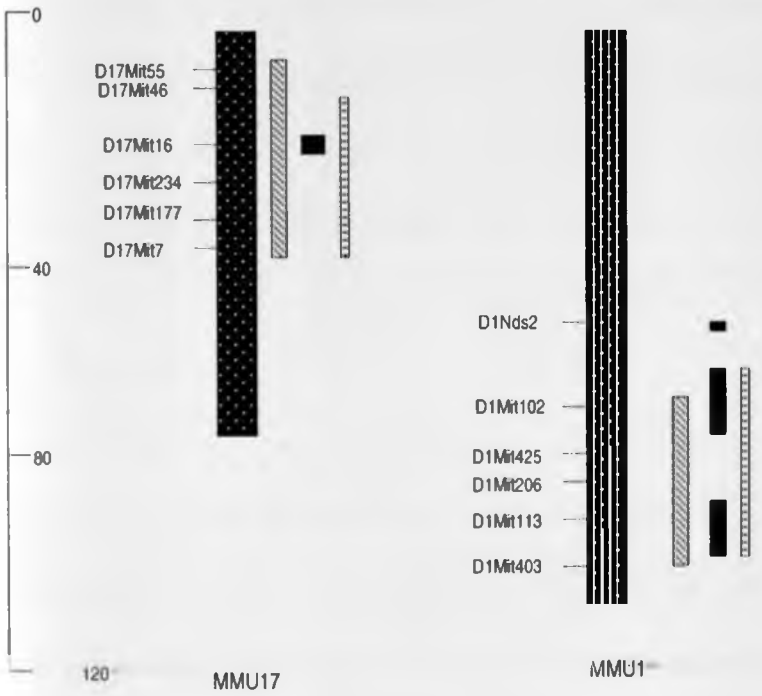

Figure 4: Summary of trypanotolerance QTL mapping in F2 129/J x C57BL/6J comparing with previously published mapped data in F2 and AIL of inbred mouse strains

Figure 4 show in summary form, the work done on mapping and fine mapping the QTLs associated with trypanotolerance in inbred mouse strains on chromosomes 1 and 17, black with white dotted bars. It illustrates the summary of progress in mapping and fine

mapping *Tir1* and *3* on F2, F6 with 95% confidence interval in all levels. Though slightly shifted to the right, *Tir1* was within the greater confidence interval previously mapped in A/J, BALB/c x C57BL/6J cross by Kemp *et al.*, (1997). The 95% confidence intervals at F2 genome-wide scan are represented by black diagonal lines bar (Kemp *et al.*, 1997), F6 Advance intercross lines with black bars (Iraqi *et al.*, 2000) and F2 129J x C57BL/6 cross is the one marked with horizontal black lines.

CHAPTER 5

DISCUSSION

5.1 Phenotypic Observation

The genetics of trypanosusceptibility in 129/J and C57BL/6J mouse strains was analyzed in F2 129/J x C57BL/6J populations. Selective genotyping was used for mapping the QTL on chromosome 1, 5 and 17. The same population developed was adequate for mapping other chromosome 2, 3 and 15 mapped earlier using FDR approach. On parental strains used, 129/J had a lower mean survival time (79 days) as compared to C57BL/6 (82 days). F2 had the highest mean survival time (158 days) which confirmed similar results found kemp et al. (1997) indicating heterosis effect found in some cases when two extreme breeds are crossed.

The survival time of parental control used 129/J and C57BL/6J, was statistically not different and the graph appeared almost the same. Previous work done using 129/J mouse strain had similar susceptibility to A/J mouse strain once challenged with *T. congolense*. In this particular study, the 129/J mouse strain behaved phenotypically less like the C57BL/6J. This observation is postulated to the fact that 129/J mouse strain was obtained from Jackson Laboratory and maintained at ILRI small animal for five years before using in this experiment. As a result of this, 129/J might have acquired some form of immunity. One possible cause might have been due to exposure to higher altitude with different pressures resulting to changes in their blood chemical structures. Also, the fact that they were maintained at ILRI small animal for five years with open environment facility, possibility of acquiring some form of immunity (acquired immunity) was high. This may

explain why, the two parental strains, when challenged with trypanosomosis, their % survival pattern were similar.

5.2 Reciprocal Crossing Effect in F2

F2 129/J x C57BL/6J population was produced on four different F1 groups as indicated in Table1. The groups A, B, C and D were as a result of reciprocal crossing of F1 mice used to generate the resource population resulting in four different subgroups within the F2 group. From the four groups, group B had the highest mean survival time of 188.7 days compared to group C which had the lowest survival mean time of 132.6 days. However, from the ANOVA test, all the groups were found to be statistically different.

This observation is suggestive of a genetic effect of the parental founders which is partially associated with imprinting and interaction (epistasis) effects between original founders. Some evidence suggests that expression of some genes depended on their parent of origin. Newborn inherit genes from their parents. Many of such genes actually remember which parent they came from and they behave accordingly through a process called imprinting. About two dozen human imprinted genes have been identified (Edward, 2000). Such phenomena are known to occur in animals as well e.g. in sheep (Charlier, *et al.*, 2001). In this study, group B had all the resistant (tolerant) alleles coming from male C57BL/6 mouse strain, suggesting that sex may be a factor influencing the expression level of the gene(s) involved. Although the mechanisms underlying imprinting are not totally unravelled, this group finding demonstrates the

important role of imprinting in trypanotolerance as earlier demonstrated by Clapcott *et al.* (2000) using four backcrosses approach.

5.3 Linkage analysis

5.3.1 Maximum Likelihood analysis

F2 linkage order gave the correct marker order comparing with the published order in mouse genome database (http://www.informatics.jax.org/). Going with assumptions in various recombinant strains (Williams *et al.*, 2001) each of the F2 should have MGD map of 1cM interval in an F2 intercross, indicating genotyping accuracy. Markers that did not link during the linkage analysis were removed from further analysis. Some of the reasons postulated to have affected these markers are preferential amplification of one allele, thus leading to exaggerated homozygous (Dominant or recessive), excess double recombination's frequency not expected within closely related markers with less than 10cM distance from one another. In addition, the frequencies of such alleles showed deviations from Hardy-Weinberg expectation with frequencies that were not consistent with effects of selection for genotyping on markers close to QTL. It was, therefore, concluded that these markers had genotyping errors, hence were duly excluded from further analyses. Some minor differences were observed between the results of this study and the published map order of some microsatellite used (Hamvas *et al.* 1998; Kozak and Stephenson, 1998) and the results from *de novo* mapping on the basis of genotyping. But selective genotyping may explain the slight changes (shift) observed, as selective genotyping is expected to create a substantial upward bias in estimates of QTL effects (Lander and Botstein, 1989; Ronin *et al.* 1998).

The longer survival time exhibited by F2 129/J x C57BL/6J compared to C57BL/6J parental line suggests a heterosis effect due to genetic background from both founders playing a major role in defining trypanotolerance. The resistant allele carried by the C57BL/6 parent appeared to be dominant influencing most of the phenotypic trait observed. The observation that many of the susceptible individuals among the F2 mice were more resistant than the parental 129/J mice, may suggest that other minor genes also modulated the response to infection. A number of modifier genes possibly within these other minor QTLs may be controlling this trait, especially by enhancing the heterotic vigour. Such heterotic vigour has been observed in all previous crossbred populations between A/J, BALB/c and C57BL/6J (Kemp *et al.*, 1997; Iraqi *et al.*, 2000) with the modifier genes possibly coming from the susceptible strain.

Alleles from other minor QTLs, those detected using FDR approach may account for this effect. Epistasis, where non-appearance of a characteristic is determined by one gene, because it has been suppressed by the activity of another gene might also have been operating in this case. For example, the susceptibility gene from 129/J may have been suppressed by the resistant allele coming from C57BL/6J. The observed phenotypic effect would also be due to an additive effect, where both gene effects were added to one another, thus leading to observed longer survival time in F2. The additive effect represents mostly small effects of alleles of many genes influencing a particular trait while the dominant effect result from the interaction of alleles at the same locus and is the basis of heterosis in crossbreeding programs. Epistatic effects result from the interaction

of alleles at different loci on the same or different chromosome. This genetic effect is not as well understood or as predictable as are additive and dominant effects.

The results of regression analysis carried out on the web based QTL express were comparable to those obtained with maximum likelihood results as analyzed by Mapmaker/QTL (Figures 3a, b and c). The QTLs *Tir1* and *Tir3* mapped on Chr 17 and 1 QTLs, *Tir1* and *Tir3* comprised a single locus with LOD scores of 3.276 and 2.588 respectively. *Tir1* and *3* mapped in 129/J x C57BL/6J cross was weaker than that observed in A/J, BALB/c x C67BL/6J cross previously mapped by Kemp *et al.* (1997). This is consistent with survival data of 129/J x C57BL/6J cross and may partly explain why the LOD scores were low compared to A/J, BALB/c whose mean survival was significantly different from C57BL/6J.

