Analysing the Genetic Diversity of Ixodes ricinus ticks using Multilocus Sequence Typing

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Analysing the Genetic Diversity of *Ixodes ricinus* ticks using Multilocus Sequence Typing

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Dedication

This thesis is dedicated to my wonderful husband Steve, who has made me endless cups of tea, put up with the late nights and all the grouchy behaviour

All my love xx

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Publications

Pietzsch, M. E., Mitchell, R., Jameson, L. J., Morgan, C., Medlock, J. M., Collins, D., Chamberlain, J. C., Gould, E. A., Hewson, R., Taylor, M. A. and Leach, S. (2008). Preliminary evaluation of exotic tick species and exotic pathogens imported on migratory birds into the British Isles. *Veterinary Parasitology*, 155, pp.328-32. (published in maiden name Mitchell)

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Contents

Abbreviations

Abbreviation	Expanded
12s	*12s* ribosomal RNA
AIC	Akaike Information Criterion
aLRT	appropriate Likelihood Ratio Test
ATL	Animal tissue Lysis
ATP	Adenosine tri-phosphate
atp6	ATP synthase F0 subunit 6
BP	before present
CCHF	Crimean Congo Haemorrhagic Fever
coi	Cytochrome C oxidase subunit I
coii	Cytochrome C oxidase subunit II
coiii	Cytochrome C oxidase subunit III
CR	Control region
cytB	Cytochrome B
DLV	Double locus variant
GTR	General Time Reversible
IGS	Intergenic spacer region
LB	Lyme borreliosis
LIV	Louping-ill virus
ML	Maximum likelihood
MLEE	Multilocus enzyme electrophoresis
MLST	Multilocus Sequence Typing
mtMLST	Mitochondrial Multilocus Sequence Typing
MY	million years
NNI	Nearest Neighbour Interchange
NRDB	Non-redundant database
PCR	Polymerase Chain Reaction
RI	raggedness index
rRNA	ribosomal ribonucleic acid
s.l.	sensu lato
SH-like	Shimodaira-Hasegawa like
SLV	Single locus variant

SPR	Sub-tree Pruning and Regrafting
SSD	sum of squared deviations
ST	Sequence type
TAE	Tris Acetate EDTA buffer
TBE	Tick Borne Encephalitis
TLV	Triple locus variant
tRNA	transfer ribonucleic acid
TROSPA	Ticks receptor of Outer Surface Protein A

Abstract

Ixodes ricinus is the most important human-biting tick in Europe and the principal vector of Lyme borreliosis. In addition, this hard tick species transmits a large number of microbial pathogens that are of importance to animal and human health. Little is known about the diversity and genetic population structure of *I. ricinus* across Europe. Genetic diversity of these tick populations may have implications on disease transmission. *I. ricinus* primers were designed for a number of mitochondrial genes and a Multilocus Sequence Typing-like Scheme (MLST) was devised. This was termed mitochondrial MLST (mtMLST). MLST has so far mainly been used for typing microbes, and the development of a MLST scheme for an arthropod vector is novel. Understanding the geographic structure of *I. ricinus* populations, in combination with studies regarding the migration of tick-borne microbial infections, e.g. Lyme borreliosis, is likely to illuminate important processes in the evolution and spread of tick-borne diseases.

1 General introduction

1.1 *Ixodes ricinus* ticks

Ixodes ricinus is the most important human-biting tick in Europe and the most important vector of diseases after mosquitoes (Parola & Raoult, 2001). This chapter reviews current research and understanding of *I. ricinus* ticks and tick-borne diseases and the uses of modern molecular techniques to resolve the complex interactions that *I. ricinus* ticks, and other related species, have with the environment.

1.1.1 The distribution of Ixodes ricinus *ticks*

The sheep tick *Ixodes ricinus*, also known as the castor bean tick, is common in many parts of Central and Western Europe. As the principal vector of Lyme borreliosis (J. S. Gray *et al.*, 2002), this species has received special research attention on its distribution and climate preferences. *I. ricinus* is the most common tick in Northwest Europe and can be found in deciduous forests in much of the Western Palaearctic (Figure 1). This range extends from central Scandinavia in the North, as far South as the Atlas mountains in Morocco, from Portugal in the West and as far East as parts of Russia (Hillyard, 1996). The range of *I. ricinus* overlaps with the range of the eastern taiga tick (J. S. Gray, 1998), *Ixodes persulcatus* (shown in Figure 1).

