BATS AND THEIR ENDOPARASITES: CHARACTERISING PIPISTRELLE INFECTIONS AND TOLL-LIKE RECEPTOR (TLR2 AND TLR4) GENE VARIATIONS

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Table of Contents:

Table of Figures:

Table of Tables:

Statement of Authorship:

I hereby certify that I am the sole owner of this thesis. The work contained in this thesis has not been previously submitted for a degree or diploma at any other University or Institution. To the best of my knowledge, this thesis contains no material previously published or written by another author except where due reference has been made in accordance with standard referencing practices.

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Abstract:

Bats are unique mammals since they are able to fly and due to their crucial ecosystem roles, they are designated as keystone species. However, in many parts of the world, it is difficult to study bats due to the existence of protective legislation caused by their threatened status. Consequently, bat endoparasite studies are limited and even less is known about the bat immune system. To address this paucity of knowledge, this study was conducted using 99 pipistrelle bats (*Pipistrellus pipistrellus*, n=93 and *P. pygmaeus*, n=6 bats) that were obtained opportunistically from the Greater Manchester and Lancashire region between September 2005 and September 2008. These bats were infected with several species of helminths and protozoan parasites as previously described (Lord, 2010; Dodd et al., 2014).

The data within this thesis describes further characterisation of the protozoan infections in this pipistrelle population through development of PCR-based molecular typing tools. This approach has allowed the molecular differentiation between *Trypanosoma dionisii* and *T. vespertilionis* infections, confirmed that all eimerian infections were caused by *Eimeria rioarribaensis* and also confirmed that *Bartonella* sp. infection is most likely to be non-zoonotic. In addition, *Cryptosporidium sp.* and *Borrelia* sp. infection data is presented; the former being the first report in a UK bat. Analysis of the infection profiles with respect to bat genotyping data (Dodd et al., 2014) shows that the parasites are randomly distributed with the exception of the *E. rioarribaensis* infections which appear to cluster in a sub-population of pipistrelles that are genetically more homogeneous.

Since Toll-like receptors (TLRs) are an important element of the mammalian innate immune system, a PCR strategy was developed to isolate TLR4 and TLR2 genes from the pipistrelle bats (n=59). The TLR4 sequences were highly variable at the amino acid level (haplotypes, n=42), and a phylogenetic analysis of the protein sequences showed that they clustered into 7 major groups. Analysis of infection profiles in these bats showed that two

TLR4 clusters appeared to correlate with susceptibility to trypanosomes (cluster 6) and *Toxoplasma gondii* (cluster 3). In addition, bats in TLR4 cluster 6 had a significantly reduced helminth burden. The TLR2 sequences were more conserved at the amino acid level (haplotypes, n=5); however, 7 bats were heterozygous at the TLR2 locus and interestingly, these correlated with a significantly reduced helminth burden.

Overall, this thesis highlights the difficulty of studying bat endoparasites and this is often confounded by the lack, or absence, of parasitic material to assist developing molecular-based tools. Despite this difficulty, interesting data have been generated with respect to the pipistrelle genetics, including Toll-like receptor variations, and eimerian, trypanosome, *T. gondii* and helminth infection profiles, and this is worthy of further detailed investigations.

1.1 General overview:

Bats (Chiroptera) most likely evolved from a shrew-like animal that climbed trees (Richardson, 2002) and they are part of the superorder Laurasiatheria, forming a sister group to the Fereuungulata (Tsagkogeorga, Parker, Stupka, Cotton, & Rossiter, 2013). Bats have become the most diverse, abundant and geographically dispersed order amongst the class Mammalia. Indeed of the estimated 4600 species of mammals, 925 species are bats (20%) and in the UK, there are 18 species of bat, of which 17 are known to breed locally (Calisher, Childs, Field, Holmes, & Schountz, 2006; Bat Conservation Trust, 2013). There are two suborders: the Yinpterochiroptera (megabats and rhinolophoid microbats) and the Yangochiroptera (all remaining microbats) and these separated approximately 64 million years ago (Teeling et al., 2005). Bats are unique mammals since they are the only ones that can truly fly and this has facilitated them forming colonies almost everywhere in the world except for Antartica and some isolated Oceanic islands. Not surprisingly, the tropics have the greatest variety of bat species; for example, 175 species are present in Indonesia, and Central and South America are home to approximately one-third of all bat species.

As bats have important roles in many environments they are designated as keystone species (Kunz, Braun de Torrez, Bauer, Lobova, & Fleming, 2011; Mehr et al., 2011; Scott, McLaren, Jones, & Harris, 2010). Indeed, some plants depend in part, or wholly, on bats to pollinate their flowers or spread their seeds and insectivorous bats help to control insect populations by eating them. For example, a common pipistrelle can eat up to 3000 tiny insects in a single night (Kunz et al., 2011; Mehr et al., 2011; Scott et al., 2010). Due to such ecosystem roles, in the UK and other countries, bats act as an 'indicator' species since any significant changes in bat populations can indicate changes to other aspects of biodiversity (Mehr et al., 2011; Scott et al., 2010).

Given this importance, bats are protected by legislation in many areas of the world. This provides bats some protection against threats such as habitat loss and pesticides (Bat Conservation Trust, 2013). However, it also means that the study of bats and the infectious agents that they are host to, including parasites, is difficult.

1.2 Pipistrelle bats:

In the UK, all bats (Table 1.1) are insectivorous and the most numerous species is *Pipistrellus pipistrellus*, the common pipistrelle, which has become well-adapted to urbanized environments where it is often found roosting in crevices around the outside of houses and buildings. Although worldwide there are many species of pipistrelle bat (Richardson, 2002), in the UK there is just three species; the common pipistrelle, the soprano pipistrelle (*P. pygma*eus) and the Nathusius' pipistrelle (*P. nathusii*). Morphological differentiation of these pipistrelle species is not trivial and indeed, the former two were only recognized as separate species following studies on their biology in the mid-1990s (Barratt, Deaville, Burland, & Bruford, 1997; Jones & Van Parijs, 1993; Park, Altringham, & Jones, 1996). The common and soprano pipistrelles (Figure 1.1) are most readily distinguished with the use of a bat detector since *P. pipistrellus* echolocates at 45 kHz while *P. pygmaeus* echolocates at 55 kHz (Bat Conservation Trust, 2013).

Table 1.1: UK bat species (Bat Conservation Trust, 2013)

UK bat species	General comments	Numbers in UK
Alcathoe bats (*Myotis alcathoe*)	Confirmed as resident in 2002; looks similar to whiskered and Brandt's bat species	No data
Barbastelle bats (*Barbastella barbastellus*)	A rare and distinctive species with a pug- like face and a large, wide ears	5000 (Harris, Morris, Wray, & Yalden, 1995)
Bechstein's bats (*Myotis bechsteinii*)	One of the rare species, found in England and South east Wales	No data

Brown long- eared bats (*Plecotus auritus*)	Has sensitive hearing due to a very large ears	245,000 (Harris et al., 1995)
Brandt's bats (*Myotis brandtii*)	Similar to whiskered bat species, being separated as distinct species in 1970	30,000 (Harris et al., 1995)
Common pipistrelle (*Pipistrellus pipistrellus*)	The most common species in the UK, weigh around 5 g	2,430,000 (Battersby, 2005)
Daubenton's bat (*Myotis daubentonii*)	Known as ' water bats' , can pray on insects from the water surface by using their large feet or tail	560,000 (Harris et al., 1995)
Greater horseshoe bat (*Rhinolophus ferrumequinum*)	Has a unique horseshoe- shaped noseleaf	>6600 (Battersby, 2005)
Grey long-eared bat (*Plecotus austriacus*)	Slightly larger than the brown long-eared bats, has a dark face	50,000 (Battersby, 2005)
Leisler's bat (*Nyctalus leisleri*)	Known as 'hairy-armed bats'	No data
Lesser horseshoe bat (*Rhinolophus hipposideros*)	Can cover the body completely with its wings while resting	No data
Nathusius' pipistrelle (*Pipistrellus nathusii*)	Classed as a resident in 1997	16,000 (Battersby, 2005)
Natterer's bat (*Myotis nattereri*)	Can fly slowly since it has broad wings	148,000 (Harris et al., 1995)
Noctule bat (*Nyctalus noctula*)	The biggest bats in the UK, can fly in straight line, high and fast since it has long narrow wings	50,000 (Harris et al., 1995)
Serotine bats (*Eptesicus serotinus*)	Has broad wings and leisurely flapping flight	15,000 (Harris et al., 1995)
Soprano pipistrelle bat (*Pipistrellus pygmaeus*)	Similar to the common pipistrelle but differentiated by its high frequency echolocation calls	1,300,000 (Battersby, 2005)

Whiskered bat (*Myotis mystacinus*)	Smaller than the Brandt's bat but shares the same shaggy fur	64,000 (Harris et al., 1995)
Greater mouse- eared bat (*Myotis myotis*)	Declared extinct in 1990 but a solitary individual has been hibernating in southern England since 2002	No data

Figure 1.1: The soprano (left) and common (right) pipistrelles (Bat Conservation Trust, 2013)

Pipistrelle bats have adapted to live in proximity to humans and this had proven to be successful in terms of their survival. Indeed, in the UK, modern houses have become common places for pipistrelles to roost in during the summer and to hibernate in throughout the winter.

A study by Racey et al. (2005) found that there was a significant pattern of genetic isolation by distance in European bats, including *P. pipistrellus* and *P. pygmaeus*, suggesting that mating might occur before the autumn migration. In addition, there were differences in the genetic population structure between different colonies of the pipistrelles (Racey et al., 2007). A sub-population (n=71) of the *P. pipistrellus* bats studied in this thesis were genotyped using eleven polymorphic loci and the data indicated that the majority of the specimens

(n=59) were most likely derived from a large interbreeding group and the remainder (n=12) were of a mixed genotype origin (Dodd et al., 2014).

The average life span of a pipistrelle in Europe is 12 years (Schober & Grimmberger, 1989). Although pipistrelles are widely distributed across the UK and Europe, their population has declined in the 20th century, mainly due to agricultural intensification. Nonetheless, a study by Wickramasinghe et al. (2003), reported that the main bat species on both conventional and organic farms are the common and soprano pipistrelles.

1.2.1: Summary of work done on the South Lancashire bat set:

* Bat samples were screened for the presence of helminths using classical and molecular approaches (Lord et al, 2012).

* Bat samples were screened for the presence of protozoan parasites: *Trypanosome* sp., *Eimeria* sp., *Babesia vesperuginis* (lord, 2010), and *Toxoplasma gondii* (Dodd et al, 2012) using molecular approach.

* Bat samples were screened for the presence of *Bartonella* infections using molecular approach (Lord, 2010).

* Host genotypes were done using eleven polymorphic loci (Dodd et al, 2012).

1.3 Threats to Bats

1.3.1 Anthropogenic:

Bat populations have decreased due to habitat loss and the use of pesticides and preservatives in timber and homes where many roost (Bat Conservation Trust, 2013). Different bat species, including the pipistrelles, roost in buildings and they are in danger due to human activities such as building works (Bat Conservation Trust, 2013). Agricultural intensification is also a major cause of the decline of many bat populations because of the high level use of

agrochemicals on many farms (Wickramasinghe, Harris, Jones, & Vaughan, 2003). In addition, bats are being killed in increasing numbers due to the increasing installation of wind turbines (Cryan, 2011).

Climate change is likely to be a major cause of bat stress and hence population reduction. For example, in the previous 15 years, about 30,000 flying foxes in Australia, the biggest bats in the world, were affected by heat stress during the summer months, when the daytime temperature increased to more than 100 ^{o}F (Welbergen, Klose, Markus, & Eby, 2008). For bats that rely on nectar fruits, the extreme weather caused changes in plant flowering and this put them the bats under additional stress by creating problems with their food sources (Welbergen et al., 2008).

A number of studies have also focused on the effect of bat exposure to heavy metals such as mercury, lead and cadmium as these elements are readily transferred through insectivore food chains (Walker, Simpson, Rockett, Wienburg, & Shore, 2007). Indeed, toxic metals are bioaccumulated by insectivorous mammals and since accumulation risk correlates with age, bat populations are at risk of toxicity; however, there have been few studies carried out in bats (Walker et al., 2007). In the UK, quantifiable levels of renal mercury, lead and cadmium were reported in 272 bats from South-West England. In pipistrelle bats, levels of toxic metals (Cd, Pb) and trace metals (Cu, Zn) have recently been determined in multiple tissues of 193 pipistrelle bats using ICP-MS (Hernout et al., 2016). The data showed that 21% of the bat population contained residues of at least one metal in sufficiently high concentration to elicit a toxic effect and hence metal contamination should be considered an environmental stressor that has major impact on bat populations (Hernout et al., 2016).

1.3.2 Natural:

A major reason for the recent decline of large numbers of bats is the emergence of White Nose Syndrome (WNS). This fungal disease has caused the death of at least 1 million bats in North America since 2006 (Foley, Clifford, Castle, Cryan, & Ostfeld, 2011). The fungus grows on the faces and wings of infected bats and causes physiological perturbations, including the water-electrolyte balance and altered torpor during hibernation (Reeder et al., 2012; Warnecke et al., 2012). Indeed, in some hibernation sites in the US, bat numbers have declined between 81-97% since 2006 when the disease was initially identified (Blehert et al., 2009). The fungus associated with White Nose Syndrome, *Pseudogymnoascus destructans* (formerly *Geomyces destructans*), has also been found in some European bats. The presence of the disease has been confirmed in six European countries: France, Hungary, Switzerland and Slovakia (1–2 location(s) per country), Germany (8 sites) and in the Czech Republic (23 sites) (Puechmaille et al., 2012). In the UK, there is a need to raise awareness of WNS among wildlife workers and cavers in order to identify and respond to any positive cases quickly. As such, a pilot project is currently in progress in the UK to check bats for possible White Nose Syndrome infections (Bat Conservation Trust, 2013). The first case of this disease was confirmed in the UK in July 2013, which was in a hibernation site in South East England (Bat Conservation Trust, 2013).

Another major natural threat to bats is predation by cats. Bats can be captured and eaten by cats, or escape with injury which may subsequently lead to death. Indeed, in a recent study on the causes of death in European bats, 15% of all bat mortality in Germany was documented as a direct consequence of cat predation (Kristin Mühldorfer et al., 2011).

1.3.2.1 Viral infection:

In European bats, adenovirus (Ad-2) and the European bat lyssavirus (EBLV-1) were documented as the cause of mortality for 1.2% of bat deaths studied in Germany (Kristin

Mühldorfer et al., 2011). Moreover, the fact that bats are carriers of lyssaviruses is a cause of concern for the general public. Indeed, in the UK, two bat workers have died of rabies infection following Daubenton's bat bites that caused transmission of European bat lyssavirus type 2 (Fooks et al., 2003).

1.3.2.2 Bacterial infection:

Bacterial infections were documented as the cause of mortality in 12.5% of European bats surveyed post-mortem in Germany (Kristin Mühldorfer et al., 2011). Moreover, there was a strong correlation between the predominant bacterium, *Pasteurella* spp., and cat predation. Other bacteria noted in the bats included *Salmonella enterica*, *Staphylococcus aureus* and *Escherichia coli*. As most of the bacterial species were classified as opportunistic pathogens, it is likely that the bats that succumbed to these infections were most likely also suffering due to injury and/or a compromised immune system (Kristin Mühldorfer et al., 2011).

Although arthropod transmitted bacteria such as *Bartonella* spp. and *Borrelia* spp. commonly infect bats, it appears that most infections are relatively well tolerated (Concannon, Wynn-Owen, Simpson, & Birtles, 2005; Evans, Bown, Timofte, Simpson, & Birtles, 2009; K Mühldorfer, 2013). Nonetheless, these reports highlight the potential that bats may play in acting as a disease reservoir for other wildlife, domestic animals and potentially, humans (D'Auria et al., 2010).

1.3.2.3 Parasite infection:

Bats are host to many different helminth and protozoan infections, some of which can cause harm; for example, the piroplasm, *Babesia vesperuginis*, is reported to cause splenomegaly, a reduction in haemoglobin level, and elevated reticulocyte levels (R. Gardner & Molyneux, 1987). Also, severe intestinal trematode infection, disseminated nematode infection and renal coccidiosis can cause death to bats; albeit, the numbers reported in the German bat post-mortem study are relatively low (0.5% of total deaths) (Kristin Mühldorfer et al., 2011). As

such, most parasites appear to be well tolerated by bats which indicates a long established association between the host and the parasites (Kristin Mühldorfer et al., 2011).

1.4 Bats as reservoir of zoonotic disease:

Unlike other mammals, bats appear to harbour many viruses without appearing to suffer detrimental health effects. For example, Hendra and Nipah viruses which have high mortality rates in other mammals, including humans, are tolerated by bats (Middleton et al., 2007; Williamson, Hooper, Selleck, Westbury, & Slocombe, 2000). The reasons for this toleration are unclear; however, O'Shea et al., (2014), suggested that the ability of bats to fly, which is not exhibited by any other mammals, might play an important role in the co-existence of bats and viruses. The "flight-as-fever" hypothesis proposes that the increased metabolism and high temperature experienced during flight might act as an adjuvant of the bat immune system, providing bats a selective force against virulence and hence allowing them to control viral infections (O'Shea et al., 2014). An alternative reason for the ability of bats to tolerate different infections without being ill is the co- evolution which means that the ancient origin of bats deduced for certain infection such as henipavirus and lyssaviruses suggested a long history of cospeciations (Calisher et al, 2006). This long history of infection might play an important role in the co-existence of bats and viruses. Another alternative is the immune system of bats that seems to have better ability in infections recognition which help bat to be tolerant of many infection agents. Despite little known about the immune system in bats, the study by Zhou et al., (2016), showed that IFN-α genes were able to induce a subset of IFN-stimulated genes linked to antiviral activity and so they may be crucial to bats ability to tolerate viral infections (Zhou et al., 2016). The interferon regulatory factor 7 (IRF7), a key regulator of IFN responses, was also found to be constitutively expressed in a range of immune and non-immune cells of *P. alecto* and activated by double-stranded RNA (Zhou et al., 2011).

Indeed, there are many different viruses that can infect bats including SARS, Ebola, Nipah, Hendra, Rabies and related lyssaviruses, that can be highly pathogenic when transmitted from bats to other mammals, including humans (Calisher et al., 2006)(Table 1.2). European Bat Lyssavirus (EBLV) is responsible for rabies and the most common type present in European bats is type 1 (Calisher et al., 2006). Unlike classical rabies, the bat virus rarely infects animals other than bats (Brookes et al., 2005; Calisher et al., 2006) and across Europe, 700 bats have been confirmed to be infected with lyssavirus (Amengual, Bourhy, López-Roig, & Serra-Cobo, 2007; Calisher et al., 2006).

In the UK, European Bat Lyssavirus has been rarely detected; after a comprehensive screening programme (n=11,500), only 14 bats, all of which were Daubenton's, were confirmed positive (Johnson et al., 2016). Furthermore, The Veterinary Laboratories Agency has screened more than 6000 bats over the past 20 years and reported only 6 bats infected with EBLV (Johnson et al., 2016; Bats Conservation Trust, 2013). These infected bats were again Daubenton's and they were infected with EBLV-type 2. The VLA has never identified any rabies virus infection in pipistrelle bats, which are the most common species in the UK (Johnson et al., 2016; Bats Conservation Trust, 2013). Given the apparent low prevalence of EBLV in UK bats, it is perhaps surprising that any UK rabies cases due to bat-human transmission have occurred. However, as documented above (1.3.2.1), two bat volunteer workers have died following Daubenton's bites (Fooks et al., 2003) and hence members of the public must take precautions if they are involved in occupations that involve working with this species of bat.

With respect to bacterial infections, *Bartonella* spp. and *Borrelia* spp. are of potential zoonotic concern since they can infect bats and possibly transmit to humans via biting arthropod vectors. Recently, a study of bats in France and Spain showed that *Bartonella* infections were present in approximately 9% of examined specimens, including the species *P.*

nathusii, *N. noctula*, *M. daubentonii*, and *M. mystacinus* (Stuckey et al., 2017). The *Bartonella* sp. detected in these bats clustered together with the zoonotic species *B. mayotimonensis* (Stuckey et al., 2017). *Borrelia* infections in bats are poorly described; however, the autopsy of a pipistrelle bat from the UK showed that the specimen was likely to have died from borreliosis and that the bacterium was closely related to known human-pathogenic *Borrelia* species responsible for causing relapsing fever in humans (Evans et al., 2009).

With respect to parasites, there is increasing evidence emerging that bats act as reservoirs for transmission of both *Trypanosoma cruzi* (Hodo et al., 2016) and *Leishmania* spp. (de Oliveira et al., 2015; Kassahun et al., 2015) infections to humans.

Table1.2: Examples of viral zoonotic pathogens in bats

Zoonotic pathogen	Bat species	Country	Reference
Rabies	Brazilian bat	Sao Paulo-Brazil	(Castilho et al., 2016)
Rabies	Daubenton's bat	UK	(R. F. Johnson et al., 2016)
Nipah	*Pteropus* bat (Flying fox)	Australia	(Calisher et al., 2006)
Nipah	*Pteropus vampyrus & P. hypomelanus*	Malaysia	(Calisher et al., 2006)
Ebola (Zaire Ebola)	*Hypsignathus monstrosus, Epomops franqueti, Myonycteris torquata* (Fruit bats)	Central African Republic, West Africa	(Calisher et al., 2006; Hassanin et al., 2016)
Hendra	*Pteropus* bat	Australia	(McMichael et al., 2017)
Coronavirus	Korean bat	Korea	(Kim et al., 2016)
Coronavirus	*Pteropus* bat	Australia	(C. Smith et al., 2016)

Table1.3: Examples of bacterial zoonotic pathogens in bats

Zoonotic pathogen	Bat species	Country	Reference
Bartonella	*P. nathusii*, *N. noctula*, *M. daubentonii*, and *M. mystacinus*	France and Spain	(Stuckey et al., 2017)
Borrelia	*pipistrelle* bat	UK	(Evans et al,2009)

1.5 Bat Parasites

1.5.1 Helminths:

The majority of the studies have shown that bats are infected with a plethora of helminths including trematodes, cestodes, and nematodes (Esteban, Oltra Ferrero, & Mas-coma, 1990; Marshall & Miller, 1979; Nahhas, Yang, & Uch, 2005). The limited reports of acanthocephalsn infections in bats (Smales, 2007) is suggestive that for many hosts, these are likely to be accidental, or paratenic infections (Gibson & McCarthy, 1987).

Although gastrointestinal helminths are generally not considered pathogenic, they are known to have an important role in influencing the host immune status and might affect the overall health status of an individual animal (Maizels & Yazdanbakhsh, 2003).

1.5.1.1 Trematodes:

Most studies have shown that trematodes are the most common class of helminth in the bat gastrointestinal tract (Table 1.4) (Ricci, 1995a, Shimalov, Demyanchik, & Demyanchik, 2002). Trematode eggs exit the mammalian host in the faeces and when they hatch, the resulting miracidium infect a snail host. After a period of development in the snail, cercariae are shed into the water and these develop into encysted metacercariae (infective stage) in a second intermediate host; for bat infections, this is most likely to be insect larvae. As such, the bat will subsequently become infected once the insect larvae mature into adults that then become part of the bat diet.

Many different trematode species have been reported in bats. For example, the study by Shimalov, Demyanchik, & Demyanchik, (2002) reported *Allasogonoporu amphoraeformisin* in *Myotis nattereri*, *Lecithodendrium linstowi* in *M. daubentonii*, and *Plagiorchis* spp. and *P. vespertilionis* in *E. serotinus* and *M. daubentonii*. Trematodes isolated from the gastrointestinal tracts of different pipistrelle bats are highlighted in Table 1.4.

1.5.1.2 Tapeworms:

Cestode eggs exit the host in the faeces and then can infect another host via direct ingestion, or, the eggs may develop in the environment into coracidium larval stages. The latter can be eaten by intermediate hosts whereupon the parasite develops into a procercoid larva. Following ingestion by a further intermediate host, the parasite develops into the infective (plerocercoid) stage which then infects the definitive host via the ingestion route. For bats, the precise route of tapeworm infection is not described though it is most likely to involve ingestion of infected insects.

Several studies have reported tapeworm infections in different bat species. For example, the study by Shimalov et al., (2002) described isolation of *Vampirolepis skrjabinariana* from five infected *Eptesicus serotinus* bats. A more recent study reported *Vampirolepis balsaci* in a *Myotis myotis* bat from Germany (Frank et al., 2015). The *Vampirolepis* tapeworm has also been reported in a number of pipistrelle bats (Table 1.4).

1.5.1.3 Nematodes:

Nematode life-cycles can be either direct, involving ingestion of eggs, or skin penetration by infective larvae, or indirect and require transmission of infective larvae by biting insects. Precise life-cycle details of bat nematodes are unknown. An example of a direct life-cycle bat infective nematode is the strongylid, *Molinostrongylus alatus*, recorded at high intensity

in *Myotis* bats (Frank et al., 2015). The bat onchocercid filarial nematode *Litomosa chiropterorum*, isolated from miniopterid bats, is an example of an indirect life-cycle bat infective nematode (Junker et al., 2009). Pipistrelle bats can be infected with nematodes transmitted by both direct and indirect routes (Table 1.4).

Table 1.4: Summary of helminth infections of pipistrelle bats. The Table was generated by searching the Host-Parasite Database at The Natural History Museum, London (Gibson, Bray, & Harris, 2005).

Parasite group	Genus	Species	Host	Locality	Reference
Acanthocephalans	*Macracanthorhynchus*	*hirudinaceus*	*Pipistrellus kuhli*	Europe	(Lanza, 1999)
Cestodes	*Hymenolepis*	*acuta*	*Pipistrellus*	Freshwater & Terrestrial - no area specified	(Nama, 1990)
	Hymenolepis	*pipistrelli*	*Pipistrellus pipistrellus, P. kuhli*	Spain + Andalusia, Iraq	(Botella, Sanchez, & Esteban, 1993; J Guillermo Esteban, Amengual, & Cobo, 2001; Nama, 1990)
	Hymenolepis	*sandgroundi*	*Pipistrellus nanus*	Ethiopian Region, Zimbabwe	(Nama, 1990)
	Staphylocystis	*syrdariensis*	*P. pipistrellus, P. pipistrellus bactrianus*	USSR (CIS), Uzbekistan	(Lanza, 1999)
	Vampirolepis	*acuta*	*Pipistrellus nathusii, P. pipistrellus*	Europe	(Lanza, 1999)
	Vampirolepis	*magnirost-ellata*	*P. pipistrellus*	Armenia	(Lanza, 1999)
	Vampirolepis	*molani*	*P. kuhli*	Iraq	(Sawada, 1990)
	Vampirolepis	*skrjabinar-iana*	*P. pipistrellus, P. kuhli, P. nathusii*	European USSR (CIS)	(Lanza, 1999)

	Vampirolepis	*urawaensiss*	*Pipistrellus abramus*	Japan, Taiwan	(Sawada, 1990)
	Raillietina	*sp.*	*P. kuhli, Pipistrellus kuhli ikhwanius*	Iraq	(Lanza, 1999)
Nematodes	*Litomosa*	*beshkovi*	*P. nathusii*	Bulgaria	(Lanza, 1999)
	Litomosa	*filaria*	*P. kuhli*	Europe	(Lanza, 1999)
	Litomosa	*ottavianii*	*P. pipistrellus*	Spain + Andalusia	(Botella et al., 1993; Lanza, 1999)
	Thelandros	*alatus*	*P. kuhli*	Iraq	(Lanza, 1999)
	Physaloptera	*brevivaginata*	*P. kuhli*	Algeria, Iraq	(Lanza, 1999)
	Physaloptera	*myotis*	*P. nathusii*	Europe	(Lanza, 1999)
	Physaloptera	*sp.*	*P. pipistrellus*	Hungary	(Lanza, 1999)
	Pseudophysalopte-ra	*sp.*	*P. kuhli*	Iraq	(HASSAN, SALIH, & ABDULLAH, 1993; Lanza, 1999)
	Longibucca	*eptesica*	*Pipistrellus subflavus*	Virginia	(Measures, 1994)
	Seuratum	*Mucronatum*	*P. pipistrellus*	Europe	(Lanza, 1999)
	Agamospirura	*sp.*	*P. pipistrellus*	Europe, Ukraine, incl. Moldavia	(Lanza, 1999)
	Ascarops	*strongylina*	*P. pipistrellus*	Europe	(Lanza, 1999)
	Physocephalus	*sexalatus*	*P. kuhli, P. nathusii, P. pipistrellus*	Europe	(Lanza, 1999)
	Spirocerca	*lupi*	*P. kuhli*	Europe	(Lanza, 1999)
	Capillaria	*italica*	*P. pipistrellus*	Europe	(Lanza, 1999)
	Capillaria	*neopulchra*	*P. nathusii*	Europe	(Lanza, 1999)
	Capillaria	*palmata*	*P. subflavus*	Louisiana	(Lotz & Font, 1991)
	Capillaria	*pipistrelli*	*Pipistrellus javanicus abramus*	Japan	(Lanza, 1999)
	Capillaria	*romana*	*P. pipistrellus*	Europe	(Lanza, 1999)
	Molinostrongylus	*alatus*	*P. pipistrellus*	Europe	(Lanza, 1999)
	Molinostrongylus	*rhinolophi*	*P. pipistrellus*	Palearctic Region	(Lanza, 1999)
	Molinostrongylus	*skrjabini*	*P. pipistrellus*	Palearctic Region, European USSR (CIS)	(Lanza, 1999)

	Molinostrongylus	*vespertilionis*	*P. nathusii, P. pipistrellus*	Europe, European USSR (CIS), Hungary, Ukraine, incl. Moldavia, Bulgaria	(GENOV, Stoykova-Hajinikolova, & MÉSZÊROS, 1992; Lanza, 1999; Matskási, Mészáros, Murai, & Gubányi, 1996; V. Tkach & Sharpilo, 1988)
Trematodes	*Anchitrema*	*Sanguineum*	*P. kuhli*	Freshwater & Terrestrial - no area specified	(Lanza, 1999)
	Brachylaima	*aristotelis*	*P. pipistrellus*	Europe	(Lanza, 1999)
	Heterophyes	*Heterophyes*	*P. kuhli*	Southern Yemen	(Lanza, 1999)
	Acanthatrium	*eptesici*	*P. subflavus*	Indiana	(Pistole, 1988)
	Acanthatrium	*pipistrelli*	*P. subflavus*	Indiana, Louisiana, Minnesota	(Lotz & Font, 1991; Pistole, 1988)
	Allassogonoporus	*amphoraef-ormis*	*P. kuhli*	Ukraine, incl. Moldavia	(V. Tkach, 2000; V. V. Tkach, Littlewood, Olson, Kinsella, & Swiderski, 2003)
	Allassogonoporus	*marginalis*	*P. subflavus*	Indiana	(Pistole, 1988)
	Lecithodendrium	*duboisi*	*P. abramus*	China	(Qu & Gong, 1992)
	Lecithodendrium	*granulosum*	*P. kuhli, P. nathusii, P. pipistrellus, Pipistrellus savii*	Italy, Europe, European USSR (CIS), Ukraine, Hungary	(Ricci, 1995a)
	Lecithodendrium	*linstowi*	*P. kuhli, P. nathusii, P. pipistrellus, P. abramus*	China, Ukraine, incl. Moldavia, Italy, European USSR (CIS)Europe, Spain + Andalusia, Hungary	(Botella et al., 1993; Qu & Gong, 1992; Ricci, 1995a)

	Lecithodendrium	*longitudinalle*	*P. abramus*	China	(Qu & Gong, 1992)
	Lecithodendrium	*macrostomum*	*P. abramus*	China	(Qu & Gong, 1992)
	Lecithodendrium	*microrchle*	*P. abramus*	China	(Qu & Gong, 1992)
	Lecithodendrium	*multiglandum*	*P. abramus*	China	(Qu & Gong, 1992)
	Lecithodendrium	*mystacini*	*P. pipistrellus*	Spain + Andalusia	(Botella et al., 1993)
	Lecithodendrium	*petalinum*	*P. abramus*	China	(Qu & Gong, 1992)
	Lecithodendrium	*rohdei*	*P. abramus*	China	(Qu & Gong, 1992)
	Lecithodendrium	*rysavy*	*P. kuhli, P. nathusii, P. pipistrellus*	USSR (CIS), Ukraine, incl. Moldavia,	(Lanza, 1999)
	Lecithodendrium	*semen*	*P. abramus*	China	(Qu & Gong, 1992)
	Lecithodendrium	*shanghaiense*	*P. abramus*	China	(Qu & Gong, 1992)
	Lecithodendrium	*sinense*	*P. abramus*	China	(Qu & Gong, 1992)
	Lecithodendrium	*skrjabini*	*P. nathusii*	European USSR (CIS), Georgia	(Lanza, 1999)
	Lecithodendrium	*spathulatum*	*P. abramus*	China	(Qu & Gong, 1992)
	Lecithoporus	*macralaimus*	*P. kuhli, P. nathusii*	European USSR (CIS)	(Lanza, 1999)
	Limatulum	*oklahomense Macy*	*P. subflavus*	Louisiana	(Lotz & Font, 1991)
	Mesodendrium	*macrostomum*	*P. abramus*	Japan	(Shimazu, 1923, 1995)
	Mesodendrium	*spathulatumm*	*P. abramus*	Japan	(Shimazu, 1923, 1995)
	Ochoterenatrema	*breckenridgei*	*P. subflavus*	Indiana	(Pistole, 1988)
	Ochoterenatrema	*diminutum*	*P. subflavus*	Indiana, Louisiana	(Lotz & Font, 1991; Pistole, 1988)
	Ochoterenatrema	*labda*	*P. subflavus*	Louisiana	(Guzmán-Cornejo, García-Prieto, Pérez-Ponce de León, & Morales-Malacara, 2003; Lotz & Font, 1991)
	Ophiosacculus	*mehelyi*	*P. pipistrellus*	Europe	(Lanza, 1999)

	Parabascus	*duboisi*	*P. pipistrellus*	Ukraine, incl. Moldavia, Europe	(Lanza, 1999)
	Parabascus	*lepidotus*	*P. kuhli, P. nathusii, P. pipistrellus*	European USSR (CIS), Ukraine, incl. Moldavia, Europe	(Lanza, 1999; V. Tkach, 2000)
	Parabascus	*semisqua-mosus*	*P. kuhli, P. nathusii, P. pipistrellus*	Ukraine, incl. Moldavia, Europe, Spain + Andalusia	(Botella et al., 1993; V. Tkach, 2000; V. V. Tkach et al., 2003)
	Paralecithodendrium	*singularium*	*P. subflavus*	Indiana	(Pistole, 1988)
	Prosthodendrium	*ascidia*	*P. kuhli, P. nathusii, P. pipistrellus*	European USSR (CIS), Europe, Spain + Andalusia, Hungary	(Botella et al., 1993)
	Prosthodendrium	*chilostomum*	*P. kuhli, P. nathusii, P. pipistrellus*	Europe, European USSR (CIS), Ukraine, incl. Moldavia, Hungary	(V. Tkach, 2000)
	Prosthodendrium	*ilei*	*P. nathusii, P. pipistrellus*	Ukraine, incl. Moldavia, Europe	(V. Tkach & Sharpilo, 1988)
	Prosthodendrium	*longiforme*	*P. kuhli, P. abramus*	Ukraine, incl. Moldavia, China	(Qu & Gong, 1992; V. Tkach, 2000)
	Prosthodendrium	*megacotyle*	*Pipistrell-us javanicus , P. pipistrell-us*	Japan, Europe	(Lanza, 1999)
	Prosthodendrium	*mehrai*	*P. abramus*	China	(Qu & Gong, 1992)
	Prosthodendrium	*ovimagnosum*	*P. abramus*	China	(Qu & Gong, 1992)
	Prosthodendrium	*travassosi*	*P. kuhli, P. nathusii*	European USSR (CIS)	(Lanza, 1999)
	Prosthodendrium	*glandulosum*	*P. kuhli*	Freshwater & Terrestrial - no area specified	(Lanza, 1999)
	Prosthodendrium	*nyctali*	*P. pipistrellus*	Kazakstan, USSR (CIS)	(Lanza, 1999)

	Prosthodendrium	*skrjabini*	*P. nathusii*	USSR (CIS)	(Lanza, 1999)
	Prosthodendrium	*gerhardi*	*Pipistrellus dormeri*	India	(GANDHI, 1989)
	Prosthodendrium	*longiforme*	*P. kuhli*	Europe	(Lanza, 1999)
	Prosthodendrium	*macnabi*	*Pipistrellus mimus mimus*	India	(GANDHI, 1989)
	Prosthodendrium	*minus*	*P. kuhli*	India	(Lanza, 1999)
	Prosthodendrium	*mizellei*	*P. mimus mimus*	India	(GANDHI, 1989)
	Prosthodendrium	*parvouterus*	*P. nathusii, P. pipistrellus*	Europe	(Lanza, 1999)
	Prosthodendrium	*pyramidum*	*P. kuhli*	Egypt, Southern Yemen	(Lanza, 1999)
	Prosthodendrium	*urna*	*P. kuhli*	Freshwater & Terrestrial - no area specified	(Lanza, 1999)
	Pycnoporus	*heteroporus*	*P. kuhli, P. savii, P. pipistrellus*	Italy, Ukraine, incl. Moldavia, Spain + Andalusia, Hungary	(Ricci, 1995a; V. Tkach, 2000; V. V. Tkach et al., 2003)
	Pycnoporus	*macrolaimus*	*P. kuhli*	Ukraine, incl. Moldavia	(V. Tkach, 2000)
	Pycnoporus	*mansouri*	*P. kuhli*	Egypt	(Lanza, 1999)
	Pycnoporus	*megacotyle*	*P. kuhli*	Ukraine, incl. Moldavia	(V. Tkach, 2000; V. V. Tkach et al., 2003)
	Pycnoporus	*skarbilovichi*	*P. abramus*	China	(Lanza, 1999)
	Pycnoporus	*transversus*	*P. abramus*	Japan	(Shimazu, 1923, 1995)
	Pycnoporus (Lecithoporus)	*macrolaimus*	*P. nathusii, P. pipistrellus P. kuhli*	Europe	(Lanza, 1999)
	Pycnoporus	*acetabulatus*	*P. pipistrellus P. kuhli*	Spain + Andalusia, Hungary, Ukraine, incl. Moldavia, Palearctic Region	(Botella et al., 1993; Sharpilo & Iskova, 1989)

	Pycnoporus	*heteroporus*	*P. pipistrellus P. kuhli*	Spain + Andalusia, Hungary, Ukraine, incl. Moldavia, Palearctic Region	(Botella et al., 1993; Sharpilo & Iskova, 1989)
	Mesotretes	*peregrinus*	*P. pipistrellus*	Spain + Andalusia, Europe	(Botella et al., 1993)
	Plagiorchis	*koreanus Ogata*	*P. pipistrellus P. kuhli*	Ukraine, incl. Moldavia, Europe	(V. Tkach, 2000)
	Plagiorchis	*micracanthos*	*Pipistrellus hesperus, Pipistrellus subflavus subflavus*	Nevada, Nebraska	(Alberta. Alberta Agriculture, Development, & KENNEDY, 1988)
	Plagiorchis	*muelleri*	*P. pipistrellus*	Ukraine, incl. Moldavia	(V. Tkach, 2000)
	Plagiorchis	*vespertilionis*	*P. pipistrellus P. kuhli, P. subflavus, P. nathusii*	Ukraine, incl. Moldavia, European USSR (CIS), Italy, Indiana, Freshwater & Terrestrial - no area specified	(Pistole, 1988; Ricci, 1995a)
	Plagiorchis	*pipistrelli-cola*	*Pipistrellus mimus*	Freshwater & Terrestrial - no area specified	(Ricci, 1995a)
	Urotrema	*scabridum braun*	*P. subflavus*	Louisiana, Freshwater & Terrestrial - no area specified	(Guzmán-Cornejo et al., 2003; Lotz & Font, 1991)

1.5.1.4 UK Helminths:

The study conducted at The University of Salford on the population of Lancashire pipistrelles analysed further in this thesis showed 68 out of 90 bats (76% prevalence) were infected with at least 1 species of helminth (Lord, Parker, Parker, & Brooks, 2012). All the helminths were digenean trematodes and 5 species were found in the 68 infected specimens: *Lecithodendrium linstowi* (80.4%), *L. spathulatum* (19.6%), *Prosthodendrium sp.* (35.3%), *Plagiorchis koreamus* (29.4) and *Pycnoporus heteoporus* (9.8%). Statistical modelling of the data from the study showed there was no difference in overall prevalence between the sexes but interestingly, the male bat infections appeared to be more aggregated than that of females, and also less abundant. The statistical modelling also showed that there was a significant increase in prevalence and abundance throughout the period from September 2005 to September 2009, indicating that environmental factors can be important in regulating infections (Lord, 2010).

1.5.2 Protozoa:

Protozoa are microscopic one-celled organisms that can be free-living or parasitic in nature. They can infect humans and animals and the infection levels vary from asymptomatic to life threatening; virulence is dependent upon the species and also, the strain (Fenchel, 2013). There are several types of protozoan parasite that can infect bats such as the haematozoa *Polychromophilus*, *Trypanosoma* and *Babesia*, and also, gastrointestinal parasites such as *Eimeria*.

1.5.2.1 Coccidia:

Coccidiosis is a parasitic infection of the intestinal tract caused by coccidian protozoans from the genera *Eimeria*, *Isospora*, *Toxoplasma*, *Cryptosporidium*, and *Sarcosystis* (Duszynski, Scott, Aragon, Leach, & Perry, 1999). The disease is transmitted between animals either by ingestion of infected faeces, or infected tissue. Most of the cases are asymptomatic; however, young or immunocompromised animals might have severe symptoms, including diarrhea which can escalate to death (Duszynski et al., 1999). Coccidia can infect different animals, including humans, birds, and livestock and infections are species specific. Coccidiosis is an economically important disease of cattle, sheep, goat, pigs, poultry, and rabbits in which the intestine, the liver and the kidneys (renal coccidiosis) can be affected.

Bats appear to be a host for multiple species of eimerian parasites. The study by Duszynski (1999), found that 29 out of 404 individual bats, representing 20 different species, were infected with eimerians, including 6 new species (Duszynski et al., 1999). A number of other bat parasite studies, from the Middle East and also North America, have also reported bat eimerian infections (Alyousif, Al-Dakhil, & Al-Shawa, 1999; McAllister, Burt, Seville, & Robison, 2011; McAllister, Seville, & Roehrs, 2012). More recently, Cryptosporidium parasites have been isolated from *Eptesicus fuscus* in the USA and a pipistrelle bat from Czech Republic (Kváč et al., 2015).

Toxoplasma gondii is another coccidian parasite that has recently been detected in bats. Two bat species, the insectivorous *Molossus molossus*, and the haematophagous common vampire bat *Desmodus rotundus*, were shown to be infected by *T. gondii* in Brazil and the parasites were isolated and genotyped with a set of PCR-RFLP markers (Cabral et al., 2013). A study in China of 626 bats, representing 10 different species, showed that *T. gondii* was detected in bats at a prevalence of 6.1 % (Jiang et al., 2014).

1.5.2.2 Kinetoplastids:

Kinetoplastids are a group of flagellate parasites which are responsible for different diseases in humans, other animals and even plants. The kinetoplastids include trypanosomes and Leishmania which are human pathogens causing devastating health impacts that include human African trypanosomiasis, Chagas disease and leishmaniasis (Stuart et al., 2008).

A number of different trypanosomatids and Leishmania species have been detected in bats, including parasites highly related to the causative agent of Chagas disease, *Trypanosoma cruzi*, designated genotype TcBat (Marcili et al., 2009; Pinto, Kalko, Cottontail, Wellinghausen, & Cottontail, 2012). Moreover, study of a human *T. cruzi* infection has confirmed that it was comprised of a mixed infection with *T. cruzi* discrete typing unit I and the *T. cruzi* of bat origin, TcBat (Ramírez et al., 2014). Recent studies have also confirmed that *Leishmania infantum, L. amazonensis* (de Oliveira et al., 2015), and *L. mexicana* (Berzunza-Cruz et al., 2015) are present in a range of bat species. Such studies clearly highlight that bats may act as important reservoirs of zoonotic infection by kinetoplastids.

1.5.2.2.1: Trypanosoma:

There are three types of stercorarian trypanosomes that can infect bats; the Herpetosoma, *Schizotrypanum*, and *Megatrypanum* (Gardner, 1986; Molyneux, 1991). The majority of bat infections are caused by *Schizotrypanum* and *Megatrypanum* and Cimex is the only known vector of these parasites (Molyneux, 1991). The *Schizotrypanum* are considered to be the most interesting because of morphological similarity to *Trypanosoma cruzi* which infects humans. However, *in vitro* study of the *Schizotrypanum* is restricted due their lack of infectivity to common laboratory animals (Molyneux, 1991).

Recently, Hamilton et al. (2012) proposed the bat seeding hypothesis which argued that the common ancestor of the *T. cruzi* clade was a bat trypanosome and that these bat

trypanosomes diversified and became geographically widespread due to host migration. Various trypanosomes within this group independently switched from bats to terrestrial mammals to initiate seeding of the terrestrial lineage of the ancestral *T. cruzi* clade (Hamilton, Teixeira, & Stevens, 2012).

1.5.2.2.1.1 Schizotrypanum:

The data from Hoare (1972), showed that bat *Schizotrypanum* (subgenus of Trypanosoma) can be differentiated from *T. cruzi* if large numbers of parasites are examined but if small numbers are examined, it cannot provide the basis for distinguishing between these parasites. *Schizotrypanum* trypomastigotes are C or S- shaped in blood smears and usually have a large terminal, or sub-terminal kinetoplast that can prevent the pointed end from being detected in stained preparations. The parasites have a free flagellum and the undulating membrane is usually not conspicuous. Also, the nucleus is usually found midway along the body of the organism. The length of bat trypanosomes is 14-24 um (Hoare, 1972). The description of two bat trypanosomes, *T. hedricki* and *T. myoti*, were explained by Bower and Woo in 1981. This description can apply to bloodstream forms of chronic infection with bat trypanosomes. Bower and Woo (1981), found that there are long slender trypomastigotes, which are present in the blood of the infected bats (Bower & Woo, 1981). They found trypomastigotes of *T. hedricki* and *T. myoti* approximately 15 days post infection by inoculation of culture forms. The slender forms have an elongated nucleus, a free flagellum and a kinetoplast, which is distant from the posterior end. The same forms were found in individuals with acute infection of *T. cruzi* (Brener, 1969; Howells & Chiari, 1975).

Amastigotes of *T. dionisii*, *T. hedricki*, and *T. myoti* in culture are similar to amastigotes of *T. cruzi* which are spherical and about 3 µm in diameter. Epimastigotes of these three parasite species vary in shape and size when observed in blood agar, or insect tissue culture medium. Bower and Woo (1981) stated that distinguishing between *T. dionisii*, *T. myoti*, *T.*

hedricki, and *T. vespertilionis* can be done by measuring the size of epimastigotes; *T. vespertilionis* is large in blood agar culture. Also, the distance between the nucleus and kinetoplast is greater in *T. vespertilionis* than the other three species. Another feature is the yellow-green pigments, which can be found in *T. myoti*, *T. hedricki* and *T. dionisii* when these are grown in blood agar media, whereas this is not found with *T. vespertilionis.* However, these pigments can be seen when the parasites are grown in medium 199 containing foetal calf serum (J. Baker, Miles, Godfrey, & Barrett, 1978).

Metacyclic stages of bat *Schizotrypanum* are also important in species identification. *Trypanosoma dionisii* usually develops dimorphic metacyclic forms; these may be short broad, or long thin forms, when the parasite is maintained in blood agar cultures. In contrast, *T. vespertilionis* develops only short broad metacyclic trypomastigotes and these forms are usually with a terminal, or sub terminal kinetoplast. *T. hedricki* and *T. myoti* have the same metacyclic forms which are long and thin (J. Baker & Thompson, 1971; R. Gardner & Molyneux, 1988a).

1.5.2.2.1.2 Megatrypanum:

There are two types of bat *Megatrypanum* that were defined by Hoare (1972): *T. megadermae* and *T. heybergi*. The *T. megadermae* kinetoplast is usually near the posterior end and it is narrow and the nucleus fills the central part of the body. In contrast, parasites of *T. heybergi* are broader and the kinetoplast and nucleus are positioned close together. There are different *Megatrypanum* that have been described such as *T. megadermae* which measures 41.2 µm in length, *T. incertum* measures 25.8 µm, *T. magnusi* measures 27.5 µm, and *T. lizae* measures 20-45 µm in length (Bandyopadhyay, Ray, & Dasgupta, 1982; Deane, Sarjeant, & Fernandez, 1978; Marinkelle, 1979). However, there have been few studies on the biology of *Megatrypanum* species of bats. Indeed, no dividing stages of *T. incertum* were found when a bat was euthanised three days after infection with *T. incertum* (Deane et al.,

1978; R. Gardner & Molyneux, 1988b; Marinkelle, 1979). Another study failed to reveal any dividing trypanosomes of *T. pessoai* which is a *T. heybergi*- like trypanosome (R. Gardner & Molyneux, 1988b; Molyneux, 1991).

1.5.2.3 UK Bat Protozoa:

A small number of classical parasitological studies have confirmed that UK bats are infected with haematozoa, including trypanosomes, *B. vesperuginis* and the haemosporidian *Polychromophilus murinus* (see references within Lord and Brooks, 2014). The most extensive bat parasite study conducted in the UK was carried out at The University of Salford in the 1980s (491 bats, representing 12 species) and many haematozoan parasites were recorded (R. A. Gardner, 1986). The most prevalent haematozoan, the *Schizotrypanum*, was detected in approximately 35% of pipistrelles examined and also, in several other bat species (R. Gardner & Molyneux, 1988b). The Megatrypanum, *T. incertum*, was also detected in pipistrelles; most of which were captured from Aberdeenshire (R. Gardner & Molyneux, 1988b). Gametocytes of the haemosporidian *Polychromophilus murinus* were found in one-third of *Myotis daubentonii* bats and the parasite was recorded in the wingless blood sucking Nycteribiid fly which likely explains the restricted host range (R. Gardner, Molyneux, & Stebbings, 1987). The piroplasm *Babesia vesperuginis* was noted in the blood of pipistrelles and *M. mystacinus* and these bats exhibited high reticulocyte counts, lowered haemoglobin levels and splenomegaly (R. Gardner et al., 1987; R. A. Gardner, 1986). A veterinary pathology study of 245 UK bats has also confirmed that severe babesiosis can, in rare cases, result in bat mortality (Simpson, 2000). The vector of *B. vesperuginis* is reported as the soft tick, *Argas vespertilionis* (R. Gardner & Molyneux, 1988b).

One of the few molecular studies carried out on UK bat haemoparasites used a PCR based strategy to confirm the presence of *B. vesperuginis*, *T. dionisii* and the haemobacterium *Bartonella* spp. in a number of bat species, including pipistrelles, from South West England

(Concannon et al., 2005). More recently, a molecular study of bat trypanosomes in southern England confirmed that *T. dionisii* was most prevalent; indeed, only a single *T. vespertilionis* infection was reported and this was in a *N. noctula* bat exhibiting a *T. dionisii*/*T.vespertilionis* mixed infection (Hamilton, Cruickshank, Stevens, Teixeira, & Mathews, 2012). Further genetic examination of these *T. dionisii* parasites confirmed that they included new genotypes: *T. dionisii* group A was present in pipistrelle bats and was identical to previously described trypanosomes; however, *T. dionisii* group B (New 1) was reported in Serotine and Whiskered bats and *T. dionisii* group B (New 2) was isolated from Noctule bats (Hamilton, Cruickshank, et al., 2012). Since the *T. dionisii* group B genotypes were genetically closer to South American strains of *T. dionisii* than they were to *T. dionisii* group A, Hamilton et al. (2012) proposed that ancient bat movements are most likely to be responsible for the observed dispersal of these trypanosomes. Indeed, such ancient bat movements are most likely a major contributor to the bat seeding hypothesis proposed to explain the evolutionary history of *T. cruzi* (Hamilton, Teixeira, et al., 2012).

An extensive study of bats from Lancashire, recently carried out at Salford University, has confirmed the presence of trypanosomes, *B. vesperuginis*, *Bartonella* sp., *Eimeria* sp. (Lord, 2010) and *T. gondii* (Dodd et al., 2014) in pipistrelles. Since this bat population was the subject of this thesis work, further details of these infections will be presented in Chapter 3.

1.6 The Mammalian Immune System: an overview

The mammalian immune system is a remarkable complex of different biochemical processes to ensure an efficient recognition and destruction of pathogen threats to host viability (Dunkelberger & Song, 2010). Mammals have evolved humoral (antibody-mediated) and cellular defense networks (Flajnik & Kasahara, 2010) that together provide an adaptive immune response to assist the innate immune system in protecting against invasive pathogens (Medzhitov, 2007). The key features of the adaptive and innate immune systems are highlighted in Table 1.4.

Table1.5: Summary of the key differences between innate and adaptive immunity

Innate immunity	Adaptive immunity
Physical and chemical barriers, phagocytic leukocytes, dendritic cells, natural killer cells, and plasma proteins (complement).	B cells, which mature into antibody secreting plasma cells. T cells, which mature into T-helper and cytotoxic T cells.
Always present.	Normally silent until activated.
Recognises any foreign pathogens: bacteria, viruses, fungi, and parasites.	Recognises highly specific antigens.
Fast response time.	Slow response time: can take 1-2 weeks.
No memory cells.	Memory cells facilitate a more rapid response upon re-exposure.

1.6.1 General innate immune system features:

The innate immune response is an evolutionary ancient system that gives multicellular organisms a quick and immediate defense against different pathogens without requiring prior exposure to these pathogens (Male, Brostoff, Routh, & Roitt, 2013; Vasselon & Detmers, 2002). The importance of the innate immune system is to recognize different structures that are present in large group of microorganisms, to activate a suitable mechanism to rapidly kill these microorganisms, and then, to activate and orientate the adaptive immune response through lymphocyte expansion (Male et al., 2013; Medzhitov & Janeway, 2000). The innate immune system is activated after a pathogen penetrates the host's physical barriers and it provides a non-specific response to a wide range of pathogens (Male et al., 2013; Medzhitov & Janeway, 2000). The innate immune response is complex and consists of biochemical and

cellular pathways which have the ability to recognize a pathogen, actively remove it and then to activate the adaptive immune response. One of the important elements of the innate immune response is transmembrane molecules that interact with microbial organisms and signal to the intracellular compartment that a cellular response is required (Male et al., 2013; Medzhitov & Janeway, 2000). Furthermore, the innate immune system recognizes the pathogen by detecting markers on them, which triggers the secretion of signaling molecules that attract other immune cells to try to fight the infection.

Phagocytes are a type of innate immune cell that ingests and degrades pathogens by expressing receptors that are able to detect pathogen associated molecular patterns (PAMPs). PAMPs are molecules that are absent from vertebrates but they are found in microorganisms (Male et al., 2013; Medzhitov & Janeway, 2000). Toll-like receptors (TLRs), which are a family of pattern recognition receptors that interact with PAMPs, are really important since they play a vital role in pathogen sensing and act as the first line of defense against infections. TLRs are located at the surface of the innate immune cells, or within the endosomes inside cells, and they recognize different PAMPs. Upon recognition, TLRs dimerise and this initiates a biochemical cascade to alert other cells to the presence of a pathogen (Male et al., 2013; Medzhitov & Janeway, 2000). Indeed, TLR interaction with PAMPs results in signaling events that activate genes that produce cytokines, such as tumor necrosis factor (TNF-α) interleukins (IL)1-6 and interferons (INF) for secretion from dendritic cells, macrophages, mast cells, and neutrophils (Male et al., 2013; Medzhitov & Janeway, 2000). Cytokines are short-lived but they can act on multiple cellular targets to assist defense against the infection. For example, macrophages secrete TNF-α which acts on vascular walls to facilitate entry of complement and antibodies into tissues to attack an infection; IL-1β assists immune cells exiting the blood and entering tissues and also, it activates lymphocyte cells and IL-6 activates lymphocytes and promotes antibody production (Male et al., 2013;

Medzhitov & Janeway, 2000). In addition, interferons are particularly important in limiting the spread of certain viral infections. IFN-α and IFN-β are secreted by infected cells and IFN-γ is released by activated T-helper cells (TH_1) (Male et al., 2013).

1.6.1.1 Toll-like receptors (TLRs):

Toll-like receptors (TLRs) are a group of receptor proteins that have a major role in the innate immune response (Netea, Brown, Kullberg, & Gow, 2008). In a process called pattern recognition, they have the ability to detect pathogen molecules by binding to pathogen-associated molecular patterns (PAMPs) (Vasselon & Detmers, 2002). TLRs are found in a variety of different cells including dendritic cells, monocytes, neutrophils and macrophages (Figure 1.2) (Netea et al., 2008).

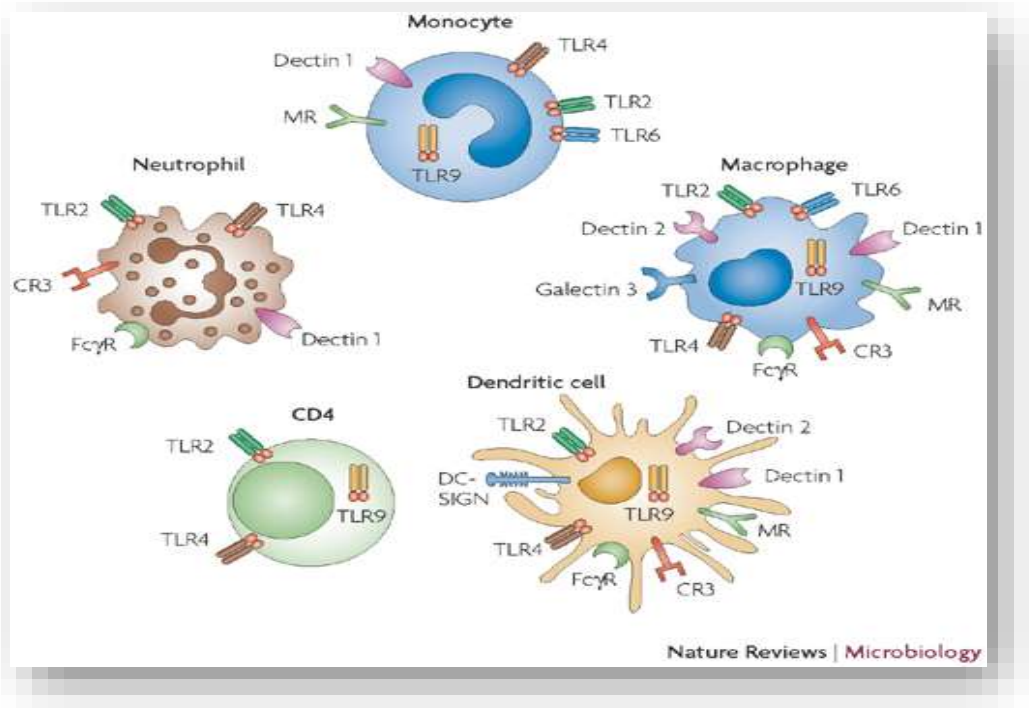

Figure1.2: Toll-like receptors associated with different innate immune response cells (Netea et al., 2008).

There are at least 10 different TLR genes in mammalian species; for example, humans have 10 (TLRs 1-10) and mice have 12 (TLRs 1-9 and TLRs 11, 12 and 13) (Hopkins & Sriskandan, 2005). The majority of TLRs exist as homodimers; however, TLR2 may form heterodimers with TLR 1 or 6 (McClure & Massari, 2014). Each TLR has a specific ligand, location and outcome and it has evolved to be able to detect a specific type of infection (Figure 1.3). In addition, the TLR may require accessory molecules to function; for example, TLR4 requires ligand binding protein (LBP), myeloid differentiation protein-2 (MD-2) and CD-14 to assist it binding to bacterial lipopolysaccharide (McClure & Massari, 2014).

TLRs 1, 2, 6, and 10, reside on plasma membranes and have the ability to detect components of microbial cell walls and membranes such as lipoproteins and peptidoglycans. TLR4 and TLR5 are also localised to the plasma membrane and recognise bacterial lipopolysaccharide (LPS) and flagellin respectively. In contrast, TLR9 is localised within the cell and detects bacterial DNA. TLRs 3, 7, 8 and 11 are also intracellular, being expressed within endosomal and lysosomal compartments (Hopkins & Sriskandan, 2005). With the exception of TLR3, all TLRs require Myeloid Differentiation Factor (MyD88) for signalling. For TLRs 4 and 2, not only is MyD88 needed but also, the cooperation of Mal (MyD88-adaptor-like)/ TIRAP (TIR domain-containing Adaptor Protein) is required for signalling (McClure & Massari, 2014). This ultimately leads to activation of NF-$_k$B and mitogen activated protein kinases (MAPKs) and then production of inflammatory cytokines and chemokines, mucins, defensins and type-II IFNs (McClure & Massari, 2014). The endosomal associated TLRs 7, 8 and 9 are also able to promote type-I IFN production via the TNF receptor associated factor protein TRAF3 (McClure & Massari, 2014). A MyD88-independent pathway is triggered by TLRs 3 and 4 and also, potentially by TLR2 (McClure & Massari, 2014). For TLRs 3 and 4, this involves signalling via the TIR domain-containing adaptor protein inducing interferon-β (TRIF), with the TLR4 pathway also requiring activation of TRAM (TRIF-related adaptor

molecule) (McClure & Massari, 2014). The MyD88-independent pathway leads to not only production of inflammatory mediators, mucins, defensins and type-II IFNs, but also, type-1 IFNs and IL-10 (McClure & Massari, 2014). With regard to parasitic infections, different TLRs are involved in recognitions of parasitic infections and activating the immune system. For example, study of *Leishmania major* infection in TLR4 knockout mice has shown that TLR4 activates iNOS (inducible nitric oxide synthase) which leads to NO synthesis and parasite death (Kropf et al., 2004). *T. cruzi* glycosylphosphatidylinositol (GPI) anchors have been shown to be potent and effective initiators of TLR2 expression in Chinese hamster ovary cells transfected with TLR2 (Campos et al., 2001). Another study showed the importance of TLR9 in visceral leishmaniasis through NK cells activation. TLR9 is required for NK activation and this really important in the production of IL-12 by DCs which leads to good prognosis (Schleicher et al., 2007). In *T. gondii* infections, TLR2 and TLR4 might play an important role of the acute *T. gondii* infection (Peng et al, 2016). Other studies showed that TLR9 play an important role in the parasite recognition and also might play an important role in congenital toxoplasma infection (Wucicka, Wilczynski, & Nowakowska, 2013). Application of live *Schistosoma mansoni* larvae, or soluble preparations derived from these larvae, to macrophages has shown that cytokine production is dependent upon activation of TLR4 (Jenkins, Hewitson, Ferret-Bernard, & Mountford, 2005). Schistosomal lysophosphatidylserine has also been shown to activate dendritic cells via TLR2 signaling and this may contribute to polarisation of the immune response, via expansion of T-regulatory cells, to elicit the fibrotic, tissue destructive liver pathology associated with this parasite (Layland, Rad, Wagner, & Da Costa, 2007; van der Kleij et al., 2002).

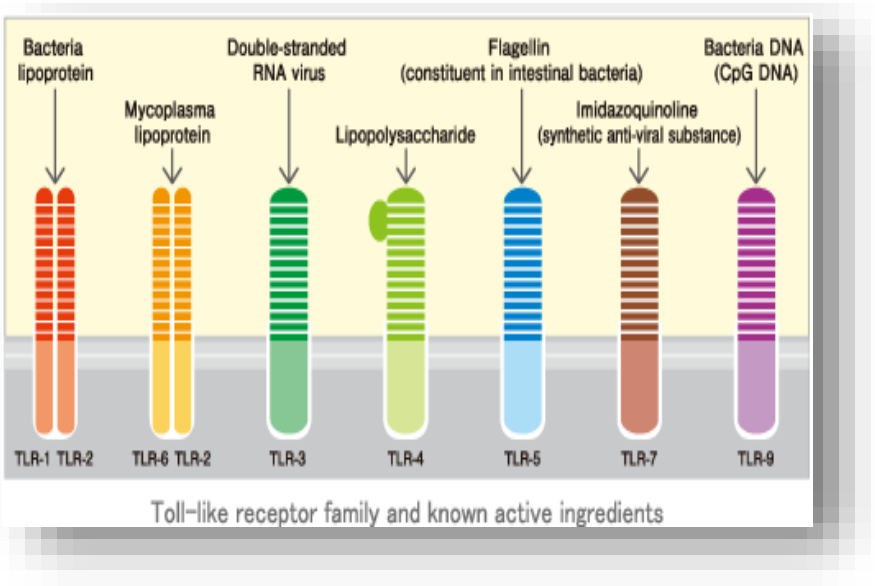

Figure 1.3: TLRs 1-7 and TLR-9 and their potential targets (Japan Science and Technology Agency, 2009).

Many different studies have shown the importance of TLRs. For example, a polymorphism of TLR2 (G2258A), can down-regulate the inflammatory response to bacterial components and this correlates with an increase in the susceptibility to TB, especially in Asian and European individuals (Y. Zhang et al., 2013). In contrast, a TLR6 polymorphism (C745T) has been shown to correlate with a reduced risk of TB infection (Zhang et al., 2013).

1.6.2 The bat immune system:

Bats have the basic cellular components of the mammalian immune system: B cells, T cells, macrophages, lymphocytes, neutrophils, eosinophils, basophils and dendritic cells (Baker, Schountz, & Wang, 2013; Sarkar & Chakravarty, 1991; Turmelle, Jackson, Green, McCracken, & Rupprecht, 2010). However, detailed studies of the bat immune system are relatively few compared to those of humans and other mammals such as rodents and hence much is still to be understood about how bats interact with infectious agents. With the notable exception of White Nose Syndrome, bats appear to tolerate many infections and this has led to the “flight-as-fever” hypothesis being proposed that argues that the increased

metabolism and high temperature experienced during flight may act as an adjuvant of the bat immune system to help control infections (O'Shea et al., 2014). Indeed, genome analysis of the Australian black flying fox *Pteropus alecto* and the vespertilionid *Myotis davidii* has provided evidence for positive selection on genes involved in DNA damage and repair (eg. p53, LIG4 and BRCA2) and innate immunity (eg. IL18, IFN-γ, IFNAR1 and IRAK4) lending support to the proposal that evolution of flight has shaped the bat genome to allow tolerance to viral infections (Zhang et al., 2013). The genome study also revealed that relative to humans, bats have lost (i) the PYHIN locus that is important for sensing microbial DNA and forming inflammasomes, (ii) killer cell lectin-like receptors and killer cell immunoglobulin-like receptors, both associated with NK cells and (iii) genes of the leukocyte receptor complex in *P. alecto* have failed to expand, as observed for humans and also *M. davidii* (Zhang et al., 2013). This data clearly indicates that intriguing differences have occurred following separation of the Yinpterochiroptera from the Yangochiroptera and also between bats and other mammals that could have profound implications for immunity.

Studies on a number of bat species show that the immunoglobulins IgM, IgE, IgA and multiple diversified IgG subclasses are expressed and that IgD expression may be restricted to insectivorous bats (Butler et al., 2011). Further analysis of immunoglobulin genes has revealed that in the pteropid bat, *P. alecto*, the antigen binding site of the variable region of the heavy chain is unusually rich in arginine and alanine residues whilst also having a lower proportion of tyrosines relative to other mammals; such differences may alter the antibody-antigen binding characteristics (Baker et al., 2013). It is likely that maternal IgG is transferred to bat offspring trans-placentally (Philbey et al., 2008) and also via the trans-mammary route (Butler & Kehrli, 2004). The study of maternal antibodies to Hendra virus in a population of *P. alecto* showed that they had a half-life of 52 days and provided immunity to the pups for approximately 8.5 months (Epstein et al., 2013).

The ability to culture immortalized cell lines from *P. alecto* (Crameri et al., 2009), has established this bat species as a model system to investigate immunological responses in bats. Since interferons are pivotal to a rapid innate immune response in mammals, these have been investigated in some detail in the *P. alecto* system. Type-3 IFNs have been shown to be differentially expressed in *P. alecto* and upregulated upon viral challenges (Zhou et al., 2011). In contrast, type-1 IFNs were not upregulated following viral challenges (Zhou et al., 2011). The type-1 IFN family in *P. alecto* is contracted relative to other mammals; however, three of the type-1 IFN-α genes appeared to be constitutively active in bat tissues and cells and not affected by viral infection (Zhou et al., 2016). Interestingly, these IFN-α genes were able to induce a subset of IFN-stimulated genes linked to antiviral activity and so they may be crucial to bats ability to tolerate viral infections (Zhou et al., 2016). The interferon regulatory factor 7 (IRF7), a key regulator of IFN responses, was also found to be constitutively expressed in a range of immune and non-immune cells of *P. alecto* and activated by double-stranded RNA (Zhou et al., 2011). Use of siRNA technology to knockdown IRF7 in *P. alecto* kidney cells resulted in enhanced viral replication, confirming the importance of IRF7 in the innate antiviral response in bats. Bone marrow-derive dendritic cells and macrophages have recently been successfully isolated and cultured from *P. alecto* (Zhou et al., 2016) and this should now facilitate more detailed investigations of how the bat adaptive immune system responds to challenges. These approaches will complement genome-scale analyses being carried out on several bat species. For example, the major histocompatibility complex (MHC) I locus of *P. alecto* and *Eptesicus fuscus* has been studied and the data reveals that bat MHC-1 genes have an unusual insertion within their peptide binding grooves which may impact the efficiency and diversity of antigen presentation to T cells and hence contribute to control of viral replication (Ng et al., 2016). The transcriptome of *P. alecto* is also being investigated and this has revealed insight into the expression of approximately 500 immune

genes representative of both the innate and adaptive systems (Papenfuss et al., 2012). The advances made with establishing cell lines in *P. alecto* have facilitated the establishment of cell lines from a number of other species (Biesold et al., 2011; Eckerle et al., 2014; He et al., 2014; Mourya et al., 2013) including *Myotis myotis* (He et al., 2014). The *M. myotis* brain derived cell line has allowed a detailed analysis of lyssavirus infection in a natural reservoir host and revealed that the pattern recognition receptors RIG-1 and MDA-5 are highly upregulated following rabies infection, which is indicative of an IFN response (He et al., 2014).

With regard to immunology studies in other bat species, Stockmaier et al. (2015) tried to understand the acute phase immune reaction to a standard lipopolysaccharide challenge in Pallas's mastiff bats (*Molossus molossus*) and found that challenged bats, in stark contrast to other mammals, showed no leucocytosis or fever responses. The reasons for this unusual finding remain speculative but might be related to a potential trade-off between bat gene families that reduce metabolic stresses associated with flight and genes involved in immune responses (Stockmaier, Dechmann, Page, & O'Mara, 2015). For example, *M. molossus* TLR4 haplotypes may exist that have lowered affinity for LPS and hence may either reduce, or eliminate a fever response (Stockmaier et al., 2015).

Bats have a complement system that can be readily assessed by assaying plasma proteins for microbicidal activity (Baker et al., 2013). Assessment of the complement system of hibernating little brown bats, *M. lucifugus*, at sites affected by White Nose Syndrome highlighted how the bat immune response can be impacted by both hibernation and also, by exposure to *Pseudogymnoascus destructans*, the causative agent of WNS (Moore et al., 2011). The bats complement activity was shown to be greatest against a gram-negative bacteria (*Escherichia coli*) than a gram-positive bacteria (*Staphylococcus aureus*) and to be least effective against a fungus (*Candida albicans*) (Moore et al., 2011). Furthermore, bats

hibernating at WNS-affected sites showed significantly elevated complement activities against *Escherichia coli* and *Staphylococcus aureus* and significantly lowered complement activity against the fungus *Candida albicans* when compared to bats hibernating at sites not affected by WNS (Moore et al., 2011). Interestingly, the microbicidal responses varied significantly across the hibernation period; however, the observed pattern for *E. coli* differed to that for *S. aureus* (Moore et al., 2011). The body condition of the bats, as measured by body mass index (BMI) was significantly reduced across the hibernation period in bats at WNS-affected sites compared to the unaffected sites (Moore et al., 2011). Moreover, at the WNS-affected sites, BMI was significantly higher during early hibernation compared to later stages and there was a significant and positive association between microbicidal activity of the plasma against *E. coli* and *C. albicans* and the BMI during the late hibernation period (Moore et al., 2011).

M. lucifugus bats from WNS-affected sites also showed significantly elevated leukocyte levels and significantly lowered IL-4 and antioxidant levels (Moore at al., 2013). Upon transcript analysis of lung tissue in *P. destructans*-infected *M. lucifugus* it was subsequently shown that transcripts for the antimicrobial peptide cathelicidin, was significantly elevated, indicating a specific immune response to the fungus (Rapin et al., 2014). Moreover, the cytokines TNF-α, IL-10 and IL-23 were also significantly elevated in the fungal-infected bats, suggesting that a defense response involving NFk-B and Th2 may be initiated (Rapin et al., 2014).

1.6.2.1 Bat TLRs:

A TLR gene study was carried out in *P. alecto* and the data confirmed that this bat has the same set of TLR (TLR1-10) genes as humans (Cowled et al., 2011). Comparison of the data to the draft genome data of the related pteropid, *P. vampyrus*, confirmed that the latter also contained the same complement of TLR genes and that the majority were located within a single exon (Cowled et al., 2011). Analysing the non-synonymous to synonymous nucleotide substitutions of the *P. alecto* TLRs to those in the human, cow and mouse genomes showed that there was weak negative selection upon the genes and hence likely evolutionary selection to conserve binding specificities (Cowled et al., 2011). Expression of viral sensing TLRs 3, 7, 8, and 9 was analysed in ten tissues from wild *P. alecto* bats and the data showed TLR3 was strongly expressed in liver, TLRs 7, 8 and 9 were strongly expressed in peripheral blood mononuclear cells and TLRs 8 and 9 were strongly expressed in the spleen (Cowled et al., 2011). The brain, kidneys and heart expressed low levels of the four TLRs (Cowled et al., 2011). The transcriptome study by Papenfuss et al. (2012) confirmed that all 10 TLRs of *P. alecto* were indeed expressed in the tissues/cells analysed: the spleen, lymph nodes and peripheral blood leukocytes.

TLRs 3, 7 and 9 have also been analysed in the fruit bat *Rousettus leschenaultia* and the data revealed that TLR3 was expressed highly in the liver whilst TLR 7 and 9 was most highly expressed in the spleen (Iha et al., 2010).

1.7 Study Aims/Hypothesis:

Bats appear able to tolerate high levels of many infectious agents, including parasites. However, given the difficulties associated with studying bats, there are not surprisingly few endoparasite studies in these hosts. Bat immunology is also relatively poorly understood compared to other mammals and most studies have focused upon interactions between hosts and viruses. To this end, work in this thesis is aimed at providing a comprehensive description of protozoan and bacterial infections in a population of pipistrelle bats from Lancashire that have already been well-studied; particularly at the level of helminth infections (Lord et al., 2012). The resulting infection data will then be analysed with respect to the host genetics; firstly by profiling the infections with respect to host genotype data generated in an earlier study (Dodd et al., 2014) and secondly, by investigating the innate immune system of these bats. With respect to this, isolation and sequencing of pipistrelle TLR genes (TLR2 and TLR4) will be carried out with a view to correlating parasite infection profiles to any TLR haplotypes. The study hypothesis is that host genetics, including innate immunity genes, are likely to influence infection outcomes and hence TLR gene variations will be observed in the bat population. Given the opportunistic sampling of hosts from the wild and hence multiple associated confounding factors, it is difficult to predict whether, or not, there might be a link between TLR haplotype and parasite infection profile. Nonetheless, the study will address the hypothesis that a correlation might exist between the observed bat parasite infection profiles and particular TLR variants.

Materials and Methods

2.1 Bioinformatics and Primer design

2.1.1 Microparasites:

GenBank data (http://www.ncbi.nlm.nih.gov/) was analysed for bat *Schizotrypanum* DNA sequences and *Eimeria* spp. in order to design suitable primers for PCR based diagnosis of bat infections. The oligonucleotide sequences are shown in Table 2.1. A set of primers were designed for distinguishing between *T. dionisii* and *T. vespertilionis* using a nested PCR approach. These primer sequences were based upon the 18S rRNA trypanosome primers used by Lord (2010). In addition, two new sets of primers were designed based upon the 18S rRNA gene sequences of trypanosomes to differentiate between *T. dionisii* and *T. vespertilionis* using a nested PCR approach. PCR primers were also designed to amplify *T. dionisii* GAPDH gene in order to sub- type this parasite using semi- nested PCR approach. Another set of oligonucleotide primers were designed based upon the 18S rRNA gene of *Eimeria* spp. in order to screen the bats for this coccidian infection. A further set of oligonucleotide primers were used to screen the bats for *Cryptosporidium* spp. by targeting the 18srRNA gene of this coccidian parasite. Two oligonucleotide primer sets were used to screen the bats for *Bartonella* spp. and *Borrelia* spp. infections based upon the citrate synthase (*gltA*) and 16S rRNA genes respectively of these bacteria using a single round PCR strategy.

For primer design, all the sequences were aligned using Clustal W (http://www.ebi.ac.uk/Tools/msa/clustalo/) and regions of high conservation were scrutinized closely for primer design with the exception of the trypanosomes, were sequence differences were utilised for primer design. All oligonucleotide sequences were checked using Primer3 (http://primer3.ut.ee/) in order to ensure minimal design parameters which include annealing temperature and GC levels, were met.

Table 2.1: Parasite and bacterial oligonucleotide primers.

Organism	Target gene	Forward primer sequence and name	Reverse primer sequence and name	Product size	Ref
T. vespertilionis	18S rRNA	TVF: 5'CGTCACACTTCCACGTGTGTCA3'	TVR: 5'TTAAAGGCCTCCGCTGGAA3'	312 bp	(Lord, 2010)
T. dionisii	18S rRNA	TryF: 5'CTAAGGCGCAATGGTTTAGTCCC3'	TryR: 5'GCGACGCGTGAAGATGGG3'	402 bp	
T. dionisii	GAPDH	GAPF: 5'ATATGAACACGGACGCGGAGT3'	GAPR: 5'CGCGCCAGTCCTTCAACG3' GAPRn: 5'CTGGGGTTGTACTCATGGTGG3'	525 bp 355 bp	
Eimeria spp.	18S rRNA	EimF: 5'CATAGTAACCGAACGGATCGC 3' EimFn: 5'AACGGGGAATTAGGGTTCGA 3'	EimR: 5'CTTCCTTGCGTTAGACACGC3' EimRn: 5'CCCCAGAACCCAGAGACTTT3'	1500 bp 753 bp	(Lord, 2010)
Cryptosporidium spp.	18S rRNA	F2: 5'-GACATA TCA TTC AAG TTT CTG ACC-3' F1: 5'-CCTATC AGC TTT AGA CGG TAG G-3'	R2: 5'-CTG AAG GAGTAA GGA ACA ACC-3' R1: 5'-TCT AAG AAT TTCACC TCT GAC TG-3'	600 bp	(Ryan et al., 2003)
Bartonella spp.	citrate synthase gene (gltA)	781F: 5'GGGGACCAGCTCATGGTGG3' 443F: 5'GCTATGTCTGCATTCTATCA3'	1137R: 5'AATGCAAAAAGAACAGTAAACA3'	400 bp	(Birtles & Raoult, 1996; Norman, Regnery, Jameson, Greene, & Krause, 1995)
Borrelia spp.	16S rRNA	BorF: 5′-AGCCTTTAAAGCTTCGCTTGTAG-3′	BorR: 5′-GCCTCCCGTAGGAGTCTGG-3′	120 bp	(Sokhna et al., 2013)

2.1.2: TLRs:

A similar approach was adopted for designing of PCR primers to amplify *P. pipistrellus* TLR4 and TLR2 genes. GenBank data within NCBI (http://www.ncbi.nlm.nih.gov/) and Ensembl (http://www.ensembl.org/index.html) was analysed for any chiropteran TLR4 and TLR2 gene sequences and these extracted and TLR4 and TLR2 gene alignments were carried out using Clustal W (http://www.ebi.ac.uk/Tools/msa/clustalo/). Regions of high conservation were scrutinized closely for PCR primer design and then primer sequences (Table 2.2) were validated using Primer3 (http://primer3.ut.ee/) to ensure minimal criteria which include annealing temperature and GC levels, were met.

Table2.2: Primer sequences for PCR amplification of pipistrelle *TLR4* and *TLR2* genes.

Target gene	Forward primer sequence and name	Reverse primer sequence and name	Estimated product size
TLR4	TLR4F: 5'CTTGAGTTTCTAGATCTCAGTA3'	TLR4R: 5'AAAGTCTCTGTAGTGAAGGC3'	1000 bp
TLR4	TLR4-2F: 5'GGAAACCCTATCCAGAGTTTAGC3'	TLR4-2 R: 5'AATTGCCAGCCATTTTCAAGAC3'	1114 bp
TLR2	TLR2F: 5'ATGCCACATGCTTTGTGGA3' *	TLR2R: 5'TCCAGGTAGGTCTTGGTGTT3' *	1100 bp
TLR2	TLR2Fn: 5'CTGAGAGATACTCATTTGGA3' *	TLR2Rn: 5'CTTCTCCAGTTTCTTCTAAC3' *	500 bp
TLR2	TLR2gapF: 5'GAGACATTAACAATACGGAGG3'	TLR2gapR: 5'GTTCAAATACTTCATCCTTTCTG3'	400 bp

* Designed by Arianne Lovey (MSc student, University of Salford)

The Smart programme was used to make gene models from the pipistrelle TLR4 and TLR2 sequences (http://smart.embl-heidelberg.de/). Predicted glycosylation sites were determined using the GlycoEP predictor (http://www.imtech.res.in/raghava/glycoep/submit.html). Mega 6 (http://www.megasoftware.net/) was used for creating phylogenetic trees. Different

methods were used to construct different trees, Neighbour- Joining tree, Minimum- Evolution tree, and UPGMA tree. Bootstrap method for was selected for all the trees with 500 replications (commonly used in many studies). Also, p- distance model was used to build the tree. This was used to measure the distance between the sequences (commonly used in many studies). Two outcome of each tree type was produced, one without outgroup and the other one with outgroup.

2.2 DNA Extraction:

DNA extraction was carried out using a spin column protocol (Isolate II Genomic DNA Kit, Bioline). Briefly, 25 mg of bat tissue, or organ (e.g. heart, spleen or intestine according to the parasite being investigated), was cut into small pieces with a sterile scalpel blade and placed into a 1.5 ml microcentrifuge tube. 180µl of lysis buffer GL and 20µl of Proteinase K was added to the sample and lysis at was allowed to proceed at 56^{o}C for 1-3 hours. The sample was vortexed for 15 seconds then 200µl of G3 buffer was added, and the sample was vortexed thoroughly and incubated for 10 minutes at 70 ^{o}C. 210µl of absolute ethanol was then added and the sample was vortexed and transferred into an isolate II spin column placed in a 2 ml collection tube and centrifuged for 1 minute at 11.6 xg (Fisher Scientific/AccuSpin Micro17). The collection tube was discarded and the isolate II column was placed into a new collection tube. 500µl of GW1 buffer was added to the column and then it was centrifuged for 1 minute at 11.6 xg (Fisher Scientific/AccuSpin Micro17). The collection tube was discarded and the isolate II column was placed into another collection tube and 600µl of GW2 buffer was added and centrifuged for a further 1 minute at 11.6 xg (Fisher Scientific/AccuSpin Micro17). Again, the collection tube was discarded and the isolate II column was placed into a clean 1.5 microcentrifuge tube. 100µl of AE buffer was pipetted onto the membrane directly, and after incubation for 1 minute at room temperature, it was

centrifuged for 1 minute at 11.6 xg (Fisher Scientific/AccuSpin Micro17) to elute the purified DNA. The eluted DNA was stored at -20^{o}C until required for further analysis.

2.3 Polymerase Chain Reaction (PCR):

2.3.1 *Schizotrypanum*, Eimeria, and Cryptosporidium PCRs:

The PCR parameters used to detect *T. dionisii, T. vespertilionis, Cryptosporidium* spp. and *Eimeria* spp. DNA from the bat tissues are summarised in Table 2.3.

2.3.2: Bartonella and Borrelia PCRs:

The PCR parameters used to detect *Bartonella* spp. and *Borrelia* spp. DNA are summarised in Table 2.3. The only exception compared to the protozoan PCRs is the use of 2x MyTaq Red mix (Bioline) for the bacterial PCRs.

2.3.3 *TLR4* and *TLR2* PCRs:

The PCR parameters used to detect pipistrelle TLR4 and TLR2 genes are summarised in Table 2.3.

2.3.8 PCR optimisation:

All the PCRs were optimised with respect to temperature and Mg^{2+} concentration in order to improve target quantity and specificity. Essentially a temperature gradient was set up a cross the thermocycler plate in order to determine the optimal primer annealing temperature and then a dilution series of Mg^{2+} was employed in order to further enhance the recovery of PCR product.

Table 2.3: PCR cycling parameters utilised for microparasite and TLR gene amplifications.

Primer combinations	Target gene	Mg^{2+} (50 mM)	Primers (10 pmol/ µl)	Taq polymerase (5 units/µl) (Bioline)	Initial denaturation (Time/Temp)	Denaturation (Time/Temp)	Annealing (Time/Temp)	Extension(Time/ Temp)	No. of cycles	Final extension (Time/Temp)
TvF/TvR	18SrRNA	2.5 µl	2.5 µ1	0.5 µl	5 min/ 94 °C	30 sec/ 94 °C	30 sec/ 66 °C	30 sec/ 72 °C	35	10min/ 72 °C
TryF/TryR	18SrRNA	2.5 µl	2.5 µ1	0.5 µl	5 min/ 94 °C	30 sec/ 94 °C	30 sec/ 61 °C	30 sec/ 72 °C	35	10min/ 72 °C
EimF/EimR EimFn/EimRn	18SrRNA	2.5 µl	2.5 µ1	1.25 µl	5 min/ 94 °C	30 sec/ 94 °C	30 sec/ 64 °C	50 sec/ 72 °C	35	10min/ 72 °C
F2/R2 F1/R1	18SrRNA	2.5 µl	2.0 µ1	0.5 µl	5 min/ 94 °C	30 sec/ 94 °C	30 sec/ 56 °C	30 sec/ 72 °C	45	7min/ 72 °C
443F/1137R 781F/1137R	citrate synthase gene (gltA)	My red taq mix	1.0 µ1	My red taq mix	5 min/ 94 °C	10 sec/ 95 °C	20 sec/ 50 °C	50 sec/ 72 °C	35	10min/ 72 °C
BorF/BorR	16SrRNA	My re taq mix	1.0 µ1	My red taq mix	5 min/ 94 °C	30 sec/ 94 °C	30 sec/ 51 °C	30 sec/ 72 °C	35	10min/ 72 °C
TLR4F/TLR4R	TLR4	1.5 µ1	2.5 µ1	0.5 µl	5 min/ 94 °C	30 sec/ 94 °C	30 sec/ 54 °C	60 sec/ 72 °C	35	10min/ 72 °C
TLR4-2F/TLR4-2R	TLR4	1.5 µ1	2.5 µ1	0.5 µl	5 min/ 94 °C	30 sec/ 94 °C	30 sec/ 58 °C	80 sec/ 72 °C	35	10min/ 72 °C
TLR2-2F/TLR2-2R	TLR2	1.5 µ1	2.5 µ1	0.5 µl	5 min/ 94 °C	30 sec/ 94 °C	30 sec/ 53 °C	60 sec/ 72 °C	35	10min/ 72 °C
TLR2Fn/TLR2Rn TLR2gapF/TLR2gaPR	TLR2	1.5 µ1	2.5 µ1	0.5 µl	5 min/ 94 °C	30 sec/ 94 °C	30 sec/ 53 °C	30 sec/ 72 °C	35	10min/ 72 °C

* All PCRs were carried out using the MultiGene OptiMax (Labnet International Inc.)