The mapped QTLs confirmed that 129/J mouse strains carry the susceptible alleles at *Tir1* and *3* which were previously mapped in A/J and BALB/c mouse strains on both F2 (Kemp *et al.*, 1997) and F6-AIL (Iraqi *et al.*, 2000) populations. This finding illustrates further the importance of these two QTLs (*Tir1* and *Tir3*) in conferring trypanotolerance trait.

Chr5 (*Tir2*) was not confirmed to exist in F2 129J x C57BL/6J cross as previously mapped in F2 and F6 by Kemp *et al.* (1997) and Iraqi *et al.* (2000), respectively. Lack of confirmation of chromosome 5 QTL on the *Tir2* region might be due to small genetic variation between 129/J and C57BL/6J hence resulting to less significant LOD scores. In addition, it was postulated that this apparent loss of *Tir2* from F2 129J x C57BL/6 cross may have been due to an allele in chromosome 5 within C57BL/6 and 129J mouse strains having the same function, meaning chromosome 5 in 129/J does not carry the susceptible allele of trypanotolerance. This could have been due to the fact that it might have been inherited from either one of three wild mouse strains where the inbred lines were developed from and or that an allele in C57BL/6 in this particular locus is not yet completely developed.

Other possible scenarios might be that the two mouse strains have got the same allele at the same locus, (taking into account that the two strains are both developed from the three wild mouse strains), but with different time of gene function onset or differing on their gene action. The result might also be due to variance of the recombination number (Darvasi and Soller, 1995), within each chromosome location of the two mouse strains due to differences in their chromosome sizes. Earlier studies also showed no evidence of chromosome 5 QTL in (BALB/cJ x C57BL/6) F6 population and it was proposed that it could have been due to loss of the allele due to many recombination events during the development of the AIL (Iraqi *et al.*, 2000). In this study, this is unlikely since at F2 level, recombinations are very limited (Darvasi and Soller, 1995). Another intriguing possibility is that 129/J does not possess the susceptible allele at these loci.

It might also reflect that mouse generate different outcomes in a given environment; meaning not only one allele is in control of the trait, but in synergy with many other minor genes within the chromosome.

5.4 Genetic Effect Estimation

QTLs are subject to dominance, additive and epistatic genetic effects. The additive effect represents mostly small effects of alleles of many genes influencing a particular trait while the dominant effect result from the interaction of alleles at the same locus and is the basis of heterosis in crossbreeding programs. Epistatic effects result from the interaction of alleles at different loci on the same chromosome. The genetic effects revealed significant interactions between *Tir1* and *Tir3*, which were detected as changes in gene action. Positive estimates indicated that alleles from resistant C57Bl/6J mouse strain contributed to the trait by increasing the survival time as found on the genetic estimates where by the additive effect was found to be thrice the dominance effect (Table 2).

5.5 False Discovery for mapping QTLs of small effects

Chromosomes 2, 3 and 15 did not show significant QTL presence, as their LOD scores were too far below the threshold level, although, False Discovery Rate (FDR) studies had identified QTL at these Chromosomes (MacLeod *et al.*, 2002). The False Discovery Rate was proposed by Benjamini and Hochberg (1995) to control errors caused by multiple hypothesis testing. FDR control methods limit the expected proportion of false rejections

within the class of rejected null hypotheses, averaged over multiple experiments, including those where no significant results were found.

FDR control was first applied to QTL mapping by Weller *et al.* (1988). In FDR analysis, normally the threshold is flexible and peaks can be picked at LOD score bellow two. The advantages of least square (LS) interval mapping is that it uses chromosome-wide permutation tests, thus allows for QTLs with LOD score that is less than 2 . On the other hand, maximum likelihood assumes that only regions with LOD scores greater than 2 are significant. Using this statistic threshold means that only QTL of large effect are reported, while QTL of small effects remain undetected. LS also uses F-statistic threshold at 5% and 1% to declare a QTL significant a criterion suggested by Lander and Kruglyak (1995). From this, it is then possible that the genome-wide (maximum-likelihood) approach used in the analysis was too restrictive whereby the LOD score of 2 in the analysis was too high to detect QTL with very small effects (LOD score bellow two), and or that the F2 resource population used in this study was small, less than 200 in number of mice genotyped.

Alternatively, in Chrs 2, 3 and 15 examined, the markers selected for the analysis were few and less well spaced than anticipated and so a true peak may have been missed or may have lead to a LOD score of bellow two, hence the conclusion that there were no QTL.

5.6 Haplotype Analysis in Fine mapping

Mapping of quantitative trait loci (QTL) for susceptibility to diseases in animal models is a powerful tool in identifying genes that may be relevant to specific trait of interest. With the availability of complete mouse genome sequence, SNP analysis allows the identification of potential candidates of various QTL mapped using mouse models. In addition to improving mapping resolution significantly in this study, the QTL mapped in this study, together with previously mapped region in A/J, BALB/c x C57BL/6J crosses were systematically analysed for SNP across five different strains of mouse 129/J, A/J, BALB/c, C3H/J and C57BL/6J. C3H/J mouse strain was shown to carry the susceptible alleles of *Tir1* and *Tir3* QTL mapped on chromosomes 17 and 1 respectively (Amwayi MSc. Thesis 2006). The SNP analyses revealed a degree of complexity at the *Tir1* locus that was not evident in genome-wide scan approach.

Where as, genome-wide scan approach had shown a single region of significance spanning a CI of 20cM, small haplotype block (mosaic) regions were revealed at the *Tir1* locus as previously described by Bonhomme *et al.* (1987) with haplotype approach. All the haplotype block regions fell within the greater QTL interval mapped in F2 population earlier and are, therefore, likely to represent loci that were detected as one large QTL, but could not be separated, in the previous study. *Tir3* which had CI of 53cM was also resolved in several but smaller haplotype (mosaic) block regions with *Tir3a* ranging from 94.18Mb-96.20Mb, *Tir3b* from 128.42Mb-128.43Mb and *Tir3c* which had the largest region ranging from 171.139Mb-176.483Mb all within the congenic mapped region.

By haplotype approach, only regions where there was consistence within the susceptible mouse strains, but different from C57BL/6 were picked to be a SNP. This meant a change in single nucleotide polymorphism from say A⇒T or G⇒C, which in essence could mean a change in the amino acid coding leading to a change in protein function. This change in nucleotide polymorphism could be the underlying factor that is causing the resistance or susceptibility in different mouse strains. Use of haplotype-sharing approach as a high resolution mapping tool helped increase the resolution of the QTLs leading to a number of possible candidate genes

5.7 Possible candidate genes

The possible candidate genes identified within the candidate fine mapped region for *Tir1* ranged from 32.48Mb-32.60Mb, while in *Tir3* the candidate fine mapped region, *Tir3a* ranged from 94.18Mb-96.20Mb, *Tir3b* from 128.42Mb-128.43Mb and *Tir3c* with the largest region ranged from 171.139Mb-176.483Mb, as indicated in appendix 5; most of these genes are involved in host response to various pathogenic infections and might be playing a role in trypanosomosis infection. Some genes are both found in Chrs 1 and 17, thus indicating possible interaction on those two QTLs on genes involved in trypanosomosis response. The knowledge on their interaction pathways might identify genes underlying QTL determining host resistance, tolerance and susceptibility to infection with *T. congolense* in mice and cattle.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The results indicate that apparently complex quantitative disease resistance trait (trypanotolerance) is in large part controlled by loci on two chromosomes, Chrs 1 and 17 respectively. The study indicates that there is considerable variation in resistance to trypanosomosis among inbred mice and much of this variation is genetic in origin. Understanding the genetic, host-pathogen interaction and immunological basis of resistance to parasitic infection is necessary for a comprehensive explanation of disease resistance. The exploitation of genetic variation for the identification of the genes conferring resistance to trypanosomosis infection could increase the accuracy of improving genetically superior animals.

a) From the results, the largest effect was found on chromosome 1 with a LOD score of 3.026. An additional significant effect was found on chromosome 17 with a LOD score of 2.58.

b) The 129/J mouse strain phenotypically known to be susceptible to *T. congolense* challenge (Morrison and Murray, 1979), and suspected to carry susceptible alleles at *Tir1, Tir2,* and *Tir3* loci as earlier observed in Balb/c and A/J mouse strains (Kemp *et al.*,1997; Iraqi *et al.*, 2000); did not carry the susceptible allele at *Tir*2. This would mean that, at this locus, the alleles are the same between the 129/J and C57BL/6J mouse strains having the same function, possibly explaining the close relationship (in origin) between the two mouse strains. In essence, additional

work need to be done to ascertain if this is the case in this particular chromosome 5.

c) Chromosomes 2, 3 and 15 did not show significant QTL presence, as their LOD scores were too far below the threshold level of LOD score 2. This indicates that the 129/J mouse strain has got no susceptible allele at the three chromosomes. Alternatively the QTLs present are of minor effect and thus can not be detected using the Mapmaker/QTL software.