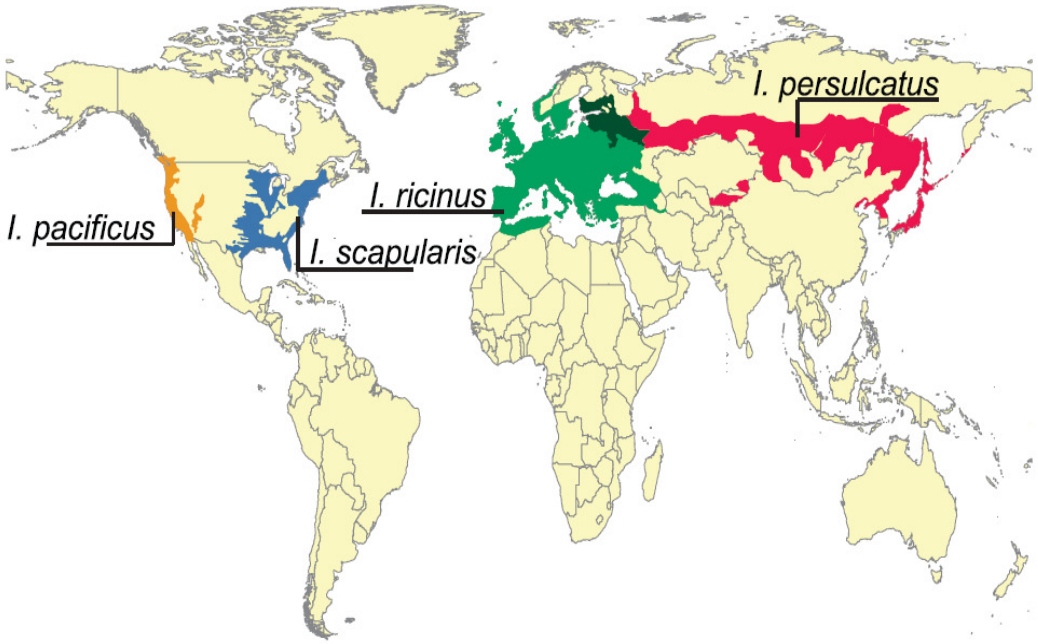

Figure 1: The approximate geographic distribution of four members of the *Ixodes ricinus* tick species complex (Swanson *et al.*, 2006)

Unlike nidiculous ticks, such as *Ixodes hexagonus* (Hillyard, 1996), who quest in the burrows of their hosts, *I. ricinus* ticks utilise the herbage layer and leaf litter of deciduous forests to quest for hosts (Lees, 1948; Osterkamp *et al.*, 1999). These ectoparasites are highly prone to desiccation and therefore, humidity represents a severely limiting factor on questing behaviour and thus, affects population densities (Randolph & Storey, 1999; Estrada-Pena *et al.*, 2006). In continental Europe *I. ricinus* is found more commonly in deciduous woodland, whereas in the UK, *I. ricinus* ticks can also be found in sheltered meadows and hillsides, as the humidity levels are higher in the UK (Eisen & Lane, 2002).

Habitat prediction maps have been extensively investigated for *I. ricinus* ticks across Europe (Estrada-Pena, 2006; Estrada-Pena *et al.*, 2006) and to some extent in the UK (Medlock *et al.*, 2008) although the majority of work is done in Continental Europe. *I. ricinus* inhabit most areas apart from mountainous and high-altitude areas (Hillyard, 1996). Suitable areas for the habitation of ticks in Europe have been identified as having different ecological factors, which leads to differentiated populations and clustering of ticks according to ecological meaning (Estrada-Pena, 2006).

1.1.2 The Classification of Ixodes ricinus *ticks*

Ticks are an ancient group of organisms thought to have originated in the mid-Cretaceous period (145-65 MYA) (Mans *et al.*, 2002). Other reports suggest that ticks first originated in the Devonian period (416-359 MYA) and are thought to have been the first group to evolve blood-feeding capabilities, the main evidence for this being that one tick species, *Amblyomma rotundatum*, feeds on *Bufo marinus*, a species of giant toad (Oliver *et al.*, 1993).

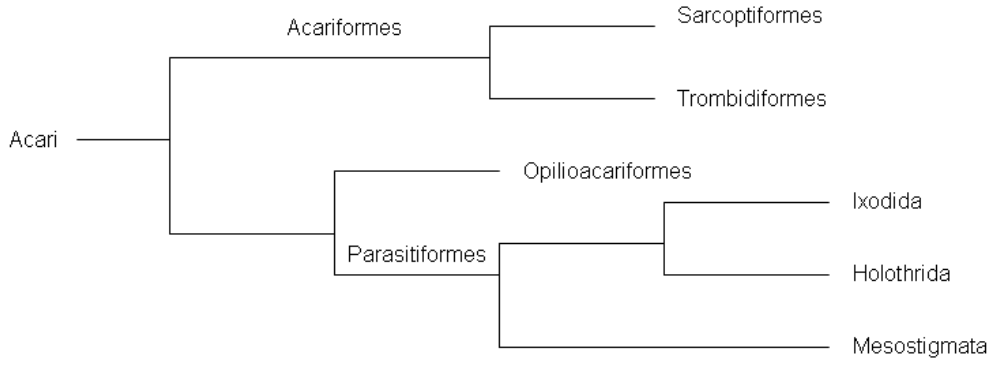

Figure 2: Phylogeny of the Subclass Acari. Based on the Tree of Life web Project (Maddison & Schulz, 2007).

Ixodes ricinus ticks belong to phylum Arthropoda, Class Arachnida, Subclass Acari (Figure 2), Superorder Parasitiformes, Order Ixodida, Family Ixodidae (shown in detail in Figure 3), Genus *Ixodes*. All species of ticks are grouped into three families (the Subclass Acari is summarised in Figure 2). These are the Argasidae, which constitutes 186 species, Nuttalliellidae containing only one species (*Nuttalliella namaqua*) and Ixodidae, the family of *Ixodes ricinus,* which currently has 692 species (Jongejan & Uilenberg, 2004). Ticks share the order with mites (Holothyrida and Mesostigmata) and a small collection of rarer large mites (Opiliacarida). The Ixodidae is split into two groups. The Prostriata group contains the genus *Ixodes* and the Metastriata group, which contains all other genera in Ixodidae.