2.4 Agarose gel electrophoresis:

1% (w/v), or 2% (w/v), agarose gels (Bioline) were prepared to check the specificity of the PCR end product. To prepare a 1% gel, 0.3 g of agarose was placed in a 250 ml conical flask and 30 ml of 1x TBE buffer (Bioline) was added and then the mixture was heated in a microwave oven for 30 s at maximum power. The mixture was then swirled and heated for a

further 30 s to ensure that all the agarose powder had dissolved. The melted agarose was then placed onto a shaker and allowed to cool to approximately 50^{o}C. Then, 30µl of Gel Red (Biotium) was added, the mixture was gently swirled and the gel then poured into the gel casting tray using casting dams and a comb. After solidification the gel was then placed into the electrophoresis tank, covered with 1x TBE buffer (Bioline) and the casting dams and comb were removed. Samples were prepared (10µl of sample + 5µl of loading dye (Bioline) and carefully aliquoted into the wells of the gel. Electrophoresis was allowed to proceed at 70 volts and after an appropriate period of time, the DNA was visualised using the UV transilluminator (SynGene). All gel images were saved as tiff files.

2.5 DNA concentration/purity:

Recovery of all DNA (bat and purified PCR products) was assessed with the NanoDrop spectrophotometer (ThermoFisher Scientific). The Nanodrop was blanked using distilled water. 1µl of the DNA was then added and absorbance readings at 260 nm and 280 nm were recorded. Also, an estimate of the purity of the DNA was determined using the ratio of the A260/A280 readings.

2.6 pGEM-T Easy vector cloning of PCR products:

5µl of 2x Rapid Ligation Buffer, 1µl of pGEM-T Easy vector (Promega), 1µl of T4 DNA Ligase and 2µl of PCR product were mixed and incubated for 1 hour at room temperature. After 1 hour, 100µl of *E. coli* competent cells (α-select, silver efficiency) (Bioline) was added to the pGEM-T easy reaction mix and the tube was incubated on ice for 30 minutes. The tube was then placed into a water bath at 42^{o}C for 45 s and then placed again on ice for 2 minutes. 900µl of LB broth (Sigma-Aldrich) was added and the cells were shaken at 200 rpm for 1 hour at 37^{o}C. The cells were subsequently spread, using a sterilised spreader, onto LB agar plates containing 100µg/ml of ampicillin (Sigma-Aldrich). In addition, 10µl of 100mM IPTG (filter sterilised) (Bioline) and 40µl of 200mg/ml X-gal (in DMSO) (Promega) were also

spread onto the surface of the LB agar plate. Plates were inverted and incubated at 37°C overnight. White colonies were subsequently picked and grown to stationary phase in order to be further analysed by plasmid mini prep and restriction enzyme digestion.

2.7 Plasmid mini prep:

Plasmid mini preps were carried out to evaluate the white colonies (2.6) using the spin column protocol (Isolate II Plasmid Mini Kit) (Bioline). Briefly, 1-5 ml of stationary phase *E. coli* culture was pelleted for 30 s at 6,000 x g (Fisher Scientific/AccuSpin Micro17). 250µl of resuspension buffer P1 was added and the cell pellet was re-suspended by gentle pipetting. 250µl of lysis buffer P2 was added to the sample and mixed by gently inverting the tube 6-8 times. The sample was then incubated at room temperature for 5 minutes, or until the lysate appeared clear. 300µl of neutralizing buffer P3 was then added and mixed thoroughly by inverting the tube 6-8 times. After centrifugation for 5 minutes at 11.6 xg (Fisher Scientific/ AccuSpin Micro17) the clear supernatant was transferred into an isolate II plasmid mini prep spin column which was then placed in a 2 ml collection tube and centrifuged for 1 minute at 11.6 xg (Fisher Scientific/AccuSpin Micro17). The collection tube was discarded and the column was placed into a new collection tube. 500µl of PW1 buffer was added to the column and then it was centrifuged for 1 minute at 11.6 xg (Fisher Scientific/AccuSpin Micro17). The collection tube was again discarded and the column placed into another collection tube. 600µl of PW2 buffer was added and the column was then centrifuged for a further 1 minute at 11.6 xg (Fisher Scientific/AccuSpin Micro17). The column was placed into a collection tube and centrifuged for 2 minutes at 11.6 xg (Fisher Scientific/AccuSpin Micro17) to remove residual ethanol. The column was then placed into a sterile 1.5 ml microcentrifuge tube and 50µl of elution buffer was pipetted directly onto the membrane. After incubation for 1 minute at room temperature, the sample was centrifuged for 1 minute at 11.6 xg (Fisher

Scientific/AccuSpin Micro17) to elute the purified DNA. The plasmid DNA was stored at -20^{o}C until required for further analysis.

2.8 Restriction enzyme digestion:

To check for successful cloning of PCR products in the pGEM-T easy plasmid the purified plasmids were subjected to restriction enzyme digestion (New England Biolabs). The 20 µl reaction mixture consisted of 2.0 µl 10x Buffer 3.1, 0.5 µl *Nco*I (10 units/µl), 1.0 µl plasmid DNA and 16.5 µl H_2O. The reaction mixture was incubated at 37^{o}C for 1 hour and then prepared for analysis by agarose gel electrophoresis.

2.9 DNA sequencing:

PCR products were purified using spin column technology, according to the manufacturer's instructions (Isolate II PCR and gel kit, Bioline). The concentration of DNA was measured using the NanoDrop spectrophotometer (2.5) and if necessary, it was adjusted using PCR grade water to the recommended concentration for DNA sequence analysis (Source BioScience). When possible, both forward and reverse primer sequencing was carried out and if required, any conflict was resolved by repeating the sequencing reaction. All the sequences were done directly from the purified PCR products of the pipistrelle bats samples except the first *T. vespertilionis* sequence which was done from cloned products due to the lack of the genomic DNA provided from Prof. Patrick Hamilton (University of Exeter).

All DNA sequencing was carried out by Source BioScience and the data were analysed by Finch TV (http://officialsite.pp.ua/?p=2958497), aligned by Clustal W (http://www.ebi.ac.uk/Tools/msa/clustalo/), and further analysed using Blast tools (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch) to check for other highly similar sequences.

2.10 Host/Parasite data:

Host and known parasite data for the pipistrelles were extracted from Lord (2010). Moreover, the final known locations of the bat specimens (Lord, 2010) were uploaded to Google maps (https://www.google.co.uk/maps/@53.5009883,-2.2676135,14z). Additionally, the pipistrelle genotyping data was extracted from Dodd et al. (2014).

2.11 Statistics:

Statistical analyses of the data were carried out using Minitab 16 (licensed to The University of Salford). Different tests were used to evaluate the significant of the data such as Fisher exact test, Chi- square test, and t-test. *P*-value was used to check the significant of the data for these test (p-value< 0.05 considers significant).

2.12 ethical reviews:

All the ethical reviews were done before this project when the samples first acquired by Jennifer Lord.

3. Results: Infection Data

A recent study of 100 pipistrelle bats (*P. pipistrellus* and *P. pygmaeus*), opportunistically sampled, where the bats, where found either dead or severely injured and then euthanised by the South Lancashire Bat Conservation Group, from sites across North West England (South Lancashire/Greater Manchester), showed that they were infected with a plethora of digenean trematodes (see Chapter 1, section 1.5.1.4) (Lord, 2010; Lord et al., 2012) and protozoans (Lord, 2010).

In the molecular based analysis carried out by Lord (2010), 37 of the pipistrelle bats (37%) were confirmed infected with trypanosomes and the majority of these (n=29) were designated as *T. dionisii* (Jennifer S Lord, 2010). However, absence of a *T. dionisii* positive control in the Lord (2010) study means that this species designation remains questionable. Additional analysis confirmed that 23 % of the pipistrelle bats were positive by PCR for *Babesia vesperuginis*, 19% were infected with *Eimeria* sp., and two bats were infected with the haemobacterium *Bartonella* sp. (Lord, 2010). The spleen sizes of the *B. vesperuginis* infected adult bats were significantly greater than the spleens of the uninfected bats (Jennifer S Lord, 2010), as noted elsewhere (Gardner et al., 1987). Further statistical analyses confirmed that there were no significant differences in the prevalences of *Trypanosoma* spp., *B. vesperuginis* or *Eimeria* sp. with respect to host sex, age and year of collection (Lord, 2010).

The Lancashire bat collection was also screened for the presence of *T. gondii* using highly sensitive and specific SAG1-PCR detection and the prevalence was reported as 10%; this was the first, and to date only report, of *T. gondii* in British bats (Dodd et al., 2014). Furthermore, a sub-population of the pipistrelles were genotyped using eleven polymorphic microsatellite

loci and the data showed that 83% of the bats were derived from one interbreeding population whilst the remaining 17% had mixed origins (Dodd et al., 2014). There appeared to be no correlation between the bat genotype and the *T. gondii* infection status (Dodd et al., 2014).

Based on the previous data (Dodd et al., 2014; Jennifer S Lord, 2010; Lord et al., 2012), the aims of this component of the thesis are to address the following unresolved areas: (i) to determine *Schizotrypanum* species identity in the pipistrelles by developing a reliable PCR strategy that is able to discriminate between *T. dionisii* and *T. vespertilionis*, (ii) to confirm the species identity of all 19 eimerian infections in the pipistrelle population, (iii) to develop a PCR screening approach based upon the *Bartonella* citrate synthase gene to determine the species of this bacterium in the pipistrelles, (iv) to develop PCR screening approach for detecting *Cryptosporidium* spp., and (v) *Borrelia* infections in the bat population and (vi) to analyse the parasite infection profiles with respect to host genotype data. Aims (i-v) will provide a more detailed picture of the protozoan and bacterial infections within the pipistrelle bats. Aim (vi) will allow the significance of the host genotype in conferring resistance/susceptibility to infection to be addressed.

3.1: B- tubulin PCR for DNA validity:

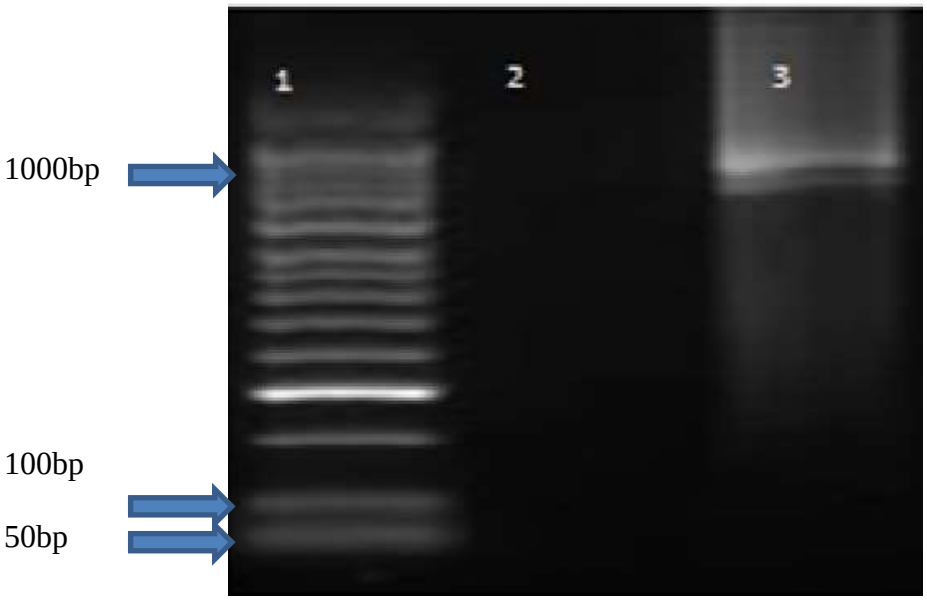

Figure3.1: Agarose gel (1%) showing pipistrelle bat B- tubulin PCR. 1, 50bp Hyperladder; 2, negative control (H_2O); 3, bat DNA samples extracted from heart (bat codes: SA606)

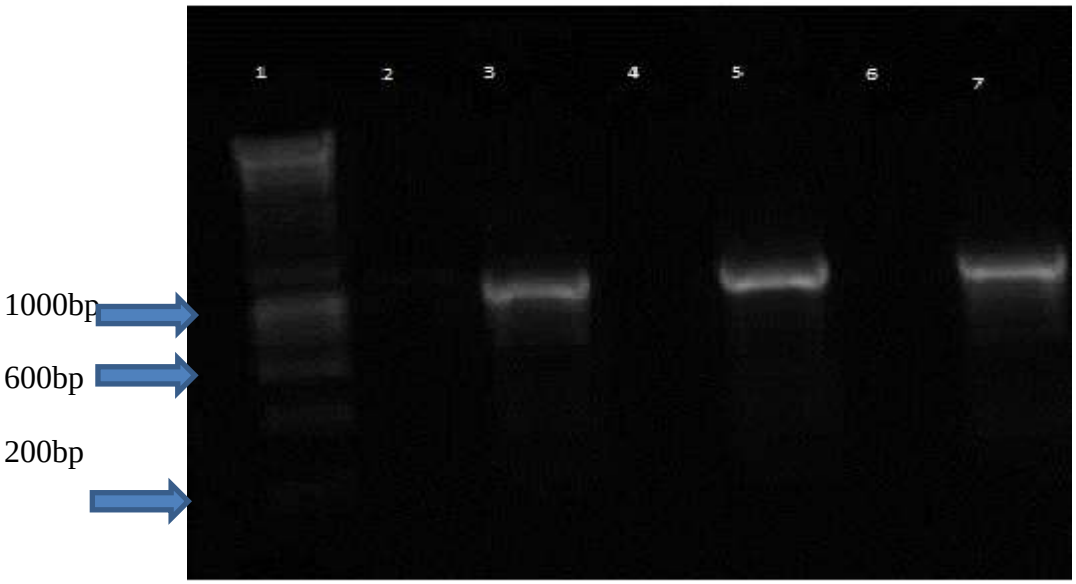

Figure3.2: Agarose gel (1%) showing pipistrelle bat B- tubulin PCR. 1, 1kb Hyperladder; 2,4,6, negative controls (H_2O); 3,5,7, bat DNA samples extracted from heart (bat codes: JL650, P605, SP677)

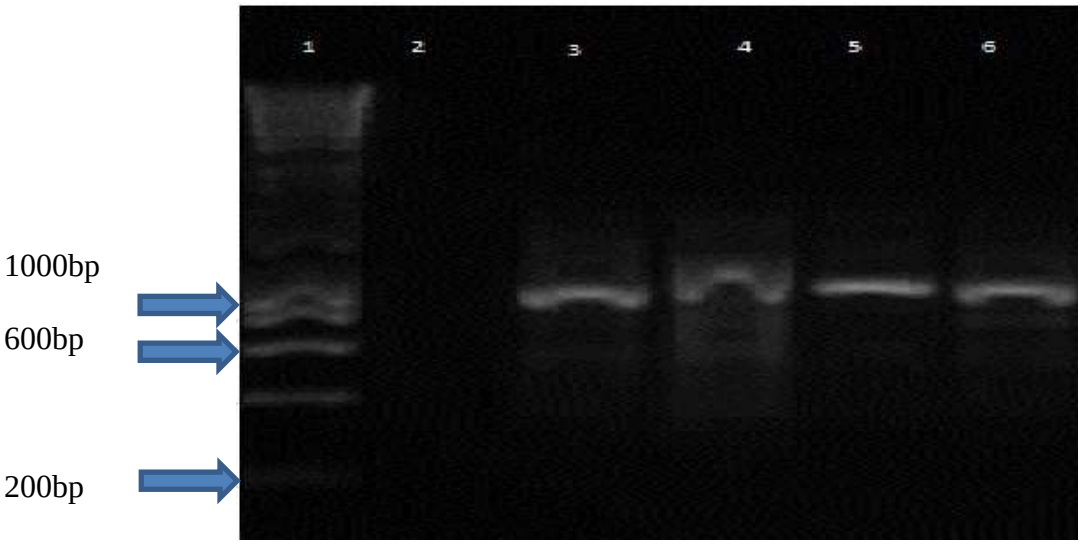

Figure3.3: Agarose gel (1%) showing pipistrelle bat B- tubulin PCR. 1, 1kb Hyperladder; 2, negative control (H_2O); 3-6, bat DNA samples extracted from heart (bat codes: SP852, SP682, JL719)

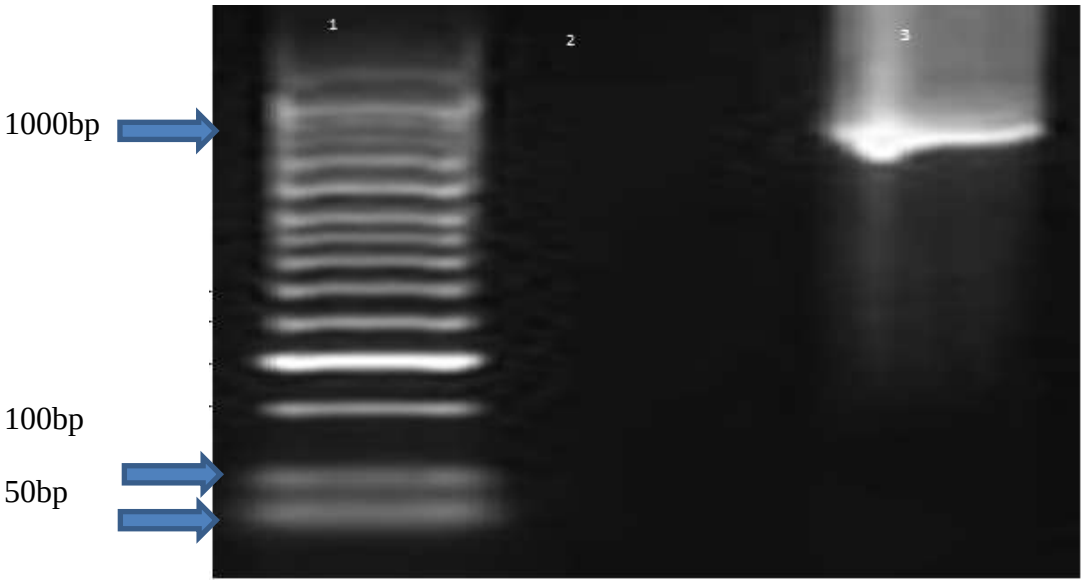

Figure3.4: Agarose gel (1%) showing pipistrelle bat B- tubulin PCR. 1, 50bp Hyperladder; 2, negative control (H_2O); 3, bat DNA samples extracted from heart (bat codes: F745)

After the DNA extraction, random samples were selected to check the integrity of the DNA using the B- tubulin primers. As the gel shows (Figure3.1- 3.4), all the samples from pipistrelle bats were successfully produced a PCR product at the expected size (1000 bp) with the B- tubulin primers which means the DNA is good to use for further analysis.

3.2 Bat trypanosomes

3.2.1 The *Schizotrypanum*:

Primers were designed for distinguishing between *T. dionisii* and *T. vespertilionis* using a nested PCR approach that targeted the 18S rRNA gene of these trypanosomes (Lord, 2010).

Panel A:

```
T.vesp  1     GTCATATGCTTGTTTCAAGGACTTAGCCATGCATGCCTCAGAATCACTGCATTGCAGGAA  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
T.dion  1     GTCATATGCTTGTTTCAAGGACTTAGCCATGCATGCCTCAGAATCACTGCATTGCAGGAA  60
```

Panel B:

```
T.vesp 956   CAGTGTGACAAGCGGCCGGGTGCT-CT-T-TC-C-C-CCTT--C-G-G-G--GGGACGCA  1002
             ||||||||||||||| |||||  |  | |  | | | ||||  | | | |  | | | ||
T.dion 958   CAGTGTGACAAGCGGCTGGGTGATGATATCCCACACACCTTCACTGCGTGTTGTGGCACA  1017

T.vesp 1003  CTCGTCGCCTTTGTCGGAAATCCGCGCCGGCTGCGGCTGTGTGCGTCACACTTCCACGTG  1062
             ||||||||||||  |||||||||     |  || ||  ||| || |  ||||   |  |
T.dion 1018  CTCGTCGCCTTTGGGGGAAATCCG----TG--GC-GC--TGT-CGACGGACTT---C--G  1062

T.vesp 1063  TGTCACACGCGCCCTGCCTGCGCCTTCCGGCAACTCACGGCATCCAGGAATGAAGGAGGG  1122
              ||| ||    | | || | ||||||||  ||||||||||||||||||||||||||||
T.dion 1063  -GTCCCATCTTCAC-GCGT-CGCCTTCCCTCAACTCACGGCATCCAGGAATGAAGGAGGG  1119
```

Panel C:

```
T.vesp 1361  ATCAATTTACGTGCATATTCTTTACGGTCCCCGCT-TTCCAGCGGAGGCCTTTAACGGGA  1419
             |  ||||||||||||||||||  ||||| || | || | |||  ||||||||||||||
T.dion 1360  AATAATTTACGTGCATATTCTTTTTGGTCCTCGTTCTTAC-GCGTGGGCCTTTAACGGGA  1418
```

Panel D:

```
T.vesp 2140  AAAGTTCACCGATATTTCTTCAATAGAGGAAGCAAAAGTC  2179
             |||||||||||||||||||||||||||||||||||||||
T.dion 2139  AAAGTTCACCGATATTTCTTCAATAGAGGAAGCAAAAGTC  2178
```

Figure 3.5: Clustal W alignment for different regions of the Schizotrypanum 18S rRNA gene sequences extracted from NCBI GenBank: *T. dionisii* (gi|4468750|), *T. vespertilionis* (gi: |4468775|): Panels A & D: Green highlights generic primer binding sites (TgF and TgR), Panels B & C: red shows the *T. dionisii* primer binding sites (TdF and TdR) and purple represents the *T. vespertilionis* primer binding sites. Additional primers were designed for *T. dionisii* and their annealing sites are shown in Panel B & C using brown and blue colouration. The full sequence alignment can be obtained from Appendix 1.

3.2.2 Schizotrypanum PCRs:

The specificity of the *Schizotrypanum* primers was assessed using purified genomic DNA extracted from *T. dionisii* and *T. vespertilionis* (kindly provided by Dr. Patrick Hamilton, University of Exeter). The PCR was carried out as described (2.3.1) and the products subjected to agarose gel electrophoresis (Figure 3.6).

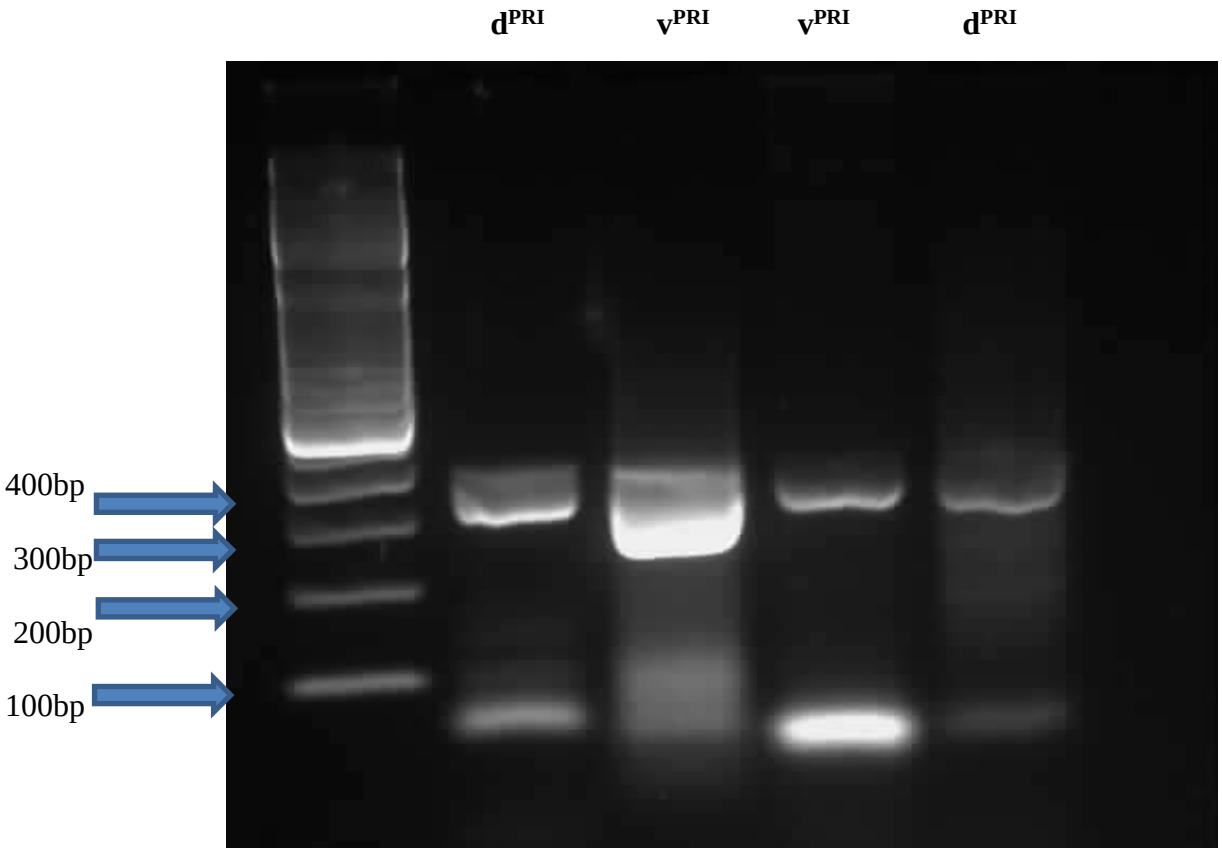

Figure 3.6: Agarose gel (1%) showing the *T. dionisii* and *T. vespertilionis* 18S rRNA PCR products. 1, 100bp hyperladder; 2 and 3, *T. dionisii* genomic DNA target; 4 and 5, *T. vespertilionis* genomic DNA target. d^{PRI} indicates primers designed to be specific for *T. dionisii*; v^{PRI} indicates primers designed to be specific for *T. vespertilionis*. *Footnote*: primer annealing was 52°C.

Based on the published 18S rRNA sequences and the expected primer binding sites, the anticipated sizes of PCR products for *T. dionisii* and *T. vespertilionis* were 332bp and 312bp respectively. The agarose gel (Figure 3.6) showed that the *T. vespertilionis* PCR might be specific because there was a product of the expected size (lane 4) and there was absence of product of this size when amplification was attempted using the *T. vespertilionis* primers with *T. dionisii* target DNA (lane 3). Although there was strong multi- band (non- specific) when the PCR was attempted using the *T. vespertilionis* primers with the T. dionisii target DNA (lane 3), none of the bands were at the expected product size and this then resolved by optimaisiting the temperature and the $MgCl_2$ as shown below (section 3.2.3). The *T.*

vespertilionis PCR product was cloned into the pGEM-T easy plasmid and subsequent DNA sequence analysis confirmed that it was 100% identical to the *T. vespertilionis* 18S rRNA gene sequence deposit in GenBank (AJ009166.1) (Figure 3.7 & Table 3.1). Due to lack of *T. vespertilionis* genomic DNA, this plasmid was subsequently used as the *T. vespertilionis* positive control for PCRs attempting to discriminate between *Schizotrypanum* infections in the bats.

Although the *T. dionisii* primer set produced a PCR product of the expected size (Figure 3.6, lane 2), this product was also observed when the primers were used with *T. vespertilionis* DNA (lane 5). As such, these *T. dionisii* primers were considered not to be specific and hence they were not used to screen bat samples.

```
T.vesp          -----------------------------------GACGCACTCGTCGCCTTTGTCGGA        24
AJ009166.1      TGACAAGCGGCCGGGTGCTCTTTCCCCCTTCGGGGGGACGCACTCGTCGCCTTTGTCGGA        1020
                                                   ***********************

T.vesp          AATCCGCGCCGGCTGCGGCTGTGTGCGTCACACTTCCACGTGTGTCACACGCGCCCTGCC        84
AJ009166.1      AATCCGCGCCGGCTGCGGCTGTGTGCGTCACACTTCCACGTGTGTCACACGCGCCCTGCC        1080
                ************************************************************

T.vesp          TGCGCCTTCCGGCAACTCACGGCATCCAGGAATGAAGGAGGGTAGTTCGGGGGAGAACGT        144
AJ009166.1      TGCGCCTTCCGGCAACTCACGGCATCCAGGAATGAAGGAGGGTAGTTCGGGGGAGAACGT        1140
                ************************************************************

T.vesp          ACTGGTGCGTCAGAGGTGAAATTCTTAGACCGCACCAAGACGAACTACAGCGAAGGCATT        204
AJ009166.1      ACTGGTGCGTCAGAGGTGAAATTCTTAGACCGCACCAAGACGAACTACAGCGAAGGCATT        1200
                ************************************************************

T.vesp          CTTCAAGGATACCTTCCTCAATCAAGAACCAAAGTGTGGGGATCGAAGATGATTAGAGAC        264
AJ009166.1      CTTCAAGGATACCTTCCTCAATCAAGAACCAAAGTGTGGGGATCGAAGATGATTAGAGAC        1260
                ************************************************************

T.vesp          CATTGTAGTCCACACTGCAAACGATGACACCCATGAATTGGGGAGTTTTTGGTCGTTAGG        324
AJ009166.1      CATTGTAGTCCACACTGCAAACGATGACACCCATGAATTGGGGAGTTTTTGGTCGTTAGG        1320
                ************************************************************

T.vesp          CGAGGTCGGGTTCATCTCGCTCCTCGTCTCGCCAATGAATATCAATTTACGTGCATATTC        384
AJ009166.1      CGAGGTCGGGTTCATCTCGCTCCTCGTCTCGCCAATGAATATCAATTTACGTGCATATTC        1380
                ************************************************************

T.vesp          TTTACGGTCCCCGCTTTCCAGCGGAGGCCTTTAACGGGAATATCCTCAGCACGTTATCTG        444
AJ009166.1      TTTACGGTCCCCGCTTTCCAGCGGAGGCCTTTAACGGGAATATCCTCAGCACGTTATCTG        1440
                ************************************************************

T.vesp          ACTTCTTCACGCGAAAGCTTTGAGGTTACAG-----------------------------        475
AJ009166.1      ACTTCTTCACGCGAAAGCTTTGAGGTTACAGTCTCAGGGGGGAGTACGTTCGCAAGAGTG        1500
                ****************************
```

Figure 3.7: Clustal W alignment of the sequence of the 18S rRNA PCR product derived from *T. vespertilionis* genomic DNA with the *T. vespertilionis* 18S rRNA sequence deposited in GenBank (AJ009166.1).

Table 3.1: BlastN summary data for the *T. vespertilionis* 18S rRNA PCR product.

Highly similar sequence	Max score	Total score	Query cover	E value	Iden	GenBank Accession number
Trypanosoma vespertilionis 18S rRNA gene, isolateP14	857	857	100%	0.0	100%	AJ009166.1
Trypanosoma conorhini 18S ribosomal RNA gene, partial sequence	693	693	94%	4e-163	91%	KP899113.1

3.2.3 PCR optimisations using the *T. vespertilionis* 18S rRNA plasmid construct:

Prior to screening bat samples, the *T. vespertilionis* primer set was optimised to improve the recovery of specific PCR product. The optimisation was carried out using the pGEM-T Easy plasmid construct containing the 18S rRNA fragment due to lack of *T. vespertilionis* genomic DNA.

3.2.3.1 Temperature gradient:

The effect of temperature on the performance of the *T. vespertilionis* PCR was assessed between 60-68°C. The Mg^{2+} concentration was set at 1.5 mM throughout this temperature optimisation as recommended by the manufacturer.

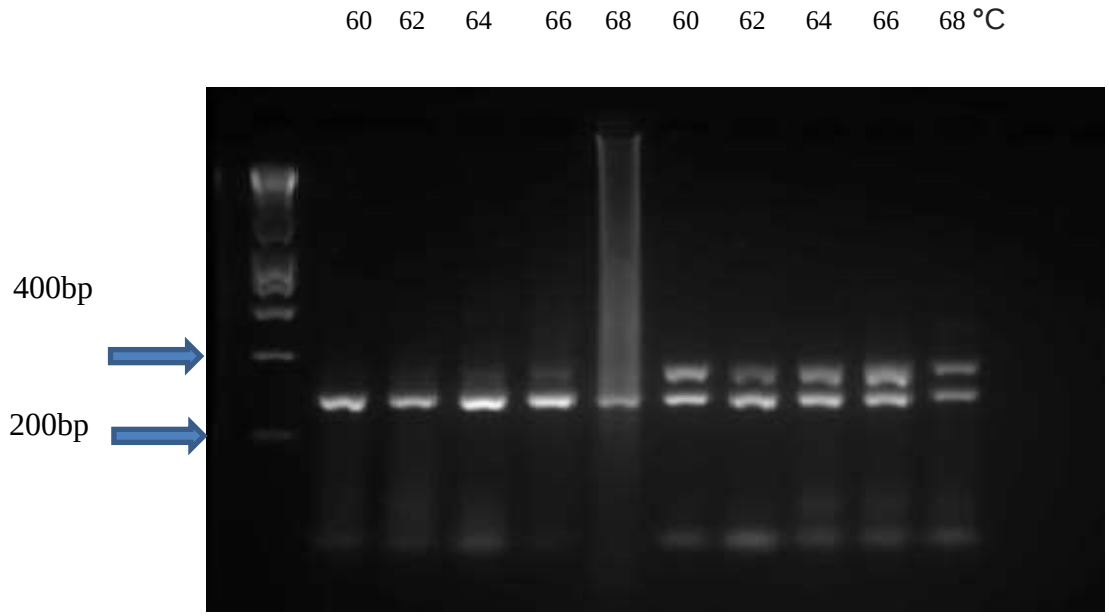

Figure 3.8: Agarose gel (1%) showing temperature gradient optimisation of the T. vespertilionis primers between 60 ^{0}C and 68 ^{0}C. 1, 1kb hyperladder; 2 – 6, *T. vespertilionis* 18S rRNA plasmid DNA target with *T. vespertilionis* primers used at the incremental annealing temperatures shown; 7 – 11, *T. dionisii* genomic DNA target with *T. vespertilionis* primers used at the incremental annealing temperatures shown.

From the above (Figure 3.8), it appeared that 66°C (lane 5) was the optimal temperature for PCR amplification of a *T. vespertilionis* 18S rRNA PCR product. Although there was a PCR product with the *T. dionisii* genomic DNA controls (lanes 7-11), this consistently appeared as a doublet and hence was distinguishable from the *T. vespertilionis* specific product.

3.2.3.2 $MgCl_2$ optimization:

To further optimize the *T. vespertilionis* reaction, PCRs were carried out in the presence of a $MgCl_2$ concentration gradient that ranged from 0.5- 1.5 mM.

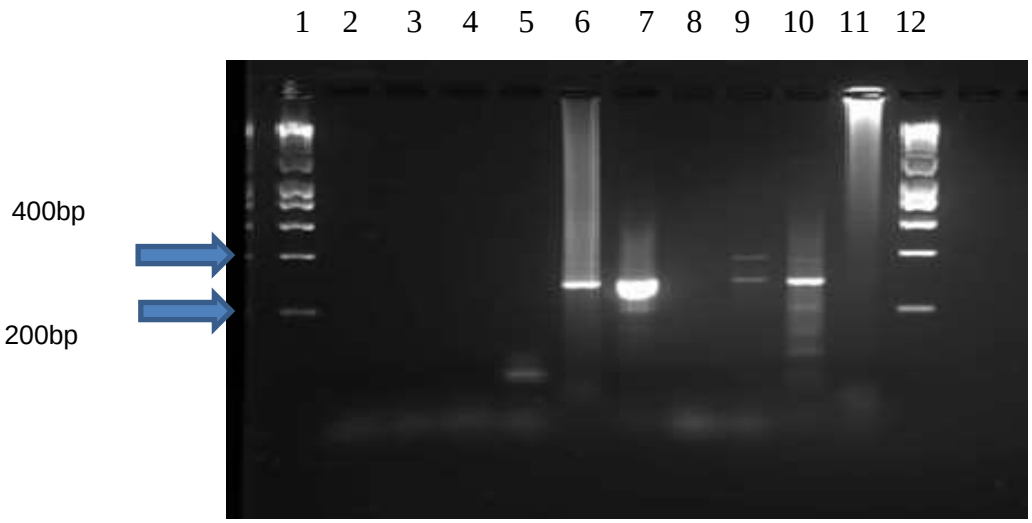

Figure 3.9: Agarose gel (1%) showing MgCl2 optimisation of the T. vespertilionis PCR. 1, 1kb hyperladder; 2 – 6, *T. vespertilionis* 18S rRNA plasmid DNA target with *T. vespertilionis* primers at incremental (0.25 mM) $MgCl_2$ concentrations between 0.5 and 1.5 mM); 7 - 11, *T. dionisii* genomic DNA target with *T. vespertilionis* primers at incremental (0.25 mM) $MgCl_2$ concentrations between 0.5 and 1.5 mM; lane 12, 1kb hyperladder. *Footnote*: the primer annealing temperature was kept at a constant 66°C for all reactions.

As shown above (Figure 3.9), the optimal Mg^{2+} concentration for PCR amplification of the *T. vespertilionis* 18S rRNA gene was 1.5 mM (lane 6). Importantly, the combination of 66°C primer annealing with 1.5 mM Mg^{2+} also enhanced the specificity of the primers since no product was produced with the *T. dionisii* genomic DNA using these conditions (lane11).

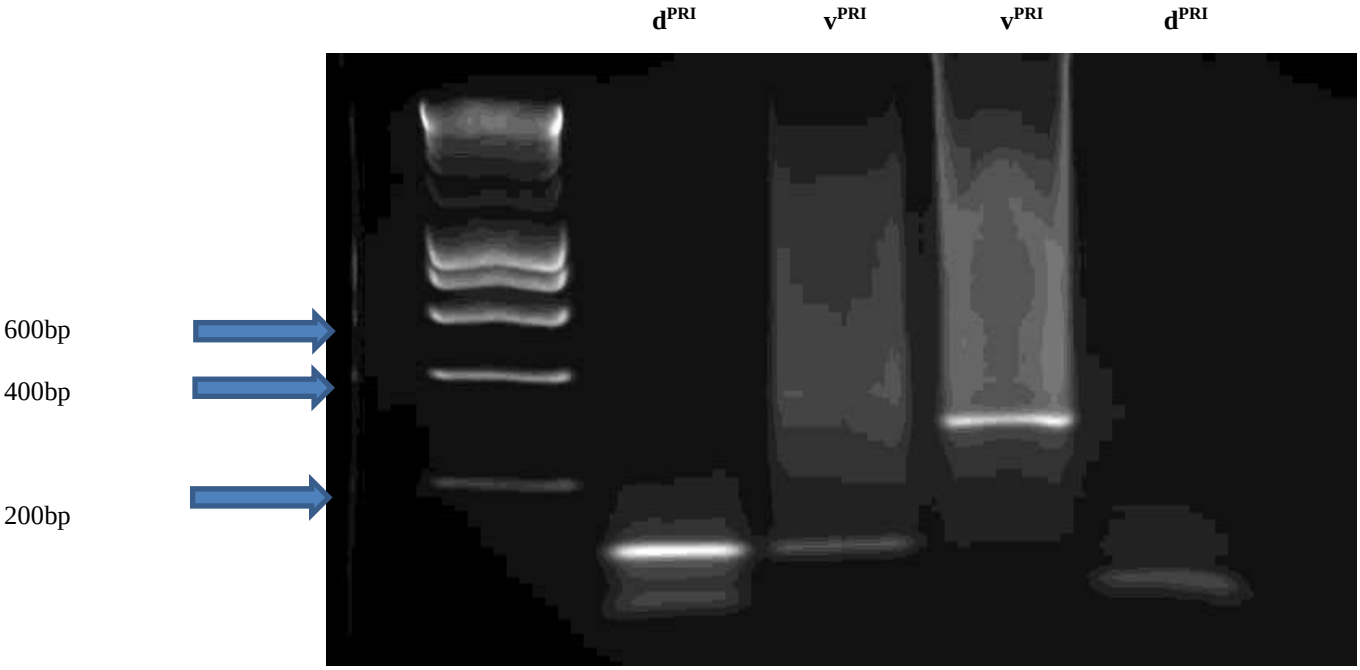

Figure 3.10: Agarose gel (1%) confirming the specificity of the *T. vespertilionis* 18S rRNA PCR at the optimised cycling conditions (66oC primer annealing & 1.5 mM Mg2+) using genomic DNA targets. 1, 1kb hyperladder; 2 and 3, *T. dionisii* DNA target; 4 and 5, *T. vespertilionis* DNA target. d^{PRI} indicates primers designed to be specific for *T. dionisii*; v^{PRI} indicates primers designed to be specific for *T. vespertilionis.*

To further confirm the specificity of the optimised *T. vespertilionis* reaction, the PCRs were repeated using the optimal cycling conditions with *T. vespertilionis* and *T. dionisii* genomic DNAs (Figure 3.10). The expected 312bp *T. vespertilionis* product was produced specifically with *T. vespertilionis* genomic DNA (lane 4). As observed previously (Figure 3.6), no PCR product was generated using the *T. vespertilionis* primers with *T. dionisii* genomic DNA (lane 3). The *T. vespertilionis* PCR product was sequenced to confirm reaction specificity and the results were as previously documented (Figure 3.7 & Table 3.1). Repeated attempts to optimise the specificity of the *T. dionisii* primer set proved unsuccessful and hence primer re-design was undertaken (see section 3.2.5).

3.2.4 Screening Bat Samples with the *T. vespertilionis* Primers

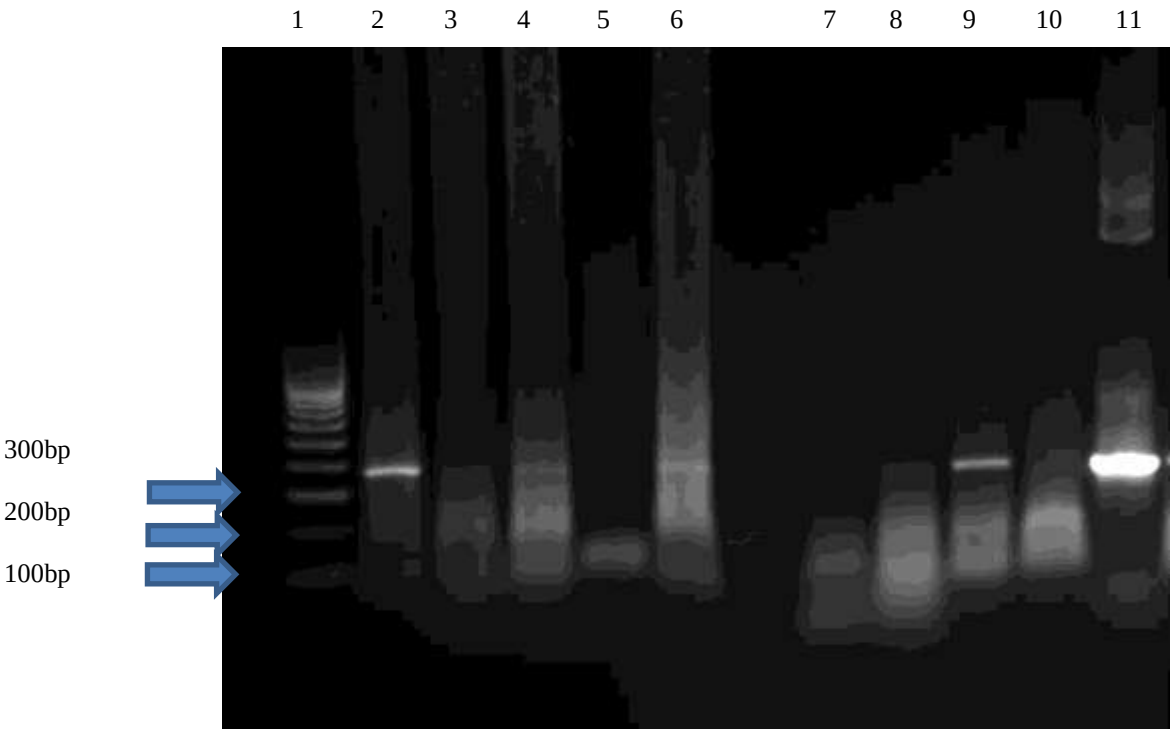

Figure 3.11(A): A representative agarose (1%) gel image showing analysis of *T. vespertilionis* 18S rRNA PCR products derived from bat heart DNA samples. 1, 100bp hyperladder; 2-10, bat DNA samples extracted from heart; 11, +ve control (*T. vespertilionis* 18S rRNA PCR product cloned into the pGEM-T Easy plasmid). Positive reactions have occurred in lanes 2, 4, 6, and 9 (bat codes: SA606, JL658, S679, JL709).

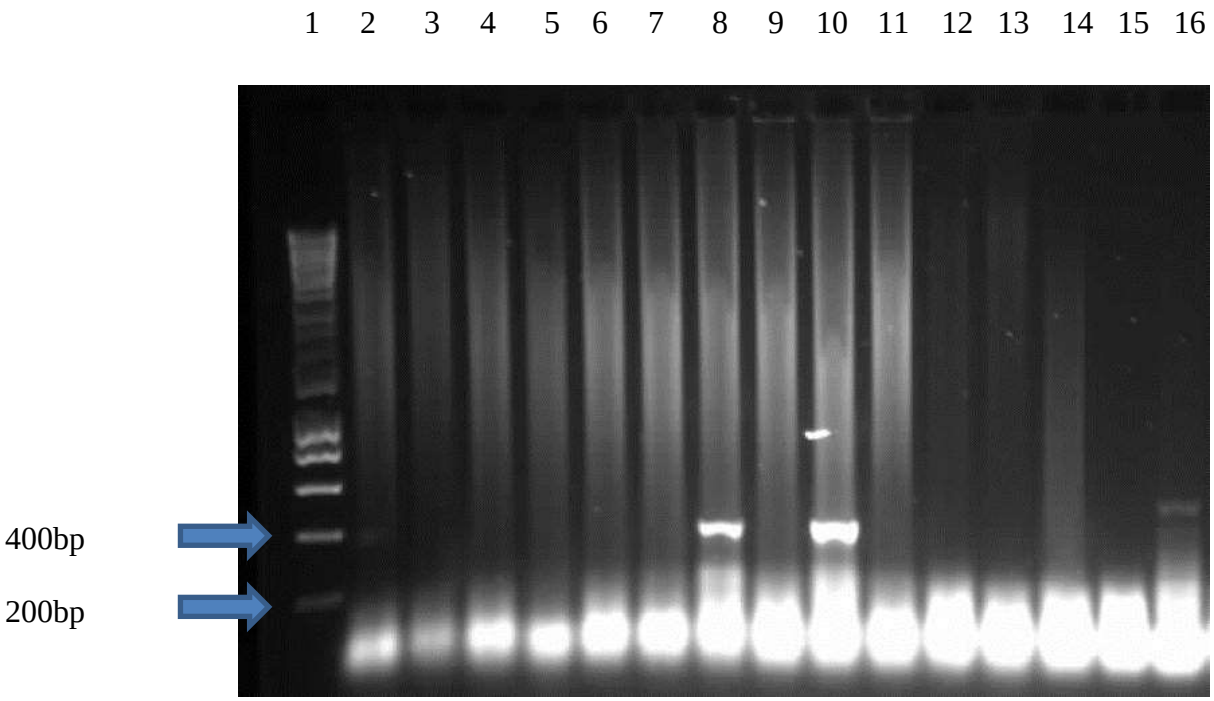

Figure 3.11(B): A representative agarose (1%) gel image showing analysis of *T. vespertilionis* 18S rRNA PCR products derived from bat spleen DNA samples. 1, 1kb hyperladder; 2-15, bat DNA samples extracted from spleen; 16, +ve control (*T. vespertilionis* PCR product cloned into the pGEM T-Easy plasmid). Positive reactions have occurred in lanes 8 and 10 (bat codes: SA606, JL709).

Bat heart and spleen DNA samples were screened using the optimised *T. vespertilionis* PCR conditions and 4 samples were positive for *T. vespertilionis* infection as shown (Figure 3.11 (A), lanes 2, 4, 6, 9 & Figure 3.11 (B), lanes 8, 10). Two of the samples were positive for both bat heart and spleen DNA targets (codes: SA606, JL709). The other two bats (codes: JL658, S679) were only diagnosed as positive by screening bat heart DNA. The PCR products were purified and DNA sequencing was performed (Figure 3.12).

```
AJ009151.1      CTCGTCGCCTTTGGGGGAAATCCGTGGCGCTGTCG------------------ACGGACT      1059
SA606           ----------------------------------------GCGTCACACTTCCACGTG      18
AJ009166.1      CTCGTCGCCTTTGTCGGAAATCCGCGCCGGCTGCGGCTGTGTGCGTCACACTTCCACGTG      1062
                                                                      *

AJ009151.1      TCGGTCCCATCTTCACGCGTCGCCTTCCCTCAACTCACGGCATCCAGGAATGAAGGAGGG      1119
SA606           TGTCACACGCGTCCTGCCTGCGCCTTCCGGCAACTCACGGCATCCAGGAATGAAGGAGGG      78
AJ009166.1      TGTCACACGCGCCCTGCCTGCGCCTTCCGGCAACTCACGGCATCCAGGAATGAAGGAGGG      1122
                *    * *     *   *  ********  ******************************

AJ009151.1      TAGTTCGGGGGAGAACGTACTGGTGCGTCAGAGGTGAAATTCTTAGACCGCACCAAGACG      1179
SA606           TAGTTCGGGGGAGAACGTACTGGTGCGTCAGAGGTGAAATTCTTAGACTGCACCAAGACG      138
AJ009166.1      TAGTTCGGGGGAGAACGTACTGGTGCGTCAGAGGTGAAATTCTTAGACCGCACCAAGACG      1182
                ********************************************** ***********

AJ009151.1      AACTACAGCGAAGGCATTCTTCAAGGATACCTTCCTCAATCAAGAACCAAAGTGTGGGGA      1239
SA606           AACTACAGCGAAGGCATTCTTCAAGGATACCTTCCTCAATCAAGAACCAAAGTGTGGGGA      198
AJ009166.1      AACTACAGCGAAGGCATTCTTCAAGGATACCTTCCTCAATCAAGAACCAAAGTGTGGGGA      1242
                ************************************************************

AJ009151.1      TCGAAGATGATTAGAGACCATTGTAGTCCACACTGCAAACGATGACACCCATGAATTGGG      1299
SA606           TCGAAGATGATTAGAGACCATTGTAGTCCACACTGCAAACGATGACACCCATGAATTGGG      258
AJ009166.1      TCGAAGATGATTAGAGACCATTGTAGTCCACACTGCAAACGATGACACCCATGAATTGGG      1302
                ************************************************************

AJ009151.1      GAGTTTTTGGTCGTTTAGGCGTGGTCGGGTTCACCCCGCTCCTCGTCTCGCCAATGAATG      1359
SA606           GAGTTTTTGGTCGTTAT-GCGAGGTCGGGTTCATCTCGCTCCTCGTCTCGCCAATGAAT-      316
AJ009166.1      GAGTTTTTGGTCGTTAG-GCGAGGTCGGGTTCATCTCGCTCCTCGTCTCGCCAATGAAT-      1360
                ***************   *** *********** * ***********************
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AJ009151.1      AATAATTTACGTGCATATTCTTTTTGGTCCTCGTTCTTACGCGTGGGCCTTTAACGGGAA      1419
SA606           ATCAATTTACGTGCATATTCTTTACGGTCCCCGCTTTCCAGCGGAGGCCTTTAAC-----      371
AJ009166.1      ATCAATTTACGTGCATATTCTTTACGGTCCCCGCTTTCCAGCGGAGGCCTTTAACGGGAA      1420
                *  ******************  ***** ** * *   ***  **********
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Figure 3.12: Clustal W alignment of a representative *T. vespertilionis* 18S rRNA PCR product derived from bat specimen SA606 with *T. dionisii* (AJ009151.1) and *T. vespertilionis* (AJ009166.1) 18S rRNA sequences extracted from NCBI GenBank.

Table 3.2: BlastN summary data for the *T. vespertilionis* 18S rRNA PCR product.

Highly similar sequence	Max score	Total score	Query cover	E value	Iden	GenBank Accession number
Trypanosoma vespertilionis 18S rRNA gene, isolate P14	669	669	100%	0.0	99%	AJ009166.1
Trypanosoma conorhini 18S ribosomal RNA gene, partial sequence	586	586	94%	1e-163	97%	KP899113.1
Trypanosoma dionisii 18S rRNA gene, isolate P3	507	507	89%	2e-147	90%	AJ009151.1

The sequence of all four PCR products (derived from bats: SA606, JL658, SP679, and JL709) were identical to each other and most similar to the 18S rRNA of *T. vespertilionis* isolate P14 (Stevens, Noyes, Dover, & Gibson, 1999). However, the pipistrelle derived 18S rRNA sequences were not 100% identical to the GenBank deposit (Table 3.2); two nucleotide changes were noted at positions 1074 and 1171 of the P14 isolate (Figure 3.12).

3.2.5 *Trypanosoma dionisii* PCR primer re-design:

The lack of *T. dionisii* and *T. vespertilionis* DNA sequences deposited in GenBank precluded the targeting of other genes for *Schizotrypanum* species identification and hence additional *T. dionisii* 18S rRNA primers were designed in an attempt to improve the specificity of the analysis using the nested PCR approach.

```
AJ009166.1      TTCGTAGTTGAATTGTGGGCCTTCGAGGCGCAATGGTTTAGTCCCGTCCACTTCGGATTG        716
AJ009151.1      TTCGTAGTTGAATTGTGGGCCTCTAAGGCGCAATGGTTTAGTCCCATCCACTTCGGATTG        718
                ********************   ******************** **************

AJ009166.1      GTGACCCATGCCCTTGAGGTCCGTGAACACTCAGAAACAAAAAACACGGGAGTGGTACCT        776
AJ009151.1      GTGACCCATGCCCTTGTGGTCCGTGAACACTCAGAAACAAAAAACACGGGAGTGGTACCC        778
                *************** ****************************************

AJ009166.1      TT-CTGATTTCCGCATGTCATGCATGCCAGGGGGCGCCCGTGATTTTTTACTGTGACTAA        835
AJ009151.1      TTTCTGATTCTCGCATGTCATGCATGCCAGGGGGCGCCCGT-GATTTTTACTGTGACTAA        837
                ** ******  ******************************   ****************

AJ009166.1      AAAAGTGTGACCAAAGCAGTCATTCGACTTGAATTAGAAAGCATGGGATAACAAAGGAGC        895
AJ009151.1      AAAAGTGTGACCAAAGCAGTCATTCGACTTGAATTAGAAAGCATGGGATAACAAAGGAGC        897
                ************************************************************

AJ009166.1      AGCCTATGGGCCACCGTTTCGGCTTTTGTTGGTTTTAAAAGTCCATTGGAGATTATGGGG        955
AJ009151.1      AGCCTATGGGCCACCGTTTCGGCTTTTGTTGGTTTTAAAAGTCCATTGGAGATTATGGGG        957
                ************************************************************

AJ009166.1      CAGTGTGACAAGCGGCCGGGTGCTCTTTCCCCCTTCGGGGGGACGCA-------------        1002
AJ009151.1      CAGTGTGACAAGCGGCTGGGTGATGATATCCCACACACCTTCACTGCGTGTTGTGGCACA        1017
                *************** ***** *  *  ***   *      **

AJ009166.1      CTCGTCGCCTTTGTCGGAAATCCGCGCCGGCTGCGGCTGTGTGCGTCACACTTCCACGTG        1062
AJ009151.1      CTCGTCGCCTTTGGGGGAAATCCGTGGCGCTGTCGA------------------CGGACT        1059
                *************  ********* * **    **                   *

AJ009166.1      TGTCACACGCGCCCTGCCTGCGCCTTCCGGCAACTCACGGCATCCAGGAATGAAGGAGGG        1122
AJ009151.1      TCGGTCCCATCTTCACGCGTCGCCTTCCCTCAACTCACGGCATCCAGGAATGAAGGAGGG        1119
                *    * *     *   *  ********  ******************************
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Figure 3.13: Clustal W alignment for a region of the Schizotrypanum 18S rRNA genes extracted from NCBI GenBank: *T. dionisii* (AJ009151.1) and *T. vespertilionis* (AJ009166.1). Red text shows the *T. dionisii* primer binding sites (TrypF and TrypR). The full sequence alignment can be obtained from Appendix 1.

```
FN599058.1      -------ACTGCCATGGCGTTGACGGGAGCGGGGGATTAGGGTTCGATTCCGGAGAGGGA 53
AJ009166.1      GTAGTGGACTGCCATGGCGTTGACGGGAGCGGGGGATTAGGGTTCGATTCCGGAGAGGGA 417
AJ009151.1      GTAGTGGACTGCCATGGCGTTGACGGGAGCGGGGGATTAGGGTTCGATTCCGGAGAGGGA 418
AJ009152.1      GTAGTGGACTGCCATGGCGTTGACGGGAGCGGGGGATTAGGGTTCGATTCCGGAGAGGGA 418
                       **************************************************

FN599058.1      GCCTGAGAAATAGCTACCACTTCTACGGAGGGCAGCAGGCGCGCAAATTGCCCAATGTCA 113
AJ009166.1      GCCTGAGAAATAGCTACCACTTCTACGGAGGGCAGCAGGCGCGCAAATTGCCCAATGTCA 477
AJ009151.1      GCCTGAGAAATAGCTACCACTTCTACGGAGGGCAGCAGGCGCGCAAATTGCCCAATGTCA 478
AJ009152.1      GCCTGAGAAATAGCTACCACTTCTACGGAGGGCAGCAGGCGCGCAAATTGCCCAATGTCA 478
                ************************************************************

FN599058.1      AAAAAACACGATGAGGCAGCGAAAAGAAATAGAGCCGACAGTGCTTTTGCATTGTCGTTT 173
AJ009166.1      AAAAAAAACGATGAGGCAGCGAAAAGAAATAGAGCCGACAGTGCTT-TGCATTGTCGTTT 536
AJ009151.1      AAAAAAAACGATGAGGCAGCGAAAAGAAATAGAGCCGACAGTGCTTTTGCATTGTCGTTT 538
AJ009152.1      AAAAAAAACGATGAGGCAGCGAAAAGAAATAGAGCCGACAGTGCTTTTGCATTGTCGTTT 538
                ****** ************************************* **************

FN599058.1      TCAATGGGGGATATTTAAACCCATCCAAAATCGAGTAACAATTGGAGGACAAGTCTGGTG 233
AJ009166.1      TCAATGGGGGATATTTAAACCCATCCAAAATCGAGTAACAATTGGAGGACAAGTCTGGTG 596
AJ009151.1      TCAATGGGGGATATTTAAACCCATCCAAAATCGAGTAACAATTGGAGGACAAGTCTGGTG 598
AJ009152.1      TCAATGGGGGATATTTAAACCCATCCAAAATCGAGTAACAATTGGAGGACAAGTCTGGTG 598
                ************************************************************

FN599058.1      CCAGCACCCGCGGTAATTCCAGCTCCAAAAGCGTATATTAATGCTGTTGCTGTTAAAGGG 293
AJ009166.1      CCAGCACCCGCGGTAATTCCAGCTCCAAAAGCGTATATTAATGCTGTTGCTGTTAAAGGG 656
AJ009151.1      CCAGCACCCGCGGTAATTCCAGCTCCAAAAGCGTATATTAATGCTGTTGCTGTTAAAGGG 658
AJ009152.1      CCAGCACCCGCGGTAATTCCAGCTCCAAAAGCGTATATTAATGCTGTTGCTGTTAAAGGG 658
                ************************************************************
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FN599058.1      TTCGTAGTTGAATTGTGGGCCTCTATAAGGCGCAATGGTTTAGTCCCATCCACTTCGGAT 353
AJ009166.1      TTCGTAGTTGAATTGTGGGCCTT--CGAGGCGCAATGGTTTAGTCCCGTCCACTTCGGAT 714
AJ009151.1      TTCGTAGTTGAATTGTGGGCCTC--TAAGGCGCAATGGTTTAGTCCCATCCACTTCGGAT 716
AJ009152.1      TTCGTAGTTGAATTGTGGGCCTC--TAAGGCGCAATGGTTTAGTCCCATCCACTTCGGAT 716
                **********************     ******************** ************

FN599058.1      TGGTGACCCATGCCCTTGTGGTCCGTGAACACTCAGAAACAAGAAACACGGGAGTGGTAC 413
AJ009166.1      TGGTGACCCATGCCCTTGAGGTCCGTGAACACTCAGAAACAAAAAACACGGGAGTGGTAC 774
AJ009151.1      TGGTGACCCATGCCCTTGTGGTCCGTGAACACTCAGAAACAAAAAACACGGGAGTGGTAC 776
AJ009152.1      TGGTGACCCATGCCCTTGTGGTCCGTGAACACTCAGAAACAAAAAACACGGGAGTGGTAC 776
                ****************** ********************** ****************

FN599058.1      CCTTTCTGATTTTCGCATGTCATGCATGCCAGGGGGCGCCCGT-GATTTTTACTGTGACT 472
AJ009166.1      CTTT-CTGATTTCCGCATGTCATGCATGCCAGGGGGCGCCCGTGATTTTTTACTGTGACT 833
AJ009151.1      CCTTTCTGATTCTCGCATGTCATGCATGCCAGGGGGCGCCCGT-GATTTTTACTGTGACT 835
AJ009152.1      CCTTTCTGATTCTCGCATGTCATGCATGCCAGGGGGCGCCCGT-GATTTTTACTGTGACT 835
                * ** ******  ******************************   **************

FN599058.1      AAAAAAGTGTGACCAAAGCAGTCATTCGACTTGAATTAGAAAGCATGGGATAACAAAGGA 532
AJ009166.1      AAAAAAGTGTGACCAAAGCAGTCATTCGACTTGAATTAGAAAGCATGGGATAACAAAGGA 893
AJ009151.1      AAAAAAGTGTGACCAAAGCAGTCATTCGACTTGAATTAGAAAGCATGGGATAACAAAGGA 895
AJ009152.1      AAAAAAGTGTGACCAAAGCAGTCATTCGACTTGAATTAGAAAGCATGGGATAACAAAGGA 895
                ************************************************************

FN599058.1      GCAGCCTATGGGCCACCGTTTCGGCTTTTGTTGGTTTTAAAAGTCCATTGGAGATTATGG 592
AJ009166.1      GCAGCCTATGGGCCACCGTTTCGGCTTTTGTTGGTTTTAAAAGTCCATTGGAGATTATGG 953
AJ009151.1      GCAGCCTATGGGCCACCGTTTCGGCTTTTGTTGGTTTTAAAAGTCCATTGGAGATTATGG 955
AJ009152.1      GCAGCCTATGGGCCACCGTTTCGGCTTTTGTTGGTTTTAAAAGTCCATTGGAGATTATGG 955
                ************************************************************

FN599058.1      GGCAGTGTGACAAGCGGCTGGGTGATGATATCCCACACAACACACCTTCACTGCGTGTTT 652
AJ009166.1      GGCAGTGTGACAAGCGGCCGGGTGCTCTTTCCCCCTTCGGGGGGACGCA---------- 1002
AJ009151.1      GGCAGTGTGACAAGCGGCTGGGTGATGATATCCCACACACCTTCACTGCGTG-------- 1007
AJ009152.1      GGCAGTGTGACAAGCGGCTGGGTGATGATATCCCACACACCTTCACTGCGTG-------- 1007
                ****************** ***** *  *  ***   *       *

FN599058.1      TTGTGTGGCACACTCGTCGCCTTTGGGGGAAATTCGTGGCGCTG---------------- 696
AJ009166.1      ------------CTCGTCGCCTTTGTCGGAAATCCGCGCCGGCTGCGGCTGTGTGCGTCA 1050
AJ009151.1      --TTGTGGCACACTCGTCGCCTTTGGGGGAAATCCGTGGCGCTGTCGA------------ 1053
AJ009152.1      --TTGTGGCACACTCGTCGCCTTTGGGGGAAATCCGTGGCGCTGTCGA------------ 1053
                            *************  ****** ** * **

FN599058.1      TTGACACGGACTTCGGTCCCATCTTCACGCGTCGCCTTCCTTCAACTCACGGCATCCAGG 756
AJ009166.1      CACTTCCACGTGTGTCACACGCGCCCTGCCTGCGCCTTCCGGCAACTCACGGCATCCAGG 1110
AJ009151.1      ------CGGACTTCGGTCCCATCTTCACGCGTCGCCTTCCCTCAACTCACGGCATCCAGG 1107
AJ009152.1      ------CGGACTTCGGTCCCATCTTCACGCGTCGCCTTCCCTCAACTCACGGCATCCAGG 1107
                      *     *    * *     *   *  ********  ******************

FN599058.1      AATGAAGGAGGGTAGTTCGGGGGAGAACGTACTGGTGCGTCAGAGGTGAAATTCTTAGAC 816
AJ009166.1      AATGAAGGAGGGTAGTTCGGGGGAGAACGTACTGGTGCGTCAGAGGTGAAATTCTTAGAC 1170
AJ009151.1      AATGAAGGAGGGTAGTTCGGGGGAGAACGTACTGGTGCGTCAGAGGTGAAATTCTTAGAC 1167
AJ009152.1      AATGAAGGAGGGTAGTTCGGGGGAGAACGTACTGGTGCGTCAGAGGTGAAATTCTTAGAC 1167
                ************************************************************

FN599058.1      CGCACCAAGACGAACTACAGCGAAGGCATTCTTCAAGGATACCTTCCTCAATCAAGAACC 876
AJ009166.1      CGCACCAAGACGAACTACAGCGAAGGCATTCTTCAAGGATACCTTCCTCAATCAAGAACC 1230
AJ009151.1      CGCACCAAGACGAACTACAGCGAAGGCATTCTTCAAGGATACCTTCCTCAATCAAGAACC 1227
AJ009152.1      CGCACCAAGACGAACTACAGCGAAGGCATTCTTCAAGGATACCTTCCTCAATCAAGAACC 1227
                ************************************************************

FN599058.1      AAAGTGTGGGGATCGAAGATGATTAGAGACCATTGTAGTCCACACTGCAAACGATGACAC 936
AJ009166.1      AAAGTGTGGGGATCGAAGATGATTAGAGACCATTGTAGTCCACACTGCAAACGATGACAC 1290
AJ009151.1      AAAGTGTGGGGATCGAAGATGATTAGAGACCATTGTAGTCCACACTGCAAACGATGACAC 1287
AJ009152.1      AAAGTGTGGGGATCGAAGATGATTAGAGACCATTGTAGTCCACACTGCAAACGATGACAC 1287
                ************************************************************

FN599058.1      CCATGAATTGGGGAGTTTTTGGTCGTTTAGGCGTGGTCGGGTTTACCCCGCTCCATCGTC 996
AJ009166.1      CCATGAATTGGGGAGTTTTTGGTCGTTA-GGCGAGGTCGGGTTCATCTCGCT-CCTCGTC 1348
AJ009151.1      CCATGAATTGGGGAGTTTTTGGTCGTTTAGGCGTGGTCGGGTTCACCCCGCT-CCTCGTC 1346
AJ009152.1      CCATGAATTGGGGAGTTTTTGGTCGTTTAGGCGTGGTCGGGTTCACCCCGCT-CCTCGTC 1346
                **************************  **** ********* * * **** * *****

FN599058.1      TCGCCAATGAATGAATAATTTACGTGCATATTCTTTTTGGTCCTCGTTTATTTTTTTTAT 1056
AJ009166.1      TCGCCAATGAAT-ATCAATTTACGTGCATATTCTTTACGGTCCCCG-------CTTTCCA 1400
AJ009151.1      TCGCCAATGAATGAATAATTTACGTGCATATTCTTTTTGGTCCTCG-------TTCTTAC 1399
AJ009152.1      TCGCCAATGAATGAATAATTTACGTGCATATTCTTTTTGGTCCTCG-------TTCTTAC 1399
                ************ *  ********************  ***** **        * *
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FN599058.1      GCGTGGGCCTTTAACGGGAATATCCTCAGCACGTTATCTGACTTCTTTACGCGAAAGCTT 1116
AJ009166.1      GCGGAGGCCTTTAACGGGAATATCCTCAGCACGTTATCTGACTTCTTCACGCGAAAGCTT 1460
AJ009151.1      GCGTGGGCCTTTAACGGGAATATCCTCAGCACGTTATCTGACTTCTTCACGCGAAAGCTT 1459
AJ009152.1      GCGTGGGCCTTTAACGGGAATATCCTCAGCACGTTATCTGACTTCTTCACGCGAAAGCTT 1459
                ***  ************************************** ************

FN599058.1      TGAGGTTACAGTCTCAGGGGGGAGTACGTTCGCAAGAGTGAAACTTAAAGAAATTGACGG 1176
AJ009166.1      TGAGGTTACAGTCTCAGGGGGGAGTACGTTCGCAAGAGTGAAACTTAAAGAAATTGACGG 1520
AJ009151.1      TGAGGTTACAGTCTCAGGGGGGAGTACGTTCGCAAGAGTGAAACTTAAAGAAATTGACGG 1519
AJ009152.1      TGAGGTTACAGTCTCAGGGGGGAGTACGTTCGCAAGAGTGAAACTTAAAGAAATTGACGG 1519
                ************************************************************

FN599058.1      AATGGCACCACAAGACGTGGAGCGTGCGGTTTAATTTGACTCAACACGGGGAACTTTACC 1236
AJ009166.1      AATGGCACCACAAGACGTGGAGCGTGCGGTTTAATTTGACTCAACACGGGGAACTTTACC 1580
AJ009151.1      AATGGCACCACAAGACGTGGAGCGTGCGGTTTAATTTGACTCAACACGGGGAACTTTACC 1579
AJ009152.1      AATGGCACCACAAGACGTGGAGCGTGCGGTTTAATTTGACTCAACACGGGGAACTTTACC 1579
                ************************************************************

FN599058.1      AGATCCGGACAGGGTGAGGATTGACAGATTGAGTGTTCTTTCTCGATCCCCTGAATGGTG 1296
AJ009166.1      AGATCCGGACAGGGTGAGGATTGACAGATTGAGTGTTCTTTCTCGATCCCCTGAATGGTG 1640
AJ009151.1      AGATCCGGACAGGGTGAGGATTGACAGATTGAGTGTTCTTTCTCGATCCCCTGAATGGTG 1639
AJ009152.1      AGATCCGGACAGGGTGAGGATTGACAGATTGAGTGTTCTTTCTCGATCCCCTGAATGGTG 1639
                ************************************************************

FN599058.1      GTGCATGGCCGCTTTTGGTCGGTGGAGTGATTTGTTTGGTTGATTCCGTCAACGGACGAG 1356
AJ009166.1      GTGCATGGCCGCTTTTGGTCGGTGGAGTGATTTGTTTGGTTGATTCCGTCAACGGACGAG 1700
AJ009151.1      GTGCATGGCCGCTTTTGGTCGGTGGAGTGATTTGTTTGGTTGATTCCGTCAACGGACGAG 1699
AJ009152.1      GTGCATGGCCGCTTTTGGTCGGTGGAGTGATTTGTTTGGTTGATTCCGTCAACGGACGAG 1699
                ************************************************************

FN599058.1      ATCCAAGCTGCCCAGTAGGATTCAGAATTGCCCATAGGATAGCAATCCCTTCCGCGGGTT 1416
AJ009166.1      ATCCAAGCTGCCCAGTAGGATTCAGAATTGCCCATAGGATAGCAATCCCTTCCGCGGGTT 1760
AJ009151.1      ATCCAAGCTGCCCAGTAGGATTCAGAATTGCCCATAGGATAGCAATCCCTTCCGCGGGTT 1759
AJ009152.1      ATCCAAGCTGCCCAGTAGGATTCAGAATTGCCCATAGGATAGCAATCCCTTCCGCGGGTT 1759
                ************************************************************

FN599058.1      TTACCCAAGGGGGGGCGGTATTCGTTTGT------------------------------- 1445
AJ009166.1      TTACCCAAGGGGGGGCGGTATTCGTTTGTATCCTTCTCTGCGGGATTCCTTGTTTCGCGC 1820
AJ009151.1      TTACCCAAGGGGGGGCGGTATTCGTTTGTATCCTTCTCTGCGGGATTCCTTGTTTTGCGC 1819
AJ009152.1      TTACCCAAGGGGGGGCGGTATTCGTTTGTATCCTTCTCTGCGGGATTCCTTGTTTTGCGC 1819
                ****************************
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Figure3.14: Clustal W alignment for a region of the Schizotrypanum 18S rRNA genes extracted from NCBI GenBank: *T. dionisii* (AJ009151.1/ FN599058.1/ AJ009152.1) and T. *vespertilionis* (AJ009166.1). Red text shows the *T. dionisii* primer binding sites (TrypF and TrypR).

After aligning the other two sequences for the *T. dionisii* the primer sites were checked, it appeared no differences between the sequences of *T. dionisii* and the primers were the sites were the best sites to design primers from since it has the most variability to distinguish between the two Schizotrypanum species.

Due to the lack of *T. dionisii* genomic DNA, it was not possible to establish an optimised set of PCR cycling parameters for the new *T. dionisii* primers. Consequently, bat heart and

spleen DNA samples were screened by PCR using the predicted Primer3 optimal annealing temperature (61^{o}C). The expected *T. dionisii* 18S rRNA PCR product size was 402bp.

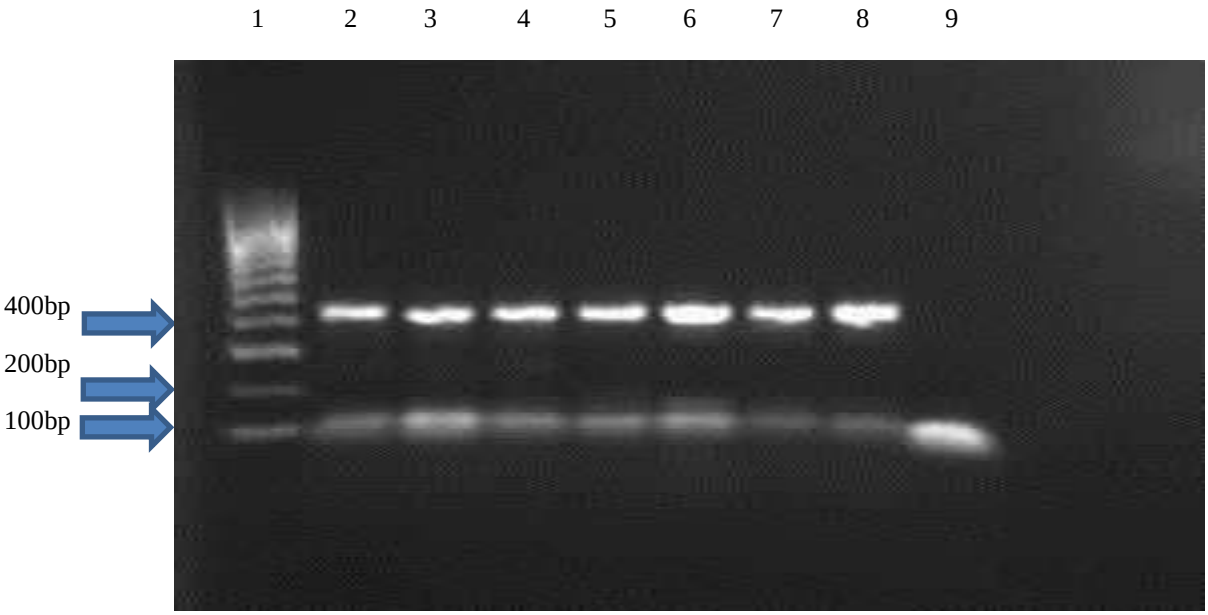

Figure 3.15 (A): A representative agarose (1%) gel image showing analysis of *T. dionisii* 18S rRNA PCR products derived from bat heart DNAs. 1, 100bp hyperladder; 2-8, bat DNA samples extracted from heart (bat codes: SP670, SP677, PB601, JL650, FP751, JL714, P605); 9, negative control DNA (bat code: SA606; confirmed positive for *T. vespertilionis*)

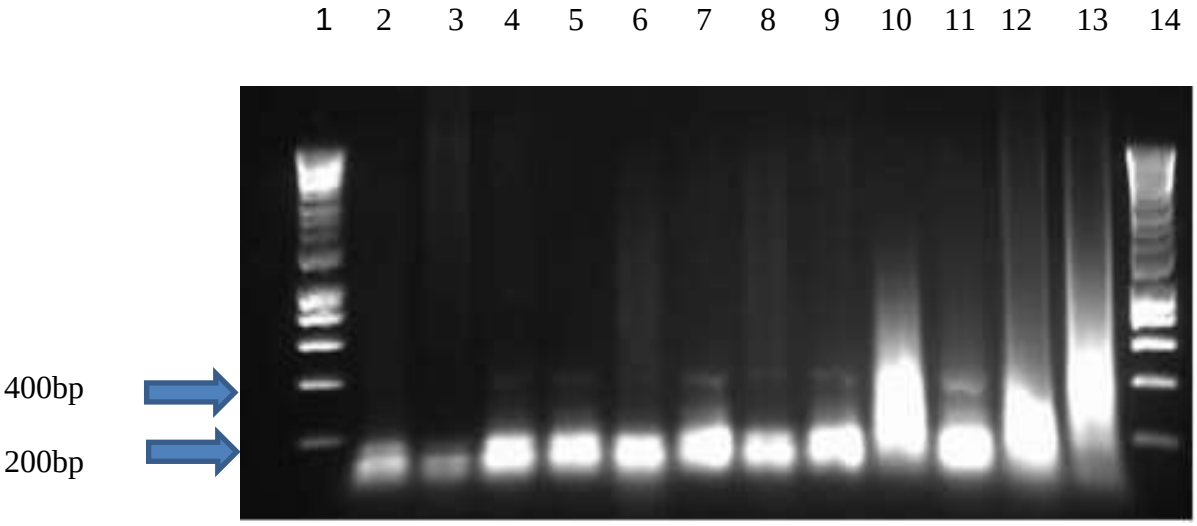

Figure 3.15 (B): A representative agarose (1%) gel image showing analysis of *T. dionisii* 18S rRNA PCR products derived from bat spleen DNAs. 1, 1kb hyperladder; 2, negative control (bat code: SA606; confirmed positive for *T. vespertilionis*); 3-13, bat DNA samples extracted from spleen (bat codes: PB601, JL650, FP751, JL714, SP670, JL708, SP677, JL627, P605, JL648, JL652); 14, 1kb hyperladder.

When comparing the heart and spleen PCRs, 90% of the bat samples (n=9) were positive for both heart and spleen infection. The remaining *T. dionisii* infection (n=1) was determined on the basis of a positive PCR amplification from just bat heart DNA (Figure 3.15(A, B)). The PCR products were purified and following DNA sequencing, all were identical to each other and to the 18S rRNA of *T. dionisii* isolate P3 (Stevens et al., 1999) (Figure 3.16 & Table 3.3).

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CLUSTAL O(1.2.1) multiple sequence alignment


AJ009166.1      TTCGTAGTTGAATTGTGGGCCTTCGAGGCGCAATGGTTTAGTCCCGTCCACTTCGGATTG        716
P605            ----------------------TCTAAGGCGCAATGGTTTAGTCCCATCCACTTCGGATTG        39
AJ009151.1      TTCGTAGTTGAATTGTGGGCCTCTAAGGCGCAATGGTTTAGTCCCATCCACTTCGGATTG        718
                                      *   ******************** **************

AJ009166.1      GTGACCCATGCCCTTGAGGTCCGTGAACACTCAGAAACAAAAAACACGGGAGTGGTACCT        776
P605            GTGACCCATGCCCTTGTGGTCCGTGAACACTCAGAAACAAAAAACACGGGAGTGGTACCC        99
AJ009151.1      GTGACCCATGCCCTTGTGGTCCGTGAACACTCAGAAACAAAAAACACGGGAGTGGTACCC        778
                **************** ****************************************
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AJ009166.1      TT-CTGATTTCCGCATGTCATGCATGCCAGGGGGCGCCCGTGATTTTTTACTGTGACTAA        835
P605            TTTCTGATTCTCGCATGTCATGCATGCCAGGGGGCGCCCGT-GATTTTTACTGTGACTAA        158
AJ009151.1      TTTCTGATTCTCGCATGTCATGCATGCCAGGGGGCGCCCGT-GATTTTTACTGTGACTAA        837
                ** ******  ****************************   ****************


AJ009166.1      AAAAGTGTGACCAAAGCAGTCATTCGACTTGAATTAGAAAGCATGGGATAACAAAGGAGC        895
P605            AAAAGTGTGACCAAAGCAGTCATTCGACTTGAATTAGAAAGCATGGGATAACAAAGGAGC        218
AJ009151.1      AAAAGTGTGACCAAAGCAGTCATTCGACTTGAATTAGAAAGCATGGGATAACAAAGGAGC        897
                ************************************************************


AJ009166.1      AGCCTATGGGCCACCGTTTCGGCTTTTGTTGGTTTTAAAAGTCCATTGGAGATTATGGGG        955
P605            AGCCTATGGGCCACCGTTTCGGCTTTTGTTGGTTTTAAAAGTCCATTGGAGATTATGGGG        278
AJ009151.1      AGCCTATGGGCCACCGTTTCGGCTTTTGTTGGTTTTAAAAGTCCATTGGAGATTATGGGG        957
                ************************************************************


AJ009166.1      CAGTGTGACAAGCGGCCGGGTGCTCTTTCCCCCTTCGGGGGGACGCACTCGTCGCCTTTG        1015
P605            CAGTGTGACAAGCGGCTGGGTGATGATATCCCACACACCTTCACTGCGTGTTGTGGCACA        338
AJ009151.1      CAGTGTGACAAGCGGCTGGGTGATGATATCCCACACACCTTCACTGCGTGTTGTGGCACA        1017
                *************** ***** *  *  ***   *      **


AJ009166.1      TCGGAAATCCGCGCCGGCTGCGGCTGTGTGCGTCACACTTCCACGTGTGTCACACGCGCC        1075
P605            CTCGTCGCCTTTGGGGGAAATCCGTGGCGCTGTCGACGGACTTCGGTCCCATCTTCACGC        398
AJ009151.1      CTCGTCGCCTTTGGGGGAAATCCGTGGCGCTGTCGACGGACTTCGGTCCCATCTTCACGC        1077
                        *   *           *      **         **


AJ009166.1      CTGCCTGCGCCTTCCGGCAACTCACGGCATCCAGGAATGAAGGAGGGTAGTTCGGGGGAG        1135
P605            GTCGC-------------------------------------------------------        403
AJ009151.1      GTCGCCTTCCCTCAACTCACGGCATCCAGGAATGAAGGAGGGTAGTTCGGGGGAGAACGT        1137
                 *  *
```

Figure 3.16: Clustal W alignment of a representative *T. dionisii* 18S rRNA PCR product derived from bat specimen P605 with the *T. dionisii* (AJ009151.1) and *T. vespertilionis* (AJ009166.1) 18S rRNA sequences deposited in NCBI GenBank.

Table 3.3: BlastN summary data for the *T. dionisii* 18S rRNA PCR product derived from pipistrelle P605.

Highly similar sequence	Max score	Total score	Query cover	E value	Iden	GenBank Accession number
Trypanosoma dionisii 18S rRNA gene, isolate P3	745	745	100%	0.0	100%	AJ009151.1
Trypanosoma dionisii culture-collection TCC/USP:495 18S ribosomal RNA gene, complete sequence	723	723	100%	0.0	99%	FJ001667.2
Trypanosoma vespertilionis 18S rRNA gene, isolate P14	508	508	85%	5e-148	91%	AJ009166.1

Another set of PCR primers were designed for *T. dionisii* sub-typing using a semi-nested PCR approach that targeted the GAPDH gene of this bat trypanosome.

```
gi|313209097|emb|FN599054.1|      ----------------------GGTCGATATGAACACGGACGCGGAGTATTTTGCATACCA      39
gi|313209103|emb|FN599056.1|      --------ACGTCGTGGCGGTGGTCGATATGAACACGGACGCGGAGTACTTTGCGTACCA      52
gi|313209100|emb|FN599055.1|      GGAGATTGACGTCGTGGCGGTGGTCGATATGAACACGGACGCGGAGTACTTTGCGTACCA      60
                                                ************************** ***** *****

gi|313209097|emb|FN599054.1|      GCTGCGCTACGACACCGTGCACGGCAAGTTCAAGTACACGGTGACGACGGCGAAGAGCAA      99
gi|313209103|emb|FN599056.1|      GATGCGTTACGACACCGTGCATGGTAAGTTCAAGTACACGGTGACGACGACGAAGAGCAA      112
gi|313209100|emb|FN599055.1|      GATGCGTTACGACACCGTGCATGGTAAGTTCAAGTACACGGTGACGACGACGAAGAGCAA      120
                                  * **** ************** ** ************************ **********

gi|313209097|emb|FN599054.1|      CCCCTCCGTGACTAAGGACGACACACTCGTGGTGAATGGCCACCGCATTCTGTGCGTGAA      159
gi|313209103|emb|FN599056.1|      CCTCTCCGTGGCGAAGGATGACACACTTGTGGTGAATGGCCATCGCATTCTGTGCGTGAA      172
gi|313209100|emb|FN599055.1|      CCTCTCCGTGGCGAAGGATGACACACTTGTGGTGAATGGCCATCGCATTCTGTGCGTGAA      180
                                  ** ******* * ***** ******** ************** *****************

gi|313209097|emb|FN599054.1|      GGCGCAGCGCAACCCGGCGGATCTCCCGTGGGGCAAGCTTGGTGTGGAGTATGTAATTGA      219
gi|313209103|emb|FN599056.1|      GGCGCAGCGCAATCCGGCGGATCTCCCGTGGGGCAAGCTTGGTGTGGAGTATGTAATTGA      232
gi|313209100|emb|FN599055.1|      GGCGCAGCGCAATCCGGCGGATCTCCCGTGGGGCAAGCTTGGTGTGGAGTATGTAATTGA      240
                                  ************ ************************************************

gi|313209097|emb|FN599054.1|      GTCAACGGGTCTGTTCACTGCCAAGGTGGCGGCGGAGGGCCACCTGCGTGGCGGTGCACG      279
gi|313209103|emb|FN599056.1|      GTCAACAGGCCTGTTCACTGCCAAGACGGCGGCGGAGGGCCACCTGCGCGGCGGTGCACG      292
gi|313209100|emb|FN599055.1|      GTCAACAGGCCTGTTCACTGCCAAGACGGCGGCGGAGGGCCACTTGCGCGGCGGTGCACG      300
                                  ****** ** ***************  **************** **** ************

gi|313209097|emb|FN599054.1|      GAAGGTCATCATCAGCGCGCCCGCCTCTGGTGGCGCCAAGACACTCGTGATGGGCGTGAA      339
gi|313209103|emb|FN599056.1|      GAAGGTCATCATCAGCGCCCCCGCCTCTGGTGGCGCCAAGACACTCGTGATGGGCGTGAA      352
gi|313209100|emb|FN599055.1|      GAAGGTCATCATCAGCGCCCCCGCCTCTGGTGGCGCCAAGACACTCGTGATGGGCGTGAA      360
                                  ****************** ****************************************

gi|313209097|emb|FN599054.1|      CCACCATGAGTACAACCCCAGTGAGCACCACGTGGTCTCGAACGCGTCATGCACGACCAA      399
gi|313209103|emb|FN599056.1|      CCACCATGAGTACAACCCCAGTGAGCACCATGTGGTGTCGAACGCGTCGTGCACGACCAA      412
gi|313209100|emb|FN599055.1|      CCACCATGAGTACAACCCCAGTGAGCACCATGTGGTGTCGAACGCGTCGTGCACGACCAA      420
                                  ****************************** ***** *********** ***********

gi|313209097|emb|FN599054.1|      TTGTCTTGCGCCCATTGTGCATGTCCTGGTGAAGGAGGGCTTTGGCGTGCAGACCGGCCT      459
gi|313209103|emb|FN599056.1|      TTGTCTTGCGCCCATTGTGCATGTTCTGGTGAAGGAGGGCTTTGGCGTGCAGACCGGCCT      472
gi|313209100|emb|FN599055.1|      TTGTCTTGCGCCCATTGTGCATGTCCTGGTGAAGGAGGGCTTTGGCGTGCAGACCGGCCT      480
                                  ************************ ************************************

gi|313209097|emb|FN599054.1|      CATGACGACGATCCACTCGTACACGGCAACACAAAAGACGGTGGACGGCGTGTCGTTGAA      519
gi|313209103|emb|FN599056.1|      CATGACGACGATCCACTCGTACACGGCAACACAGAAGACGGTGGATGGTGTGTCGTTGAA      532
gi|313209100|emb|FN599055.1|      CATGACGACGATCCACTCGTACACGGCAACACAGAAGACGGTGGATGGTGTGTCGTTGAA      540
                                  ********************************** *********** ** ***********

gi|313209097|emb|FN599054.1|      GGACTGGCGCGGCGGTCGTGCGGCTGCGGTGAACATCATTCCAAGCACGACTGGTGCGGC      579
gi|313209103|emb|FN599056.1|      GGACTGGCGCGGCGGTCGTGCGGCTGCGGTGAACATCATTCCGAGCACGACTGGTGCGGC      592
gi|313209100|emb|FN599055.1|      GGACTGGCGCGGCGGTCGTGCGGCTGCGGCGAACATCATTCCGAGCACGACTGGTGCGGC      600
                                  ***************************** ************ *****************
```

Figure 3.17: Clustal W sequence alignment of a region of the *T. dionisii* GAPDH gene extracted from NCBI GenBank: *T. dionisii* A (FN599054.1), *T. dionisii* B (FN599056.1), *T. dionisii* B (FN599055.1). Red text shows the *T. dionisii* primer binding sites (GAPF, GAPR and GAPRn). The full sequence alignment can be obtained from Appendix 1.