Genomic scans have proved extremely useful for determining the rough chromosomal location of QTLs. However, the imprecision with whom genome screens localize QTLs presents difficulties in in-depth studies of gene(s) that are responsible for the phenotypic effects and for assessing potential pleiotropism. Theoretical and practical analyses suggest that QTLs can be mapped to a confidence interval of ~10-30 cM using standard genetic analysis.

a) The results from this study show the synergies between classical (congenic) genetic studies and genomic tools such as the SNP database in dissecting the genetic basis of disease in mouse model. In addition, this study shows that genetic approaches are still the best in gene identification for trypanotolerance to allow for MAS and MAI breeding methods in livestock improvement programs. This study indicates that, this approach of combining both the genome-wide scan and SNP increases the mapping resolution of the QTL region. It is a powerful tool to narrow down the gene list in otherwise a too large QTL.

b) It is now possible to identify the comparative chromosomal regions of these QTLs in livestock to consider future possibility of improving control strategies. These may include breeding for resistant or tolerant livestock, development of vaccines, or identification of new trypanocidal drugs.

c) The study also indicates that it is not possible to map QTLs with minor effect using convectional genome-wide search as found with chromosomes 2, 3 and 15, that had earlier been mapped using FDR approach.

6.2 Recommendations

Based on the results of this study, further study is required to specifically identify and analyze genes within the fine mapped region. Different molecular techniques can be implored, for example, confirmation of candidates' genes by establishing suitable candidates where SNP could result in an amino acid alteration and where there is plausible link to the aetiology or pathology of the disease.

DNA Microarray technology is another advanced molecular technique that can be used. This involves the study of gene expression in different developmental stages, under different environmental conditions and in different genotypes, and is a key approach in the gene discovery process. Studies of physiological pathways, including disease pathogenesis, have previously been hampered by the ability to examine only a few proteins or genes at a time. DNA microarrays provide a means of "expression profiling" thousands of transcripts simultaneously by means of densely arrayed cDNA fragments. Analysis of mRNA expression using DNA microarrays can now be exploited to examine the relationship between host and pathogen in much greater detail than was possible previously. Sequencing of the now confirmed genes, further genetic data and functional assays profiling is needed to establish their function in relation to trypanosomosis.

Specifically, the following further investigations need to be undertaken:-

1) Understand host-pathogen interactions and host responses - at the genome level - in response to trypanosomiasis.

2) Identify genes underlying QTL determining host resistance, tolerance and susceptibility to infection with *T. congolense* in mice and cattle.

3) Identify host and parasite genes and molecular pathways involved in the whole disease response in different mouse strain.

4) Develop methodology and informatics tools such as Comparative mapping for generation of meaningful expression information from a range of cell types and mixes of cell types in response to trypanosomosis infection as generated by the DNA microarray.

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APPENDICES

APPENDIX 1

Extraction of high molecular weight DNA from mouse tail using the urea lysate method

Chop 1-1.5 cm of mouse tail in tinny pieces into a 2ml Eppendorf tube.

Add 0.8ml of 8M Urea pH7.6, mix gently

Incubate at 37°c overnight in a waterbath

Pippete out the 0.8ml lysate into a new tube using a large bore pippete tip

Add an equal volume of Phenol: Chlorform: Isoamyl alcohol (25:24:1) into the lysate

Mix gently on wing rollers for 30 min

Spin in microfuge 12000rpm for 2 min at RT

Recover the clear supernatant into a new Eppendorf tube

Repeat step 5-8

Add an equal volume of Chlorform only and repeat step 6-8

Add $1/10^{th}$ volume 3M Sodium Acetate pH 5.2, followed by 2 volumes of ice-cold absolute Ethanol

Mix by gentle inversion over 1 min and leave to precipitate at RT for 1 hour

Aspirate the supernatant gently and discard

Add 1 ml of 70% Ethanol to the pellet, mix gently for 10 min on wing roller

Spin in microfuge at 12000rpm 2 min at RT and discard the supernatant

Dry the pellet under vacuum for ~5 min. DO NOT OVER DRY

Resuspend the pellet in 200µl T.E. with RNAase dissolved

APPENDIX 2

Number of SSLP Loci studied per chromosome and their distances in cM

Chromosome 1

Loci	MGD-Position (cM)	Sequence	Average Product size
D1Mit68	9	TGTGCTCTCTGGCAAAACAC CGTTATGTGCCTACCACCCT	174
D1Mit70	17.8	CCAAGTCAAGTCATCATATGATTT CCTGTGTGCCCTGCTTAATT	181
D1mit232	20.8	TTGTCCTCTGACCTTGCAGA TCCCCCTTCATTTCCTCTTT	142
D1Mit303	34.8	GGTTTCTATTTCGGTTCTCGG TCTGTGCTGCAAAACAGAGG	128
D1Mit216	49.7	GGGAGACAACAAATAATCATATTGC AGAGGTGGGTCCTGGAAACT	120
D1Mit94	64.0	CGACTTCCCTTGATGTCCAT TTTGTGTTGTGCAGTCTGTCTG	154
D1Mit425	81.6	CAAAAAAACAACACATTTTACTTTCA ACTTTGTATTTCACATGATGTCCTG	121
D1Mit206	95.8	TGAGGCACCTTTGTATTCAGC CCAGATGTCTTTGAACATTCTCC	126
D1Mit17	106.3	GTGTCTGCCTTTGCACCTTT CTGCTGTCTTTCCATCCACA	167

Chromosome 2

Loci	MGD-Position (cM)	Sequence	Average Product size
D2Mit92	41.4	TGTATGCACAGGTATTTCCCC TGAGGAAAGGGGATAAAATTTG	149
D2Mit300	50.3	TGTGCACACTGTGGTCATACA TTAGGCTATTTCTACCTGTGTAGAACC	106
D2Mit304	73.0	AAGCAGGTGTCGCTGATTG AGAAGATGGACCGAGGGG	118
D2Mit194	81.4	TGGAATTCCAAAGTCAAGGG GGGAAGAATGGGGGAAGTTA	114

Chromosome 3

Loci	MGD-Position (cM)	Sequence	Average Product size
D3Mit230	38.3	GAATGGCCAGGGTAAAATCA TTGAACTCCAAACTTGAGACCA	147
D3Mit107	55	ACCTCCAGTCAGTGTCTGCA TCTTGCACACTTGCACACAA	197
D3Mit127	70	CCTTCTGACAAGCAGGATTTG TTTCTAGCATCTCCAAGCAGG	175
D3Mit19	87.6	CAGCCAGAGAGGAGCTGTCT GAACATTGGGGTGTTTGCTT	159

Chromosome 5

Loci	MGD-Position (cM)	Sequence	Average Product size
D5Mit66	17.0	CACGCCCAGCTATGAAAAG TGCCCTCAAAGCAGTATGC	214
D5Mit255	34.0	CCCTGTGCTCTGGATTAGTTG TCAAGACCAGCATCAAACCA	118
D5Mit200	36.0	CACAGAGAAGAATCTGCGAGC TTGCAAAGTGTTTTAATCAGTATTTG	108
D5Mit201	42.0	GAGGACTCCTTCGATTTCCC TTCCTAAGCAGGAACTGACCA	110
D5Mit113	42.0	ACAGTATTTTCTTTTTCCAAGTGTG CAAAGACTCTAGGTGTGACCCC	105
D5Mit259	45.0	ATGATCAAAACATTACTCCCTTCC ACAAAGTCAGGAGAAATGTGCA	130
D5Mit20	52.0	TGAATCTGTGGCCAAATGAA CTTTGCCAGAGCAGCCAT	154
D5Mit172	53.0	TTCACAGAAAGCAAACAGTTACTATT AGAACTGATTCCAGTAAGGTGTCC	145
D5Mit157	57.0	TAGGTATGTGGGCTTGCACA TGGCTGCTGAATTTTAGCG	124
D5Mit242	66.0	AAATTTCAGGATTGAGTTCTCACC TATGTCAAATGCATGCACCC	145
D5Mit168	78.0	CAGGTGACAGTTGTTCTCTTCC CATGCATGAACACACATCACA	150
D5Mit122	85.0	AGATTCTTGTGAAAGGAATTCTCG TTCTTGTTTACTTTCTTTTTCGGG	158

Chromosome 15

Loci	MGD-Position (cM)	Sequence	Average Product size
D15Mit179	10.8	TGTGAAAAGTTTGTACCATACAAATC CACTTGTGCCTCTGTATGCG	147
D15Mit138	15.4	TTCAATTCCCTTTTGTCAAATG CAAGACCCTAGATTCAGTCTACCC	147
D15Mit105	47.9	ACTGGCTTATCTAGCATTCTCCC CATATTGTCTTATCAGCCATGTCC	122