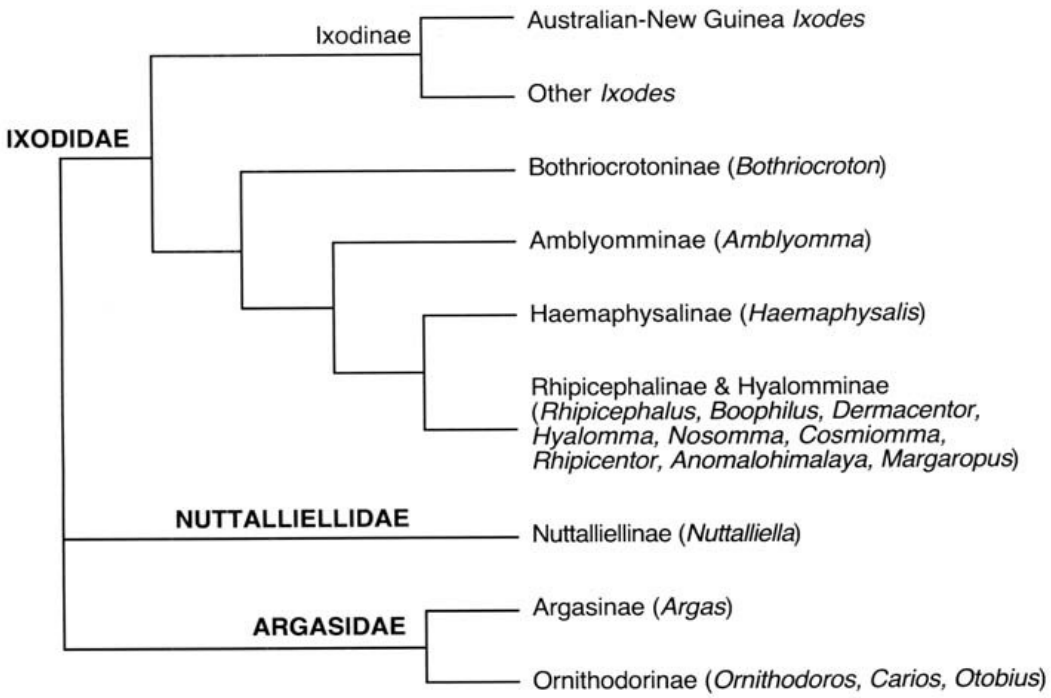

Figure 3: The Suborder Ixodidae (Barker & Murrell, 2004)

I. ricinus along with 13 other species (Figure 4) belongs to the *Ixodes persulcatus* species complex (which has also been called the *Ixodes ricinus* species complex (Xu *et al*., 2003)) which was originally conceived in 1971 (Filippova, 1971). Several other species have been added through molecular analysis and morphological determination, as well as some being omitted. Twenty-one species are currently considered part of the complex considering all current research (Xu *et al*., 2003). A summary of these species is shown in Table 1.

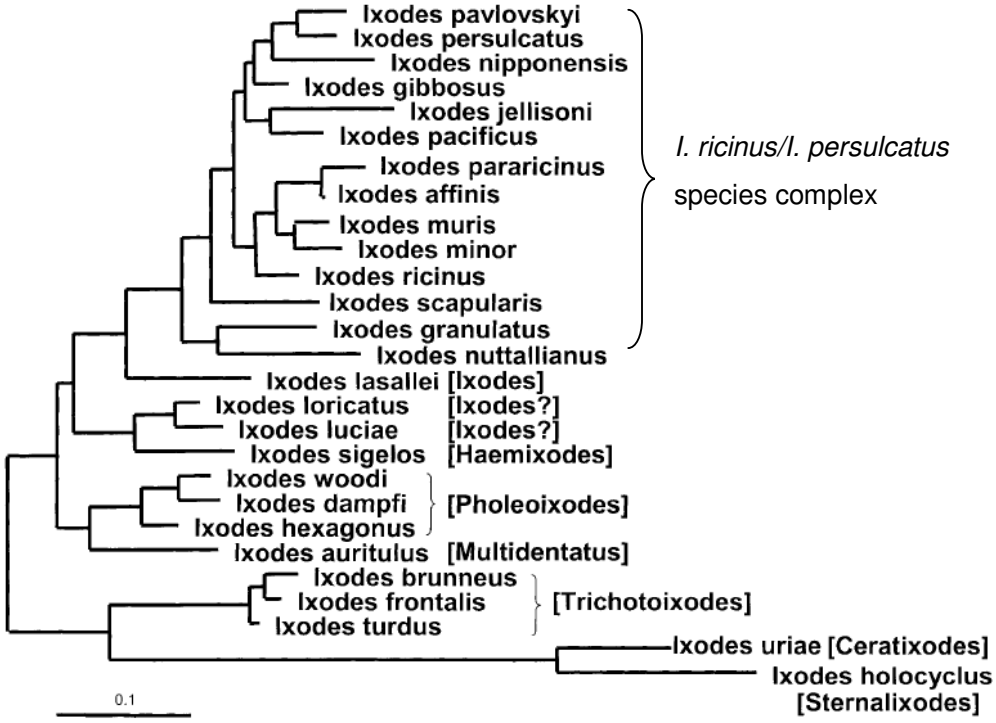

Figure 4: The *Ixodes persulcatus*/*Ixodes ricinus* species complex. Based upon a phylogenetic analysis by Xu *et al.* (2003).

The classification of ticks in the family Ixodidae was first based on ecological, morphological and biological characteristics (reviewed in Nava *et al*., 2009). Since the molecular biology revolution, molecular markers have transformed tick phylogenies with nuclear and mitochondrial DNA dominating the current markers used.