Again, due to the lack of *T. dionisii* genomic DNA, it was not possible to establish an optimised set of PCR cycling parameters for the *T. dionisii* GAPDH primers. Consequently, bat heart and spleen DNA samples were screened by PCR using the predicted Primer3 optimal annealing temperature (60^{o}C).

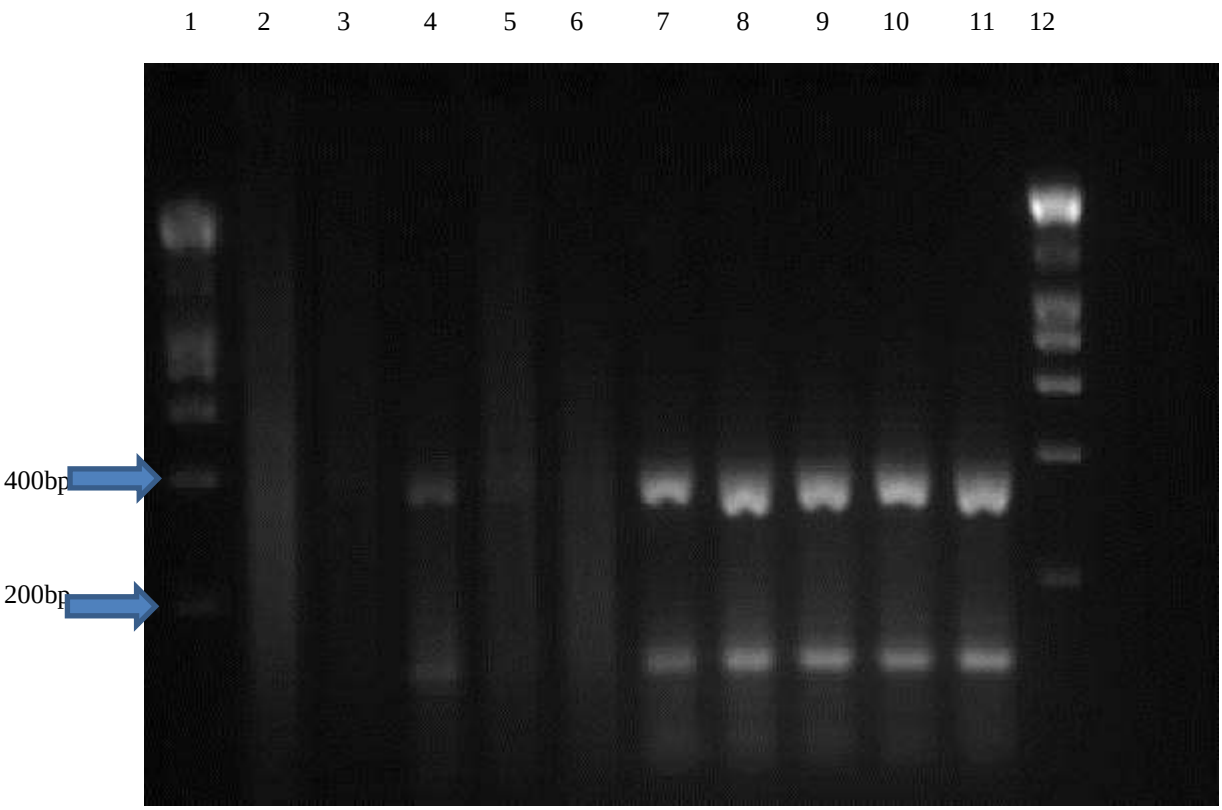

Figure 3.18(A): A representative agarose (1%) gel image showing analysis of *T. dionisii* GAPDH PCR products derived from bat spleen DNAs. 1, 1kb hyperladder; 2, negative control (bat code: SA606; confirmed positive for *T. vespertilionis*); 3-11, bat DNA samples extracted from spleen (bat codes: PB601, JL650, FP751, JL714, SP670, SP677, P605, JL648, JL654); 12, 1kb hyperladder.

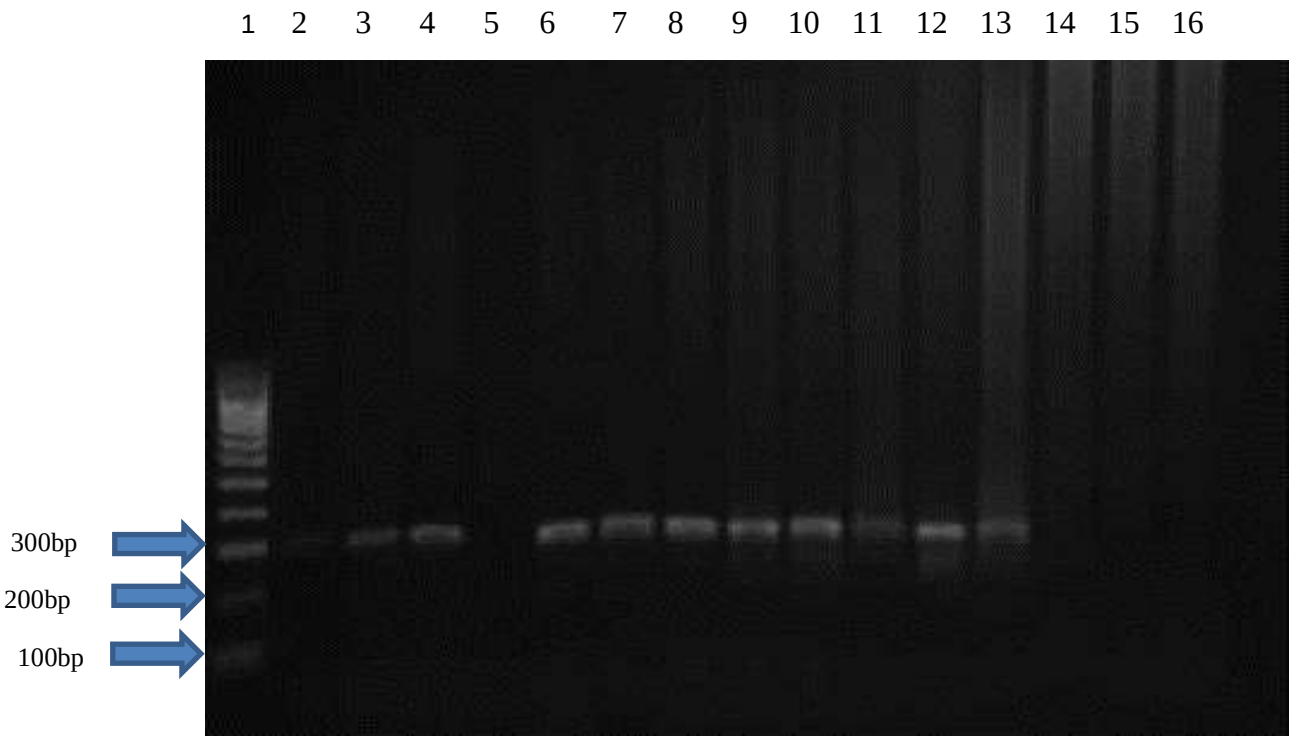

Figure 3.18(B): A representative agarose (1%) gel image showing analysis of *T. dionisii* GAPDH PCR products derived from bat heart samples. 1, 100bp hyperladder; 2-15, bat DNA samples extracted from heart (bat codes: JH802, PB601, JL650, FP751, JL714, SP670, SP677, P605, JL648, JL654, SP649, JL648, F711, JL613); 16, negative control (bat code: SA606; confirmed positive for *T. vespertilionis*).

The expected PCR product of 355bp was produced in a total of 33 samples; this included the 10 that were positive with the 18S rRNA primers (Figure 3.18(A, B)). As observed for the 18S rRNA PCR approach, approximately 90% of the *T. dionisii* infections (n=30) were determined on the basis of PCR product amplification from both heart and spleen DNA targets and the remainder (n=3) were derived from just bat heart DNA samples.

All the PCR products were purified and DNA sequencing was carried out. The resulting data confirmed that 26 GAPDH PCR products were identical to each other and to the GAPDH gene of *T. dionisii* strain Z3126 (*T. dionisii* A) (Hamilton, Cruickshank, et al., 2012) (Figure 3.19 & Table 3.4). Unfortunately, it was not possible to recover good quality GAPDH

sequence data from a small number of the bats (codes: JL627, JL651, JL652, JL708, JL654, JL640, GH606) and hence the *T. dionisii* strain type for these infections remains unknown.

```
CLUSTAL O(1.2.3) multiple sequence alignment

JL654          ----TATATGAACACGGACGCGGAGTATTTTGCATACCAGCTGCGCTACGACACCGTGCA        56
FN599054.1     GGTCGATATGAACACGGACGCGGAGTATTTTGCATACCAGCTGCGCTACGACACCGTGCA        60
                    ************************************************

JL654          CGGCAAGTTCAAGTACACGGTGACGACGGCGAAGAGCAACCCCTCCGTGACTAAGGACGA        116
FN599054.1     CGGCAAGTTCAAGTACACGGTGACGACGGCGAAGAGCAACCCCTCCGTGACTAAGGACGA        120
               ************************************************************

JL654          CACACTCGTGGTGAATGGCCACCGCATTCTGTGCGTGAAGGCGCAGCGCAACCCGGCGGA        176
FN599054.1     CACACTCGTGGTGAATGGCCACCGCATTCTGTGCGTGAAGGCGCAGCGCAACCCGGCGGA        180
               ************************************************************



JL654          TCTCCCGTGGGGCAAGCTTGGTGTGGAGTATGTAATTGAGTCAACGGGTCTGTTCACTGC        236
FN599054.1     TCTCCCGTGGGGCAAGCTTGGTGTGGAGTATGTAATTGAGTCAACGGGTCTGTTCACTGC        240
               ************************************************************

JL654          CAAGGTGGCGGCGGAGGGCCACCTGCGTGGCGGTGCACGGAAGGTCATCATCAGCGCGCC        296
FN599054.1     CAAGGTGGCGGCGGAGGGCCACCTGCGTGGCGGTGCACGGAAGGTCATCATCAGCGCGCC        300
               ************************************************************

JL654          CGCCTCTGGTGGCGCCAAGACACTCGTGATGGGCGTGAACCACCATGAGTACAACCCCAG        356
FN599054.1     CGCCTCTGGTGGCGCCAAGACACTCGTGATGGGCGTGAACCACCATGAGTACAACCCCAG        360
               ************************************************************
```

Figure 3.19: Clustal W alignment of a representative T. dionisii GAPDH PCR product derived from bat specimen JL654 with a fragment of the GAPDH gene from *T. dionisii* strain Z3126 (GenBank accession number FN599054.1).

Table 3.4: BlastN summary data for the *T. dionisii* GAPDH PCR product derived from pipistrelle JL654.

Highly similar sequence	Max score	Total score	Query cover	E value	Iden	GenBank Accession number
Trypanosoma dionisii partial gapdh gene for glyceraldehyde phosphate dehydrogenase, strain Z3126	656	656	100%	0.0	100%	FN599054.1
Trypanosoma dionisii glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) gene, partial cds	656	656	100%	0.0	100%	FJ649494.1
Trypanosoma dionisii partial gapdh gene for glyceraldehyde phosphate dehydrogenase, isolate gnash	545	545	99%	2e-151	94%	FN599056.1
Trypanosoma dionisii partial gapdh gene for glyceraldehyde phosphate dehydrogenase, isolate x842	540	540	99%	2e-151	94%	FN599055.1

3.3 Bat Eimeria:

The Lord (2010) study showed that 19 pipistrelle bats were infected with eimerian parasites and DNA sequence data for one 18S rRNA derived PCR product was reported to be 99.8% identical to *E. rioarribaensis*. The other 18 eimerian parasite PCR products were not purified and sequenced. Therefore, to gain further insight into the pipistrelle eimerian infection, 18S rRNA PCR amplifications were repeated on purified bat intestinal DNA samples. The expected PCR product of 800 bp was amplified in the 19 pipistrelles documented by Lord (2010) as being infected with *Eimeria* sp. (Figure 3.20). Moreover, samples reported as eimerian-free by Lord (2010), were also screened by PCR to confirm that they were indeed negative (lanes 2, 3). All the eimerian 18S rRNA PCR products were purified, DNA sequencing was performed and the resulting data was aligned with other eimerian 18S rRNA sequences extracted from GenBank (Figure 3.21 & Table 3.5).

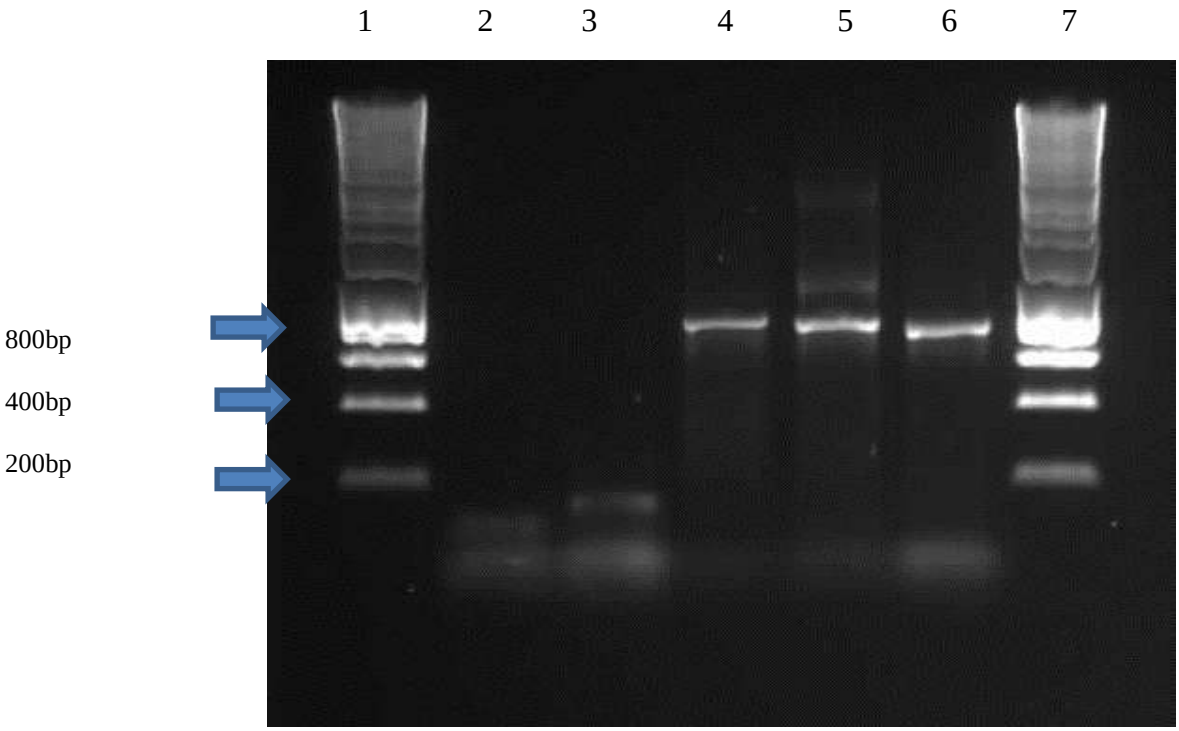

Figure 3.20: Analysis of bat eimerian 18S rRNA PCR products by agarose (1.0%) gel electrophoresis. 1, 1kb hyperladder; 2-3, negative control bats shown previously (Jennifer S Lord, 2010) to not be infected with *Eimeria* sp. (codes: JL645, JL705); 4-6, representative bat samples (SP852, SP682, JL719) with primers designed to be specific for eimerian 18S rRNA PCR amplification; 7, 1kb hyperladder.

```
CLUSTAL 2.1 multiple sequence alignment

Q993645.1       CGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGA  338
JL719            -------TTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGA 53
AF307877.1       CGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGA 360
                        **************************************************

JQ993645.1       AGGCAGCAGGCGCGCAAATTACCCAATGAAAACAGTTTCGAGGTAGTGACGAGAAATAAC 398
JL719            AGGCAGCAGGCGCGCAAATTACCCAATGAAAACAGTTTCGAGGTAGTGACGAGAAATAAC 113
AF307877.1       AGGCAGCAGGCGCGCAAATTACCCAATGAAAACAGTTTCGAGGTAGTGACGAGAAATAAC 420
                 ************************************************************

JQ993645.1       AATACAGGGCATTTTATGCTCTGTAATTGGAATGATGGGAATGTAAAACCCTCTCAGAGT 458
JL719            AATACAGGGCATTTTATGCTCTGTAATTGGAATGATGGGAATGTAAAACCCTCTCAGAGT 173
AF307877.1       AATACAGGGCATTTTATGCTCTGTAATTGGAATGATGGGAATGTAAAACCCTCTCAGAGT 480
                 ************************************************************

JQ993645.1       AACAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGTGTAT 518
JL719            AACAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGTGTAT 233
AF307877.1       AACAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGTGTAT 540
                 ************************************************************

JQ993645.1       ATTAGAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATTTCTGTCGTGGTCATCCGGTACC 578
JL719            ATTAGAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATTTCTGTCGTGGTCATCCGGTACC 293
AF307877.1       ATTAGAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATTTCTGTCGTGGTCATCCGGTACC 600
                 ************************************************************
```

```
JQ993645.1      GCCCGTATGGGTGTGCACCTGGTTTGACCTCGGCTTTCTTCCGGTAGCCTTCCGCGCTTC 638
JL719           GCCCGTATGGGTGTGCACCTGGTTTGACCTCGGCTTTCTTCCGGTAGCCTTCCGCGCTTC 353
AF307877.1      GCCCGTATGGGTGTGCACCTGGTTTGACCTCGGCTTTCTTCCGGTAGCCTTCCGCGCTTC 660
                ************************************************************

JQ993645.1      ACTGCGTGGTTGGTGTTCCGGAACTTTTACTTTGAGAAAAATAGAGTGTTTCAAGCAGGC 698
JL719           ATTGCGTGGTTGGTGTTCCGGAACTTTTACTTTGAGAAAAATAGAGTGTTTCAAGCAGGC 413
AF307877.1      ATTGCGTGGTTGGTGTTCCGGAACTTTTACTTTGAGAAAAATAGAGTGTTTCAAGCAGGC 720
                * ************************************************************

JQ993645.1      TTGTCGCCCTGAATACTTCAGCATGGAATAATAAGATAGGACCTTGGTTCTATTTTGTTG 758
JL719           TTGTCGCCCTGAATACTTCAGCATGGAATAATAAGATAGGACCTTGGTTCTATTTTGTTG 473
AF307877.1      TTGTCGCCCTGAATACTTCAGCATGGAATAATAAGATAGGACCTTGGTTCTATTTTGTTG 780
                ************************************************************

JQ993645.1      GTTTCTAGGACCAAGGTAATGATTAATAGGGACAGTTGGGGGCATTCGTATTTAACTGTC 818
JL719           GTTTCTAGGACCAAGGTAATGATTAATAGGGACAGTTGGGGGCATTCGTATTTAACTGTC 533
AF307877.1      GTTTCTAGGACCAAGGTAATGATTAATAGGGACAGTTGGGGGCATTCGTATTTAACTGTC 840
                ************************************************************

JQ993645.1      AGAGGTGAAATTCTTAGATTTGTTAAAGACGAACTACTGCGAAAGCATTTGCCAAGGATG 878
JL719           AGAGGTGAAATTCTTAGATTTGTTAAAGACGAACTACTGCGAAAGCATTTGCCAAGGATG 593
AF307877.1      AGAGGTGAAATTCTTAGATTTGTTAAAGACGAACTACTGCGAAAGCATTTGCCAAGGATG 900
                ************************************************************

JQ993645.1      TTTTCATTAATCAAGAACGACAGTAGGGGGTTTGAAGACGATTAGATACCGTCGTAATCT 938
JL719           TTTTCATTAATCAAGAACGACAGTAGGGGGTTTGAAGACGATTAGATACCGTCGTAATCT 653
AF307877.1      TTTTCATTAATCAAGAACGACAGTAGGGGGTTTGAAGACGATTAGATACCGTCGTAATCT 960
                ************************************************************

JQ993645.1      CTACCATAAACTATGCCGACTAGAGATAGGGAAACGCCTACCTTGGCTTCTCCTGCACCT 998
JL719           CTACCATAAACTATGCCGACTAGAGATAGGGAAATGCCTACCTTGGCTTCTCCTGCACCT 713
AF307877.1      CTACCATAAACTATGCCGACTAGAGATAGGGAAATGCCTACCTTGGCTTCTCCTGCACCT 1020
                ********************************* ************************

JQ993645.1      CATGAGAAATCAAAGTCTCTGGGTTCTGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAA 1058
JL719           CATGAGAAATCAAAGTCTCTGGGTTCTGGGG---------------------------- 744
AF307877.1      CATGAGAAATCAAAGTCTCTGGGTTCTGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAA 1080
                ******************************
```

Figure 3.21: Clustal W sequence alignment of a representative bat eimerian 18S rRNA PCR product derived from bat specimen JL719 with fragments of the 18S rRNA gene from *E. rioarribaensis* (AF307877.1) and *E. cahirinensis* isolate NFS (JQ993645.1) extracted from NCBI GenBank.

Table 3.5: BlastN summary data for the pipistrelle eimerian 18S rRNA PCR products

Highly similar sequence	Max score	Total score	Query cover	E value	Iden	GenBank Accession number
Eimeria rioarribaensis 18S ribosomal RNA gene, partial sequence	1375	1375	100%	0.0	100%	AF307877.1
Eimeria cahirinensis isolate NFS 18S ribosomal RNA gene, partial sequence	1363	1363	100%	0.0	99%	JQ993645.1

The resulting sequence data confirmed that all 19 PCR products were identical to each other and also, were 100% identical to the 18S rRNA of *E. rioarribaensis.*

3.4 Bat Cryptosporidium:

At initiation of this study, no bat cryptosporidium infection had been reported in any of the pipistrelle bats sample panel. Given that bats are host to the coccidian *Eimeria* spp., it seemed reasonable to propose that they may also harbour cryptosporidium parasites. As such, the Lancashire pipistrelle collection was screened for *Cryptosporidium* spp., using a PCR approach that targeted the parasite 18S rRNA gene. To develop this diagnostic approach, *Cryptosporidium ubiquitum* genomic DNA was used as a positive control (kindly provided by Eljelani Salim, PhD student, University of Salford). The expected PCR product of 600 bp was amplified from 14 out of 92 (15%) pipistrelle bats (Figure 3.22). The PCR products were purified, DNA sequencing performed, and the resulting data was aligned with other *Cryptosporidium* spp. 18S rRNA sequences available in GenBank (Figure 3.23 & Table 3.6).

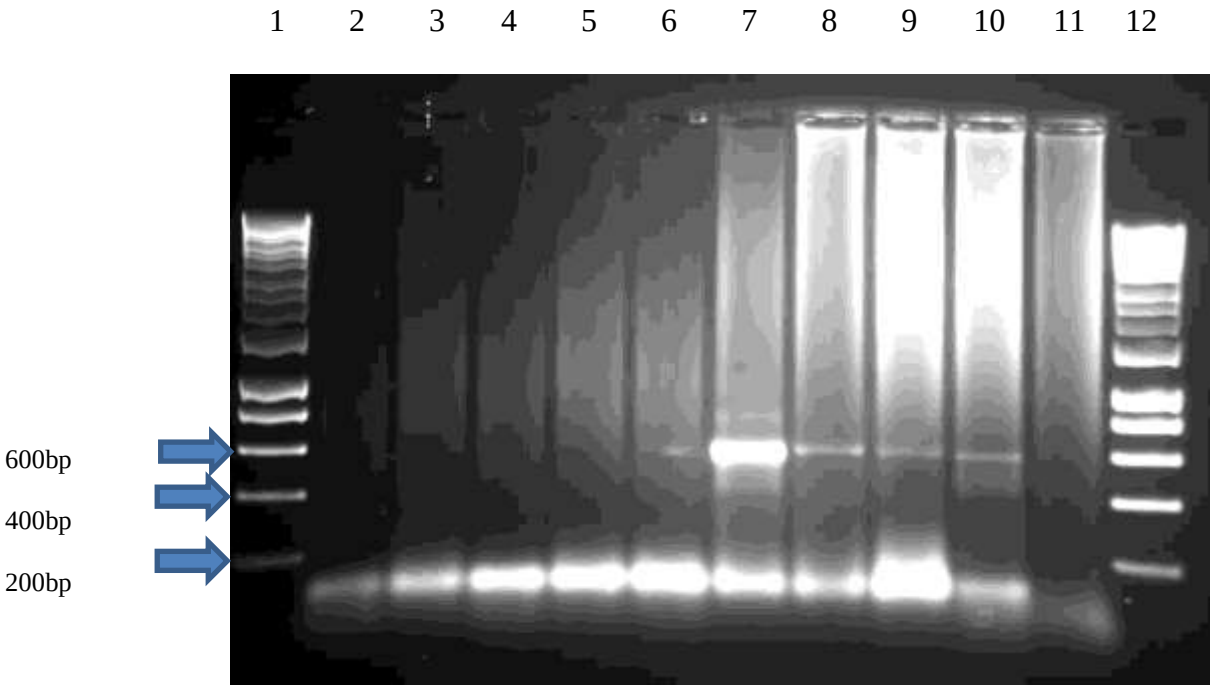

Figure 3.22: Analysis of bat *Cryptosporidium* spp. 18S rRNA PCR products by agarose (1.0%) gel electrophoresis. 1, 1kb hyperladder; 2-9, representative bat samples (codes: S682, F745, F802, JL714, F721, C802, F546, S680 respectively) with primers designed to be specific for the *Cryptosporidium* spp. 18S rRNA gene; 10, positive control (*C. ubiquitum*); 11, negative control (H_2O); 12, 1kb hyperladder.

```
CLUSTAL O (1.2.3) multiple sequence alignment


F546            ----------------------------------------------------TCCTATCA        8
KR819168.1      TCATAATAACTTTACGGATCACATTTTTTGTGACATATCATTCAAGTTTCTGACCTATCA        60
                                                                   *******

F546            GCTTTAGACGGTAGGGTATTGGCCTACCGTGGCAATGACGGGTAACGGGGAATTAGGGTT        68
KR819168.1      GCTTTAGACGGTAGGGTATTGGCCTACCGTGGCAATGACGGGTAACGGGGAATTAGGGTT        120
                ************************************************************

F546            CGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGC        128
KR819168.1      CGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGC        180
                ************************************************************

F546            AAATTACCCAATCCTAATACAGGGAGGTAGTGACAAGAAATAACAATACAGGACTTTAAA        188
KR819168.1      AAATTACCCAATCCTAATACAGGGAGGTAGTGACAAGAAATAACAATACAGGACTTTA-A        239
                ************************************************************ *

F546            CAGTTTTGTAATTGGAATGAGTTAAGTATAAACCCCTTTACAAGTATCAATTGGAGGGCA        248
KR819168.1      CAGTTTTGTAATTGGAATGAGTTAAGTATAAACCCCTTTACAAGTATCAATTGGAGGGCA        299
                ************************************************************

F546            AGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCA        308
KR819168.1      AGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCA        359
                ************************************************************

F546            GTTAAAAAGCTCGTAGTTGGATTTCTGTTAATAGTTTATATATAATGTCTCGTACATTTA        368
KR819168.1      GTTAAAAAGCTCGTAGTTGGATTTCTGTTAATAGTTTATATATAATGTCTCGTACATTTA        419
                ************************************************************
```

```
F546            TATAATATTAACATAATTCATATTACTATTTTTTATAGTATATGAAACTTTACTTTGAGA        428
KR819168.1      TATAATATTAACATAATTCATATTACTATT---TTTAGTATATGAAACTTTACTTTGAGA        476
                ******************************   * ************************

F546            AAATTAGAGTGCTTAAAGCAGGCTATAGCCTTGAATACTTCAGCATGGAATAATATTAAA        488
KR819168.1      AAATTAGAGTGCTTAAAGCAGGCTATTGCCTTGAATACTTCAGCATGGAATAATATTAAA        536
                ************************* ********************************

F546            GATTTTTATCTTTCTTATTGGTTCTAAGATAGAAATAATGATTAATAGGGACAGTTGGGG        548
KR819168.1      GATTTTTATCTTTCTTATTGGTTCTAAGATAGAAATAATGATTAATAGGGACAGTTGGGG        596
                ************************************************************

F546            GCATTTGTATTTAACAGTCAGAGGTGAAATTCTTAAAA----------------------        586
KR819168.1      GCATTTGTATTTAACAGTTAGAGGTGAAATTCTTAGATTTGTTAAAGACAAACTAGTGCG        656
                ****************** *************** *
```

Figure 3.23: Clustal W sequence alignment of a representative bat *Cryptosporidium* 18S rRNA PCR product derived from an intestinal DNA preparation from bat specimen F546 with the 18S rRNA sequence from Cryptosporidium sp. bat genotype IV isolate 13973CZ (KR819168.1).

Table 3.6: BlastN summary data for the *Cryptosporidium* 18S rRNA PCR products derived from pipistrelle bat F546

Highly similar sequence	Max score	Total score	Query cover	E value	Iden	GenBank Accession number
Cryptosporidium sp. bat genotype IV isolate 13973CZ small subunit ribosomal RNA gene, partial sequence	1038	1038	100%	0.0	99%	KR819168.1
Cryptosporidium canis isolate CHF8 18S ribosomal RNA gene, partial sequence	990	990	99%	0.0	97%	KU608308.1

All 14 pipistrelle 18S rRNA PCR products were identical to each other and most similar to the 18S rRNA of *Cryptosporidium sp.* bat genotype IV isolate 13973CZ (Kváč et al., 2015).

3.5 Bat bacterial infections

3.5.1 Bartonella:

To further characterize the two Bartonella infections in the South Lancashire bat collection described by Lord (2010) using a 16S rRNA gene targeting approach, a PCR was developed using primers specific for the Bartonella citrate synthase gene (Norman et al., 1995). After successful amplification of the expected 400 bp PCR product from the 2 Bartonella infected bats (codes: JL726 and SP817), the PCR products (Figure 3.24) were purified and DNA sequencing performed. The resulting data (Figure 3.25) was aligned with other Bartonella citrate synthase gene sequences deposited in GenBank. A random selection of bat samples reported by Lord (2010) as not infected with *Bartonella* were also screened by PCR and the data confirmed the negative infection status of these samples.

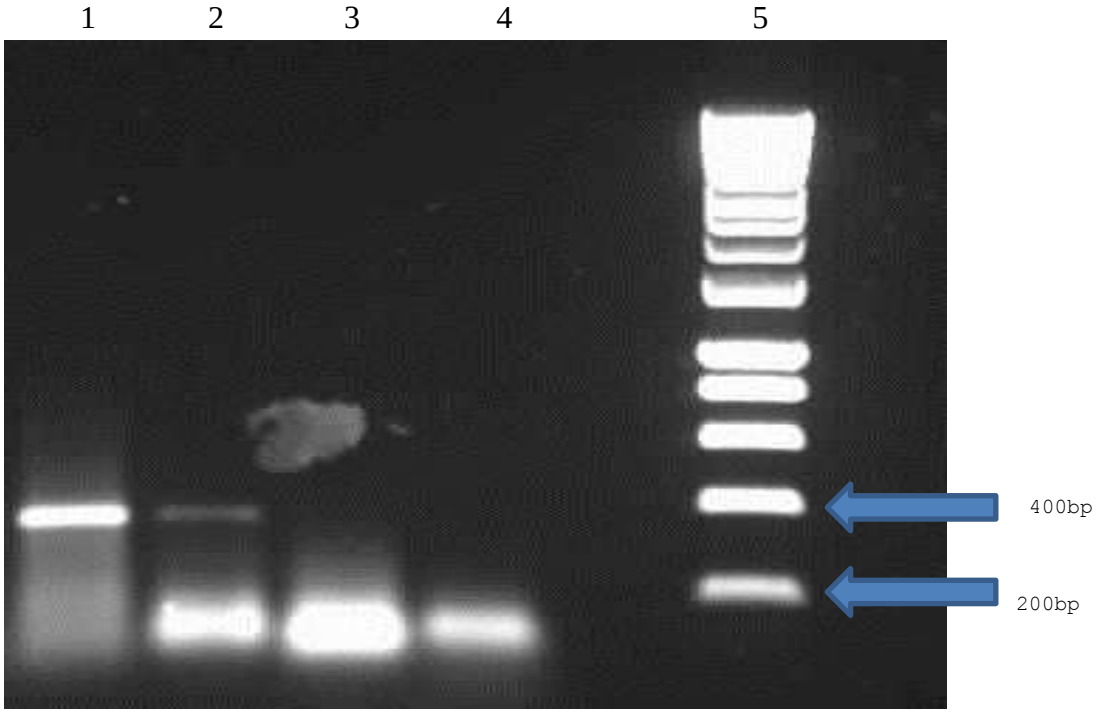

Figure 3.24: Analysis of the bat Bartonella citrate synthase (gltA) PCR products by agarose (1%) gel electrophoresis. 1-2, bat samples (J726, SP817); 3-4, negative controls (purified DNA from bats JL613 and JL706 shown by Lord (2010) to be uninfected with *Bartonella*); 5, 1kb hyperladder.

```
  CLUSTAL O (1.2.3) multiple sequence alignment

J726            GGGACCAGCTCATGGTGGAGCCAATGAAGCATGCCTAAAGATGCTACAAGAAATAGGTTC 60
AJ871614.1      ------------------AGCCAATGAAGCATGCCTAAAGATGCTACAAGAAATAGGTTC 42
KF003137.1      ------------------AGCCAATGAAGCATGCCTAAAGATGCTACAAGAAATAGGTTC 42
                                  ****************************************

J726            CGTTAAGAGAATTCCTGAATTCATTGCACGTGCAAAAGATAAAAATGATCCTTTCCGCCT 120
AJ871614.1      CGTTAAGAGAATTCCTGAATTCATTGCACGTGCAAAAGATAAAAATGATCCTTTCCGCCT 102
KF003137.1      CGTTAAGAGAATTCCTCAATTCATTGCACGTGCAAAAGATAAAAATGATCCTTTCCGCCT 102
                *************** ****************************************

J726            CATGGGCTTTGGTCATCGAGTCTATAAAAATTATGACCCACGTGCAAAAATCATGCAACA 180
AJ871614.1      CATGGGCTTTGGTCATCGAGTCTATAAAAATTATGACCCACGTGCAAAAATCATGCAACA 162
KF003137.1      GATGGGCTTTGGTCATCGAGTCTATAAAAATTATGACCCACGTGCAAAAATCATGCAACA 162
                 ************************************************************

J726            AACCTGCCATGATGTTTTAAAAGAACTAAATATTCAAGATGATTTGCTTCTTGACATCGC 240
AJ871614.1      AACCTGCCATGATGTTTTAAAAGAACTAAATATTCAAGATGATTTGCTTCTTGACATCGC 222
KF003137.1      AACCTGCCATGATGTTTTAAAAGAACTAAATATTCAAGATGATCCGCTTCTTGACATCGC 222
                ****************************************  ***************

J726            TATAGAGCTTGAGAAAATCGCTTTAAATGATGAATATTTTGTTGAGAAAAAGCTTTATCC 300
AJ871614.1      TATAGAGCTTGAGAAAATCGCTTTAAATGATGAATATTTTGTTGAGAAAAAGCTTTATCC 282
KF003137.1      TATAGAACTTGAGAAAATCGCCTTAAATGATGAATATTTTGTTGAGAAAAAGCTTTATCC 282
                ****** ************** ************************************

J726            GAATGTTGATTTCTATTCTGGCATTACATTAAAAGCTCTAGGCTTTCCAACTGAAATGTT 360
AJ871614.1      GAATGTTGATTTCTATTCTGGCATTACATTAAAAGCTCTAGGCTTTCCAACTGAAA---- 338
KF003137.1      AAATGTTGATTTCTATTCTGGCATTACATTAAAAGCTCTAGGCTTTCCAACCGAAA---- 338
                 ************************************************* ****

J726            TACTGTTCTTTTTGCATAA       379
AJ871614.1      ------------------        338
KF003137.1      ------------------        338
```

Figure 3.25: Clustal W sequence alignment of the bat Bartonella citrate synthase PCR product derived from bat specimen J726 with partial citrate synthase gene sequences from an uncultured *Bartonella* sp. (isolate M207) (AJ871614.1) and an uncultured *Bartonella* sp. clone NB-1.2 (KF003137.1).

Table 3.7: BlastN summary data for the *Bartonella* gltA PCR products

Highly similar sequence	Max score	Total score	Query cover	E value	Iden	GenBank Accession number
Uncultured *Bartonella sp.* partial gltA gene for citrate synthase, isolate M207	625	625	89%	0.0	100%	AJ871614.1
Uncultured *Bartonella sp.* clone NB-1.2 citrate synthase (gltA) gene, partial cds	580	580	89%	0.0	98%	KF003137.1

Both of the pipistrelle-derived *Bartonella* citrate synthase DNA sequences were identical to each other and also, to *Bartonella* sp. isolate M207, an uncultured isolate derived from a British bat (Concannon et al., 2005).

3.5.2 Borrelia:

To screen for Borrelia infections in the South Lancashire bat collection, PCR was performed on the 16S rRNA gene using Borrelia specific primers. A positive control (*Borrelia borgdorferi sensu stricto* culture) was kindly provided by Jessica Hall (PhD student, University of Salford). One out of 100 bat spleen samples (bat code: JL709) amplified the expected 120 bp PCR product that was indicative of a Borrelia infection (Figure 3.26). The PCR product was sequenced and the resulting data was analysed with respect to other *Borrelia* spp. 16S rRNA sequences deposited in GenBank (Figure 3.27).

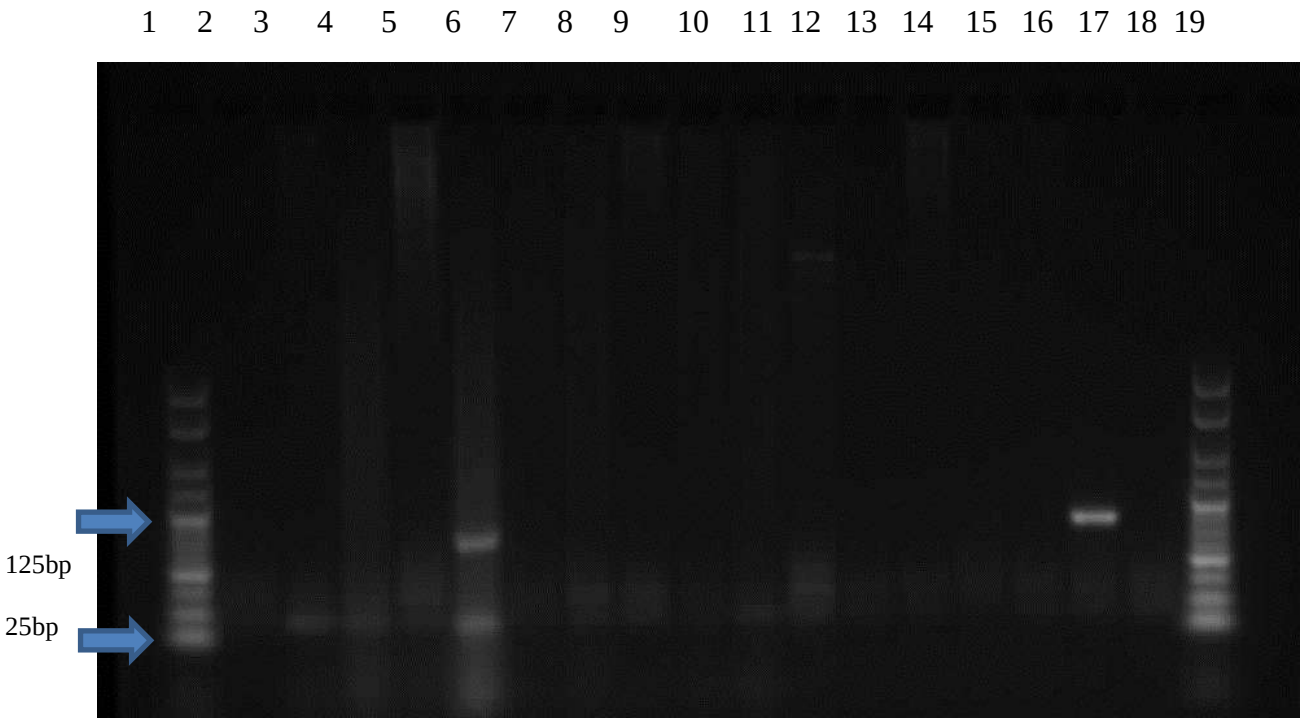

Figure 3.26: Analysis of bat Borrelia 16S rRNA PCR products by agarose gel (2.0%) electrophoresis. 1, 25bp hyperladder; 2-16, bat spleen DNA samples; 17, positive control (*B. borgdorferi sensu stricto* culture); 18, negative control (H_2O); 19, 25bp hyperladder.

```
CLUSTAL 2.1 multiple sequence alignment

JL709           TAAGTAGCCGGCCTGAGAGGGTGAACGGTCACACTGGAACTGAGATACGGTCCAGACTCC  97
KF395229.1      TAAGTAGCCGGCCTGAGAGGGTGAACGGTCACACTGGAACTGAGATACGGTCCAGACTCC 241
KF395228.1      TAAGTAGCCGGCCTGAGAGGGTGAACGGTCACACTGGAACTGAGATACGGTCCAGACTCC 234
FJ868583.1      TAAGTAGCCGGCCTGAGAGGGTGAACGGTCACACTGGAACTGAGATACGGTCCAGACTCC 265
DQ469888.1      TAAGTAGCCGGCCTGAGAGGGTGAACGGTCACACTGGAACTGAGATACGGTCCAGACTCC 272
CP005851.2      TAAGTAACCGGCCTGAGAGGGTGAACGGTCACACTGGAACTGAGATACGGTCCAGACTCC 330
                ****** **************************************************
```

```
JL709           TACGGGAGGCA   108
KF395229.1      TACGGGAGGCA   252
KF395228.1      TACGGGAGGCA   245
FJ868583.1      TACGGGAGGCA   276
DQ469888.1      TACGGGAGGCA   283
KU672548.1      TACGGGAGGCA   341
                ***********
```

Figure 3.27: Clustal W sequence alignment of the bat *Borrelia* 16S rRNA PCR product derived from bat specimen JL709 with partial 16S rRNA sequences extracted from GenBank as follows: *Borrelia* sp. F3 (KF395229.1), *Borrelia* sp. F17 (KF395228.1), *Borrelia* sp. CPB1 (FJ868583.1), *B. afzelii* strain Mng 3602 (DQ469888.1) and *B. garinii* strain Ip-6322 (KU672548.1).

Table 3.8: BlastN summary data for the *Borrelia* 16S rRNA PCR product.

Highly similar sequence	Max score	Total score	Query cover	E value	Iden	GenBank accession number
Borrelia sp. F3 16S ribosomal RNA gene, partial sequence	132	132	91%	9e-28	100%	KF395229.1
Borrelia sp. F17 16S ribosomal RNA gene, partial sequence	132	132	91%	9e-28	100%	KF395228.1
Borrelia sp. CPB1 16S ribosomal RNA gene, partial sequence	132	132	91%	9e-28	100%	FJ868583.1
Borrelia afzelii strain Mng 3602 16S ribosomal RNA gene, partial sequence	132	132	91%	9e-28	100%	DQ469888.1
Borrelia garinii strain Tom 2903 16S ribosomal RNA gene, partial sequence	132	132	91%	9e-28	100%	DQ469880.1
Borrelia afzelii gene for 16S rRNA, partial sequence, strain:U001	132	132	91%	9e-28	100%	AB035406.1
Borrelia garinii gene for 16S rRNA, partial sequence, strain:F641	132	132	91%	9e-28	100%	AB035392.1
Borrelia garinii gene for 16S rRNA, partial sequence, strain:F548	132	132	91%	9e-28	100%	AB035391.1

The sequence analysis showed that the pipistrelle derived Borrelia infection was 100% identical to eight 16S rRNA Borrelia sequences deposited in GenBank; one of these was derived from an isolate (CPB1) responsible for causing fatal borreliosis in a British bat (Evans et al., 2009).

3.6 Further analyses of the pipistrelle protozoan and bacterial infections

The protozoan and bacterial infections presented above (sections 3.1-3.4), and also, data for the piroplasm *Babesia vesperuginis* (Jennifer S Lord, 2010) and *Toxoplasma gondii* (Dodd et al., 2014), are summarised at the individual bat level in the table below (Table 3.9).

Table 3.9: Summary of the 70 protozoan and bacterial infections of the Lancashire pipistrelle bats. *Footnote*: single infection (red), double infections (black), triple infections (purple), quadruple infections (orange), quintuple infections (blue) and "mixed" genotype bats (Dodd et al, 2014) (brown).

Bat code	*T.v*	*T.d*	*Eimeria rioarrib -aensis*	*Bartonella* sp.	*Cryptosporidium* sp.	*Borrelia* sp.	*B. vesperuginis* (Jennifer S Lord, 2010)	*Toxoplasma gondii* (Dodd et al, 2014)
SP649	_	+	_	_	_	_	_	_
SP670	_	+	_	_	_	_	_	_
SP672	_	+	_	_	_	_	_	_
JL640	_	+	_	_	_	_	_	_
SP677	_	+	_	_	_	_	_	_
JL645	_	+	_	_	_	_	_	_
PB601	_	+	_	_	_	_	_	_
JL650	_	+	_	_	_	_	_	_
GH606	_	+	_	_	_	_	_	_
JL659	_	+	_	_	_	_	_	_
JL705	_	+	_	_	_	_	_	_

Bat code	*T.v*	*T.d*	*Eimeria rioarrib -aensis*	*Bartonella* sp.	*Cryptosporidium* sp.	*Borrelia* sp.	*B. vesperuginis* (Lord, 2010)	*Toxoplasma gondii* (Dodd et al, 2014)
JL710	–	+	–	–	–	–	–	–
JL708	–	+	–	–	–	–	–	–
FP751	–	+	–	–	–	–	–	–
JL714	–	+	–	–	–	–	–	–
FP761	–	+	–	–	–	–	–	–
JH802	–	+	–	–	–	–	–	–
SP842	–	+	–	–	–	–	–	–
JL658	+	–	–	–	–	–	–	–
SP679	+	–	–	–	–	–	–	–
JL624	–	–	+	–	–	–	–	–
JL628	–	–	+	–	–	–	–	–
JL656	–	–	+	–	–	–	–	–
SP682	–	–	+	–	–	–	–	–
FP745	–	–	+	–	–	–	–	–
SP819	–	–	+	–	–	–	–	–
JL723	–	–	+	–	–	–	–	–
FP546	–	–	–	–	+	–	–	–
JL707	–	–	–	–	+	–	–	–
JL706	–	–	–	–	+	–	–	–
JL718	–	–	–	–	+	–	–	–
GH708	–	–	–	–	+	–	–	–
CS804	–	–	–	–	+	–	–	–
JL612	–	–	–	–	–	–	+	–
SP666	–	–	–	–	–	–	+	–
JL704	–	–	–	–	–	–	+	–
FP712	–	–	–	–	–	–	+	–
FP801	–	–	–	–	–	–	+	–
JL722	–	–	–	–	–	–	+	–
GH704	–	–	–	–	–	–	+	–
CS801	–	–	–	–	–	–	+	–

Bat code	*T.v*	*T.d*	*Eimeria rioarrib -aensis*	*Bartonella* sp.	*Cryptosporidium* sp.	*Borrelia* sp.	*B. vesperuginis* (Lord, 2010)	*Toxoplasma gondii* (Dodd et al, 2014)
MD802	–	–	–	–	–	–	+	–
PH604	–	–	–	–	–	–	–	+
PB602	–	–	–	–	–	–	–	+
SA07?	–	–	–	–	–	–	–	+
JL613	–	–	–	–	–	–	–	+
JL651	–	+	–	–	–	–	+	–
JL652	–	+	–	–	–	–	+	–
JL654	–	+	–	–	–	–	+	–
JL648	–	+	–	–	–	–	+	–
JL627	–	+	–	–	–	–	+	–
FP711	–	+	–	–	–	–	+	–
SP668	–	–	+	–	–	–	+	–
JL719	–	–	+	–	–	–	+	–
SP852	–	–	+	–	–	–	+	–
SP655	–	–	+	–	–	–	+	–
CS610	–	+	+	–	–	–	–	–
SA606	+	–	+	–	–	–	–	–
JL709	+	–	–	–	–	+	–	–
FP744	–	+	–	–	+	–	–	–
SP684	–	+	–	–	+	–	–	–
SP681	–	–	+	–	+	–	–	–
JL726	–	+	–	+	–	–	–	–
FP802	–	–	–	–	–	–	+	+
CS802	–	+	+	–	+	–	–	–
SP680	–	+	+	–	+	–	–	–
SP817	–	+	+	+	–	–	–	–
JL647	–	–	+	–	+	–	+	–
SA605	–	+	–	–	+	–	+	+
P605	–	+	+	–	+	–	+	+
Total	4	33	19	2	14	1	23	7

In total, the molecular diagnostic approach undertaken confirmed that 70 of the 99 pipistrelles (prevalence = 70.7%) were infected with a microparasite (Table 3.9). These infections were significantly more likely to be due to a protozoan parasite (total protozoans detected = 100) than with the Bacteria *Bartonella* and *Borrelia* (total detected = 3). Furthermore, the bats appeared to be more commonly infected with blood protozoans (total detected trypanosomes and piroplasms = 60) than with coccidian parasites (total detected = 40). The most prevalent protozoan in the bats was *T. dionisii* and this was observed approximately 8-times more frequently than the related *T. vespertilioni*s.

Previous genotyping work on the pipistrelles had shown that the majority of the bats were from one interbreeding population and the remainder were of mixed origin (Dodd et al., 2014). When analysing the infection data at the level of these two populations, 59 infected bats were representative of the single interbreeding population (Table 3.10) and 12 infected individuals were of mixed genetic origin (Table 3.11). Not surprisingly given the respective numbers of bats in the two populations, a greater variety of infections, including multiple co-infections, were observed in the single interbreeding group (Table 3.10). The two most commonly encountered parasites responsible for single infections in the single interbreeding group of bats, *T. dionisii* and *B. vesperuginis*, were also found to be the most common combination representative of a double infection (Table 3.10). Interestingly, the coccidian parasites *Eimeria* and *Cryptosporidium*, were more often found as a component of a co-infection than as single infections within the single interbreeding group of bats; this appeared to not be the case with the other protozoans (Table 3.10). Also of possible note was the observed infection profile within the mixed genotype group of bats; no *Eimeria* parasites were observed within this population and also, equal numbers of *T. dionisii*, *B. vesperuginis* and *Cryptosporidium* single infections were observed (Table 3.11). Given the relative

numbers of bats in the two populations (n=59 and n=12), it appears that the mixed genotype group has an over-representation of single piroplasm and *Cryptosporidium* infections compared to the single interbreeding population of bats.

Table 3.10: Infection profiles within the single interbreeding population of pipistrelles.

Infection load	Number of samples	Infection profile
Single infection	37 bats (52%)	*T. dionisii*: 16 bats *T. vespertilionis*: 2 bats *Eimeria*: 6 bats *Cryptosporidium*: 4 bats *B. vesperuginis*: 7 bats *Toxoplasma*: 2 bats
Double infections	16 bats (22%)	*T. dionisii* + *B. vesperuginis*: 5 bats *Eimeria*+ *B. vesperuginis*: 4 bats *T. dionisii*+ *Eimeria*: 1 bat *T. vespertilionis*+ *Eimeria*: 1 bat *T. dionisii*+ *Cryptosporidium*: 2 bats *Eimeria*+ *Cryptosporidium*: 1 bat *T. dionisii*+ *Bartonella*: 1 bat *Toxoplasma*+ *B. vesperuginis*: 1 bat
Triple infections	4 bats (5%)	*Cryptosporidium*+ *Eimeria*+ *T. dionisii*: 2 bats *T. dionisii*+ *Bartonella*+ *Eimeria*: 1 bat *Cryptosporidium*+ *Eimeria*+ *B. vesperuginis*: 1 bat
Quadruple infections	1 bat (1%)	*Cryptosporidium*+ *Toxoplasma*+ *T. dionisii*+ *B. vesperuginis*
Quintuple infections	1 bat (1%)	*Cryptosporidium*+ *Eimeria*+ *T. dionisii*+ *B. vesperuginis*+ *Toxoplasma*

Table 3.11: Infection profiles within the mixed genotype pipistrelles.

Infection load	Number of samples	Infection profile
Single infection	8 bats	*T. dionisii*: 2 bats *Cryptosporidium*: 2 bats *B. vesperuginis*: 2 bats *Toxoplasma*: 2 bat
Double infections	2 bats	*T. dionisii* + *Vesperuginis*: 1 bat *T. vespertilionis*+ *Borrelia*: 1 bat

3.6.1 Infections and the environment:

The locations where the bats were found/recovered were extracted from the Lord (2010) study and plotted (Appendix 2). This analysis showed that the bat infections appeared to be scattered across South Lancashire and Greater Manchester and hence geographic location had limited, if any, impact upon the observed infection profiles. Furthermore, bats lacking a microparasite infection were also dispersed across the study area, again, indicative of the environment having limited, if any, impact upon infection status.

The trypanosome and coccidian infection data was also analysed with respect to the season of host acquisition. This analysis showed there was no statistical significance to the seasonal infection data for the *T. dionisii*, *T. vespertilionis*, *E. rioarribaensis*, and *Cryptosporidium* infections (Table 3.12).

Table 3.12: Seasonal protozoan infection profiles in the bats. *Footnote:* Winter: Dec-Feb; Spring: Mar- May; Summer: June- Aug; Autumn: Sept- Nov.

	Spring	Summer	Autumn	Winter	χ^2 *p*-value
T. vespertilionis	0	1.6% (1/66)	13% (3/23)	0	> 0.05
T. dionisii	27% (3/11)	30.3% (20/66)	43.5% (10/23)	0	> 0.05
Cryptosporidium	27% (3/11)	6% (4/66)	26% (6/23)	0	> 0.05
E. rioarribaensis	9% (1/11)	13.6% (9/66)	39% (9/23)	0	> 0.05
Total (infected +non-infected)	11	66	23	0	

3.6.2. Infections and host factors:

The trypanosome and coccidian infection data was analysed with respect to host gender and age. Regarding gender, the majority of the infected bats were male (n=46); however, there was no statistical significance between prevalence of infection between the sexes (Table 3.13). when the total male and female were checked, the majority of the pipistrelles were male (n= 62) whereas the remaining was females (n= 37) which reflect to the ratio of the infected pipistrelle showed the majority of the infected pipistrelles were males. With respect to host age, the majority of the infected bats were adults and there was no statistical significance between the prevalence of infection between these adults and the juvenile pipistrelles (Table 3.14).

Table 3.13: Gender profiles of the pipistrelle infections. *Footnote*: Baby pipistrelles were excluded from this age analysis.

Gender	Male	Female	χ^2 *p*-value
Total	46	21	> 0.05
T. vespertilionis	6.5%	4.7%	> 0.05
T. dionisii	47.8%	21.7%	> 0.05
Cryptosporidium	15%	28.5%	> 0.05
E. rioarribaensis	30%	19%	> 0.05

Table 3.14: Age profiles of the pipistrelle infections.

Age group	Adult	Juvenile	Baby	χ^2 *p*-value (adult/juvenile)
T. vespertilionis	7.4%	0	0	> 0.05
T. dionisii	46.2%	53.8%	33.3%	> 0.05
Cryptosporidium	18.5%	23%	33.3%	> 0.05
E. rioarribaensis	27.7%	23%	33.3%	> 0.05
Total	54	13	3	> 0.05

3.6.2.1 Infections and host genotype:

The potential influence of host genetic background upon infection outcome was noted earlier (Tables 3.10 and 3.11) and hence the bat genotype data, determined from microsatellite analysis of 11 polymorphic loci (Dodd et al. 2014), was analysed with respect to parasite infections.

Interestingly, when the bats that showed no protozoan and helminth infections were analysed with respect to genotype, all these non-infected bats were from the single interbreeding population. However, when analysed statistically, there was no significant correlation between the host genotype and the parasite-free status of the bats (*p*-value = 0.055).

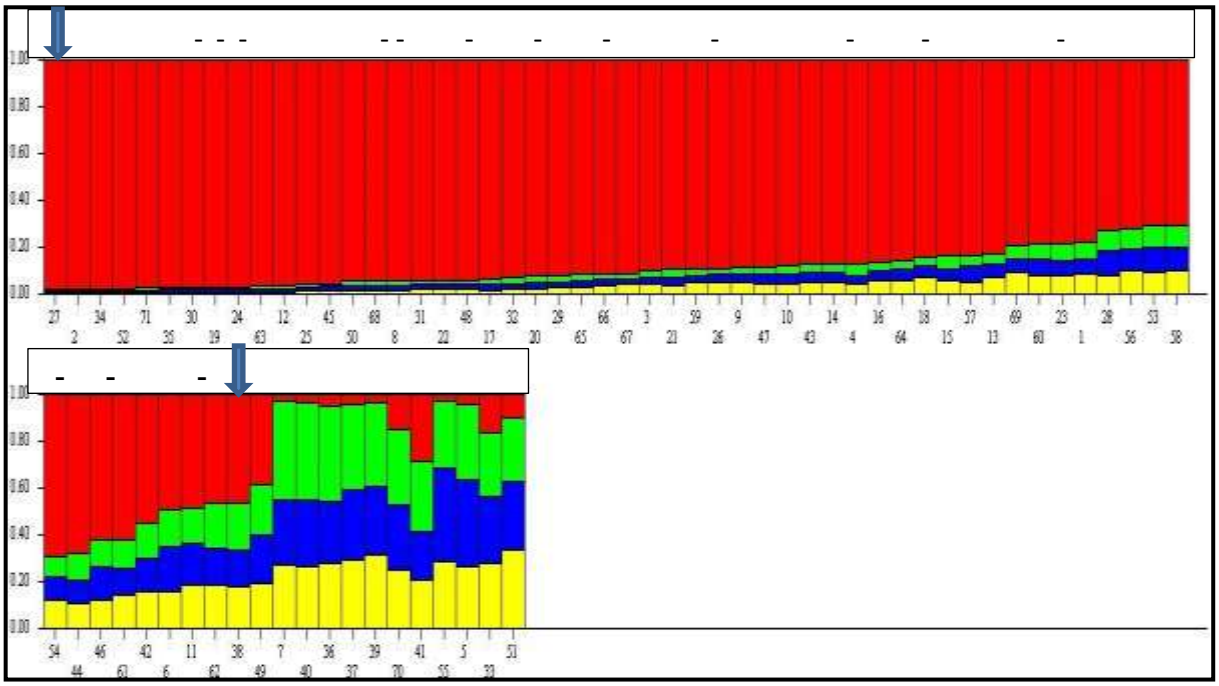

Figure 3.28: The population structure of the pipistrelles (Dodd et al, 2014) highlighting the non-infected (protozoan and helminth) individuals (red text below):

1 = CS/06/10, 2 = CS/08/01, 3 = CS/08/02, 4 = CS/08/A, 5 = FP/05/46, 6 = FP/07/11, 7 = FP/07/12, 8 = FP/07/13, 9 = FP/07/21, 10 = FP/07/37, 11 = FP/07/42, 12 = FP/07/44, 13 = FP/07/45, 14 = FP/07/47, 15 = FP/07/51, 16 = FP/08/02, 17 = GH/06/06, 18 = GH/07/09, 19 = GH/07/10, 20 = JH/07/01, 21 = JH/08/02, 22 = JL/06/12, 23 = JL/06/13, 24 = JL/06/15, 25 = JL/06/24, 26 = JL/06/26, 27= JL/06/27, 28 = JL/06/28, 29 = JL/06/40, 30 = JL/06/42, 31 = JL/06/45, 32 = JL/06/47, 33 = JL/06/54, 34 = JL/06/56, 35 = JL/06/59, 36 = JL/07/04, 37 = JL/07/07, 38 = JL/07/08, 39 = JL/07/09, 40 = JL/07/10, 41 = JL/07/11, 42 = JL/07/12, 43 = JL/07/14, 44 = JL/07/18, 45 = JL/07/23, 46 = JL/07/25, 47 = MD/08/02, 48 = MH/08/02, 49 = PB/06/01, 50 = PB/06/02, 51 = PH/06/04, 52 = PH/06/05, 53 = SA/06/05, 54 = SA/06/07, 55 = SA/07/U, 56 = SP/06/49, 57 = SP/06/55, 58 = SP/06/68, 59 = SP/06/70, 60 = SP/06/72, 61 = SP/06/77, 62 = SP/06/79, 63 = SP/06/80, 64 = SP/06/81, 65 = SP/06/82, 66 = SP/06/83, 67 = SP/06/84, 68 = SP/08/16, 69 = SP/08/17, 70 = SP/08/18, 71 = SP/08/19.

Footnote: the single interbreeding group is represented by bats designated between the arrows and the mixed population is represented by bats outside the arrows.

When analysing the eimerian infections with respect to the bat genotype data (Figure 3.29), it was noted that all the infected bats were from the single interbreeding population and this correlation was statistically significant *(p*-value=0.02).

The *T. dionisii*, *Cryptosporidium* and *B. vesperuginis* infection profiles did not correlate with the bat genotype data (*p*-values > 0.05) (Appendix 4). Furthermore, when all double protozoan infections (18 bats) and multi-protozoan infections (6 bats) were also analysed with respect to host genotype, again, no statistically significant correlations were observed (*p*-values > 0.05). Finally, with respect to the helminth infection data reported by Lord (2010), all five species of trematodes were also analysed with respect to the bat genotype data and again, no significant correlations were noted (*p*-values > 0.05).

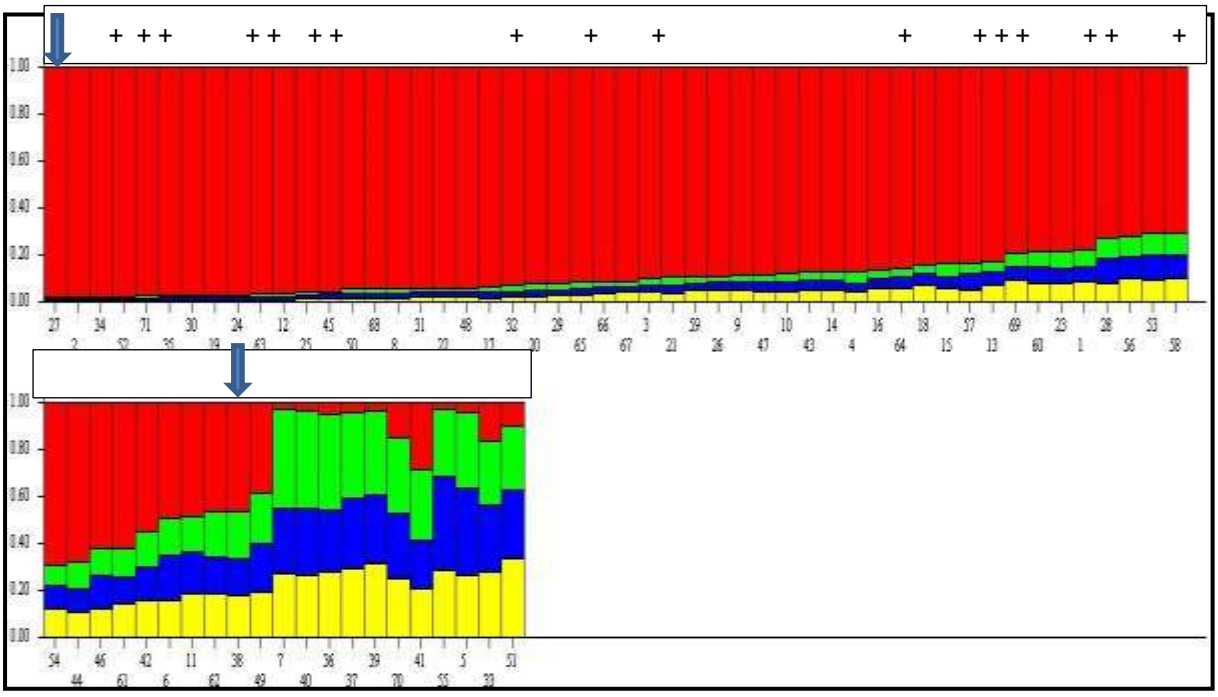

Figure 3.29: The population structure of the pipistrelles (Dodd et al, 2014) highlighting the *E. rioarribaensis* infections (red text below):

1 = CS/06/10, 2 = CS/08/01, 3 = CS/08/02, 4 = CS/08/A, 5 = FP/05/46, 6 = FP/07/11, 7 = FP/07/12, 8 = FP/07/13, 9 = FP/07/21, 10 = FP/07/37, 11 = FP/07/42, 12 = FP/07/44, 13 = FP/07/45, 14 = FP/07/47, 15 = FP/07/51, 16 = FP/08/02, 17 = GH/06/06, 18 = GH/07/09, 19 = GH/07/10, 20 = JH/07/01, 21 = JH/08/02, 22 = JL/06/12, 23 = JL/06/13, 24 = JL/06/15, 25 = JL/06/24, 26 = JL/06/26, 27= JL/06/27, 28 = JL/06/28, 29 = JL/06/40, 30 = JL/06/42, 31 = JL/06/45, 32 = JL/06/47, 33 = JL/06/54, 34 = JL/06/56, 35 = JL/06/59, 36 = JL/07/04, 37 = JL/07/07, 38 = JL/07/08, 39 = JL/07/09, 40 = JL/07/10, 41 = JL/07/11, 42 = JL/07/12, 43 = JL/07/14, 44 = JL/07/18, 45 = JL/07/23, 46 = JL/07/25, 47 = MD/08/02, 48 = MH/08/02, 49 = PB/06/01, 50 = PB/06/02, 51 = PH/06/04, 52 = PH/06/05, 53 = SA/06/05, 54 = SA/06/07, 55 = SA/07/U, 56 = SP/06/49, 57 = SP/06/55, 58 = SP/06/68, 59 = SP/06/70, 60 = SP/06/72, 61 = SP/06/77, 62 = SP/06/79, 63 = SP/06/80, 64 = SP/06/81, 65 = SP/06/82, 66 = SP/06/83, 67 = SP/06/84, 68 = SP/08/16, 69 = SP/08/17, 70 = SP/08/18, 71 = SP/08/19.

Footnote: the single interbreeding group is represented by bats designated between the arrows and the mixed population is represented by bats outside the arrows.

3.7 Discussion

The molecular diagnostic data presented in this Chapter provides an extension to earlier studies on protozoans and helminths in the pipistrelle population from North West England (Dodd et al., 2014; Lord, 2010; Lord et al., 2012). As a consequence, the data offers the opportunity to begin to address the parasite community composition and potential roles that the environment and host genetics might have in shaping the observed infection profiles.

3.7.1 Trypanosome (*Schizotrypanum*):

Upon initiation of this study, data in the Lord (2010) thesis showed that 37 bats were infected with trypanosomes. At least 30 of these infections were proposed to be due to *T. dionisii*; however, lack of positive control DNAs for *T. dionisii* and *T. vespertilionis* did not allow absolute confirmation of the Schizotrypanum infections in the bats (Lord, 2010). Unfortunately, no archived cultures of *T. dionisii* and *T. vespertilionis* exist and given the protected status of British bats, it was beyond the scope of this thesis to acquire bat blood and attempt to establish cultures. However, acquisition of a small amount of genomic DNAs from Dr Patrick Hamilton (University of Exeter) allowed attempts at optimisation of an 18S rRNA PCR approach that was discriminatory between *T. dionisii* and *T. vespertilionis*. It was apparent from the recovery of PCR products that the detection rate was greater for both species of trypanosome when bat heart DNA preparations were screened compared to bat spleen DNA preparations. This most likely reflects that the bat heart has a greater capacity to hold residual blood than the capillary network of the spleen. As such, a lack of PCR product derived from spleen DNA, when a product has been generated from a heart DNA preparation, is likely to indicate that the limits of PCR detection using the spleen have been breached. Sequencing the 18S rRNA PCR products confirmed that 4 trypanosome infections were due to *T. vespertilionis*; albeit, two nucleotide variations were consistently observed relative to

the 18S rRNA sequence of the *T. vespertilionis* P14 isolate derived from a pipistrelle bat in England (Baker, 1974; Stevens et al., 1999). Given that there are no further *T. vespertilionis* sequences deposited in GenBank, it is not possible to comment further on these nucleotide variations. Sequencing the *T. dionisii* 18S rRNA PCR products confirmed that they were identical to the *T. dionisii* P3 isolate derived from a pipistrelle bat from England (Baker & Thompson, 1971; Stevens et al., 1999). However, the number of bats confirmed to be infected with *T. dionisii* was less than anticipated based upon the Lord (2010) study and hence an alternative strategy, using a semi-nested approach to target PCR amplification of the *T. dionisii* glyceraldehyde phosphate dehydrogenase (GAPDH) gene was undertaken. This approach not only confirmed the 10 *T. dionisii* infections based upon the 18S rRNA PCR strategy but it extended the detection of *T. dionisii* to a total of 33 bats. Moreover, the majority of the PCR products (=79%) produced good quality sequence data that confirmed that these *T. dionisii* infections were identical to the P3 isolate (J. Baker & Thompson, 1971; Hamilton, Cruickshank, et al., 2012) and also, the Z3126 isolate derived from a soprano pipistrelle from Wytham, Oxfordshire (Hamilton, Cruickshank, et al., 2012). Interestingly, none of the GAPDH sequence data provided evidence for the presence of *T. dionisii* strain B in the pipistrelles. *T. dionisii* strain B, a representative South American strain, was recently discovered in Noctule, Serotine and Whiskered bats in the UK but was not reported present in 26 pipistrelle specimens (Hamilton, Cruickshank, et al., 2012). Since *T. dionisii* strain A, the representative European strain that includes isolates P3 and Z3126, was the only sequence type confirmed in the South Lancashire pipistrelles, then this data, in conjunction with the Hamilton et al., (2012) report, may indicate some host specificity associated with the *T. dionisii* strains and pipistrelle bats.

Overall, the trypanosome infected bats reported in this thesis using the combination of 18S rRNA and GAPDH PCR approaches was identical to the *Trypanosoma* spp. infections reported by Lord (2010) using only the 18S rRNA strategy. At 37% prevalence, the trypanosome infections in the South Lancashire pipistrelles (including 3 soprano pipistrelles) is approximately the same as that reported (35%) by Gardner (1986) following blood smear inspections of 206 *P. pipistrellus* specimens sampled from across the UK. However, this prevalence data differs remarkably to the single *T. dionisii* infection in 36 Cornish pipistrelles examined by Concannon et al. (2005) using a PCR diagnostic assay that targeted the 18S rRNA gene. This difference may be partly explained by contrasting PCR performances. However, it more likely reflects a difference in transmission dynamics between these two areas of the UK. Such differences could be explored by further studies of the bats and the known intermediate host responsible for trypanosome transmission, *Cimex pipistrelli* (Gardner & Molyneux, 1988a).

Since *T. dionisii* infections in the South Lancashire pipistrelles were observed approximately 8-times more frequently than *T. vespertilionis*, it is likely that transmission of the former species is favoured. However, data in the literature to support this is sparse; the extensive study by Gardner (1986) documented *Schizotrypanum* infections and did not distinguish between *T. dionisii* and *T. vespertilionis*. Much earlier reports provide data on *T. vespertilionis* infections occurring more frequently than *T. dionisii* (see Lord & Brooks, 2014); however, relatively few hosts are examined. The most recent analysis though documented 16 trypanosome infections in 4 species of UK bats, including pipistrelles, and 15 of these were reported as *T. dionisii* and only 1 as *T. vespertilionis* (Hamilton, Cruickshank, et al., 2012). Interestingly, the *T. vespertilionis* infection was described in a Noctule bat that was also infected with *T. dionisii* (Hamilton, Cruickshank, et al., 2012). Given that very few

bats have been confirmed with *T. vespertilionis* infections, it is not surprising that co-infections with both trypanosome species are rare and this is indeed corroborated by the infection data within this thesis.

When the trypanosome infections were analysed with respect to the season of host acquisition, it showed no statistical significance to the seasonal infection data for the *T. dionisii* and *T. vespertilionis* infections. Moreover, there was no significant correlation between the bat genotype and whether, or not, the specimen was infected with trypanosomes.

3.7.2 Eimeria:

The eimerian infection in 19 South Lancashire bats reported by Lord (2010) was based upon PCR amplification of the 18S rRNA gene. Subsequent DNA sequencing was only performed on one PCR product and it was shown to be 99.8% identical to the 18S rRNA gene of *E. rioarribaensis*, an eimerian parasite previously reported to infect myotid bats from North America (Duszynski et al., 1999; Zhao, Duszynski, & Loker, 2001). PCR screening carried out in this thesis confirmed the eimerian infection status of the South Lancashire bats described by Lord (2010). Moreover, all eimerian 18S rRNA PCR products were subjected to DNA sequencing and the data confirmed that all the sequences were identical to *E. rioarribaensis*. As such, the one nucleotide difference between the South Lancashire bat derived eimerian 18S rRNA product and that of *E. rioarribaensis* reported by Lord (2010) must have been a PCR or sequencing artefact. To our knowledge, the data constitutes the first record of an eimerian parasite in British bats and indeed, the first record of an eimerian in a common, or soprano pipistrelle.

With regard to seasonal effect, the data showed high prevalence in autumn and summer which reflect to the condition that is needed for the oocyst to develop such as high humidity.

The infected bats are expected to be infected for several days which might preclude the identification of seasonal effect.

On analyzing the eimerian infection data with respect to host genotype it was apparent that all the infected hosts were from the single interbreeding population. Moreover, this infection profile was statistically significant and hence indicative of bats from the mixed genotype population having a degree of resistance to eimerian infection. This is further supported by the lack of evidence of an environmental affect since the bats lacking eimerian infection were geographically dispersed across the study region and therefore presumably as exposed to the risk of acquiring an infection as the rest of the population.

Not surprisingly, there is no published study on the host genetics of bat-*Eimeria* spp. interactions. Given the problems encountered in the poultry industry with coccidiosis and the knowledge that different breeds of chicken display differences in resistance/susceptibility to eimerian parasites (Bumstead, Bumstead, Rothwell, & Tomley, 1995; Bumstead & Millard, 1987; Emara et al., 2002; Johnson & Edgar, 1986), then some insight can be gained from avian immunogenomic studies. Of particular interest is the quantitative trait loci (QTL) analysis of an F2 cross carried out in chickens with *E. tenella* resistant and susceptible lines (Pinard-van der Laan et al., 2009). The resulting QTL data highlighted a number of candidate genes that may provide resistance to *E. tenell*a infection in chickens, including innate immunity (TLR7) and inflammatory response genes (Pinard-van der Laan et al., 2009).

3.7.3 Cryptosporidium:

There have been few studies on *Cryptosporidium* spp. in bats (Dubey, Hamir, Sonn, & Topper, 1998; Kváč et al., 2015; Morgan et al., 1999; Wang et al., 2013; Ziegler, Wade, Schaaf, Chang, & Mohammed, 2007) and hence the role of bats in parasite transmission is largely unknown. However, the detection of the human infective *C. parvum* and *C. muris* in

myotid bats (Kváč et al., 2015; Zahedi, Paparini, Jian, Robertson, & Ryan, 2016) and common pipistrelles (Kváč et al., 2015) raises the possibility that bats may act as important reservoirs of *Cryptosporidium* spp. and hence this is worthy of further study.

The molecular data presented in this chapter presents the first evidence that UK bats are infected with *Cryptosporidium* sp. Based upon the 18S rRNA sequence data, the parasite was most closely related to a *Cryptosporidium* sp. bat genotype IV isolate derived from common pipistrelles studies in the Czech Republic (Kváč et al., 2015).

There appeared to be no significant association of *Cryptosporidium* sp. infection in the South Lancashire pipistrelles with environmental or host parameters. The infection data does however show that bats infected with *Cryptosporidium* sp. are often also infected with *E. rioarribaensis* (6/15, 40%) which may indicate a common route of infection for these two coccidians.

3.7.4 Bacterial infections:

The *Bartonella* sp. infection reported by Lord (2010) in 2 bats was based upon PCR amplification of the 16S-23S rRNA internal transcribed spacer (ITS) region; one amplicon was sequenced and it appeared to cluster closely with rodent-associated *Bartonella* spp. However, the absence of a bat-associated *Bartonella* spp. ITS sequence in the phylogram precluded a more detailed analysis. As such, to further describe these two *Bartonella* sp. infections the bacterial citrate synthase gene was PCR amplified and the resulting products were sequenced. The data showed that the bat Bartonella citrate synthase sequences were identical to the citrate synthase sequence from an uncultured *Bartonella* sp. that had been isolated from a Cornish pipistrelle (Concannon et al., 2005). As proposed by Concannon et al., (2005), based upon a phylogeny of *Bartonella* spp. citrate synthase sequences, including those derived from the Cornish bats, the data in this chapter supports the proposal that bat-

associated *Bartonella* sp. are all closely associated with the bat host and may form strains of the same species.

The low prevalence of *Bartonella* sp. in the South Lancashire pipistrelles contrasts with the 18% prevalence (18/491) of bartonellae reported in the extensive study of UK bats carried out in the 1980s (Gardner et al., 1987; Gardner, 1986). However, it is similar to the 8% *Bartonella* sp. infection rate reported in the Cornish bats (Concannon et al., 2005).

The molecular data in this chapter also confirm that *Borrelia* sp. is present in UK bats, as noted in an earlier report describing fatal borreliosis in a female pipistrelle from Cornwall (Evans et al., 2009). Indeed, the 16S rRNA gene fragment data from the South Lancashire pipistrelle was identical to the 16S rRNA sequence deposited from the *Borrelia* sp. isolate derived from the Cornish pipistrelle (Evans et al., 2009). As noted (Evans et al., 2009), the Cornish bat derived *Borrelia* sp. was closely related to other *Borrelia* spp. known to cause relapsing fever in Africa and Asia and hence bats may act as a reservoir of spirochetes that are potentially of public health concern. Unfortunately, the sequence data generated from the South Lancashire bat was of insufficient length to provide greater insight since the fragment was also identical to other *Borrelia* sp. isolates from questing Gulf Coast ticks (Lee et al., 2014) and also, to *B. garinii* and *B. afzelii* isolates; these species are known to cause human Lyme borreliosis (Nadelman & Wormser, 1998; Picken et al., 1998; Richter, Schlee, Allgöwer, & Matuschka, 2004). As such, it would be worthwhile carrying out a more detailed analysis of the *Borrelia* sp. infection described in the adult male pipistrelle acquired from Tottington, Greater Manchester. Although this specimen died in captivity, there was no evidence to suggest that the death was due to borreliosis (personal communication, Jennifer S. Lord).

3.7.5 Co-infections:

Based on the genotyping data carried out on a subset the bat population (n=71) (Dodd et al., 2014), the majority of the bats were from one interbreeding group (n=59) and the remainder were designated as being of mixed origin (n=12). Given the numbers representative of the two populations, it is not surprising that a greater variety of infections were found in the single interbreeding group. As such, it is not possible to state that the mixed genotype bat group has a greater level of resistance to parasite infections than the single interbreeding group. There does not appear to be any strong evidence for negative interactions between any of the parasites. The coccidians *E. rioarribaensis* and *Cryptosporidium* sp. were often components of a co-infection and this may reflect that they are commonly found to co-exist in the environment; quite possibly the roost environment.

4. Pipistrelle Toll-like receptors (TLRs): *TLR2* and *TLR4*:

Toll-like receptors (TLRs) play an important role in the outcome of parasite infections in vertebrate hosts. For example, study of *Leishmania major* infection in TLR4 knockout mice has shown that TLR4 activates iNOS (inducible nitric oxide synthase) which leads to NO synthesis and parasite death (Kropf et al., 2004). *T. cruzi* glycosylphosphatidylinositol (GPI) anchors have been shown to be potent and effective initiators of TLR2 expression in Chinese hamster ovary cells transfected with TLR2 (Campos et al., 2001). Moreover, macrophages from TLR2 knockout mice were unable to respond to *T. cruzi* GPI anchors via the network of cytokine expression observed with control macrophages (Campos et al., 2001). TLR2 knockout mice study has also highlighted the importance of TLR2 in defence against *T. gondii* infection since the TLR2-deficient animals died 8 days post-infection with an avirulent strain of the parasite (Mun et al., 2003). The levels of expression of TLR2 and TLR4 have also been assessed in the brains of mice infected with *Acanthamoeba* spp. and the resulting data confirmed that these TLRs are highly expressed in neurons, glial cells, and endothelial cells within the neocortex 2 days post-infection (Wojtkowiak-Giera et al., 2016). Perhaps most interestingly with respect to human infections with a parasite, a study of monocyte expression in children from Malawi reported that TLR2 and TLR4 expression levels were significantly lower in severe malaria cases compared to control groups (Mandala et al., 2016).

As illustrated above, it is becoming increasingly clear that TLR2 and TLR4 have important roles in protection of mammals against parasite infection. However, the role of these TLRs in bats is less well understood; indeed, there is no sequence data currently available for TLR2 or TLR4 from a pipistrelle bat. As such, the main aim of this chapter of the thesis is to use a PCR-based strategy to isolate pipistrelle TLR2 and TLR4 gene sequences. This gene

isolation strategy will that act as a platform for further analyses, in Chapter 5, of TLR2 and TLR4 gene variation in the population of South Lancashire bats.

4.1 The *P. pipistrelle TLR 4* gene:

4.1.1 Bioinformatics:

Given the absence of a published, or unpublished, TLR4 gene sequence from pipistrelle bats, the TLR4 genes from other bat species were obtained from GenBank and aligned in order to facilitate PCR primer design to conserved regions (Figure 4.1). The full length TLR4 gene in bats was approximately 2.5 kb; however, due to high levels of species variation at the 5' and 3' ends of the gene, it was not possible to design PCR primers to amplify the full-length pipistrelle TLR4 gene. Instead, a set of PCR primers were designed to allow amplification of two overlapping PCR products (TLR4 F - TLR4 R and TLR4 2F - TLR4 2R) which were anticipated to yield approximately 2 kb of novel *P. pipistrellus* TLR4 gene sequence (Figure 4.1).