Chromosome 17

Loci	MGD-cM	Sequence	Average Product size
D17Mit175	17.7	TGGAAATCGGAGCCTCTG TTGGAAAAGGTTGAGAGTAGATCA	109
D17Mit68	24.5	GTCCTGACATCATGCTTTGTG CTACCGTTTGGAAGGCTGAG	130
D17Mit117	29.4	AGTCCATTTATCGGGGGC TTTAATGGCACATCTGGCAA	122
D17Mit184	38.5	TGCACTACCCAAACATGCAT ACTTCTGACAGGAAGCATCCA	135
D17Mit93	44.5	TGTCCTTCGAGTGTTTGTGTG TCCCCGGTGAATGAGTTATC	153
D17Mit155	55.7	TGAGAAGGTTGGGTTTATATATTTAGG CGATCATTTCCTTGCAACCT	140
D17Mit123	56.7	CACAAGGAGGGAGCCTGTAG CACCGTAAGAGTCTAATAATAAGGGG	133

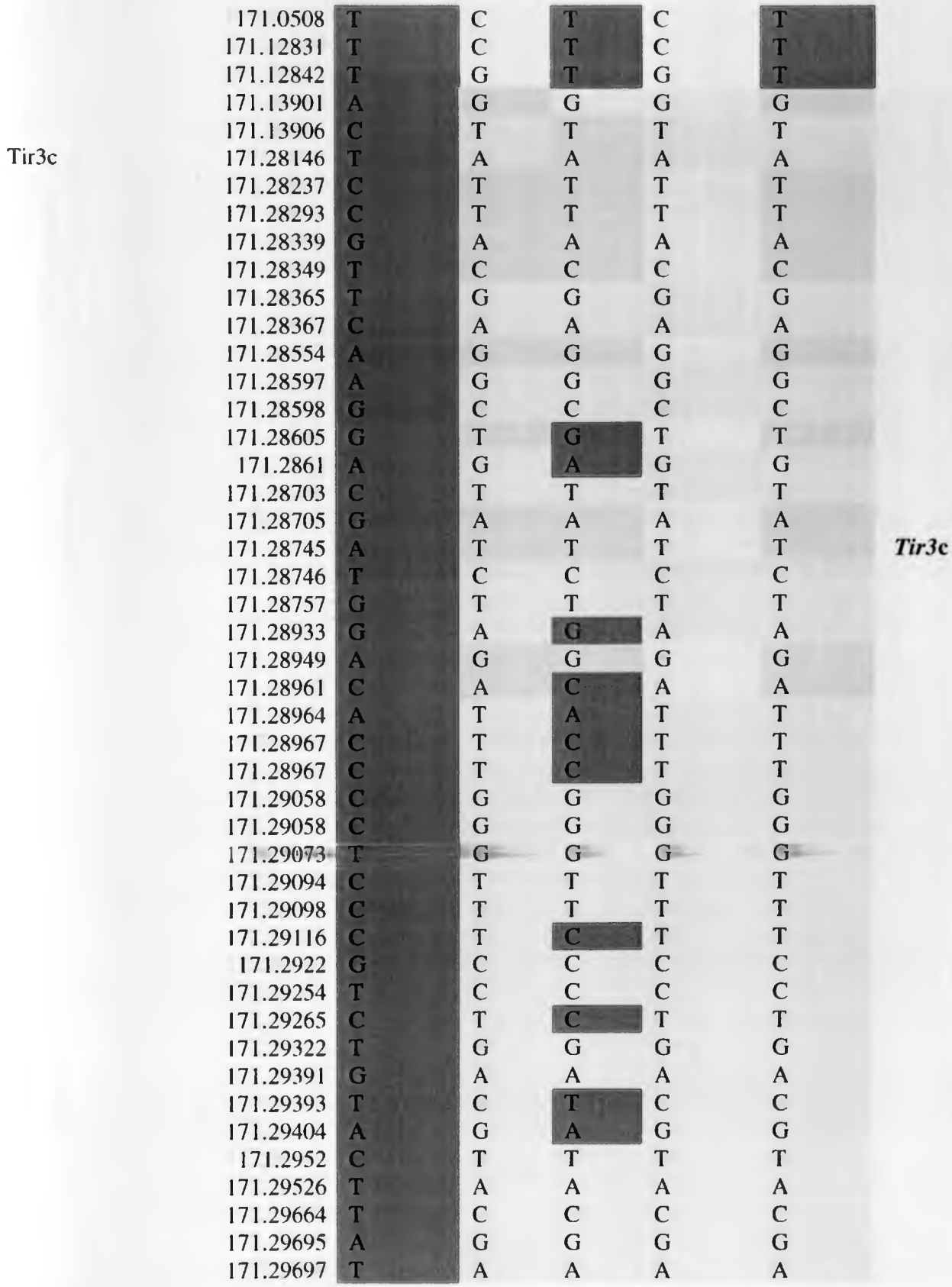
APPENDIX 3

Shared Haplotype regions (SNP)

a) Haplotype sharing region and markers within on the *Tir3a, b and C* loci

Marker	cM	MBp	C57BL/6J	129/J	A/J	C3H/HeJ	BALB/cJ	
D1mit60	58.7cM	93.408454	A	G	A	G	G	
		93.629047	C	A	C	A	A	
		93.78423	G	A	G	G	A	
		93.845713	G	A	A	A	A	
		93.956664	A	T	A	A	A	
		94.009617	A	C	A	A	A	
		94.048725	G	A	G	G	G	
		94.085598	G	G	A	A	A	
		94.177642	C	G	G	G	G	
		94.285431	A	G	A	A	A	
		94.29068	A	G	A	A	A	
		94.442652	A	A	A	A	A	
		94.513188	G	G	G	G	G	
		94.597448	G	G	G	G	C	
		94.606759	G	A	A	A	G	
		94.69709	A	A	A	A	A	
		94.833667	A	G	A	A	G	
		94.835864	A	G	G	G	G	***Tir3a***
		94.877395	C	A	A	A	A	
		94.976871	A	A	C	C	C	
		94.987719	C	C	A	A	C	
		95.012192	G	A	A	A	A	
		95.082296	G	C	C	C	C	
		95.23595	G	G	A	A	A	
		95.354952	G	G	A	A	A	
		95.465877	A	G	G	G	G	
		95.561126	G	G	A	A	A	
		95.674089	G	G	A	A	A	
		95.839236	A	A	G	G	G	
		95.902135	G	A	A	A	A	
		96.059376	G	A	A	A	A	
		96.119584	A	G	G	G	G	
		96.198573	A	G	G	G	G	
		96.254625	C	A	A	A	C	
		96.385929	G	G	G	G	A	
		96.450869	G	G	G	G	C	
		96.494265	G	G	G	G	C	
		96.55782	A	A	A	A	A	

		96.583201	C	C	A	A	C	
		96.583261	A	A	C	C	C	
		96.684289	C	C	C	C	A	
		96.836231	A	A	G	G	G	
		96.929983	A	A	C	C	C	
		97.027095	T	T	A	A	T	
		97.136948	G	G	A	A	G	
		97.177674	A	A	G	G	A	
		97.235903	G	G	A	A	A	
		97.311355	G	G	G	G	G	
		97.373615	G	G	G	G	G	
		97.47895	A	A	A	A	A	
		97.540905	A	C	C	C	A	
		97.541429	A	G	G	G	A	
		97.580711	G	G	A	A	G	
		125.69576	G	T	T	T		
D1mit139 (126.3)	65	125.6958	G	A	A	A	A	
		127.42152	A	G	G	G		
D1mit286	67	128.42609	C	T	T	T	T	
		128.42613	G	A	A	A	A	
		128.42631	G	A	A	A	A	
		128.42651	G	A	A	A	A	
		128.42654	T	C	C	C	C	*Tir3b*
		128.4269	T	G	G	G	G	
		128.42801	C	T	T	T		
		128.4281	T	C	C	C	C	
		128.42811	C	A	A	A		
		128.42812	A	G	G	G	G	
		128.42819	T	G	G	G	G	
		128.4282	C	A	A	A		
		128.42859	C	T	T	T	T	
		128.42866	T	G	G	G	G	
		128.42881	A	T	T	T	T	
		128.42894	C	T	T	T	T	
		128.42911	A	G	G	G	G	
Tir3b		128.42918	A	G	G	G	G	
		128.42928	A	C	C	C	C	
		134.99777	T	C	C	C	T	
		134.99782	C	G	G	G	C	
		134.99794	A	C	C	C	A	
		171.04601	A	T	A	T	A	
		171.04622	C	A	C	A	C	
		171.04921	C	T	C	T	C	
		171.0505	A	G	A	G	A	
		171.0506	C	T	C	T	C	