Mitochondrial DNA has frequently been used for the systematics of ticks due to its advantages of being maternally inherited, infrequently recombining (Barr *et al*., 2005; Filipowicz *et al*., 2008) and ease of amplification by polymerase chain reaction (PCR) due to a high number of copies per cell. Caporale *et al.* (1995) used 16S mitochondrial DNA splitting members of the *I. persulcatus* complex into two clades. Other studies have been performed using 16S (Black & Piesman, 1994) but a study performed by Xu *et al.* (2003) provides one of the most comprehensive analyses using 11 species from the *I. persulcatus* complex and 16 other *Ixodes* species. In this study, 16S mitochondrial genes were used to delineate the origins of the *I. persulcatus* species complex, finding it to be paraphyletic.

Nuclear markers have also proved popular for studying ticks. Microsatellites (Kempf *et al*., 2009), and nuclear ribosomal DNA (Black *et al*., 1997; Mangold *et al*., 1998a;

Dobson & Barker, 1999; Klompen *et al.*, 2007) have proved to be instrumental in the study of the Acari subclass.

Table 1: A list of ticks from the *Ixodes persulcatus* species complex

Genus	Species	Reference(s)
Ixodes	*affinis*	(Oliver, 1996; Kierans *et al.*, 1999)
Ixodes	*asanumai*	(Hoogstraal, 1978)
Ixodes	*dantatus*	(Oliver, 1996)
Ixodes	*gibbosus*	(Kierans *et al.*, 1999)
Ixodes	*hyatti*	(Kierans *et al.*, 1999)
Ixodes	*jellisoni*	(Kierans *et al.*, 1999; Eisen & Lane, 2002)
Ixodes	*kashmiricus*	(Kierans *et al.*, 1999)
Ixodes	*kazakstani*	(Kierans *et al.*, 1999)
Ixodes	*minor*	(Oliver, 1996)
Ixodes	*muris*	(Lacombe *et al.*, 1999; Scott *et al.*, 2001; Eisen & Lane, 2002)
Ixodes	*neotomae*	(Oliver, 1996)
Ixodes	*nipponensis*	(Kierans *et al.*, 1999)
Ixodes	*nuttallianus*	(Kierans *et al.*, 1999)
Ixodes	*pacificus*	(Kierans *et al.*, 1999)
Ixodes	*parariciniis*	(Beldomenico *et al.*, 2004)
Ixodes	*pavlovskyi*	(Kierans *et al.*, 1999)
Ixodes	*persulcatus*	(Kierans et al., 1999)
Ixodes	*ricinus*	(Kierans et al., 1999)
Ixodes	*scapularis*	(Kierans et al., 1999)
Ixodes	*sinensis*	(Kierans *et al.*, 1999; Sun *et al.*, 2003)
Ixodes	*spinipalpis*	(Oliver, 1996)

1.1.3 The basic anatomy of Ixodes ricinus

Ixodes ricinus ticks are hard-bodied ectoparasites, commonly known as castor bean ticks. The anatomy of a hard tick is shown in Figure 5. Larval ticks have three pairs of legs and can therefore be distinguished from nymphs and adults easily as these have four pairs of legs. *I. ricinus* nymphs are 1.3-1.5mm long. Adult females are 3.0-3.6mm in length compared to the marginally smaller male tick measuring 2.4-2.8mm. Once fed to repletion, adult females can reach 1.1cm in length (Hillyard, 1996).

I. ricinus ticks can be distinguished from other *Ixodes* species according to several distinct features. The legs of *I. ricinus* ticks have moderate thickness and length unlike *Ixodes vespertillionis,* which have elongated appendages longer than the body. The capitulum, also known as a 'false head' has two porose areas either side of the hypostome on the dorsal surface. *I. ricinus* also lack lateral spurs on the basal segment of the palps, the palps are relatively long with the second and third segments of the palps as long or longer than the width of the capitulum. The internal spur on coxa I is elongated, the external spur is short and the base of the capitulum on the ventral surface has no auriculae (projects either side of the capitulum on the ventral surface).