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gi|558135472|ref|XM_006091085.      TTAATGTGGCTCACAATCTAATTGATTCCTTCAAGTTACCGGACTATTTT 698
gi|554578862|ref|XM_005880935.      TTAATGTGGCTCACAATCTAATTGATTCCTTCAAGTTACCGGACTATTTT 698
gi|584056807|ref|XM_006772885.      TTAATGTGGCTCACAATCTAATTGATTCCTTCAAGTTACCGGACTATTTT 698
gi|641721271|ref|XM_008152116.      TTAATGTGGCTCACAATCTAATCGATTCCTTCAAGTTACCGAACTATTTT 510
gi|588480441|ref|NM_001290172.      TTAATGTGGCTCACAATCTTATTGATTCCTTCAAGTTACCTGAATATTTT 626
                                    ****************** ** ****************  * ******

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gi|584056807|ref|XM_006772885.      TCTAACCTGCCTAACCTGGAGCACTTGGATCTTTCCAATAACAAGATCCG 748
gi|641721271|ref|XM_008152116.      TCTAACCTGCCTAACCTGGAGCACTTGGACCTTTCCAATAATAAGATTCG 560
gi|588480441|ref|NM_001290172.      TCTAACCTGTCCGACCTGGAGCACTTAGACCTTTCCAATAACAAGATCCA 676
                                    ********* *  ************* ** *********** ***** *

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gi|584056807|ref|XM_006772885.      CAATATTTACCATGAAGACTTGCAGGTTTTACATCAAATGCCCTCATTCA 798
gi|641721271|ref|XM_008152116.      AAATATTTACCATGAAGACTTGCAGGTTTTACATCAAATGCCCTCATTCA 610
gi|588480441|ref|NM_001290172.      AACTATTTGTCATAAAGACCTACAGGTTCTACATCAAATGCCCCCATCCA 726
                                     * *****  *** ***** * ****** *************  *** **

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gi|584056807|ref|XM_006772885.      AACTCTCCTTAGACCTGTCCCTCAACCCTTTAGACTTTATTCAACCAGGT 848
gi|641721271|ref|XM_008152116.      AACTCTCTTTAGACCTGTCCCTCAACCCTTTAGACTTTATCCAACCAGGT 660
gi|588480441|ref|NM_001290172.      AACTCTCTTTAGACTTGTCCCTGAACCCTTTAGACTTCATCCAACGAGGT 776
                                    ******* ****** ******* ************** ** **** ****

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gi|554578862|ref|XM_005880935.      GCCTTTGAAAAAATTAAGCTCCATGAACTGACTTTGAGAAGTAATTTTGA 898
gi|584056807|ref|XM_006772885.      GCCTTTGAAAAAATTAAGCTCCATGAACTGACTTTGAGAAGTAATTTTGA 898
gi|641721271|ref|XM_008152116.      GCCTTTGAAAAAATTAAGCTCCATGAACTGACTTTGAGAAGTAATTTTGA 710
gi|588480441|ref|NM_001290172.      GCCTTTAAAGAAATTAAGCTCCATGAACTAACTTTGAGAAGTAATTTTAA 826
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gi|584056807|ref|XM_006772885.      TAGTCCAGAGGTCATGAAAATGTGTATTCAAGGTCTGGCTGGTTTAAAGA 948
gi|641721271|ref|XM_008152116.      TAGTGCAGAGGTCATGAAAACGTTTATTCAAGGTCTGGCAGGTTTAAAGA 760
gi|588480441|ref|NM_001290172.      CAGTACAGATGTAATGAAAACTTGTGTTCAAGGCCTCGCTGGCTTAAAAA 876
                                     *** **** ** *******  * * ******* ** ** ** ***** *

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gi|584056807|ref|XM_006772885.      TCAATCGGTTGATTCTGGGAGAATTTAAAAATGAAAGAACCTTAGTAAAC 998
gi|641721271|ref|XM_008152116.      TCAAACGGCTGATTCTGGGAGAATTTAAAAATGAAAGGATCTTAGTAAAC 810
gi|588480441|ref|NM_001290172.      TCAATCGTTTGGTTCTAGGAGAATTTAAAAATGAAAGAGCCATAAAACAT 926
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gi|584056807|ref|XM_006772885.      TTCAACAATTCTGCCCTGGAGGGTCTGTGCAATTTGACCATTGAAGAATT 1048
gi|641721271|ref|XM_008152116.      TTGGACAAATCTGCCCTGGAGGAACTGTGTAATTTGACCATTGAAGAATT 860
gi|588480441|ref|NM_001290172.      TTTGACAAATCTGCCATGGAGGGACTGTGCAATTTGACCATTGACGAATT 976
                                    **  **** ****** ******  ***** ************** *****

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gi|554578862|ref|XM_005880935.      CCGGATAGCACACTTCGATGAGTTTCCAGGGGATGATCTTGGCTTTTTAA 1098
gi|584056807|ref|XM_006772885.      CCGGATAGCACGCTTCAATGAGTTTCCAGGGGATGATCTTGGCTTTTTAA 1098
gi|641721271|ref|XM_008152116.      CCGGATAGCACACTTCCAAGACTTTCCAGAGGATTACCTTGGCTTTTTAA 910
gi|588480441|ref|NM_001290172.      CCGGATGACATACTTCGATGACTTCTCAGAGGATGTTATTAACTTTTTTA 1026
                                    ******  **  **** * ** **  *** ****    **  ****** *

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gi|554578862|ref|XM_005880935.      ATTGTTTGGCAGATGCTTCTACAATATCTCTTGTGAGTCTATATTTAGAT 1148
gi|584056807|ref|XM_006772885.      ATTGTTTGGCAGATGCTTCTACAATATCTCTTGTGAGTCTATATTTAGAC 1148
gi|641721271|ref|XM_008152116.      ATTGTTTGGCAGATGCTTCTGCAATATCTCTGGTGAGTCTGAATATAGAC 960
gi|588480441|ref|NM_001290172.      ATTGTTTGGCAAATGTTTCTACAATTTCTCTGGTGGGTCTGTATTTAAAC 1076
                                    ******** ** * * **** **** *****  ** ****  ** ** *

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gi|641721271|ref|XM_008152116.      TAATTGTAAATTTGAACATTTTCCTACATTGGAGCTTACCTTTCTCAAGC 1060
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gi|584056807|ref|XM_006772885.      AGTTCATTTTCACTGCCAACAAAGGTATTACCACTTTTACTGAAGTTAAT 1298
gi|641721271|ref|XM_008152116.      AGTTTGTTTTCACTGACAACAAAGGTATTACCACTTTTACTGAAGTTAAT 1110
gi|588480441|ref|NM_001290172.      AGTTGGTTTTCACTGCCAACAGGGGTATGAGCACTTTTACTGAAGTTAAA 1226
                                    ****  ********* *****  ***** * ********** *******

                                              TLR4F
                                          ------------>
gi|558135472|ref|XM_006091085.      CTACCAAACCTTGAGTTTCTAGATCTCAGTAAAAATGGCTTGAGTTACAA 1348
gi|554578862|ref|XM_005880935.      CTACCAAACCTTGAGTTTCTAGATCTCAGTAGAAATGGCTTGAGTTTCAA 1348
gi|584056807|ref|XM_006772885.      CTACCAAACCTTGAGTTTCTAGATCTCAGTAGAAATGGCTTGAGTTTCAA 1348
gi|641721271|ref|XM_008152116.      CTAAGAAACCTTGAGTTTCTAGATCTCAGTAGTAATGGCTTGAGTTTCAA 1160
gi|588480441|ref|NM_001290172.      CTACCAAAACTTGAGTTTCTAGATCTCAGTAGAAATAGTTTGAGTTTCAA 1276
                                    ***  *** **********************  *** * ******* ***

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gi|554578862|ref|XM_005880935.      GTCCTGCTGCTCTCACCGTGATTTTGGGACAACCCGACTGAAACACTTAG 1398
gi|584056807|ref|XM_006772885.      GTCCTGCTGCTCTCACCGTGATTTTGGGACAACCCGACTGAAACACTTAG 1398
gi|641721271|ref|XM_008152116.      GTCTTGCTGCTCTCACCGTGATTTTGGGACAACCCAACTGAAACACTTAA 1210
gi|588480441|ref|NM_001290172.      GAGTTGCTGTTCTCGCACTTTTTGGGGGACAACTAGACTGAAACACTTAG 1326
                                    *   ***** **** *  *  **  ********   *************

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gi|588480441|ref|NM_001290172.      ATCTGAGCTTTAATGATGTTATTACCATGAGCTCAAACTCCTTGGGCTTA 1376
                                    ******* ** ***  * ****** *****  ** **** * *** ****

gi|558135472|ref|XM_006091085.      GAGCAACTAGAACGTCTGGATTTCCAGCATTCCACTCTGAAACAGGCCAG 1498
gi|554578862|ref|XM_005880935.      GAGCAACTAGAACATCTGGATTTCCAGCATTCCACTCTGAAACAGGCCAG 1498
gi|584056807|ref|XM_006772885.      GAGCAACTAGAACATCTGGATTTCCAGCATTCCACTCTGAAACAGGCCAG 1498
gi|641721271|ref|XM_008152116.      GAGCAACTAGAACGACTGGATTTCCAGCATTCCACTTTGAAACAGGCCAG 1310
gi|588480441|ref|NM_001290172.      GAGCAACTAAAATATCTGGATTTCCAGCATTCCAATTTGAAACAGGCCAG 1426
                                    ********* **   ****************** * *************

gi|558135472|ref|XM_006091085.      TGATTTTTCAGTATTCCTCTCACTCAAAAATCTCCTTTACCTTGATATCT 1548
gi|554578862|ref|XM_005880935.      TGATTTTTCAGTATTCCTCTCACTCAAAAATCTCCTTTACCTTGATATCT 1548
gi|584056807|ref|XM_006772885.      TGATTTTTCAGTATTCCTCTCACTCAAAAATCTCCTTTACCTTGATATCT 1548
gi|641721271|ref|XM_008152116.      TACTTTTTCAATATTCCTCTCACTCAAAAACCTCCTTTACCTTGATATCT 1360
gi|588480441|ref|NM_001290172.      TGATTTTTCGGTATTCCTATCACTCAAAAACCTACTTTACCTTGATATTT 1476
                                    *  ******  ******* *********** ** ************** *

gi|558135472|ref|XM_006091085.      CTTACACTAACACCAAGATTGTCTTCCTGCGCATCTTTGATGGCTTGATC 1598
gi|554578862|ref|XM_005880935.      CTTACACTAACACCAAGATTGTCTTCCTGGGCATCTTTGATGGCTTGATC 1598
gi|584056807|ref|XM_006772885.      CTTACACTAACACCAAGATTGTCTTCCTGGGCATCTTTGATGGCTTGATC 1598
gi|641721271|ref|XM_008152116.      CTTACACTAACATCCAGATTGTCTTCAAGGGCATCTTTGATGGCTTGATC 1410
gi|588480441|ref|NM_001290172.      CTTATACTCGCATCCGAATCATCTTCCATGGCATCTTTGACGGCTTGTTC 1526
                                    **** ***  ** *   **  *****    ********** ****** **

                                              TLR4-2R
                                          <------------
gi|558135472|ref|XM_006091085.      AGCCTCCAAGTCTTGAAAATGGCTGGCAATTCTTTTCAGGATGCACTCC- 1647
gi|554578862|ref|XM_005880935.      AGCCTCCAAGTCTTGAAAATGGCTGGCAATTCTTTTCAGGATGCACTCC- 1647
gi|584056807|ref|XM_006772885.      AGCCTCCAAGTCTTGAAAATGGCTGGCAATTCTTTTCAGGATGCACTCC- 1647
gi|641721271|ref|XM_008152116.      AGCCTCCAAGTCTTGAAAATGGCTGGCAATTCCTTTCAGGATGCATTCC- 1459
gi|588480441|ref|NM_001290172.      AGCCTCGAAGTCTTGAAAATGGCTGGCAATTCTTTTCAGGACA-ACTCCG 1575
                                    ****** ************************* ********   * ***

gi|558135472|ref|XM_006091085.      TTCCAAATATCTTCAGAGATCTGACTCAGTTGACTGAACTGGACCTCTCT 1697
gi|554578862|ref|XM_005880935.      TCCCAAATATCTTCAGAGATCTGACTCAGTTGACTGACCTGGACCTCTCT 1697
gi|584056807|ref|XM_006772885.      TCCCAAATATCTTCAGAGACCTGACTCAGTTGACTGTCCTGGACCTCTCT 1697
gi|641721271|ref|XM_008152116.      TTCCAAATGTCTTCAGAGATCTGACTCAGTTGACTATCCTGGACCTCTCT 1509
gi|588480441|ref|NM_001290172.      TTCCAAATATCTTCAAAGCGCTGACTAACTTAACCTTCCTGGACCTCTCT 1625
                                    * ****** ****** **  ****** * ** **    ************
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gi|558135472|ref|XM_006091085.      CAGTGTCAACTGGAACGGGTGTCCCAGGAGGCATTTGGCTCACTCCTTAG 1747
gi|554578862|ref|XM_005880935.      CAGTGTCAACTGGAACAGGTGTCCCAGGAGGCATTTGGCTCACTCCTTAG 1747
gi|584056807|ref|XM_006772885.      CAGTGTCAACTAGAACGGGTGTCCCAGGAGGCATTTGGCTCACTCCTTAG 1747
gi|641721271|ref|XM_008152116.      CAGTGTCAATTGGAACAGGTGTCTCCGGAGGCATTCAGCTCACTCCTTAG 1559
gi|588480441|ref|NM_001290172.      AATTGCCAGCTAGAACGAGTGTCCCAGGCGGCATTTGGCTCACTCGTTAA 1675
                                     * ** **  * ****  ***** * ** ******  ******** ***


gi|558135472|ref|XM_006091085.      ACTCCAGGTGCTAAATATGAGTCACAACCACCTCTTGTCCTTGGATATGC 1797
gi|554578862|ref|XM_005880935.      ACTCCAGGTGCTAAATATGAGTCACAATCACCTCTTGTCCTTGGATATGC 1797
gi|584056807|ref|XM_006772885.      ACTCCAGGTGCTAAATATGAGTCACAACCACCTCTTGTCCTTGGATATGC 1797
gi|641721271|ref|XM_008152116.      ACTCGAGGTGCTAAATATGAGTCACAACCACCTCTTGTCCTTGGATATGC 1609
gi|588480441|ref|NM_001290172.      ACTTAAGTCACTAAATATGAGTCACAACCACCTTTTATCCTTGGATCTAT 1725
                                    ***  **   ***************** ***** ** ********* *


gi|558135472|ref|XM_006091085.      TTCCTTTTAAAAATCTC---TCTCTCCGGGTTCTAGACTGTAGTTTTAAC 1844
gi|554578862|ref|XM_005880935.      TTCCTTATAAAAATCTC---TCTCTCCGGGTTCTAGATTGCAGTTTTAAC 1844
gi|584056807|ref|XM_006772885.      TTCCTTATAAAAATCTC---TCTCTCCGGGTTCTAGACTGCAGTTTTAAC 1844
gi|641721271|ref|XM_008152116.      TTCCTTACAAAAATCTC---CCTCTCTCGGTTCTAGACTGCAGTTTTAAC 1656
gi|588480441|ref|NM_001290172.      TTCCTTATAAACTTCCCCACTCTCTCCAGGATCTGGACTGCAGTTTTAAT 1775
                                    ******  ***  ** *    *****  ** *** ** ** ********


gi|558135472|ref|XM_006091085.      CGTATAGTGGCCGCCAATGGGCAGGAACTACAGCATTTTCCAAGCAATGT 1894
gi|554578862|ref|XM_005880935.      CGTATAGTGGCCGCCAATGGGCAGGAACTACAACATTTTCCAAGCAATGT 1894
gi|584056807|ref|XM_006772885.      CGTATAGTGGCCGCCAATGGGCAGGAACTACAACATTTTCCAAGCAATGT 1894
gi|641721271|ref|XM_008152116.      CGTATAGTGGCCGCCAATGGGCAGGAACTACAGCATTTTCCAAGCAATGT 1706
gi|588480441|ref|NM_001290172.      CGCATAGTGGCCTCCAATAGGCAAGAACTACAGCATTTTCCAAGTAATCT 1825
                                    ** ********* ***** **** ******** ********** *** *


gi|558135472|ref|XM_006091085.      AACTTCCTTAAACCTGAACCAGAATAACTTTGCTTGTGTTTGTGAACACA 1944
gi|554578862|ref|XM_005880935.      AACTTCCTTAAATCTGACCCAGAATAACTTTGCTTGTGTTTGTGAACACA 1944
gi|584056807|ref|XM_006772885.      AACTTCCTTAAATCTGACCCAGAATAACTTTGCTTGTGTTTGTGAACACA 1944
gi|641721271|ref|XM_008152116.      AACTTCCTTACATCTGACCCAGAATGACTTTGCTTGTGTTTGTGAACACA 1756
gi|588480441|ref|NM_001290172.      AACTTCCTTAAATCTCACTGGGAATGACTTTGCTTGCATTTGTGAACACC 1875
                                    ********** * ** *    **** **********  ***********


gi|558135472|ref|XM_006091085.      TGCGCTTCCTGCAGTGGGTCCAGGATCACAGGCACATCTTGGTGGGAGCT 1994
gi|554578862|ref|XM_005880935.      TGCGCTTCCTGCAGTGGGTCCAGGATCACAGGCGCATCTTGGTGGGAGCT 1994
gi|584056807|ref|XM_006772885.      TGCGTTTCCTGCAGTGGGTCCAAGATCACAGGCGCATCTTGGTGGGCGCT 1994
gi|641721271|ref|XM_008152116.      TGCGTTTCCTGCAGTGGGTCCAGGACCACAGGAGCATCTTGGTGGGAGCT 1806
gi|588480441|ref|NM_001290172.      AGAGTTTTCTGCAGTGGGTCAAGGACCACAGGCACCTCTTGGTGGGAGTT 1925
                                     * * ** ************ * ** ******  * ********** * *


gi|558135472|ref|XM_006091085.      GAACACATGATGTGTGAGAAACCTTTAGCTATGCAGGGTGTGCCTGTGCT 2044
gi|554578862|ref|XM_005880935.      GAACACATGATGTGTGAGAAACCTTTAGCTATGCAGGGTGTGCCTGTGCT 2044
gi|584056807|ref|XM_006772885.      GAACACATGATGTGTGAGAAACCTTTAGCTATGCAGGGCGTGCCTGTGCT 2044
gi|641721271|ref|XM_008152116.      GAACACATGATGTGTAAGACACCTTTAGCTATGCAGGGTGTGCCTGTGCT 1856
gi|588480441|ref|NM_001290172.      ACACAAATGGTGTGTGTGAAACCTTTAGATATGCAGGGTGTGCCTGTACT 1975
                                      *** *** *****  ** ******** ********* ******** **


gi|558135472|ref|XM_006091085.      CAGTTTTAGAAATGCCACCTGCCAGATGAGCAAAACTATCATTAGTGTGT 2094
gi|554578862|ref|XM_005880935.      CAGTTTTAGAAATGCCACCTGCCAGATGAGCAAAACTATCATTAGTGTGT 2094
gi|584056807|ref|XM_006772885.      CAGTTTTAGAAATGCCACCTGCCAGATGAGCAAAACTATCATTAGTGCGT 2094
gi|641721271|ref|XM_008152116.      CAGTTTTAGAAACACCACCTGCCAGATGAACAAAACTGTCATTAGTGTGT 1906
gi|588480441|ref|NM_001290172.      CAGTTTTAGAAATGCCACCTGTCCGATGAGCAAGACTGTCATTAGTGTGT 2025
                                    ************  ******* * ***** *** *** ********* **


gi|558135472|ref|XM_006091085.      CAGTTCTCTCAGTACTCGTGGTATCTGTAGCCGCAGTTCTGGTCTACAAG 2144
gi|554578862|ref|XM_005880935.      CAGTTCTCTCAGTACTCGTGGTATCTGTAGCCGCAGTTCTGGTCTACAAG 2144
gi|584056807|ref|XM_006772885.      CAGTTCTCTCAGTACTCGTGGTCTCTGTAGCCGTAGTTCTGGTCTACAAG 2144
gi|641721271|ref|XM_008152116.      CCGTTCTCTCAGTACTCGTGGTATCTGTGGCTGCAGTTCTGGTCTACAAG 1956
gi|588480441|ref|NM_001290172.      CGGTTCTCACTGTGCTTGTGGTATCTGTGGTAGCAGTTCTGGTTTATAAG 2075
                                    * ****** * ** ** ***** ***** *  * ********* ** ***


gi|558135472|ref|XM_006091085.      TTCTATTTCCACCTGATGCTTCTGGCTGGCTGCAAAAAGTATGGCAAAGG 2194
gi|554578862|ref|XM_005880935.      TTCTACTTCCACCTGATGCTTCTGGCTGGCTGCAAAAAGTATGGCAAAGG 2194
gi|584056807|ref|XM_006772885.      TTCTATTTCCACCTGATGCTTCTGGCTGGCTGCAAAAAATATGGCAAAGG 2194
gi|641721271|ref|XM_008152116.      TTCTATTTCCACCTGATGCTTCTGGCTGGCTGCAAAAGGTACAGCAAAGG 2006
gi|588480441|ref|NM_001290172.      TTCTATTTCCACCTGATGCTTCTTGCTGGCTGCAAAAAGTATGGCAGAGG 2125
                                    ***** ***************** *************  **  *** ***


gi|558135472|ref|XM_006091085.      GGAAAGCACCTACGATGCCTTTGTCATCTACTCCAGCCATGATGAGGACT 2244
gi|554578862|ref|XM_005880935.      GGAAAGCACCTACGATGCCTTTGTCATCTACTCCAGCCATGATGAGGACT 2244
gi|584056807|ref|XM_006772885.      GGAAAGCACCTACGATGCCTTTGTCATCTACTCCAGCCATGATGAGGACT 2244
gi|641721271|ref|XM_008152116.      GGACAGCACTTATGATGCCTTTGTCATCTACTCCAGCCACGATGAGGACT 2056
gi|588480441|ref|NM_001290172.      TGAAAGCACCTATGATGCCTTTGTTATTTACTCAAGCCAGGATGAGGACT 2175
                                     ** ***** ** ************ ** ***** ***** **********
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gi|558135472|ref|XM_006091085.      GGGTGAGGAATGAGTTGGTGAAGAACTTGGAGGAGGGAGTCCCCCCCTTT 2294
gi|554578862|ref|XM_005880935.      GGGTGAGGAATGAGTTGGTGAAGAACTTGGAGGAGGGGGTCCCCCCCTTT 2294
gi|584056807|ref|XM_006772885.      GGGTGAGGAATGAGTTGGTGAAGAACTTGGAGGAGGGGGTACCCCCTTTT 2294
gi|641721271|ref|XM_008152116.      GGGTGAGGAATGAGTTGGTAAAGAACTTGGAGGAGGGGGTACCCCCCTTT 2106
gi|588480441|ref|NM_001290172.      GGGTGAGGAATGAGTTGGTAAAGAACTTGGAGGAGGGGGTGCCCCCCTTT 2225
                                    ****************** ********** ** ***** ***

gi|558135472|ref|XM_006091085.      CAGCTCTGCCTTCACTACAGAGACTTTATCCCTGGTGTGGCCATTGCTGC 2344
gi|554578862|ref|XM_005880935.      CAGCTCTGCCTTCACTACAGAGACTTTATCCCTGGCGTGGCCATTGCTGC 2344
gi|584056807|ref|XM_006772885.      CAGCTCTGCCTTCACTACAGAGACTTTATCCCTGGCGTGGCCATTGCTGC 2344
gi|641721271|ref|XM_008152116.      CAGCTCTGCCTTCACTACAGAGACTTTATCCCTGGCGTGGCCATTGCTGC 2156
gi|588480441|ref|NM_001290172.      CAGCTCTGCCTTCACTACAGAGACTTTATTCCTGGTGTGGCCATTGCTGC 2275
                                    **************************** ***** **************
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TLR4R

Figure4.1: Clustal W sequence alignment of bat *TLR4* sequences: gi|558135472| *TLR4* of *Myotis lucifugus* mRNA, gi|554578862| *TLR4* of *Myotis brandtii* mRNA, gi|584056807| *TLR4* of *Myotis davidii* mRNA, gi|588480441| *TLR4* of *Pteropus alecto* mRNA, gi|641721271| *TLR4* of *Eptesicus fuscus* mRNA. Note: The full sequence alignment can be obtained from the Appendix 1. Highly conserved regions utilised for primer design are highlighted with arrows. The introns are expected to be in the 5' and 3' ends based on *M. brandtii* annotated TLR4 sequence.

4.1.2 *TLR4* PCR:

The ability of the TLR4 PCR primers to amplify a PCR product of the expected size was assessed using pipistrelle spleen DNA samples as shown below (Figures 4.2 and 4.3).

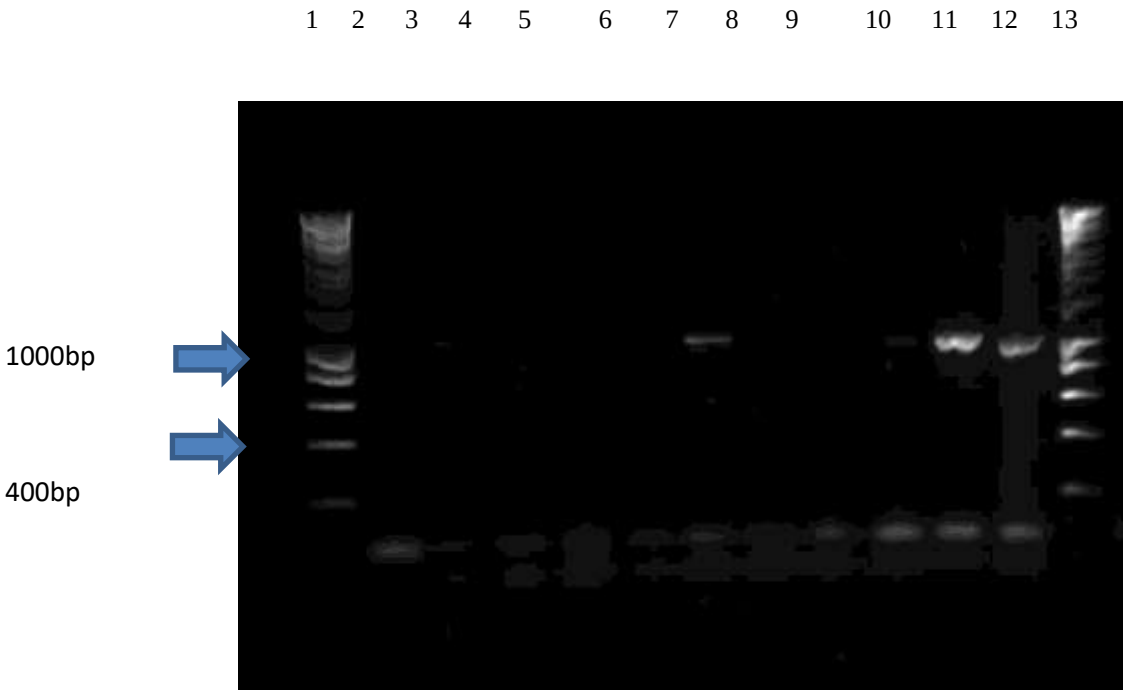

Figure 4.2: A representative agarose gel (1%) showing PCR products derived from *P. pipistrellus* using the TLR4 F/R primer combination. 1, 1kb hyperladder; 2, negative control (H_2O); 3-12, bat DNA samples (codes: S682, JH701, G704, J706, J711, F745, C801, F801, SP817, S846); 13, 1kb hyperladder. *Footnote:* primer annealing temperature was 54^{o}C and 2.5 µl Mg^{2+} was used in the PCR.

The TLR4 F/R primer combination generated a PCR product of the expected size (1014bp) for 59 of the bat specimens (Figure 4.2). In addition, the TLR4-2F/R primer combination also produced a PCR product of the expected size (1226bp) for 59 of the bat specimens (Figure 4.3).

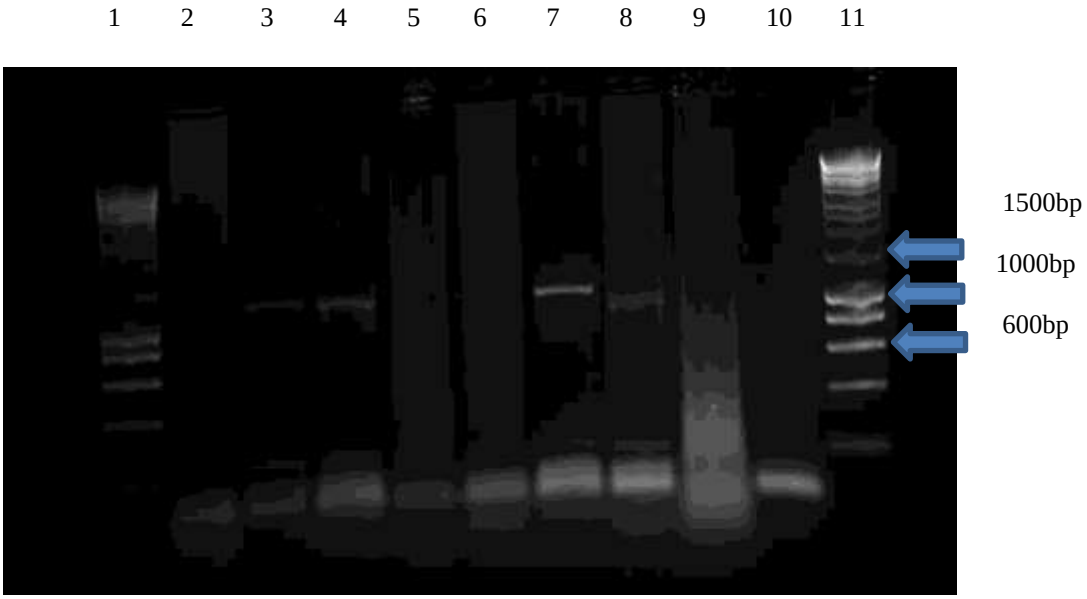

Figure 4.3: A representative agarose gel (1%) showing PCR products derived from *P. pipistrellus* using the TLR4-2F/R primer combination. 1, 1kb hyperladder; 2-9, bat DNA samples (codes: S682, JH701, F745, G704, C801, SP817, S846, F801); 10, negative control (H_2O); 11, 1kb hyperladder. *Footnote:* primer annealing temperature was 58^oC and 2.5 µl Mg^{2+} was used in the PCR.

In total, 59 of the bat specimens produced TLR4 PCR amplification products for each primer set and these were purified, sequenced and the resulting data was manually assembled into one contiguous TLR4 sequence for further analysis. If there is any PCR problem that was encountered with any of the products, the PCR will be repeated for a second time to solve the problem and to make sure a right product was amplified. One of the sequences, derived from the *P. pipistrellus* (individual S818) and a further sequence from *P. pygmaeus* (J649), were aligned and compared with TLR4 sequences from other bat species (Figure 4.4). The resulting data confirmed the PCR strategy for isolation of the pipistrelle TLR4 gene was successful and the *P. pipistrellus* and *P. pygmaeus* sequences were highly similar, being 98% identical to each other. With respect to TLR4 sequences from other bats, the BlastN analysis showed that the *P. pipistrellus* TLR4 sequence was more highly conserved than that of *P.*

pygmaeus and also, for both pipistrelles, the TLR4 gene sequences showed more homology, as expected, to the vespertilionids than to the fruit bat *P. alecto* (Tables 4.1 and 4.2). Repeated attempts were made to try and obtain TLR4 PCR products from the remaining 36 bats. However, despite purifying more DNA and also, changing the PCR conditions, it proved impossible to generate any further products that were visible by agarose gel electrophoresis.

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CLUSTAL O(1.2.4) multiple sequence alignment


S818              --------------------GAGTTTANCCCCNGGANNTTT---TCTGACTATNAGTTTA   37
J649              ------------------------------------------------------------   0
XM_008152116.1    TTGACAGGAAACCCTATCCAGAGTTTAGCCCCAGGAGCCTTTTCTGGACTACCAAGTTTA   381
XM_005880935.2    TTGACAGGAAACCCTATCCAGAGTTTAGCCCCGGGAGCCTTTTCTGGACTGCCAAGTTTA   600


S818              CAGNCCTGGGTGGC-TGGGGAGCCAACCTAGCATCTCTAGAGGACTTCCCCATGGCNGAC   96
J649              -------------G-TGTAAGANAAAATTANTGTCTCTTAGGGACTTCCCCATGGCAGAT   46
XM_008152116.1    CAGACACTGGTGGCTGTGGAGACAAACCTAGCCTCTCTAGAGGACTT--CCCCATCACAC   439
XM_005880935.2    CAGACACTGGTGGCTGTGGAGACAAACCTAGCATCGCTAGAGGACTT--CCCCATCAGAC   658
                                       **  **   ** **   ******  **    *  *


S818              ATCTGTAATCCTTGAAGGAGCTTAATGTGGCTCACAATCTAATCGATTCCTTCAAGTTAC   156
J649              ANNTTAATCCCTTGATAGAGCTTAATGTGGCTCACAATCTAATCGATTCCTTCAAGTTAC   106
XM_008152116.1    ATCTGAAATCCTTGAAGGAGCTTAATGTGGCTCACAATCTAATCGATTCCTTCAAGTTAC   499
XM_005880935.2    ATCTGAAAACCTTGAAGGAGCTTAATGTGGCTCACAATCTAATTGATTCCTTCAAGTTAC   718
                  *  *  *  ******  ************************* ****************


S818              CGGACTATTTTTCTAACCTGCCTAACCTGGAACACTTGGACCTTTCTAATAATAAGATTC   216
J649              CGGACTATTTTTCTAACCTGCCTAACCTGGAACACTTGGACCTTTCTAATAATAAGATTC   166
XM_008152116.1    CGAACTATTTTTCTAACCTGCCTAACCTGGAGCACTTGGACCTTTCCAATAATAAGATTC   559
XM_005880935.2    CGGACTATTTTTCTAACCTGCCTAACCTGGAGCACTTGGATCTTTCCAATAACAAGATCC   778
                  ** ***************************** ******** ***** ***** ***** *


S818              GAAAAATTTACCATGAAGACTTGCAGGTTTTACATCAAATGCCCTCATTCAAACTCTCTT   276
J649              GAAAAATTTACCATGAAGACTTGCAGGTTTTACATCAAATGCCCTCATTCAAACTCTCTT   226
XM_008152116.1    GAAATATTTACCATGAAGACTTGCAGGTTTTACATCAAATGCCCTCATTCAAACTCTCTT   619
XM_005880935.2    GCAATATTTACCATGAAGACTTGCAGGTTTTACATCAAATGCCTTCATTCAAACTCTCCT   838
                  * ** ************************************ ************** *


S818              TAGACCTGTCCCTCAACCCTTTAGACTTTATCCAACCAGGTGCCTTTGAAAAAATTAAGC   336
J649              TAGACCTGTCCCTCAACCCTTTAGACTTTATCCAACCAGGTGCCTTTGAAAAAATTAAGC   286
XM_008152116.1    TAGACCTGTCCCTCAACCCTTTAGACTTTATCCAACCAGGTGCCTTTGAAAAAATTAAGC   679
XM_005880935.2    TAGACCTGTCCCTCAACCCTTTAGACTTTATTCAACCAGGTGCCTTTGAAAAAATTAAGC   898
                  ****************************** ****************************


S818              TCCATGAACTGACTTTGAGAAGTAATTTTGATAGTAAAAAGGTCATGAAAACATGTATTC   396
J649              TCCATGAACTGACTTTGAGAAGTAATTTTGATAGTAAAAAGGTCATGAAAACATGTATTC   346
XM_008152116.1    TCCATGAACTGACTTTGAGAAGTAATTTTGATAGTGCAGAGGTCATGAAAACGTTTATTC   739
XM_005880935.2    TCCATGAACTGACTTTGAGAAGTAATTTTGATAGTCCAGAGGTCATGAAAATGTGTATTC   958
                  ********************************  * ************  * *****


S818              AAGGTCTGGCAGGTTTAAAGATCAATCGGCTGATTCTAGGAGAATTTAAAAATGAAAGGA   456
J649              AAGGTCTGGCAGGTTTAAAGATCAATCGGCTGATTCTAGGAGAATTTAAAAATGAAAGGA   406
XM_008152116.1    AAGGTCTGGCAGGTTTAAAGATCAAACGGCTGATTCTGGGAGAATTTAAAAATGAAAGGA   799
XM_005880935.2    AAGGTCTGGCTGGTTTAAAGATCAATCGGTTGATTCTGGGAGAATTTAAAAATGAAAGAA   1018
                  ********** ************** *** ******* ******************* *
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S818              ACTTAGTAGACTTGGACAAATCTGCCCTGGAGGAACTGTGCAACTTGACCATTGATGAAT   516
J649              ACTTAGTAGACTTGGACAAATCTGCCCTGGAGGAACTGTGCAACTTGACCATTGATGAAT   466
XM_008152116.1    TCTTAGTAAACTTGGACAAATCTGCCCTGGAGGAACTGTGTAATTTGACCATTGAAGAAT   859
XM_005880935.2    ACTTAGTAAACTTCAACAAATCTGCCCTGGAGGGTCTGTGCAATTTGACCATTGAAGAAT   1078
                   ******* ****  ******************  ***** ** ********** ****

S818              TCCGGATAGCACACTTCCAAGACTTTCCAGAGGATTGCCGTGGCTTTTTAAATTGTCTGG   576
J649              TCCGGATAGCACACTTCCAAGACTTTCCAGAGGATTGCCGTGGCTTTTTAAATTGTCTGG   526
XM_008152116.1    TCCGGATAGCACACTTCCAAGACTTTCCAGAGGATTACCTTGGCTTTTTAAATTGTTTGG   919
XM_005880935.2    TCCGGATAGCACACTTCGATGAGTTTCCAGGGGATGATCTTGGCTTTTTAAATTGTTTGG   1138
                  ***************** * ** ******* ****   * ***************** ***

S818              CAGATGCTTCTGCAGTATCTCTGATGAGTCTGAAAATAGGCAGGCTAGAAAGCCTTCCAA   636
J649              CAGATGCTTCTGCAGTATCTCTGATGAGTCTGAAAATAGGCAGGCTAGAAAGCCTTCCAA   586
XM_008152116.1    CAGATGCTTCTGCAATATCTCTGGTGAGTCTGAATATAGACAGGCTAGAAAGCCTTCCAA   979
XM_005880935.2    CAGATGCTTCTACAATATCTCTTGTGAGTCTATATTTAGATGAGCTAAAAATCTTTCCAA   1198
                  *********** ** *******  *******  *  ***    **** *** * ******

S818              CAGGTTTCAAATGGCAGTACTTAAAATTGTCTAATTGTAAATTTCAAGATTTCCCTACAT   696
J649              CAGGTTTCAAATGGCAGTACTTAAAATTGTCTAATTGTAAATTTCAAGATTTCCCTACAT   646
XM_008152116.1    AAGGTTTCAAATGGCAATACTTAAACTTGACTAATTGTAAATTTGAACATTTTCCTACAT   1039
XM_005880935.2    AAGGTTTCAAATGGCAATACTTAAATTTGTCTAAATGTATATTTGAACATTTTCCTACAT   1258
                   *************** ******** *** **** **** **** ** **** *******

S818              TGGAGCTTACCTTTCTCAAGCAATTTATTTTCACTGCCAACAAAGTTATTAACCACTTTT   756
J649              TGGAGCTTACCTTTCTCAAGCAATTTATTTTCACTGCCAACAAAGTTATTAACCACTTTT   706
XM_008152116.1    TGGAGCTTACCTTTCTCAAGCAGTTTGTTTTCACTGACAACAAAGGTATTACCACT--TT   1097
XM_005880935.2    TGGAGCTTACCTTTCTCAAGCAGTTTGTTTTCACTGCCAACAAAGGTATTACCACT--TT   1316
                  ********************** *** ********* ******** ***** *     **

S818              AACTAAACTTAATCTAAGAAACCTTGAGTTTCTAGATCTCAGTAGAAAATGGCTTGAGTT   816
J649              AACTAAACTTAATCTAAGAAACCTTGAGTTTCTAGATCTCAGTAGAAAATGGCTTGAGTT   766
XM_008152116.1    TACTGAAGTTAATCTAAGAAACCTTGAGTTTCTAGATCTCAGTAGT-AATGGCTTGAGTT   1156
XM_005880935.2    TACTGAAGTTAATCTACCAAACCTTGAGTTTCTAGATCTCAGTAGA-AATGGCTTGAGTT   1375
                   *** ** ********  ***************************  *************

S818              TCAAGTCTTGCTGCTCTGACCGTGATTTTGGGACAACCCGACTGAAACACTTAGATCTGA   876
J649              TCAAGTCTTGCTGCTCTGACCGTGATTTTGGGACAACCCGACTGAAACACTTAGATCTGA   826
XM_008152116.1    TCAAGTCTTGCTGCTCTCACCGTGATTTTGGGACAACCCAACTGAAACACTTAAATCTGA   1216
XM_005880935.2    TCAAGTCCTGCTGCTCTCACCGTGATTTTGGGACAACCCGACTGAAACACTTAGATCTGA   1435
                  ******* ********* ********************** ************* ******

S818              GCTTCAATAGTATTATTACCAATGACTTCAAACTTTCGTGGGCTTAGAGCAAAATAGAAC   936
J649              GCTTCAATAGTATTAATACCAATGACTTCAAACTTTCATGGGCTTAAGAGCAAATAGAAC   886
XM_008152116.1    GCTTCAATAGTATTATTACCATGACTTCAAACTT---CGTGGGCTTAGAGCAACTAGAAC   1273
XM_005880935.2    GCTTCAATAATATTATTATCATGACTTCAAACTT---CTTGGGCTTAGAGCAACTAGAAC   1492
                  ********* ***** ** **     *  **  *      **  *      ** ******

S818              ATCTGGATTTCCAGCATTCCACTTTGAGACAGGCCAGTACTTTTTCAGTATTCCTCTCAC   996
J649              ATCTGGATTTCCAGCATTCCACTTTGAGACAGGCCAGTACTTTTTCAGTATTCCTCTCAC   946
XM_008152116.1    GACTGGATTTCCAGCATTCCACTTTGAAACAGGCCAGTACTTTTTCAATATTCCTCTCAC   1333
XM_005880935.2    ATCTGGATTTCCAGCATTCCACTCTGAAACAGGCCAGTGATTTTTCAGTATTCCTCTCAC   1552
                    ********************** *** **********  ******* ************

S818              TCAAAAACCTCCTTTACCTTGATATCTCTTACACTGACATCAAGATTGTCTTCCAGGGCA   1056
J649              TCGAAAACCTCCTTTACCTTGATATCTCTTACACTGACATCAAATTTGTCTTCCAGGGCA   1006
XM_008152116.1    TCAAAAACCTCCTTTACCTTGATATCTCTTACACTAACATCCAGATTGTCTTCAAGGGCA   1393
XM_005880935.2    TCAAAAATCTCCTTTACCTTGATATCTCTTACACTAACACCAAGATTGTCTTCCTGGGCA   1612
                  ** **** ************************** *** * *  ********  *****

S818              TCTTTGATGGCTTGATCAGCCTCCAAGTCTTAAAAATTGGCTGGCAATTCCTTTCCAGGA   1116
J649              TCTTTGATGGCTTGATCAGCCTCCAAGTCTTAAAAATGGCTGGCAATTCCTTTTCAGGAT   1066
XM_008152116.1    TCTTTGATGGCTTGATCAGCCTCCAAGTCTTGAAAATGGCTGGCA-ATTCCTTTCAGGAT   1452
XM_005880935.2    TCTTTGATGGCTTGATCAGCCTCCAAGTCTTGAAAATGGCTGGCA-ATTCTTTTCAGGAT   1671
                  ****************************** ***** *   *    * * ****  *
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S818              TGCATTCCTCCAAATATTTTCAGAGATCTGACTCAGTTGACTGTCCTGGACCTCTCTCAG   1176
J649              GCATTCCTTCCAAATATTTTCAGAGATCTGACTCAGTTGACTGTCCTGGACCTCTCTCAG   1126
XM_008152116.1    GCATTCCTTCCAAATGTCTTCAGAGATCTGACTCAGTTGACTATCCTGGACCTCTCTCAG   1512
XM_005880935.2    GCACTCCTCCCAAATATCTTCAGAGATCTGACTCAGTTGACTGACCTGGACCTCTCTCAG   1731
                      * *  ****** * ************************  ****************

S818              TGTCAACTGGAACAGGTGTCCCCAGAGGCATTCGGCTCACTCCTTAGACTCCAGGTGCTA   1236
J649              TGTCAACTGGAACAGGTGTCCCCAGAGGCATTCGGCTCACTCCTTAGACTCCAGGTGCTA   1186
XM_008152116.1    TGTCAATTGGAACAGGTGTCTCCGGAGGCATTCAGCTCACTCCTTAGACTCGAGGTGCTA   1572
XM_005880935.2    TGTCAACTGGAACAGGTGTCCCAGGAGGCATTTGGCTCACTCCTTAGACTCCAGGTGCTA   1791
                  ****** ************* *  ********  ***************** ********

S818              AATATGAGTCACAACCACCTCTTGTCCTTGGATATGCTTCCTTATAAAAATCTCTCTCTC   1296
J649              AATATGAGTCACAACCACCTCTTGTCCTTGGATATGCTTCCTTATAAAAATCTCTCTCTC   1246
XM_008152116.1    AATATGAGTCACAACCACCTCTTGTCCTTGGATATGCTTCCTTACAAAAATCTCCCTCTC   1632
XM_005880935.2    AATATGAGTCACAATCACCTCTTGTCCTTGGATATGCTTCCTTATAAAAATCTCTCTCTC   1851
                  ************** ***************************** ********* *****

S818              TGGCTTCTAGACTACAGTTTTAACCGTATAGTGGCCGCCAATGGGCAGGAACTACAGCAT   1356
J649              TGGCTTCTAGACTACAGTTTTAACCGTATAGTGGCCGCCAATGGGCAGGAACTACAGCAT   1306
XM_008152116.1    TCGGTTCTAGACTGCAGTTTTAACCGTATAGTGGCCGCCAATGGGCAGGAACTACAGCAT   1692
XM_005880935.2    CGGGTTCTAGATTGCAGTTTTAACCGTATAGTGGCCGCCAATGGGCAGGAACTACAACAT   1911
                    * ******* * ****************************************** ***

S818              ATTCCAAGCAATGTAACTTCGTTAAATCTGACCCAGAATGACTTTGCTTGTGTTTGTGAA   1416
J649              ATTCCAAGCAATGTAACTTCGTTAAATCTGACCCAGAATGACTTTGCTTGTGTTTGTGAA   1366
XM_008152116.1    TTTCCAAGCAATGTAACTTCCTTACATCTGACCCAGAATGACTTTGCTTGTGTTTGTGAA   1752
XM_005880935.2    TTTCCAAGCAATGTAACTTCCTTAAATCTGACCCAGAATAACTTTGCTTGTGTTTGTGAA   1971
                   ******************* *** ************** ********************

S818              CACATGTGTTTCCTGCAGTGGGTCCAGGACCACAGGCGCATCTTGGTGGGAGCTGAACAC   1476
J649              CACATGCGTTTCTTGCAGTGGGTCCAGGACCACAGGCGCATCTTGGTGGGAGCTGAACAC   1426
XM_008152116.1    CACATGCGTTTCCTGCAGTGGGTCCAGGACCACAGGAGCATCTTGGTGGGAGCTGAACAC   1812
XM_005880935.2    CACATGCGCTTCCTGCAGTGGGTCCAGGATCACAGGCGCATCTTGGTGGGAGCTGAACAC   2031
                  ****** * *** ***************** ****** ************************

S818              ATGATGTGTAAGACACCGTTAGCTATGCAGGGTGTGCCTGTGCTCAGTTTTAGAAAACAC   1536
J649              ATGATGTGTAAGACACCGTTAGCTATGCAGGGTGTGCCTGTGCTCAGTTTTAGAAAACAC   1486
XM_008152116.1    ATGATGTGTAAGACACCTTTAGCTATGCAGGGTGTGCCTGTGCTCAGTTTTAGAA-ACAC   1871
XM_005880935.2    ATGATGTGTGAGAAACCTTTAGCTATGCAGGGTGTGCCTGTGCTCAGTTTTAGAA-ATGC   2090
                  ********* *** *** ************************************ *  *

S818              CACCTGCCAGATGAACAAAACTGTCATTAGTGTGTCAGTTCTCTCAGTACTCATAGTATC   1596
J649              CACCTGCCAGATGAACAAAACTGTCATTAGTGTGTCAGTTCTCTCAGTACTCATAGTATC   1546
XM_008152116.1    CACCTGCCAGATGAACAAAACTGTCATTAGTGTGTCCGTTCTCTCAGTACTCGTGGTATC   1931
XM_005880935.2    CACCTGCCAGATGAGCAAAACTATCATTAGTGTGTCAGTTCTCTCAGTACTCGTGGTATC   2150
                  ************** ******* ************* *************** * *****

S818              TGTGGCTGCAGTTCTGGTCTACAAGTTCTATTTCCACCTGATGCTTCTGGCTGGCTGCAG   1656
J649              TGTGGCTGCAGTTCTGGTCTACAAGTTCTATTTCCACCTGATGCTTCTGGCTGGCTGCAG   1606
XM_008152116.1    TGTGGCTGCAGTTCTGGTCTACAAGTTCTATTTCCACCTGATGCTTCTGGCTGGCTGCAA   1991
XM_005880935.2    TGTAGCCGCAGTTCTGGTCTACAAGTTCTACTTCCACCTGATGCTTCTGGCTGGCTGCAA   2210
                  *** ** ************************ ****************************

S818              AAAGGTACGGCAAAGGGGACAGCATGTACGATGCCTTTGTCATCTACTCCAAGCCATGAT   1716
J649              AAAGGTACGGCAAAGGGGACAGCATGTACGATGCCTTTGTCATCTACTCCAGCCCATGAT   1666
XM_008152116.1    AAGGT-ACAGCAAAGGGGACAGCACTTATGATGCCTTTGTCATCTACTCCAGCCACGATG   2050
XM_005880935.2    AAAGT-ATGGCAAAGGGGAAAGCACCTACGATGCCTTTGTCATCTACTCCAGCCATGATG   2269
                  ** *  *  ********** ****  ** **********************  *

S818              GAGGACTGGGTTGAGGAATGAGTTGGTGAAGAACTTGGAGGAGGGGGTNCCCCCCTTTTN   1776
J649              GAGGACTGGGGTGAGGAATGAGTTNGTGAANANNTTGGAGGAGGGGGTACCCCCCTTTCA   1726
XM_008152116.1    --AGGACTGGGTGAGGAATGAGTTGGTAAAGAACTTGGAGGAGGGGGTACCCCCCTTTCA   2108
XM_005880935.2    --AGGACTGGGTGAGGAATGAGTTGGTGAAGAACTTGGAGGAGGGGGTCCCCCCCTTTCA   2327
                    *    ** ************* ** ** *  ************** *********
```

```
S818                NNCTCTGCCTTCACTACA-----------------------------------------   1794
J649                GCTCNTGCCTTCACTACAANAAAACT---------------------------------   1752
XM_008152116.1      GCTCTGCCTT-CACTACAGAGACTTTATCCCTGGCGTGGCCATTGCTGCCAACATCATCC   2167
XM_005880935.2      GCTCTGCCTT-CACTACAGAGACTTTATCCCTGGCGTGGCCATTGCTGCCAACATCATCC   2386
                          * * *******
```

Figure 4.4: Clustal W sequence alignment of *TLR4* PCR products derived from one *P. pipistrellus* bat (code: S818), one *P. pygmaeus* bat (code: J649) with *TLR4* gene sequences from *Eptesicus fuscus* (XM_008152116.1) and *Myotis brandtii* (XM_005880935.2).

Table 4.1: BlastN summary data for the *TLR4* gene product derived from *P. pipistrellus*.

Highly similar sequence	Max score	Total score	Query cover	E value	Iden	GenBank accession number
Eptesicus fuscus toll-like receptor 4 (TLR4), mRNA	2680	2680	100%	0.0	94%	XM_008152116.1
Myotis brandtii toll-like receptor 4 (TLR4), mRNA	2453	2453	100%	0.0	91%	XM_005880935.2
Myotis lucifugus toll-like receptor 4 (TLR4), mRNA	2414	2414	100%	0.0	91%	XM_006091085.2
Myotis davidii toll like receptor 4 (TLR4), mRNA	2399	2399	100%	0.0	91%	XM_015569901.1
Pteropus alecto toll like receptor 4 (TLR4), mRNA	1504	1504	100%	0.0	82%	NM_001290172.1

Table 4.2: BlastN summary data for the *TLR4* gene product derived from *P. pygmaeus*.

Highly similar sequence	Max score	Total score	Query cover	E value	Iden	GenBank accession number
Eptesicus fuscus toll-like receptor 4 (TLR4), mRNA	2569	2569	100%	0.0	91%	XM_008152116.1
Myotis brandtii toll-like receptor 4 (TLR4), mRNA	2350	2350	100%	0.0	88%	XM_005880935.2
Myotis lucifugus toll-like receptor 4 (TLR4), mRNA	2316	2316	100%	0.0	88%	XM_006091085.2
Myotis davidii toll like receptor 4 (TLR4), mRNA	2294	2294	100%	0.0	88%	XM_015569901.1
Pteropus alecto toll like receptor 4 (TLR4), mRNA	1476	1479	100%	0.0	80%	NM_001290172.1

The translated nucleotide sequences of the pipistrelle TLR4 genes were aligned with TLR4 protein sequence from *Eptesicus fuscus* to reveal high levels of conservation (Figure 4.5). Indeed, the common and soprano pipistrelle TLR4 protein sequences were 95% identical to each other and the predicted N-glycosylation sites were conserved between the vespertilionids. As predicted based upon nucleotide conservation, the common pipistrelle TLR4 protein sequence was slightly more conserved relative to the TLR4 sequences of other bats than that of the soprano pipistrelle (Tables 4.3 and 4.4).

```
CLUSTAL O(1.2.4) multiple sequence alignment

J649                ------------------------------------------------------------   0
S818                ------------------------------------------------------------   0
XP_008150338.1      MRLTRLAGTLLPAMAFLSCLRPESWDPCVQVVPNVTYQCMELNLYTIPDNIPTTTKNLDL   60

J649                ------------------------------------------------------------   0
S818                ------------------------------------------------------EFXPXX   6
XP_008150338.1      SFNPLRHLGSHSFSNFSELQVLDLSRCEIQKIEDDAYQGLKHLSILILTGNPIQSLAPGA   120

J649                ------------VRXNXCLLGTSPWQIXKSLIELNVAHNLIDSFKLPDYFSNLPNLEHLD   48
S818                FSDY-XFTXLGGWGANLASLEDFPIXHLKSLKELNVAHNLIDSFKLPDYFSNLPNLEHLD   65
XP_008150338.1      FSGLPSLQTLVAVETNLASLEDFPITHLKSLKELNVAHNLIDSFKLPNYFSNLPNLEHLD   180
                                  * . *   *    *** ***************:************

J649                LSNNKIRKIYHEDLQVLHQMPSFKLSLDLSLNPLDFIQPGAFEKIKLHELTLRSNFDSKK   108
S818                LSNNKIRKIYHEDLQVLHQMPSFKLSLDLSLNPLDFIQPGAFEKIKLHELTLRSNFDSKK   125
XP_008150338.1      LSNNKIRNIYHEDLQVLHQMPSFKLSLDLSLNPLDFIQPGAFEKIKLHELTLRSNFDSAE   240
                    *******:************************************************ :

J649                VMKTCIQGLAGLKINRLILGEFKNERNLDDLDKSALEELCNLTIDEFRIAHFQDFPEDCR   168
S818                VMKTCIQGLAGLKINRLILGEFKNERNLDDLDKSALEELCNLTIDEFRIAHFQDFPEDCR   185
XP_008150338.1      VMKTFIQGLAGLKIKRLILGEFKNERILVNLDKSALEELCNLTIEEFRIAHFQDFPEDYL   300
                    **** *********:*********** * :**************:*************

J649                GFLNCLADASAVSLMSLKIGRLESLPTGFKWQYLKLSNCKFQDFPTLELTFLKQFIFTAN   228
S818                GFLNCLADASAVSLMSLKIGRLESLPTGFKWQYLKLSNCKFQDFPTLELTFLKQFIFTAN   245
XP_008150338.1      GFLNCLADASAISLVSLNIDRLESLPKGFKWQYLNLTNCKFEHFPTLELTFLKQFVFTDN   360
                    ***********:**:**:*.******.*******:*:****:.************:** *

J649                KVITTFTKLNLRNLEFLDLSRNGLSFKSCCSDRDFGTTRLKHLDLSFIVLHDMFKLSWAL   288
S818                KVITTFTKLNLRNLEFLDLSRNGLSFKSCCSDRDFGTTRLKHLDLSFNSIITMTSNFVGL   305
XP_008150338.1      KGITTFTEVNLRNLEFLDLSSNGLSFKSCCSHRDFGTTQLKHLNLSFNSIITMTSNFVGL   420
                    * *****::*********** **********.******:****:***  :  * .   .*

J649                ESKENIWISSIPLEDRPVLFQSSHSLKTSFTLISLTLTSRFVFQGIFDGLISLQVLKMAG   348
S818                EQIEHLDFQHSTLERQASTFSVFLSLKNLLYLDISYTDIKIVFQGIFDGLISLQVLKMAG   365
XP_008150338.1      EQLERLDFQHSTLK-QASTFSIFLSLKNLLYLDISYTNIQIVFKGIFDGLISLQVLKMAG   479
                    *. *.: :.   *: :   *.   ***. : *       ::**:****************
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J649                NSFQDAFLPNIFRDLTQLTVLDLSQCQLEQVSPEAFGSLLRLQVLNMSHNHLLSLDMLPY   408
S818                NSFQDAFLPNIFRDLTQLTVLDLSQCQLEQVSPEAFGSLLRLQVLNMSHNHLLSLDMLPY   425
XP_008150338.1      NSFQDAFLPNVFRDLTQLTILDLSQCQLEQVSPEAFSSLLRLEVLNMSHNHLLSLDMLPY   539
                    **********:********:***************.*****:*****************

J649                KNLSLWLLDYSFNRIVAANGQELQHIPSNVTSLNLTQNDFACVCEHMRFLQWVQDHRRIL   468
S818                KNLSLWLLDYSFNRIVAANGQELQHIPSNVTSLNLTQNDFACVCEHMCFLQWVQDHRRIL   485
XP_008150338.1      KNLPLSVLDCSFNRIVAANGQELQHFPSNVTSLHLTQNDFACVCEHMRFLQWVQDHRSIL   599
                    *** * :** ***************:*******:************* ********* **

J649                VGAEHMMCKTPLAMQGVPVLSFRNTTCQMNKTVISVSVLSVLIVSVAAVLVYKFYFHLML   528
S818                VGAEHMMCKTPLAMQGVPVLSFRNTTCQMNKTVISVSVLSVLIVSVAAVLVYKFYFHLML   545
XP_008150338.1      VGAEHMMCKTPLAMQGVPVLSFRNTTCQMNKTVISVSVLSVLVVSVAAVLVYKFYFHLML   659
                    ****************************************:*****************

J649                LAGCRKKVRQRGQHVRCLCHLLQPHGMNEDWGEEEVVENXGGGGTPLSAXAFTTXK----   584
S818                LAGCRRKVYGKGDSMYDAFVIYSSHGHDEDWVRNELVKNLEEGXPPFXXLPSL-------   598
XP_008150338.1      LAGCKR--YSKGDSTYDAFVIYSS--HDEDWVRNELVKNLEEGVPPFQLCLHYRDFIPGV   715
                    ****::    :*:       : .    :*** .:*:*:*   *  *:

J649                ------------------------------------------------------------   584
S818                ------------------------------------------------------------   598
XP_008150338.1      AIAANIIQEGFHKSRKVIVVVSQHFIQSRWCIFEYEIAQTWQFLSSHAGIIFIVLQKVEK   775


J649                ------------------------------------------------------------   584
S818                ------------------------------------------------------------   598
XP_008150338.1      SLLRQQVELYRLLSRNTYLEWEDSALGRHIFWRRLRKALLDGKPWSPEGTVDAEVSQDET   835


J649                ----        584
S818                ----        598
XP_008150338.1      MTSF        839
```

Figure 4.5: Clustal W sequence alignment of *TLR4* amino acid sequences from one *P. pipistrellus* bat (code: S818), one *P. pygmaeus* bat (code: J649) and *Eptesicus fuscus*: XP_008150338.1. Predicted N-glycosylation sites are shown in red font.

Table 4.3: BlastP summary data for the *TLR4* gene product derived from *P. pipistrellus*.

Highly similar sequence	Max score	Total score	Query cover	E value	Iden	GenBank accession number
toll-like receptor 4 [*Eptesicus fuscus*]	994	994	100%	0.0	89%	XP_008150338.1
toll-like receptor 4 [*Myotis brandtii*]	962	962	100%	0.0	87%	XP_005880997.1
toll-like receptor 4 [*Myotis davidii*]	952	952	100%	0.0	86%	XP_015425387.1
toll-like receptor 4 [*Myotis lucifugus*]	941	941	100%	0.0	85%	XP_006091147.1
toll-like receptor 4 precursor [*Pteropus alecto*]	809	809	100%	0.0	74%	NP_001277101.1

Table 4.4: BlastP summary data for the *TLR4* gene product derived from *P. pygmaeus*.

Highly similar sequence	Max score	Total score	Query cover	E value	Iden	GenBank accession number
toll-like receptor 4 [*Eptesicus fuscus*]	816	816	100%	0.0	86%	XP_008150338.1
toll-like receptor 4 [*Myotis brandtii*]	793	793	100%	0.0	84%	XP_005880997.1
toll-like receptor 4 [*Myotis davidii*]	784	784	100%	0.0	83%	XP_015425387.1
toll-like receptor 4 [*Myotis lucifugus*]	774	774	100%	0.0	82%	XP_006091147.1
toll-like receptor 4 precursor [*Pteropus alecto*]	650	650	100%	0.0	70%	NP_001277101.1

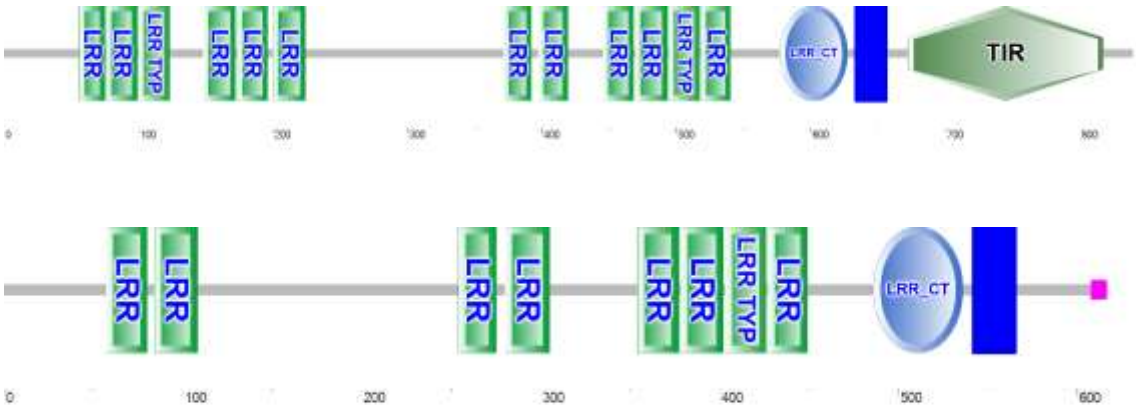

Figure 4.6: Domain structures of the *TLR4* protein from *E. fuscus* (above) and *P. pipistrellus* (code: S818) (below). The blue block represents the transmembrane domain and TIR is the cytoplasmic Toll/IL-IR domain. *Footnote*: *P. pygmaeus* (code: J649) has the same *TLR4* domain structure as shown for *P. pipistrellus*.

Comparing the domain models for the pipistrelle and *E. fuscus* TLR4 proteins revealed, as expected, based upon positioning of the PCR primers, that the pipistrelle protein lacked the cytoplasmic Toll/IL-IR domain and also, a number of the leucine rich repeats associated with the extracellular domain (Figure 4.6).

4.2 The pipistrelle *TLR2*:

An attempt was made to PCR amplify the gene encoding TLR2 in the pipistrelle bats. Oligonucleotide primers were designed using a similar strategy to that presented for the amplification of the pipistrelle TLR4 gene and this allowed some preliminary sequence data to be generated at the 5' end of the pipistrelle TLR2 gene by Arianne Lovey (MSc student, University of Salford). This preliminary sequence data was then used to design further overlapping oligonucleotide primers (TLR2Fn/TLR2Rn, TLR2.2F/TLR2.2R and TLR2gapF/TLR2gapR) (Figure 4.7) to allow the majority of the pipistrelle TLR2 gene to be PCR amplified (Figures 4.8-4.10).

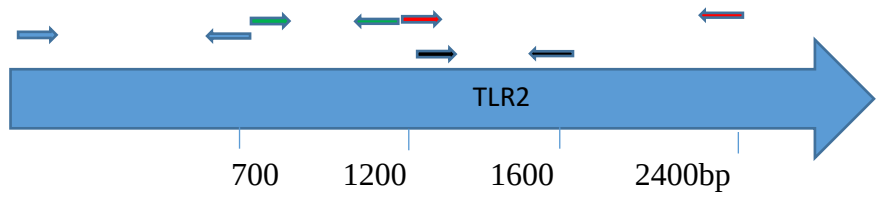

Figure 4.7: PCR primer combinations and binding sites used to amplify the pipistrelle *TLR2* gene. *Footnote*: primer combinations used to amplify products were as follows: 1 (TLR2F/ TLR2R), 2 (TLR2.2F/TLR2.2R), 3 (TLR2Fn/TLR2Rn) and 4 (TLR2gapF/TLR2gapR); 1-3 were designed by Arianne Lovey (MSc student, University of Salford). 1 = blue; 2 = red; 3 = green and 4 = black.

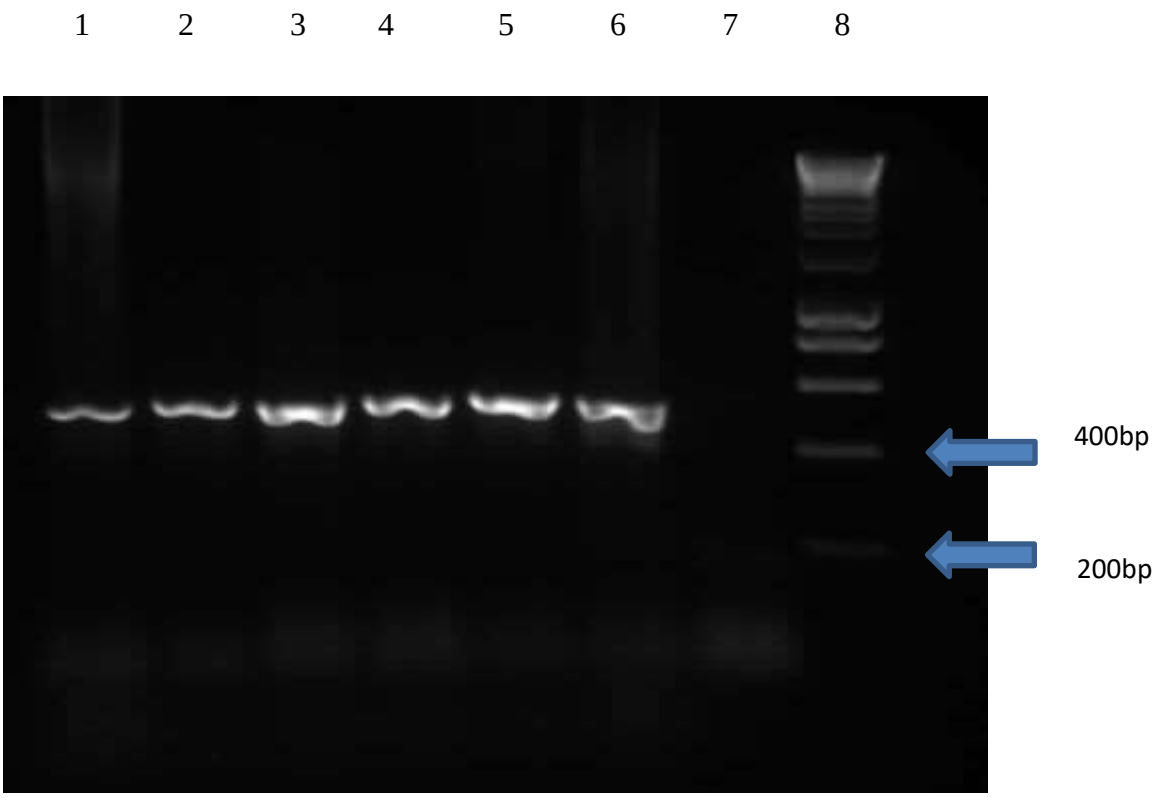

Figure 4.8: Representative agarose (1%) gel image showing PCR amplification of the *P. pipistrellus TLR2* gene fragment (500bp) derived from primers TLR2Fn/ TLR2Rn (combination 3 in Figure 4.7). 1-6, bat samples (bat codes: JL628, JL647, FP737, S607, J707, SA7?); 7, negative control (H_2O); 8, 1kb hyperladder.

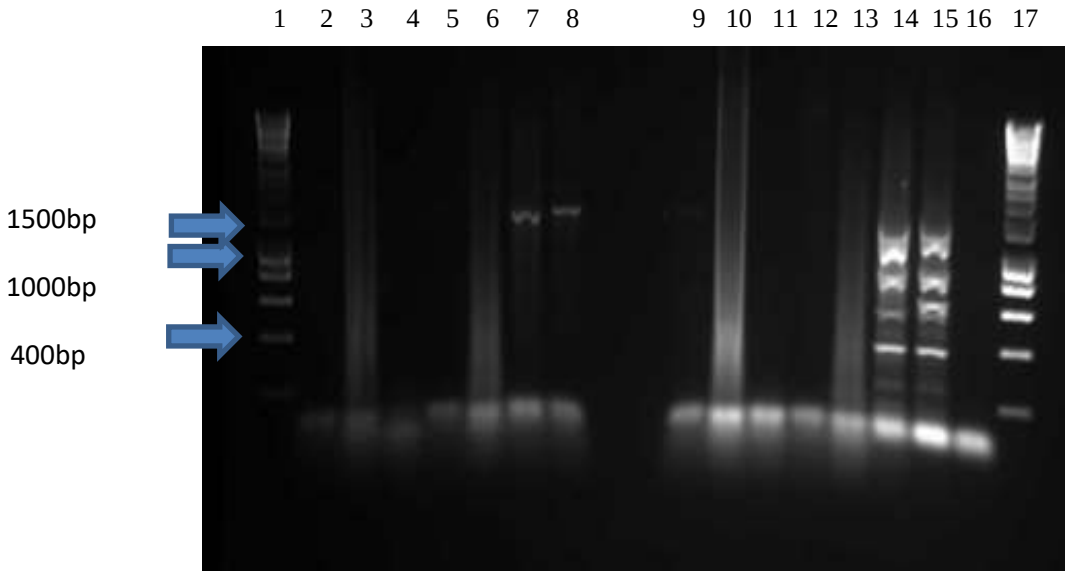

Figure 4.9: Representative agarose (1%) gel image showing PCR amplification of *P. pipistrellus TLR2* gene fragment (1200bp) derived from primers TLR2.2F/TLR2.2R (combination 2 in Figure 4.7). 1, 1kb hyperladder; 2-15, bat samples; 16, negative control (H_2O); 17, 1kb hyperladder. Positive samples are shown in lanes 7, 8 and 9 (corresponding to bats JL628, JL647, FP737 respectively). *Footnote*: 24 PCR TLR2.2F/TLR2.2R products were generated in this thesis work and 35 were produced by Arianne Lovey (MSc student, University of Salford).

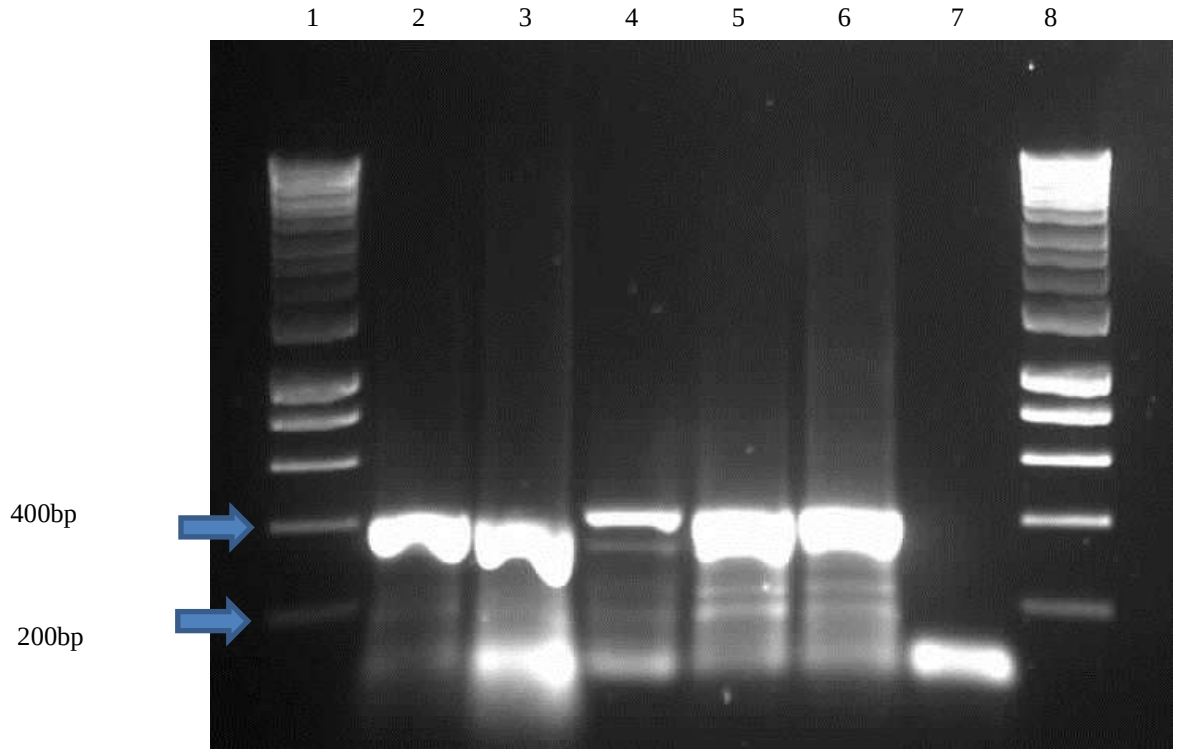

Figure 4.10: Representative agarose (1%) gel image showing PCR amplification of *P. pipistrellus TLR2* gene fragments (400bp) derived from primers TLR2gapF/TLR2gapR (combination 4 in Figure 4.7). 1, 1kb hyperladder; 2-6, bat samples (codes: Jl628, JL647, FP737, SA607, J707); 7, negative control (H_2O); 8, 1kb hyperladder.

In total, PCR products were successfully amplified from 59 bats for each primer combination. All PCR products were purified, DNA sequencing was performed and the resulting data was manually assembled into one contiguous TLR2 sequence, based upon the overlapping regions, for further analysis. The TLR2 gene sequence from one *P. pipistrellus* (code: S818) and one *P. pygmaeus* (code: J649) were aligned with other vespertilionid bat TLR2 gene sequences and the resulting data confirmed that the pipistrelle TLR2 gene isolation strategy had been successful. Indeed, the common and soprano TLR2 gene sequences were almost identical to each other; 2 nucleotides differences were apparent at positions 1620 and 1653 of

the pipistrelle sequences, and they were highly conserved to the TLR2 gene sequences from other bats (Figure 4.11 and Table 4.5).

```
CLUSTAL O(1.2.4) multiple sequence alignment


S818              ------------------------------------------------------------      0
J649              ------------------------------------------------------------      0
XM_008144148.1    ------------------------------------------------------------      0
XM_014543903.1    AACATTCAGTAATGAAATAAAGGTATAAGGATTAGTAAAGGGAAATAAAACCATCACCTA      60


S818              ------------------------------------------------------------      0
J649              ------------------------------------------------------------      0
XM_008144148.1    ------------------------------------------------------------      0
XM_014543903.1    GCTGCTGATATGATTGTATACATAGGAAAATGTTTTTTTTTAAACCTACAAACTATGGAA      120


S818              ------------------------------------------------------------      0
J649              ------------------------------------------------------------      0
XM_008144148.1    ------------------------------------------------------------      0
XM_014543903.1    TTACTAAGTTAGTTCAGCAAGGTTGCTCAATGCATGGTAAATGTATACAATATGCTCTCA      180


S818              ------------------------------------------------------------      0
J649              ------------------------------------------------------------      0
XM_008144148.1    -------------------------------------------TCACGGGACGATGCCACA      18
XM_014543903.1    ACCACAATCACTCACTTGAGCCTCTTTTATTTGTAGGTTGAATCACGGGACCATGCCACA      240


S818              ------------------------TGGGGACCGTAATCAGCCTGTTCAAGGAAGGGGCCCN      37
J649              ------------------------TGGGGACCGTAATCAGCCTGTTCAAGGAAGGGGCCCN      37
XM_008144148.1    TGCTTTGTGGACAGCGTGGGTCTTGGGGAGCGTAATCAGCCTGTTCGAGGAAGGGGCCCC      78
XM_014543903.1    TGCTTTGTGGACACTGTGGGTCTTGGGGACCGTCATCAGCCTGTTCAAGGAAGGGGCCCC      300
                                        ****** *** ************ ************


S818              TGATCAGGCTT--TTC-CTCTGACTTGTGACCCCACGGGGGTCTGCGATGGCCACTCCAG      94
J649              TGATCAGGCTT--TTC-CTCTGACTTGTGACCCCACGGGGGTCTGCGATGGCCACTCCAG      94
XM_008144148.1    TGATCAGGCT---TCTCCTCTGACTTGTGACCCCACTGGGGTCTGCGATGGCCACGCCAG      135
XM_014543903.1    TGATCAGGCT---TCTTCTCTGACTTGTGACCCCACTGGGATCTGCGATGGCCACTCCAG      357
                  ********** ***   ****************** *** ************** ****


S818              ATCTTTAATCTCCATCCCCTCAGGGCTCACGGCAACTGTGACGAGCCTCGACCTGTCCAA      154
J649              ATCTTTAATCTCCATCCCCTCAGGGCTCACGGCAACTGTGACGAGCCTCGACCTGTCCAA      154
XM_008144148.1    ATCTTTAATCTCCATCCCCTCCGGGCTCATGGCAACTGTGAAGAGCCTCGACCTGTCCAA      195
XM_014543903.1    ATCTTTAATCTCCATCCCCTCAGGGCTCATGGCAACTGTAAAGAGCCTCGACCTGTCCAA      417
                  ********************* ******* ********* * ******************


S818              CAACAAGATCGCCTATGTCAGCAACAGCGACCTGCGGATGTGTGTGAACCTCAGGGCTCT      214
J649              CAACAAGATCGCCTATGTCAGCAACAGCGACCTGCGGATGTGTGTGAACCTCAGGGCTCT      214
XM_008144148.1    CAACAAGATCGCCTACGTCAGCAACAGTGACCTGCGGATGTGTGTGAACCTCAAGGCTCT      255
XM_014543903.1    CAACAAGATCACCTATGTCAGCAACAGCGACCTGCGGATGTGTGTGAACCTCAAGGCTCT      477
                  ********** **** *********** ************************ ******


S818              GAGGCTGGGATCCAATAGCATTGACACGATAGAGGAAGATTCCTTTTTCTCCCTGGGGAG      274
J649              GAGGCTGGGATCCAATAGCATTGACACGATAGAGGAAGATTCCTTTTTCTCCCTGGGGAG      274
XM_008144148.1    GAGGCTGGGATCCAATAGCATTGACACGATAGAGGAAGATTCCTTTTTCTCCCTGGGGAG      315
XM_014543903.1    GAGGCTGGGATCCAATAACATTGACACGATAGAGGAAGATTCCTTTTTCTCCCTGGGGAG      537
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S818              TCTTGAACATTTGGACTTATCCTATAATCACTTAGCTAATTTATCAGCCTCCTGGTTCAG      334
J649              TCTTGAACATTTGGACTTATCCTATAATCACTTAGCTAATTTATCAGCCTCCTGGTTCAG      334
XM_008144148.1    TCTTGAACATTTGGACTTATCCTATAATCACTTATCTATTTTATCAGCCTCCTGGTTCAG      375
XM_014543903.1    TCTGGAACATTTGGACTTATCCTATAATCTCTTACCTAATTTATCAGCCTCCTGGTTCAG      597
                  *** ************************ **** *** ********************


S818              GCCTCTTACTTCCTTGAACGTCTTAAACTTATTGGGAAACCCTTACAAAACACTTGGGAA      394
J649              GCCTCTTACTTCCTTGAACGTCTTAAACTTATTGGGAAACCCTTACAAAACACTTGGGAA      394
XM_008144148.1    GCCCCTTACTTCCTTGAACTTCTTAAACTTACTGGGAAACCCTTACAAAACACTTGGGAA      435
XM_014543903.1    GCCCCTGACTTCCTTGAACTTCTTAAACTTACTGGGAAACCCTTACAAAACACTCGGGAA      657
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S818              AACACCTCTTTTTTCTCATCTCACCAAATTGCGAATCCTAAAAGTAGGACATAGTTACCT      454
J649              AACACCTCTTTTTTCTCATCTCACCAAATTGCGAATCCTAAAAGTAGGACATAGTTACCT      454
XM_008144148.1    AACATCTCTTTTTTCTCATCTCACCAAGCTGCGAATCCTAAAAGTAGGACATAGTTACCA      495
XM_014543903.1    AACATCTCTTTTTTCTCATCTCACCAACTTGCGAATCCTAAAAGTAGGACATAGTTACCA      717
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S818              CTTCACTGAAATTCAGGAAAAGGATTTTGTTGGGCTAACTTTTCTCAAAGAGCTTGAGAT      514
J649              CTTCACTGAAATTCAGGAAAAGGATTTTGTTGGGCTAACTTTTCTCAAAGAGCTTGAGAT      514
XM_008144148.1    CTTCACTGAAATTCAGGAAAAGGATTTTGTGGGGCTAACGTTTCTTAAGGAGCTTGAGAT      555
XM_014543903.1    CTTCACTGAAATTCAGGAAAAGGATTTTGTTGGGCTGACTGTTCTTAAGGAACTTGAGAT      777
                  ****************************** ***** **  **** ** ** ********


S818              CGATGCTTCCAATCTCCAGAAGTATGCGCCTAGGAGTTTGAAGGTGATTCAGAACATCAG      574
J649              CGATGCTTCCAATCTCCAGAAGTATGCGCCTAGGAGTTTGAAGGTGATTCAGAACATCAG      574
XM_008144148.1    TGATGCTTCCAATCTCCAGAAGTATGCGCCAAGGAGTTTGAAGTTGATTCAGAACATCAG      615
XM_014543903.1    TGATGCTTCCAATCTCCAGAAGTATGGGGCAAACAGTTTGAAGGTGATTCAGAACATCAG      837
                   ************************* * * *  ********* ****************


S818              CCACCTGATCCTTCACATGAAGCAGCCCACTTTCTTGATGAAGATTTCTGAGGATCTTTT      634
J649              CCACCTGATCCTTCACATGAAGCAGCCCACTTTCTTGATGAAGATTTCTGAGGATCTTTT      634
XM_008144148.1    CCACCTGATCCTTCGGGTGAAGCAGCCTACTTTCTTGCTGGAGATTTCTGTAGATCTTTT      675
XM_014543903.1    CCACCTGATCCTTCATATGAAGCAGCCTGCTTTCTTGCCGGAGATTTTTGTAGATCTTGT      897
                  **************   **********  ********  * ****** **  ****** *


S818              AAGTTCCTTGGGACATTTGGAACTGAGAGATACTCATTTGGACAATTTCCATTTTTCAAA      694
J649              AAGTTCCTTGGGACATTTGGAACTGAGAGATACTCATTTGGACAATTTCCATTTTTCAAA      694
XM_008144148.1    AAGTTCCTTGGGACATTTGGAACTGAGAGATACTCATTTGGACACTTTCCATTTTTCAAT      735
XM_014543903.1    AAGTTCCTTGGAACATTTGGAACTGAGAGATACTCGTTTGGGCACTTTCCGTTTTTCAAA      957
                  *********** ********************** ***** ** ***** ********


S818              AGTATCCACCAATGAAACCAAGACCATTAAAAAGTTCACCTTTAGAAATGTGAAGATCAC      754
J649              AGTATCCACCAATGAAACCAAGACCATTAAAAAGTTCACCTTTAGAAATGTGAAGATCAC      754
XM_008144148.1    AGTATCCACCAATGAAACCAAGACAATTAAAAAGTTCACCTTTAGAAATGTGAAAATCAC      795
XM_014543903.1    AGTATCCACCAATGAAACCAAGACAATTAAAAAGTTCACCTTTAGAAATGTGGAAATCAC      1017
                  ********************** ************************** * *****


S818              AGATGAAGGTTTTAATGAAATGGTGAAACTGTTGAATCATGTTTCTGAAATATTAGATGT      814
J649              AGATGAAGGTTTTAATGAAATGGTGAAACTGTTGAATCATGTTTCTGAAATATTAGATGT      814
XM_008144148.1    GGATGAAGGTTTTAACGAAATGGTGAAACTGCTGAATTATGTTTCTGAAATCGTAGATGT      855
XM_014543903.1    GGATGAAAGTTTTAATGAGATGGTGAGACTGTTGAATTATGGTTCTGACATATTAGATGT      1077
                   ****** ******* ** ******* **** ***** *** ****** **  *******


S818              GGAATTTGATAGCTGCACCCTCAATGGAATTGGTGATTTTGACATAACTGTTATGGACAC      874
J649              GGAATTTGATAGCTGCACCCTCAATGGAATTGGTGATTTTGACATAACTGTTATGGACAC      874
XM_008144148.1    GGAATTTGATAGCTGCACCCTCAATGGAATTGGTGATTTTGACACAACTGCTATGGACAC      915
XM_014543903.1    GGAGTTTGATAGCTGCACCCTTGATGGAGTTGGTGATTTTGACC---CTGCTATGGACAC      1134
                  *** *****************  ***** **************    *** *********


S818              AAATAAAGATATAAGTAAAATAGAGACATTAACAATACGGAGGTTGTATATTCCAAATTT      934
J649              AAATAAAGATATAAGTAAAATAGAGACATTAACAATACGGAGGTTGTATATTCCAAATTT      934
XM_008144148.1    AAATAAAGATATAAGTAAAATACAGACATTAACAATACGGAGGTTGTATATTCCATATTT      975
XM_014543903.1    AAATAAAGATGTCAGTAAAATAGAGACATTAACAATACGGAGGTTGTATATTCCACATTT      1194
                  ********** * ********* ********************************* ****


S818              TTACTCATTTTATGATCTGAGCAGTTTATATTCACTTACTGGAACAGTTAAGAGAATCAC      994
J649              TTACTCATTTTATGATCTGAGCAGTTTATATTCACTTACTGGAACAGTTAAGAGAATCAC      994
XM_008144148.1    TTACTTATTTTCTGATCTGAGCAGTTTATATTCACTTACTGGAACAGTTAAAAGAATCAC      1035
XM_014543903.1    TTACTCATTTTATGATCTGAGGAGTTTATATTCACTTACTGGAACAGTTAAAAGAATCAC      1254
                  ***** ***** ********* ****************************** ********


S818              GATAGAAAGCAGTAAGGTTTTCCTAGTTCCTTGTTCACTTTCGCAACACTTAAAATCATT      1054
J649              GATAGAAAGCAGTAAGGTTTTCCTAGTTCCTTGTTCACTTTCGCAACACTTAAAATCATT      1054
XM_008144148.1    AATAGAAAGCAGTAAGGTTTTCCTAGTTCCTTGTTCACTTTCGCAACACTTAAAATCATT      1095
XM_014543903.1    AATAGAAAACAGTAAGGTTTTCCTAGTTCCTTGTTTACTTTCGCAACACTTAAAATCATT      1314
                   ******* ************************* ************************


S818              AGAATATTTGGACCTCAATGGCAACTTAATAGTTGAAAACTCATTGACAAACGCAGCCTG      1114
J649              AGAATATTTGGACCTCAATGGCAACTTAATAGTTGAAAACTCATTGACAAACGCAGCCTG      1114
XM_008144148.1    AGAATATTTGGACCTCAGTGGCAACTTGATAGTTGAAAACTTATTGAAAAACGCAGCCTG      1155
XM_014543903.1    GGAATATTTGGACCTCAGTGGCAACTTAATAGTGGAAAACTCATTGAAAAACGCAGCCTG      1374
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S818              TGAGTATGCCTGGCCCTCCCTGCAAACCTTAATCTTGAGGCAGAATCATCTGAGGTCGTT      1174
J649              TGAGTATGCCTGGCCCTCCCTGCAAACCTTAATCTTGAGGCAGAATCATCTGAGGTCGTT      1174
XM_008144148.1    TGAGTATGCCTGGCCCTCCCTGCAAACCTTAATCTTAAGGCAGAATCATTTGAGGTCGTT      1215
XM_014543903.1    TGAGTATGCCTGGCCCTCCCTGCAAACCTTAATTTTAAGGCAGAATCATTTGAGATCGTT      1434
                  ********************************** ** ************ **** *****


S818              AGAAGAAACTGGAGAAGTTTTGCTTACTCTGAAAAACCTGACTAACCTTGATATCAGCAA      1234
J649              AGAAGAAACTGGAGAAGTTTTGCTTACTCTGAAAAACCTGACTAACCTTGATATCAGCAA      1234
XM_008144148.1    AGAAAAAACTGGAGAAATTTTGCTTACTCTGAAAAGTCTGACTAACCTTGATATCAGCAA      1275
XM_014543903.1    AGAACAAACCGGAGAAACTTTGCTTACTCTGAAAAGTCTGACTAACCTTGATATCAGTAA      1494
                  **** **** ******  *****************  ******************** **


S818              GAATAATTTCCATCCTATATCTAAAACTTGTCAGTGGCCAGAAAGGATGAAGTATTTGAA      1294
J649              GAATAATTTCCATCCTATATCTAAAACTTGTCAGTGGCCAGAAAGGATGAAGTATTTGAA      1294
XM_008144148.1    GAATAATTTCCACCCTATATCTAAAACTTGTCAGTGGCCAGAAAGGATGAAATGTTTGAA      1335
XM_014543903.1    GAATAATTTCCATCCTATATCTAAAACTTGTCAGTGGCCAGAAAAGATGACATGTTTGAA      1554
                  ************ ******************************** *****  * ******


S818              CTTATCCAATACAAGAATACAGAGTTTAACCAAATGCATTCCTCAGACGCTGGAAGTTTT      1354
J649              CTTATCCAATACAAGAATACAGAGTTTAACCAAATGCATTCCTCAGACGCTGGAAGTTTT      1354
XM_008144148.1    CTTATCCAATACAAGAATACAGAGTTTAACCAAATGCATTCCTCAGACACTGGAAGTTTT      1395
XM_014543903.1    CTTATCCAGTACAAGAATACAGAGTTTAACCAAATGCATTCCTCAGAAACTGGAAATTTT      1614
                  ******** ************************************  ****** ****


S818              AGATGTTAGCAATAATAGCCTCAGTTCGTTTTCGTTGACTATGCCACAACTCAGAGAACT      1414
J649              AGATGTTAGCAATAATAGCCTCAGTTCGTTTTCGTTGACTATGCCACAACTCAGAGAACT      1414
XM_008144148.1    GGATGTTAGCAATAACAGCCTCAGTTCCTTTTCGTTGACTCTGCCACAACTCAGAGAACT      1455
XM_014543903.1    AGATGTTAGCAACAACAGCCTCAGTTCGTTTTCGTTGACGATGCCACAACTCAGAGAACT      1674
                   *********** ** *********** ***********  ********************


S818              TTATATTTCCGGAAATAGGTTGAAGACTCTACCAGATGCCTCCTCCTTACCCATGTTACT      1474
J649              TTATATTTCCGGAAATAGGTTGAAGACTCTACCAGATGCCTCCTCCTTACCCATGTTACT      1474
XM_008144148.1    TTATATTTCCGGAAATAAGTTGAAGACTCTACCAGATGCCTCCTCCTTACCCATGTTACT      1515
XM_014543903.1    TTATATTTCCGGAAATAAGTTGAAGACTCTACCAGATGCTTCCTCCTTACCCATGTTACT      1734
                  ***************** ******************** ********************


S818              CGTCATGAGAATCAGCAGAAATACAATAAATACGTTCTCTAAGGAGCAACTTGATTCGTT      1534
J649              CGTCATGAGAATCAGCAGAAATACAATAAATACGTTCTCTAAGGAGCAACTTGATTCGTT      1534
XM_008144148.1    AATCATGAGAATCAGCAGAAATACAATAAATACTTTCTCCAAGGAGCAACTTGATTCTTT      1575
XM_014543903.1    AGTCATGAGAATCAGCAGAAATACAATAAATACTTTCTCTAAGGAGCAACTTGATTCTTT      1794
                    ******************************** ***** ***************** **
S818              TAAAAAACTGAAGACTTTGGAAGCTGGCAGCAACAGTTTCATCTGTTCCTGCGAATTCCT      1594
J649              TAAAAAACTGAAGACTTTGGAAGCTGGCAGCAACAGTTTCATCTGTTCCTGCGAATTCCT      1594
XM_008144148.1    TAAAACATTGAAGACTTTGGAAGCTGGCAGCAACAATTTCATCTGTTCCTGTGAATTCCT      1635
XM_014543903.1    TCAAAAACTGAAGACTTTGGAAGCTGGCAGCAACAATTTCATCTGTTCCTGTGAATTCCT      1854
                  * *** * ************************** *************** ********


S818              GTCCTTTACTCAGGGGCAGCAAGCACTGGCCCAAGTCCTGGTCGACTGGCCAGAAAACTA      1654
J649              GTCCTTTACTCAGGGGCAGCAAGCAATGGCCCAAGTCCTGGTCGACTGGCCAGAAAACAA      1654
XM_008144148.1    GTCCTTTACTCAGGGGCACCAAGCCCTGGCCCAAGTCCTGACCGACTGGCCAGAACACTA      1695
XM_014543903.1    GTCCTTCACTCAGGGGCAGCCAGCACTGGCCCAAGTCCTGATCGACTGGCCAGAAAACTA      1914
                  ****** *********** * ***  **************  ************* ** *


S818              CCTGTGCGATTCCCCATCCCATGTGCGGGGCCAGCGGGTGCAAGACACTCACCTCTCGGT      1714
J649              CCTGTGCGATTCCCCATCCCATGTGCGGGGCCAGCGGGTGCAAGACACTCACCTCTCGGT      1714
XM_008144148.1    CCTGTGTGATTCTCCATCCCATGTGCGGGGCCAGCGGGTGCGGGACACTCATCTCTCGGC      1755
XM_014543903.1    CCTGTGTGATTCTCCATCCCATGTGCGGGGCCAGCGGGTGCAGGACACTCATCTCTCGGC      1974
                  ****** ***** ***************************  ******** *******


S818              TTCTGAGTGCCACAGGGTGGCTGTGGTGTCTGCTGTGTGCTGTGCCCTTTTCCTGCTGAT      1774
J649              TTCTGAGTGCCACAGGGTGGCTGTGGTGTCTGCTGTGTGCTGTGCCCTTTTCCTGCTGAT      1774
XM_008144148.1    TTCTGAGTGCCACAGGGTGGCTGTGGTGTCTGCCGTATGCTGTGCCCTTTTCCTGCTGAT      1815
XM_014543903.1    TTCTGAGTGCCACAGGGTGGCTCTGGTGTCTGCCGTATGCTGTGCCCTTTTCCTGCTGAT      2034
                  ********************** ********** ** ************************


S818              CCTGCTCACTGGGGTTCTGTGCCACCGTTTCCATGGCCTGTGGTACATGAAGATGATGTG      1834
J649              CCTGCTCACTGGGGTTCTGTGCCACCGTTTCCATGGCCTGTGGTACATGAAGATGATGTG      1834
XM_008144148.1    CCTGCTCGCTGGGGTTCTGTGCCACCGTTTCCATGGCCTGTGGTACATGAAAATGATGTG      1875
XM_014543903.1    CCTGCTCGCTGGGGTTCTGTGCCACCGTTTCCATGGCCTGTGGTACATGAAAATGATGTG      2094
                  ******* ****************************************** ********


S818              GGCCTGGCTCCAGGCCAAAAGGAAGCCCAGGAGAGCCCCCCCGAGGGACCTCAGTTACGA      1894
J649              GGCCTGGCTCCAGGCCAAAAGGAAGCCCAGGAGAGCCCCCCCGAGGGACCTCAGTTACGA      1894
XM_008144148.1    GGCCTGGCTCCAGGCCAAAAGGAAGCCCAGGAGAGCCCCCCAGAGGGACCTCTGTTATGA      1935
XM_014543903.1    GGCCTGGCTTCAGGCCAAAAGGAAGCCCAAGCGAGCCCCCCAGAGGGACCTCTGTTATGA      2154
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S818                CGCCTTTGTGTCTTACAGCGAGCAGGATTCCCACTGGGTGGAGAACCTGATGGTCCAGGA      1954
J649                CGCCTTTGTGTCTTACAGCGAGCAGGATTCCCACTGGGTGGAGAACCTGATGGTCCAGGA      1954
XM_008144148.1      CGCCTTTGTGTCTTACAGTGAGCAGGACTCCCACTGGGTGGAGAACCTGATGGTCCAGGA      1995
XM_014543903.1      CGCCTTTGTGTCTTACAGTGAGCAGGATTCCCACTGGGTGGAGAACCTGATGGTCCAGGA      2214
                    ****************** ******** ********************************

S818                GCTGGAGCACTTCGACCCTCCCTTCAAGCTGTGTCTTCATAAGCGGGACTTTGTTCCCGG      2014
J649                GCTGGAGCACTTCGACCCTCCCTTCAAGCTGTGTCTTCATAAGCGGGACTTTGTTCCCGG      2014
XM_008144148.1      GCTGGAGCACTTCAACCCTCCCTTTAAGCTGTGTCTTCATAAGCGGGACTTCGTTCCTGG      2055
XM_014543903.1      GCTGGAGCACTTCAACCCTCCCTTTAAGCTGTGTCTTCATAAGCGGGACTTTGTTCCTGG      2274
                    ************* ********** ************************* ***** **

S818                CAAGTGGATTATTGACAATATCATCGACTCCATCGAAAAGAGCCACAAAACCATCTTCGT      2074
J649                CAAGTGGATTATTGACAATATCATCGACTCCATCGAAAAGAGCCACAAAACCATCTTCGT      2074
XM_008144148.1      CAAATGGATTATTGACAATATCATTGACTCCATCGAAAAGAGCCACAAAACCATCTTCGT      2115
XM_014543903.1      CAAATGGATTATTGACAACATCATTGACTCCATCGAAAAGAGCCACAAAACCATCTTCGT      2334
                    *** ************** ***** ************************************

S818                GCTTTCCGAGAACTCGTGAAGA----------------------------------------      2096
J649                GCTTTCCGAGAACTCGTGAAGA----------------------------------------      2096
XM_008144148.1      GCTTTCCGAGAACTTTGTGAAGAGCGAGTGGTGCAAGTACGAGCTGGACTTCTCTCATTT      2175
XM_014543903.1      GCTTTCCGAGAACTTTGTGAAGAGCGAGTGGTGCAAGTACGAACTGGACTTCTCCCATTT      2394
                    **************     *

S818                ------------------------------------------------------------      2096
J649                ------------------------------------------------------------      2096
XM_008144148.1      TCGCCTCTTTGATGAGAACGATGATGCTGCCATCCTCGTTCTGCTGGAGCCCCTGGAGAA      2235
XM_014543903.1      TCGCCTCTTTGATGAGAACAACGATGCCGCCATTCTCGTTCTGCTGGAGCCCCTGGAGAA      2454

S818                ------------------------------------------------------------      2096
J649                ------------------------------------------------------------      2096
XM_008144148.1      GAAGGCCATTCCCCAGCGTTTCTGTAAGCTGCGCAAGATCATGAACACCAAGACCTACCT      2295
XM_014543903.1      GAAGGCCATTCCCCAGCGTTTCTGTAAGCTGCGCAAGATCATGAACACCAAGACCTACCT      2514

S818                ------------------------------------------------------------      2096
J649                ------------------------------------------------------------      2096
XM_008144148.1      GGAGTGGCCCACTGATGAAACTCAGCAGGAGGGGTTCTGGTTCAATTTGAGAACTGCAAT      2355
XM_014543903.1      GGAGTGGCCCACTGATGAAACCCAGCAGGAGGGGTTCTGGTTAAATTTGAGAACTGCAAT      2574

S818                ------------------------------------------    2096
J649                ------------------------------------------    2096
XM_008144148.1      AAAGTCCTAAGTTCCTTCATTAAAGGCCAGTCTTGGACTG--    2395
XM_014543903.1      AAAGTCCTAAGATCCTTCATTAAAGGTCAGTCTTAGACTGGT    2616
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Figure 4.11: Clustal W alignment of the *TLR2* gene sequences derived from *P. pipistrellus* (code: S818) and *P. pygmaeus* (code: J649) with the *TLR2* mRNA sequences from *E. fuscus* (XM_008144148.1) and M. brandtii (XM_014543903.1). *Footnote*: Arianne Lovey (MSc student, University of Salford) was responsible for *all* the pipistrelle sequence data between 1-700 bp and also, 35 bat sequences between 1200-2096bp.