Tir3c
171.0508 T C T C T
171.12831 T C T C T
171.12842 T G T G T
171.13901 A G G G G
171.13906 C T T T T
171.28146 T A A A A
171.28237 C T T T T
171.28293 C T T T T
171.28339 G A A A A
171.28349 T C C C C
171.28365 T G G G G
171.28367 C A A A A
171.28554 A G G G G
171.28597 A G G G G
171.28598 G C C C C
171.28605 G T G T T
171.2861 A G A G G
171.28703 C T T T T
171.28705 G A A A A
171.28745 A T T T T
171.28746 T C C C C
171.28757 G T T T T
171.28933 G A G A A
171.28949 A G G G G
171.28961 C A C A A
171.28964 A T A T T
171.28967 C T C T T
171.28967 C T C T T
171.29058 C G G G G
171.29058 C G G G G
171.29073 T G G G G
171.29094 C T T T T
171.29098 C T T T T
171.29116 C T C T T
171.2922 G C C C C
171.29254 T C C C C
171.29265 C T C T T
171.29322 T G G G G
171.29391 G A A A A
171.29393 T C T C C
171.29404 A G A G G
171.2952 C T T T T
171.29526 T A A A A
171.29664 T C C C C
171.29695 A G G G G
171.29697 T A A A A
Tir3c

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171.29709	C	A	C	A	A
171.29712	A	G	G	G	G
171.29764	G	G	A	A	G
171.30673	A	G	A	G	G
171.3083	A	G	A	G	G
171.30845	C	C	C	T	C
171.30845	C	C	C	T	C
171.31008	T	T	T	A	T
171.31014	C	C	C	T	C
171.31035	T	G	G	G	G
171.31042	A	G	G	G	G
171.31053	T	T	T	C	T
171.31106	C	G	G	G	G
171.31116	G	A	A	A	A
171.31248	G	G	G	A	G
171.3125	A	G	G	G	G
171.31276	T	A	A	A	A
171.3128	T	T	T	C	T
171.31304	A	A	A	G	A
171.31335	T	C	C	C	C
171.31743	A	G	G	G	G
171.31745	T	C	C	C	C
171.31748	G	G	G	A	G
171.31748	T	T	T	C	T
172.34324	C	T	T	T	T
172.3433	C	T	T	T	T
172.34332	A	G	G	G	G
172.34335	G	A	A	A	A
172.34336	A	G	G	G	G
172.34344	C	T	T	T	T
172.39329	G	C	C	C	C
172.3933	G	A	A	A	A
172.39382	A	G	G	G	G
172.39383	T	C	C	C	C
172.39383	T	C	C	C	C
172.39383	T	C	C	C	C
172.39432	G	A	A	A	A
172.39432	G	A	A	A	A
172.39433	G	A	A	A	A
172.39444	C	G	G	G	G
172.39444	C	G	G	G	G
172.39725	C	A	A	A	A
172.39749	C	G	G	G	G
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172.3976	T	C	C	C	C

172.3976	T	C	C	C	C
172.3976	T	C	C	C	C
172.39805	G	C	C	C	C
172.39805	G	A	A	A	A
172.39943	A	G	G	G	G
172.40048	G	A	A	A	A
172.40111	C	T	T	T	T
172.40126	A	G	G	G	G
172.40126	G	T	T	T	T
172.40134	C	T	T	T	T
172.40136	T	C	C	C	C
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172.40143	C	T	T	T	T
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172.40169	G	T	T	T	T
172.40169	G	T	T	T	T
172.40169	D	G	G	G	G
172.40175	A	G	G	G	G
172.40177	G	A	A	A	A
172.40178	C	A	A	A	A
172.40183	G	A	A	A	A
172.40224	A	G	G	G	G
172.40235	G	A	A	A	A
172.4024	A	G	G	G	G
172.40241	D	A	A	A	A
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172.4025	C	T	T	T	T
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172.441	C	T	T	T	T
172.44102	C	G	G	G	G
172.44109	A	G	G	G	G
172.44146	A	C	C	C	C
172.44161	T	C	C	C	C
172.44547	G	A	A	A	A
172.44567	T	A	A	A	A
172.44605	C	T	T	T	T
173.08759	C	T	C	T	T
173.0876	G	A	A	A	A
173.08764	T	C	T	C	C
173.35073	C	C	C	T	C
173.35081	A	A	A	G	A

173.35112	C	T	T	T	T
173.35189	A	G	G	G	G
173.3519	C	T	T	T	T
173.35224	T	C	C	C	C
173.35231	A	C	C	C	C
173.35242	C	C	C	T	C
173.3552	A	G	G	G	G
173.35522	A	A	A	A	A
173.35541	G	C	C	C	C
173.35835	T	C	T	C	C
173.35838	C	T	T	T	T
173.83597	T	C	C	C	C
173.84155	C	T	C	T	T
173.84158	T	A	A	A	A
173.84936	A	G	G	G	G
173.85505	T	C	C	C	C
173.8575	T	C	C	C	C
173.86651	T	C	C	C	C
173.86665	C	G	C	G	G
173.87388	T	A	A	A	A
173.87403	T	C	C	C	C
173.87434	A	G	A	G	G
173.87444	A	C	A	C	C
173.87629	A	G	G	G	G
173.87635	C	T	C	T	T
173.88125	A	T	G	T	T
174.00283	G	A	A	A	A
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174.00288	A	G	G	G	G
174.00296	A	G	G	G	G
174.003	C	G	G	G	G
174.00309	C	G	G	G	G
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174.02752	T	C	T	C	C
174.02753	A	G	A	G	G
174.02756	C	T	C	T	T
174.02767	T	G	G	G	G
174.02767	C	T	C	T	T
174.02769	T	A	A	A	A
174.02771	A	C	C	C	C
174.02776	A	G	G	G	G
174.03176	G	T	T	T	T
174.03192	C	A	C	A	A
174.03211	G	T	T	T	T

174.04005	T	G	G	G	G
174.04783	G	A	G	A	A
174.0492	A	G	A	G	G
174.05056	A	C	C	C	C
174.05066	G	C	C	C	C
174.05067	C	G	G	G	G
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174.05067	G	A	A	A	A
174.05074	G	A	A	A	A
174.05128	C	T	T	T	T
174.05131	T	A	A	A	A
174.05136	T	A	A	A	A
174.05158	G	A	G	A	A
174.05244	G	C	G	C	C
174.05259	A	G	G	G	G
174.05402	C	T	C	T	T
174.0544	A	G	G	G	G
174.05448	A	G	A	G	G
174.05583	C	G	G	G	G
174.05614	G	A	G	A	A
174.05615	C	T	T	T	T
174.0576	C	T	T	T	T
174.05798	A	G	A	G	G
174.0611	C	G	C	G	G
174.06185	A	G	G	G	G
174.06319	G	A	A	A	A
174.0646	C	T	T	T	T
174.06466	A	G	A	G	G
174.06472	C	T	T	T	T
174.06765	A	G	G	G	G
174.06769	G	T	T	T	T
174.07453	A	G	G	G	G
174.07467	A	C	C	C	C
174.07467	A	C	C	C	C
174.0748	G	C	C	C	C
174.07486	T	G	G	G	G
174.07728	C	A	A	A	A
174.07734	T	C	C	C	C
174.07738	A	G	G	G	G
174.07739	C	T	T	T	T
174.07749	A	G	G	G	G
174.07983	T	A	A	A	A
174.07991	C	T	T	T	T
174.08208	A	G	G	G	G
174.0822	A	G	G	G	G
174.08221	A	T	T	T	T