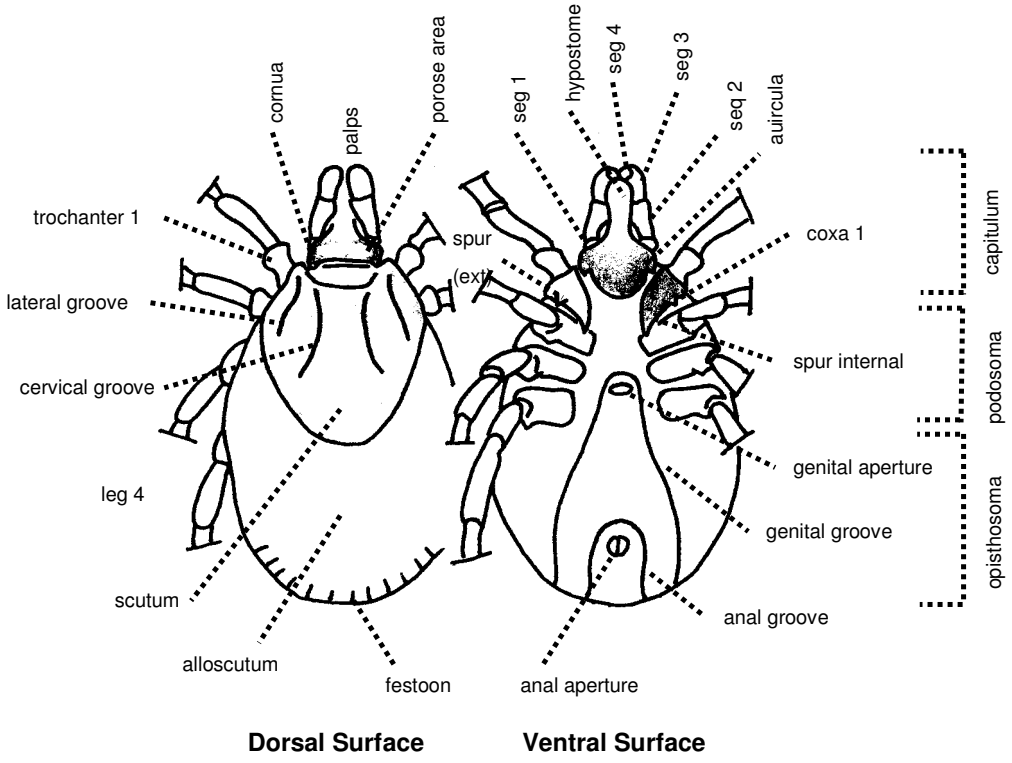

Figure 5: General tick anatomy (adapted from Hillyard, 1996)

1.1.4 Life cycle of Ixodes ricinus

The lifecycle of ticks is complex and in Europe, typically lasts for 2-3 years, but can take up to six (Hillyard, 1996) (shown in Figure 6). The ecological situation of the ticks can have a significant impact on the developmental rate. Large differences were noted

in the developmental rate of *I. ricinus* populations by Estrada-Pena *et al.* (2004) due to local variations in climate. Indeed, other areas of Europe also have exhibited variations in developmental rate due to climatic variables (Randolph *et al.*, 2002).

The developmental cycle begins with the hatching of the clutch of eggs to the larval stage. Larvae seek small mammal hosts such as mice and voles and ground feeding birds (Hillyard, 1996; J. S. Gray, 1998; Kierans *et al.*, 1999), and take a blood meal. Engorgement of larvae can take several days. Once fully engorged larvae will detach from their host and may fall into the undergrowth to moult. Highly specific abiotic factors determine the success of tick survival and questing. High humidity is considered one of the most significant indicator of increased survival (Randolph & Storey, 1999) as ticks are prone to desiccation. If these conditions are not met, ticks may enter a period called diapause, slowing metabolic activity until preferable conditions are encountered. To survive over these extended periods, ticks use fat reserves to maintain essential metabolic functions during this sedentary state (Belozerov, 1982).