Table 4.5: BlastN summary data for the *TLR2* gene derived from *P. pipistrellus* (code: S818). *Footnote*: the BlastN data for the *P. pygmaeus* (code: J649) *TLR2* gene was identical to that shown below.

Highly similar sequence	Max score	Total score	Query cover	E value	Iden	Gen bank #
Eptesicus fuscus toll-like receptor 2 (TLR2), mRNA	3264	3264	100%	0.0	95%	XM_008144148.1
Myotis brandtii toll-like receptor 2 (TLR2), mRNA	3107	3107	100%	0.0	93%	XM_014543903.1
Myotis lucifugus toll-like receptor 2 (TLR2), mRNA	3079	3079	100%	0.0	93%	XM_014456089.1
Myotis davidii toll like receptor 2 (TLR2), mRNA	3007	3007	100%	0.0	93%	XM_006770106.2
Pteropus alecto toll like receptor 2 (TLR2), mRNA	2239	2239	100%	0.0	86%	XM_006906255.2

Not surprisingly given the above, the translated nucleotide sequences of the pipistrelle TLR2 genes were almost identical; the two nucleotide change resulted in an amino acid change at position 541 and 553 of the protein sequences (Figure 4.12). The pipistrelle TLR2 amino acid sequences were highly similar to the TLR2 proteins of *E. fuscus* and *M. brandtii* (Table 4.6). The predicted N-glycosylation sites in the *E. fuscus* TLR2 protein were conserved in

the pipistrelles; in addition, a further two N-glycosylation sites were predicted in the pipistrelle proteins (Figure 4.12).

```
CLUSTAL O(1.2.4) multiple sequence alignment

S818              ----------XGTVISLFKEGAXDQAFPLTCDPTGVCDGHSRSLISIPSGLTATVTSLDL      50
J649              ----------XGTVISLFKEGAXDQAFPLTCDPTGVCDGHSRSLISIPSGLTATVTSLDL      50
XP_008142370.1    MPHALWTAWVLGSVISLFEEGAPDQASPLTCDPTGVCDGHARSLISIPSGLMATVKSLDL      60
                            *:*****:*** *** ************:********** ***.****

S818              SNNKIAYVSNSDLRMCVNLRALRLGSNSIDTIEEDSFFSLGSLEHLDLSYNHLANLSASW      110
J649              SNNKIAYVSNSDLRMCVNLRALRLGSNSIDTIEEDSFFSLGSLEHLDLSYNHLANLSASW      110
XP_008142370.1    SNNKIAYVSNSDLRMCVNLKALRLGSNSIDTIEEDSFFSLGSLEHLDLSYNHLSILSASW      120
                  ******************:**********************************: *****

S818              FRPLTSLNVLNLLGNPYKTLGKTPLFSHLTKLRILKVGHSYLFTEIQEKDFVGLTFLKEL      170
J649              FRPLTSLNVLNLLGNPYKTLGKTPLFSHLTKLRILKVGHSYLFTEIQEKDFVGLTFLKEL      170
XP_008142370.1    FRPLTSLNFLNLLGNPYKTLGKTSLFSHLTKLRILKVGHSYHFTEIQEKDFVGLTFLKEL      180
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S818              EIDASNLQKYAPRSLKVIQNISHLILHMKQPTFLMKISEDLLSSLGHLELRDTHLDNFHF      230
J649              EIDASNLQKYAPRSLKVIQNISHLILHMKQPTFLMKISEDLLSSLGHLELRDTHLDNFHF      230
XP_008142370.1    EIDASNLQKYAPRSLKLIQNISHLILRVKQPTFLLEISVDLLSSLGHLELRDTHLDTFHF      240
                  ****************:*********::******::** *****************.****

S818              SKVSTNETKTIKKFTFRNVKITDEGFNEMVKLLNHVSEILDVEFDSCTLNGIGDFDITVM      290
J649              SKVSTNETKTIKKFTFRNVKITDEGFNEMVKLLNHVSEILDVEFDSCTLNGIGDFDITVM      290
XP_008142370.1    SIVSTNETKTIKKFTFRNVKITDEGFNEMVKLLNYVSEIVDVEFDSCTLNGIGDFDTTAM      300
                  * ********************************:****:**************** *.*

S818              DTNKDISKIETLTIRRLYIPNFYSFYDLSSLYSLTGTVKRITIESSKVFLVPCSLSQHLK      350
J649              DTNKDISKIETLTIRRLYIPNFYSFYDLSSLYSLTGTVKRITIESSKVFLVPCSLSQHLK      350
XP_008142370.1    DTNKDISKIQTLTIRRLYIPYFYLFSDLSSLYSLTGTVKRITIESSKVFLVPCSLSQHLK      360
                  *********:********** ** * **********************************

S818              SLEYLDLSGNLIVENSLTNAACEYAWPSLQTLILRQNHLRSLEETGEVLLTLKNLTNLDI      410
J649              SLEYLDLSGNLIVENSLTNAACEYAWPSLQTLILRQNHLRSLEETGEVLLTLKNLTNLDI      410
XP_008142370.1    SLEYLDLSGNLIVENLLKNAACEYAWPSLQTLILRQNHLRSLEKTGEILLTLKSLTNLDI      420
                  *************** *.************************:***:*****.******

S818              SKNNFHPISKTCQWPERMKYLNLSNTRIQSLTKCIPQTLEVLDVSNNSLSSFSLTMPQLR      470
J649              SKNNFHPISKTCQWPERMKYLNLSNTRIQSLTKCIPQTLEVLDVSNNSLSSFSLTMPQLR      470
XP_008142370.1    SKNNFHPISKTCQWPERMKCLNLSNTRIQSLTKCIPQTLEVLDVSNNSLSSFSLTLPQLR      480
                  ****************** ************************************:****

S818              ELYISGNRLKTLPDASSLPMLLVMRISRNTINTFSKEQLDSFKKLKTLEAGSNSFICSCE      530
J649              ELYISGNRLKTLPDASSLPMLLVMRISRNTINTFSKEQLDSFKKLKTLEAGSNSFICSCE      530
XP_008142370.1    ELYISGNKLKTLPDASSLPMLLIMRISRNTINTFSKEQLDSFKTLKTLEAGSNNFICSCE      540
                  *******:**************:********************.*********.******

S818              FLSFTQGQQALAQVLVDWPENYLCDSPSHVRGQRVQDTHLSVSECHRVAVVSAVCCALFL      590
J649              FLSFTQGQQAMAQVLVDWPENYQCDSPSHVRGQRVQDTHLSVSECHRVAVVSAVCCALFL      590
XP_008142370.1    FLSFTQGHQALAQVLTDWPEHYLCDSPSHVRGQRVRDTHLSASECHRVAVVSAVCCALFL      600
                  *******:** ****.****:* ************:*****.******************

S818              LILLTGVLCHRFHGLWYMKMMWAWLQAKRKPRRAPPRDLSYDAFVSYSEQDSHWVENLMV      650
J649              LILLTGVLCHRFHGLWYMKMMWAWLQAKRKPRRAPPRDLSYDAFVSYSEQDSHWVENLMV      650
XP_008142370.1    LILLAGVLCHRFHGLWYMKMMWAWLQAKRKPRRAPQRDLCYDAFVSYSEQDSHWVENLMV      660
                  ****:****************************** ***.******************

S818              QELEHFDPPFKLCLHKRDFVPGKWIIDNIIDSIEKSHKTIFVLSENS-R-----------      698
J649              QELEHFDPPFKLCLHKRDFVPGKWIIDNIIDSIEKSHKTIFVLSENS-R-----------      698
XP_008142370.1    QELEHFNPPFKLCLHKRDFVPGKWIIDNIIDSIEKSHKTIFVLSENFVKSEWCKYELDFS      720
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S818              ------------------------------------------------------------      698
J649              ------------------------------------------------------------      698
XP_008142370.1    HFRLFDENDDAAILVLLEPLEKKAIPQRFCKLRKIMNTKTYLEWPTDETQQEGFWFNLRT      780

S818              ----    698
J649              ----    698
XP_008142370.1    AIKS    784
```

Figure 4.12: Clustal W alignment of the *TLR2* amino acid sequences from *P. pipistrellus* (code: S818) and *P. pygmaeus* (code: J649) with the *TLR2* protein from *E. fuscus*: XP_008142370.1. *Footnote*: Predicted N-glycosylation sites are highlighted in emboldened red font.

Table 4.6: BlastP summary data for the *TLR2* amino acid sequence derived from *P. pipistrellus* (code: S818). *Footnote*: the BlastP data for the *P. pygmaeus* (code: J649) *TLR2* sequence was identical to that shown below.

Highly similar sequence	Max score	Total score	Query cover	E value	Iden	Gen bank #
toll-like receptor 2 [*Eptesicus fuscus*]	1301	1301	100%	0.0	93%	XP_008142370.1
toll-like receptor 2 [*Myotis brandtii*]	1257	1257	100%	0.0	90%	XP_014399389.1
toll-like receptor 2 [*Myotis lucifugus*]	1236	1236	100%	0.0	90%	XP_006081868.1
toll-like receptor 2 [*Myotis davidii*]	1233	1233	100%	0.0	89%	XP_006770169.2
toll-like receptor 2 precursor [*Pteropus alecto*]	1134	1134	100%	0.0	81%	XP_006906317.1

Unfortunately, due to lack of conservation at the 5' and 3' ends of the TLR2 gene between the different bat species, it was not possible to isolate the full TLR2 gene sequence for the common and soprano pipistrelles. Nonetheless, based upon a functional domain analysis, the pipistrelle proteins showed high conservation with the *E. fuscus* TLR2 protein, lacking only the cytoplasmic Toll/IL-IR domain and also, one leucine rich repeat associated with the extracellular domain (Figure 4.13).

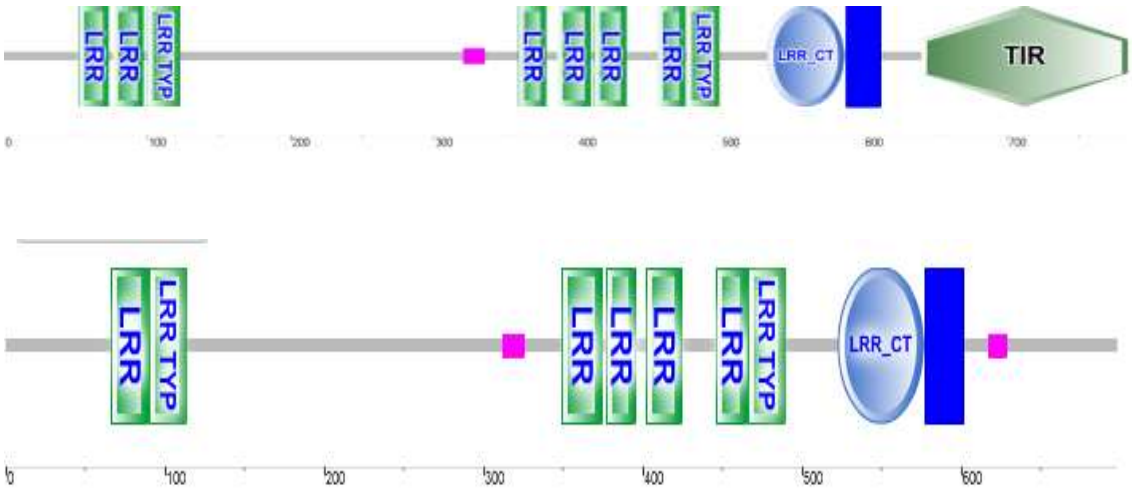

Figure 4.13: Domain structures of the *TLR2* protein from *E. fuscus* (above) and *P. pipistrellus* (code: S818) (below). The blue block represents the transmembrane domain and TIR is the cytoplasmic Toll/IL-IR domain. *Footnote*: *P. pygmaeus* (code: J649) has the same *TLR2* domain structure as shown for *P. pipistrellus*.

Discussion

The aim of this chapter was to characterise pipistrelle genes encoding TLR2 and TLR4 by using a PCR-based approach. Given the absence of a pipistrelle genome sequence, or publications on pipistrelle TLRs, the approach was dependent upon aligning TLR sequences from a small number of vespertilionid bats and the fruit bat *P. alecto* in order to design PCR primers to evolutionary conserved regions. As a result, it was not possible to provide full gene sequence data for pipistrelle TLR2 and TLR4 since the 5' and 3' regions of these genes were less well conserved between the bat species.

Nonetheless, the gene isolation approach proved successful and allowed isolation of both TLR4 and TLR2 gene sequences from the common and the soprano pipistrelles. Fortuitously, both pipistrelle TLRs appeared to lack intron sequences since the introns are expected to be at the 5' and 3' ends of the gene and it was not possible to design primers in these two regions due to lack of conservation between the bat species in these two regions. The predicted introns were based on searching the *M. brandtii* annotated genome for TLR4 and TLR2 mRNA sequences which indicates that this myotid bat introns are located at the 5' ans3' regions of the TLR genes. As noted in the literature, bats have smaller genomes than other mammals (Seim et al., 2013; J. D. Smith & Gregory, 2009) and this may reflect evolutionary events associated with metabolism and flight (Hughes & Hughes, 1995).

The common and soprano pipistrelle TLR2 and TLR4 sequences were most similar to each other and they were also highly similar to the other vespertilionid TLR4 and TLR2 sequences. Inspection of the TLR4 sequence showed that one internal PCR primer binding site (TLR4-F) was 100% conserved with the pipistrelle sequences whilst the other (TLR4-2R) showed 7/23 (30%) mismatches. It is not possible to comment on the conservation of the external PCR primer binding sites. However, it is quite likely that even though sequences of

high conservation between the bat species were used to design PCR primers, the pipistrelle sequences at the primer binding regions may not have been a 100% match. Indeed, variability of the pipistrelle TLR4 sequences between individual bats (Chapter 5) and specifically, at the PCR primer binding sites, probably explains the failure to amplify TLR4 PCR products from 36 of the 95 (38%) bats. Although the pipistrelle TLR2 is more conserved than TLR4 to other bat sequences, a similar explanation of TLR2 gene variability at the PCR primer binding sites is the likely explanation for a failure to amplify TLR2 PCR products from these bats.

Assuming that the pipistrelle TLR sequences are most similar to those of *E. fuscus*, as indicated by Blast analysis, then the gene isolation approach has revealed approximately 71% of the pipistrelle TLR4 gene and 89% of the pipistrelle TLR2 gene. Importantly, for both pipistrelle TLRs, the sequences encompass the majority of the external domains, including the leucine-rich regions, involved in interactions with pathogen associated molecular patterns (Ng & Xavier, 2011). Predicted N-glycosylation sites, also potentially involved in recognition of pathogen associated molecular patterns, were also conserved between TLR4 of the pipistrelles and *E. fuscus* TLR4. In addition, the pipistrelle TLR2 proteins appeared to have two additional N-glycosylation sites relative to the big brown bat TLR2.

In conclusion, a PCR-based gene isolation strategy has allowed isolation and sequence characterisation of large fragments of the *P. pipistrellus* and *P. pygmaeus* TLR2 and TLR4 genes. This strategy forms the basis for sequencing the pipistrelle TLR2 and TLR4 genes in the majority of the South Lancashire bat population under study. The data describing TLR2 and TLR4 gene variations in the bat population, that then allows an attempt to correlate parasite infection profiles to TLR haplotypes, is presented in Chapter 5.

5. TLR gene variations and parasite infection profiles

5.1 TLR2 and TLR4: roles in helminth infections:

Analysis of cytokine responses in ex vivo monocytes derived from African children exposed to gastrointestinal nematode infection has highlighted the role that these parasites may have in modulating innate immune responses to pathogens (Jackson et al., 2006). Other work has even highlighted the role of parasite endosymbionts; for example, the major Wolbachia surface protein (WSP) of filarial nematode endosymbionts is able to initiate activation of TLR2 and TLR4 expression using a reporter gene assay with transfected human embryonic kidney 293 (HEK293) cells (Brattig et al., 2004). Moreover, WSP stimulates an inflammatory immune response in murine macrophages and dendritic cells (DCs), again through TLR4 and TLR2, as confirmed by using mouse mutants (Brattig et al., 2004).

Major excretory-secretory (ES) products of helminths are known to influence immunomodulatory outcomes to infection and ES-62 of the rodent filarial nematode *Acanthocheilonema viteae* has been shown, using mouse mutants, to activate a MyD88-dependent TLR4 signalling pathway that leads to suppression of macrophage and dendritic cell responses (Goodridge et al., 2005). Interestingly, it appears that TLR4 may act via a novel mechanism that might involve another TLR and possibly, suppression of MyD88 to other TLRs (Goodridge et al., 2005).

Application of live *Schistosoma mansoni* larvae, or soluble preparations derived from these larvae, to macrophages has shown that cytokine production is dependent upon activation of TLR4 (Jenkins, Hewitson, Ferret-Bernard, & Mountford, 2005). Schistosomal lysophosphatidylserine has also been shown to activate dendritic cells via TLR2 signalling

and this may contribute to polarisation of the immune response, via expansion of T-regulatory cells, to elicit the fibrotic, tissue destructive liver pathology associated with this parasite (Layland, Rad, Wagner, & Da Costa, 2007; van der Kleij et al., 2002). Indeed, bone marrow-derived macrophages from mice with *S. mansoni* egg-induced pulmonary granulomas have a greater response to TLR2 and TLR3 activation than control mice (Joshi, Raymond, Coelho, Kunkel, & Hogaboam, 2008).

A study of the rat tapeworm *Hymenolepis diminuta* has revealed high expression of TLR4 and TLR2 in the rodent colon and jejunum 6-8 days post-infection (Kosik-Bogacka et al., 2012). This up regulation of TLR2 and TLR4 expression may be important in the pathomechanism of hymenolepidosis and hence is worthy of further study (Kosik-Bogacka et al., 2012).

Mouse mutants have been used to examine the role of TLRs in *Heligmosomoides polygyrus* infection. Interestingly, MyD88 mutants showed increased immunity to *H. polygyrus* infection whereas, TLR2, TLR4, TLR5, and TLR9 mutants were unable to exhibit enhanced expulsion of *H. polygyrus* (Reynolds et al., 2014). The systemic response to the related nematode, *H. bakeri,* was also assessed in cultured splenocytes derived from infected mouse strains and the data showed an upregulation of TLR2, TLR4 and TLR9-mediated cytokine responses in a manner that was strain and parasite exposure dependent (Friberg et al., 2013). Further study of innate immune responses in the wood mouse, *Apodemus sylvaticus,* showed through statistical modelling, that a significant amount of TLR2 variation in the natural population over time could be explained by exposure to and hence the transmission dynamics of *H. polygyrus* and also, the pinworm *Syphacia stroma* and the digenean fluke *Brachylaima recurva* (Friberg et al., 2013). Importantly, the latter study was carried out on a natural

population and there is an increased need, as argued elsewhere (Friberg et al., 2010; Pederson & Babayan, 2011), to investigate immunological responses in natural populations to complement laboratory-based investigations (Friberg, Bradley, & Jackson, 2010; Pedersen & Babayan, 2011).

Based on the growing recognition that TLR2 and TLR4 have important roles in helminth (above) and protozoan (see section 4.1) infections the aims of this chapter are: (i) to characterise the TLR2 and TLR4 sequence variation across the pipistrelle population and (ii) to analyse the TLR4 and TLR2 amino acid changes with respect to the known parasite infection profiles in the pipistrelle bats.

5.2 *TLR4*:

5.2.1 Sequence analysis:

After using the PCR-based strategy to isolate 59 individual pipistrelle TLR4 sequences (Chapter 4), the DNA (Figure 5.1) and translated amino acid (Figure 5.2) sequences were aligned using Clustal W. The DNA sequences showed that there were 42 TLR4 haplotypes at the gene level and this translated into 42 different protein sequences. These haplotypes were identified from the phylogenetic tree; each different branch was counted as one haplotype, which showed 42 haplotypes are present within the TLR4 sequences.

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 multiple sequence alignment:

S818   ---------------GAGTTTANCCCCNGGANNTTTTCTGACTATNAGTTTACAGNCCTG 45
S819   ----------------------------------------------------CANTG 5
J628   ------------------------------------------------------------ 0
S817   -----------------TCAGAGTTTAGCCCCNGNNNTTTTCTGACTNTNANNTNCAGAC 43
S815   -----------------TCAGAGTTTAGCCCCNGNNNTTTTCTGACTNTNANNTNCAGAC 43
J649   ------------------------------------------------------------ 0
J656   ----------------------------------TGGACTANTCAANGTTTACAGACNN 25

S818   GGTGGCTGGGGAGCC--AACCTAGCATCTCTAGAGGACTTCCCCATGGCNGACATCTGTA 103
S819   NGNGGNTGNGAGACA--AACCTAGCATCCNTAGAGGACTTCCCCATGGCAGACATATGAA 63
J628   ------------------------------TAGGGACTTGANCCATGGCAGGNTGTAAAA 30
S817   TGGGGTGGGTGGGGGACAACCTAGCATCTCTAGAGGACTTCCCCATGGCAGACATCTGAA 103
S815   TGGGGTGGGTGGGGGACAACCTAGCATCTCTAGAGGACTTCCCCATGGCAGACATCTGAA 103
J649   -------GTGTAAGANAAAATTANTGTCTCTTAGGGACTTCCCCATGGCAGATANNTTAA 53
J656   NGGTGGCNGGGGAAGAAGAACTGGTGGCTCTTAGGGACTTCGCCATGGCAGTAAAGTAAA 85
                                                *    ****** *        *


S818   ATCCTTGAAGGAGCTTAATGTGGCTCACAATCTAATCGATTCCTTCAAGTTACCGGACTA 163
S819   ATCCTTGATGGAACTTAATGTGGCTCACAATCTAATCGATTCCTTCAAGTTACCGGACTA 123
J628   ATCCNTGTTAGAGCTTAATGTGGCTCACAATCTAATCGATTCCTTCAAGTTACCGGACTA 90
S817   ATCCTTGAAGGAGCTTAATGTGGCTCACAATCTAATCGATTCCTTCAAGTTACCGGACTA 163
S815   ATCCTTGAAGGAGCTTAATGTGGCTCACAATCTAATCGATTCCTTCAAGTTACCGGACTA 163
J649   TCCCTTGATAGAGCTTAATGTGGCTCACAATCTAATCGATTCCTTCAAGTTACCGGACTA 113
J656   TCCCTTGATAGAGCTTAATGTGGCTCACAATCTAATCGATTCCTTCAAGTTACCGGACTA 145
            **   ** ********************************************


S818   TTTTTCTAACCTGCCTAACCTGGAACACTTGGACCTTTCTAATAATAAGATTCGAAAAAT 223
S819   TTTTTCTCACCTGCCTAACCGGGAACTATTGGACCTTTCTAATAATAAGATTCGAAAAAT 183
J628   TTTTTCTAACCTGCCTAACCTGGAACACTTGGACCTTTCTAATAATAAGATTCGAAAAAT 150
S817   TTTTTCTAACCTGCCTAACCTGGAACACTTGGACCTTTCTAATAATAAGATTCGAAAAAT 223
S815   TTTTTCTAACCTGCCTAACCTGGAACACTTGGACCTTTCTAATAATAAGATTCGAAAAAT 223
J649   TTTTTCTAACCTGCCTAACCTGGAACACTTGGACCTTTCTAATAATAAGATTCGAAAAAT 173
J656   TTTTTCTAACCTGCCTAACCTGGAACACTTGGACCTTTCTAATAATAAGATTCGAAAAAT 205
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S818   TTACCATGAAGACTTGCAGGTTTTACATCAAATGCCCTCATTCAAACTCTCTTTAGACCT 283
S819   TTACCATGAAGACTTGCAGGTTTTACATCAAATGCCCTCATTCAAACTCTCTTTAGACCT 243
J628   TTACCATGAAGACTTGCAGGTTTTACATCAAATGCCCTCATTCAAACTCTCTTTAGACCT 210
S817   TTACCATGAAGACTTGCAGGTTTTACATCAAATGCCCTCATTCAAACTCTCTTTAGACCT 283
S815   TTACCATGAAGACTTGCAGGTTTTACATCAAATGCCCTCATTCAAACTCTCTTTAGACCT 283
J649   TTACCATGAAGACTTGCAGGTTTTACATCAAATGCCCTCATTCAAACTCTCTTTAGACCT 233
J656   TTACCATGAAGACTTGCAGGTTTTACATCAAATGCCCTCATTCAAACTCTCTTTAGACCT 265
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S818   GTCCCTCAACCCTTTAGACTTTATCCAACCAGGTGCCTTTGAAAAAATTAAGCTCCATGA 343
S819   GTCCCTCAACCCTTTAGACTTTATCCAACCAGGTGCCTTTGAAAAAATTAAGCTCCATGA 303
J628   GTCCCTCAACCCTTTAGACTTTATCCAACCAGGTGCCTTTGAAAAAATTAAGCTCCATGA 270
S817   GTCCCTCAACCCTTTAGACTTTATCCAACCAGGTGCCTTTGAAAAAATTAAGCTCCATGA 343
S815   GTCCCTCAACCCTTTAGACTTTATCCAACCAGGTGCCTTTGAAAAAATTAAGCTCCATGA 343
J649   GTCCCTCAACCCTTTAGACTTTATCCAACCAGGTGCCTTTGAAAAAATTAAGCTCCATGA 293
J656   GTCCCTCAACCCTTTAGACTTTATCCAACCAGGTGCCTTTGAAAAAATTAAGCTCCATGA 325
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S818   ACTGACTTTGAGAAGTAATTTTGATAGTAAAAAGGTCATGAAAACATGTATTCAAGGTCT 403
S819   ACTGACTTTGAGAAGTAATTTTGATAGTAAAAAGGTCATGAAAACATGTATTCAAGGTCT 363
J628   ACTGACTTTGAGAAGTAATTTTGATAGTACAGAGGTCATGAAAACATGTATTCAAGGTCT 330
S817   ACTGACTTTGAGAAGTAATTTTGATAGTACAGAGGTCATGAAAACATGTATTCAAGGTCT 403
S815   ACTGACTTTGAGAAGTAATTTTGATAGTACAGAGGTCATGAAAACATGTATTCAAGGTCT 403
J649   ACTGACTTTGAGAAGTAATTTTGATAGTAAAAAGGTCATGAAAACATGTATTCAAGGTCT 353
J656   ACTGACTTTGAGAAGTAATTTTGATAGTAAAAAGGTCATGAAAACATGTATTCAAGGTCT 385
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S818   GGCAGGTTTAAAGATCAATCGGCTGATTCTAGGAGAATTTAAAAATGAAAGGAACTTAGT 463
S819   GGCAGGTTTAAAGATCAATCGGCTGATTCTAGGAGAATTTAAAAATGAAAGGAACTTAGT 423
J628   GGCAGGTTTAAAGATCAATCGGCTGATTCTAGGAGAATTTAAAAATGAAAGGAACTTAGT 390
S817   GGCAGGTTTAAAGATCAATCGGCTGATTCTAGGAGAATTTAAAAATGAAAGGAACTTAGT 463
S815   GGCAGGTTTAAAGATCAATCGGCTGATTCTAGGAGAATTTAAAAATGAAAGGAACTTAGT 463
J649   GGCAGGTTTAAAGATCAATCGGCTGATTCTAGGAGAATTTAAAAATGAAAGGAACTTAGT 413
J656   GGCAGGTTTAAAGATCAATCGGCTGATTCTAGGAGAATTTAAAAATGAAAGGAACTTAGT 445
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S818   AGACTTGGACAAATCTGCCCTGGAGGAACTGTGCAACTTGACCATTGATGAATTCCGGAT 523
S819   AGACTTGGACAAATCTGCCCTGGAGGAACTGTGCAACTTGACCATTGATGAATTCCGGAT 483
J628   AGACTTGGCCAAATCTGCCCTGGAGGAACTGTGCAACTTGACCATTGATGAATTCCGGAT 450
S817   AGACTTGGCCAAATCTGCCCTGGAGGAACTGTGCAACTTGACCATTGATGAATTCCGGAT 523
S815   AGACTTGGCCAAATCTGCCCTGGAGGAACTGTGCAACTTGACCATTGATGAATTCCGGAT 523
J649   AGACTTGGACAAATCTGCCCTGGAGGAACTGTGCAACTTGACCATTGATGAATTCCGGAT 473
J656   AGACTTGGACAAATCTGCCCTGGAGGAACTGTGCAACTTGACCATTGATGAATTCCGGAT 505
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S818   AGCACACTTCCAAGACTTTCCAGAGGATTGCCGTGGCTTTTTAAATTGTCTGGCAGATGC 583
S819   AGCACACTTCCAAGACTTTCCAGAGGATTGCCGTGGCTTTTTAAATTGTCTGGCAGATGC 543
J628   AGCACACTTCCAAAACTTTTCAAAGGATTACCGTGGCTTTTTAAATTGTCTGGCAGATGC 510
S817   AGCACACTTCCAAAACTTTTCAAAGGATTACCGTGGCTTTTTAAATTGTCTGGCAGATGC 583
S815   AGCACACTTCCAAAACTTTTCAAAGGATTACCGTGGCTTTTTAAATTGTCTGGCAGATGC 583
J649   AGCACACTTCCAAGACTTTCCAGAGGATTGCCGTGGCTTTTTAAATTGTCTGGCAGATGC 533
J656   AGCACACTTCCAAGACTTTCCAGAGGATTGCCGTGGCTTTTTAAATTGTCTGGCAGATGC 565
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S818   TTCTGCAGTATCTCTGATGAGTCTGAAAATAGGCAGGCTAGAAAGCCTTCCAACAGGTTT 643
S819   TTCTGCAGTATCTCTGATGAGTCTGAAAATAGGCAGGCTAGAAAGCCTTCCAACAGGTTT 603
J628   TTCTGCAGTATCTCTGATGAGTCTGCATATAGACAGGCTAGAAAGCCTTCCAACAGGTTT 570
S817   TTCTGCAGTATCTCTGATGAGTCTGCATATAGACAGGCTAGAAAGCCTTCCAACAGGTTT 643
S815   TTCTGCAGTATCTCTGATGAGTCTGCATATAGACAGGCTAGAAAGCCTTCCAACAGGTTT 643
J649   TTCTGCAGTATCTCTGATGAGTCTGAAAATAGGCAGGCTAGAAAGCCTTCCAACAGGTTT 593
J656   TTCTGCAGTATCTCTGATGAGTCTGAAAATAGGCAGGCTAGAAAGCCTTCCAACAGGTTT 625
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S818   CAAATGGCAGTACTTAAAATTGTCTAATTGTAAATTTCAAGATTTCCCTACATTGGAGCT 703
S819   CAAATGGCAGTACTTAAAATTGTCTAATTGTAAATTTCAAGATTTCCCTACATTGGAGCT 663
J628   CAAATGGCAATACTTAAAATTGTCTAATTGTAAATTTAAAGATTTCCCTACATTGGAGCT 630
S817   CAAATGGCAATACTTAAAATTGTCTAATTGTAAATTTAAAGATTTCCCTACATTGGAGCT 703
S815   CAAATGGCAATACTTAAAATTGTCTAATTGTAAATTTAAAGATTTCCCTACATTGGAGCT 703
J649   CAAATGGCAGTACTTAAAATTGTCTAATTGTAAATTTCAAGATTTCCCTACATTGGAGCT 653
J656   CAAATGGCAGTACTTAAAATTGTCTAATTGTAAATTTCAAGATTTCCCTACATTGGAGCT 685
       ************************************************************

S818   TACCTTTCTCAAGCAATTTATTTTCACTGCCAACAAAGTTATTAACCACTTTTAACTAAA 763
S819   TACCTTTCTCAAGCAATTTATTTTCACTGCCAACAAAGTTATTAACCACTTTTAACTAAA 723
J628   TACCTTTCTCAAGCAGTTTGTTTTCACTGCCAACAAAGGTATTACCCACTTTTAACTGAA 690
S817   TACCTTTCTCAAGCAGTTTGTTTTCACTGCCAACAAAGGTATTACCCACTTTTAACTGAA 763
S815   TACCTTTCTCAAGCAGTTTGTTTTCACTGCCAACAAAGGTATTACCCACTTTTAACTGAA 763
J649   TACCTTTCTCAAGCAATTTATTTTCACTGCCAACAAAGTTATTAACCACTTTTAACTAAA 713
J656   TACCTTTCTCAAGCAATTTATTTTCACTGCCAACAAAGTTATTAACCACTTTTAACTAAA 745
       *************** *** ****************** ***** ***************

S818   CTTAATCTAAGAAACCTTGAGTTTCTAGATCTCAGTAGAAAATGGCTTGAGTTTCAAGTC 823
S819   CTTAATCTAAGAAACCTTGAGTTTCTAGATCTCAGTAGAAAATGGCTTGAGTTTCAAGTC 783
J628   GTTAATCTAAGAAACCTTGAGTTTCTAGATCTTAGTAGAAAATGGCTTGAGTTTCAAGTC 750
S817   GTTAATCTAAGAAACCTTGAGTTTCTAGATCTTAGTAGAAAATGGCTTGAGTTTCAAGTC 823
S815   GTTAATCTAAGAAACCTTGAGTTTCTAGATCTTAGTAGAAAATGGCTTGAGTTTCAAGTC 823
J649   CTTAATCTAAGAAACCTTGAGTTTCTAGATCTCAGTAGAAAATGGCTTGAGTTTCAAGTC 773
J656   CTTAATCTAAGAAACCTTGAGTTTCTAGATCTCAGTAGAAAATGGCTTGAGTTTCAAGTC 805

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S818   TTGCTGCTCTGACCGTGATTTTGGGACAACCCGACTGAAACACTTAGATCTGAGCTTCAA 883
S819   TTGCTGCTCTGACCGTGATTTTGGGACAACCCGACTGAAACACTTAGATCTGAGCTTCAA 843
J628   TTGCTGCTCTCGCCGTGATTTTGGGACAACCCAACTGAAACACTTAGATCTGAGCTTCAA 810
S817   TTGCTGCTCTCGCCGTGATTTTGGGACAACCCAACTGAAACACTTAGATCTGAGCTTCAA 883
S815   TTGCTGCTCTCGCCGTGATTTTGGGACAACCCAACTGAAACACTTAGATCTGAGCTTCAA 883
J649   TTGCTGCTCTGACCGTGATTTTGGGACAACCCGACTGAAACACTTAGATCTGAGCTTCAA 833
J656   TTGCTGCTCTGACCGTGATTTTGGGACAACCCGACTGAAACACTTAGATCTGAGCTTCAA 865
       ***********   ********************** ****************************

S818   TAGTATTATTACCAATGACTTCAAACTTTCGTGGGCTTAGAGCAAAATAGAACATCTGGA 943
S819   TAGTATTATTTCTANGAGTTTTAAACTTTCGTGGGCTTAGAGCAAAATAGAACATCTGGA 903
J628   TAGTATTATTACCATGACTTTCAAACTTCGTGGGGTTAAGAGCAAAATAGAACATCTGGA 870
S817   TAGTATTATTACCATGACTTTCAAACTTCGTGGGGTTAAGAGCAAAATAGAACATCTGGA 943
S815   TAGTATTATTACCATGACTTTCAAACTTCGTGGGGTTAAGAGCAAAATAGAACATCTGGA 943
J649   TAGTATTAATACCAATGACTTCAAACTTTCATGGGCTTAAGAGCAAATAGAACATCTGGA 893
J656   TAGTATTATTACCAATGACTTCAAACTTTCGTGGGCTTAGAGCAAAATAGAACATCTGGA 925
       ********************************     *****  ***   ******************

S818   TTTCCAGCATTCCACTTTGAGACAGGCCAGTACTTTTTCAGTATTCCTCTCACTCAAAAA 1003
S819   TTTCCAGCATTCCACTTTGAGACAGGCCAGTACTTTTTCAGTATTCCTCTCACTCGAAAA 963
J628   TTTCCAGCATTCCACTTTGAGACAGGCCAGTACTTTTTCAGTATTCCTCTCACTCAAAAA 930
S817   TTTCCAGCATTCCACTTTGAGACAGGCCAGTACTTTTTCAGTATTCCTCTCACTCAAAAA 1003
S815   TTTCCAGCATTCCACTTTGAGACAGGCCAGTACTTTTTCAGTATTCCTCTCACTCAAAAA 1003
J649   TTTCCAGCATTCCACTTTGAGACAGGCCAGTACTTTTTCAGTATTCCTCTCACTCGAAAA 953
J656   TTTCCAGCATTCCACTTTGAGACAGGCCAGTACTTTTTCAGTATTCCTCTCACTCGAAAA 985
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S818   CCTCCTTTACCTTGATATCTCTTACACTGACATCAAGATTGTCTTCCAGGGCATCTTTGA 1063
S819   CTCCTTTTACCTTGATATCTCTTACACTGACATCAAGATTGTCTTCCAGGGCATCTTTGA 1023
J628   CCTCCTTTACCTTGATATCTCTTACACTGACATCAAGATTGTCTTCCAGGGCATCTTTGA 990
S817   CCTCCTTTACCTTGATATCTCTTACACTGACATCAAGATTGTCTTCCAGGGCATCTTTGA 1063
S815   CCTCCTTTACCTTGATATCTCTTACACTGACATCAAGATTGTCTTCCAGGGCATCTTTGA 1063
J649   CCTCCTTTACCTTGATATCTCTTACACTGACATCAAATTTGTCTTCCAGGGCATCTTTGA 1013
J656   CCTCCTTTACCTTGATATCTCTTACACTGACATCAAGATTGTCTTCCAGGGCATCTTTGA 1045
       ************************************************************

S818   TGGCTTGATCAGCCTCCAAGTCTTAAAAATTGGCTGGCAATTCCTTTCCAGGATGCATTC 1123
S819   TGGCTTGGTCAGCCTCCAAGTCTTAAAAAATGGCTGGCAATTCCTTTCAGGATGCATTCC 10838
J628   TGGCTTGATCAGCCTCCAAGTCTTAAAAATGGCTGGCAATTCCTTTTCAGGATGCATTCC 1050
S817   TGGCTTGATCAGCCTCCAAGTCTTAAAAATGGCTGGCAATTCCTTTTCAGGATTCATTCC 1123
S815   TGGCTTGATCAGCCTCCAAGTCTTAAAAATGGCTGGCAATTCCTTTTCAGGATTCATTCC 1123
J649   TGGCTTGATCAGCCTCCAAGTCTTAAAAATGGCTGGCAATTCCTTTTCAGGATGCATTCC 1073
J656   TGGCTTGGTCAGCCTCCAAGTCTTAAAAATGGCTGGCAATTCCTTTTCAGGATGCATTCC 1105
       ************************************  **  * * * *****  *        *

S818   CTCCAAATATTTTCAGAGATCTGACTCAGTTGACTGTCCTGGACCTCTCTCAGTGTCAAC 1183
S819   TTCCAAATATTTTCAGAGATCTGACTCAGTTGACTGTCCTGGACCTCTCTCAGTGTCAAC 1143
J628   TTCCAAATATTTTCAGAGATCTGACTCAGTTGACTGTCCTGGACCTCTCTCAGTGTCAAC 1110
S817   TTCCAAATATTTTCAGAGATCTGACTCAGTTGACTGTCCTGGACCTCTCTCAGTGTCAAC 1183
S815   TTCCAAATATTTTCAGAGATCTGACTCAGTTGACTGTCCTGGACCTCTCTCAGTGTCAAC 1183
J649   TTCCAAATATTTTCAGAGATCTGACTCAGTTGACTGTCCTGGACCTCTCTCAGTGTCAAC 1133
J656   TTCCAAAATTTTCAGAGATCTGACTCAGTTGACTGTCCTGGACCTCTCTCAGTGTCAACT 1165
        ************                  *

S818   TGGAACAGGTGTCCCCAGAGGCATTCGGCTCACTCCTTAGACTCCAGGTGCTAAATATGA 1243
S819   TGGAACAGGTGTCCCCAGAGGCATTCGGCTCACTCCTTAGACTCCAGGTGCTAAATATGA 1203
J628   TGGAACAGGTGTCCCCAGAGGCATTCGGCTCACTCCTTAGACTCCAGGTGCTAAATATGA 1170
S817   TGGAACAGGTGTCCCCAGAGGCATTCGGCTCACTCCTTAGACTCCAGGTGCTAAATATGA 1243
S815   TGGAACAGGTGTCCCCAGAGGCATTCGGCTCACTCCTTAGACTCCAGGTGCTAAATATGA 1243
J649   TGGAACAGGTGTCCCCAGAGGCATTCGGCTCACTCCTTAGACTCCAGGTGCTAAATATGA 1193
J656   GGAACAGGTGTCCCCAGAGGCATTCGGCTCACTCCTTAGACTCCAGGTGCTAAATATGAG 1225
        * *         ***

S818   GTCACAACCACCTCTTGTCCTTGGATATGCTTCCTTATAAAAATCTCTCTCTCTGGCTTC 1303
S819   GTCACAACCACCTCTTGTCCTTGGATATGCTTCCTTATAAAAATCTCTCTCTCTGGCTTC 1263
J628   GTCACAACCACCTCTTGTCCTTGGATATGCTTCCTTATAAAAATCTCTCTCTCTGGCTTC 1230
S817   GTCACAACCACCTCTTGTCCTTGGATATGCTTCCTTATAAAAATCTCTCTCTCTGGCTTC 1303
S815   GTCACAACCACCTCTTGTCCTTGGATATGCTTCCTTATAAAAATCTCTCTCTCTGGCTTC 1303
J649   GTCACAACCACCTCTTGTCCTTGGATATGCTTCCTTATAAAAATCTCTCTCTCTGGCTTC 1253
J656   TCACAACCACCTCTTGTCCTTGGATATGCTTCCTTATAAAAATCTCTCTCTCTGGCTTCT 1285
                                           ****

S818   TAGACTACAGTTTTAACCGTATAGTGGCCGCCAATGGGCAGGAACTACAGCATATTCCAA 1363
S819   TAGACTACAGTTTTAACCGTATAGTGGCCGCCAATGGGCAGGAACTACAGCATATTCCAA 1323
J628   TAGACTACAGTTTTAACCGTATAGTGGCCGCCAATGGGCAGGAACTACAGCATATTCCAA 1290
S817   TAGACTACAGTTTTAACCGTATAGTGGCCGCCAATGGGCAGGAACTACAGCATATTCCAA 1363
S815   TAGACTACAGTTTTAACCGTATAGTGGCCGCCAATGGGCAGGAACTACAGCATATTCCAA 1363
J649   TAGACTACAGTTTTAACCGTATAGTGGCCGCCAATGGGCAGGAACTACAGCATATTCCAA 1313
J656   AGACTACAGTTTTAACCGTATAGTGGCCGCCAATGGGCAGGAACTACAGCATATTCCAAG 1345
               *** * *        *  *  * *   **    *           *   *

S818   GCAATGTAACTTCGTTAAATCTGACCCAGAATGACTTTGCTTGTGTTTGTGAACACATGT 1423
S819   GCAATGTAACTTCGTTAAATCTGACCCAGAATGACTTTGCTTGTGTTTGTGAACACATGT 1383
J628   GCAATGTAACTTCGTTAAATCTGACCCAGAATGACTTTGCTTGTGTTTGTGAACACATGC 1350
S817   GCAATGTAACTTCGTTAAATCTGACCCAGAATGACTTTGCTTGTGTTTGTGAACACATGT 1423
S815   GCAATGTAACTTCGTTAAATCTGACCCAGAATGACTTTGCTTGTGTTTGTGAACACATGT 1423
J649   GCAATGTAACTTCGTTAAATCTGACCCAGAATGACTTTGCTTGTGTTTGTGAACACATGC 1373
J656   CAATGTAACTTCGTTAAATCTGACCCAGAATGACTTTGCTTGTGTTTGTGAACACATGTG 1405
        *     *  *   *  **      **   *     **        **    *
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S818   GTTTCCTGCAGTGGGTCCAGGACCACAGGCGCATCTTGGTGGGAGCTGAACACATGATGT 1483
S819   GTTTCCTGCAGTGGGTCCAGGACCACAGGCGCATCTTGGTGGGAGCTGAACACATGATGT 1443
J628   GTTTCCTGCAGTGGGTCCAGGACCACAGGCGCATCTTGGTGGGAGCTGAACACATGATGT 1410
S817   GTTTCCTGCAGTGGGTCCAGGACCACAGGCGCATCTTGGTGGGAGCTGAACACATGATGT 1483
S815   GTTTCCTGCAGTGGGTCCAGGACCACAGGCGCATCTTGGTGGGAGCTGAACACATGATGT 1483
J649   GTTTCTTGCAGTGGGTCCAGGACCACAGGCGCATCTTGGTGGGAGCTGAACACATGATGT 1433
J656   TTTCCTGCAGTGGGTCCAGGACCACAGGCGCATCTTGGTGGGAGCTGAACACATGATGTG 1465
        ** *       **   *    *    *         *  **      *

S818   GTAAGACACCGTTAGCTATGCAGGGTGTGCCTGTGCTCAGTTTTAGAAAACACCACCTGC 1543
S819   GTAAGACACCGTTAGCTATGCAGGGTGTGCCTGTGCTCAGTTTTTAGAAAACACCACCTG 1503
J628   GTAAGACACCGTTAGCTATGCAGGGTGTGCCTGTGCTCAGTTTTAGAAAACACCACCTGC 1470
S817   GTAAGACACCGTTAGCTATGCAGGGTGTGCCTGTGCTCAGTTTTAGAAAACACCACCTGT 1543
S815   GTAAGACACCGTTAGCTATGCAGGGTGTGCCTGTGCTCAGTTTTAGAAAACACCACCTGT 1543
J649   GTAAGACACCGTTAGCTATGCAGGGTGTGCCTGTGCTCAGTTTTAGAAAACACCACCTGC 1493
J656   TAAGACACCGTTAGCTATGCAGGGTGTGCCCTGTGCTCAGTTTTAGAAAACACCACCTGT 1525
              *   *          **     ***************    ***   *   *

S818   CAGATGAACAAAACTGTCATTAGTGTGTCAGTTCTCTCAGTACTCATAGTATCTGTGGCT 1603
S819   CAGATGAACAAAACTGTCATTAGTGTGTCAGTTCTCTCNNTACTCATAGTATCTGTGGCT 1563
J628   CAGATGAACAAAACTGTCATTAGTGTGTCAGTTCTCTCAGTACTCATAGTATCTGTGGCT 1530
S817   CAGATGAACAAAACTGTCATTAGTGTGTCAGTTCTCTCAGTACTCATAATATCTGTGGCT 1603
S815   CAGATGAACAAAACTGTCATTAGTGTGTCAGTTCTCTCAGTACTCATAATATCTGTGGCT 1603
J649   CAGATGAACAAAACTGTCATTAGTGTGTCAGTTCTCTCAGTACTCATAGTATCTGTGGCT 1553
J656   CAGATGAACAAAACTGTCATTAGTGTGTCAGTTCTCTCAGTACTCATAATATCTGTGGCT 1585
       ****************************************   **********************

S818   GCAGTTCTGGTCTACAAGTTCTATTTCCACCTGATGCTTCTGGCTGGCTGCAGAAAGGTA 1663
S819   GCAGTTCTGGTCTACAAGTTCTATTTCCACCTGATGCTTCTGGCTGGCTGCAGAAANGTA1623
J628   GCAGTTCTGGTCTACAAGTTCTATTTCCACCTGATGCTTCTGGCTGACTGCAGAAAGGTA 1590
S817   GCAGTTCTGGTCTACAAGTTCTATTTCCACCTGATGCTTCTGGCTGGCTGCAGAAAGGTA 1663
S815   GCAGTTCTGGTCTACAAGTTCTATTTCCACCTGATGCTTCTGGCTGGCTGCAGAAGGTAC 1663
J649   GCAGTTCTGGTCTACAAGTTCTATTTCCACCTGATGCTTCTGGCTGGCTGCAGAAAGGTA 1613
J656   GCAGTTCTGGTCTACAAGTTCTATTTCCACCTGATGCTTCTGGCTGACTGCAGAAAGGTA 1645
       ************************************************** *********** *

S818   CGGCAAAGGGGACAGCATGTACGATGCCTTTGTCATCTACTCCAAGCCATGATGAGGACT 1723
S819   CGGCAAAAGGGACAGCATGTACGATGCCTTTGTCATCTACTCCAAGCCATGATGAGGACT 168
J628   CGGCAAAGGGGACAGCATGTACGATGCCTTTGTCATCTACTCCAAGCCATGATGAGGACT 1650
S817   CGGCAAAGGGGACAGCATGTACGATGCCTTTGTCATCTACTCCAAGCCATGATGAGGACT 1723
S815   GGCAAAAGGGACAGCATGTAAGATGCCTTTTCATCTACTCCAGCCATGAAGAGGACTGGG 1723
J649   CGGCAAAGGGGACAGCATGTACGATGCCTTTGTCATCTACTCCAGCCCATGATGAGGACT 1673
J656   CGGCAAAGGGGACAGCATGTACGATGCCTTTGTCATCTACTCCAAGCCATGATGAGGACT 1705
       **** ******          *       ***


S818   GGGTTGAGGAATGAGTTGGTGAAGAACTTGGAGGAGGGGGTNCCCCCCTTTTNNNCTCTG 1783
S819   GGGTTGAGGAATGAGTTGGTGAAGAACTTGGAGGANGGGGNACCCCCCTTTCNNNTNTGC1743
J628   GGGTTGAGGAATGAGTTGGTGAAGAACTTNNNGAGGGGTGCCCCCCTTTCAGCTCTGCCT 1710
S817   GGGTTGAGGAATGAGTTGGTGAAGAACTTGGNNGAGGGGGTNCCCCCCTTTCAGCTCTGC 1783
S815   TGAGGAATGAGTTGTGAAGAACTTGGANGANGGGGTACCCCCCTTTCAGCTCTGCCTTCA 1783
J649   GGGGTGAGGAATGAGTTNGTGAANANNTTGGAGGAGGGGGTACCCCCCTTTCAGCTCNTG 1733
J656   GGGTGAGGAATGAATTTGGTGAAGAACTTGGAGGANGGGGTNNCCCCCTTTCAGCTCTGC 1765
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S818   CCTTCACTACA---------    1794
S819   CTT----------------    1746
J628   TCACTAC----------      1717
S817   CTTCACTACNAGNANAC--    1800
S815   CTACA---------         1788
J649   CCTTCACTACAANAAAACT-    1752
J656   CTTCACTACNAGAGACTT      1783
```

Figure 5.1: Clustal W DNA sequence alignment for the pipistrelle *TLR4* gene derived from 7 bats. *Footnote:* Bat J649, a soprano pipistrelle, was free of protozoan and helminth infections. Only 7 sequences shown as these are representative of the diversity (see Figure 5.3)

```
 multiple sequence alignment:


S818   ~~~~~~~~~~~~~~~EFXPXXFSDYXFTXLGGWGANLASLEDFPIXHLKSLKELNVAHNL 45
S819   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~XXXXXDKPSIXRGLPHQTYKSLMELNVAHNL 31
S817   ~~~~~~~~~~~~~~~SEFSPXXFLTXXXRLGWVGDNLASLEDFPIRHLKSLKELNVAHNL 45
J628   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~RDLXMXVNKSXLELNVAHNL 20
J656   ~~~~~~~~~~~~~~~~~~~~GLXXVYRXXWXGKKNWWLLGTSPWQ~SKSLIELNVAHNL 38
S815   ~~~~~~~~~~~~~~~SEFSPXXFLTXXXRLGWVGDNLASLEDFPIRHLKSLKELNVAHNL 45
J649   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~VRXNXCLLGTSPWQIXKSLIELNVAHNL 28
                                                          ********

S818   IDSFKLPDYFSNLPNLEHLDLSNNKIRKIYHEDLQVLHQMPSFKLSLDLSLNPLDFIQPG 105
S819   IDSFKLPDYFSHLPNRELLDLSNNKIRKIYHEDLQVLHQMPSFKLSLDLSLNPLDFIQPG 91
S817   IDSFKLPDYFSNLPNLEHLDLSNNKIRKIYHEDLQVLHQMPSFKLSLDLSLNPLDFIQPG 105
J628   IDSFKLPDYFSNLPNLEHLDLSNNKIRKIYHEDLQVLHQMPSFKLSLDLSLNPLDFIQPG 80
J656   IDSFKLPDYFSNLPNLEHLDLSNNKIRKIYHEDLQVLHQMPSFKLSLDLSLNPLDFIQPG 98
S815   IDSFKLPDYFSNLPNLEHLDLSNNKIRKIYHEDLQVLHQMPSFKLSLDLSLNPLDFIQPG 105
J649   IDSFKLPDYFSNLPNLEHLDLSNNKIRKIYHEDLQVLHQMPSFKLSLDLSLNPLDFIQPG 88
       ************************************************************

S818   AFEKIKLHELTLRSNFDSKKVMKTCIQGLAGLKINRLILGEFKNERNLDDLDKSALEELC 165
S819   AFEKIKLHELTLRSNFDSKKVMKTCIQGLAGLKINRLILGEFKNERNLDDLDKSALEELC 151
J656   AFEKIKLHELTLRSNFDSKKVMKTCIQGLAGLKINRLILGEFKNERNLDDLDKSALEELC 158
J628   AFEKIKLHELTLRSNFDSTEVMKTCIQGLAGLKINRLILGEFKNERNLVDLAKSALEELC 140
S817   AFEKIKLHELTLRSNFDSTEVMKTCIQGLAGLKINRLILGEFKNERNLVDLAKSALEELC 165
S815   AFEKIKLHELTLRSNFDSTEVMKTCIQGLAGLKINRLILGEFKNERNLVDLAKSALEELC 165
J649   AFEKIKLHELTLRSNFDSKKVMKTCIQGLAGLKINRLILGEFKNERNLDDLDKSALEELC 148
       ******************  ****************************** ********

S818   NLTIDEFRIAHFQDFPEDCRGFLNCLADASAVSLMSLKIGRLESLPTGFKWQYLKLSNCK 225
S819   NLTIDEFRIAHFQDFPEDCRGFLNCLADASAVSLMSLKIGRLESLPTGFKWQYLKLSNCK 211
J656   NLTIDEFRIAHFQDFPEDCRGFLNCLADASAVSLMSLKIGRLESLPTGFKWQYLKLSNCK 218
J628   NLTIDEFRIAHFQNFSKDYRGFLNCLADASAVSLMSLHIDRLESLPTGFKWQYLKLSNCK 200
S817   NLTIDEFRIAHFQNFSKDYRGFLNCLADASAVSLMSLHIDRLESLPTGFKWQYLKLSNCK 225
S815   NLTIDEFRIAHFQNFSKDYRGFLNCLADASAVSLMSLHIDRLESLPTGFKWQYLKLSNCK 225
J649   NLTIDEFRIAHFQDFPEDCRGFLNCLADASAVSLMSLKIGRLESLPTGFKWQYLKLSNCK 208
       ************************************************************
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S818   FQDFPTLELTFLKQFIFTANKVITTFTKLNLRNLEFLDLSRNGLSFKSCCSDRDFGTTRL 285
S819   FQDFPTLELTFLKQFIFTANKVITTFTKLNLRNLEFLDLSRNGLSFKSCCSDRDFGTTRL 271
J656   FQDFPTLELTFLKQFIFTANKVITTFTKLNLRNLEFLDLSRNGLSFKSCCSDRDFGTTRL 278
J628   FKDFPTLELTFLKQFVFTANKGITTFTEVNLRNLEFLDLSRKWLEFQVLLLSPDFWDNPT 260
S817   FKDFPTLELTFLKQFVFTANKGITTFTEVNLRNLEFLDLSRNGLSFKSCCSRRDFGTTQL 285
S815   FKDFPTLELTFLKQFVFTANKGITTFTEVNLRNLEFLDLSRNGLSFKSCCSRRDFGTTQL 285
J649   FQDFPTLELTFLKQFIFTANKVITTFTKLNLRNLEFLDLSRNGLSFKSCCSDRDFGTTRL 268
       ************************** ************ *        ***

S818   KHLDLSFNSIITMTSNFVGLEQIEHLDFQHSTLERQASTFSVFLSLKNLLYLDISYTDIK 345
S819   KHLDLSFNSIISMVLNFVGLEQIEHLDFQHSTLERQASTFSVFLSLENLLLPDYLFTTSR 331
J656   KHLDLSFNSIITMTSNFVGLEQIEHLDFQHSTLERQASTFSVFLSLENLLYLDISYTDIK 338
J628   ETLRSELQSYYTMTSNFVGLEQIEHLDFQHSTLERQASTFSVFLSLKNLLYLDISYTDIK 320
S817   KHLDLSFNSIITMTSNFVGLEQIEHLDFQHSTLERQASTFSVFLSLKNLLYLDISYTDIK 345
S815   KHLDLSFNSIIPMLQTSWALESKENIWISSIPLEDRPVLFQYSSHSKTSFTLISLTLTSR 345
J649   KHLDLSFIVLHDMFKLSWALESKENIWISSIPLEDRPVLFQSSHSLKTSFTLISLTLTSR 328

                  *      **  *

S818   IVFQGIFDGLISLQVLKMAGNSFQDAFLPNIFRDLTQLTVLDLSQCQLEQVSPEAFGSLL 405
S819   LSSRASLMAWSASKSLKMAGNSFQDAFLPNIFRDLTQLTVLDLSQCQLEQVSPEAFGSLL 391
J656   IVFQGIFDGLVSLQVLKMAGNSFQDAFLPNIFRDLTQLTVLDLSQCQLEQVSPEAFGSLL 398
J628   IVFQGIFDGLISLQVLKMAGNSFQDAFLPNIFRDLTQLTVLDLSQCQLEQVSPEAFGSLL 380
S817   IVFQGIFDGLISLQVLKMAGNSFQDSFLPNIFRDLTQLTVLDLSQCQLEQVSPEAFGSLL 405
S815   LSSRASLMALSASKVLKMAGNSFQDSFLPNIFRDLTQLTVLDLSQCQLEQVSPEAFGSLL 405
J649   FVFQGIFDGLISLQVLKMAGNSFQDAFLPNIFRDLTQLTVLDLSQCQLEQVSPEAFGSLL 388
                      ************************************************

S818   RLQVLNMSHNHLLSLDMLPYKNLSLWLLDYSFNRIVAANGQELQHIPSNVTSLNLTQNDF 465
S819   RLQVLNMSHNHLLSLDMLPYKNLSLWLLDYSFNRIVAANGQELQHIPSNVTSLNLTQNDF 451
J656   RLQVLNMSHNHLLSLDMLPYKNLSLWLLDYSFNRIVAANGQELQHIPSNVTSLNLTQNDF 458
J628   RLQVLNMSHNHLLSLDMLPYKNLSLWLLDYSFNRIVAANGQELQHIPSNVTSLNLTQNDF 440
S817   RLQVLNMSHNHLLSLDMLPYKNLSLWLLDYSFNRIVAANGQELQHIPSNVTSLNLTQNDF 465
S815   RLQVLNMSHNHLLSLDMLPYKNLSLWLLDYSFNRIVAANGQELQHIPSNVTSLNLTQNDF 465
J649   RLQVLNMSHNHLLSLDMLPYKNLSLWLLDYSFNRIVAANGQELQHIPSNVTSLNLTQNDF 448

       ************************************************************

S818   ACVCEHMCFLQWVQDHRRILVGAEHMMCKTPLAMQGVPVLSFRNTTCQMNKTVISVSVLS 525
S819   ACVCEHMCFLQWVQDHRRILVGAEHMMCKTPLAMQGVPVLSFRNTPPARNKTKLLVCQFS 511
J656   ACVCEHMCFLQWVQDHRRILVGAEHMMCKTPLAMQGVPVLSFRNTTCQMNKTVISVSVLS 518
J628   ACVCEHMRFLQWVQDHRRILVGAEHMMCKTPLAMQGVPVLSFRNTTCQMNKTVISVSVLS 500
S817   ACVCEHMCFLQWVQDHRRILVGAEHMMCKTPLAMQGVPVLSFRNTTCQMNKTVISVSVLS 525
S815   ACVCEHMCFLQWVQDHRRILVGAEHMMCKTPLAMQGVPVLSFRNTTCQMNKTVISVSVLS 525
J649   ACVCEHMRFLQWVQDHRRILVGAEHMMCKTPLAMQGVPVLSFRNTTCQMNKTVISVSVLS 508
       ********************************************** ***   *   *

S818   VLIVSVAAVLVYKFYFHLMLLAGCRRKVYGKGDSMYDAFVIYSSHGHDEDWVRNELVKNL 585
S819   VYSYLVAAVLVYKFYFHLMLSGWLQRKVRQKGQHVRCLCHLLQPHGHDEGLGEEEVGEEL 571
J656   VLIISVAAVLVYKFYFHLMLLADCRRKVYGKGDSMYDAFVIYSSHGHDEDWVRNELVKNL 578
J628   VLIVSVAAVLVYKFYFHLMLLADCRRKVYGKGGQHVRCLCHLLQPGHDEGLGEEEVGEEL 560
S817   VLIISVAAVLVYKFYFHLMLLAGCRRKVYGKGDSMYDAFVIYSSHGHDEDWVRNELVKNL 585
S815   VLIISVAAVLVYKFYFHLMLLAGCRRKVYGKGDSMYDAFVIYSSHGHDEDWVRNELVKNL 585
J649   VLIVSVAAVLVYKFYFHLMLLAGCRKKVRQRGQHVRCLCHLLQPHGMNEDWGEEEVVENX 568
       *     ***************
```

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S818   EEGXPPFXXLPSL~~~ 598
S819   GGXXTPLXXXP~~~~~ 582
J656   EXGXXPFQLCLHYXRL 594
J628   XEG~CPPFSSAFT~~~ 572
S817   XEG~XPPFQLCLHYXX 600
S815   XXGVPPFQL~CLHY~~ 596
J649   GGGGTPLSAXAFTTXK 584
```

Figure 5.2: Clustal W amino acid sequence alignment of *TLR4* derived from 7 pipistrelle bats. Glycosylation sites are highlighted in bold. *Footnote:* Bat J649, a soprano pipistrelle, was free of protozoan and helminth infections. Only 7 sequences shown as these are representative of the diversity (see Figure 5.3)

A phylogenetic tree was assembled using 54 of the TLR4 protein sequences (data from baby bats was excluded since the baby immune system is not fully developed and babies are excluded from infection analysis) in order to more closely examine the relationships between the different sequences (Figure 5.3). The resulting Neighbor-Joining phylogram showed that the TLR4 sequences positioned into 7 clusters; albeit, the bootstrap support for some of the clusters was relatively weak (Figure 5.3 and Table 5.1). Assembly of the data using UPGMA and minimum-evolution approaches also produced phylograms that positioned the TLR4 protein sequences into the 7 clusters (data not shown). Overall, the TLR4 amino acid sequence identities between the different pipistrelles ranged from 85 -100%. Moreover, outgroup sequences were added to the phylogenetic tree in order to have better view of the different clusters and check whether there might be any change in these clusters and the relationships between the TLR4 sequences and the phylogenetic tree with the outgroups showed the same outcome (Figure5.4).

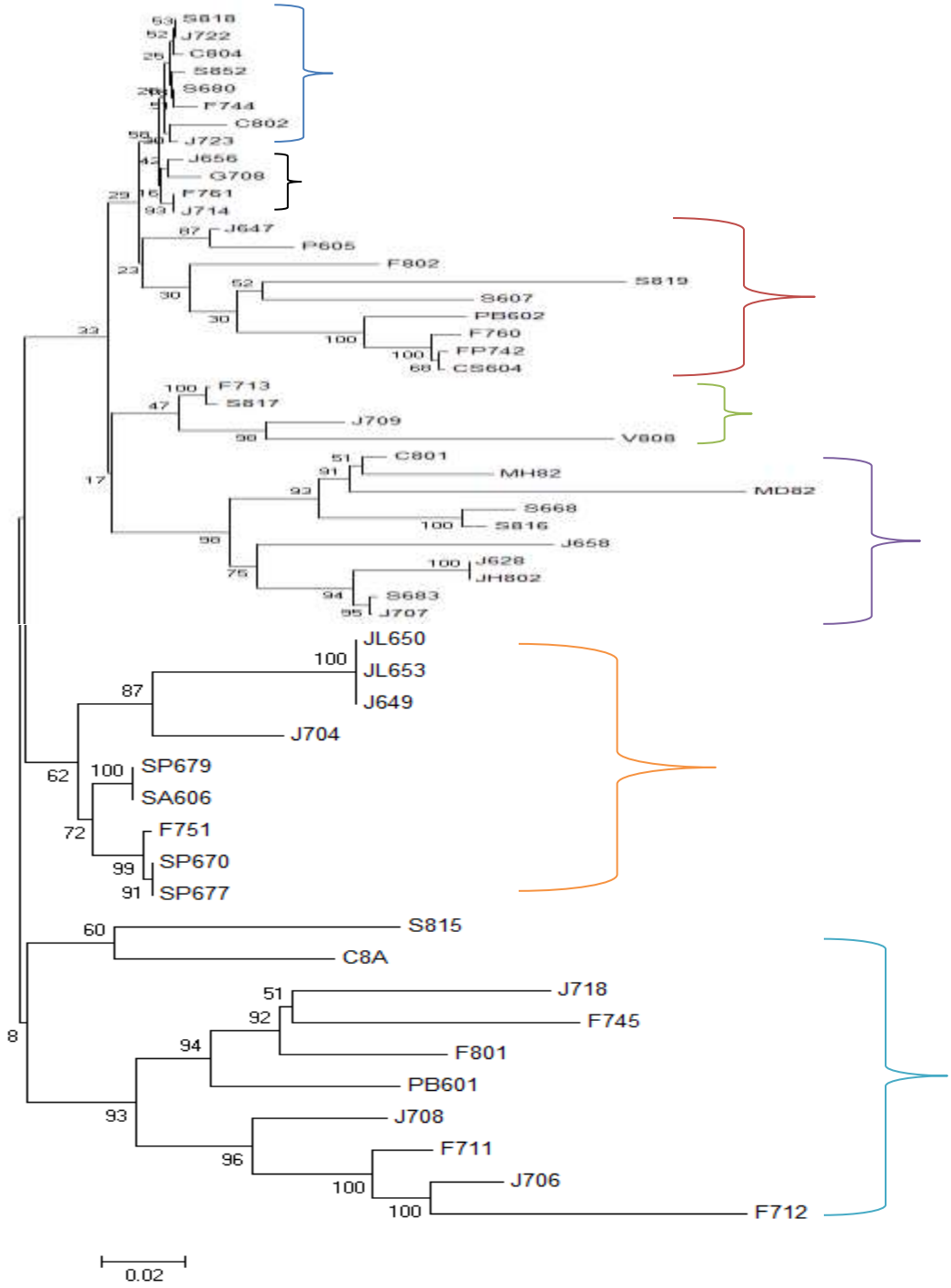


Figure 5.3: Neighbor-Joining phylogenetic tree of the pipistrelle *TLR4* protein sequences. Bootstrap support values (%) are shown on the nodes. Clusters as follows: Blue = 1, Black = 2, Red = 3, Green = 4, Purple = 5, Orange = 6 and Light Blue = 7. *Footnote:* Babies are excluded from the analysis and the soprano pipistrelles are within cluster 6 (codes: J649, JL650 and JL653).

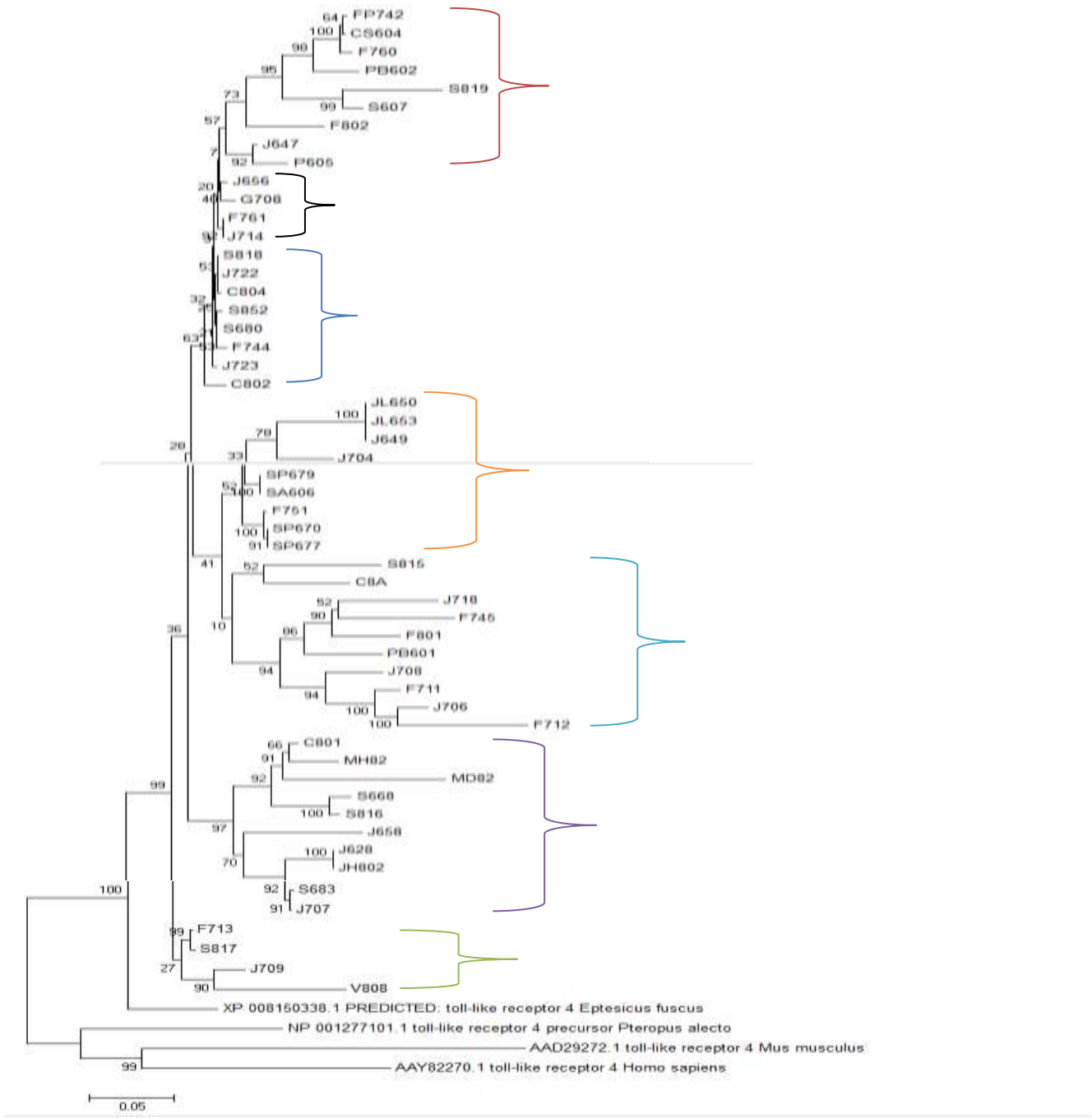


Figure 5.4: Neighbor-Joining phylogenetic tree of the pipistrelle TLR4 protein sequences with different TLR4 outgroups sequences. Bootstrap support values (%) are shown on the nodes. Clusters as follows: Blue = 1, Black = 2, Red = 3, Green = 4, Purple = 5, Orange = 6 and Light Blue = 7. *Footnote:* Babies are excluded from the analysis and the soprano pipistrelles are within cluster 6 (codes: J649, JL650 and JL653).

Table 5.1: *TLR4* cluster frequencies in the pipistrelles

TLR4 clusters	Frequency	Bat codes
1	15%	S818, J722, C804, S852, S680, F744, C802, J723
2	7%	J656, G708, F761, J714
3	16%	J647, P605, F802, S819, S607, PB602, F760, FP742, CS604
4	7%	F713, S817, J709, V808
5	18%	C801, MH82, MD82, S668, S816, J658, J628, JH802, S683, J707
6	16%	J649, JL650, JL653, J704, SP679, SA606, F751, SP670, SP677
7	18%	S815, C8A, J718, F745, F801, PB601, J708, F711, J706, F712

With respect to the bat genotyping data (Dodd et al, 2014), the TLR4 gene was sequenced in 6 of the 12 bats reported to be of mixed genotype origin and these bats TLR4 sequences were dispersed across 5 of the 7 TLR4 clusters: cluster 1 (code: S818), cluster 4 (code: J709), cluster 5 (code: J707), cluster 6 (code: J704) and cluster 7 (codes: PB601 and F712). This is not surprising given that the genotyping was based upon microsatellite analysis of 11 polymorphic loci and hence it is more representative of genetic homo-/heterogeneity than the single locus TLR4 gene.

5.2.2 *TLR4* clusters and parasite infections:

As the genetically mixed bats were not infected with eimerians and this seemed a significant observation (Chapter 3), it would appear, given the divergence of TLR4 sequences derived from these bats, to exclude a role for TLR4 in conferring resistance to *E. rioarribaensis*.

The parasite infection profiles were analysed with respect to the pipistrelle TLR4 clusters to explore further any potential correlations. Interestingly, 3 bats within TLR4 cluster 3 (F802, P605, PB602) were recorded as being infected with *T. gondii* (Dodd et al., 2014) and there

were no further bats within the TLR4-sequenced subset positive for *T. gondii* infection. This profile of *T. gondii* infection was significant (Fisher's exact test: $p = 0.003$). Also, TLR4 cluster 6 contained six bats with trypanosome infections (*T.dionisii* and *T. vespertilionis*) whereas 13 additional trypanosome infections were dispersed across the six other clusters (TLR4 clusters 1 and 7: 3 infected bats; TLR4 clusters 2, 4 and 5: 2 infected bats; TLR4 cluster 3: 1 infected bat). This profile of trypanosome infections was significant (Fisher's exact test: $p = 0.043$). The infection profiles of the other protozoan parasites were not significant when assessed against TLR4 cluster.

With respect to helminth burden (Lord et al., 2012), the mean intensity of helminth infection across the 54 pipistrelles for which a TLR4 sequence was derived was 56 ± 65. Within each TLR4 cluster the mean helminth burden ranged from 21 (cluster 6) to 99 (cluster 7) (Table 5.2) and interestingly, there appeared to be a significantly lower mean worm intensity for bats within TLR4 cluster 6 (*t*-test, $p = 0.04$). The t-test was done by comparing worm burden for cluster 6 against worm burden for all the other clusters because of the sample size, some clusters has low number of samples. So, t-test was the appropriate test to use.

Table 5.2: worm burden in the seven *TLR4* clusters

cluster #	worm burden
1	55/ +/- 48
2	55/ +/- 51
3	42/ +/- 75
4	56/ +/-45
5	50/ +/- 68
6	21/ +/- 47
7	99/ +/- 75

Upon inspecting the primary amino acids sequences within TLR4 clusters 3 and 6 and comparing these to the TLR4 protein sequences from the other clusters, it was not possible to state with any confidence if specific amino acids might be responsible for conferring host susceptibility to *T. gondii*, or trypanosomes, or resistance to high helminth burdens. Indeed, there were many TLR4 amino acid changes noted and some of these changes were very common, for example; K332E, I356V and C473R occurred in 17, 16 and 26 of the pipistrelles respectively (Table 5.3). Moreover, these changes were apparent within the different Leucine Rich Repeat (LRR) regions of the pipistrelle TLR4 protein (Table 5.3). In contrast, and perhaps importantly, the seven predicted N-glycosylation sites (positions: 166, 411, 427, 454, 459, 509, 515) were completely conserved in all the pipistrelle TLR4 proteins sequences.

Table 5.3: Summary of all the non-synonymous amino acids changes observed in the pipistrelle *TLR4* gene sequences (babies excluded from the analysis). *Footnotes*: All the amino acid positions are based on the S818 sequence in Figure 5.2. *P. pygmaeus* bats (included in cluster 6) are highlighted in red font, Cluster 3 in Blue, and Cluster 6 in Green.