174.08223	T	G	G	G	G
174.09033	T	G	G	G	G
174.09037	A	G	G	G	G
174.09037	T	G	G	G	G
174.09038	A	C	C	C	C
174.09039	G	A	A	A	A
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174.09097	A	T	T	T	T
174.09103	C	T	T	T	T
174.09113	C	A	A	A	A
174.09114	T	A	A	A	A
174.09116	A	T	T	T	T
174.09196	A	G	G	G	G
174.0921	A	G	G	G	G
174.09212	T	C	C	C	C
174.0923	C	A	A	A	A
174.10112	A	G	G	G	G
176.47401	T	C	C	C	C
176.47411	A	G	G	G	G
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176.48276	T	A	A	A	A
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176.48278	A	G	G	G	G
176.48349	A	G	G	G	G
176.48371	C	T	T	T	T
176.48378	C	G	G	G	G
176.48387	T	T	T	G	T
176.48388	T	T	T	A	T
176.48394	C	C	C	T	C
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176.484	G	G	G	A	G
176.4856	A	A	A	T	A
176.48562	G	G	G	A	G
176.48574	A	A	A	C	A
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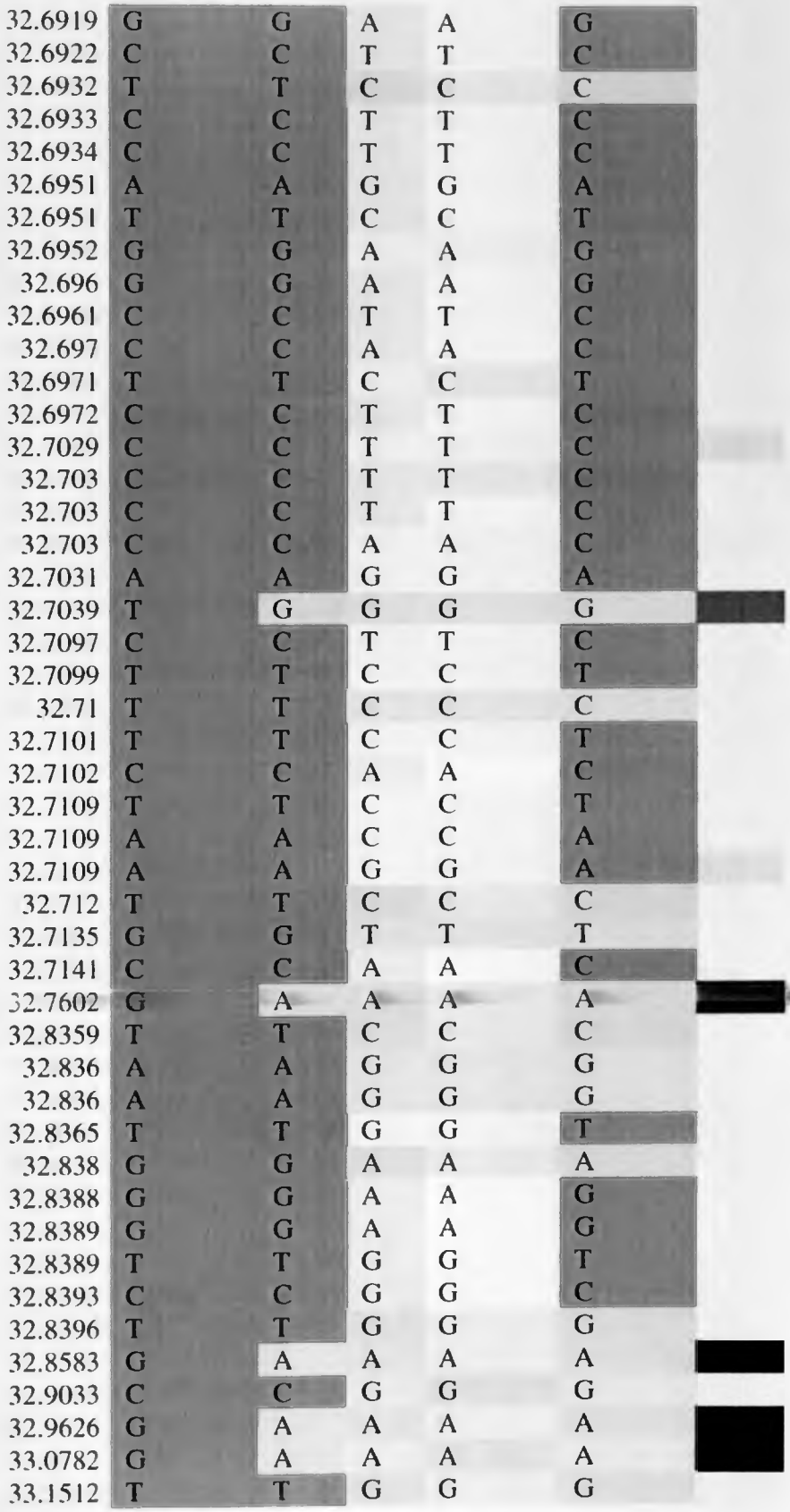
176.48575	G	G	G	A	G
176.48577	G	G	G	A	G
176.48665	C	C	C	T	C
176.48665	G	G	G	A	G
176.48767	T	T	T	C	T
176.48795	T	T	T	C	T
176.48872	G	G	G	C	G
176.48986	G	G	G	A	G
176.49115	G	G	G	A	G

		176.49126	G	G	G	A	G	
		176.49195	T	T	T	G	T	
		182.65801	C	C	C	T	C	
		182.69163	G	G	G	G	A	
D1mit362	106	191.80383	G	G	G	A	G	

***Tir1* conserved haplotype region**

Marker	cM	MBp	C57BL/6J	129/J	A/J	C3H/HeJ	BALB/cJ	
D17Mit16	**17.4**	32.0718	A	A	G	A	G	
		32.1124	G	G	A	G	A	
		32.1887	A	A	G	A	G	
		32.2469	G	G	A	A	G	
		32.2661	A	A	G	A	G	
		32.3657	A	C	C	C	C	F6
		32.4031	C	G	G	G	G	
		32.4141	C	C	A	A	C	
		32.4363	C	C	T	T	C	
		32.4513	T	T	G	G	G	
		32.4726	T	T	C	C	C	
		32.4726	A	A	C	C	C	
		32.4726	T	T	G	G	G	
		32.4726	C	C	G	G	G	
		32.4726	C	C	T	T	T	
		32.4726	C	C	T	T	T	
		32.4726	C	C	A	A	A	
		32.4726	A	A	G	G	G	
		32.4727	T	T	G	G	G	
		32.4727	C	C	G	G	G	
		32.4727	G	G	C	C	C	
		32.4727	G	G	A	A	A	
		32.4727	T	T	C	C	C	
		32.4727	A	A	G	G	G	
		32.4727	A	A	T	T	T	
		32.4727	C	C	T	T	T	
		32.4727	C	C	G	G	G	
		32.4727	G	G	C	C	C	
		32.4727	T	T	C	C	C	
		32.4728	G	G	A	A	A	
		32.4728	A	A	T	T	T	
		32.4728	A	A	C	C	C	
		32.4728	T	T	G	G	G	
		32.4728	T	T	C	C	C	
		32.4753	A	A	C	C	C	
		32.4837	A	G	G	G	G	F6