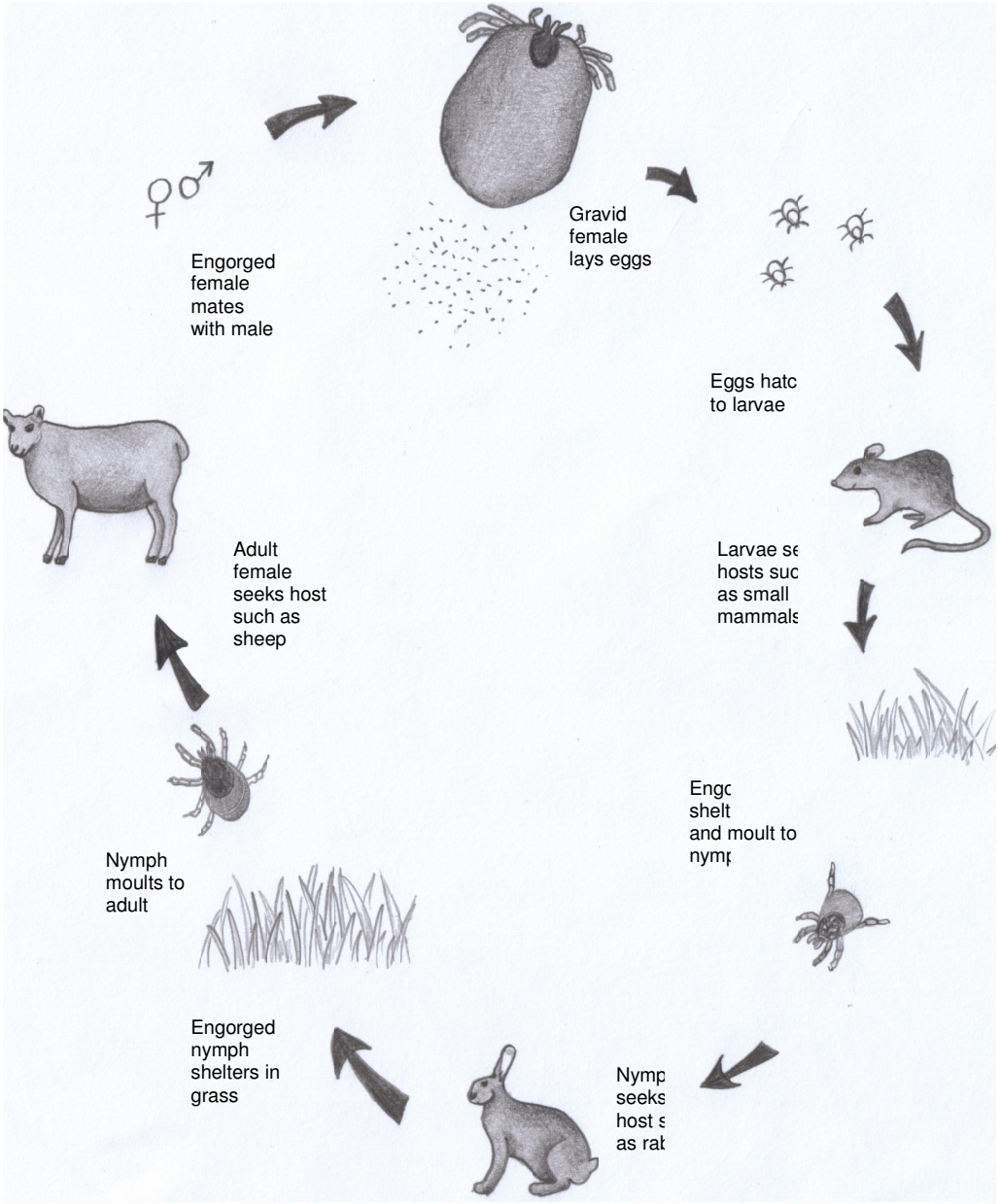

Figure 6: Life cycle of the sheep tick, *Ixodes ricinus* (adapted from Hillyard, 1996)

Digestion of the first blood meal leads to moulting to the nymphal life-stage. The nymph quests for slightly larger birds and mammals than nymphs, including rabbits, squirrels and occasionally, humans (J. S. Gray, 1998). The ticks will feed for several days, and detach from the host once fully engorged, and thus will moult to the adult final stage. Adult ticks will quest for large birds and mammals, the most common and most important is deer (J. S. Gray, 1998; Clutton-Brock *et al.*, 2004). Despite these differences regarding host preferences dependent on the tick stage, ticks are

essentially generalists and will parasitize nearly all land vertebrates apart from amphibians (Kierans *et al.*, 1999).

Deer are the reproductive hosts of *I. ricinus* and an individual host can feed hundreds of adult ticks (Clutton-Brock *et al.*, 2004; Scharlemann *et al.*, 2008). In the UK in the last thirty years many different deer species have shown large population expansions (Ward, 2005). These population expansions have possibly led to the expansion of ticks (Stafford *et al.*, 2003; Ostfeld *et al.*, 2006; Scharlemann *et al.*, 2008).

Questing is a sedentary process with the tick migrating short distances vertically to the tip of grass stems and leaf litter (Randolph & Storey, 1999). Ticks do not move great distances horizontally (Eisen & Lane, 2002). Hosts are detected by body heat, CO_2, odours, light changes and mechanical vibrations (Lees, 1948; Osterkamp *et al.*, 1999). The seasonal activity of *I. ricinus* as previously mentioned is highly dependent on local climatic variations. Generally, in Southern regions of the UK, the peak of questing activity occurs at the end of spring into the middle of summer, and has diminished to low activity by the middle of autumn. Tick populations from central and Northern regions of the UK are more like Continental Europe with a bi-modal activity peak of nymphs in spring and autumn. Activity peaks of adults and larvae are seen in spring and autumn (reviewed in Kurtenbach *et al.*, 2006). Although the questing activity of *I. ricinus* ticks is not normally associated with the winter months, evidence has been seen that suggests tick activity is at a low level, rather than completely absent (Dautel *et al.*, 2008).

1.2 Diseases transmitted by ticks

Ticks are responsible for the transmission of a variety of microorganisms including bacteria, viruses and protozoa and toxin associated diseases. Ticks are recognised as second only to mosquitoes as influential vectors for the transmission of disease (Parola & Raoult, 2001). This section examines the main viral and bacterial infections ticks are known to transmit.

1.2.1 Borrelia burgdorferi *s.l.*

The spirochaete *Borrelia burgdorferi* sensu lato (s.l.) is the most common vector-borne pathogen in temperate climates (Smith et al., 2000), causing Lyme borreliosis (LB) in humans (J. S. Gray *et al.*, 2002). Named after the town of Old Lyme in Connecticut, United States of America (USA), where it was discovered in the mid-1970s (Steere *et*

al., 1977b), this spirochaete has become a paradigm model for studying the emergence of vector-borne diseases.

B. burgdorferi s.l. forms a species complex of 16 named species (Postic *et al*., 1998; Masuzawa *et al*., 2005; Chu *et al*., 2008; Margos *et al*., 2009; Rudenko, Golovchenko, Grubhoffer *et al*., 2009; Rudenko, Golovchenko, Lin *et al*., 2009). A large number of different vertebrate host species are known to be involved in the maintenance cycles of the microparasite. The different species of *B. burgdorferi* s.l. in Eurasia are specialized to different hosts, such as small rodents or passerine bird species (Kurtenbach *et al*., 1998c; Kurtenbach *et al*., 2002). The relative abundance of these microparasite species is therefore a result of the structure of the vertebrate host community (Etti *et al*., 2003).

Lyme disease is the clinical manifestation of *B. burgdorferi* s.l. Cases of Lyme disease have increased in the UK, although this may be due to increased awareness and greater access to diagnostic facilities and treatment. The increase of Lyme disease in humans in England and Wales has been documented by the Health Protection Agency and is shown in Figure 7. In order to transmit *Borrelia* spirochaetes, ticks are required to inject the bacteria directly into the host through a bite. Larvae can acquire the spirochaetes from vertebrate carriers (discussed in section 1.1.4), while nymphal stages transmit the bacteria to susceptible hosts. Therefore, the life cycle of tick-borne microparasites is extended compared to microparasites transmitted by insect vectors e.g. mosquitoes.