LRRs	Position	Amino acids change	Number of samples	Frequency of change	Bat code
	253	K ➔ E	12	20%	V808, C8A, S815, J709, S817, F713, JH802, S683, J707, J706, J628, F712
LRR3	254	L➔ V	12	20%	V808, C8A, S815, J709, S817, F713, JH802, S683, J707, J706, F712
		L ➔ T	5	8%	MD82, S668, S816, C801, MH82
	256	L ➔S	5	8%	MD82, S668, S816, C801, MH82
	257	R➔K	5	8%	MD82, S668, S816, C801, MH82
	258	N ➔K	5	8%	MD82, S668, S816, C801, MH82
	259	L➔P	5	8%	MD82, S668, S816, C801, MH82
	261	F ➔ V	5	8%	MD82, S668, S816, C801, MH82
	262	L ➔S	5	8%	MD82, S668, S816, C801, MH82
	263	D ➔R	5	8%	MD82, S668, S816, C801, MH82
	265	S ➔Q	5	8%	MD82, S668, S816, C801, MH82
	267	N➔K	9	15%	MD82, S668, S816, C801, MH82, J628, JH802, S683, J707
	268	G➔W	9	15%	MD82, S668, S816, C801, MH82, J628, JH802, S683, J707
	270	E➔S	9	15%	MD82, S668, S816, C801, MH82, J628, JH802, S683, J707
	272	K➔Q	9	15%	MD82, S668, S816, C801, MH82, J628, JH802, S683, J707
	273	S➔V	10	17%	MD82, S668, S816, C801, MH82, J628, JH802, S683, J707, J658
	274	L➔C	10	17%	MD82, S668, S816, C801, MH82, J628, JH802, S683, J707, J658
	275	L➔C	11	18%	MD82, S668, S816, C801, MH82, J628, JH802, S683, J707, J658, F712
LRR4	281	G➔W	11	18%	J628, J658, MD82, JH802, S683, J707, S668, S816, C801, MH82, F712
	282	T ➔D	11	18%	J628, J658, MD82, JH802, S683, J707, S668, S816, C801, MH82, F712
	283	T➔N	11	18%	J628, J658, MD82, JH802, S683, J707, S668, S816, C801, MH82, F712
	284	R➔Q	7	12%	V808, C8A, S815, J709, S817, F713, J706
		R ➔ P	11	18%	J628, J658, MD82, JH802, S683, J707, S668, S816, C801, MH82, F712
	285	L ➔T	11	18%	J628, J658, MD82, JH802, S683, J707, S668, S816, C801, MH82, F712
	286	K ➔ E	11	18%	J628, J658, MD82, JH802, S683, J707, S668, S816, C801, MH82, F712
	287	H ➔ T	11	18%	J628, J658, MD82, JH802, S683, J707, S668, S816, C801, MH82, F712

	289	D ➔R	11	18%	J628, J658, MD82, JH802, S683, J707, S668, S816, C801, MH82, F712
	290	L ➔S	11	18%	J628, J658, MD82, JH802, S683, J707, S668, S816, C801, MH82, F712
	291	S ➔ E	11	18%	J628, J658, MD82, JH802, S683, J707, S668, S816, C801, MH82, F712
	292	F ➔ L	11	18%	J628, J658, MD82, JH802, S683, J707, S668, S816, C801, MH82, F712
	293	N➔ Q	11	17%	J628, J658, MD82, JH802, S683, J707, S668, S816, C801, MH82, F712
		N ➔ I	8	18%	F711, **J649**, J704, SP679, J708, **JL650**, **JL653**, SA606
	295	I ➔ Y	11	18%	J628, J658, MD82, JH802, S683, J707, S668, S816, C801, MH82, F712
		I ➔ L	6	10%	**J649**, J704, SP679, **JL650**, **JL653**, SA606
	296	I ➔ Y	11	18%	J628, J658, MD82, JH802, S683, J707, S668, S816, C801, MH82, F712
	297	T ➔P	10	17%	S668, C801, S815, SP679, SA606, J704, SP670, SP677, F751, C802
	299	T ➔L	10	17%	S668, C801, S815, SP679, SA606, J704, SP670, SP677, F751, C802
	300	S ➔ Q	10	17%	S668, C801, S815, SP679, SA606, J704, SP670, SP677, F751, C802
	301	N ➔T	10	17%	S668, C801, S815, SP679, SA606, J704, SP670, SP677, F751, C802
	302	F ➔ S	10	17%	S668, C801, S815, SP679, SA606, J704, SP670, SP677, F751, C802
		F➔ L	7	12%	F712, J708, J706, F711, C8A, V808, PB601
	303	V ➔W	13	22%	S668, C801, S815, SP679, SA606, J704, SP670, SP677, F751, C802, **J649**, **JL650**, **JL653**
		V➔R	6	10%	F712, J708, J706, F711, C8A, PB601
	304	G ➔A	13	20%	S668, C801, S815, SP679, SA606, J704, SP670, SP677, F751, C802, **J649**, **JL650**, **JL653**
	308	I ➔ N	8	13%	J706, F712, F745, F711, F801, PB601, J708, J718
		I ➔ K	10	17%	J704, **J649**, **JL650**, **JL653**, SP679, SA606, F751, C8A, SP670, SP677
	309	E ➔ R	8	13%	J706, F712, F745, F711, F801, PB601, J708, J718
	310	H➔ T	8	13%	J706, F712, F745, F711, F801, PB601, J708, J718
		H➔ N	11	18%	J704, **J649**, **JL650**, **JL653**, SP679, SA606, F751, C8A, SP670, SP677, S815
	311	L ➔ S	8	13%	J706, F712, F745, F711, F801, PB601, J708, J718
		L ➔ I	11	18%	J704, **J649**, **JL650**, **JL653**, SP679, SA606, F751, C8A, SP670, SP677, S815
	312	D ➔G	8	13%	J706, F712, F745, F711, F801, PB601, J708, J718
		D➔W	11	18%	J704, **J649**, **JL650**, **JL653**, SP679, SA606, F751, C8A, SP670, SP677, S815
	314	Q➔ P	8	13%	J706, F712, F745, F711, F801, PB601, J708, J718
		Q➔ S	13	22%	J704, **J649**, **JL650**, **JL653**, SP679, SA606, F751, C8A, SP670, SP677, S815, S668, S816
	315	H ➔A	8	13%	J706, F712, F745, F711, F801, PB601, J708, J718
		H ➔S	11	18%	J704, **J649**, **JL650**, **JL653**, SP679, SA606, F751, C8A, SP670, SP677, S815

	316	S ➔ F	7	13%	J706, F712, F745, F711, F801, PB601, J708
		S ➔ I	11	18%	J704, **J649**, **JL650**, **JL653**, SP679, SA606, F751, C8A, SP670, SP677, S815
	317	T ➔H	8	13%	J706, F712, F745, F711, F801, PB601, J708, J718
		T ➔P	11	18%	J704, **J649**, **JL650**, **JL653**, SP679, SA606, F751, C8A, SP670, SP677, S815
	318	L➔F	8	13%	J706, F712, F745, F711, F801, PB601, J708, J718
	320	R ➔T	8	13%	J706, F712, F745, F711, F801, PB601, J708, J718
		R ➔D	13	22%	J704, **J649**, **JL650**, **JL653**, SP679, SA606, F751, C8A, SP670, SP677, S815, S668, S816
	322	A ➔P	14	22%	J704, **J649**, **JL650**, **JL653**, SP679, SA606, F751, C8A, SP670, SP677, S815, S668, S816, J718
		A ➔Q	7	12%	J706, F712, F745, F711, F801, PB601, J708
LRR5	328	F➔ S	14	22%	J704, **J649**, **JL650**, **JL653**, SP679, SA606, F751, C8A, SP670, SP677, S815, S668, S816, J718
		F➔ P	7	12%	J706, F712, F745, F711, F801, PB601, J708
	332	K➔ E	17	28%	J647, MD82, J656, F802, F761, PB602, S819, F760, J714, P605, S607, S816, C8A, J709, G708, FP742, CS604
	334	L➔ S	8	13%	J718, J704, F801, S815, **J649**, **JL650**, **JL653**, JH802
	335	L➔ F	8	13%	J718, J704, F801, S815, **J649**, **JL650**, **JL653**, JH802
	336	Y➔ T	8	13%	J718, J704, F801, S815, **J649**, **JL650**, **JL653**, JH802
	338	D➔ I	9	15%	J718, J704, F801, S815, **J649**, **JL650**, **JL653**, JH802, PB601
	341	Y➔ T	11	18%	J718, J706, F711, F712, F801, **J649**, **JL650**, **JL653**, S815, JH802, PB601
	342	T➔L	11	18%	J718, J706, F711, F712, F801, **J649**, **JL650**, **JL653**, S815, JH802, PB601
	343	D➔ T	11	18%	J718, J706, F711, F712, F801, **J649**, **JL650**, **JL653**, S815, JH802, PB601
	344	I➔ S	11	18%	J718, J706, F711, F712, F801, **J649**, **JL650**, **JL653**, S815, JH802, PB601
	345	K➔ R	8	18%	J718, J706, F711, F712, **J649**, **JL650**, **JL653**, S815
LRR6	356	I➔ V	16	27%	J647, MD8, J656, F802, F761, PB602, F760, J714, P605, J704, C8A, J709, PB601, G708, FP742, CS604
	357	S➔ A	8	13%	J718, J706, F711, F712, S819, F745, F801, S815
	358	L➔ S	8	13%	J718, J706, F711, F712, S819, F745, F801, S815
	359	Q➔ K	8	13%	J718, J706, F711, F712, S819, F745, F801, S815
	360	V➔ S	8	13%	J718, J706, F711, F712, S819, F745, F801, S815
	363	M➔W	8	13%	J718, J706, F711, F712, S819, F745, F801, S815
	364	A➔ L	8	13%	J718, J706, F711, F712, S819, F745, F801, S815
	365	G➔ A	8	13%	J718, J706, F711, F712, S819, F745, F801, S815
	366	N➔ I	8	13%	J718, J706, F711, F712, S819, F745, F801, S815
	367	S➔ P	8	13%	J718, J706, F711, F712, S819, F745, F801, S815
LRRCT	473	C➔ R	26	44%	F712, J658, S683, J707, JH802, F745, S607, PB602, FP742, F760, CS604, V808, J706, F711, **J649**, **JL650**, **JL653**, SP679, P605, J714, S852,

					F801, MD82, F761, J628, SA606
	510	T ➔ H	5	9%	J718, S607, FP742, CS604, S819
	514	M ➔D	6	10%	J718, S607, FP742, F760, PB602, CS604,
	516	K ➔Q	5	9%	J718, FP742, F760, PB602, CS604
	517	T ➔N	5	9%	J718, FP742, F760, PB602, CS604
	518	V ➔C	6	10%	J718, FP742, F760, PB602, CS604, C8A

When the number of TLR4 amino acid changes was compared between the bats, it was apparent that the *T. gondii* susceptible bats in cluster 3 had limited sequence changes (mean = 5.8 ±3.2). In contrast, the trypanosome susceptible/helminth "resistant" bats in cluster 6 had a much greater number of TLR4 amino acid changes (mean = 22.8 ±3.8). Overall, the mean number of TLR4 amino acid changes (relative to the sequence for bat S818) was 16.2 ±13.1. Only bats in TLR4 cluster 1 (mean number of amino acid changes = 0.13 ±0.35), and so most similar to bat S818, showed a significantly different (p <0.05) number of residue changes relative to the other TLR4 clusters. Although 2 bats within TLR4 cluster 1 lacked protozoans (codes: S818 and C804), the remainder had trypanosome, *B. vesperuginis* and/or eimerian infections and hence there was nothing unusual about the parasite profile within these bats relative to the rest of the population under study.

Table 5.4: Number of amino acid changes observed in the pipistrelle *TLR4* sequences relative to bat S818. *Footnote: P. pygmaeus* bats are highlighted in red.

Sample code	# of Multiple changes	Cluster #
S852	1	1
J647, J656, F802, G708	2	3,2,3,2
P605, J714, F761, S817	3	3,2,2,4
F713, S607	4	4,3
V808, J709	5	4,4
F760, C802, PB602	7	3,1,3
FP742, CS604	8	3,3
S819	11	3
J708, J658	17	7,5
F751, SP670, SP677, PB601	18	6,6,6,7
C8A	19	7
F745	23	7
SA606, S683, J628, SP679	24	6,5,5,6
J649, JL650, JL653	25	6,6,6
J707, J704	28	5,6

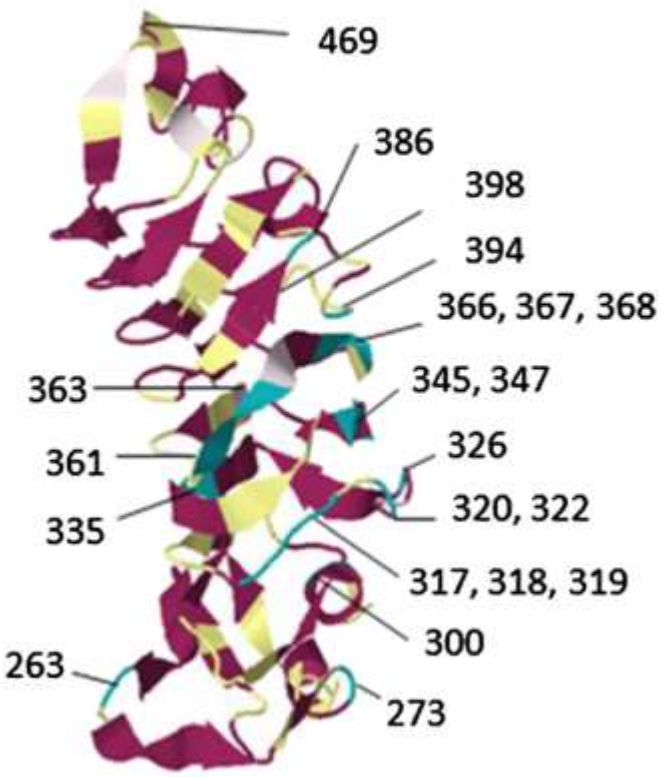
MH82	30	5
F711, F801	31	7,7
JH802	32	5
MD82, J706	33	5,7
S816, J718	34	5,7
F712, C801	37	5,7
S815	38	7
S668	41	5

5.2.3 Evolutionary conservation of *TLR4*:

A recent study of TLR4 sequence variation in 23 species of wild rodent highlighted that specific amino acids residues are under positive selection and hence are evolutionarily conserved, most probably due to a role in interacting with pathogen associated molecular patterns (Fornuskova et al., 2013). Consequently, this model of the rodent TLR4 (Figure 5.5) was used to scrutinized the pipistrelle TLR4 sequences by sequence alignment (Figure 5.6). The resulting data showed that of the 11 rodent TLR4 amino acid residues under positive selection, three sites were completely conserved in all the pipistrelle TLR4 sequences (E^{319}, F^{369} and I^{440}) (Table 5.5). Moreover, the rodent sequences also included F and I at the positions equivalent to F^{369} and I^{440} in the bat TLR4. Although the rodent species do not have a glutamate residue equivalent to E^{319} of the pipistrelle TLR4, this site is variable across the rodents and a similar residue, D^{347}, is present. In total, only 2 of the 11 sites noted as being under positive selection in the rodent TLR4 did not have a conserved amino acid residue in the pipistrelles: I/V^{335} and $A/K/Q/R/G/L/E^{361}$. This may be indicative of a difference between the interactions of rodent TLR4s and pipistrelle TLR4s with their respective ligands.

Of the 7 predicted bat N-glycosylation sites, N427, N454 and N515 (numbering based on bat S818) are not conserved in the rodent sequences, being replaced with Q, S and R residues respectively. This may further reflect differences between the bat and rodent TLR4s.

(a)



Codon	Residue Variety
263	R, K
273*	H, R, P
300	D, N, Y, V
317	S, H, D, P, Y
318	I, L, V
319	Q, R, K, E
320	H, Q, Y
322	A, K, G, E
326	S, N, K, R
335*	I, V
345*	A, T, P, K
347*	H, S, T, D, N, R, K
361*	A, Q, K, R, G, L, E
362	S, H, T, D, P
363*	T, M, I, L
366*	D, R, K, G
367	A, Q, T, D, R, K
368*	M, L, V
386	S, T, I, R
394*	F, M, L, V
398*	S, D, N, I, G
469*	A, T, I, G, V

Figure 5.5: Model of the *Rattus norvegicus TLR4* ligand binding region highlighting amino acid positions under variable evolutionary selection: turquoise = most variable; violet = most conserved; white = average conservation; yellow = insufficient data. Codons with the asterisk have been identified as being under positive selection(Fornůsková et al., 2013).

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 multiple sequence alignment:

S818         ----------------------------EFXPXXFSDYXFTXLGGWGANLASLEDFPIXH 32
J649         -----------------------------------------------VRXNXCLLGTSPWQIX 16
J628         ------------------------------------------------------RDLXMXVN 8
S815         ----------------------------SEFSPXXFLTXXXRLGWVGDNLASLEDFPIRHL 33
S817         ----------------------------SEFSPXXFLTXXXRLGWVGDNLASLEDFPIRHL 33
J656         ------------------------------------GLXXVYRXXWXGKKNWWLLGTSPWQ 25
S819         ---------------------------------------XXXXXDKPSIXRGLPHQTY 19
KC811688.1   CEIETIEDKAWHGLNQLSTLVLTGNPIKSFSPGSFSGLTNLENLVAVETKMTSLEGFHIG 60
KC811609.1   CEIETIEDKAWHGLHQLSTLVLTGNPIKSFSPGSFSGLTNLENLVAVETKLTSLEGFHIG 60

S818         -----------------LKSLKELNVAHNLIDSFKLPDYFSNLPNLEHLDLSNNKIRKIY 75
J649         -----------------KSLIELNVAHNLIDSFKLPDYFSNLPNLEHLDLSNNKIRKIY 58
J628         -----------------KSXLELNVAHNLIDSFKLPDYFSNLPNLEHLDLSNNKIRKIY 50
S815         -----------------KSLKELNVAHNLIDSFKLPDYFSNLPNLEHLDLSNNKIRKIY 75
S817         -----------------KSLKELNVAHNLIDSFKLPDYFSNLPNLEHLDLSNNKIRKIY 75
J656         -----------------SKSLIELNVAHNLIDSFKLPDYFSNLPNLEHLDLSNNKIRKIY 68
S819         -----------------KSLMELNVAHNLIDSFKLPDYFSHLPNRELLDLSNNKIRKIY 61
KC811688.1   ----------------QLISLKKLNVAHNLIHSFKLPEYFSNLTNLEHVDLSYNYIQTIS 104
KC811609.1   ----------------QLITLKKLNVAHNLIHSFKLPEYFSNLTNLEYVDLSYNYIQTIS 104
                              ************** *** * ***  *** * *
```

```
S818          HEDLQVLHQMPSFKLSLDLSLNPLDFIQPGAFEKIKLHELTLRSNFDSKKVMKTCIQGLA 135
J649          HEDLQVLHQMPSFKLSLDLSLNPLDFIQPGAFEKIKLHELTLRSNFDSKKVMKTCIQGLA 118
J628          HEDLQVLHQMPSFKLSLDLSLNPLDFIQPGAFEKIKLHELTLRSNFDSTEVMKTCIQGLA 110
S815          HEDLQVLHQMPSFKLSLDLSLNPLDFIQPGAFEKIKLHELTLRSNFDSTEVMKTCIQGLA 135
S817          HEDLQVLHQMPSFKLSLDLSLNPLDFIQPGAFEKIKLHELTLRSNFDSTEVMKTCIQGLA 135
J656          HEDLQVLHQMPSFKLSLDLSLNPLDFIQPGAFEKIKLHELTLRSNFDSKKVMKTCIQGLA 128
S819          HEDLQVLHQMPSFKLSLDLSLNPLDFIQPGAFEKIKLHELTLRSNFDSKKVMKTCIQGLA 121
KC811688.1    VKDLQFLRENPQVNLSLDLSLNPIDSIQAQAFQGIRLHELTLRSNFNSSNVLKMCLQNMT 164
KC811609.1    VKDLQFLRENPQVNLSLDLSLNPIDSIQAQAFQGIRLHELTLRSNFNSSNVLKMCLQNMT 164
                *** *   *   ***************      * ********** *  * * * *

S818          GLKINRLILGEFKNERNLDDLDKSALEELCNLTIDEFRIAHFQDFPEDCRGFLNCLADAS 195
J649          GLKINRLILGEFKNERNLDDLDKSALEELCNLTIDEFRIAHFQDFPEDCRGFLNCLADAS 178
J628          GLKINRLILGEFKNERNLVDLAKSALEELCNLTIDEFRIAHFQNFSKDYRGFLNCLADAS 170
S815          GLKINRLILGEFKNERNLVDLAKSALEELCNLTIDEFRIAHFQNFSKDYRGFLNCLADAS 195
S817          GLKINRLILGEFKNERNLVDLAKSALEELCNLTIDEFRIAHFQNFSKDYRGFLNCLADAS 195
J656          GLKINRLILGEFKNERNLDDLDKSALEELCNLTIDEFRIAHFQDFPEDCRGFLNCLADAS 188
S819          GLKINRLILGEFKNERNLDDLDKSALEELCNLTIDEFRIAHFQDFPEDCRGFLNCLADAS 181
KC811688.1    GLHVHRLILGEFKNERNLESFDRSVMEGLCNVSIDEFRLTYINHFSDDIYN-LNCLANIS 223
KC811609.1    GLHVHRLILGEFKNERNLESFDRSVMEGLCNVTIDEFRLTYINHFSDDIYN-LNCLANVS 223
              **   **************    *  * ***  ******     *       *****  *

S818          AVSLMSLKIGRLESLPTGFKWQYLKLSNCKFQDFPTLELTFLKQFIFTANKVITTFTKLN 255
J649          AVSLMSLKIGRLESLPTGFKWQYLKLSNCKFQDFPTLELTFLKQFIFTANKVITTFTKLN 238
J628          AVSLMSLHIDRLESLPTGFKWQYLKLSNCKFKDFPTLELTFLKQFVFTANKGITTFTEVN 230
S815          AVSLMSLHIDRLESLPTGFKWQYLKLSNCKFKDFPTLELTFLKQFVFTANKGITTFTEVN 255
S817          AVSLMSLHIDRLESLPTGFKWQYLKLSNCKFKDFPTLELTFLKQFVFTANKGITTFTEVN 255
J656          AVSLMSLKIGRLESLPTGFKWQYLKLSNCKFQDFPTLELTFLKQFIFTANKVITTFTKLN 248
S819          AVSLMSLKIGRLESLPTGFKWQYLKLSNCKFQDFPTLELTFLKQFIFTANKVITTFTKLN 241
KC811688.1    AMSFTGVHIKHIADVPRHFKWQSLSIIRCHLKPFPKLSLPFLKSWTLTTNREDISFGQLA 283
KC811609.1    XMSFTGVYLKHIADVPRHFKWQSLSIIRCHLKPFPKLSLPFLKSWTLTTNREDISFGQLA 283
                *               **** *    *       * * ***    * *     *

S818          LRNLEFLDLSRNGLSFKSCCSDRDFGTTRLKHLDLSFNSIITMTSNFVGLEQIEHLDFQH 315
J649          LRNLEFLDLSRNGLSFKSCCSDRDFGTTRLKHLDLSFIVLHDMFKLSWALESKENIWISS 298
J628          LRNLEFLDLSRKWLEFQVLLLSPDFWDNPTETLRSELQSYYTMTSNFVGLEQIEHLDFQH 290
S815          LRNLEFLDLSRNGLSFKSCCSRRDFGTTQLKHLDLSFNSIIPMLQTSWALESKENIWISS 315
S817          LRNLEFLDLSRNGLSFKSCCSRRDFGTTQLKHLDLSFNSIITMTSNFVGLEQIEHLDFQH 315
J656          LRNLEFLDLSRNGLSFKSCCSDRDFGTTRLKHLDLSFNSIITMTSNFVGLEQIEHLDFQH 308
S819          LRNLEFLDLSRNGLSFKSCCSDRDFGTTRLKHLDLSFNSIISMVLNFVGLEQIEHLDFQH 301
KC811688.1    LPSLRYLDLSRNAMSFRGCCSYSDFGTNNLKYLDLSFNGVILMSANFMGLEELEYLDFQH 343
KC811609.1    LPSLRYLDLSRNAMSFRGCCSYSDVGTNGLKYLDLSFNGVILMSANFMGLEELEYLDFQH 343
              ** *  ******   *       *                  *       *

S818          STLERQASTFSVFLSLKNLLYLDISYTDIKIVFQGIFDGLISLQVLKMAGNSFQDAFLPN 375
J649          IPLEDRPVLFQSSHSLKTSFTLISLTLTSRFVFQGIFDGLISLQVLKMAGNSFQDAFLPN 358
J628          STLERQASTFSVFLSLKNLLYLDISYTDIKIVFQGIFDGLISLQVLKMAGNSFQDAFLPN 350
S815          IPLEDRPVLFQYSSHSKTSFTLISLTLTSRLSSRASLMALSASKVLKMAGNSFQDSFLPN 375
S817          STLERQASTFSVFLSLKNLLYLDISYTDIKIVFQGIFDGLISLQVLKMAGNSFQDSFLPN 375
J656          STLERQASTFSVFLSLENLLYLDISYTDIKIVFQGIFDGLVSLQVLKMAGNSFQDAFLPN 368
S819          STLERQASTFSVFLSLENLLLPDYLFTTSRLSSRASLMAWSASKSLKMAGNSFQDAFLPN 361
KC811688.1    STLK-KVTEFSVFLSLEKLLYLDISYTNTKIDFDGIFLGLISLNTLKMAGNSFKDNTLSN 401
KC811609.1    STLK-KVTEFSVFLSLEKLLYLDISYTNTRIDFDGIFLGLISLNTLKMAGNSFKDNTLSN 401
                                                        ****** *  * *

S818          IFRDLTQLTVLDLSQCQLEQVSPEAFGSLLRLQVLNMSHNHLLSLDMLPYKNLS-LWLLD 434
J649          IFRDLTQLTVLDLSQCQLEQVSPEAFGSLLRLQVLNMSHNHLLSLDMLPYKNLS-LWLLD 417
J628          IFRDLTQLTVLDLSQCQLEQVSPEAFGSLLRLQVLNMSHNHLLSLDMLPYKNLS-LWLLD 409
S815          IFRDLTQLTVLDLSQCQLEQVSPEAFGSLLRLQVLNMSHNHLLSLDMLPYKNLS-LWLLD 434
S817          IFRDLTQLTVLDLSQCQLEQVSPEAFGSLLRLQVLNMSHNHLLSLDMLPYKNLS-LWLLD 434
J656          IFRDLTQLTVLDLSQCQLEQVSPEAFGSLLRLQVLNMSHNHLLSLDMLPYKNLS-LWLLD 427
S819          IFRDLTQLTVLDLSQCQLEQVSPEAFGSLLRLQVLNMSHNHLLSLDMLPYKNLS-LWLLD 420
KC811688.1    VFTNTTNLTFLDLSKCQLEQISRGVFDTLYRLQLLNMSHNNLLFLDPSHYKQLYSLRTLD 463
KC811609.1    VFTNTTNLTFLDLSKCQLEQISWGVFDTLYRLQLLNMSHNNLLFLDPSHYKQLYSLRTLD 463
               *   * ** **** ***** *   *  * ********** ** **   ** *  *  **

S818          YSFNRIVAANGQELQHIPSNVTSLNLTQNDFACVCEHMCFLQWVQDHRRILVGAEHMMCK 494
J649          YSFNRIVAANGQELQHIPSNVTSLNLTQNDFACVCEHMRFLQWVQDHRRILVGAEHMMCK 477
J628          YSFNRIVAANGQELQHIPSNVTSLNLTQNDFACVCEHMRFLQWVQDHRRILVGAEHMMCK 469
S815          YSFNRIVAANGQELQHIPSNVTSLNLTQNDFACVCEHMCFLQWVQDHRRILVGAEHMMCK 494
S817          YSFNRIVAANGQELQHIPSNVTSLNLTQNDFACVCEHMCFLQWVQDHRRILVGAEHMMCK 494
J656          YSFNRIVAANGQELQHIPSNVTSLNLTQNDFACVCEHMCFLQWVQDHRRILVGAEHMMCK 487
S819          YSFNRIVAANGQELQHIPSNVTSLNLTQNDFACVCEHMCFLQWVQDHRRILVGAEHMMCK 480
KC811688.1    CSFNRIETSKG-ILQHFPKSLAVFNLTNNSVACICEYQNFLQWVKDQKMFLVNVEQMKCA 521
KC811609.1    CSFNRIETSKG-ILQHFPKSLAVFNLTNNSVACICEYQNFLQWVKDQKMFLVNVEQMKCA 521
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S818          TPLAMQGVPVLSFRNTTCQMNKTVISVSVLSVLIVSVAAVLVYKFYFHLMLLAGCRRKVY 554
J649          TPLAMQGVPVLSFRNTTCQMNKTVISVSVLSVLIVSVAAVLVYKFYFHLMLLAGCRKKVR 537
J628          TPLAMQGVPVLSFRNTTCQMNKTVISVSVLSVLIVSVAAVLVYKFYFHLMLLADCRRKVY 529
S815          TPLAMQGVPVLSFRNTTCQMNKTVISVSVLSVLIISVAAVLVYKFYFHLMLLAGCRRKVY 554
S817          TPLAMQGVPVLSFRNTTCQMNKTVISVSVLSVLIISVAAVLVYKFYFHLMLLAGCRRKVY 554
J656          TPLAMQGVPVLSFRNTTCQMNKTVISVSVLSVLIISVAAVLVYKFYFHLMLLADCRRKVY 547
S819          TPLAMQGVPVLSFRNTPPARNKTKLLVCQFSVYSYLVAAVLVYKFYFHLMLSGWLQRKVR 540
KC811688.1    SPIDMKASLVLDFTNSTCYIYKTIISVSVVSVLVVATVAFLIYHFYFHLILIAGCKK--Y 579
KC811609.1    SPIDMKASLVLDFTNSTCYIYKTIISVSVVSVLVVATVAFLIYHFYFHLILIAGCKK--Y 579
               *  *    **** *      **   *    *      * *   ***** *

S818          GKG-DSMYDAFVIYSSHGHDEDWVRNELVKNLEEG-XPPFXX---LPSL---------- 598
J649          QRG-QHVRCLCHLLQPHGMNEDWGEEEVVENXGGG-GTPLSAXAFTTXK---------- 584
J628          GKGGQHVRCLCHLLQP-GHDEGLGEEEVGEELXEG-CPPFSSAFT--------------- 572
S815          GKG-DSMYDAFVIYSSHGHDEDWVRNELVKNLXXG-VPPFQL-CLHY------------ 598
S817          GKG-DSMYDAFVIYSSHGHDEDWVRNELVKNLXEG-XPPFQL-CLHYXX---------- 600
J656          GKG-DSMYDAFVIYSSHGHDEDWVRNELVKNLEXG-XXPFQLCLHYXRL---------- 594
S819          QKG-QHVRCLCHLLQPHGHDEGLGEEEVGEELGGX-XTPLXX----XP------------ 582
KC811688.1    SRG-ESIYDAFVIYSS--QNEDWVRNELVKNLEEG-VPRFQLCLHYRDFIPGVAIAANII 635
KC811609.1    NRG-ESIYDAFVIYSS--QNEDWVRNELVKNLEEG-VPRFQLCLHYRDFIPGVAIAANII 635
                              *     *    *

S818          ---------------------------------------------------------- 598
J649          ---------------------------------------------------------- 584
J628          ---------------------------------------------------------- 572
S815          ---------------------------------------------------------- 598
S817          ---------------------------------------------------------- 600
J656          ---------------------------------------------------------- 594
S819          ---------------------------------------------------------- 582
KC811688.1    QEGFHKSRKVIVVVSRHFIQSRWCIFEYEIAQTWQFLSSRSGIIFIVLEKVEKSLLRQQV 695
KC811609.1    QEGFHKSRKVIVVVSKHFIQSRWCIFEYEIAQTWQFLSSRSGIIFIVLEKVEKSLLRQQV 695


S818          ---------------------------------------------------- 598
J649          ---------------------------------------------------- 584
J628          ---------------------------------------------------- 572
S815          ---------------------------------------------------- 598
S817          ---------------------------------------------------- 600
J656          ---------------------------------------------------- 594
S819          ---------------------------------------------------- 582
KC811688.1    ELYRLLSRNTYLEWEDNALGRHIFWRRLKKALLDGKALNPDETSEEEQEATTLT 749
KC811609.1    ELYRLLSRNTYLEWEDNALGRHIFWRRLKKALLDGKALNPDGTSEEEQETTTFT 749
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Figure 5.6: Clustal W alignment of representative pipistrelle *TLR4* sequences (one from each cluster) with the *TLR4* sequence from Rattus norvegicus (KC811688.1) and the greater bandicoot rat, *Bandicota indica* (KC811609.1) (Fornuskova et al, 2013). *Footnote*: J649 and S819 are representative of *TLR4* clusters 6 and 3 respectively.

Table 5.5: *TLR4* amino acid variability in the pipistrelles at the positions identified by Fornuskova et al., (2013) as being under positive selection in rodents. *Footnote*: all the numbered positions in pipistrelle bats are based upon the *TLR4* sequence in bat specimen S818.

Amino acid variability/positions	Amino acid in pipistrelles/positions	Bat codes
H, R, P/ 273*	P, N/245	MD82, all the other pipistrelles
I, V/335*	E, R, S/289=LRR4	J718, F711, F712, F745, PB601, J708, J706, S668, S816, F751, V808, all the other pipistrelles
A,T,P,K/345*	T, P, H/317	S818, J647, S680, MD82, F761, J656, F802, F711, PB602, J722, J628, J658, S683, S819, F760, J714, P605, C802, S607, F713, J723, F744, S852, C804, JH802, J707, C801, MH82, J709, V808, G708, FP742, CS604, S817, J704, J649, JL650, JL653, SA606, SP679, F751, C8A, SP670, SP677, S815, J718, J706, F711, F712, F745, F801
H, S, T, D, N, R, K /347*	E/319	All the 54 pipistrelles
A, K, Q, R, G, L, E /361*	N, T, P/334=LRR5	S818, J647, S680, MD82, F761, J656, F802, PB602, J722, J628, J658, S683, S819, F760, P605, C802, S607, F713, SA606, SP679, J723, F751, F744, S852, C804, JH802, J707, C801, MH82, J709, V808, C8A, G708, FP742, CS604, S817, SP670, SP677, J718, J704, F801, J649, JL650, JL653, S815, J706, F711, F712, F745, PB601
T,M,I,L/363*	F, L/336=LRR5	J718, J704, F801, S815, J649, JL650, JL653, all the other pipistrelles
D, R, K, G/366*	I, Y, D/339=LRR5	J718, F801, S815, J649, JL650, JL653, PB601, J706, F711, F712, all the other pipistrelles

M, L, V/368*	L, F, S/441=LRR5	J718, S819, F745, J718, F801, S815, J649, JL650, JL653, PB601, J706, F711, F712, all the other pipistrelles
F, M, L, V/394*	F/369=LRR6	All the 54 pipistrelles
S, D,N,I,G/398*	S, H, A/371=LRR6	C802, S815, S852, S817, J706, F711, F712, all the other pipistrelles
A,T,I,G,V/469*	I/440	All the 54 pipistrelles

5.3 *TLR2*

5.3.1 Sequence analysis:

Following the PCR-based isolation strategy (Chapter 4) 59 pipistrelle TLR2 gene fragments were subjected to DNA sequencing and the resulting data, including the translated amino acid sequences were aligned using Clustal W. The TLR2 DNA sequences were highly conserved amongst the pipistrelles; there were 5 haplotypes that generated 5 different protein sequences and the representative sequences are shown below (Figures 5.7 and 5.8).

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S818  ~~~~~~~~~~~~~~~~~~~~~~~~TGGGGACCGTAATCAGCCTGTTCAAGGAAGGGGCCCN  37
JL613 ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ 0
JL647 ATGCTTGTGGACAGTGTGGGTCTTGGGGACCGTAATCAGCCTGTTCAAGGAAGGGGCCCN 60
J707  ATGCTTGTGGACAGTGTGGGTCTTGGGGACCGTAATCAGCCTGTTCAAGGAAGGGGCCCN 60
J649  ~~~~~~~~~~~~~~~~~~~~~~~~TGGGGACCGTAATCAGCCTGTTCAAGGAAGGGGCCCN 37

S818  TGATCAGGCTTTTCCTCTGACTTGTGACCCCACGGGGGTCTGCGATGGCCACTCCAGATC 97
JL613 ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ATC 3
JL647 NGATCAGGCTTTTCCTCTGACTTGTGACCCCACGGGGGTCTGCGATGGCCACTCCAGATC 120
J707  NGATCAGGCTTTTCCTCTGACTTGTGACCCCACGGGGGTCTGCGATGGCCACTCCAGATC 120
J649  TGATCAGGCTTTTCCTCTGACTTGTGACCCCACGGGGGTCTGCGATGGCCACTCCAGATC 97
                                                              ***

S818  TTTAATCTCCATCCCCTCAGGGCTCACGGCAACTGTGACGAGCCTCGACCTGTCCAACAA 157
JL613 TTTGATCTCCATCCCCTCCGGGCTCACGGCAACTGTGACGAGCCTCGACCTGTCCAACAA 63
JL647 TTTAATCTCCATCCCCTCAGGGCTCACGGCAACTGTGACGAGCCTCGACCTGTCCAACAA 180
J707  TTTAATCTCCATCCCCTCAGGGCTCACGGCAACTGTGACGAGCCTCGACCTGTCCAACAA 180
J649  TTTAATCTCCATCCCCTCAGGGCTCACGGCAACTGTGACGAGCCTCGACCTGTCCAACAA 157
      *** ************************************************************

S818  CAAGATCGCCTATGTCAGCAACAGCGACCTGCGGATGTGTGTGAACCTCAGGGCTCTGAG 217
JL613 CAAGATCGCCTATGTCAGCAACAGCGACCTGCGGATGTGTGTGAACCTCAGGGCTCTGAG 123
JL647 CAAGATCGCCTATGTCAGCAACAGCGACCTGCGGATGTGTGTGAACCTCAGGGCTCTGAG 240
J707  CAAGATCGCCTATGTCAGCAACAGCGACCTGCGGATGTGTGTGAACCTCAGGGCTCTGAG 240
J649  CAAGATCGCCTATGTCAGCAACAGCGACCTGCGGATGTGTGTGAACCTCAGGGCTCTGAG 217
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S818   GCTGGGATCCAATAGCATTGACACGATAGAGGAAGATTCCTTTTTCTCCCTGGGGAGTCT 277
JL613  GCTGGGATCCAATAGCATTGACACGATAGAGGAAGATTCCTTTTTCTCCCTGGGGAGTCT 183
JL647  GCTGGGATCCAATAGCATTGACACGATAGAGGAAGATTCCTTTTTCTCCCTGGGGAGTCT 300
J707   GCTGGGATCCAATAGCATTGACACGATAGAGGAAGATTCCTTTTTCTCCCTGGGGAGTCT 300
J649   GCTGGGATCCAATAGCATTGACACGATAGAGGAAGATTCCTTTTTCTCCCTGGGGAGTCT 277
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S818   TGAACATTTGGACTTATCCTATAATCACTTAGCTAATTTATCAGCCTCCTGGTTCAGGCC 337
JL613  TGAACATTTGGACTTATCCTATAATCACTTAGCTAATTTATCAGCCTCCTGGTTCAGGCC 243
JL647  TGAACATTTGGACTTATCCTATAATCACTTAGCTAATTTATCAGCCTCCTGGTTCAGGCC 360
J707   TGAACATTTGGACTTATCCTATAATCACTTAGCTAATTTATCAGCCTCCTGGTTCAGGCC 360
J649   TGAACATTTGGACTTATCCTATAATCACTTAGCTAATTTATCAGCCTCCTGGTTCAGGCC 337
       ************************************************************

S818   TCTTACTTCCTTGAACGTCTTAAACTTATTGGGAAACCCTTACAAAACACTTGGGAAAAC 397
JL613  TCTTACTTCCTTGAACGTCTTAAACTTACTGGGAAACCCTTACAAAACACTTGGGAAAAC 303
JL647  TCTTACTTCCTTGAACGTCTTAAACTTATTGGGAAACCCTTACAAAACACTTGGGAAAAC 420
J707   TCTTACTTCCTTGAACGTCTTAAACTTATTGGGAAACCCTTACAAAACACTTGGGAAAAC 420
J649   TCTTACTTCCTTGAACGTCTTAAACTTATTGGGAAACCCTTACAAAACACTTGGGAAAAC 397
       ************************************************************

S818   ACCTCTTTTTTCTCATCTCACCAAATTGCGAATCCTAAAAGTAGGACATAGTTACCTCTT 457
JL613  ACCTCTTTTTTCTCATCTCACCAAATTGCGAATCCTAAAAGTAGGACATAGTTACCTCTT 363
JL647  ACCTCTTTTTTCTCATCTCACCAAATTGCGAATCCTAAAAGTAGGACATAGTTACCTCTT 480
J707   ACCTCTTTTTTCTCATCTCACCAAATTGCGAATCCTAAAAGTAGGACATAGTTACCTCTT 480
J649   ACCTCTTTTTTCTCATCTCACCAAATTGCGAATCCTAAAAGTAGGACATAGTTACCTCTT 457
       ************************************************************

S818   CACTGAAATTCAGGAAAAGGATTTTGTTGGGCTAACTTTTCTCAAAGAGCTTGAGATCGA 517
JL613  CACTGAAATTCAGGAAAAGGATTTTGTTGGGCTAACTTTTCTCAAAGAGCTTGAGATCGA 423
JL647  CACTGAAATTCAGGAAAAGGATTTTGTTGGGCTAACTTTTCTCAAAGAGCTTGAGATCGA 540
J707   CACTGAAATTCAGGAAAAGGATTTTGTTGGGCTAACTTTTCTCAAAGAGCTTGAGATCGA 540
J649   CACTGAAATTCAGGAAAAGGATTTTGTTGGGCTAACTTTTCTCAAAGAGCTTGAGATCGA 517
       ************************************************************

S818   TGCTTCCAATCTCCAGAAGTATGCGCCTAGGAGTTTGAAGGTGATTCAGAACATCAGCCA 577
JL613  TGCTTCCAATCTCCAGAAGTATGCGCCTAGGAGTTTGAAGGTGATTCAGAACATCAGCCA 483
JL647  TGCTTCCAATCTCCAGAAGTATGCGCCTAGGAGTTTGAAGGTGATTCAGAACATCAGCCA 600
J707   TGCTTCCAATCTCCAGAAGTATGCGCCTAGGAGTTTGAAGGTGATTCAGAACATCAGCCA 600
J649   TGCTTCCAATCTCCAGAAGTATGCGCCTAGGAGTTTGAAGGTGATTCAGAACATCAGCCA 577
       ************************************************************

S818   CCTGATCCTTCACATGAAGCAGCCCACTTTCTTGATGAAGATTTCTGAGGATCTTTTAAG 637
JL613  CCTGATCCTTCACATGAAGCAGCCCACTTTCTTGATGAAGATTTCTGAGGATCTTTTAAG 543
JL647  CCTGATCCTTCACATGAAGCAGCCCACTTTCTTGATGAAGATTTCTGAGGATCTTTTAAG 660
J707   CCTGATCCTTCACATGAAGCAGCCCACTTTCTTGATGAAGATTTCTGAGGATCTTTTAAG 660
J649   CCTGATCCTTCACATGAAGCAGCCCACTTTCTTGATGAAGATTTCTGAGGATCTTTTAAG 637
       ************************************************************

S818   TTCCTTGGGACATTTGGAACTGAGAGATACTCATTTGGACAATTTCCATTTTTCAAAAGT 697
JL613  TTCCTTGGGACATTTGGAACTGAGAGATACTCATTTGGACAATTTCCATTTTTCAAAAGT 603
FP737  TTCCTTGGGACATTTGGAACTGAGAGATACTCATTTGGACAATTTCCATTTTTCAAAAGT 720
J707   TTCCTTGGGACATTTGGAACTGAGAGATACTCATTTGGACAATTTCCATTTTTCAAAAGT 720
J649   TTCCTTGGGACATTTGGAACTGAGAGATACTCATTTGGACAATTTCCATTTTTCAAAAGT 697
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S818   ATCCACCAATGAAACCAAGACCATTAAAAAGTTCACCTTTAGAAATGTGAAGATCACAGA 757
JL613  ATCCACCAATGAAACCAAGACCATTAAAAAGTTCACCTTTAGAAATGTGAAGATCACAGA 663
JL647  ATCCACCAATGAAACCAAGACCATTAAAAAGTTCACCTTTAGAAATGTGAAGATCACAGA 780
J707   ATCCACCAATGAAACCAAGACCATTAAAAAGTTCACCTTTAGAAATGTGAAGATCACAGA 780
J649   ATCCACCAATGAAACCAAGACCATTAAAAAGTTCACCTTTAGAAATGTGAAGATCACAGA 757
       ************************************************************

S818   TGAAGGTTTTAATGAAATGGTGAAACTGTTGAATCATGTTTCTGAAATATTAGATGTGGA 817
JL613  TGAAGGTTTTAATGAAATGGTGAAACTGTTGAATCATGTTTCTGAAATATTAGATGTGGA 723
JL647  TGAAGGTTTTAATGAAATGGTGAAACTGTTGAATCATGTTTCTGAAATATTAGATGTGGA 840
J707   TGAAGGTTTTAATGAAATGGTGAAACTGTTGAATCATGTTTCTGAAATATTAGATGTGGA 840
J649   TGAAGGTTTTAATGAAATGGTGAAACTGTTGAATCATGTTTCTGAAATATTAGATGTGGA 817
       ************************************************************

S818   ATTTGATAGCTGCACCCTCAATGGAATTGGTGATTTTGACATAACTGTTATGGACACAAA 877
JL613  ATTTGATAGCTGCACCCTCAATGGAATTGGTGATTTTGACATAACTGTTATGGACACAAA 783
JL647  ATTTGATAGCTGCACCCTCAATGGAATTGGTGATTTTGACATAACTGTTATGGACACAAA 900
J707   ATTTGATAGCTGCACCCTCAATGGAATTGGTGATTTTGACATAACTGTTATGGACACAAA 900
J649   ATTTGATAGCTGCACCCTCAATGGAATTGGTGATTTTGACATAACTGTTATGGACACAAA 877
       ************************************************************

S818   TAAAGATATAAGTAAAATAGAGACATTAACAATACGGAGGTTGTATATTCCAAATTTTTA 937
JL613  TAAAGATATAAGTAAAATAGAGACATTAACAATACGGAGGTTGTATATTCCAAATTTTTA 843
JL647  TAAAGATATAAGTAAAATAGAGACATTAACAATACGGAGGTTGTATATTCCAAATTTTTA 960
J707   TAAAGATATAAGTAAAATAGAGACATTAACAATACGGAGGTTGTATATTCCAAATTTTTA 960
J649   TAAAGATATAAGTAAAATAGAGACATTAACAATACGGAGGTTGTATATTCCAAATTTTTA 937
       ************************************************************

S818   CTCATTTTATGATCTGAGCAGTTTATATTCACTTACTGGAACAGTTAAGAGAATCACGAT 997
JL613  CTCATTTTATGATCTGAGCAGTTTATATTCACTTACTGGAACAGTTAAGAGAATCACGAT 903
JL647  CTCATTTTATGATCTGAGCAGTTTATATTCACTTACTGGAACAGTTAAGAGAATCACGAT 1020
J707   CTCATTTTATGATCTGAGCAGTTTATATTCACTTACTGGAACAGTTAAGAGAATCACGAT 1020
J649   CTCATTTTATGATCTGAGCAGTTTATATTCACTTACTGGAACAGTTAAGAGAATCACGAT 997
       ************************************************************

S818   AGAAAGCAGTAAGGTTTTCCTAGTTCCTTGTTCACTTTCGCAACACTTAAAATCATTAGA 1057
JL613  AGAAAGCAGTAAGGTTTTCCTAGTTCCTTGTTCACTTTCGCAACACTTAAAATCATTAGA 963
JL647  AGAAAGCAGTAAGGTTTTCCTAGTTCCTTGTTCACTTTCGCAACACTTAAAATCATTAGA 1080
J707   AGAAAGCAGTAAGGTTTTCCTAGTTCCTTGTTCACTTTCGCAACACTTAAAATCATTAGA 1080
J649   AGAAAGCAGTAAGGTTTTCCTAGTTCCTTGTTCACTTTCGCAACACTTAAAATCATTAGA 1057
       ************************************************************

S818   ATATTTGGACCTCAATGGCAACTTAATAGTTGAAAACTCATTGACAAACGCAGCCTGTGA 1117
JL613  ATATTTGGACCTCAATGGCAACTTAATAGTTGAAAACTCATTGACAAACGCAGCCTGTGA 1023
JL647  ATATTTGGACCTCAATGGCAACTTAATAGTTGAAAACTCATTGACAAACGCAGCCTGTGA 1140
J707   ATATTTGGACCTCAATGGCAACTTAATAGTTGAAAACTCATTGACAAACGCAGCCTGTGA 1140
J649   ATATTTGGACCTCAATGGCAACTTAATAGTTGAAAACTCATTGACAAACGCAGCCTGTGA 1117
       ************************************************************

S818   GTATGCCTGGCCCTCCCTGCAAACCTTAATCTTGAGGCAGAATCATCTGAGGTCGTTAGA 1177
JL613  GTATGCCTGGCCCTCCCTGCAAACCTTAATCTTGAGGCAGAATCATCTGAGGTCGTTAGA 1083
JL647  GTATGCCTGGGCCTCCCTGCAAACCTTAATCTTGAGGCAGAATCATCTGAGGTCGTTAGA 1200
J707   GTATGCCTGGCCCTCCCTGCAAACCTTAATCTTGAGGCAGAATCATCTGAGGTCGTTAGA 1200
J649   GTATGCCTGGCCCTCCCTGCAAACCTTAATCTTGAGGCAGAATCATCTGAGGTCGTTAGA 1177
       ************************************************************
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S818   AGAAACTGGAGAAGTTTTGCTTACTCTGAAAAACCTGACTAACCTTGATATCAGCAAGAA 1237
JL613  AGAAACTGGAGAAGTTTTGCTTACTCTGAAAAACCTGACTAACCTTGATATCAGCAAGAA 1143
JL647  AGAAACTGGAGAAGTTTTGCTTACTCTGAAAAACCTGACTAACCTTGATATCAGCAAGAA 1260
J707   AGAAACTGGAGAAGTTTTGCTTACTCTGAAAAACCTGACTAACCTTGATATCANCAAGAA 1260
J649   AGAAACTGGAGAAGTTTTGCTTACTCTGAAAAACCTGACTAACCTTGATATCAGCAAGAA 1237
       ************************************************************

S818   TAATTTCCATCCTATATCTAAAACTTGTCAGTGGCCAGAAAGGATGAAGTATTTGAACTT 1297
JL613  TAATTTCCATCCTATATCTAAAACTTGTCAGTGGCCAGAAAGGATGAAGTATTTGAACTT 1203
JL647  TAATTTCCATCCTATATCTAAAACTTGTCAGTGGCCAGAAAGGATGAAGTATTTGAACTT 1320
J707   TAATTTCCATCCTATATCTAAAACTTGTCAGTGGCCAGAAAGGATGAAGTATTTGAACTT 1320
J649   TAATTTCCATCCTATATCTAAAACTTGTCAGTGGCCAGAAAGGATGAAGTATTTGAACTT 1297
       ************************************************************

S818   ATCCAATACAAGAATACAGAGTTTAACCAAATGCATTCCTCAGACGCTGGAAGTTTTAGA 1357
JL613  ATCCAATACAAGAATACAGAGTTTAACCAAATGCATTCCTCAGACGCTGGAAGTTTTAGA 1263
JL647  ATCCAATACAAGAATACACAGTTTAACCAAATGCATTCCTCAGACGCTGGAAGTTTTAGA 1380
J707   ATCCAATACAAGAATACAGAGTTTAACCAAATGCATTCCTCAGACGCTGGAAGTTTTAGA 1380
J649   ATCCAATACAAGAATACAGAGTTTAACCAAATGCATTCCTCAGACGCTGGAAGTTTTAGA 1357
       ************************************************************

S818   TGTTAGCAATAATAGCCTCAGTTCGTTTTCGTTGACTATGCCACAACTCAGAGAACTTTA 1417
JL613  TGTTAGCAATAATAGCCTCAGTTCGTTTTCGTTGACTATGCCACAACTCAGAGAACTTTA 1323
JL647  TGTTAGCAATAATAGCCTCAGTTCGTTTTCGTTGACTATGCCACAACTCAGAGAACTTTA 1440
J707   TGTTAGCAATAACAGCCTCAGTTCGTTTTCGTTGACTATGCAACAACTCAGAGAACTTGA 1440
J649   TGTTAGCAATAATAGCCTCAGTTCGTTTTCGTTGACTATGCCACAACTCAGAGAACTTTA 1417
       ************ *************************** *************** *

S818   TATTTCCGGAAATAGGTTGAAGACTCTACCAGATGCCTCCTCCTTACCCATGTTACTCGT 1477
JL613  TATTTCCGGAAATAGGTTGAAGACTCTACCAGATGCCTCCTCCTTACCCATGTTACTCGT 1383
JL647  TATTTCCGGAAATAGGTTGAAGACTCTACCAGATGCCTCCTCCTTACCCATGTTACTCGT 1500
J707   TATTTCCGGAAATAGGTTGAAGACTCTACCAGATGCCTCCTCCTTACCCATGTTACTCGT 1500
J649   TATTTCCGGAAATAGGTTGAAGACTCTACCAGATGCCTCCTCCTTACCCATGTTACTCGT 1477
       ************************************************************

S818   CATGAGAATCAGCAGAAATACAATAAATACGTTCTCTAAGGAGCAACTTGATTCGTTTAA 1537
JL613  CATGAGAATCAGCAGAAATACAATAAATACGTTCTCTAAGGAGCAACTTGATTCGTTTAA 1443
JL647  CATGAGAATCAGCAGAAATACAATAAATACGTTCTCTAAGGAGCAACTTGATTCGTTTAA 1560
J707   CATGAGAATCAGCAGAAATACAATAAATACGTTCTCTAAGGAGCAACTTGATTCGTTTAA 1560
J649   CATGAGAATCAGCAGAAATACAATAAATACGTTCTCTAAGGAGCAACTTGATTCGTTTAA 1537
       ************************************************************

S818   AAAACTGAAGACTTTGGAAGCTGGCAGCAACAGTTTCATCTGTTCCTGCGAATTCCTGTC 1597
JL613  AAAACTGAAGACTTTGGAAGCTGGCAGCAACAGTTTCATCTGTTCCTGCGAATTCCTGTC 1503
JL647  AAAACTGAAGACTTTGGAAGCTGGCAGCAACAGTTTCATCTGTTCCTGCGAATTCCTGTC 1620
J707   AAAACTGAAGACTTTGGAAGCTGGCAGCAACAGTTTCATCTGTTCCTGCGAATTCCTGTC 1620
J649   AAAACTGAAGACTTTGGAAGCTGGCAGCAACAGTTTCATCTGTTCCTGCGAATTCCTGTC 1597
       ************************************************************

S818   CTTTACTCAGGGGCAGCAAGCACTGGCCCAAGTCCTGGTCGACTGGCCAGAAAACTACCT 1657
JL613  CTTTACTCAGGGGCAGCAAGCACTGGCCCAAGTCCTGGTCGACTGGCCAGAAAACTACCT 1563
JL647  CTTTACTCAGGGGCAGCAAGCACTGGCCCAAGTCCTGGTCGACTGGCCAGAAAACTACCT 1680
J707   CTTTACTCAGGGGCAGCAAGCACTGGCCCAAGTCCTGGTCGACTGGCCAGAAAACTACCT 1680
J649   CTTTACTCAGGGGCAGCAAGCAATGGCCCAAGTCCTGGTCGACTGGCCAGAAAACAACCT 1657
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S818   GTGCGATTCCCCATCCCATGTGCGGGGCCAGCGGGTGCAAGACACTCACCTCTCGGTTTC 1717
JL613  GTGCGATTCCCCATCCCATGTGCGGGGCCAGCGGGTGCAAGACACTCACCTCTCGGTTTC 1623
JL647  GTGCGATTCCCCATCCCATGTGCGGGGCCAGCGGGTGCAAGACACTCACCTCTCGGTTTC 1740
J707   GTGCGATTCCCCATCCCATGTGCGGGGCCAGCGGGTGCAAGACACTCACCTCTCGGTTTC 1740
J649   GTGCGATTCCCCATCCCATGTGCGGGGCCAGCGGGTGCAAGACACTCACCTCTCGGTTTC 1717
       ************************************************************

S818   TGAGTGCCACAGGGTGGCTGTGGTGTCTGCTGTGTGCTGTGCCCTTTTCCTGCTGATCCT 1777
JL613  TGAGTGCCACAGGGTGGCTGTGGTGTCTGCTGTGTGCTGTGCCCTTTTCCTGCTGATCCT 1683
JL647  TGAGTGCCACAGGGTGGCTGTGGTGTTTGCTGTGTGCTGTGCCCTTTTCCTGCTGATCCT 1800
J707   TGAGTGNCACAGGGTGGCTGTGGTGTCTGCTGTGTGCTGTGCCCTTTTCCTGCTGATCCT 1800
J649   TGAGTGCCACAGGGTGGCTGTGGTGTCTGCTGTGTGCTGTGCCCTTTTCCTGCTGATCCT 1777
       ************************* ******************************** 

S818   GCTCACTGGGGTTCTGTGCCACCGTTTCCATGGCCTGTGGTACATGAAGATGATGTGGGC 1837
JL613  GCTCACTGGGGTTCTGTGCCACCGTTTCCATGGCCTGTGGTACATGAAGATGATGTGGGC 1743
JL647  GCTCACTGGGGTTCTGTGCCACCGTTTCCATGGCCTGTGGTACATGAAGATGATGTGGGC 1860
J707   GCTCACTGGGGTTCTGTGCCACCGTTTCCATGGCCTGTGGTACATGAAGATGATGTGGGC 1860
J649   GCTCACTGGGGTTCTGTGCCACCGTTTCCATGGCCTGTGGTACATGAAGATGATGTGGGC 1837
       ************************************************************

S818   CTGGCTCCAGGCCAAAAGGAAGCCCAGGAGAGCCCCCCCGAGGGACCTCAGTTACGACGC 1897
JL613  CTGGCTCCAGGCCAAAAGGAAGCCCAGGAGAGCCCCCCCGAGGGACCTCAGTTACGACGC 1803
JL647  CTGGCTCCAGGCCAAAAGGAAGCCCAGGAGAGCCCCCCCGAGGGACCTCTGTTACGACGC 1920
J707   CTGGCTCCAGGCCAAAAGGAAGCCCAGGAGAGCCCCCCCGAGGGACCTCTGNTACGACGC 1920
J649   CTGGCTCCAGGCCAAAAGGAAGCCCAGGAGAGCCCCCCCGAGGGACCTCAGTTACGACGC 1897
       ************************************************************

S818   CTTTGTGTCTTACAGCGAGCAGGATTCCCACTGGGTGGAGAACCTGATGGTCCAGGAGCT 1957
JL613  CTTTGTGTCTTACAGCGAGCAGGATTCCCACTGGGTGGAGAACCTGATGGTCCAGGAGCT 1863
JL647  CTTTGTGTCTTACAGCGAGCAGGATTCCCACTGGGTGGAGAACCTGATGGTCCAGGAGCT 1980
J707   CTTTGTGTCTTACAGCGAGCAGGATTCCCACTGGGTGGAGAACCTGATGGTCCAGGAGCT 1980
J649   CTTTGTGTCTTACAGCGAGCAGGATTCCCACTGGGTGGAGAACCTGATGGTCCAGGAGCT 1957
       ************************************************************

S818   GGAGCACTTCGACCCTCCCTTCAAGCTGTGTCTTCATAAGCGGGACTTTGTTCCCGGCAA 2017
JL613  GGAGCACTTCGACCCTCCCTTCAAGCTGTGTCTTCATAAGCGGGACTTTGTTCCCGGCAA 1923
JL647  GGAGCACTTCGACCCTCCCTTCAAGCTGTGTCTTCATAAGCGGGACTTTGTTCCCGGCAA 2040
J707   GGAGCACTTCGACCCTCCCTTCAAGCTGTGTCTTCATAAGCGGGACTTTGTTCCCGGCAA 2040
J649   GGAGCACTTCGACCCTCCCTTCAAGCTGTGTCTTCATAAGCGGGACTTTGTTCCCGGCAA 2017
       ************************************************************

S818   GTGGATTATTGACAATATCATCGACTCCATCGAAAAGAGCCACAAAACCATCTTCGTGCT 2077
JL613  GTGGATTATTGACAATATCATCGACTCCATCGAAAAGAGCCACAAAACCATCTTCGTGCT 1983
JL647  GTGGATTATTGACAATATCATCGACTCCATCGAAAAGAGCCACAAAACCATCTTCGTGCT 2100
J707   GTGGATTATTGACAATATCATCGACTCCATCGAAAAGAGCCACAAAACCATCTTCGTGCT 2100
J649   GTGGATTATTGACAATATCATCGACTCCATCGAAAAGAGCCACAAAACCATCTTCGTGCT 2077
       ************************************************************
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S818   TTCCGAGAACTCGTGAAGA~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ 2096
JL613  TTCCGAGAACTCGTGAAGA~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ 2002
JL647  TTCCGAGAACTTGTGAAG~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ 2118
J707   TTCCGAGAACTTGTGAAGAGCGCGTGGTGCAAGTACGAGCTGGACTTCTCCCATTTCGCN 2160
J649   TTCCGAGAACTCGTGAAGA~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ 2096
       *********** ******
```

Figure 5.7: Clustal W DNA sequence alignment of representative pipistrelle TLR2 gene sequences. Other bat TLR2 sequences not shown since they were identical to one of the above. *Footnotes:* Bat J649 was a soprano pipistrelle and it was not infected with helminths or protozoans. Some of the above data were derived from Arianne Lovey (MSc student, University of Salford) as indicated in Chapter 4.

```
S818   ~~~~~~~~XGTVISLFKEGAXDQAFPLTCDPTGVCDGHSRSLISIPSGLTATVTSLDLSN 52
JL613  ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~IFVSIPSGLTATVTSLDLSN 20
JL647  XCLWTVWVLGTVISLFKEGAXDQAFPLTCDPTGVCDGHSRSLISIPSGLTATVTSLDLSN 60
J707   XCLWTVWVLGTVISLFKEGAXDQAFPLTCDPTGVCDGHSRSLISIPSGLTATVTSLDLSN 60
J649   ~~~~~~~~XGTVISLFKEGAXDQAFPLTCDPTGVCDGHSRSLISIPSGLTATVTSLDLSN 52
                                                 *****************

S818   NKIAYVSNSDLRMCVNLRALRLGSNSIDTIEEDSFFSLGSLEHLDLSYNHLA**N**LSASWFR 112
JL613  NKIAYVSNSDLRMCVNLRALRLGSNSIDTIEEDSFFSLGSLEHLDLSYNHLA**N**LSASWFR 80
JL647  NKIAYVSNSDLRMCVNLRALRLGSNSIDTIEEDSFFSLGSLEHLDLSYNHLA**N**LSASWFR 120
J707   NKIAYVSNSDLRMCVNLRALRLGSNSIDTIEEDSFFSLGSLEHLDLSYNHLA**N**LSASWFR 120
J649   NKIAYVSNSDLRMCVNLRALRLGSNSIDTIEEDSFFSLGSLEHLDLSYNHLA**N**LSASWFR 112
       ************************************************************

S818   PLTSLNVLNLLGNPYKTLGKTPLFSHLTKLRILKVGHSYLFTEIQEKDFVGLTFLKELEI 172
JL613  PLTSLNVLNLLGNPYKTLGKTPLFSHLTKLRILKVGHSYLFTEIQEKDFVGLTFLKELEI 140
JL647  PLTSLNVLNLLGNPYKTLGKTPLFSHLTKLRILKVGHSYLFTEIQEKDFVGLTFLKELEI 180
J707   PLTSLNVLNLLGNPYKTLGKTPLFSHLTKLRILKVGHSYLFTEIQEKDFVGLTFLKELEI 180
J649   PLTSLNVLNLLGNPYKTLGKTPLFSHLTKLRILKVGHSYLFTEIQEKDFVGLTFLKELEI 172
       ************************************************************

S818   DASNLQKYAPRSLKVIQ**N**ISHLILHMKQPTFLMKISEDLLSSLGHLELRDTHLDNFHFSK 232
JL613  DASNLQKYAPRSLKVIQ**N**ISHLILHMKQPTFLMKISEDLLSSLGHLELRDTHLDNFHFSK 200
JL647  DASNLQKYAPRSLKVIQ**N**ISHLILHMKQPTFLMKISEDLLSSLGHLELRDTHLDNFHFSK 240
J707   DASNLQKYAPRSLKVIQ**N**ISHLILHMKQPTFLMKISEDLLSSLGHLELRDTHLDNFHFSK 240
J649   DASNLQKYAPRSLKVIQ**N**ISHLILHMKQPTFLMKISEDLLSSLGHLELRDTHLDNFHFSK 232
       ************************************************************

S818   VST**N**ETKTIKKFTFRNVKITDEGFNEMVKLLNHVSEILDVEFDSCTLNGIGDFDITVMDT 292
JL613  VST**N**ETKTIKKFTFRNVKITDEGFNEMVKLLNHVSEILDVEFDSCTLNGIGDFDITVMDT 260
JL647  VST**N**ETKTIKKFTFRNVKITDEGFNEMVKLLNHVSEILDVEFDSCTLNGIGDFDITVMDT 300
J707   VST**N**ETKTIKKFTFRNVKITDEGFNEMVKLLNHVSEILDVEFDSCTLNGIGDFDITVMDT 300
J649   VST**N**ETKTIKKFTFRNVKITDEGFNEMVKLLNHVSEILDVEFDSCTLNGIGDFDITVMDT 292
       ************************************************************

S818   NKDISKIETLTIRRLYIPNFYSFYDLSSLYSLTGTVKRITIESSKVFLVPCSLSQHLKSL 352
JL613  NKDISKIETLTIRRLYIPNFYSFYDLSSLYSLTGTVKRITIESSKVFLVPCSLSQHLKSL 320
JL647  NKDISKIETLTIRRLYIPNFYSFYDLSSLYSLTGTVKRITIESSKVFLVPCSLSQHLKSL 360
J707   NKDISKIETLTIRRLYIPNFYSFYDLSSLYSLTGTVKRITIESSKVFLVPCSLSQHLKSL 360
J649   NKDISKIETLTIRRLYIPNFYSFYDLSSLYSLTGTVKRITIESSKVFLVPCSLSQHLKSL 352
       ************************************************************
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S818   EYLDLSGNLIVENSLTNAACEYAWPSLQTLILRQNHLRSLEETGEVLLTLKNLTNLDISK 412
JL613  EYLDLSGNLIVENSLTNAACEYAWPSLQTLILRQNHLRSLEETGEVLLTLKNLTNLDISK 380
JL647  EYLDLSGNLIVENSLTNAACEYAWPSLQTLILRQNHLRSLEETGEVLLTLKNLTNLDISK 420
J707   EYLDLSGNLIVENSLTNAACEYAWPSLQTLILRQNHLRSLEETGEVLLTLKNLTNLDISK 420
J649   EYLDLSGNLIVENSLTNAACEYAWPSLQTLILRQNHLRSLEETGEVLLTLKNLTNLDISK 412
       ************************************************************

S818   NNFHPISKTCQWPERMKYLNLSNTRIQSLTKCIPQTLEVLDVSNNSLSSFSLTMPQLREL 472
JL613  NNFHPISKTCQWPERMKYLNLSNTRIQSLTKCIPQTLEVLDVSNNSLSSFSLTMPQLREL 440
JL647  NNFHPISKTCQWPERMKYLNLSNTRIQSLTKCIPQTLEVLDVSNNSLSSFSLTMPQLREL 480
J707   NNFHPISKTCQWPERMKYLNLSNTRIQSLTKCIPQTLEVLDVSNNSAGSFRLQQPQFRFL 480
J649   NNFHPISKTCQWPERMKYLNLSNTRIQSLTKCIPQTLEVLDVSNNSLSSFSLTMPQLREL 472
       ************************************************  ** ***

S818   YISGNRLKTLPDASSLPMLLVMRISRNTINTFSKEQLDSFKKLKTLEAGSNSFICSCEFL 532
JL613  YISGNRLKTLPDASSLPMLLVMRISRNTINTFSKEQLDSFKKLKTLEAGSNSFICSCEFL 500
JL647  YISGNRLKTLPDASSLPMLLVMRISRNTINTFSKEQLDSFKKLKTLEAGSNSFICSCEFL 540
J707   YISGNRLKTLPDASSLPMLLVMRISRNTINTFSKEQLDSFKKLKTLEAGSNSFICSCEFL 540
J649   YISGNRLKTLPDASSLPMLLVMRISRNTINTFSKEQLDSFKKLKTLEAGSNSFICSCEFL 532
       ************************************************************

S818   SFTQGQQALAQVLVDWPENYLCDSPSHVRGQRVQDTHLSVSECHRVAVVSAVCCALFLLI 592
JL613  SFTQGQQALAQVLVDWPENYLCDSPSHVRGQRVQDTHLSVSECHRVAVVSAVCCALFLLI 560
JL647  SFTQGQQALAQVLVDWPENYLCDSPSHVRGQRVQDTHLFVSECHRVAVVFAVCCALFLLI 600
J707   SFTQGQQALAQVLVDWPENYLCDSPSHVRGQRVQDTHLSVSECHRVAVVFAVCCALFLLI 600
J649   SFTQGQQAMAQVLVDWPENYQCDSPSHVRGQRVQDTHLSVSECHRVAVVSAVCCALFLLI 592
       ******** *********** ***************** ********************

S818   LLTGVLCHRFHGLWYMKMMWAWLQAKRKPRRAPPRDLSYDAFVSYSEQDSHWVENLMVQE 652
JL613  LLTGVLCHRFHGLWYMKMMWAWLQAKRKPRRAPPRDLSYDAFVSYSEQDSHWVENLMVQE 620
JL647  LLTGVLCHRFHGLWYMKMMWAWLQAKRKPRRAPPRDLCYDAFVSYSEQDSHWVENLMVQE 660
J707   LLTGVLCHRFHGLWYMKMMWAWLQAKRKPRRAPPRDLXYDAFVSYSEQDSHWVENLMVQE 660
J649   LLTGVLCHRFHGLWYMKMMWAWLQAKRKPRRAPPRDLSYDAFVSYSEQDSHWVENLMVQE 652
       ************************************************************