		32.5239	C	T	T	T	C
		32.5241	T	A	A	A	A
		32.5241	A	G	G	G	G
		32.5241	C	D	D	D	D
		32.5308	T	T	C	C	C
		32.5309	C	C	T	T	T
		32.5371	C	C	G	G	G
		32.5908	A	G	G	G	G
		32.611	C	C	T	T	C
		32.6124	A	A	G	G	G
		32.6139	T	T	C	C	C
		32.614	T	T	C	C	C
		32.614	T	T	C	C	T
		32.614	T	T	G	G	T
		32.6141	G	G	C	C	C
		32.6172	C	C	G	G	G
		32.6363	G	G	A	A	G
		32.6369	C	C	T	T	C
		32.6681	A	A	G	G	G
		32.6681	C	C	T	T	C
		32.6681	A	A	G	G	G
		32.6682	C	C	T	T	T
		32.6686	A	A	G	G	G
		32.6687	G	G	T	T	G
		32.6762	T	T	C	C	C
		32.6763	C	C	A	A	A
		32.6763	T	T	C	C	C
		32.6763	G	G	T	T	T
		32.6763	T	T	C	C	C
		32.6764	G	G	A	A	A
		32.6764	C	C	T	T	T
		32.6764	C	C	T	T	T
		32.6764	G	G	A	A	A
D17Mit214	**18.7**	32.6857	T	T	C	C	C
		32.6859	C	C	T	T	C
		32.6889	T	T	C	C	C
		32.6889	A	A	G	G	A
		32.6896	T	T	G	G	T
		32.6896	C	C	T	T	C
		32.6896	G	G	C	C	G
		32.6899	T	T	C	C	T
		32.6906	G	G	C	C	G
		32.6906	G	G	A	A	G
		32.6906	G	G	A	A	G
		32.6906	T	T	C	C	C
		32.6918	C	C	T	T	C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32.6934 C C T T C
32.6951 A A G G A
32.6951 T T C C T
32.6952 G G A A G
32.696 G G A A G
32.6961 C C T T C
32.697 C C A A C
32.6971 T T C C T
32.6972 C C T T C
32.7029 C C T T C
32.703 C C T T C
32.703 C C T T C
32.703 C C A A C
32.7031 A A G G A
32.7039 T G G G G
32.7097 C C T T C
32.7099 T T C C T
32.71 T T C C C
32.7101 T T C C T
32.7102 C C A A C
32.7109 T T C C T
32.7109 A A C C A
32.7109 A A G G A
32.712 T T C C C
32.7135 G G T T T
32.7141 C C A A C
32.7602 G A A A A
32.8359 T T C C C
32.836 A A G G G
32.836 A A G G G
32.8365 T T G G T
32.838 G G A A A
32.8388 G G A A G
32.8389 G G A A G
32.8389 T T G G T
32.8393 C C G G C
32.8396 T T G G G
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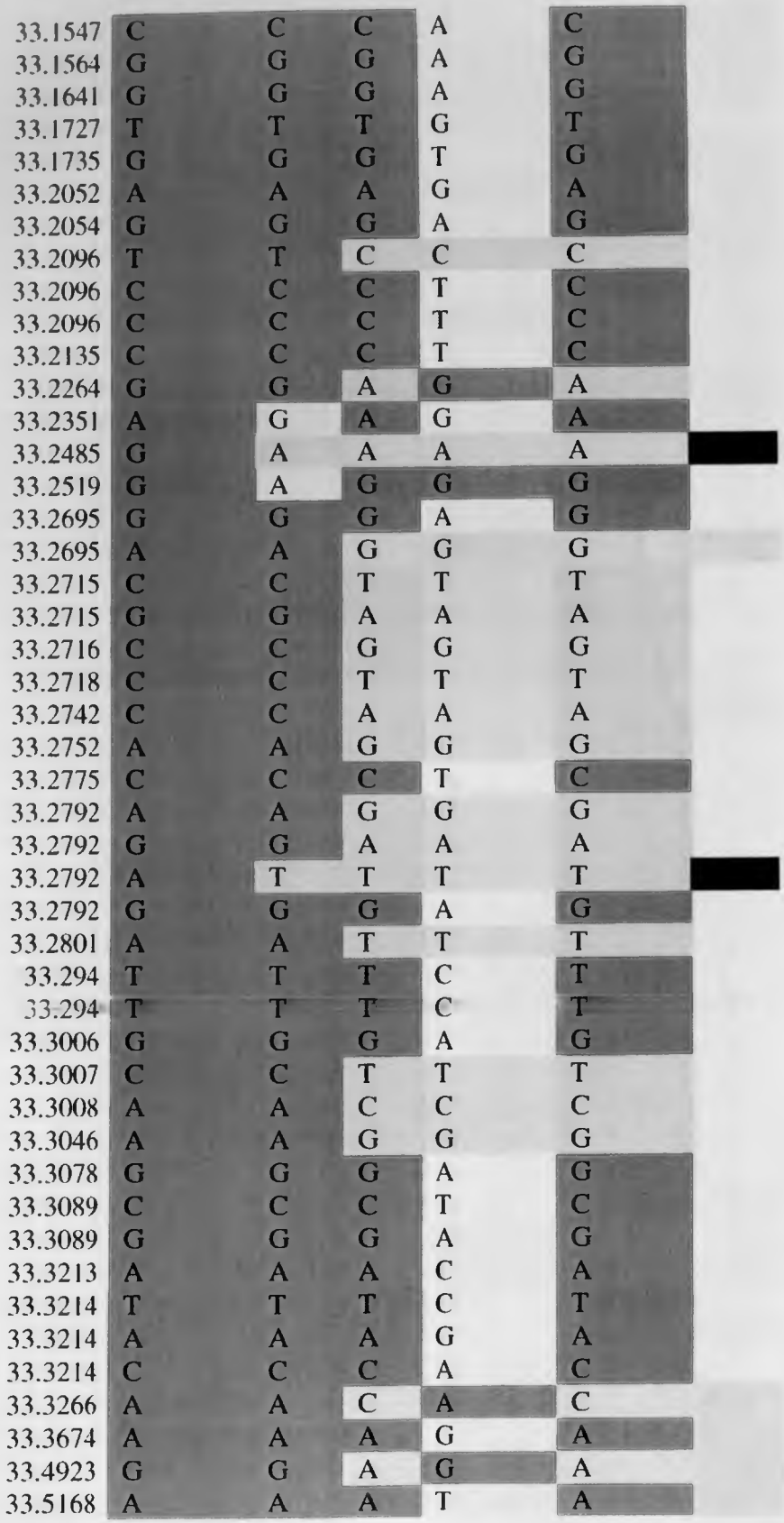
32.9033 C C G G G
32.9626 G A A A A
33.0782 G A A A A
33.1512 T T G G G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33.1735 G G G T G
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33.2054 G G G A G
33.2096 T T C C C
33.2096 C C C T C
33.2096 C C C T C
33.2135 C C C T C
33.2264 G G A G A
33.2351 A G A G A
33.2485 G A A A A
33.2519 G A G G G
33.2695 G G G A G
33.2695 A A G G G
33.2715 C C T T T
33.2715 G G A A A
33.2716 C C G G G
33.2718 C C T T T
33.2742 C C A A A
33.2752 A A G G G
33.2775 C C C T C
33.2792 A A G G G
33.2792 G G A A A
33.2792 A T T T T
33.2792 G G G A G
33.2801 A A T T T
33.294 T T T C T
33.294 T T T C T
33.3006 G G G A G
33.3007 C C T T T
33.3008 A A C C C
33.3046 A A G G G
33.3078 G G G A G
33.3089 C C C T C
33.3089 G G G A G
33.3213 A A A C A
33.3214 T T T C T
33.3214 A A A G A
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33.4923 G G A G A
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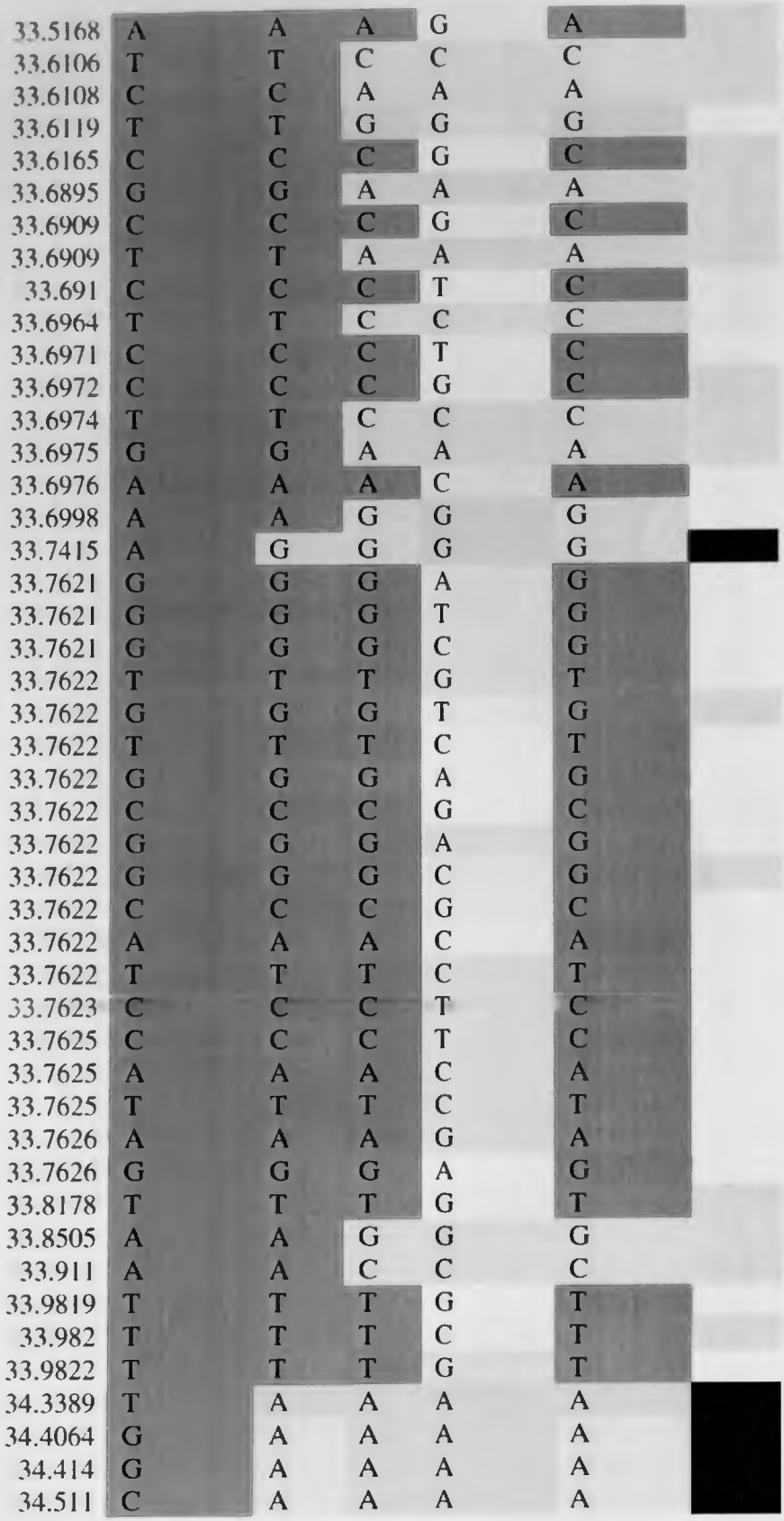
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33.6165 C C C G C
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33.6909 C C C G C
33.6909 T T A A A
33.691 C C C T C
33.6964 T T C C C
33.6971 C C C T C
33.6972 C C C G C
33.6974 T T C C C
33.6975 G G A A A
33.6976 A A A C A
33.6998 A A G G G
33.7415 A G G G G
33.7621 G G G A G
33.7621 G G G T G
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33.7622 G G G A G
33.7622 C C C G C
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33.7622 C C C G C
33.7622 A A A C A
33.7622 T T T C T
33.7623 C C C T C
33.7625 C C C T C
33.7625 A A A C A
33.7625 T T T C T
33.7626 A A A G A
33.7626 G G G A G
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33.8505 A A G G G
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33.9819 T T T G T
33.982 T T T C T
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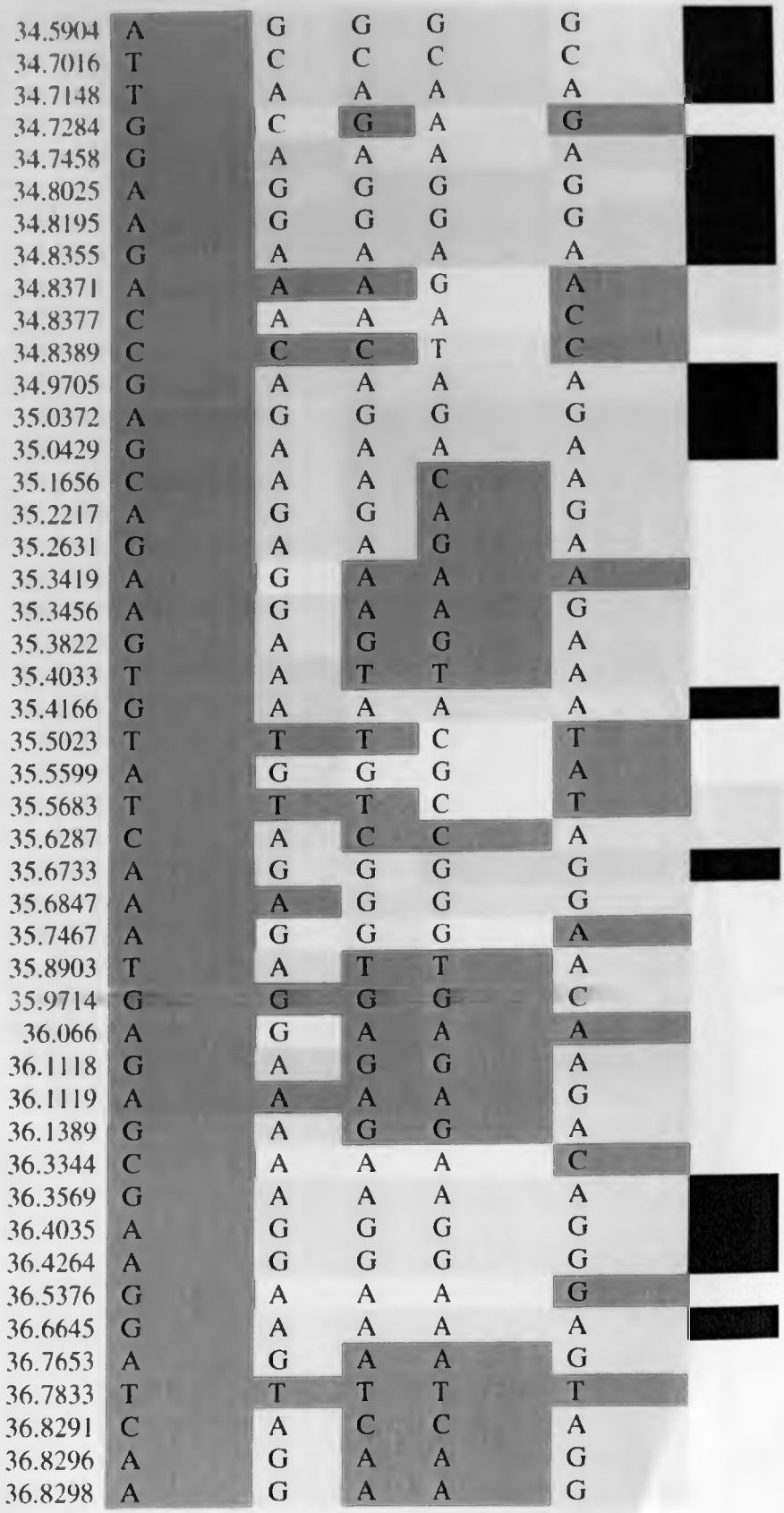