The transmission dynamics of *Borrelia* is complex due to a host complement interaction with the different species of *Borrelia*. *Borrelia garinii* and *Borrelia valaisiana* are maintained in the environment by birds, mostly passerine species (Humair *et al*., 1993; Gern *et al*., 1998; J. S. Gray, 1998; Kurtenbach *et al*., 1998a; Kurtenbach *et al*., 1998b; Hanincova *et al*., 2003b). *Borrelia afzelii* is maintained in the environment by many rodent species (J. S. Gray, 1998; Hanincova *et al*., 2003a). Host complement interactions leads to the lysis of non-complementary species of bacteria, which are introduced into hosts (Kurtenbach *et al*., 1998c; Kurtenbach *et al*., 2002).

Spirochaete infections of *Borrelia* are not transmitted from mother to egg (transovarial) (Magnarelli *et al*., 1987; Schoeler & Lane, 1993; Toutoungi & Gern, 1993). Thus, questing nymphs do not carry *Borrelia* infections. The infection prevalence of *B. burgdorferi* s.l. is highly dependent upon the maintenance hosts available and microclimate and therefore subject to fluctuations according to local conditions.

Investigations in the UK have indicated an infection prevalence of *Borrelia* of approximately 5% (Vollmer *et al.*, 2010).

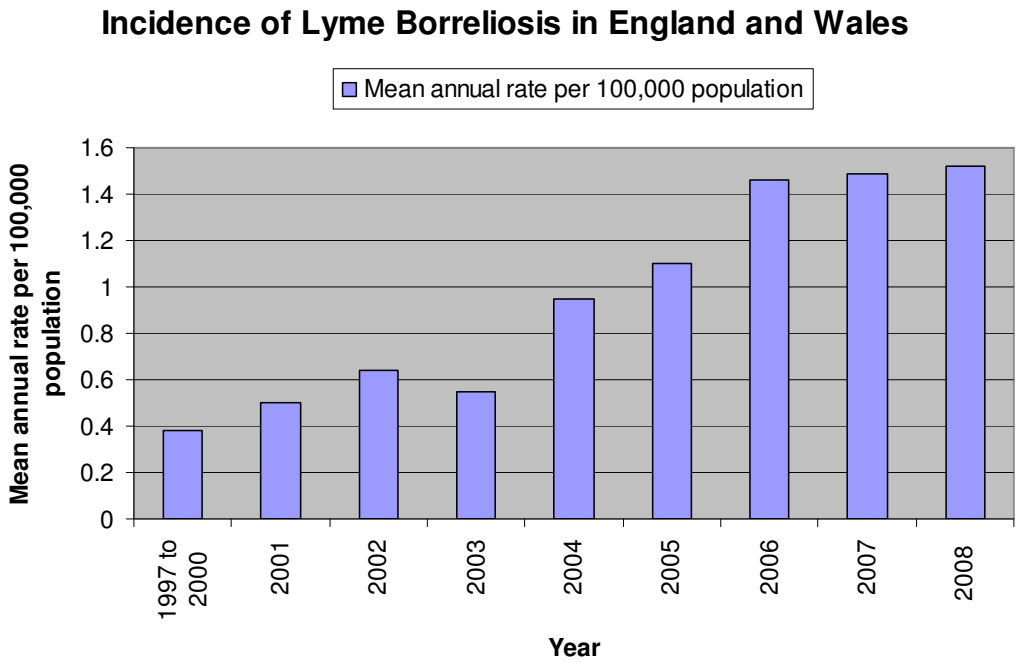

Figure 7: A bar chart showing the incidence of Lyme borreliosis cases in England and Wales documented by the Health Protection Agency (Health Protection Agency, 2010)

1.2.2 Tick borne Flaviviruses - Tick Borne Encephalitis

Flaviviruses belong to the family *Flaviviridae* which has over 70 recognised viruses. One of the most medically significant viruses, which in endemic to Europe belonging to this group is Tick Borne Encephalitis (TBE). As well as being one of the most widely spread viruses in Europe it is also capable of a fatality rate of 20%, although rates of 3% are more common depending on the strain of the virus in the outbreak, making it one of the most dangerous infections in Europe and Asia (Gritsun *et al.*, 2003). Annually it is estimated that TBE causes at least 11,000 human cases of encephalitis in Europe. These infections are usually acquired through a tick bite although there have been reports of TBE contracted through infected goat milk (Kohl *et al.*, 1996; Labuda *et al.*, 2002).

Incidence of TBE in Europe is undergoing a shift in geographical areas it affects. Climate change resulting in milder winters and earlier springs has been implicated for the rise in cases in Sweden (Lindgren & Gustafson, 2001), Norway (Skarpaas *et al.*,

2004), Germany (Hemmer *et al*., 2005), Denmark (Skarphédinsson *et al*., 2005) and Finland (Han *et al*., 2002). Conversely, data from the Netherlands fails to provide evidence for a TBE reservoir in ticks or wildlife (van der Poel *et al*., 2005).

The only know Flavivirus, which is endemic to the UK, is thought to be the Louping Ill virus (LIV). The virus is common in upland areas and moors including North-Yorkshire, Scotland, parts of Wales (Gaunt *et al*., 1997) and also in Dartmoor (Twomey *et al*., 2001). Despite being common in the wild and recognised for over the last 200 years (McGuire *et al*., 1998) the virus remains under researched.