S818   LEHFDPPFKLCLHKRDFVPGKWIIDNIIDSIEKSHKTIFVLSENS~R~~~~~~~~~~~~~ 698
JL613  LEHFDPPFKLCLHKRDFVPGKWIIDNIIDSIEKSHKTIFVLSENS~R~~~~~~~~~~~~~ 667
JL647  LEHFDPPFKLCLHKRDFVPGKWIIDNIIDSIEKSHKTIFVLSENFVK~~~~~~~~~~~~~ 706
J707   LEHFDPPFKLCLHKRDFVPGKWIIDNIIDSIEKSHKTIFVLSENFVKSAWCKYELDFSHF 720
J649   LEHFDPPFKLCLHKRDFVPGKWIIDNIIDSIEKSHKTIFVLSENS~R~~~~~~~~~~~~~ 698
       ****************************************
```

Figure 5.8: Clustal W amino acid sequence alignment of TLR2 derived from 5 pipistrelle bats. Glycosylation sites are highlighted in bold. *Footnotes:* Bat J649, a soprano pipistrelle, was free of protozoan and helminth infections. Only 5 sequences shown as these are representative of the diversity (see Figure 5.9).

The translated amino acid sequences showed that the pipistrelle TLR2 sequences were between 99% and 100% identical. Not surprisingly therefore, the 6 predicted N-glycosylation sites were fully conserved in all the 59 pipistrelle TLR2 sequences. However, interestingly, 7 of the pipistrelles showed heterozygosity in the TLR2 gene sequences and these all resulted in a non-synonymous amino acid change (Table 5.6). Moreover, one of these bats, F744, showed heterozygosity at two positions within the TLR2 gene. Closer inspection of the changes in relation to the gene model (Figure 4.13) highlighted that only two variable positions; F/S^{582} (bat JL628) and H/Q^{566} (bat F744), were within Leucine rich repeat regions (LRRs).

Table 5.6: Summary of the pipistrelle TLR2 heterozygosity observed in the bat population. *Footnotes*: all the numbered positions are based on bat S818 (see Figures 5.6 and 5.7). Data derived from Arianne Lovey (MSc student, University of Salford).

Nucleotide position	Nucleotide variations	Amino acids	Bat code
156	A/G	K/E^{52}	SP649
468	T/C	I/S^{156}	SA07?
741	C/T	R/S^{247}	S607
1746	T/C	F/S^{582}	JL628
1698/2025	T/A- T/A	H/Q^{566} I/M^{675}	F744
1794	A/C	L/M^{598}	J722
1884	T/A	D/E^{628}	JH802

All the TLR2 heterozygotes were determined by Arianne Lovey (MSc student, University of Salford). This was determined from both the forward and reverse sequence where double peaks were found when analysing the sequences which indicate that heterozygote is present in these TLR2 sequences. When comparing the heterozygosity found in the pipistrelle TLR2 gene with the TLR4 gene where no heterozygotes were found in any of the pipistrelle sequences, the data showed it is statistically significant (Fisher's exact test: $p = 0.012$).

On analysing the phylogeny of the TLR2 sequences, without taking account of the heterozygotic changes, it was apparent that they could be organised into 5 clusters. Most of the clusters had high bootstrap support, although cluster 1, with 45 bats, had relatively low bootstrap support (Figure 5.9). The frequency of each TLR2 cluster is shown in Table 5.7 and all the soprano bats were positioned with cluster 4. Most of the mixed genotype bats (Dodd et al., 2014) were not surprisingly positioned within TLR2 cluster 1. Analysis of the TLR2 sequences using UPGMA and minimum-evolution approaches also positioned the sequences into 5 clusters (data not shown). Moreover, phylogenetic tree was done with including different outgroup sequences and the outcome of the phylogeny showed the same number of clusters like without outgroups (Figure 5.10).

Figure 5.9: Neighbor-Joining phylogenetic tree of the pipistrelle TLR2 protein sequences. Bootstrap support values (%) are shown on the nodes. *Footnotes:* Babies are excluded from this analysis. Arrows show the pipistrelles heterozygotic for TLR2.

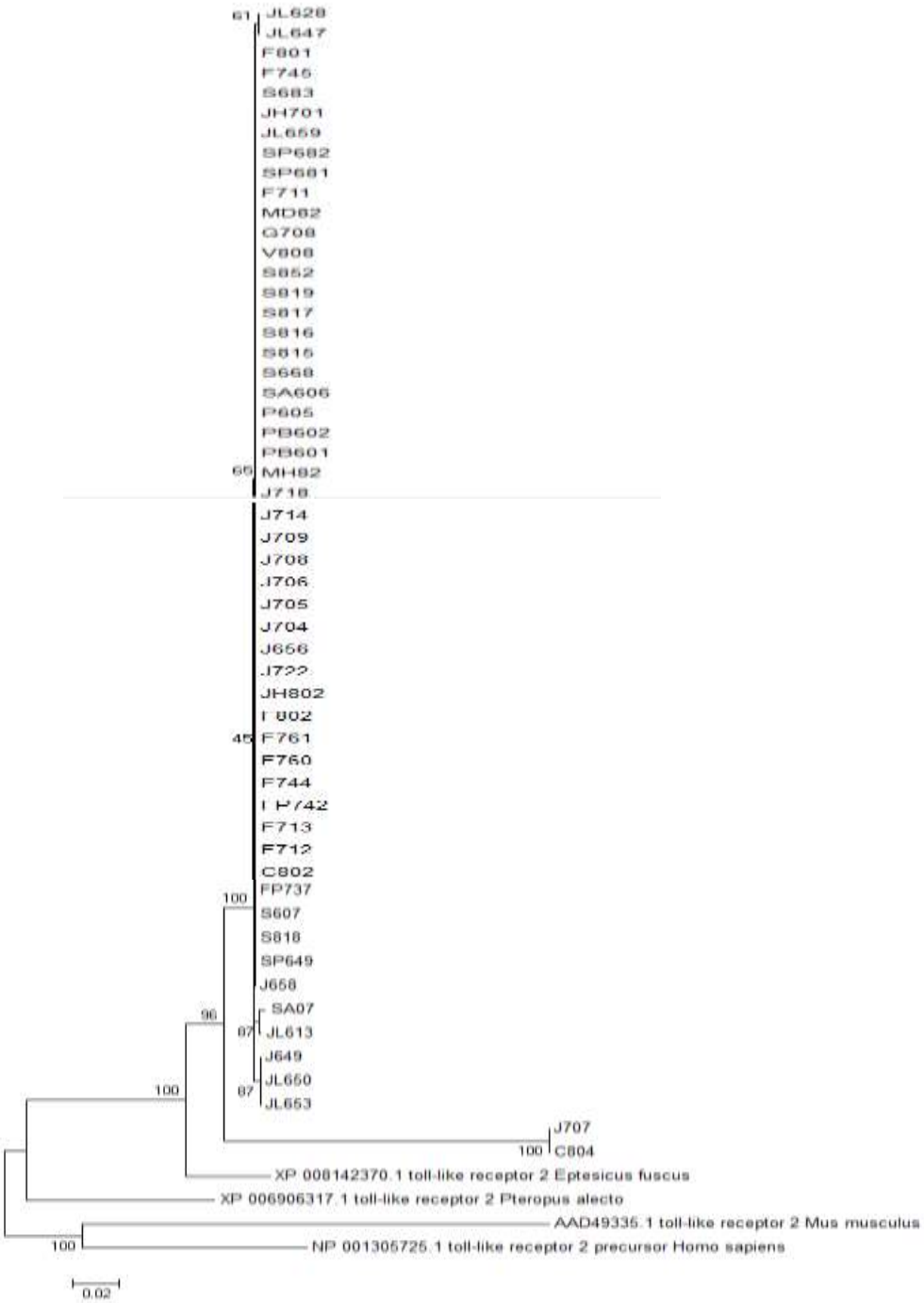

Figure 5.10: Neighbor-Joining phylogenetic tree of the pipistrelle TLR2 protein sequences with different TLR2 outgroups sequences. Bootstrap support values (%) are shown on the nodes. *Footnotes:* Babies are excluded from this analysis.

Table 5.7: TLR2 cluster frequencies

TLR2 clusters	Frequency	Bat codes
1	%86	All the other 45 pipistrelles
2	3%	SA07, JL613
3	3%	JL628, JL647
4	5%	J649, JL650, JL653
5	3%	J707, C804

5.3.2 TLR2 variations and parasite infections:

Given the dominance of TLR2 cluster 1, it was not possible to do any statistically meaningful analysis of the parasite infection profiles based upon cluster origin. Furthermore, very few amino acid changes were observed between TLR2 sequences of individual bats and hence a meaningful analysis of potential roles of any of these changes was not possible (Table 5.8).

Table 5.8: Summary of the amino acid changes observed in the TLR2 gene of 54 pipistrelle bats. *Footnote*: all the positions are based on the TLR2 sequence of bat S818 (see Figure 5.7).

Position	Amino acid change	Number of samples	Frequency of changes	Bat code
33	S ➔ I	2	3%	SA7?, JL613
34	L ➔ F	2	3%	SA7?, JL613
473	T ➔ Q	2	3%	J707, C804
474	M ➔ Q	2	3%	J707, C804
477	L ➔ F	2	3%	J707, C804
579	S ➔ F	2	3%	JL628, JL647

A subset of bats were heterozygous at the TLR2 locus (Table 5.6) and inspection of their parasite infections revealed that the mean helminth intensity of these bats (27.1 ±25) was significantly less than the mean helminth intensity of the TLR2 homozygotes (59.5 ±66) (*t*-test, *p*-value= 0.027). As the locations of acquisition for the TLR2 heterozygotes were scattered across the South Lancashire/Greater Manchester region then it is likely that this

helminth infection data cannot be explained by environmental differences. The low numbers of TLR2 heterozygous bats precluded a statistically meaningful analysis of whether, or not, these bats had any interesting protozoan infection differences compared to the homozygous bats.

5.4 TLR4/2 chimeric proteins

5.4.1 Sequence analysis:

In order to account for potential simultaneous expression and action of both TLR4 and TLR2 genes in defence against parasite infection, respective TLR amino acid sequences were truncated and then fused to generate chimeric TLR4/2 sequences for a subset of 45 bats. Phylogenetic analysis of the TLR4/2 chimeric sequences showed that 6 clusters were formed; albeit, bootstrap support for cluster 1 is less convincing (Figure 5.11). Six clusters were also formed when the sequences were analysed using UPGMA and minimum-evolution approaches (data not shown).

One less cluster is formed using the TLR4/2 sequences than is formed by TLR4 alone (Figure 5.3); this is most probably a consequence of the latter analysis including 9 additional sequences and also, that TLR2 sequences are so well conserved that they have little overall impact on the phylogeny. The soprano pipistrelles are positioned within cluster 4, the most frequent TLR4/2 cluster (Table 5.9). Five of the TLR2 heterozygotes are represented in the phylogeny and these bats (S607, F744/J722 and JL628/JH802) are localized to TLR4/2 clusters 1, 3 and 6 respectively.

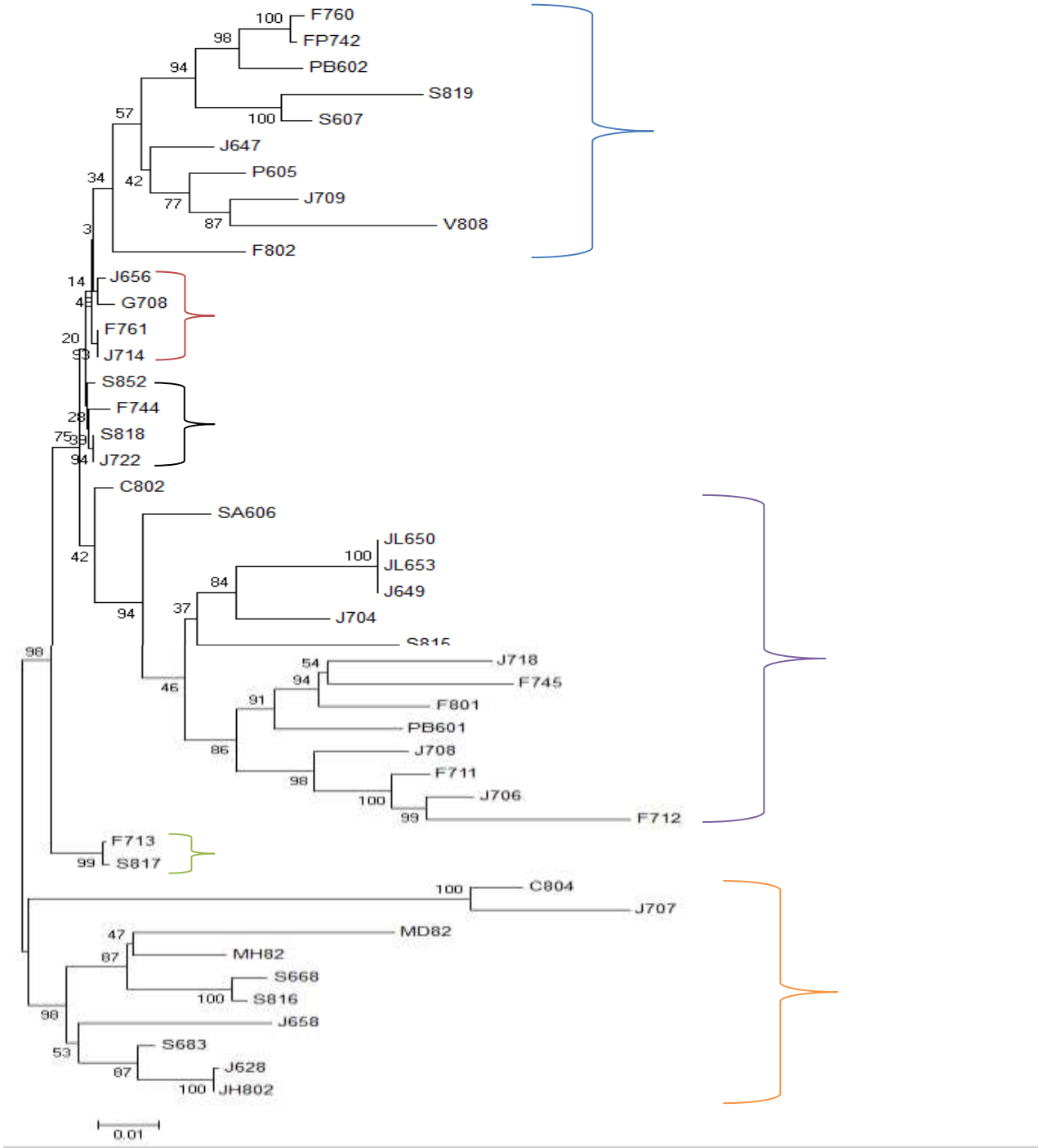

Figure 5.11: Neighbor-Joining phylogenetic tree of the pipistrelle TLR4/2 chimeric protein sequences. Bootstrap support values (%) are shown on the nodes. Blue = 1, Red = 2, Black = 3, Purple = 4, Green = 5, Orange = 6. *Footnotes:* TLR2 heterozygotic sequences were analysed as for Figure 5.8. Babies are excluded from this analysis.

Table 5.9: TLR4/2 cluster frequencies in the pipistrelles

TLR4/2 clusters	Frequency	Bat codes
1	22.2%	J647, P605, F802, S819, S607, PB602, F760, FP742, J709, V808
2	8.8%	J656, G708, F761, J714
3	8.8%	S818, J722, S852, F744
4	33.3%	J649, JL650, JL653, J704, SA606, C802, S815, J718, F745, F801, PB601, J708, F711, J706, F712
5	4.4%	F713, S817
6	22.2%	MH82, MD82, S668, S816, J658, J628, JH802, S683, J707, C804

5.4.2 TLR4/2 variations and parasite infections:

As reported earlier for the TLR4 analysis (5.2.2), the *T. gondii* infected bats (F802, P605, PB602) was limited to a single cluster (TLR4/2 cluster 1) and this infection profile was statistically significant (Fisher's exact test, *p*-value = 0.008). However, the earlier significance associated with TLR4 cluster 6 and both trypanosome infection and helminth intensity (5.2.2) were not observed when the analysis was repeated with the TLR4/2 clusters. The most likely explanation for this discrepancy is the reduced numbers of bats in the TLR4/2 phylogram.

6. Discussion:

Following on from the PCR gene isolation strategy presented in Chapter 4, the data within this chapter presents sequences for the genes encoding TLR4 and TLR2 from a considerable proportion of the pipistrelle population from South Lancashire/Greater Manchester. Although not based upon entire gene sequences, the data nonetheless highlights the variability of these TLR genes in the bat population. In particular, the pipistrelle TLR4 gene has high levels of sequence variability as 42 haplotypes are described from 59 individual bats which are extremely high level of variability can be found compared to other mammals. Moreover, the homozygosity of the TLR4 genotypes is unexpected given the random mating patterens and should be the subject of further investigation. A phylogenetic analysis of these TLR4 sequences positioned them into 7 clusters; however, bootstrap support was relatively weak for a number of the clusters. Although TLR2 variability in the bats was not as great (haplotypes = 5), there was also a small number (n = 7) of TLR2 heterozygotes. A study of TLR4 and TLR2 polymorphisms in over 4,000 individuals from a region of Ghana endemic for malaria infection identified 34 TLR4 single nucleotide polymorphisms (SNPs) and 12 TLR2 SNPs (May et al., 2010). A more limited study of TLR polymorphisms in African penguins also highlighted that TLR2 had limited diversity relative to some other TLRs (Dalton, Vermaak, Roelofse, & Kotze, 2016). As such, the reduced level of pipistrelle TLR2 diversity compare to that of TLR4 is consistent with reports in other widely diverse species and this may reflect that TLR2 is functional as a heterodimer whereas TLR4 is active as a homodimer (McClure & Massari, 2014).

As described in a number of studies (Brattig et al., 2004; Goodridge et al., 2005; Jenkins et al., 2005), TLR4 and TLR2 are important mediators of the innate immune response against parasite infections. However, other than a small number of studies that describe TLR gene sequences and gene expression in fruit bats (Cowled et al., 2011; Iha et al., 2010), there is an

absence of knowledge of how bat TLRs may contribute to the bat innate immune system. This is of much interest given the undoubted status that bats have acquired for being reservoirs of infection and particularly, viruses of zoonotic potential (O'Shea et al., 2014).

The parasite infection profiles described in the South Lancashire/Greater Manchester pipistrelles (Chapter 3; Lord, 2010; Lord et al., 2012; Dodd et al., 2014) presented an ideal opportunity to analyse TLR sequence variation and address the question of whether, or not, particular parasite infections might correlate with a TLR haplotype, or group of haplotypes. Of course, any infection profile is subject to influence from the environment as well as the host genetics; however, for the purposes of this study the bats were opportunistically obtained throughout the South Lancashire/Greater Manchester and there appeared to be no "hotspot" for infections (Chapter 3).

With respect to TLR4, it was observed that in a small group of bats (n = 9, TLR4 cluster 6) there was statistical support for susceptibility to trypanosome infection. One interpretation of the trypanosome infection data is that the other TLR4 clusters identified in the study (1-5 and 7) may be involved in mediating protection against trypanosomes. However, there were examples of bats within TLR4 clusters 1-5 and 7 with trypanosome infections; this ranged from 50% infection prevalence in clusters 3 and 4 to 11% in cluster 3. Another interpretation of the data is that as bats are capable of harbouring a multitude of infectious agents (O'Shea et al., 2014) the pipistrelle TLR4 does not provide full protection against trypanosome infection and hence TLR4 cluster 6 haplotypes are simply hyporesponsive to trypanosomes relative to the other TLR4 clusters. This might allow trypanosome parasitaemias to elevate to higher levels in these bats compared to the others and hence PCR-based detection is perhaps more robust whereas in other bats, low trypanosome parasitaemia may fail to yield a detectable PCR product. Of course, other possible explanations for the trypanosome

infections also exist; not least that the data is serendipitous and the result of an analysis based upon a phylogeny with weak bootstrap support.

Interestingly, the data also highlighted that pipistrelles within TLR4 cluster 6 had a significantly reduced helminth burden relative to the remainder of the bats. Accepting the cautionary note about the phylogram, this may indicate that TLR4 has a role in protection against helminths. Given that the bats were solely infected with digenean trematodes and the vast majority were *Lecithodendrium linstowi* (Lord et al., 2012), then it is possible that TLR4 haplotypes within cluster 6 interact with this parasite, or molecules released from it. As noted earlier (5.1), one of the most well studied trematodes, *S. mansoni*, is reported to activate mouse macrophage TLR4 (Jenkins et al., 2005).

In addition, it appeared that another small group of bats (n = 9, TLR4 cluster 3) appeared to be susceptible to *T. gondii* infection. However, the latter should be treated with an additional degree of caution since the majority of the *T. gondii* infected bats (Dodd et al., 2014) were not represented in the subgroup for which TLR4 sequence data was obtained.

For any interactions to occur between the pipistrelle TLR4s and pathogen associated molecular patterns (PAMPs) derived from the parasites then N-glycosylation sites as well as Leucine Rich Repeat (LRR) regions are likely to be of importance. To this end, complete conservation of all predicted pipistrelle N-glycosylation sites is likely to be of relevance. Furthermore, comparison of the pipistrelle TLRs to those of rodents (Fornuskova et al., 2013) highlight how most of the residues under positive selection in rodents were also conserved, or semi-conserved, in the bats. Indeed, only 2 out of 11 of these sites in the rodent had a dissimilar amino acid residue in the pipistrelles. This may be due to distinct evolutionary pressures and infectious agents associated with these different orders of mammal. In terms of the amino acid changes noted between the different pipistrelle TLR4 sequences, many of

these occurred in LRRs 3-6 and so they may well modify any interaction with parasite-derived PAMPs. However, given the large number of amino acid changes, it is not possible to conclude that a specific one, or group of them, are more likely to be of importance.

With respect to TLR2 variability in the bats, the most striking observation was that a small number of pipistrelles displayed heterozygosity and interestingly, there was statistical support for this group of bats (n = 7) having a reduced helminth burden. It seems reasonable to hypothesize that a heterozygote might have an advantage and therefore the reduced helminth burden associated with the TLR2 heterozygotes might be the expected outcome. However, there is conflicting data in the literature. For example, a 22 bp heterozygous deletion in the untranslated exon of human TLR2 is associated with protection against cerebral malaria through a mechanism likely to involve reduced TLR2 expression and hence an attenuation of the inflammatory response which would favour protection against cerebral malaria (Greene et al., 2012). Also, a human TLR2 heterozygous mutation within the intracellular Toll/IL-1 receptor domain is associated with increased likelihood of staphylococcal infection (Lorenz, Mira, Cornish, Arbour, & Schwartz, 2000) and severity of atopic dermatitis (Ahmad-Nejad et al., 2004). Only 2 of the 7 TLR2 heterozygotic changes occur within LRRs and so it is difficult to speculate as to how the pipistrelle TLR2 heterozygotes may confer some protection against enhanced helminth burdens. Nonetheless, this interesting result would be worthy of further investigation.

Finally, Lord et al. (2012) showed through statistical modelling that helminths were less abundant in the male pipistrelles and also, that helminths were more aggregated in the males. An inspection of the polymorphisms in the TLRs of the male and female bats showed that a number of TLR4 variants (N293I, I295L, V303R, I308N, I308K, E309R, H310T, L311S, D312G, Q314P, H315A, and F328P) occurred in some of the infected males but female bats with these changes were helminth-free. This interesting observation is most likely

serendipitous since it seems highly unlikely that TLR4 haplotypes may be involved in sex-linked infection outcomes.

The study hypothesis is that host genetics, including innate immunity genes, are likely to influence infection outcomes and hence TLR gene variations will be observed in the bat population. Because of the opportunistic sampling method of hosts from the wild and hence multiple associated confounding factors, it is difficult to predict whether, or not, there might be a link between TLR haplotype and parasite infection profile. Nonetheless, the study will address the hypothesis that a correlation might exist between the observed bat parasite infection profiles and particular TLR variants. After analyzing the pipistrelles TLR4 and TLR2 genes, there was high level of variability in TLR4 gene (Haplotype= 42) compared to TLR2 gene (haplotype= 5). Due to high variability found in TLR4, it was difficult to assess all of these changes as single change and as a result different clusters were assembled using phylogenetic tree. There were 7 main clusters in TLR4 gene and some of the cluster associated with the susceptibility and resistant to parasite like cluster 6 which had low worm burden but high trypanosome infections compared to the other clusters. Also, cluster 3 was the only cluster infected with *T. gondii* whereas none of the other cluster got this parasite. From this data, it is difficult to say that a specific change might have an effect of the susceptibility or resistant to a specific parasite; however, the data might suggest that some of the pipistrelles might have a degree of susceptibility or resistant to some parasitic infections when the data was analysed as clusters. With regard to TLR2 gene, it was highly conserved among pipistrelle bats and the low number of changes precluded any meaningful statistical analysis; however, heterozygotes were found in some of the pipistrelle TLR2 gene (n=7). When analysis the parasitic profile of these heterozygotes pipistrelle, they had low worm burden compared to the non- heterozygotes pipistrelles which might suggest that the heterozygotes of these samples associated with low worm infections.

6.1 Thesis conclusions:

Overall, work presented in this thesis has provided insight into microparasite infections within a pipistrelle population sampled opportunistically at sites across South Lancashire and Greater Manchester. In carrying out the work, it was clear that the lack of archived bat parasite material and also, the lack of prior molecular-based studies of bat parasites, resulted in certain difficulties; not least, with the design of trypanosome-specific PCR primers. Nonetheless, the descriptions of *T. dionisii* and *T. vespertilionis* infections in the pipistrelles are now presented with confidence and hence extend the analysis done by Lord (2010). In addition, the data produced shows that the bats are infected with the coccidians *E. rioarribaensis* and *Cryptosporidium* sp. bat genotype IV; these are new parasite descriptions for UK bats. Two bats were also confirmed infected with *Bartonella* sp. and a single bat infection with *Borrelia* sp. was noted. Taken together with the prior knowledge of *B. vesperuginis* (Lord, 2010), *T. gondii* (Dodd et al., 2014) and helminth (Lord et al., 2012) infections in these bats, it is reasonable to propose that the South Lancashire/Greater Manchester pipistrelles have provided much insight into bat parasite infections.

Indeed, the subsequent genotyping of these pipistrelles has now revealed that a major influence of the eimerian infection profile may be the genetics of the host. Indeed, this is the first report to highlight the role of bat genetics in susceptibility/resistance to parasite infection. Specifically, *E. rioarribaensis* was detected exclusively in bats likely to form a single inter-breeding group whereas the bats of mixed genetic origin in the study appeared to be genetically resistant to eimerian infection.

To explore genetic influences upon infection further, this thesis work then addressed the role of the bat innate immune system upon infection outcomes by study of TLRs. Specifically, large fragments of the pipistrelle TLR4 and TLR2 genes were PCR amplified and sequenced from a subset of the bats. As highlighted earlier in this chapter, the TLR4 genes showed

considerable diversity and a number of the bats were also heterozygous at the TLR2 locus. A correlational analysis of these gene variations with the parasite infections generated some intriguing results with respect to trypanosome and helminths that would be worthy of future investigation.

6.2 Future Directions:

This study might usefully be extended in a number of ways.

(i) Use of multivariate statistical modelling of the infection and TLR polymorphism data would provide a more robust analysis.

(ii) It would be highly useful to obtain knowledge of the expression patterns of the TLR4 and TLR2 genes in pipistrelles. This would necessitate tissue sampling and mRNA extraction from live bats (and hence would require a license). Nonetheless, at present, there is no knowledge of which pipistrelle cells/tissues express these TLRs and the conclusions in this thesis would benefit from such data.

(iii) Any TLR polymorphisms confirmed important through the multivariate statistical modelling might be further analysed via genetic study in a model organism. For example, a TLR4 or TLR2 mouse mutant could be genetically engineered to express a bat TLR gene. A subsequent infection assay might then be possible in order to support a role for the TLR variant being involved in the innate immune response to a parasite.

(iv) Establishment of a pipistrelle cell culture might also permit cell-based assays to be carried out with specific parasite molecules suspected to be important in TLR binding.

Wider approaches may also be warranted and could include the following.

(v) Bat sampling was carried out opportunistically via acquisition of dead, or injured bats that were subsequently euthanized due to extent of injury. This precluded a detailed autopsy in most instances and hence unfortunately no data is available on the potential pathologies associated with any of the infections (Lord, 2010). Also, importantly, the resulting group of pipistrelles may well not be representative of the general bat population and hence not all conclusions may be valid at the general population level. However, it would be very difficult to repeat the extensive studies carried out on the South Lancashire/Greater Manchester pipistrelles; not least because a bat license would be necessary and the justification (ie. random sampling) may be insufficient.

(vi) A small number of live bats are sampled under license for studies that usually involve virus monitoring. It would be useful to obtain blood from such sampling efforts in order to attempt culture of any of the blood parasites that might be present. This would provide a useful resource for any future downstream studies given the distinct lack of archived bat parasite resources currently available.

(vii) Efforts in this study have focused upon TLR4 and TLR2; however, it is quite likely that other bat TLRs may also be involved in innate immune responses to parasite infection. To this end, it would be worthwhile attempting to isolate and sequence further pipistrelle TLR genes.

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Appendix:

Appendix 1:

Schizotrypanum alignment:

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Query  1     GTCATATGCTTGTTTCAAGGACTTAGCCATGCATGCCTCAGAATCACTGCATTGCAGGAA  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1     GTCATATGCTTGTTTCAAGGACTTAGCCATGCATGCCTCAGAATCACTGCATTGCAGGAA  60

Query  61    TCTGCGCATGGCTCATTACATCAGACGTAATCTGCCGCAAAAATCTTGCGGTCTCCGCAA  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61    TCTGCGCATGGCTCATTACATCAGACGTAATCTGCCGCAAAAATCTTGCGGTCTCCGCAA  120

Query  121   CATTGGATAACTTGGCGAAACGCCAAGCTAATACATGAACCAACCGGACGTTCTCTGTTC  180
             |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121   CATTGGATAACTTGGCGAAACGCCAAGCTAATACATGAACCAACCGGATGTTCTCTGTTC  180

Query  181   CGGCGGCGGGGTCACACCCGCCGCCATGGGACGTCCAGCGAATGAATGAAAGTAAAACCA  240
             |||||||||||||||||||||||||||||||||||| || ||| || |||  ||||||
Sbjct  181   CGGCGGTAGGG-CA-ACCTGCTGCCATGGGACGTCCAGCGAATGAATGAAAGTAAAACCA  238

Query  241   ATGCC-C-TCACCGGCAGTAACACTCAGAAGTGTTGATTCAATTCATTCCGTGCGAAAGC  298
             |||||||||||||||||||||||||||||||||| |||||||||||| ||| | |||||
Sbjct  239   ATGCCGCATCAACGGCAGTAACACCCAGAAGTGTTGATTCAATTCATTCCGTGCGAAAGC  298

Query  299   TGGG-TTTCACACCCGGCGTCTTTTGACGAACAACTGCCCTATCAGCCAGTGATGGCCGT  357
             ||||||||| |||||||||||||||||||||||||||||||||| |||  |||| ||||
Sbjct  299   TGGGTTTTCTTACCTGGCGTCTTTTGACGAACAACTGCCCTATCAGCCAGCGATGGCCGT  358

Query  358   GTAGTGGACTGCCATGGCGTTGACGGGAGCGGGGGATTAGGGTTCGATTCCGGAGAGGGA  417
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  359   GTAGTGGACTGCCATGGCGTTGACGGGAGCGGGGGATTAGGGTTCGATTCCGGAGAGGGA  418

Query  418   GCCTGAGAAATAGCTACCACTTCTACGGAGGGCAGCAGGCGCGCAAATTGCCCAATGTCa  477
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  419   GCCTGAGAAATAGCTACCACTTCTACGGAGGGCAGCAGGCGCGCAAATTGCCCAATGTCA  478

Query  478   aaaaaaaaCGATGAGGCAGCGAAAAGAAATAGAGCCGACAGTGC-TTTGCATTGTCGTTT  536
             |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  479   AAAAAAAACGATGAGGCAGCGAAAAGAAATAGAGCCGACAGTGCTTTTGCATTGTCGTTT  538
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Query  537   TCAATGGGGGATATTTAAACCCATCCAAAATCGAGTAACAATTGGAGGACAAGTCTGGTG  596
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  539   TCAATGGGGGATATTTAAACCCATCCAAAATCGAGTAACAATTGGAGGACAAGTCTGGTG  598

Query  597   CCAGCACCCGCGGTAATTCCAGCTCCAAAAGCGTATATTAATGCTGTTGCTGTTAAAGGG  656
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  599   CCAGCACCCGCGGTAATTCCAGCTCCAAAAGCGTATATTAATGCTGTTGCTGTTAAAGGG  658

Query  657   TTCGTAGTTGAATTGTGGGCCTTCGAGGCGCAATGGTTTAGTCCCGTCCACTTCGGATTG  716
             |||||||||||||| ||||||||||||||||||||   ||||||||||||||||||||||
Sbjct  659   TTCGTAGTTGAATTGTGGGCCTCTAAGGCGCAATGGTTTAGTCCCATCCACTTCGGATTG  718

Query  717   GTGACCCATGCCCTTGAGGTCCGTGAACACTCAGAAACAAAAAAACACGGGAGTGGTACC-  775
             |||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct  719   GTGACCCATGCCCTTGTGGTCCGTGAACACTCAGAAACAAAAAAACACGGGAGTGGTACCC  778

Query  776   TTTCTGATTTCCGCATGTCATGCATGCCAGGGGGCGCCCGTGATTTTTTACTGTGACTAA  835
             ||||||||||||||| ||||||||||||||||||||||||||||||  |||||||||
Sbjct  779   TTTCTGATTCTCGCATGTCATGCATGCCAGGGGGCGCCCGTGA-TTTTTACTGTGACTAA  837

Query  836   AAAAGTGTGACCAAAGCAGTCATTCGACTTGAATTAGAAAGCATGGGATAACAAAGGAGC  895
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  838   AAAAGTGTGACCAAAGCAGTCATTCGACTTGAATTAGAAAGCATGGGATAACAAAGGAGC  897

Query  896   AGCCTATGGGCCACCGTTTCGGCTTTTGTTGGTTTTAAAAGTCCATTGGAGATTATGGGG  955
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  898   AGCCTATGGGCCACCGTTTCGGCTTTTGTTGGTTTTAAAAGTCCATTGGAGATTATGGGG  957

Query  956   CAGTGTGACAAGCGGCCGGGTGCT-CT-T-TC-C-C-CCTT--C-G-G-G--GGGACGCA  1002
             || | | |  | | | |  |||| | | |  | |  | ||||| ||||||||||||||||
Sbjct  958   CAGTGTGACAAGCGGCTGGGTGATGATATCCCACACACCTTCACTGCGTGTTGTGGCACA  1017

Query  1003  CTCGTCGCCTTTGTCGGAAATCCGCGCCGGCTGCGGCTGTGTGCGTCACACTTCCACGTG  1062
             |  |   ||||  | || |||  || ||  |     |||||||||  ||||||||||||
Sbjct  1018  CTCGTCGCCTTTGGGGGAAATCCG----TG--GC-GC--TGT-CGACGGACTT---C--G  1062

Query  1063  TGTCACACGCGCCCTGCCTGCGCCTTCCGGCAACTCACGGCATCCAGGAATGAAGGAGGG  1122
              ||||||||||||||||||||||||||||||  |||||||| | || | |    || |||
Sbjct  1063  -GTCCCATCTTCAC-GCGT-CGCCTTCCCTCAACTCACGGCATCCAGGAATGAAGGAGGG  1119
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Query  1123  TAGTTCGGGGGAGAACGTACTGGTGCGTCAGAGGTGAAATTCTTAGACCGCACCAAGACG  1182
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1120  TAGTTCGGGGGAGAACGTACTGGTGCGTCAGAGGTGAAATTCTTAGACCGCACCAAGACG  1179

Query  1183  AACTACAGCGAAGGCATTCTTCAAGGATACCTTCCTCAATCAAGAACCAAAGTGTGGGGA  1242
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1180  AACTACAGCGAAGGCATTCTTCAAGGATACCTTCCTCAATCAAGAACCAAAGTGTGGGGA  1239

Query  1243  TCGAAGATGATTAGAGACCATTGTAGTCCACACTGCAAACGATGACACCCATGAATTGGG  1302
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1240  TCGAAGATGATTAGAGACCATTGTAGTCCACACTGCAAACGATGACACCCATGAATTGGG  1299

Query  1303  GAGTTTTTGGTCG-TTAGGCGAGGTCGGGTTCATCTCGCTCCTCGTCTCGCCAATGAAT-  1360
             ||||||||||||||||||||||| | |||||||||||| ||||||| ||||||||||||
Sbjct  1300  GAGTTTTTGGTCGTTTAGGCGTGGTCGGGTTCACCCCGCTCCTCGTCTCGCCAATGAATG  1359

Query  1361  ATCAATTTACGTGCATATTCTTTACGGTCCCCGCT-TTCCAGCGGAGGCCTTTAACGGGA  1419
             |||||||||||||| ||| | || | || |||||  ||||||||||||||||||| |
Sbjct  1360  AATAATTTACGTGCATATTCTTTTTGGTCCTCGTTCTTAC-GCGTGGGCCTTTAACGGGA  1418

Query  1420  ATATCCTCAGCACGTTATCTGACTTCTTCACGCGAAAGCTTTGAGGTTACAGTCTCAGGG  1479
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1419  ATATCCTCAGCACGTTATCTGACTTCTTCACGCGAAAGCTTTGAGGTTACAGTCTCAGGG  1478

Query  1480  GGGAGTACGTTCGCAAGAGTGAAACTTAAAGAAATTGACGGAATGGCACCACAAGACGTG  1539
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1479  GGGAGTACGTTCGCAAGAGTGAAACTTAAAGAAATTGACGGAATGGCACCACAAGACGTG  1538

Query  1540  GAGCGTGCGGTTTAATTTGACTCAACACGGGGAACTTTACCAGATCCGGACAGGGTGAGG  1599
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1539  GAGCGTGCGGTTTAATTTGACTCAACACGGGGAACTTTACCAGATCCGGACAGGGTGAGG  1598

Query  1600  ATTGACAGATTGAGTGTTCTTTCTCGATCCCCTGAATGGTGGTGCATGGCCGCTTTTGGT  1659
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1599  ATTGACAGATTGAGTGTTCTTTCTCGATCCCCTGAATGGTGGTGCATGGCCGCTTTTGGT  1658
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Query  1660  CGGTGGAGTGATTTGTTTGGTTGATTCCGTCAACGGACGAGATCCAAGCTGCCCAGTAGG  1719
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1659  CGGTGGAGTGATTTGTTTGGTTGATTCCGTCAACGGACGAGATCCAAGCTGCCCAGTAGG  1718

Query  1720  ATTCAGAATTGCCCATAGGATAGCAATCCCTTCCGCGGGTTTTACCCAAgggggggCGGT  1779
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1719  ATTCAGAATTGCCCATAGGATAGCAATCCCTTCCGCGGGTTTTACCCAAGGGGGGGCGGT  1778

Query  1780  ATTCGTTTGTATCCTTCTCTGCGGGATTCCTTGTTTCGCGCAAGGTGAGATTTTGGGCAA  1839
             |||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  1779  ATTCGTTTGTATCCTTCTCTGCGGGATTCCTTGTTTTGCGCAAGGTGAGATTTTGGGCAA  1838

Query  1840  CAGCAGGTCTGTGATGCTCCTCAATGTTCTGGGCGACACGCGCACTACAATGTCAGTGAG  1899
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1839  CAGCAGGTCTGTGATGCTCCTCAATGTTCTGGGCGACACGCGCACTACAATGTCAGTGAG  1898

Query  1900  AACAAGAAAAAACGACTCTTGTCGGACCTACTTGATCAAAAGAGTGGGAAAACCCCGGAAT  1959
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1899  AACAAGAAAAAACGACTCTTGTCGGACCTACTTGATCAAAAGAGTGGGAAAACCCCGGAAT  1958

Query  1960  CACGTAGACCCACTTGGGACCGAGTATTGCAATTATTGGTCGCGCAACGAGGAATGTCTC  2019
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1959  CACGTAGACCCACTTGGGACCGAGTATTGCAATTATTGGTCGCGCAACGAGGAATGTCTC  2018

Query  2020  GTAGGCGCAGCTCATCAAACTGTGCCGATTACGTCCCTGCCATTTGTACACACCGCCCGT  2079
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2019  GTAGGCGCAGCTCATCAAACTGTGCCGATTACGTCCCTGCCATTTGTACACACCGCCCGT  2078

Query  2080  CGTTGTTTCCGATGATGGTGCAATACAGGTGATCGGACAGTCGAGTGTCTCACTTGACCG  2139
             |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2079  CGTTGTTTCCGATGATGGTGCAATACAGGTGATCGGACAGTCGAGTGTTTCACTTGACCG  2138

Query  2140  AAAGTTCACCGATATTTCTTCAATAGAGGAAGCAAAAGTC  2179
             |||||||||||||||||||||||||||||||||||||||
Sbjct  2139  AAAGTTCACCGATATTTCTTCAATAGAGGAAGCAAAAGTC  2178
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Clustal W alignment for different regions of the *Schizotrypanum* 18S rRNA gene sequence extracted from NCBI GenBank: *T. dionisii* (gi|4468750|), *T. vespertilionis* (gi: |4468775|): Panels A & D: Green highlights generic primer binding sites (TgF and TgR), Panels B & C: red shows the *T. dionisii* primer binding sites (TdF and TdR) and purple represents the *T. vespertilionis* primer binding sites. Additional primers were designed for *T. dionisii* and their annealing sites are shown in Panel B & C using brown and blue colouration.

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CLUSTAL O(1.2.1) multiple sequence alignment


gi|4468750|emb|AJ009151.1|      GTCATATGCTTGTTTCAAGGACTTAGCCATGCATGCCTCAGAATCACTGCATTGCAGGAA
gi|4468775|emb|AJ009166.1|      GTCATATGCTTGTTTCAAGGACTTAGCCATGCATGCCTCAGAATCACTGCATTGCAGGAA
                                ************************************************************

gi|4468750|emb|AJ009151.1|      TCTGCGCATGGCTCATTACATCAGACGTAATCTGCCGCAAAAATCTTGCGGTCTCCGCAA
gi|4468775|emb|AJ009166.1|      TCTGCGCATGGCTCATTACATCAGACGTAATCTGCCGCAAAAATCTTGCGGTCTCCGCAA
                                ************************************************************

gi|4468750|emb|AJ009151.1|      CATTGGATAACTTGGCGAAACGCCAAGCTAATACATGAACCAACCGGATGTTCTCTGTTC
gi|4468775|emb|AJ009166.1|      CATTGGATAACTTGGCGAAACGCCAAGCTAATACATGAACCAACCGGACGTTCTCTGTTC
                                ************************************************ ************

gi|4468750|emb|AJ009151.1|      CGGCGGTAGGGCAA--CCTGCTGCCATGGGACGTCCAGCGAATGAATGAAAGTAAAACCA
gi|4468775|emb|AJ009166.1|      CGGCGGCGGGGGTCACACCCGCCGCCATGGGACGTCCAGCGAATGAATGAAAGTAAAACCA
                                ******  ***  *  ** ** ************************************

gi|4468750|emb|AJ009151.1|      ATGCCGCATCAACGGCAGTAACACCCAGAAGTGTTGATTCAATTCATTCCGTGCGAAAGC
gi|4468775|emb|AJ009166.1|      ATGCC--CTCACCGGCAGTAACACTCAGAAGTGTTGATTCAATTCATTCCGTGCGAAAGC
                                *****   *** ************ ************************************

gi|4468750|emb|AJ009151.1|      TGGGTTTTCTTACCTGGCGTCTTTTGACGAACAACTGCCCTATCAGCCAGCGATGGCCGT
gi|4468775|emb|AJ009166.1|      TGGGTT-TCACACCCGGCGTCTTTTGACGAACAACTGCCCTATCAGCCAGTGATGGCCGT
                                ****** **  *** ************************************ *********

gi|4468750|emb|AJ009151.1|      GTAGTGGACTGCCATGGCGTTGACGGGAGCGGGGGGATTAGGGTTCGATTCCGGAGAGGGA
gi|4468775|emb|AJ009166.1|      GTAGTGGACTGCCATGGCGTTGACGGGAGCGGGGGGATTAGGGTTCGATTCCGGAGAGGGA
                                ************************************************************

gi|4468750|emb|AJ009151.1|      GCCTGAGAAATAGCTACCACTTCTACGGAGGGCAGCAGGCGCGCAAATTGCCCAATGTCA
gi|4468775|emb|AJ009166.1|      GCCTGAGAAATAGCTACCACTTCTACGGAGGGCAGCAGGCGCGCAAATTGCCCAATGTCA
                                ************************************************************

gi|4468750|emb|AJ009151.1|      AAAAAAAACGATGAGGCAGCGAAAAGAAATAGAGCCGACAGTGCTTTTGCATTGTCGTTT
gi|4468775|emb|AJ009166.1|      AAAAAAAACGATGAGGCAGCGAAAAGAAATAGAGCCGACAGTGCTT-TGCATTGTCGTTT
                                ********************************************** **************

gi|4468750|emb|AJ009151.1|      TCAATGGGGGATATTTAAACCCATCCAAAATCGAGTAACAATTGGAGGACAAGTCTGGTG
gi|4468775|emb|AJ009166.1|      TCAATGGGGGATATTTAAACCCATCCAAAATCGAGTAACAATTGGAGGACAAGTCTGGTG
                                ************************************************************


gi|4468750|emb|AJ009151.1|      CCAGCACCCGCGGTAATTCCAGCTCCAAAAGCGTATATTAATGCTGTTGCTGTTAAAGGG
gi|4468775|emb|AJ009166.1|      CCAGCACCCGCGGTAATTCCAGCTCCAAAAGCGTATATTAATGCTGTTGCTGTTAAAGGG
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gi|4468750|emb|AJ009151.1|      TTCGTAGTTGAATTGTGGGCCTCTAAGGCGCAATGGTTTAGTCCCATCCACTTCGGATTG
gi|4468775|emb|AJ009166.1|      TTCGTAGTTGAATTGTGGGCCTTCGAGGCGCAATGGTTTAGTCCCGTCCACTTCGGATTG
                                ***********************   ******************** **************

gi|4468750|emb|AJ009151.1|      GTGACCCATGCCCTTGTGGTCCGTGAACACTCAGAAACAAAAAACACGGGAGTGGTACCC
gi|4468775|emb|AJ009166.1|      GTGACCCATGCCCTTGAGGTCCGTGAACACTCAGAAACAAAAAACACGGGAGTGGTACCT
                                *************** ****************************************

gi|4468750|emb|AJ009151.1|      TTTCTGATTCTCGCATGTCATGCATGCCAGGGGGCGCCCGT-GATTTTTACTGTGACTAA
gi|4468775|emb|AJ009166.1|      TT-CTGATTTCCGCATGTCATGCATGCCAGGGGGCGCCCGTGATTTTTTACTGTGACTAA
                                ** ******  ******************************   *****************

gi|4468750|emb|AJ009151.1|      AAAAGTGTGACCAAAGCAGTCATTCGACTTGAATTAGAAAGCATGGGATAACAAAGGAGC
gi|4468775|emb|AJ009166.1|      AAAAGTGTGACCAAAGCAGTCATTCGACTTGAATTAGAAAGCATGGGATAACAAAGGAGC
                                ************************************************************

gi|4468750|emb|AJ009151.1|      AGCCTATGGGCCACCGTTTCGGCTTTTGTTGGTTTTAAAAGTCCATTGGAGATTATGGGG
gi|4468775|emb|AJ009166.1|      AGCCTATGGGCCACCGTTTCGGCTTTTGTTGGTTTTAAAAGTCCATTGGAGATTATGGGG
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gi|4468750|emb|AJ009151.1|      CAGTGTGACAAGCGGCTGGGTGATGATATCCCACACACCTTCACTGCGTGTTGTGGCACA
gi|4468775|emb|AJ009166.1|      CAGTGTGACAAGCGGCCGGGTGCTCTTTCCCCCTTCGGGGGGACGCA-------------
                                **************** ***** *  *  ***   *      **

gi|4468750|emb|AJ009151.1|      CTCGTCGCCTTTGGGGGAAATCCGTGGCGCTGTCGA------------------CGGACT
gi|4468775|emb|AJ009166.1|      CTCGTCGCCTTTGTCGGAAATCCGCGCCGGCTGCGGCTGTGTGCGTCACACTTCCACGTG
                                *************  ********* * **    **                   *

gi|4468750|emb|AJ009151.1|      TCGGTCCCATCTTCACGCGTCGCCTTCCCTCAACTCACGGCATCCAGGAATGAAGGAGGG
gi|4468775|emb|AJ009166.1|      TGTCACACGCGCCCTGCCTGCGCCTTCCGGCAACTCACGGCATCCAGGAATGAAGGAGGG
                                *    * *     *   *  ********  ******************************

gi|4468750|emb|AJ009151.1|      TAGTTCGGGGGAGAACGTACTGGTGCGTCAGAGGTGAAATTCTTAGACCGCACCAAGACG
gi|4468775|emb|AJ009166.1|      TAGTTCGGGGGAGAACGTACTGGTGCGTCAGAGGTGAAATTCTTAGACCGCACCAAGACG
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gi|4468750|emb|AJ009151.1|      AACTACAGCGAAGGCATTCTTCAAGGATACCTTCCTCAATCAAGAACCAAAGTGTGGGGA
gi|4468775|emb|AJ009166.1|      AACTACAGCGAAGGCATTCTTCAAGGATACCTTCCTCAATCAAGAACCAAAGTGTGGGGA
                                ************************************************************

gi|4468750|emb|AJ009151.1|      TCGAAGATGATTAGAGACCATTGTAGTCCACACTGCAAACGATGACACCCATGAATTGGG
gi|4468775|emb|AJ009166.1|      TCGAAGATGATTAGAGACCATTGTAGTCCACACTGCAAACGATGACACCCATGAATTGGG
                                ************************************************************

gi|4468750|emb|AJ009151.1|      GAGTTTTTGGTCGTTTAGGCGTGGTCGGGTTCACCCCGCTCCTCGTCTCGCCAATGAATG
gi|4468775|emb|AJ009166.1|      GAGTTTTTGGTCGTTA-GGCGAGGTCGGGTTCATCTCGCTCCTCGTCTCGCCAATGAAT-
                                ***************  **** *********** * ***********************

gi|4468750|emb|AJ009151.1|      AATAATTTACGTGCATATTCTTTTTGGTCCTCGTTCTTACGCGTGGGCCTTTAACGGGAA
gi|4468775|emb|AJ009166.1|      ATCAATTTACGTGCATATTCTTTACGGTCCCCGCTTTCCAGCGGAGGCCTTTAACGGGAA
                                *  ********************  ***** ** * *   ***  ***************

gi|4468750|emb|AJ009151.1|      TATCCTCAGCACGTTATCTGACTTCTTCACGCGAAAGCTTTGAGGTTACAGTCTCAGGGG
gi|4468775|emb|AJ009166.1|      TATCCTCAGCACGTTATCTGACTTCTTCACGCGAAAGCTTTGAGGTTACAGTCTCAGGGG
                                ************************************************************

gi|4468750|emb|AJ009151.1|      GGAGTACGTTCGCAAGAGTGAAACTTAAAGAAATTGACGGAATGGCACCACAAGACGTGG
gi|4468775|emb|AJ009166.1|      GGAGTACGTTCGCAAGAGTGAAACTTAAAGAAATTGACGGAATGGCACCACAAGACGTGG
                                ************************************************************

gi|4468750|emb|AJ009151.1|      AGCGTGCGGTTTAATTTGACTCAACACGGGGAACTTTACCAGATCCGGACAGGGTGAGGA
gi|4468775|emb|AJ009166.1|      AGCGTGCGGTTTAATTTGACTCAACACGGGGAACTTTACCAGATCCGGACAGGGTGAGGA
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gi|4468750|emb|AJ009151.1|      TTGACAGATTGAGTGTTCTTTCTCGATCCCCTGAATGGTGGTGCATGGCCGCTTTTGGTC
gi|4468775|emb|AJ009166.1|      TTGACAGATTGAGTGTTCTTTCTCGATCCCCTGAATGGTGGTGCATGGCCGCTTTTGGTC
                                ************************************************************

gi|4468750|emb|AJ009151.1|      GGTGGAGTGATTTGTTTGGTTGATTCCGTCAACGGACGAGATCCAAGCTGCCCAGTAGGA
gi|4468775|emb|AJ009166.1|      GGTGGAGTGATTTGTTTGGTTGATTCCGTCAACGGACGAGATCCAAGCTGCCCAGTAGGA
                                ************************************************************

gi|4468750|emb|AJ009151.1|      TTCAGAATTGCCCATAGGATAGCAATCCCTTCCGCGGGTTTTACCCAAGGGGGGGCGGTA
gi|4468775|emb|AJ009166.1|      TTCAGAATTGCCCATAGGATAGCAATCCCTTCCGCGGGTTTTACCCAAGGGGGGGCGGTA
                                ************************************************************

gi|4468750|emb|AJ009151.1|      TTCGTTTGTATCCTTCTCTGCGGGATTCCTTGTTTTGCGCAAGGTGAGATTTTGGGCAAC
gi|4468775|emb|AJ009166.1|      TTCGTTTGTATCCTTCTCTGCGGGATTCCTTGTTTCGCGCAAGGTGAGATTTTGGGCAAC
                                ********************************** ************************

gi|4468750|emb|AJ009151.1|      AGCAGGTCTGTGATGCTCCTCAATGTTCTGGGCGACACGCGCACTACAATGTCAGTGAGA
gi|4468775|emb|AJ009166.1|      AGCAGGTCTGTGATGCTCCTCAATGTTCTGGGCGACACGCGCACTACAATGTCAGTGAGA
                                ************************************************************

gi|4468750|emb|AJ009151.1|      ACAAGAAAAACGACTCTTGTCGGACCTACTTGATCAAAAGAGTGGGAAAACCCCGGAATC
gi|4468775|emb|AJ009166.1|      ACAAGAAAAACGACTCTTGTCGGACCTACTTGATCAAAAGAGTGGGAAAACCCCGGAATC
                                ************************************************************

gi|4468750|emb|AJ009151.1|      ACGTAGACCCACTTGGGACCGAGTATTGCAATTATTGGTCGCGCAACGAGGAATGTCTCG
gi|4468775|emb|AJ009166.1|      ACGTAGACCCACTTGGGACCGAGTATTGCAATTATTGGTCGCGCAACGAGGAATGTCTCG
                                ************************************************************

gi|4468750|emb|AJ009151.1|      TAGGCGCAGCTCATCAAACTGTGCCGATTACGTCCCTGCCATTTGTACACACCGCCCGTC
gi|4468775|emb|AJ009166.1|      TAGGCGCAGCTCATCAAACTGTGCCGATTACGTCCCTGCCATTTGTACACACCGCCCGTC
                                ************************************************************

gi|4468750|emb|AJ009151.1|      GTTGTTTCCGATGATGGTGCAATACAGGTGATCGGACAGTCGAGTGTTTCACTTGACCGA
gi|4468775|emb|AJ009166.1|      GTTGTTTCCGATGATGGTGCAATACAGGTGATCGGACAGTCGAGTGTCTCACTTGACCGA
                                ********************************************** ************

gi|4468750|emb|AJ009151.1|      AAGTTCACCGATATTTCTTCAATAGAGGAAGCAAAAGTC
gi|4468775|emb|AJ009166.1|      AAGTTCACCGATATTTCTTCAATAGAGGAAGCAAAAGTC
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Clustal W alignment for a region of the *Schizotrypanum* 18S rRNA gene sequence extracted from NCBI GenBank: *T. dionisii* (gi|4468750|), *T. vespertilionis* (gi: |4468775|): red shows the *T. dionisii* primer binding sites (TrypF and TrypR

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CLUSTAL O(1.2.2) multiple sequence alignment

gi|313209097|emb|FN599054.1|      ----------------------GGTCGATATGAACACGGACGCGGAGTATTTTGCATACCA
gi|313209103|emb|FN599056.1|      --------ACGTCGTGGCGGTGGTCGATATGAACACGGACGCGGAGTACTTTGCGTACCA
gi|313209100|emb|FN599055.1|      GGAGATTGACGTCGTGGCGGTGGTCGATATGAACACGGACGCGGAGTACTTTGCGTACCA
                                              *************************** ***** *****

gi|313209097|emb|FN599054.1|      GCTGCGCTACGACACCGTGCACGGCAAGTTCAAGTACACGGTGACGACGGCGAAGAGCAA
gi|313209103|emb|FN599056.1|      GATGCGTTACGACACCGTGCATGGTAAGTTCAAGTACACGGTGACGACGACGAAGAGCAA
gi|313209100|emb|FN599055.1|      GATGCGTTACGACACCGTGCATGGTAAGTTCAAGTACACGGTGACGACGACGAAGAGCAA
                                  * **** ************** ** ************************ **********

gi|313209097|emb|FN599054.1|      CCCCTCCGTGACTAAGGACGACACACTCGTGGTGAATGGCCACCGCATTCTGTGCGTGAA
gi|313209103|emb|FN599056.1|      CCTCTCCGTGGCGAAGGATGACACACTTGTGGTGAATGGCCATCGCATTCTGTGCGTGAA
gi|313209100|emb|FN599055.1|      CCTCTCCGTGGCGAAGGATGACACACTTGTGGTGAATGGCCATCGCATTCTGTGCGTGAA
                                  ** ******* * ***** ******** ************** *****************

gi|313209097|emb|FN599054.1|      GGCGCAGCGCAACCCGGCGGATCTCCCGTGGGGCAAGCTTGGTGTGGAGTATGTAATTGA
gi|313209103|emb|FN599056.1|      GGCGCAGCGCAATCCGGCGGATCTCCCGTGGGGCAAGCTTGGTGTGGAGTATGTAATTGA
gi|313209100|emb|FN599055.1|      GGCGCAGCGCAATCCGGCGGATCTCCCGTGGGGCAAGCTTGGTGTGGAGTATGTAATTGA
                                  ************ ************************************************

gi|313209097|emb|FN599054.1|      GTCAACGGGTCTGTTCACTGCCAAGGTGGCGGCGGAGGGCCACCTGCGTGGCGGTGCACG
gi|313209103|emb|FN599056.1|      GTCAACAGGCCTGTTCACTGCCAAGACGGCGGCGGAGGGCCACCTGCGCGGCGGTGCACG
gi|313209100|emb|FN599055.1|      GTCAACAGGCCTGTTCACTGCCAAGACGGCGGCGGAGGGCCACTTGCGCGGCGGTGCACG
                                  ****** ** ***************  ***************** **** ************

gi|313209097|emb|FN599054.1|      GAAGGTCATCATCAGCGCGCCCGCCTCTGGTGGCGCCAAGACACTCGTGATGGGCGTGAA
gi|313209103|emb|FN599056.1|      GAAGGTCATCATCAGCGCCCCCGCCTCTGGTGGCGCCAAGACACTCGTGATGGGCGTGAA
gi|313209100|emb|FN599055.1|      GAAGGTCATCATCAGCGCCCCCGCCTCTGGTGGCGCCAAGACACTCGTGATGGGCGTGAA
                                  ****************** ******************************************

gi|313209097|emb|FN599054.1|      CCACCATGAGTACAACCCCAGTGAGCACCACGTGGTCTCGAACGCGTCATGCACGACCAA
gi|313209103|emb|FN599056.1|      CCACCATGAGTACAACCCCAGTGAGCACCATGTGGTGTCGAACGCGTCGTGCACGACCAA
gi|313209100|emb|FN599055.1|      CCACCATGAGTACAACCCCAGTGAGCACCATGTGGTGTCGAACGCGTCGTGCACGACCAA
                                  ****************************** ***** *********** ***********

gi|313209097|emb|FN599054.1|      TTGTCTTGCGCCCATTGTGCATGTCCTGGTGAAGGAGGGCTTTGGCGTGCAGACCGGCCT
gi|313209103|emb|FN599056.1|      TTGTCTTGCGCCCATTGTGCATGTTCTGGTGAAGGAGGGCTTTGGCGTGCAGACCGGCCT
gi|313209100|emb|FN599055.1|      TTGTCTTGCGCCCATTGTGCATGTCCTGGTGAAGGAGGGCTTTGGCGTGCAGACCGGCCT
                                  ********************** ***********************************


gi|313209097|emb|FN599054.1|      CATGACGACGATCCACTCGTACACGGCAACACAAAAGACGGTGGACGGCGTGTCGTTGAA
gi|313209103|emb|FN599056.1|      CATGACGACGATCCACTCGTACACGGCAACACAGAAGACGGTGGATGGTGTGTCGTTGAA
gi|313209100|emb|FN599055.1|      CATGACGACGATCCACTCGTACACGGCAACACAGAAGACGGTGGATGGTGTGTCGTTGAA
                                  ********************************** *********** ** ***********

gi|313209097|emb|FN599054.1|      GGACTGGCGCGGCGGTCGTGCGGCTGCGGTGAACATCATTCCAAGCACGACTGGTGCGGC
gi|313209103|emb|FN599056.1|      GGACTGGCGCGGCGGTCGTGCGGCTGCGGTGAACATCATTCCGAGCACGACTGGTGCGGC
gi|313209100|emb|FN599055.1|      GGACTGGCGCGGCGGTCGTGCGGCTGCGGCGAACATCATTCCGAGCACGACTGGTGCGGC
                                  ***************************** ************ *****************

gi|313209097|emb|FN599054.1|      GAAGGCAGTGGGCATGGTTATCCCAAGCACGCAGGGCAAGCTGACGGGCATGTCGTTTCG
gi|313209103|emb|FN599056.1|      GAAGGCGGTGGGCATGGTGATCCCAAGCACGCAGGGCAAGCTGACGGGCATGTCGTTTCG
gi|313209100|emb|FN599055.1|      GAAGGCGGTGGGCATGGTGATCCCAAGCACGCAGGGCAAGCTGACGGGCATGTCGTTTCG
                                  ****** *********** ****************************************

gi|313209097|emb|FN599054.1|      TGTCCCCACCCCGGACGTGTCCGTGGTGGACCTCACCTTCACGGCGACGCGCGATACCAG
gi|313209103|emb|FN599056.1|      TGTCCCCACCCCGGACGTGTCCGTGGTGGACCTCACCTTCACGGCGACGCGCGACACCAG
gi|313209100|emb|FN599055.1|      TGTCCCCACCCCGGACGTGTCCGTGGTGGACCTCACCTTCACGGCGACGCGCGACACCAG
                                  ********************************************************** *****

gi|313209097|emb|FN599054.1|      CATACAGGAGATTGACGCCGCGCTGAAGCGCGCGTCCAAGACGTACATGAAGGGCATTCT
gi|313209103|emb|FN599056.1|      CATACAGGAGATCGACGCTGCACTGAAGCGCGCGTCCAAGACGTACATGAAGGGCATTCT
gi|313209100|emb|FN599055.1|      CATACAGGAGATCGACGCTGCACTGAAGCGCGCGTCCAAGACGTACATGAAGGGCATTCT
                                  ************ ***** ** ************************************

gi|313209097|emb|FN599054.1|      TGGCTACACGGACGAAGAGCTTGTGAGTACGGACTTCATTAATGACAACCGCAGCTCCAT
gi|313209103|emb|FN599056.1|      TGGCTACACGGACGAAGAGCTTGTGAGCACAGACTTCATTAATGATAACCGCAGCTCCAT
gi|313209100|emb|FN599055.1|      TGGTTACACGGACGAAGAGCTTGTGAGCACAGACTTCATTAATGATAACCGCAGCTCCAT
                                  *** ********************** ** ************** **************

gi|313209097|emb|FN599054.1|      CTACGA----------------
gi|313209103|emb|FN599056.1|      CTACGACTCCAAGGCGACCTT
gi|313209100|emb|FN599055.1|      CTACGACTCCAAGGCGAC---
                                  ******
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Clustal W alignment for a region of the *T. dionisii* GAPDH gene sequence extracted from NCBI GenBank: *T. dionisii* (gi|313209097|), *T. dionisii* (gi|313209103|), *T. dionisii* (gi|313209100|): red shows the *T. dionisii* primer binding sites (GAPF, GAPR and GAPRn

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CLUSTAL 2.1 multiple sequence alignment

gi|558135472|ref|XM_006091085.   TCCAGAATGCTACGGTTGCCACTCTCACTTCCTCTTGCCCCTCACCCAGC
gi|554578862|ref|XM_005880935.   TCCAGAATGCTACGGTTGCCACTCTCACTTCCTCTTGCCCCTCACCCAGC
gi|584056807|ref|XM_006772885.   TCCAGAATGCTACGGTTGCCACTCTCACTTCCTCTTGCCCATCACCCAGC
gi|641721271|ref|XM_008152116.   --------------------------------------------------
gi|588480441|ref|NM_001290172.   --------------------------------------------------

gi|558135472|ref|XM_006091085.   GCTGCTTTGAATGCAGCATTTGTCGTGGGGCAC-GTACAAGGGAGAGAAG
gi|554578862|ref|XM_005880935.   GCTGCTTTGAATACAGCAATTTTCGTGGGGCAC-ACACAAGGGAGAGAAG
gi|584056807|ref|XM_006772885.   ACTGCTTTGAATACAGCAATTGTCTTGGGGCAC-GCACAAGGGAGAGAAG
gi|641721271|ref|XM_008152116.   --------------------------------------------------
gi|588480441|ref|NM_001290172.   --------------------GCTGTGGGGCACCGAGCAAGGGAAAGAAG

gi|558135472|ref|XM_006091085.   ACAGCAGTGCCTGGGAGGCTGCT-GGGCAGGCGGTTACTGCGCGTCATGC
gi|554578862|ref|XM_005880935.   ACAGCAGTGCCTGGGAGGCTGCT-GGGCAGGCGGTTACTGCGCGTCATGC
gi|584056807|ref|XM_006772885.   ACAGCAGTGCCTGGGAGGCTTCT-GGGCAGGCGGTTACTGCGTGTCATGC
gi|641721271|ref|XM_008152116.   --------------------------------------------------
gi|588480441|ref|NM_001290172.   ACAG---TGCCTTGGAGGCTGCCCAGGCAGGTGGTTACGGTTACACATGC

gi|558135472|ref|XM_006091085.   TGTGGCAGGGCCATTTCTGGTGGCAGACAATGCCGGGATAATGTGGCCCA
gi|554578862|ref|XM_005880935.   TGTGGCAGGGCCATTTCTGGTGGCAGACAATGCTGGGATAATGTGGCCCA
gi|584056807|ref|XM_006772885.   TGTGGCAGGGCCATTTCTGGTGGCAGACAATGCCGGGATAATGTGGCCCA
gi|641721271|ref|XM_008152116.   ----------------------------------------ATGCGGCTCA
gi|588480441|ref|NM_001290172.   TTTCACAGGGTCATTTTTGGCCACAGAAAATGCCAGGATGATGCCTCCCA
                                                                        ***   * **

gi|558135472|ref|XM_006091085.   CCCGCCTGGCTGGGATGCTGCTCCCAGCCATGGCCTTCCTCTCCTGCCTG
gi|554578862|ref|XM_005880935.   CCCGCCTGGCTGGGACGCTGCTCCCAGCCATGGCCTTCCTCTCCTGCCTG
gi|584056807|ref|XM_006772885.   CCCGCCTGGCTGGGGCTCTCCTCCCAGCCATGGCCTTCCTCTCCTGCCTG
gi|641721271|ref|XM_008152116.   CCCGCCTGGCTGGGACGCTGCTCCCAGCCATGGCCTTCCTCTCCTGCCTG
gi|588480441|ref|NM_001290172.   CCCGCCTGGCTGGGATTCTGATCCCAGCCATGGCCTTTCTTTCCTGCCTG
                                 **************   **  **************** ** *********

gi|558135472|ref|XM_006091085.   AGACCCGAGAGCTGGGACCCTTGCGTGCAGGTGGTTCCTAATGTTACTTA
gi|554578862|ref|XM_005880935.   AGACCCGAGAGCTGGGACCCTTGCGTGCAGGTGGTTCCTAACGTTACTTA
gi|584056807|ref|XM_006772885.   AGACCCGAGAGCTGGGAACCTTGCGTGCAGGTGGTTCCTAATGATACTTA
gi|641721271|ref|XM_008152116.   AGACCCGAGAGCTGGGACCCTTGTGTGCAGGTGGTTCCTAATGTTACTTA
gi|588480441|ref|NM_001290172.   AGACCTGAGAGCTGGGACCCTTGCGTGCGGGTGGTTCCTCACATTACCTA
                                 ***** *********** ***** **** ********** *   *** **

gi|558135472|ref|XM_006091085.   CCAGTGCATGGAGCTGAATCTCTACAAAATCCCTGATAACCTCCCTACAT
gi|554578862|ref|XM_005880935.   CCAGTGCATGGAGCTGAATCTCTACAAAATCCCTGATAACATCCCTACAT
gi|584056807|ref|XM_006772885.   CCAGTGCATGGAGCTGAATCTCTACAAAATCCCTGATAACATCCCTACAT
gi|641721271|ref|XM_008152116.   TCAGTGCATGGAGCTGAATCTCTACACAATCCCTGATAACATCCCTACAA
gi|588480441|ref|NM_001290172.   CCAGTGCATGGAGCTGAATCTCTACAAAATCCCCAACAACATCCCCACAT
                                  ************************* ******  * *** **** ***

gi|558135472|ref|XM_006091085.   CAATCAAGAACCTGGACTTGAGCTTTAACCCCCTGAGGCATTTAGGCAGC
gi|554578862|ref|XM_005880935.   CAATCAAGAACCTGGACTTGAGCTTTAACCCCCTGGGGCATTTAGGCAGC
gi|584056807|ref|XM_006772885.   CAATCAAGAACCTGGACTTGAGCTTTAACCCCCTGAGGCATTTAGGCAGC
gi|641721271|ref|XM_008152116.   CAACCAAGAACCTGGACCTGAGCTTTAACCCCCTGAGGCATTTAGGCAGC
gi|588480441|ref|NM_001290172.   CAGTCAAGAAACTGGATCTGAGCTTTAACCCCCTGAGACGTCTAAGCAGC
                                 **  ****** *****  ***************** * * * ** *****

gi|558135472|ref|XM_006091085.   CACAGCTTCTCCAACTTCTCAGAACTGCAGGTGCTGGATTTATCCAGGTG
gi|554578862|ref|XM_005880935.   CACAGCTTCTCCAACTTCTCAGAACTGCAGGTGCTGGATTTATCCAGGTG
gi|584056807|ref|XM_006772885.   CACAGCTTCTCCAACTTCTCAGAACTGCAGGTGCTGGATTTATCCAGGTG
gi|641721271|ref|XM_008152116.   CACAGCTTCTCCAACTTCTCAGAACTGCAGGTGCTGGATTTATCCAGGTG
gi|588480441|ref|NM_001290172.   CATATCTTCTCCAACTTCTCAGAATTGCAGGTGCTGGATTTATCTAGGTG
                                 ** * ******************* ****************** *****

gi|558135472|ref|XM_006091085.   TGAAATTCAGAAGATTGAAGATGATGCATATCAAGGCCTAAAGCATCTCT
gi|554578862|ref|XM_005880935.   TGAAATTCAGAAGGTTGAAGATGATGCATATCAAGGCCTAAAGCATCTCT
gi|584056807|ref|XM_006772885.   TGAAATTCAGAAGATTGAAGACGATGCATATCAAGGCCTAAAGCATCTCT
gi|641721271|ref|XM_008152116.   TGAAATTCAGAAGATTGAAGATGATGCATATCAAGGCCTAAAACATCTCT
gi|588480441|ref|NM_001290172.   TGAAATTGAGATGATTGAAGATGATGCATATGAGGGTCTAAACCATCTCT
                                 ******* *** * ******* ********* * ** ***** *******
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gi|558135472|ref|XM_006091085.   CCATCTTGATATTGACAGGAAACCCTATCCAGAGTTTAGCCCCGGGAGCC
gi|554578862|ref|XM_005880935.   CCATCTTGATATTGACAGGAAACCCTATCCAGAGTTTAGCCCCGGGAGCC
gi|584056807|ref|XM_006772885.   CCATCTTGATATTGACAGGAAACCCTATCCAGAGTTTAGCCCCTGGAGCC
gi|641721271|ref|XM_008152116.   CCATCTTGATATTGACAGGAAACCCTATCCAGAGTTTAGCCCCAGGAGCC
gi|588480441|ref|NM_001290172.   CCACCTTGGTATTGACAGGAAACCCTATCCAGAGTTTAGCCATGGGAGCC
                                 *** **** ********************************   ******

gi|558135472|ref|XM_006091085.   TTTTCTGGACTGCCAAGTTTACAGACACTGGTGGCTGTGGAGACAAACCT
gi|554578862|ref|XM_005880935.   TTTTCTGGACTGCCAAGTTTACAGACACTGGTGGCTGTGGAGACAAACCT
gi|584056807|ref|XM_006772885.   TTTTCTGGACTGCCAAGTTTACAGACACTGGTGGCTGTGGAGACAAACCT
gi|641721271|ref|XM_008152116.   TTTTCTGGACTACCAAGTTTACAGACACTGGTGGCTGTGGAGACAAACCT
gi|588480441|ref|NM_001290172.   TTTTCTGGACTATCAAGTTTACAGACACTGGTGGCTGTGGAGATAAACCT
                                 ***********  ****************************** ******

gi|558135472|ref|XM_006091085.   AGCATCACTAGAGGACTTCCCCATCAGACATCTGAAAACCTTGAAGGAGC
gi|554578862|ref|XM_005880935.   AGCATCGCTAGAGGACTTCCCCATCAGACATCTGAAAACCTTGAAGGAGC
gi|584056807|ref|XM_006772885.   AGCATCGCTAGAGGACTTCCCCATCAGACATCTGAAAACCTTGAAGGAGC
gi|641721271|ref|XM_008152116.   AGCCTCTCTAGAGGACTTCCCCATCACACATCTGAAATCCTTGAAGGAGC
gi|588480441|ref|NM_001290172.   AGTGTCTCTAGAGGACTTCCCCATTGGACACCTGAAAACCTTGAAGGAGC
                                 **  ** *****************   *** ****** ************

gi|558135472|ref|XM_006091085.   TTAATGTGGCTCACAATCTAATTGATTCCTTCAAGTTACCGGACTATTTT
gi|554578862|ref|XM_005880935.   TTAATGTGGCTCACAATCTAATTGATTCCTTCAAGTTACCGGACTATTTT
gi|584056807|ref|XM_006772885.   TTAATGTGGCTCACAATCTAATTGATTCCTTCAAGTTACCGGACTATTTT
gi|641721271|ref|XM_008152116.   TTAATGTGGCTCACAATCTAATCGATTCCTTCAAGTTACCGAACTATTTT
gi|588480441|ref|NM_001290172.   TTAATGTGGCTCACAATCTTATTGATTCCTTCAAGTTACCTGAATATTTT
                                 ****************** ** *****************  * ******

gi|558135472|ref|XM_006091085.   TCTAACCTGCCTAACCTGGAGCACTTGGATCTTTCCAATAACAAGATCCG
gi|554578862|ref|XM_005880935.   TCTAACCTGCCTAACCTGGAGCACTTGGATCTTTCCAATAACAAGATCCG
gi|584056807|ref|XM_006772885.   TCTAACCTGCCTAACCTGGAGCACTTGGATCTTTCCAATAACAAGATCCG
gi|641721271|ref|XM_008152116.   TCTAACCTGCCTAACCTGGAGCACTTGGACCTTTCCAATAATAAGATTCG
gi|588480441|ref|NM_001290172.   TCTAACCTGTCCGACCTGGAGCACTTAGACCTTTCCAATAACAAGATCCA
                                 ********* *  ************* ** *********** ***** *

gi|558135472|ref|XM_006091085.   CAATATTTACCATGAAGACTTGCAGGTTTTACATCAAATGCCCTCATTCA
gi|554578862|ref|XM_005880935.   CAATATTTACCATGAAGACTTGCAGGTTTTACATCAAATGCCTTCATTCA
gi|584056807|ref|XM_006772885.   CAATATTTACCATGAAGACTTGCAGGTTTTACATCAAATGCCCTCATTCA
gi|641721271|ref|XM_008152116.   AAATATTTACCATGAAGACTTGCAGGTTTTACATCAAATGCCCTCATTCA
gi|588480441|ref|NM_001290172.   AACTATTTGTCATAAAGACCTACAGGTTCTACATCAAATGCCCCCATCCA
                                  * *****  *** ***** * ****** *************  *** **

gi|558135472|ref|XM_006091085.   AACTCTCCTTAGACCTGTCCCTCAACCCTTTAGACTTTATTCAACCAGGT
gi|554578862|ref|XM_005880935.   AACTCTCCTTAGACCTGTCCCTCAACCCTTTAGACTTTATTCAACCAGGT
gi|584056807|ref|XM_006772885.   AACTCTCCTTAGACCTGTCCCTCAACCCTTTAGACTTTATTCAACCAGGT
gi|641721271|ref|XM_008152116.   AACTCTCTTTAGACCTGTCCCTCAACCCTTTAGACTTTATCCAACCAGGT
gi|588480441|ref|NM_001290172.   AACTCTCTTTAGACTTGTCCCTGAACCCTTTAGACTTCATCCAACGAGGT
                                 ******* ****** ******* ************** ** **** ****

gi|558135472|ref|XM_006091085.   GCCTTTGAAAAAATTAAGCTCCATGAACTGACTTTGAGAAGTAATTTTGA
gi|554578862|ref|XM_005880935.   GCCTTTGAAAAAATTAAGCTCCATGAACTGACTTTGAGAAGTAATTTTGA
gi|584056807|ref|XM_006772885.   GCCTTTGAAAAAATTAAGCTCCATGAACTGACTTTGAGAAGTAATTTTGA
gi|641721271|ref|XM_008152116.   GCCTTTGAAAAAATTAAGCTCCATGAACTGACTTTGAGAAGTAATTTTGA
gi|588480441|ref|NM_001290172.   GCCTTTAAAGAAATTAAGCTCCATGAACTAACTTTGAGAAGTAATTTTAA
                                 ****** ** ****************** ***************** *

gi|558135472|ref|XM_006091085.   TAGTGCAGAGGTCATGAAAACGTGTATTCAAGGTCTGGCTGGTTTAAAGA
gi|554578862|ref|XM_005880935.   TAGTCCAGAGGTCATGAAAATGTGTATTCAAGGTCTGGCTGGTTTAAAGA
gi|584056807|ref|XM_006772885.   TAGTCCAGAGGTCATGAAAATGTGTATTCAAGGTCTGGCTGGTTTAAAGA
gi|641721271|ref|XM_008152116.   TAGTGCAGAGGTCATGAAAACGTTTATTCAAGGTCTGGCAGGTTTAAAGA
gi|588480441|ref|NM_001290172.   CAGTACAGATGTAATGAAAACTTGTGTTCAAGGCCTCGCTGGCTTAAAAA
                                  *** **** ** *******  * * ******* ** ** ** ***** *

gi|558135472|ref|XM_006091085.   TCAATCGGTTGATTCTGGGAGAATTTAAAAATGAAAGAACCATAGTAAAC
gi|554578862|ref|XM_005880935.   TCAATCGGTTGATTCTGGGAGAATTTAAAAATGAAAGAAACTTAGTAAAC
gi|584056807|ref|XM_006772885.   TCAATCGGTTGATTCTGGGAGAATTTAAAAATGAAAGAACCTTAGTAAAC
gi|641721271|ref|XM_008152116.   TCAAACGGCTGATTCTGGGAGAATTTAAAAATGAAAGGATCTTAGTAAAC
gi|588480441|ref|NM_001290172.   TCAATCGTTTGGTTCTAGGAGAATTTAAAAATGAAAGAGCCATAAAACAT
                                 **** **  ** **** ********************   * **  * *

gi|558135472|ref|XM_006091085.   TTCAACAAATCTGCCCTGGAGGGTCTGTGCAATTTGACCATTGAAGAATT
gi|554578862|ref|XM_005880935.   TTCAACAAATCTGCCCTGGAGGGTCTGTGCAATTTGACCATTGAAGAATT
gi|584056807|ref|XM_006772885.   TTCAACAATTCTGCCCTGGAGGGTCTGTGCAATTTGACCATTGAAGAATT
gi|641721271|ref|XM_008152116.   TTGGACAAATCTGCCCTGGAGGAACTGTGTAATTTGACCATTGAAGAATT
gi|588480441|ref|NM_001290172.   TTTGACAAATCTGCCATGGAGGGACTGTGCAATTTGACCATTGACGAATT
                                 **  **** ****** ******  ***** ************** *****
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```
gi|558135472|ref|XM_006091085.   CCGGATAGCACACTTCGATGAGTTTCCAGGGGATGATCTTGGCTTTTTAA
gi|554578862|ref|XM_005880935.   CCGGATAGCACACTTCGATGAGTTTCCAGGGGATGATCTTGGCTTTTTAA
gi|584056807|ref|XM_006772885.   CCGGATAGCACGCTTCAATGAGTTTCCAGGGGATGATCTTGGCTTTTTAA
gi|641721271|ref|XM_008152116.   CCGGATAGCACACTTCCAAGACTTTCCAGAGGATTACCTTGGCTTTTTAA
gi|588480441|ref|NM_001290172.   CCGGATGACATACTTCGATGACTTCTCAGAGGATGTTATTAACTTTTTTA
                                 ******  **  **** * ** **  *** ****    **  ****** *

gi|558135472|ref|XM_006091085.   ATTGTTTGCCAGAGGCTTCTACAATATCTCTTATGGGTCTGTATTTAGAC
gi|554578862|ref|XM_005880935.   ATTGTTTGGCAGATGCTTCTACAATATCTCTTGTGAGTCTATATTTAGAT
gi|584056807|ref|XM_006772885.   ATTGTTTGGCAGATGCTTCTACAATATCTCTTGTGAGTCTATATTTAGAC
gi|641721271|ref|XM_008152116.   ATTGTTTGGCAGATGCTTCTGCAATATCTCTGGTGAGTCTGAATATAGAC
gi|588480441|ref|NM_001290172.   ATTGTTTGGCAAATGTTTCTACAATTTCTCTGGTGGGTCTGTATTTAAAC
                                 ******** ** * * **** **** *****  ** ****  ** ** *

gi|558135472|ref|XM_006091085.   GAGCTAAAAATCTTTCCAAAAGGTTTCAAATGGCAATACTTAAATTTGTC
gi|554578862|ref|XM_005880935.   GAGCTAAAAATCTTTCCAAAAGGTTTCAAATGGCAATACTTAAATTTGTC
gi|584056807|ref|XM_006772885.   AAGCTAAAAATCTTTCTAGAAGATTTCAAATGGCAATACTTAAAATTGTC
gi|641721271|ref|XM_008152116.   AGGCTAGAAAGCCTTCCAAAAGGTTTCAAATGGCAATACTTAAACTTGAC
gi|588480441|ref|NM_001290172.   AGGCTAGAAGTCCTTTCTAAAGATTTCAAATGGCAACACTTAAAACTGAC
                                   **** **  * **    *** ************* *******  ** *

gi|558135472|ref|XM_006091085.   TAAATGTATATTTGAACATTTTCCTACATTGGAGCTTACCTTTCTCAAGC
gi|554578862|ref|XM_005880935.   TAAATGTATATTTGAACATTTTCCTACATTGGAGCTTACCTTTCTCAAGC
gi|584056807|ref|XM_006772885.   TAAATGTAAATTTGAACATTTTCCTACATTGGACCTTACCTTTCTCAAGC
gi|641721271|ref|XM_008152116.   TAATTGTAAATTTGAACATTTTCCTACATTGGAGCTTACCTTTCTCAAGC
gi|588480441|ref|NM_001290172.   TAATTCTAAATTTGATCATTTTCCCAGGTTGGAACTTGACTCTCTCAAAA
                                 *** * ** ****** ******** *  ***** ***  ** ******

gi|558135472|ref|XM_006091085.   AGTTTGTTTTCACTGCCAACAAAGGTATTACCACTTTTACTAAAGTTAAT
gi|554578862|ref|XM_005880935.   AGTTTGTTTTCACTGCCAACAAAGGTATTACCACTTTTACTGAAGTTAAT
gi|584056807|ref|XM_006772885.   AGTTCATTTTCACTGCCAACAAAGGTATTACCACTTTTACTGAAGTTAAT
gi|641721271|ref|XM_008152116.   AGTTTGTTTTCACTGACAACAAAGGTATTACCACTTTTACTGAAGTTAAT
gi|588480441|ref|NM_001290172.   AGTTGGTTTTCACTGCCAACAGGGGTATGAGCACTTTTACTGAAGTTAAA
                                 ****  ********* *****  ***** * ********** *******

gi|558135472|ref|XM_006091085.   CTACCAAACCTTGAGTTTCTAGATCTCAGTAAAAATGGCTTGAGTTACAA
gi|554578862|ref|XM_005880935.   CTACCAAACCTTGAGTTTCTAGATCTCAGTAGAAATGGCTTGAGTTTCAA
gi|584056807|ref|XM_006772885.   CTACCAAACCTTGAGTTTCTAGATCTCAGTAGAAATGGCTTGAAACACTTAG
gi|641721271|ref|XM_008152116.   CTAAGAAACCTTGAGTTTCTAGATCTCAGTAGTAATGGCTTGAGTTTCAA
gi|588480441|ref|NM_001290172.   CTACCAAAACTTGAGTTTCTAGATCTCAGTAGAAATAGTTTGAGTTTCAA
                                 ***  *** ***********************  *** * ******* ***

gi|558135472|ref|XM_006091085.   GTCTTGCTGCTCTCACCGTGATTTTGGGACAACCCAACTGAAACACTTAA
gi|554578862|ref|XM_005880935.   GTCCTGCTGCTCTCACCGTGATTTTGGGACAACCCGACTGAAACACTTAG
gi|584056807|ref|XM_006772885.   GTCCTGCTGCTCTCACCGTGATTTTGGGACAACCCGACTGAAACACTTAG
gi|641721271|ref|XM_008152116.   GTCTTGCTGCTCTCACCGTGATTTTGGGACAACCCAACTGAAACACTTAA
gi|588480441|ref|NM_001290172.   GAGTTGCTGTTCTCGCACTTTTTGGGGGACAACTAGACTGAAACACTTAG
                                 *   ***** **** *  *  **  ********   *************

gi|558135472|ref|XM_006091085.   ATCTGAGCTTCAATAATATTATTATCATGACTTCAAACTTCTTGGGCTTA
gi|554578862|ref|XM_005880935.   ATCTGAGCTTCAATAATATTATTATCATGACTTCAAACTTCTTGGGCTTA
gi|584056807|ref|XM_006772885.   ATCTGAGTTTCAATAATATTATTATCATGACTTCGAACTTCTTGGACTTA
gi|641721271|ref|XM_008152116.   ATCTGAGCTTCAATAGTATTATTACCATGACTTCAAACTTCGTGGGCTTA
gi|588480441|ref|NM_001290172.   ATCTGAGCTTTAATGATGTTATTACCATGAGCTCAAACTCCTTGGGCTTA
                                 ******* ** ***  * ****** *****  ** **** * *** ****

gi|558135472|ref|XM_006091085.   GAGCAACTAGAACGTCTGGATTTCCAGCATTCCACTCTGAAACAGGCCAG
gi|554578862|ref|XM_005880935.   GAGCAACTAGAACATCTGGATTTCCAGCATTCCACTCTGAAACAGGCCAG
gi|584056807|ref|XM_006772885.   GAGCAACTAGAACATCTGGATTTCCAGCATTCCACTCTGAAACAGGCCAG
gi|641721271|ref|XM_008152116.   GAGCAACTAGAACGACTGGATTTCCAGCATTCCACTTTGAAACAGGCCAG
gi|588480441|ref|NM_001290172.   GAGCAACTAAAATATCTGGATTTCCAGCATTCCAATTTGAAACAGGCCAG
                                 ********* **   ****************** * *************

gi|558135472|ref|XM_006091085.   TGATTTTTCAGTATTCCTCTCACTCAAAAATCTCCTTTACCTTGATATCT
gi|554578862|ref|XM_005880935.   TGATTTTTCAGTATTCCTCTCACTCAAAAATCTCCTTTACCTTGATATCT
gi|584056807|ref|XM_006772885.   TGATTTTTCAGTATTCCTCTCACTCAAAAATCTCCTTTACCTTGATATCT
gi|641721271|ref|XM_008152116.   TACTTTTTCAATATTCCTCTCACTCAAAAACCTCCTTTACCTTGATATCT
gi|588480441|ref|NM_001290172.   TGATTTTTCGGTATTCCTATCACTCAAAAACCTACTTTACCTTGATATTT
                                 *  ******  ******* *********** ** ************** *

gi|558135472|ref|XM_006091085.   CTTACACTAACACCAAGATTGTCTTCCTGCGCATCTTTGATGGCTTGATC
gi|554578862|ref|XM_005880935.   CTTACACTAACACCAAGATTGTCTTCCTGGGCATCTTTGATGGCTTGATC
gi|584056807|ref|XM_006772885.   CTTACACTAACACCAAGATTGTCTTCCTGGGCATCTTTGATGGCTTGATC
gi|641721271|ref|XM_008152116.   CTTACACTAACATCCAGATTGTCTTCAAGGGCATCTTTGATGGCTTGATC
gi|588480441|ref|NM_001290172.   CTTATACTCGCATCCGAATCATCTTCCATGGCATCTTTGACGGCTTGTTC
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gi|558135472|ref|XM_006091085.   AGCCTCCAAGTCTTGAAAATGGCTGGCAATTCTTTTCAGGATGCACTCC-
gi|554578862|ref|XM_005880935.   AGCCTCCAAGTCTTGAAAATGGCTGGCAATTCTTTTCAGGATGCACTCC-
gi|584056807|ref|XM_006772885.   AGCCTCCAAGTCTTGAAAATGGCTGGCAATTCTTTTCAGGATGCACTCC-
gi|641721271|ref|XM_008152116.   AGCCTCCAAGTCTTGAAAATGGCTGGCAATTCCTTTCAGGATGCATTCC-
gi|588480441|ref|NM_001290172.   AGCCTCGAAGTCTTGAAAATGGCTGGCAATTCTTTTCAGGACA-ACTCCG
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gi|558135472|ref|XM_006091085.   TTCCAAATATCTTCAGAGATCTGACTCAGTTGACTGAACTGGACCTCTCT
gi|554578862|ref|XM_005880935.   TCCCAAATATCTTCAGAGATCTGACTCAGTTGACTGACCTGGACCTCTCT
gi|584056807|ref|XM_006772885.   TCCCAAATATCTTCAGAGACCTGACTCAGTTGACTGTCCTGGACCTCTCT
gi|641721271|ref|XM_008152116.   TTCCAAATGTCTTCAGAGATCTGACTCAGTTGACTATCCTGGACCTCTCT
gi|588480441|ref|NM_001290172.   TTCCAAATATCTTCAAAGCGCTGACTAACTTAACCTTCCTGGACCTCTCT
                                 * ****** ****** **  ****** * ** **    ************

gi|558135472|ref|XM_006091085.   CAGTGTCAACTGGAACGGGTGTCCCAGGAGGCATTTGGCTCACTCCTTAG
gi|554578862|ref|XM_005880935.   CAGTGTCAACTGGAACAGGTGTCCCAGGAGGCATTTGGCTCACTCCTTAG
gi|584056807|ref|XM_006772885.   CAGTGTCAACTAGAACGGGTGTCCCAGGAGGCATTTGGCTCACTCCTTAG
gi|641721271|ref|XM_008152116.   CAGTGTCAATTGGAACAGGTGTCTCCGGAGGCATTCAGCTCACTCCTTAG
gi|588480441|ref|NM_001290172.   AATTGCCAGCTAGAACGAGTGTCCCAGGCGGCATTTGGCTCACTCGTTAA
                                  * ** **  * ****  ***** * ** ******  ******** ***

gi|558135472|ref|XM_006091085.   ACTCCAGGTGCTAAATATGAGTCACAACCACCTCTTGTCCTTGGATATGC
gi|554578862|ref|XM_005880935.   ACTCCAGGTGCTAAATATGAGTCACAATCACCTCTTGTCCTTGGATATGC
gi|584056807|ref|XM_006772885.   ACTCCAGGTGCTAAATATGAGTCACAACCACCTCTTGTCCTTGGATATGC
gi|641721271|ref|XM_008152116.   ACTCGAGGTGCTAAATATGAGTCACAACCACCTCTTGTCCTTGGATATGC
gi|588480441|ref|NM_001290172.   ACTTAAGTCACTAAATATGAGTCACAACCACCTTTTATCCTTGGATCTAT
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gi|558135472|ref|XM_006091085.   TTCCTTTTAAAAATCTC---TCTCTCCGGGTTCTAGACTGTAGTTTTAAC
gi|554578862|ref|XM_005880935.   TTCCTTATAAAAATCTC---TCTCTCCGGGTTCTAGATTGCAGTTTTAAC
gi|584056807|ref|XM_006772885.   TTCCTTATAAAAATCTC---TCTCTCCGGGTTCTAGACTGCAGTTTTAAC
gi|641721271|ref|XM_008152116.   TTCCTTACAAAAATCTC---CCTCTCTCGGTTCTAGACTGCAGTTTTAAC
gi|588480441|ref|NM_001290172.   TTCCTTATAAACTTCCCCACTCTCTCCAGGATCTGGACTGCAGTTTTAAT
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gi|558135472|ref|XM_006091085.   CGTATAGTGGCCGCCAATGGGCAGGAACTACAGCATTTTCCAAGCAATGT
gi|554578862|ref|XM_005880935.   CGTATAGTGGCCGCCAATGGGCAGGAACTACAACATTTTCCAAGCAATGT
gi|584056807|ref|XM_006772885.   CGTATAGTGGCCGCCAATGGGCAGGAACTACAACATTTTCCAAGCAATGT
gi|641721271|ref|XM_008152116.   CGTATAGTGGCCGCCAATGGGCAGGAACTACAGCATTTTCCAAGCAATGT
gi|588480441|ref|NM_001290172.   CGCATAGTGGCCTCCAATAGGCAAGAACTACAGCATTTTCCAAGTAATCT
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gi|558135472|ref|XM_006091085.   AACTTCCTTAAACCTGAACCAGAATAACTTTGCTTGTGTTTGTGAACACA
gi|554578862|ref|XM_005880935.   AACTTCCTTAAATCTGACCCAGAATAACTTTGCTTGTGTTTGTGAACACA
gi|584056807|ref|XM_006772885.   AACTTCCTTAAATCTGACCCAGAATAACTTTGCTTGTGTTTGTGAACACA
gi|641721271|ref|XM_008152116.   AACTTCCTTACATCTGACCCAGAATGACTTTGCTTGTGTTTGTGAACACA
gi|588480441|ref|NM_001290172.   AACTTCCTTAAATCTCACTGGGAATGACTTTGCTTGCATTTGTGAACACC
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gi|558135472|ref|XM_006091085.   TGCGCTTCCTGCAGTGGGTCCAGGATCACAGGCACATCTTGGTGGGAGCT
gi|554578862|ref|XM_005880935.   TGCGCTTCCTGCAGTGGGTCCAGGATCACAGGCGCATCTTGGTGGGAGCT
gi|584056807|ref|XM_006772885.   TGCGTTTCCTGCAGTGGGTCCAAGATCACAGGCGCATCTTGGTGGGCGCT
gi|641721271|ref|XM_008152116.   TGCGTTTCCTGCAGTGGGTCCAGGACCACAGGAGCATCTTGGTGGGAGCT
gi|588480441|ref|NM_001290172.   AGAGTTTTCTGCAGTGGGTCAAGGACCACAGGCACCTCTTGGTGGGAGTT
                                  * * ** ************ * ** ******  * ********** * *

gi|558135472|ref|XM_006091085.   GAACACATGATGTGTGAGAAACCTTTAGCTATGCAGGGTGTGCCTGTGCT
gi|554578862|ref|XM_005880935.   GAACACATGATGTGTGAGAAACCTTTAGCTATGCAGGGTGTGCCTGTGCT
gi|584056807|ref|XM_006772885.   GAACACATGATGTGTGAGAAACCTTTAGCTATGCAGGGCGTGCCTGTGCT
gi|641721271|ref|XM_008152116.   GAACACATGATGTGTAAGACACCTTTAGCTATGCAGGGTGTGCCTGTGCT
gi|588480441|ref|NM_001290172.   ACACAAATGGTGTGTGTGAAACCTTTAGATATGCAGGGTGTGCCTGTACT
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gi|558135472|ref|XM_006091085.   CAGTTTTAGAAATGCCACCTGCCAGATGAGCAAAACTATCATTAGTGTGT
gi|554578862|ref|XM_005880935.   CAGTTTTAGAAATGCCACCTGCCAGATGAGCAAAACTATCATTAGTGTGT
gi|584056807|ref|XM_006772885.   CAGTTTTAGAAATGCCACCTGCCAGATGAGCAAAACTATCATTAGTGCGT
gi|641721271|ref|XM_008152116.   CAGTTTTAGAAACACCACCTGCCAGATGAACAAAACTGTCATTAGTGTGT
gi|588480441|ref|NM_001290172.   CAGTTTTAGAAATGCCACCTGTCCGATGAGCAAGACTGTCATTAGTGTGT
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gi|558135472|ref|XM_006091085.   CAGTTCTCTCAGTACTCGTGGTATCTGTAGCCGCAGTTCTGGTCTACAAG
gi|554578862|ref|XM_005880935.   CAGTTCTCTCAGTACTCGTGGTATCTGTAGCCGCAGTTCTGGTCTACAAG
gi|584056807|ref|XM_006772885.   CAGTTCTCTCAGTACTCGTGGTCTCTGTAGCCGTAGTTCTGGTCTACAAG
gi|641721271|ref|XM_008152116.   CCGTTCTCTCAGTACTCGTGGTATCTGTGGCTGCAGTTCTGGTCTACAAG
gi|588480441|ref|NM_001290172.   CGGTTCTCACTGTGCTTGTGGTATCTGTGGTAGCAGTTCTGGTTTATAAG
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gi|558135472|ref|XM_006091085.   TTCTATTTCCACCTGATGCTTCTGGCTGGCTGCAAAAAGTATGGCAAAGG
gi|554578862|ref|XM_005880935.   TTCTACTTCCACCTGATGCTTCTGGCTGGCTGCAAAAAGTATGGCAAAGG
gi|584056807|ref|XM_006772885.   TTCTATTTCCACCTGATGCTTCTGGCTGGCTGCAAAAAATATGGCAAAGG
gi|641721271|ref|XM_008152116.   TTCTATTTCCACCTGATGCTTCTGGCTGGCTGCAAAAGGTACAGCAAAGG
gi|588480441|ref|NM_001290172.   TTCTATTTCCACCTGATGCTTCTTGCTGGCTGCAAAAAGTATGGCAGAGG
                                 ***** ***************** *************  **  *** ***

gi|558135472|ref|XM_006091085.   GGAAAGCACCTACGATGCCTTTGTCATCTACTCCAGCCATGATGAGGACT
gi|554578862|ref|XM_005880935.   GGAAAGCACCTACGATGCCTTTGTCATCTACTCCAGCCATGATGAGGACT
gi|584056807|ref|XM_006772885.   GGAAAGCACCTACGATGCCTTTGTCATCTACTCCAGCCATGATGAGGACT
gi|641721271|ref|XM_008152116.   GGACAGCACTTATGATGCCTTTGTCATCTACTCCAGCCACGATGAGGACT
gi|588480441|ref|NM_001290172.   TGAAAGCACCTATGATGCCTTTGTTATTTACTCAAGCCAGGATGAGGACT
                                  ** ***** ** *********** ** ***** ***** **********

gi|558135472|ref|XM_006091085.   GGGTGAGGAATGAGTTGGTGAAGAACTTGGAGGAGGGAGTCCCCCCCTTT
gi|554578862|ref|XM_005880935.   GGGTGAGGAATGAGTTGGTGAAGAACTTGGAGGAGGGGGTCCCCCCCTTT
gi|584056807|ref|XM_006772885.   GGGTGAGGAATGAGTTGGTGAAGAACTTGGAGGAGGGGGTACCCCCTTTT
gi|641721271|ref|XM_008152116.   GGGTGAGGAATGAGTTGGTAAAGAACTTGGAGGAGGGGGTACCCCCCTTT
gi|588480441|ref|NM_001290172.   GGGTGAGGAATGAGTTGGTAAAGAACTTGGAGGAGGGGGTGCCCCCCTTT
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gi|558135472|ref|XM_006091085.   CAGCTCTGCCTTCACTACAGAGACTTTATCCCTGGTGTGGCCATTGCTGC
gi|554578862|ref|XM_005880935.   CAGCTCTGCCTTCACTACAGAGACTTTATCCCTGGCGTGGCCATTGCTGC
gi|584056807|ref|XM_006772885.   CAGCTCTGCCTTCACTACAGAGACTTTATCCCTGGCGTGGCCATTGCTGC
gi|641721271|ref|XM_008152116.   CAGCTCTGCCTTCACTACAGAGACTTTATCCCTGGCGTGGCCATTGCTGC
gi|588480441|ref|NM_001290172.   CAGCTCTGCCTTCACTACAGAGACTTTATTCCTGGTGTGGCCATTGCTGC
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gi|558135472|ref|XM_006091085.   CAACATCATCCAGGAAGGTTTCCACAAGAGCCGGAAGGTCATTGTGGTGG
gi|554578862|ref|XM_005880935.   CAACATCATCCAGGAAGGTTTCCACAAGAGCCGGAAGGTCATTGTGGTGG
gi|584056807|ref|XM_006772885.   CAACATCATCCAGGAAGGTTTCCACAAGAGCCGGAAGGTCATTGTGGTGG
gi|641721271|ref|XM_008152116.   CAACATCATCCAGGAAGGTTTCCACAAGAGCAGGAAGGTCATTGTGGTGG
gi|588480441|ref|NM_001290172.   CAACATCATCCAAGAAGGTTTCCACAAGAGCCGAAAGGTTATCGTCGTGG
                                 ************ ****************** * ***** ** ** ****

gi|558135472|ref|XM_006091085.   TGTCCCGGCACTTCATCCAGAGCCGATGGTGCCTCTTTGAGTACGAGATC
gi|554578862|ref|XM_005880935.   TGTCCCAGCACTTCATACAGAGCCGATGGTGCCTCTTTGAGTATGAGATC
gi|584056807|ref|XM_006772885.   TGTCCCAGCACTTCATCCAGAGCCGATGGTGCCTCTTTGAGTACGAGATC
gi|641721271|ref|XM_008152116.   TGTCCCAGCACTTCATCCAGAGCCGATGGTGCATCTTTGAATATGAGATT
gi|588480441|ref|NM_001290172.   TGTCCCAGCACTTCATCCAGAGCCGATGGTGTATTTTTGAGTATGGGATT
                                 ****** ********* **************  * ***** ** * ***

gi|558135472|ref|XM_006091085.   GCCCAGACCTGGCAGTTCCTGAGCAGTCACGCAGGCATCATCTTCATCGT
gi|554578862|ref|XM_005880935.   GCCCAGACCTGGCAGTTCCTGAGCAGTCACGCAGGCATCATCTTCATCGT
gi|584056807|ref|XM_006772885.   GCCCAGACCTGGCAGTTCCTGAGCAGTCACGCGGGCATCATCTTCATCAT
gi|641721271|ref|XM_008152116.   GCCCAGACCTGGCAGTTCCTGAGCAGTCACGCGGGCATCATCTTCATCGT
gi|588480441|ref|NM_001290172.   GCCCAGACCTGGCAGTTTCTGAGCAGTCGTGCGGGCATCATCTTCATTGT
                                 ***************** **********  ** **************  *

gi|558135472|ref|XM_006091085.   CCTGCAGAAGGTGGAGAGGTCCCTGCTCAGGCAGCAGGTGGAACTGTATC
gi|554578862|ref|XM_005880935.   CCTGCAGAAGGTGGAGAAGTCCCTGCTCAGGCAGCAGGTGGAACTGTATC
gi|584056807|ref|XM_006772885.   CCTGCAGAAGGTGGAGAAGTCCCTGCTCAGGCAGCAGGTGGAACTGTATC
gi|641721271|ref|XM_008152116.   CCTGCAGAAGGTGGAGAAATCCCTGCTCCGGCAGCAGGTGGAGCTGTATC
gi|588480441|ref|NM_001290172.   CCTACAGAAGGTGGAGAAGTCCCTGCTCCGGCAGCAGGTGGAGCTGTATC
                                 *** **************  ********* ************* *******

gi|558135472|ref|XM_006091085.   GCCTTCTCAGCAGGAACACTTACCTAGAGTGGGAGGACAGTGTCCTGGGC
gi|554578862|ref|XM_005880935.   GCCTTCTCAGCAGGAACACTTACCTAGAGTGGGAGGACAGTGTCCTGGGC
gi|584056807|ref|XM_006772885.   GCCTTCTCAGCAGGANCACTTACCTAGAGTGGGAGGACAGTGTCCTGGGC
gi|641721271|ref|XM_008152116.   GCCTTCTCAGCAGGAACACTTACCTAGAGTGGGAGGACAGTGCCCTGGGC
gi|588480441|ref|NM_001290172.   GCCTTCTCAGCAGGAACACTTACCTGGAGTGGGAGGACAGTGTCCTCGGG
                                 *************** ********* **************** *** **

gi|558135472|ref|XM_006091085.   CGGCACATCTTCTGGCGACGACTGAGAAAAGCCTTGCTGGATGGTAAGCC
gi|554578862|ref|XM_005880935.   CGGCACATCTTCTGGCGACGACTGAGAAAAGCCTTGCTGGATGGTAAGCC
gi|584056807|ref|XM_006772885.   CGGCACATCTTCTGGCGACGACTGAGAAAAGCCTTGCTGGACGGTAAGCC
gi|641721271|ref|XM_008152116.   CGGCACATCTTCTGGCGACGACTGAGAAAAGCCTTGCTGGATGGTAAGCC
gi|588480441|ref|NM_001290172.   CGGCACATCTTCTGGAGACGACTCAGAAAAGCCTTGCTAGATGGCAAACT
                                 *************** ******* ************** ** ** ** * *

gi|558135472|ref|XM_006091085.   ATGGAGTCCAGAAGGAACAGAGGATGCAGAAAGCAGCCAGGATGAAGCAA
gi|554578862|ref|XM_005880935.   ATGGAGTCCAGAAGGAACAGAGGATGCAGAAAGCAGCCAGGATGAAGCAA
gi|584056807|ref|XM_006772885.   GTGGAGTCCAGAAGGAACAGAGGGTGCAGAAAGCAGCCAGGATGAAGCAA
gi|641721271|ref|XM_008152116.   ATGGAGTCCAGAAGGAACAGTGGATGCAGAAGTCAGTCAGGATGAAACAA
gi|588480441|ref|NM_001290172.   GTGGAGTCCAGAAGGAGCAGCAGATGCAGAAAGCAGCCAGCATGAAGCAA
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gi|558135472|ref|XM_006091085.   CGACCTCCACCTAACGTACAGACACTCCTTGGGTGCCTCTTGCCCAGGTG
gi|554578862|ref|XM_005880935.   CGACCTCCACCTGACGTACAGACACTCCTCGGGTGCCTCTTGCCCAGGTG
gi|584056807|ref|XM_006772885.   CGACCTCCACCTGACGTACAGACACTCCTCGGGTGCCTCTTGGCCAGGTG
gi|641721271|ref|XM_008152116.   TGACCTCCTTCTGA-----------------------------------
gi|588480441|ref|NM_001290172.   CAATCACTATCTGAGGAGAAAAAACTCCTGAGGTGCTTCTTGTGCAGCTG
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gi|558135472|ref|XM_006091085.   CATCCAATATTTGTTCCGTTGACAATTATTAAATGCTGCAGCA-------
gi|554578862|ref|XM_005880935.   CATCCAATATTTGTTCAGTTGACAAGTATTAAATGCTGCAGTATAGCCGG
gi|584056807|ref|XM_006772885.   CATCCAATATTTGTTCAGTTGACAATTATTAAATGCTGCAGCATAGCAGG
gi|641721271|ref|XM_008152116.   --------------------------------------------------
gi|588480441|ref|NM_001290172.   GATTCATTACTTGTTCAGTTAACAAGTATTAAATGTTGCAACACTGCAAA

gi|558135472|ref|XM_006091085.   --------------------------------------------------
gi|554578862|ref|XM_005880935.   CATTGCACCAAGGGAAGGTGCTTCAGTGGTACCCGGGACACACAGGACTG
gi|584056807|ref|XM_006772885.   CATTGCACCAAGGGAAGGTGCTTCAGTGGTACCCAGGACACACAGGAC--
gi|641721271|ref|XM_008152116.   --------------------------------------------------
gi|588480441|ref|NM_001290172.   AAAAAAAAAAAAAAAAAA-------------------------------

gi|558135472|ref|XM_006091085.   --------------------------------------------------
gi|554578862|ref|XM_005880935.   CTAATCTCACAGAGTTTACAGTGTGGAGGGAATAAATACTGCGCTAAAAT
gi|584056807|ref|XM_006772885.   -TAATCTCACAGAGTTTACAGTGTGGAGGGAATAAATACTGTGATAAAAT
gi|641721271|ref|XM_008152116.   --------------------------------------------------
gi|588480441|ref|NM_001290172.   --------------------------------------------------

gi|558135472|ref|XM_006091085.   --------------------------------------------------
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gi|584056807|ref|XM_006772885.   ATAGAACCTGCAGGTGGATGTTTCAACCAACTCAGCCTAGGCATTCAGGA
gi|641721271|ref|XM_008152116.   --------------------------------------------------
gi|588480441|ref|NM_001290172.   --------------------------------------------------

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gi|584056807|ref|XM_006772885.   CAAAGAAACTCAACTCAATTCTTACCCTATATACTTGAATTATAACTAGG
gi|641721271|ref|XM_008152116.   --------------------------------------------------
gi|588480441|ref|NM_001290172.   --------------------------------------------------

gi|558135472|ref|XM_006091085.   --------------------------------------------------
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gi|584056807|ref|XM_006772885.   AGATCTGCCCTGGTAACATCAGACAAGGGCATAATTCTTCTCCTGAGCCT
gi|641721271|ref|XM_008152116.   --------------------------------------------------
gi|588480441|ref|NM_001290172.   --------------------------------------------------

gi|558135472|ref|XM_006091085.   --------------------------------------------------
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gi|584056807|ref|XM_006772885.   TTTGAATGGAAAGCACCTCATATTTTATGTGTTGGCTGCCTTGAAACAAA
gi|641721271|ref|XM_008152116.   --------------------------------------------------
gi|588480441|ref|NM_001290172.   --------------------------------------------------

gi|558135472|ref|XM_006091085.   --------------------------------------------------
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gi|584056807|ref|XM_006772885.   GTGGTTTTGTCGTTTCTACTACACTGGGCCTTTCCTCACTTTTCCCATTT
gi|641721271|ref|XM_008152116.   --------------------------------------------------
gi|588480441|ref|NM_001290172.   --------------------------------------------------

gi|558135472|ref|XM_006091085.   --------------------------------------------------
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gi|584056807|ref|XM_006772885.   CTACTGAATACAATTTAAGTTCTACGTGATGCCTCAGAAGGCTTCTAATT
gi|641721271|ref|XM_008152116.   --------------------------------------------------
gi|588480441|ref|NM_001290172.   --------------------------------------------------

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gi|584056807|ref|XM_006772885.   CAGATCCTCCCTCCACTTCAAGTCAATTCCCTCGCAAAGGTTAAAAACCT
gi|641721271|ref|XM_008152116.   --------------------------------------------------
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gi|641721271|ref|XM_008152116.   --------------------------------------------------
gi|588480441|ref|NM_001290172.   --------------------------------------------------

gi|558135472|ref|XM_006091085.   --------------------------------------------------
gi|554578862|ref|XM_005880935.   GTCATTATTAACTAATAGTCCCTGATGTAGTTTTGTTTTTATAAATTCAG
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gi|641721271|ref|XM_008152116.   --------------------------------------------------
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gi|641721271|ref|XM_008152116.   --------------------------------------------------
gi|588480441|ref|NM_001290172.   --------------------------------------------------

gi|558135472|ref|XM_006091085.   --------------------------------------------------
gi|554578862|ref|XM_005880935.   TAAGAGACATGCTGGAAATATCCATGTTTAACCAATATCTTTCGAGCAAA
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gi|641721271|ref|XM_008152116.   --------------------------------------------------
gi|588480441|ref|NM_001290172.   --------------------------------------------------

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gi|641721271|ref|XM_008152116.   --------------------------------------------------
gi|588480441|ref|NM_001290172.   --------------------------------------------------

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gi|641721271|ref|XM_008152116.   --------------------------------------------------
gi|588480441|ref|NM_001290172.   --------------------------------------------------

gi|558135472|ref|XM_006091085.   --------------------------------------------------
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gi|584056807|ref|XM_006772885.   GGAAAGTGGTGCTTATTGTAACAGAGGAGAGAAGTCTGACATCTTGGTCT
gi|641721271|ref|XM_008152116.   --------------------------------------------------
gi|588480441|ref|NM_001290172.   --------------------------------------------------

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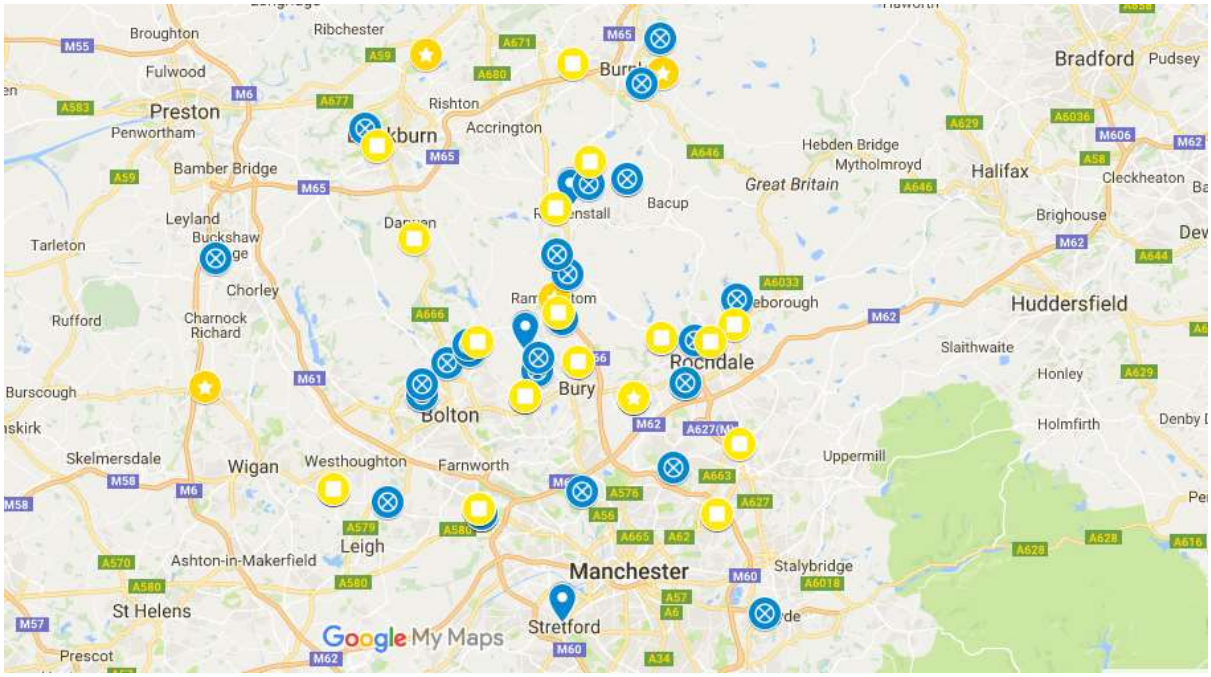
gi|641721271|ref|XM_008152116.   --------------------------------------------------
gi|588480441|ref|NM_001290172.   --------------------------------------------------

gi|558135472|ref|XM_006091085.    ------------------------------------------------
gi|554578862|ref|XM_005880935.    GGAGGGCAGCTCTTCTGGATGATCCTAGAAACAGGTGGGCTGACAC--
gi|584056807|ref|XM_006772885.    GGAGGGCAGCAATTCTGGATGATTCTAGAAACAGGTGGGCTGACAGAG
gi|641721271|ref|XM_008152116.    ------------------------------------------------
gi|588480441|ref|NM_001290172     ------------------------------------------------
```