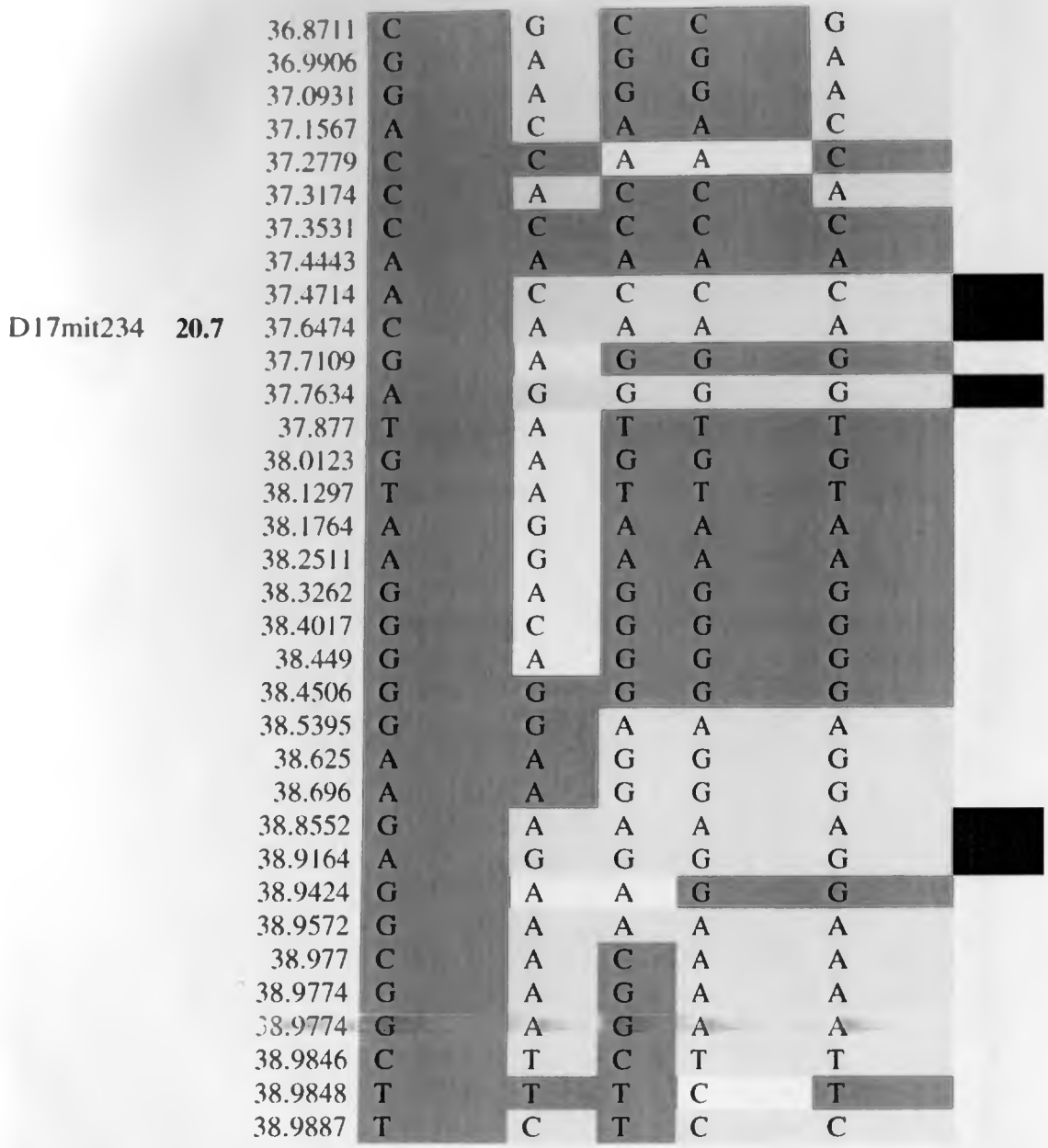
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37.1567 A C A A C
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37.3174 C A C C A
37.3531 C C C C C
37.4443 A A A A A
37.4714 A C C C C
37.6474 C A A A A
37.7109 G A G G G
37.7634 A G G G G
37.877 T A T T T
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38.1297 T A T T T
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38.4506 G G G G G
38.5395 G G A A A
38.625 A A G G G
38.696 A A G G G
38.8552 G A A A A
38.9164 A G G G G
38.9424 G A A G G
38.9572 G A A A A
38.977 C A C A A
38.9774 G A G A A
38.9774 G A G A A
38.9846 C T C T T
38.9848 T T T C T
38.9887 T C T C C