Clustal W sequence alignment of bat TLR4 sequences: gi|558135472| TLR4 of *Myotis lucifugus*, gi|554578862| TLR4 of *Myotis brandtii*, gi|584056807| TLR4 of *Myotis davidii*, gi|588480441| TLR4 of *Pteropus alecto*, gi|641721271| TLR4 of *Eptesicus fuscus*

Appendix 2:



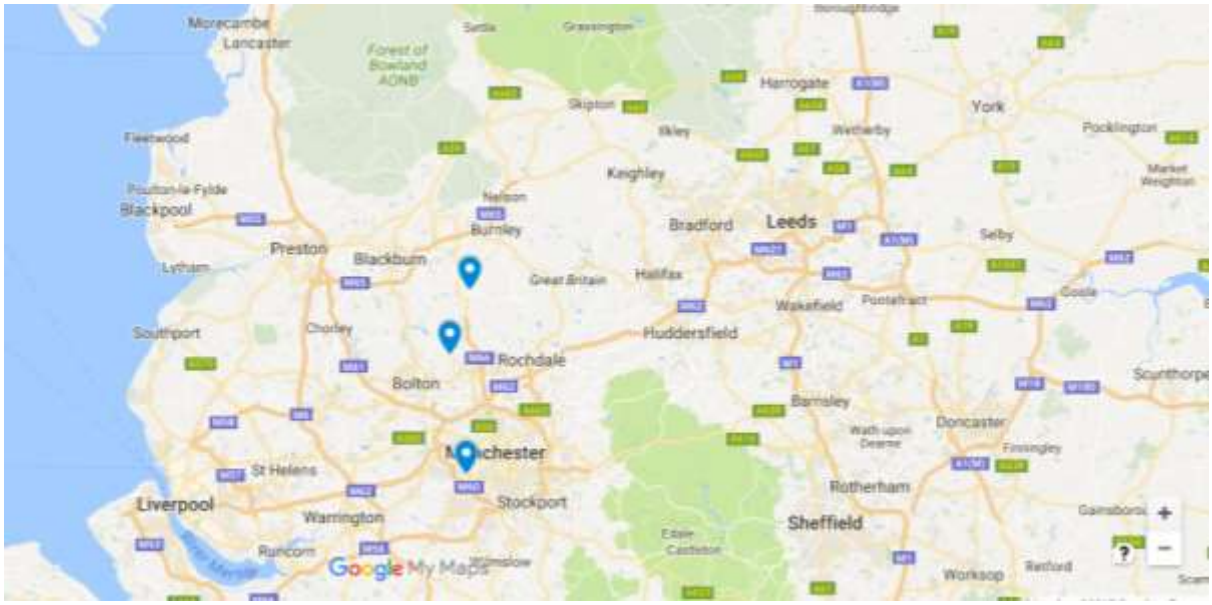
A map showing the location of pipistrelle bats infected with protozoa.

T.vespertilionis infected bats

T. dionisii infected bats

Cryptosporidium infected bats

Eimeria infected bats



location of *T. vespertilionis* infected bats .

location of *T. dionisii* infected bats

location of *Cryptosporidium* infected bats

location of *Eimeria* infected bats.

location of non- infected bats.

locations for single and mixed genotypes pipistrelle bats.

single interbreeding group

mixed genotype

A map showing the location of pipistrelle bats from TLR4 cluster 6

A map showing the location of pipistrelle bats from TLR2 heterozygotes bats

Appendix 3 :

TLR4 amino acid changes vs infection profile

Amino acid position and change	Infection profile	Infections	Chi-Square test
K 253 E	7 samples→ Helminths	*T. dionisii, Cryptosporidium, Eimeria, Bartonella, T. vespertilionis, Borrelia, L. linstowi, P. Koreanus, Prosthodendrium sp.*	p-value= 1.00
	7 samples→Protozoa		p-value= 0.6
L 254 V	7 samples→ Helminths	*T. dionisii, Cryptosporidium, Eimeria, Bartonella, T. vespertilionis, Borrelia, L. linstowi, P. Koreanus, Prosthodendrium sp.*	p-value= 1.00
	7 samples→Protozoa		p-value= 0.6
L 254 T	4 samples→Helminths	*B. vesperuginis, Eimeria, L. linstowi, P. Koreanus, Prosthodendrium sp.*	p-value= 0.7
	3 samples→Protozoa		p-value= 1.00
L 256 S	4 samples→Helminths	*B. vesperuginis, Eimeria, L. linstowi, P. Koreanus, Prosthodendrium sp.*	p-value= 0.7
	3 samples→Protozoa		p-value= 1.00
R 257 K	4 samples→Helminths	*B. vesperuginis, Eimeria, L. linstowi, P. Koreanus, Prosthodendrium sp.*	p-value= 0.7
	3 samples→Protozoa		p-value= 1.00
N 258 K	4 samples→Helminths	*B. vesperuginis, Eimeria, L. linstowi, P. Koreanus, Prosthodendrium sp.*	p-value= 0.7
	3 samples→Protozoa		p-value= 1.00
L 259 P	4 samples→Helminths	*B. vesperuginis, Eimeria, L. linstowi, P. Koreanus, Prosthodendrium sp.*	p-value= 0.7
	3 samples→Protozoa		p-value= 1.00
F 261 V	4 samples→Helminths	*B. vesperuginis, Eimeria, L. linstowi, P. Koreanus, Prosthodendrium sp.*	p-value= 0.7
	3 samples→Protozoa		p-value= 1.00
L 262 S	4 samples→Helminths	*B. vesperuginis, Eimeria, L. linstowi, P. Koreanus, Prosthodendrium sp.*	p-value= 0.7
	3 samples→Protozoa		p-value= 1.00

D 263R	4 samples→Helminths	*B. vesperuginis, Eimeria, L. linstowi, P. Koreanus, Prosthodendrium sp.*	p-value= 0.7
	3 samples→Protozoa		p-value= 1.00
S 265 Q	4 samples→Helminths	*B. vesperuginis, Eimeria, L. linstowi, P. Koreanus, Prosthodendrium sp.*	p-value= 0.7
	3 samples→Protozoa		p-value= 1.00
N 267 K	5 samples→ Helminths	*Eimeria, T. vespertilionis, B. vesperuginis, T.dionisii, Cryptosporidium, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	5 samples→ Protozoa		p-value= 0.7
G 268 W	5 samples→ Helminths	*Eimeria, T. vespertilionis, B. vesperuginis, T.dionisii, Cryptosporidium, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	5 samples→ Protozoa		p-value= 0.7
E 270 S	5 samples→ Helminths	*Eimeria, T. vespertilionis, B. vesperuginis, T.dionisii, Cryptosporidium, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	5 samples→ Protozoa		p-value= 0.7
K 272 Q	5 samples→ Helminths	*Eimeria, T. vespertilionis, B. vesperuginis, T.dionisii, Cryptosporidium, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	5 samples→ Protozoa		p-value= 0.7
S 273 V	5 samples→ Helminths	*Eimeria, T. vespertilionis, B. vesperuginis, T.dionisii, T. vespertilionis, Cryptosporidium, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	6 samples→ Protozoa		p-value= 0.7
L 274 C	5 samples→ Helminths	*Eimeria, T. vespertilionis, B. vesperuginis, T.dionisii, T. vespertilionis, Cryptosporidium, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	6 samples→ Protozoa		p-value= 0.7

L 275 C	5 samples→ Helminths	*Eimeria, T. vespertilionis, B. vesperuginis, T.dionisii, T. vespertilionis, Cryptosporidium, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	7 samples→ Protozoa		p-value= 0.7
G 281 W	5 samples→ Helminths	*Eimeria, T. vespertilionis, B. vesperuginis, T.dionisii, T. vespertilionis, Cryptosporidium, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	7 samples→ Protozoa		p-value= 0.7
T 282 D	5 samples→ Helminths	*Eimeria, T. vespertilionis, B. vesperuginis, T.dionisii, T. vespertilionis, Cryptosporidium, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	7 samples→ Protozoa		p-value= 0.7
T 283 N	5 samples→ Helminths	*Eimeria, T. vespertilionis, B. vesperuginis, T.dionisii, T. vespertilionis, Cryptosporidium, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	7 samples→ Protozoa		p-value= 0.7
R 284 Q	6 samples→ Helminths	*T. vespertilionis, Borrelia, Bartonella, Eimeria, Cryptosporidium, T. dionisii, Prosthodendrium sp L. linstowi, P. Koreanus*	p-value= 0.7
	2 samples→ Protozoa		p-value= 1.00
R 284 P	5 samples→ Helminths	*Eimeria, T. vespertilionis, B. vesperuginis, T.dionisii, T. vespertilionis, Cryptosporidium, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	7 samples→ Protozoa		p-value= 0.7

L 285 T	5 samples→ Helminths	*Eimeria, T. vespertilionis, B. vesperuginis, T.dionisii, T. vespertilionis, Cryptosporidium, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	7 samples→ Protozoa		p-value= 0.7
K 286 E	5 samples→ Helminths	*Eimeria, T. vespertilionis, B. vesperuginis, T.dionisii, T. vespertilionis, Cryptosporidium, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	7 samples→ Protozoa		p-value= 0.7
H 287 T	5 samples→ Helminths	*Eimeria, T. vespertilionis, B. vesperuginis, T.dionisii, T. vespertilionis, Cryptosporidium, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	7 samples→ Protozoa		p-value= 0.7
D 289 R	5 samples→ Helminths	*Eimeria, T. vespertilionis, B. vesperuginis, T.dionisii, T. vespertilionis, Cryptosporidium, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	7 samples→ Protozoa		p-value= 0.7
L 290 S	5 samples→ Helminths	*Eimeria, T. vespertilionis, B. vesperuginis, T.dionisii, T. vespertilionis, Cryptosporidium, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	7 samples→ Protozoa		p-value= 0.7
S 291 E	5 samples→ Helminths	*Eimeria, T. vespertilionis, B. vesperuginis, T.dionisii, T. vespertilionis, Cryptosporidium, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	7 samples→ Protozoa		p-value= 0.7
F 292 L	5 samples→ Helminths	*Eimeria, T. vespertilionis, B. vesperuginis, T.dionisii, T. vespertilionis, Cryptosporidium, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	7 samples→ Protozoa		p-value= 0.7

N 293 Q	5 samples→ Helminths	*Eimeria, T. vespertilionis, B. vesperuginis, T.dionisii, T. vespertilionis, Cryptosporidium, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	7 samples→ Protozoa		p-value= 0.7
N 293 I	2 samples→ Helminths	*T. vespertilionis, B. vesperuginis, Cryptosporidium, T. dionisii, Prosthodendrium sp L. linstowi, P. Koreanus, L. spathulatum, P. heteroporus*	p-value= 1.00
	6 samples→ Protozoa		p-value= 0.7
I 295 Y	5 samples→ Helminths	*Eimeria, T. vespertilionis, B. vesperuginis, T.dionisii, T. vespertilionis, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	7 samples→ Protozoa		p-value= 0.7
I 295 L	1 samples→ Helminths	*T. vespertilionis, B. vesperuginis, Eimeria, Prosthodendrium sp L. linstowi, P. Koreanus*	p-value= 1.00
	3 samples→ Protozoa		p-value= 1.00
I 296 Y	5 samples→ Helminths	*Eimeria, T. vespertilionis, B. vesperuginis, T.dionisii, T. vespertilionis, Cryptosporidium, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	7 samples→ Protozoa		p-value= 0.7
T 297 P	5 samples→ Helminths	*T. vespertilionis, B. vesperuginis, Cryptosporidium, Eimeria, T. dionisii, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	9 samples→ Protozoa		p-value= 0.7
T 299 L	5 samples→ Helminths	*T. vespertilionis, B. vesperuginis, Cryptosporidium, Eimeria, T. dionisii, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	9 samples→ Protozoa		p-value= 0.7

S 301 Q	5 samples→ Helminths	*T. vespertilionis, B. vesperuginis, Cryptosporidium, Eimeria, T. dionisii, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	9 samples→ Protozoa		p-value= 0.7
N 301 T	5 samples→ Helminths	*T. vespertilionis, B. vesperuginis, Cryptosporidium, Eimeria, T. dionisii, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	9 samples→ Protozoa		p-value= 0.7
F 302 S	5 samples→ Helminths	*T. vespertilionis, B. vesperuginis, Cryptosporidium, Eimeria, T. dionisii, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	9 samples→ Protozoa		p-value= 0.7
F 302 L	3 samples→ Helminths	*B. vesperuginis, T. dionisii, Prosthodendrium sp L. linstowi, P. Koreanus, L. spathulatum, P. heteroporus*	p-value= 1.00
	4 samples→ Protozoa		p-value= 1.00
V 303 W	5 samples→ Helminths	*B. vesperuginis, Eimeria, T. dionisii, T. vespertilionis, Cryptosporidium, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	10 samples→ Protozoa		p-value= 1.00
V 303 R	2 samples→ Helminths	*B. vesperuginis, T. dionisii, Prosthodendrium sp L. linstowi, P. Koreanus, L. spathulatum, P. heteroporus*	p-value= 1.00
	4 samples→ Protozoa		p-value= 1.00
G 304 A	5 samples→ Helminths	*B. vesperuginis, Eimeria, T. dionisii, T. vespertilionis, Cryptosporidium, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	10 samples→ Protozoa		p-value= 1.00

I 308 N	3 samples→ Helminths	*B. vesperuginis, Eimeria, T. dionisii, Cryptosporidium, Prosthodendrium sp L. linstowi, P. Koreanus, L. spathulatum, P. heteroporus*	p-value= 1.00
	7 samples→ Protozoa		p-value= 1.00
I 308 K	3 samples→ Helminths	*T. vespertilionis, B. vesperuginis, Eimeria, T. dionisii, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	7 samples→ Protozoa		p-value= 1.00
E 309 R	3 samples→ Helminths	*B. vesperuginis, Eimeria, T. dionisii, Cryptosporidium, Prosthodendrium sp L. linstowi, P. Koreanus, L. spathulatum, P. heteroporus*	p-value= 1.00
	7 samples→ Protozoa		p-value= 1.00
H 310 T	3 samples→ Helminths	*B. vesperuginis, Eimeria, T. dionisii, Cryptosporidium, Prosthodendrium sp L. linstowi, P. Koreanus, L. spathulatum, P. heteroporus*	p-value= 1.00
	7 samples→ Protozoa		p-value= 1.00
H 310 N	4 samples→ Helminths	*T. vespertilionis, B. vesperuginis, Eimeria, T. dionisii, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	7 samples→ Protozoa		p-value= 1.00
L 311 S	3 samples→ Helminths	*B. vesperuginis, Eimeria, T. dionisii, Cryptosporidium, Prosthodendrium sp L. linstowi, P. Koreanus, L. spathulatum, P. heteroporus*	p-value= 1.00
	7 samples→ Protozoa		p-value= 1.00
L 311 I	4 samples→ Helminths	*T. vespertilionis, B. vesperuginis, Eimeria, T. dionisii, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	7 samples→ Protozoa		p-value= 1.00

D 312 G	3 samples→ Helminths	*B. vesperuginis, Eimeria, T. dionisii, Cryptosporidium, Prosthodendrium sp L. linstowi, P. Koreanus, L. spathulatum, P. heteroporus*	p-value= 1.00
	7 samples→ Protozoa		p-value= 1.00
D 312 W	4 samples→ Helminths	*T. vespertilionis, B. vesperuginis, Eimeria, T. dionisii, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	7 samples→ Protozoa		p-value= 1.00
Q 314 P	3 samples→ Helminths	*B. vesperuginis, Eimeria, T. dionisii, Cryptosporidium, Prosthodendrium sp L. linstowi, P. Koreanus, L. spathulatum, P. heteroporus*	p-value= 1.00
	7 samples→ Protozoa		p-value= 1.00
Q 314 S	5 samples→ Helminths	*T. vespertilionis, B. vesperuginis, Eimeria, T. dionisii, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	8 samples→ Protozoa		p-value= 0.7
H 315 A	3 samples→ Helminths	*B. vesperuginis, Eimeria, T. dionisii, Cryptosporidium, Prosthodendrium sp L. linstowi, P. Koreanus, L. spathulatum, P. heteroporus*	p-value= 1.00
	7 samples→ Protozoa		p-value= 1.00
H 315 S	4 samples→ Helminths	*T. vespertilionis, B. vesperuginis, Eimeria, T. dionisii, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	7 samples→ Protozoa		p-value= 1.00
S 316 F	3 samples→ Helminths	*B. vesperuginis, Eimeria, T. dionisii, Prosthodendrium sp L. linstowi, P. Koreanus, L. spathulatum, P. heteroporus*	p-value= 1.00
	6 samples→ Protozoa		p-value= 1.00

S 316 I	4 samples→ Helminths	*T. vespertilionis, B. vesperuginis, Eimeria, T. dionisii, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	7 samples→ Protozoa		p-value= 1.00
T 317 H	3 samples→ Helminths	*B. vesperuginis, Eimeria, T. dionisii, Cryptosporidium, Prosthodendrium sp L. linstowi, P. Koreanus, L. spathulatum, P. heteroporus*	p-value= 1.00
	7 samples→ Protozoa		p-value= 1.00
T 317 P	4 samples→ Helminths	*T. vespertilionis, B. vesperuginis, Eimeria, T. dionisii, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	7 samples→ Protozoa		p-value= 1.00
L 318 F	3 samples→ Helminths	*B. vesperuginis, Eimeria, T. dionisii, Cryptosporidium, Prosthodendrium sp L. linstowi, P. Koreanus, L. spathulatum, P. heteroporus*	p-value= 1.00
	7 samples→ Protozoa		p-value= 1.00
R 320 T	3 samples→ Helminths	*B. vesperuginis, Eimeria, T. dionisii, Cryptosporidium, Prosthodendrium sp L. linstowi, P. Koreanus, L. spathulatum, P. heteroporus*	p-value= 1.00
	7 samples→ Protozoa		p-value= 1.00
R 320 D	5 samples→ Helminths	*T. vespertilionis, B. vesperuginis, Eimeria, T. dionisii, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	8 samples→ Protozoa		p-value= 0.7

A 322 P	5 samples→ Helminths	*T. vespertilionis, B. vesperuginis, Eimeria, T. dionisii, Cryptosporidium, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	9 samples→ Protozoa		p-value= 0.7
A 322 Q	3 samples→ Helminths	*B. vesperuginis, Eimeria, T. dionisii, Prosthodendrium sp L. linstowi, P. Koreanus, L. spathulatum, P. heteroporus*	p-value= 1.00
	6 samples→ Protozoa		p-value= 1.00
F 328 S	5 samples→ Helminths	*T. vespertilionis, B. vesperuginis, Eimeria, T. dionisii, Cryptosporidium, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	9 samples→ Protozoa		p-value= 0.7
F 328 P	3 samples→ Helminths	*B. vesperuginis, Eimeria, T. dionisii, Prosthodendrium sp L. linstowi, P. Koreanus, L. spathulatum, P. heteroporus*	p-value= 1.00
	6 samples→ Protozoa		p-value= 1.00
K 332 E	10 samples→ Helminths	*B. vesperuginis, Eimeria, Cryptosporidium, T. vespertilionis, T. dionisii, Toxoplasma, Borrelia, Prosthodendrium sp, L. linstowi, P. heteroporus, P. Koreanus*	p-value= 1.00
	11 samples→ Protozoa		p-value= 0.8
L 334 S	2 samples→ Helminths	*B. vesperuginis, T. dionisii, Cryptosporidium, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	4 samples→ Protozoa		p-value= 1.00

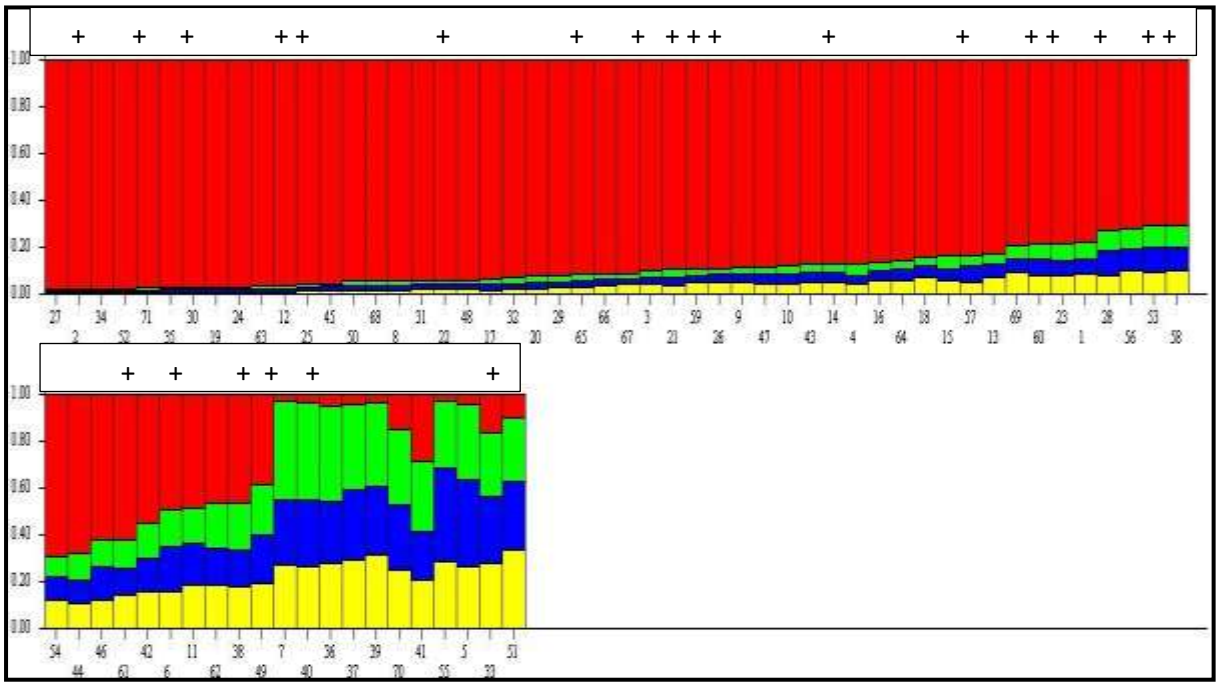
L 335 F	2 samples→ Helminths	*B. vesperuginis, T. dionisii, Cryptosporidium, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	4 samples→ Protozoa		p-value= 1.00
Y 336 T	2 samples→ Helminths	*B. vesperuginis, T. dionisii, Cryptosporidium, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	4 samples→ Protozoa		p-value= 1.00
D 338 I	2 samples→ Helminths	*B. vesperuginis, T. dionisii, Cryptosporidium, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	5 samples→ Protozoa		p-value= 1.00
Y 341T	3 samples→ Helminths	*B. vesperuginis, T. dionisii, Cryptosporidium, Prosthodendrium sp, L. linstowi, P. Koreanus, L. spathulatum*	p-value= 1.00
	7 samples→ Protozoa		p-value= 1.00
T 342 L	2 samples→ Helminths	*B. vesperuginis, T. dionisii, Cryptosporidium, Prosthodendrium sp, L. linstowi, P. Koreanus, L. spathulatum*	p-value= 1.00
	7 samples→ Protozoa		p-value= 1.00
D 343 T	2 samples→ Helminths	*B. vesperuginis, T. dionisii, Cryptosporidium, Prosthodendrium sp, L. linstowi, P. Koreanus, L. spathulatum*	p-value= 1.00
	7 samples→ Protozoa		p-value= 1.00
I 344 S	2 samples→ Helminths	*B. vesperuginis, T. dionisii, Cryptosporidium, Prosthodendrium sp, L. linstowi, P. Koreanus, L. spathulatum*	p-value= 1.00
	7 samples→ Protozoa		p-value= 1.00
K 345 R	3 samples→ Helminths	*B. vesperuginis, T. dionisii, Cryptosporidium, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	3 samples→ Protozoa		p-value= 1.00

I 356 V	9 samples→ Helminths	*B. vesperuginis, Eimeria, T. vespertilionis, T. dionisii, Cryptosporidium, Toxoplasma, Borrelia, Prosthodendrium sp, P. heteroporus, L. linstowi, P. Koreanus*	p-value= 1.00
	12 samples→ Protozoa		p-value= 1.00
S 357 A	5 samples→ Helminths	*B. vesperuginis, T. dionisii, Cryptosporidium, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	5 samples→ Protozoa		p-value= 1.00
L 358 S	5 samples→ Helminths	*B. vesperuginis, T. dionisii, Cryptosporidium, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	5 samples→ Protozoa		p-value= 1.00
Q 359 K	5 samples→ Helminths	*B. vesperuginis, T. dionisii, Cryptosporidium, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	5 samples→ Protozoa		p-value= 1.00
V 360 S	5 samples→ Helminths	*B. vesperuginis, T. dionisii, Cryptosporidium, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	5 samples→ Protozoa		p-value= 1.00
M 363 W	5 samples→ Helminths	*B. vesperuginis, T. dionisii, Cryptosporidium, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	5 samples→ Protozoa		p-value= 1.00
A 364 L	5 samples→ Helminths	*B. vesperuginis, T. dionisii, Cryptosporidium, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	5 samples→ Protozoa		p-value= 1.00

G 365 A	5 samples→ Helminths	*B. vesperuginis, T. dionisii, Cryptosporidium, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	5 samples→ Protozoa		p-value= 1.00
N 366 I	5 samples→ Helminths	*B. vesperuginis, T. dionisii, Cryptosporidium, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	5 samples→ Protozoa		p-value= 1.00
S 367 P	5 samples→ Helminths	*B. vesperuginis, T. dionisii, Cryptosporidium, Prosthodendrium sp, L. linstowi, P. Koreanus*	p-value= 1.00
	5 samples→ Protozoa		p-value= 1.00
C 473 R	11 samples→ Helminths	*B. vesperuginis, T. vespertilionis, T. dionisii, Cryptosporidium, Eimeria, Toxoplasma, L. linstowi, L. spathulatum, Prosthodendrium sp, P. koreanus, P. heteropus*	p-value= 1.00
	18 samples→ Protozoa		p-value= 0.8
T 510 H	2 samples→ Helminths	*Cryptosporidium, Eimeria, L. linstowi, P. koreanus, P. heteropus*	p-value= 1.00
	2 samples→ Protozoa		p-value= 1.00
M 514 D	3 samples→ Helminths	*Cryptosporidium, Toxoplasma, L. linstowi, L. spathulatum, P. koreanus, P. heteropus*	p-value= 1.00
	2 samples→ Protozoa		p-value= 1.00
K 516 Q	2 samples→ Helminths	*Cryptosporidium, Toxoplasma, L. linstowi, L. spathulatum*	p-value= 1.00
	2 samples→ Protozoa		p-value= 1.00

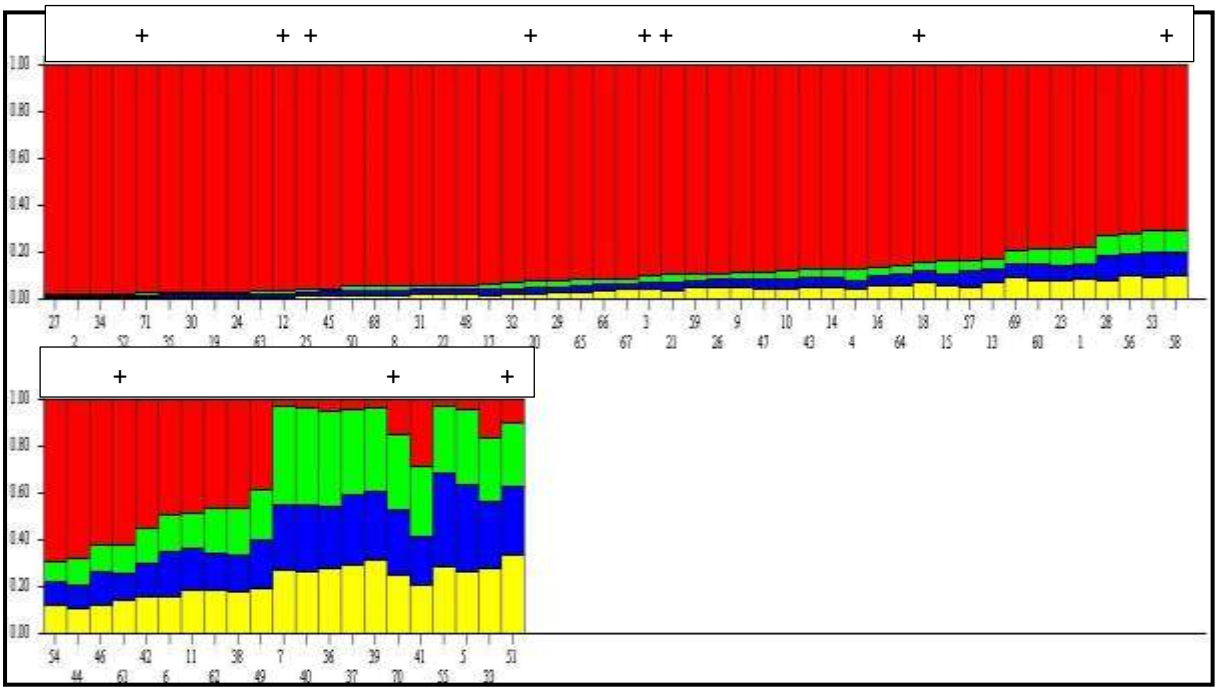
T 517 N	2 samples→ Helminths	*Cryptosporidium, Toxoplasma, L. linstowi, L. spathulatum*	p-value= 1.00
	2 samples→ Protozoa		p-value= 1.00
V 518 C	3 samples→ Helminths	*Cryptosporidium, Toxoplasma, L. linstowi, L. spathulatum*	p-value= 1.00
	2 samples→ Protozoa		p-value= 1.00

Appendix 4:



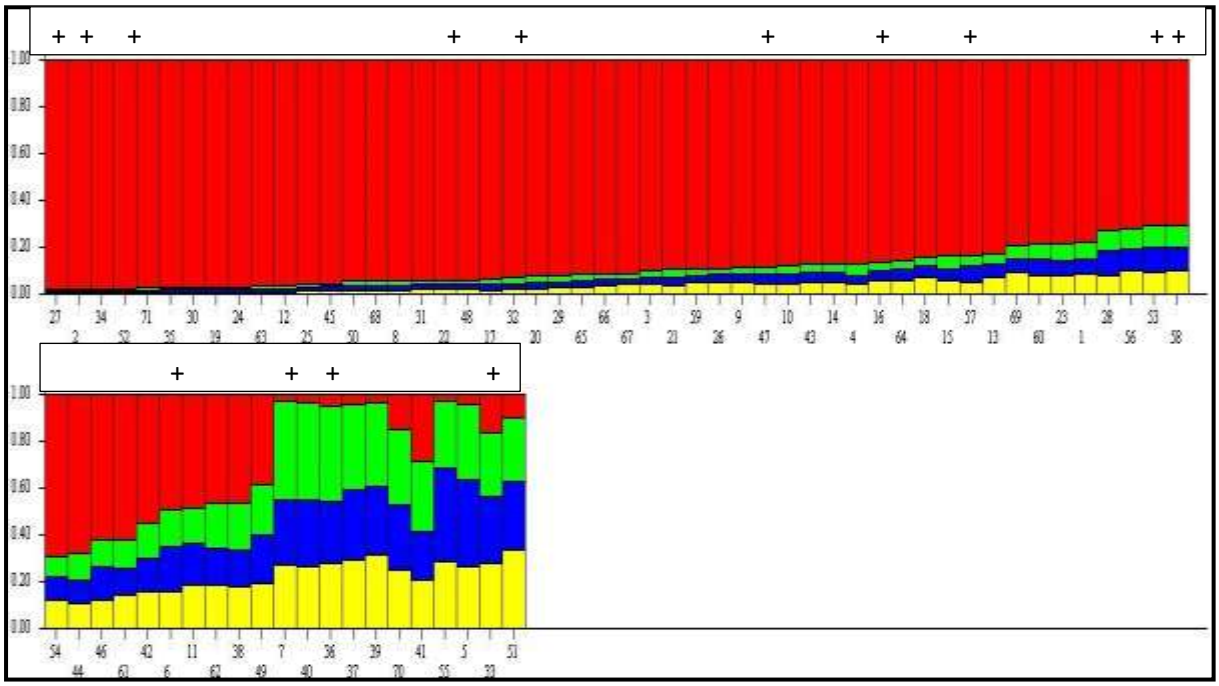
bat multilocus genotypes (Dodd et al, 2014) vs *T. dionisii* infections:

1 = CS/06/10, 2 = CS/08/01, 3 = CS/08/02, 4 = CS/08/A, 5 = FP/05/46, 6 = FP/07/11, 7 = FP/07/12, 8 = FP/07/13, 9 = FP/07/21, 10 = FP/07/37, 11 = FP/07/42, 12 = FP/07/44, 13 = FP/07/45, 14 = FP/07/47, 15 = FP/07/51, 16 = FP/08/02, 17 = GH/06/06, 18 = GH/07/09, 19 = GH/07/10, 20 = JH/07/01, 21 = JH/08/02, 22 = JL/06/12, 23 = JL/06/13, 24 = JL/06/15, 25 = JL/06/24, 26 = JL/06/26, 27= JL/06/27, 28 = JL/06/28, 29 = JL/06/40, 30 = JL/06/42, 31 = JL/06/45, 32 = JL/06/47, 33 = JL/06/54, 34 = JL/06/56, 35 = JL/06/59, 36 = JL/07/04, 37 = JL/07/07, 38 = JL/07/08, 39 = JL/07/09, 40 = JL/07/10, 41 = JL/07/11, 42 = JL/07/12, 43 = JL/07/14, 44 = JL/07/18, 45 = JL/07/23, 46 = JL/07/25, 47 = MD/08/02, 48 = MH/08/02, 49 = PB/06/01, 50 = PB/06/02, 51 = PH/06/04, 52 = PH/06/05, 53 = SA/06/05, 54 = SA/06/07, 55 = SA/07/U, 56 = SP/06/49, 57 = SP/06/55, 58 = SP/06/68, 59 = SP/06/70, 60 = SP/06/72, 61 = SP/06/77, 62 = SP/06/79, 63 = SP/06/80, 64 = SP/06/81, 65 = SP/06/82, 66 = SP/06/83, 67 = SP/06/84, 68 = SP/08/16, 69 = SP/08/17, 70 = SP/08/18, 71 = SP/08/19. Red shows positive samples.



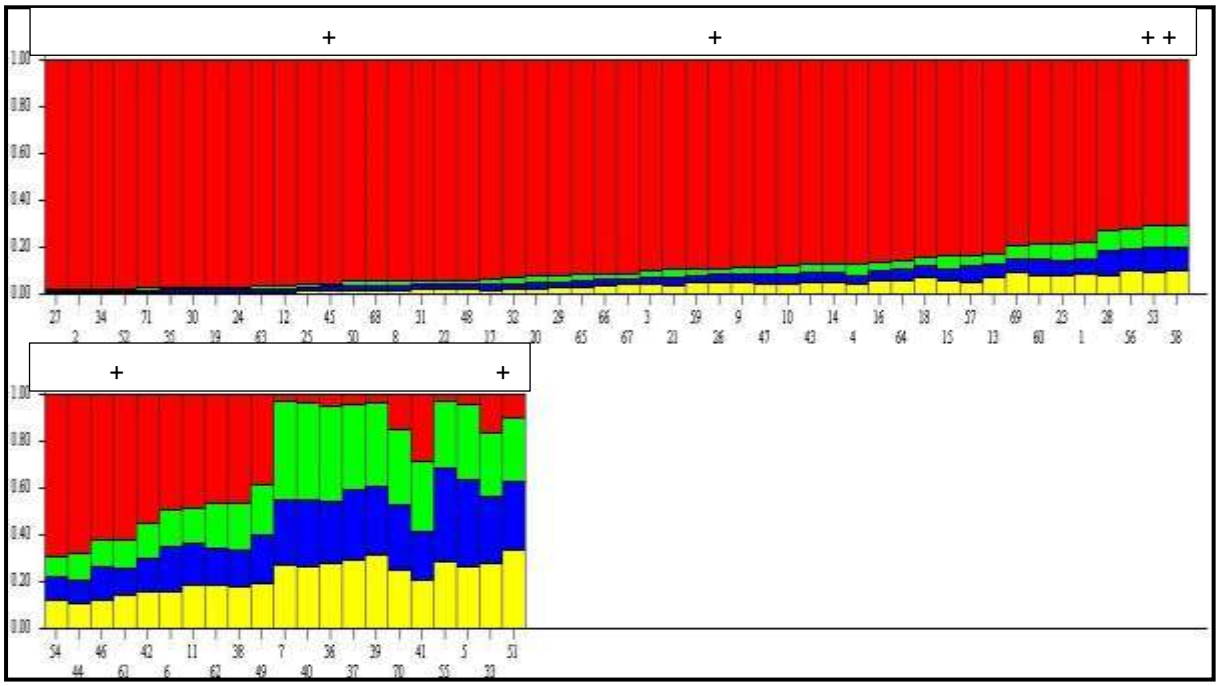
bat multilocus genotypes (Dodd et al, 2014) vs *Cryptosporidium* infections:

1 = CS/06/10, 2 = CS/08/01, 3 = CS/08/02, 4 = CS/08/A, 5 = FP/05/46, 6 = FP/07/11, 7 = FP/07/12, 8 = FP/07/13, 9 = FP/07/21, 10 = FP/07/37, 11 = FP/07/42, 12 = FP/07/44, 13 = FP/07/45, 14 = FP/07/47, 15 = FP/07/51, 16 = FP/08/02, 17 = GH/06/06, 18 = GH/07/09, 19 = GH/07/10, 20 = JH/07/01, 21 = JH/08/02, 22 = JL/06/12, 23 = JL/06/13, 24 = JL/06/15, 25 = JL/06/24, 26 = JL/06/26, 27= JL/06/27, 28 = JL/06/28, 29 = JL/06/40, 30 = JL/06/42, 31 = JL/06/45, 32 = JL/06/47, 33 = JL/06/54, 34 = JL/06/56, 35 = JL/06/59, 36 = JL/07/04, 37 = JL/07/07, 38 = JL/07/08, 39 = JL/07/09, 40 = JL/07/10, 41 = JL/07/11, 42 = JL/07/12, 43 = JL/07/14, 44 = JL/07/18, 45 = JL/07/23, 46 = JL/07/25, 47 = MD/08/02, 48 = MH/08/02, 49 = PB/06/01, 50 = PB/06/02, 51 = PH/06/04, 52 = PH/06/05, 53 = SA/06/05, 54 = SA/06/07, 55 = SA/07/U, 56 = SP/06/49, 57 = SP/06/55, 58 = SP/06/68, 59 = SP/06/70, 60 = SP/06/72, 61 = SP/06/77, 62 = SP/06/79, 63 = SP/06/80, 64 = SP/06/81, 65 = SP/06/82, 66 = SP/06/83, 67 = SP/06/84, 68 = SP/08/16, 69 = SP/08/17, 70 = SP/08/18, 71 = SP/08/19. Red shows positive samples.



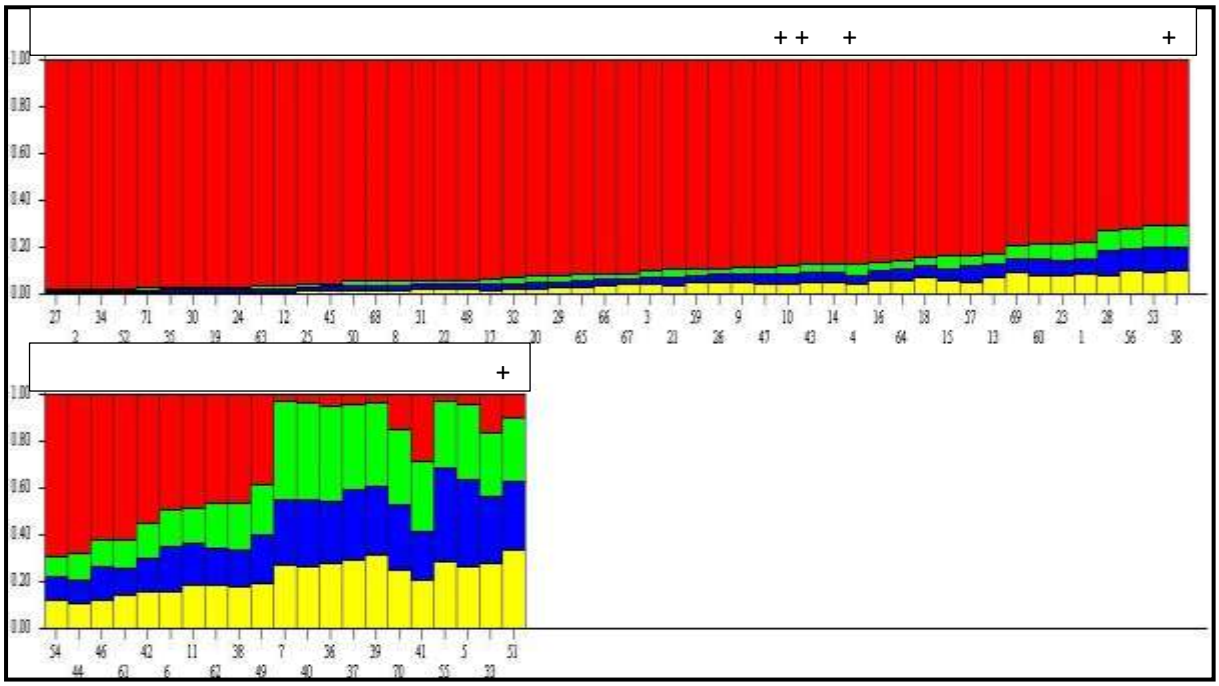
bat multilocus genotypes (Dodd et al, 2014) vs *Babesia* infections:

1 = CS/06/10, 2 = CS/08/01, 3 = CS/08/02, 4 = CS/08/A, 5 = FP/05/46, 6 = FP/07/11, 7 = FP/07/12, 8 = FP/07/13, 9 = FP/07/21, 10 = FP/07/37, 11 = FP/07/42, 12 = FP/07/44, 13 = FP/07/45, 14 = FP/07/47, 15 = FP/07/51, 16 = FP/08/02, 17 = GH/06/06, 18 = GH/07/09, 19 = GH/07/10, 20 = JH/07/01, 21 = JH/08/02, 22 = JL/06/12, 23 = JL/06/13, 24 = JL/06/15, 25 = JL/06/24, 26 = JL/06/26, 27= JL/06/27, 28 = JL/06/28, 29 = JL/06/40, 30 = JL/06/42, 31 = JL/06/45, 32 = JL/06/47, 33 = JL/06/54, 34 = JL/06/56, 35 = JL/06/59, 36 = JL/07/04, 37 = JL/07/07, 38 = JL/07/08, 39 = JL/07/09, 40 = JL/07/10, 41 = JL/07/11, 42 = JL/07/12, 43 = JL/07/14, 44 = JL/07/18, 45 = JL/07/23, 46 = JL/07/25, 47 = MD/08/02, 48 = MH/08/02, 49 = PB/06/01, 50 = PB/06/02, 51 = PH/06/04, 52 = PH/06/05, 53 = SA/06/05, 54 = SA/06/07, 55 = SA/07/U, 56 = SP/06/49, 57 = SP/06/55, 58 = SP/06/68, 59 = SP/06/70, 60 = SP/06/72, 61 = SP/06/77, 62 = SP/06/79, 63 = SP/06/80, 64 = SP/06/81, 65 = SP/06/82, 66 = SP/06/83, 67 = SP/06/84, 68 = SP/08/16, 69 = SP/08/17, 70 = SP/08/18, 71 = SP/08/19. Red shows positive samples.



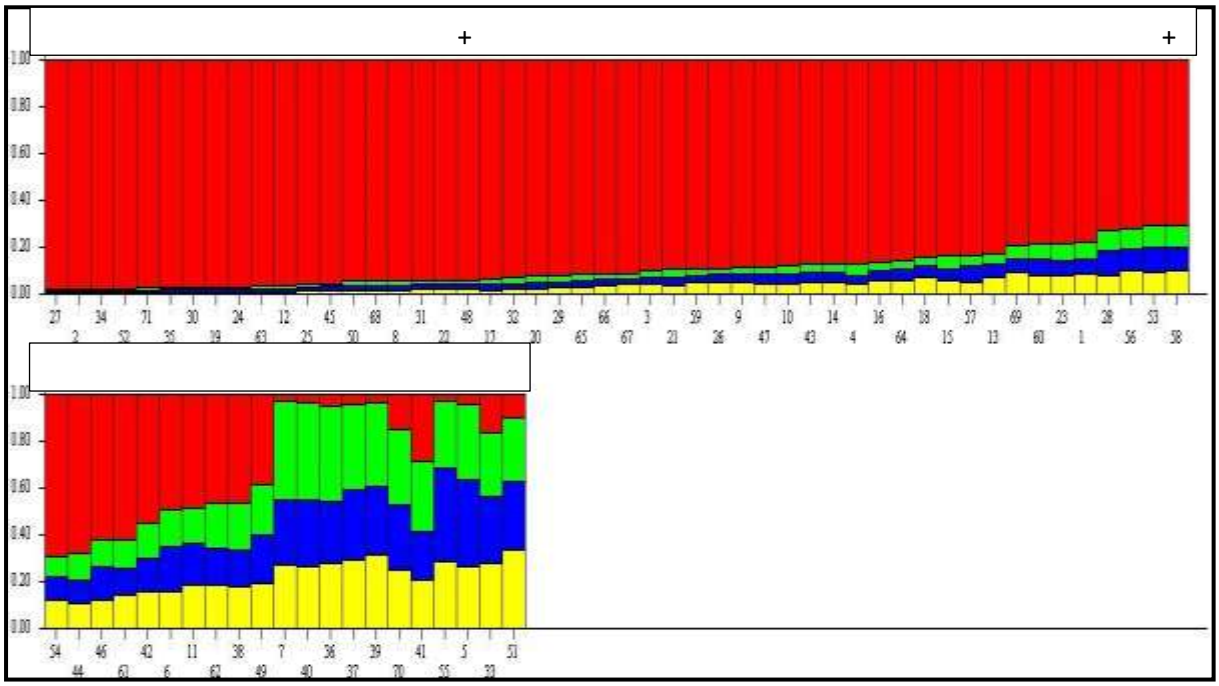
bat multilocus genotypes (Dodd et al, 2014) highlighting the TLR2 heterozygotes.

1 = CS/06/10, 2 = CS/08/01, 3 = CS/08/02, 4 = CS/08/A, 5 = FP/05/46, 6 = FP/07/11, 7 = FP/07/12, 8 = FP/07/13, 9 = FP/07/21, 10 = FP/07/37, 11 = FP/07/42, 12 = FP/07/44, 13 = FP/07/45, 14 = FP/07/47, 15 = FP/07/51, 16 = FP/08/02, 17 = GH/06/06, 18 = GH/07/09, 19 = GH/07/10, 20 = JH/07/01, 21 = JH/08/02, 22 = JL/06/12, 23 = JL/06/13, 24 = JL/06/15, 25 = JL/06/24, 26 = JL/06/26, 27= JL/06/27, 28 = JL/06/28, 29 = JL/06/40, 30 = JL/06/42, 31 = JL/06/45, 32 = JL/06/47, 33 = JL/06/54, 34 = JL/06/56, 35 = JL/06/59, 36 = JL/07/04, 37 = JL/07/07, 38 = JL/07/08, 39 = JL/07/09, 40 = JL/07/10, 41 = JL/07/11, 42 = JL/07/12, 43 = JL/07/14, 44 = JL/07/18, 45 = JL/07/23, 46 = JL/07/25, 47 = MD/08/02, 48 = MH/08/02, 49 = PB/06/01, 50 = PB/06/02, 51 = PH/06/04, 52 = PH/06/05, 53 = SA/06/05, 54 = SA/06/07, 55 = SA/07/U, 56 = SP/06/49, 57 = SP/06/55, 58 = SP/06/68, 59 = SP/06/70, 60 = SP/06/72, 61 = SP/06/77, 62 = SP/06/79, 63 = SP/06/80, 64 = SP/06/81, 65 = SP/06/82, 66 = SP/06/83, 67 = SP/06/84, 68 = SP/08/16, 69 = SP/08/17, 70 = SP/08/18, 71 = SP/08/19. Red shows Heterozygotes samples.



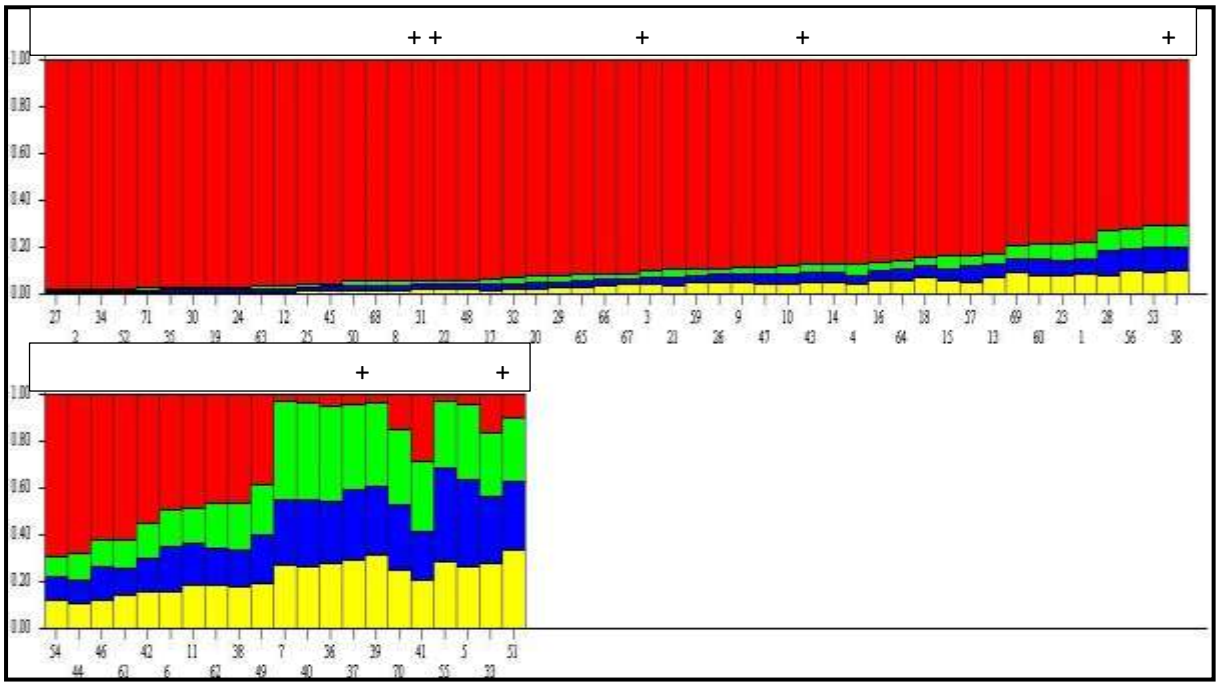
Mapping the pipistrelle TLR1 cluster to the bat multilocus genotyping data (Dodd et al, 2014).

1 = CS/06/10, 2 = CS/08/01, 3 = CS/08/02, 4 = CS/08/A, 5 = FP/05/46, 6 = FP/07/11, 7 = FP/07/12, 8 = FP/07/13, 9 = FP/07/21, 10 = FP/07/37, 11 = FP/07/42, 12 = FP/07/44, 13 = FP/07/45, 14 = FP/07/47, 15 = FP/07/51, 16 = FP/08/02, 17 = GH/06/06, 18 = GH/07/09, 19 = GH/07/10, 20 = JH/07/01, 21 = JH/08/02, 22 = JL/06/12, 23 = JL/06/13, 24 = JL/06/15, 25 = JL/06/24, 26 = JL/06/26, 27= JL/06/27, 28 = JL/06/28, 29 = JL/06/40, 30 = JL/06/42, 31 = JL/06/45, 32 = JL/06/47, 33 = JL/06/54, 34 = JL/06/56, 35 = JL/06/59, 36 = JL/07/04, 37 = JL/07/07, 38 = JL/07/08, 39 = JL/07/09, 40 = JL/07/10, 41 = JL/07/11, 42 = JL/07/12, 43 = JL/07/14, 44 = JL/07/18, 45 = JL/07/23, 46 = JL/07/25, 47 = MD/08/02, 48 = MH/08/02, 49 = PB/06/01, 50 = PB/06/02, 51 = PH/06/04, 52 = PH/06/05, 53 = SA/06/05, 54 = SA/06/07, 55 = SA/07/U, 56 = SP/06/49, 57 = SP/06/55, 58 = SP/06/68, 59 = SP/06/70, 60 = SP/06/72, 61 = SP/06/77, 62 = SP/06/79, 63 = SP/06/80, 64 = SP/06/81, 65 = SP/06/82, 66 = SP/06/83, 67 = SP/06/84, 68 = SP/08/16, 69 = SP/08/17, 70 = SP/08/18, 71 = SP/08/19. Red shows cluster1 samples.



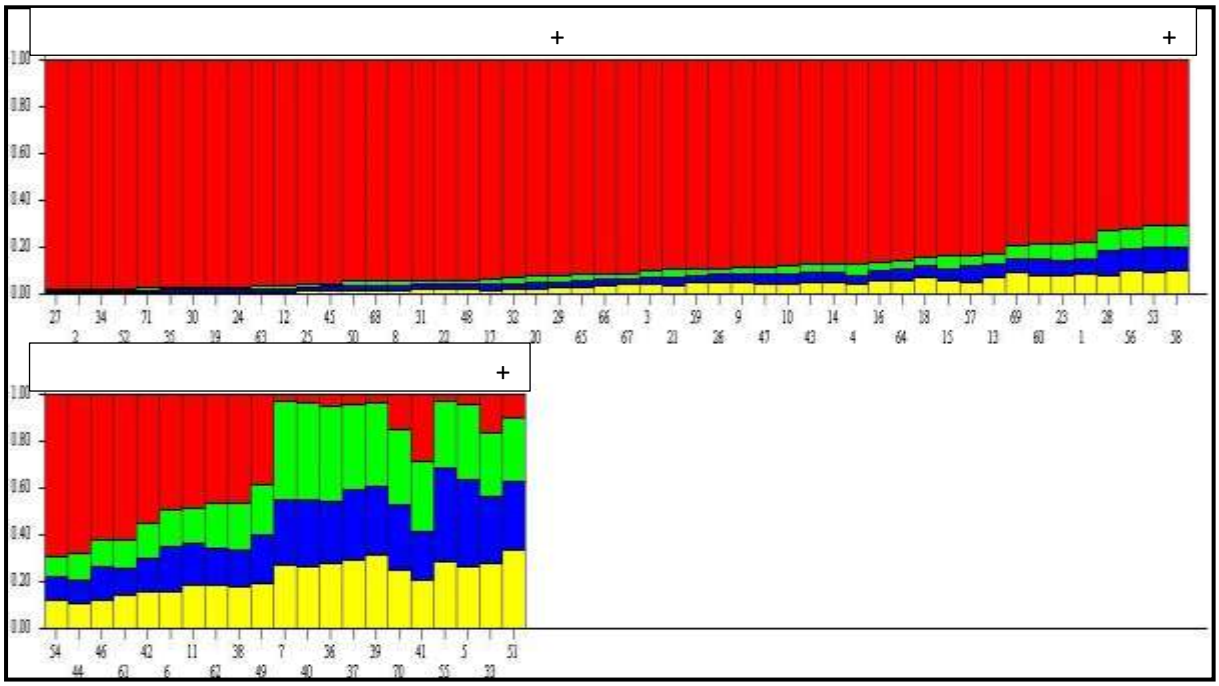
Mapping the pipistrelle TLR2 cluster to the bat multilocus genotyping data (Dodd et al, 2014).

1 = CS/06/10, 2 = CS/08/01, 3 = CS/08/02, 4 = CS/08/A, 5 = FP/05/46, 6 = FP/07/11, 7 = FP/07/12, 8 = FP/07/13, 9 = FP/07/21, 10 = FP/07/37, 11 = FP/07/42, 12 = FP/07/44, 13 = FP/07/45, 14 = FP/07/47, 15 = FP/07/51, 16 = FP/08/02, 17 = GH/06/06, 18 = GH/07/09, 19 = GH/07/10, 20 = JH/07/01, 21 = JH/08/02, 22 = JL/06/12, 23 = JL/06/13, 24 = JL/06/15, 25 = JL/06/24, 26 = JL/06/26, 27= JL/06/27, 28 = JL/06/28, 29 = JL/06/40, 30 = JL/06/42, 31 = JL/06/45, 32 = JL/06/47, 33 = JL/06/54, 34 = JL/06/56, 35 = JL/06/59, 36 = JL/07/04, 37 = JL/07/07, 38 = JL/07/08, 39 = JL/07/09, 40 = JL/07/10, 41 = JL/07/11, 42 = JL/07/12, 43 = JL/07/14, 44 = JL/07/18, 45 = JL/07/23, 46 = JL/07/25, 47 = MD/08/02, 48 = MH/08/02, 49 = PB/06/01, 50 = PB/06/02, 51 = PH/06/04, 52 = PH/06/05, 53 = SA/06/05, 54 = SA/06/07, 55 = SA/07/U, 56 = SP/06/49, 57 = SP/06/55, 58 = SP/06/68, 59 = SP/06/70, 60 = SP/06/72, 61 = SP/06/77, 62 = SP/06/79, 63 = SP/06/80, 64 = SP/06/81, 65 = SP/06/82, 66 = SP/06/83, 67 = SP/06/84, 68 = SP/08/16, 69 = SP/08/17, 70 = SP/08/18, 71 = SP/08/19. Red shows cluster2 samples.



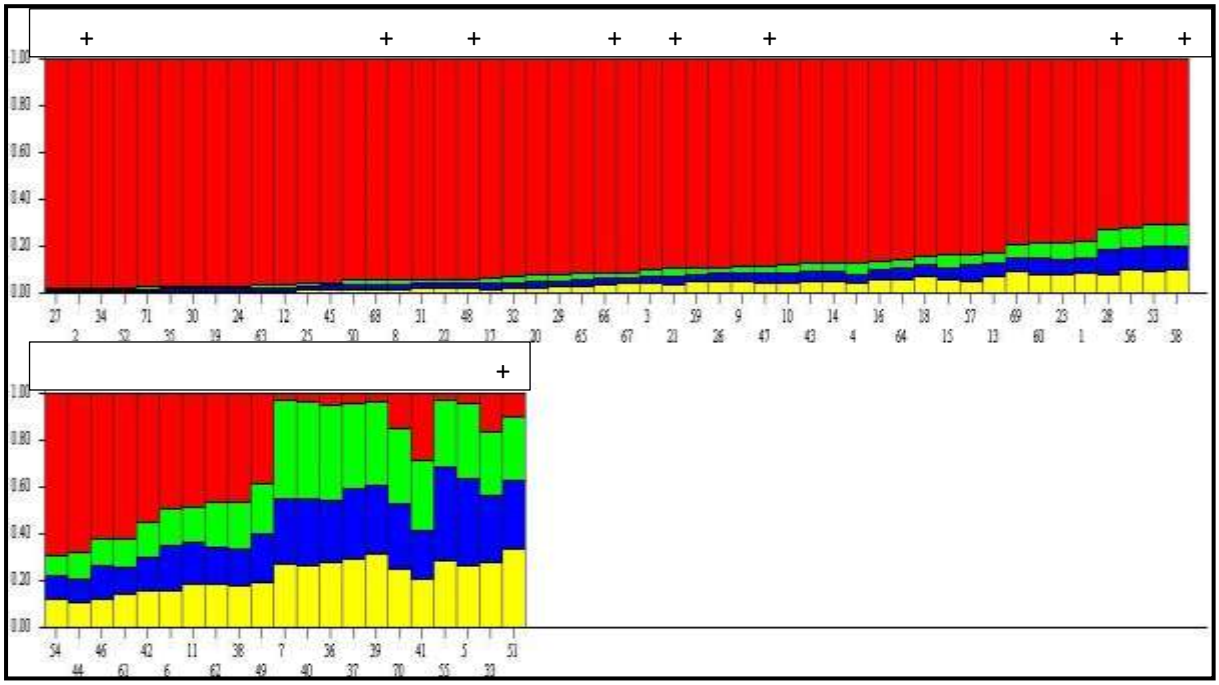
Mapping the pipistrelle TLR3 cluster to the bat multilocus genotyping data (Dodd et al, 2014).

1 = CS/06/10, 2 = CS/08/01, 3 = CS/08/02, 4 = CS/08/A, 5 = FP/05/46, 6 = FP/07/11, 7 = FP/07/12, 8 = FP/07/13, 9 = FP/07/21, 10 = FP/07/37, 11 = FP/07/42, 12 = FP/07/44, 13 = FP/07/45, 14 = FP/07/47, 15 = FP/07/51, 16 = FP/08/02, 17 = GH/06/06, 18 = GH/07/09, 19 = GH/07/10, 20 = JH/07/01, 21 = JH/08/02, 22 = JL/06/12, 23 = JL/06/13, 24 = JL/06/15, 25 = JL/06/24, 26 = JL/06/26, 27= JL/06/27, 28 = JL/06/28, 29 = JL/06/40, 30 = JL/06/42, 31 = JL/06/45, 32 = JL/06/47, 33 = JL/06/54, 34 = JL/06/56, 35 = JL/06/59, 36 = JL/07/04, 37 = JL/07/07, 38 = JL/07/08, 39 = JL/07/09, 40 = JL/07/10, 41 = JL/07/11, 42 = JL/07/12, 43 = JL/07/14, 44 = JL/07/18, 45 = JL/07/23, 46 = JL/07/25, 47 = MD/08/02, 48 = MH/08/02, 49 = PB/06/01, 50 = PB/06/02, 51 = PH/06/04, 52 = PH/06/05, 53 = SA/06/05, 54 = SA/06/07, 55 = SA/07/U, 56 = SP/06/49, 57 = SP/06/55, 58 = SP/06/68, 59 = SP/06/70, 60 = SP/06/72, 61 = SP/06/77, 62 = SP/06/79, 63 = SP/06/80, 64 = SP/06/81, 65 = SP/06/82, 66 = SP/06/83, 67 = SP/06/84, 68 = SP/08/16, 69 = SP/08/17, 70 = SP/08/18, 71 = SP/08/19. Red shows cluster3 samples.



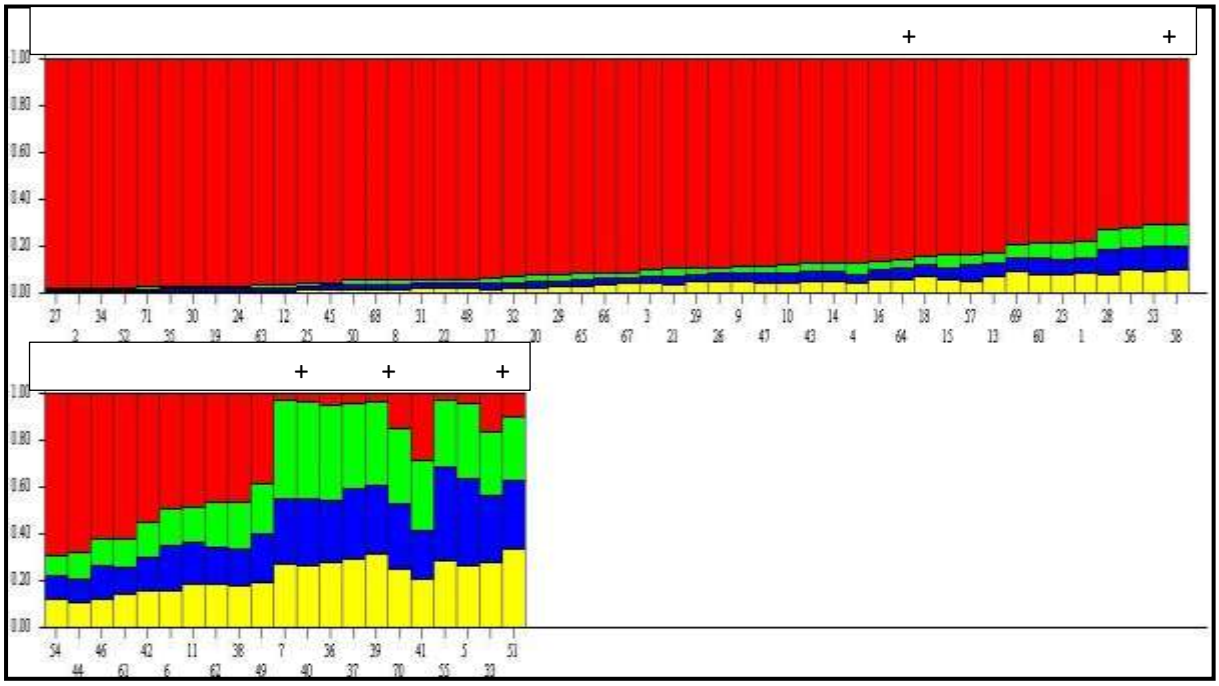
Mapping the pipistrelle TLR4 cluster to the bat multilocus genotyping (Dodd et al, 2014).

1 = CS/06/10, 2 = CS/08/01, 3 = CS/08/02, 4 = CS/08/A, 5 = FP/05/46, 6 = FP/07/11, 7 = FP/07/12, 8 = FP/07/13, 9 = FP/07/21, 10 = FP/07/37, 11 = FP/07/42, 12 = FP/07/44, 13 = FP/07/45, 14 = FP/07/47, 15 = FP/07/51, 16 = FP/08/02, 17 = GH/06/06, 18 = GH/07/09, 19 = GH/07/10, 20 = JH/07/01, 21 = JH/08/02, 22 = JL/06/12, 23 = JL/06/13, 24 = JL/06/15, 25 = JL/06/24, 26 = JL/06/26, 27= JL/06/27, 28 = JL/06/28, 29 = JL/06/40, 30 = JL/06/42, 31 = JL/06/45, 32 = JL/06/47, 33 = JL/06/54, 34 = JL/06/56, 35 = JL/06/59, 36 = JL/07/04, 37 = JL/07/07, 38 = JL/07/08, 39 = JL/07/09, 40 = JL/07/10, 41 = JL/07/11, 42 = JL/07/12, 43 = JL/07/14, 44 = JL/07/18, 45 = JL/07/23, 46 = JL/07/25, 47 = MD/08/02, 48 = MH/08/02, 49 = PB/06/01, 50 = PB/06/02, 51 = PH/06/04, 52 = PH/06/05, 53 = SA/06/05, 54 = SA/06/07, 55 = SA/07/U, 56 = SP/06/49, 57 = SP/06/55, 58 = SP/06/68, 59 = SP/06/70, 60 = SP/06/72, 61 = SP/06/77, 62 = SP/06/79, 63 = SP/06/80, 64 = SP/06/81, 65 = SP/06/82, 66 = SP/06/83, 67 = SP/06/84, 68 = SP/08/16, 69 = SP/08/17, 70 = SP/08/18, 71 = SP/08/19. Red shows cluster4 samples.



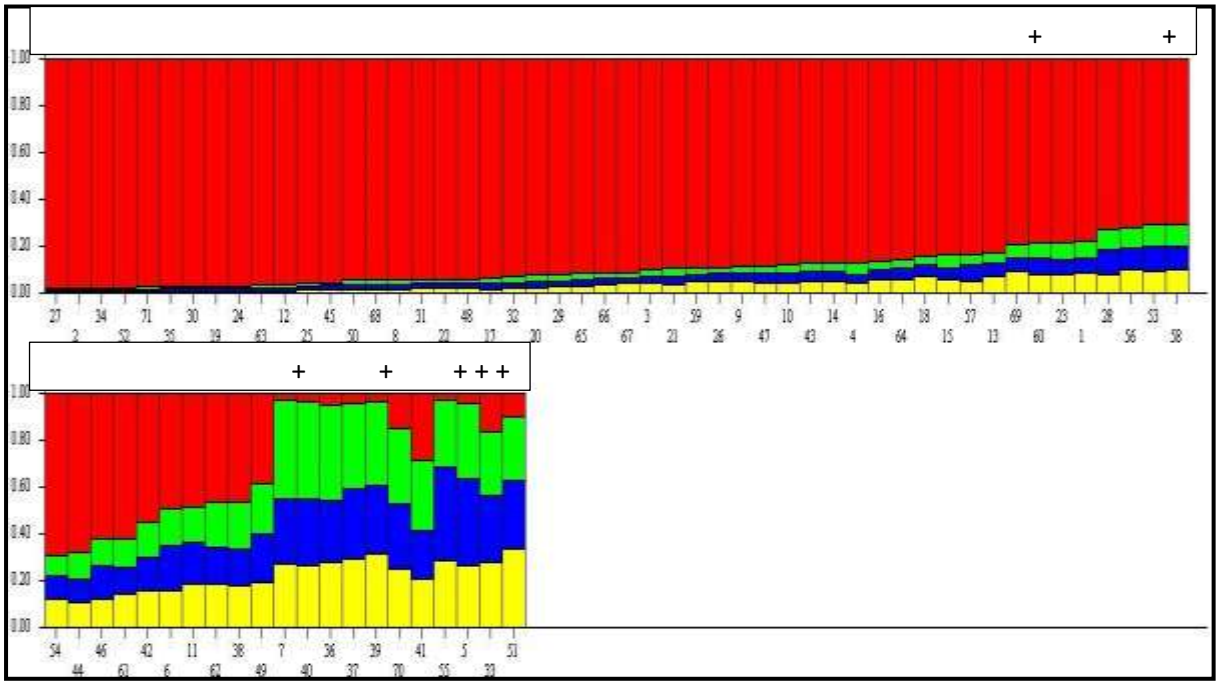
Mapping the pipistrelle TLR5 cluster to the bat multilocus genotyping data (Dodd et al, 2014).

1 = CS/06/10, 2 = CS/08/01, 3 = CS/08/02, 4 = CS/08/A, 5 = FP/05/46, 6 = FP/07/11, 7 = FP/07/12, 8 = FP/07/13, 9 = FP/07/21, 10 = FP/07/37, 11 = FP/07/42, 12 = FP/07/44, 13 = FP/07/45, 14 = FP/07/47, 15 = FP/07/51, 16 = FP/08/02, 17 = GH/06/06, 18 = GH/07/09, 19 = GH/07/10, 20 = JH/07/01, 21 = JH/08/02, 22 = JL/06/12, 23 = JL/06/13, 24 = JL/06/15, 25 = JL/06/24, 26 = JL/06/26, 27= JL/06/27, 28 = JL/06/28, 29 = JL/06/40, 30 = JL/06/42, 31 = JL/06/45, 32 = JL/06/47, 33 = JL/06/54, 34 = JL/06/56, 35 = JL/06/59, 36 = JL/07/04, 37 = JL/07/07, 38 = JL/07/08, 39 = JL/07/09, 40 = JL/07/10, 41 = JL/07/11, 42 = JL/07/12, 43 = JL/07/14, 44 = JL/07/18, 45 = JL/07/23, 46 = JL/07/25, 47 = MD/08/02, 48 = MH/08/02, 49 = PB/06/01, 50 = PB/06/02, 51 = PH/06/04, 52 = PH/06/05, 53 = SA/06/05, 54 = SA/06/07, 55 = SA/07/U, 56 = SP/06/49, 57 = SP/06/55, 58 = SP/06/68, 59 = SP/06/70, 60 = SP/06/72, 61 = SP/06/77, 62 = SP/06/79, 63 = SP/06/80, 64 = SP/06/81, 65 = SP/06/82, 66 = SP/06/83, 67 = SP/06/84, 68 = SP/08/16, 69 = SP/08/17, 70 = SP/08/18, 71 = SP/08/19. Red shows cluster5 samples.



Mapping the pipistrelle TLR6 cluster to the bat multilocus genotyping data (Dodd et al, 2014).

1 = CS/06/10, 2 = CS/08/01, 3 = CS/08/02, 4 = CS/08/A, 5 = FP/05/46, 6 = FP/07/11, 7 = FP/07/12, 8 = FP/07/13, 9 = FP/07/21, 10 = FP/07/37, 11 = FP/07/42, 12 = FP/07/44, 13 = FP/07/45, 14 = FP/07/47, 15 = FP/07/51, 16 = FP/08/02, 17 = GH/06/06, 18 = GH/07/09, 19 = GH/07/10, 20 = JH/07/01, 21 = JH/08/02, 22 = JL/06/12, 23 = JL/06/13, 24 = JL/06/15, 25 = JL/06/24, 26 = JL/06/26, 27= JL/06/27, 28 = JL/06/28, 29 = JL/06/40, 30 = JL/06/42, 31 = JL/06/45, 32 = JL/06/47, 33 = JL/06/54, 34 = JL/06/56, 35 = JL/06/59, 36 = JL/07/04, 37 = JL/07/07, 38 = JL/07/08, 39 = JL/07/09, 40 = JL/07/10, 41 = JL/07/11, 42 = JL/07/12, 43 = JL/07/14, 44 = JL/07/18, 45 = JL/07/23, 46 = JL/07/25, 47 = MD/08/02, 48 = MH/08/02, 49 = PB/06/01, 50 = PB/06/02, 51 = PH/06/04, 52 = PH/06/05, 53 = SA/06/05, 54 = SA/06/07, 55 = SA/07/U, 56 = SP/06/49, 57 = SP/06/55, 58 = SP/06/68, 59 = SP/06/70, 60 = SP/06/72, 61 = SP/06/77, 62 = SP/06/79, 63 = SP/06/80, 64 = SP/06/81, 65 = SP/06/82, 66 = SP/06/83, 67 = SP/06/84, 68 = SP/08/16, 69 = SP/08/17, 70 = SP/08/18, 71 = SP/08/19. Red shows cluster6 samples.



Mapping the pipistrelle TLR7 cluster to the bat multilocus genotyping (Dodd et al, 2014).

1 = CS/06/10, 2 = CS/08/01, 3 = CS/08/02, 4 = CS/08/A, 5 = FP/05/46, 6 = FP/07/11, 7 = FP/07/12, 8 = FP/07/13, 9 = FP/07/21, 10 = FP/07/37, 11 = FP/07/42, 12 = FP/07/44, 13 = FP/07/45, 14 = FP/07/47, 15 = FP/07/51, 16 = FP/08/02, 17 = GH/06/06, 18 = GH/07/09, 19 = GH/07/10, 20 = JH/07/01, 21 = JH/08/02, 22 = JL/06/12, 23 = JL/06/13, 24 = JL/06/15, 25 = JL/06/24, 26 = JL/06/26, 27= JL/06/27, 28 = JL/06/28, 29 = JL/06/40, 30 = JL/06/42, 31 = JL/06/45, 32 = JL/06/47, 33 = JL/06/54, 34 = JL/06/56, 35 = JL/06/59, 36 = JL/07/04, 37 = JL/07/07, 38 = JL/07/08, 39 = JL/07/09, 40 = JL/07/10, 41 = JL/07/11, 42 = JL/07/12, 43 = JL/07/14, 44 = JL/07/18, 45 = JL/07/23, 46 = JL/07/25, 47 = MD/08/02, 48 = MH/08/02, 49 = PB/06/01, 50 = PB/06/02, 51 = PH/06/04, 52 = PH/06/05, 53 = SA/06/05, 54 = SA/06/07, 55 = SA/07/U, 56 = SP/06/49, 57 = SP/06/55, 58 = SP/06/68, 59 = SP/06/70, 60 = SP/06/72, 61 = SP/06/77, 62 = SP/06/79, 63 = SP/06/80, 64 = SP/06/81, 65 = SP/06/82, 66 = SP/06/83, 67 = SP/06/84, 68 = SP/08/16, 69 = SP/08/17, 70 = SP/08/18, 71 = SP/08/19. Red shows cluster7 samples.