1.2.3 Crimean Congo Haemorrhagic Fever virus

Crimean Congo haemorrhagic fever (CCHF) virus is a tick borne haemorrhagic fever with mortality rates anywhere between 10 and 50% although occasional rates up to 80% have been reported in China (Yu-Chen *et al*., 1985). This haemorrhagic fever was first described in the modern world in Crimea when over 200 military personnel were inflicted with a severe haemorrhagic fever (Chumakov, 1945). The victims of this epidemic experienced a variety of haemorrhagic symptoms. The virus aetiology was established much later by Chumakov *et al*. (1968).

Since 2002, CCHF has emerged as a serious infection in Turkey (Yilmaz et al., 2009) leading to a fatality rate of 5%, significantly lower than in some other endemic CCHF areas. The incidence of CCHF in Turkey was highly significant in people that had been exposed to ticks and tick bites with most infections occurring from May to July. Numbers of infections have been increasing since 2002 and shows no sign of lessening. In the future it will be important to monitor tick populations in these areas to asses the risk to human health that tick numbers may have, possibly leading to a public health awareness of the risks of exposure to ticks and the diseases they may carry.

1.3 Multilocus sequence typing

1.3.1 The creation of Multilocus sequence typing

The study of bacterial populations has become a key part of understanding the spread of emerging infections and unambiguous genotyping systems have been essential to this process (Gevers *et al.*, 2005). While many different methods have been investigated to monitor the spread of pathogens (reviewed in Maiden, 2006), multilocus sequence typing (MLST) has emerged as an invaluable tool to monitor the spread of virulent bacterial strains or fungal pathogens (Maiden *et al.*, 1998; Enright *et al.*, 2000; Meyer *et al.*, 2009). Advantages are the reproducibility of typing methods between laboratories, portability and data sharing via the internet and more robust analyses than single loci can offer (Maiden *et al.*, 1998; Urwin & Maiden, 2003).

Multilocus sequence typing was first developed by Maiden *et al.* (1998) to study the epidemiology of bacterial pathogens. Multilocus enzyme electrophoresis (MLEE) was previously used as a tool to study epidemiology assessing the genetic variation of proteins that have electrophoretic properties by running these enzymes through a starch gel. As only mutations that changed the electrophoretic properties of the enzymes resulted in differentiation of alleles, many mutations are not detected. MLST uses sequence information of genes and therefore is much more sensitive to mutations, and therefore superseded MLEE due to its superior level of resolution. Since its first introduction, it has been used widely for the molecular typing of bacteria, in particular *Neisseria meningitidis* (Maiden *et al.*, 1998; Feil *et al.*, 2000a; Clarke *et al.*, 2001) and more recently, *Cryptococcus spp* (Meyer *et al.*, 2009).

1.3.2 Design of multilocus sequence typing schemes

MLST was introduced as a portable and globally accessible method for the study of bacterial pathogens. MLST schemes are individually tailored to specific bacterial species according to a set of guidelines set out by Maiden *et al.*, (1998).

Multilocus sequence typing normally uses the sequences of approximately 450bp sized fragments from usually six to seven housekeeping genes. The genes selected should be single copy genes, be nearly neutrally evolving and not be prone to recombination. Traditionally, slowly evolving housekeeping genes, which are under purifying selection, are chosen (Maiden, 2006).

The selected gene fragments are amplified from all samples by PCR and sequenced. Sequenced amplicons are trimmed according to the region of interest and traces are checked for sequencing errors. Alleles are assigned sequential numbers according to their sequence of discovery. These allele types are combined to create an allelic profile and assigned a unique identifying number called a sequence type (ST), e.g. bacteria A, B and C each had three gene fragments sequenced. For bacterial clone A, the allele types sequenced produced an allelic profile of 1, 1, 1 and were assigned ST1. Bacterial clone B was shown to be uniform for allele type 1 and 2 but dissimilar at allele three (1, 1, 2) producing an ST designation of ST2. Bacterial clone three was shown to have an allelic profile of 1, 1, 2, identical to bacterial clone 2 and is therefore given the same ST designation, ST2.

Since the advent of the twentieth century, the use of computers has revolutionised the analysis of all types of data, which the creators of MLST exploited to great effect. With the exponential increase in the volume of available data for analysis, greater computing power has also increased to cope with the analysis of such large datasets. In light of the increase in the amount of MLST data available, many analysis techniques have been developed. One of the most notable was eBURST analysis (Feil & Enright, 2004; Feil *et al*., 2004). This analysis creates clonal complexes which cluster samples with identical STs and also STs with allelic profiles that differ from other allelic profile by one, two and three loci, called single locus variant (SLV), double locus variant (DLV) and triple locus variants (TLV).

There are 25 MLST schemes described on the MLST website (www.mlst.net). One of the most recent additions to the website is the *Borrelia burgdorferi* s.l. scheme (Margos *et al*., 2008; Margos *et al*., 2009) which has delineated a new species (*Borrelia bavariensis*) and identified geographic clustering, dependent on the host association of *Borrelia* species with rodent and avian hosts (Vollmer *et al*., in press). *B. burgdorferi* s.l. has more than one version of a multilocus typing scheme. Alternative schemes have been developed by Rudenko *et al.* (2009) and Bunikis *et al.* (2004) although these schemes deviated from the normal code of gene selection, using genes belonging to different categories, including non-coding regions, highly conserved regions offering no option of differentiation and at the other end of the spectrum, hyper-variable outer surface proteins.

MLST is not a perfect scheme and does have problems. One of the identified problems is due to highly uniform housekeeping loci e.g. *Yersinia pestis* (Achtman *et*

al., 1999). The use of rapidly evolving loci could lead to resolution beyond the homogenous structure that has been seen using MLST.

1.4 Mitochondria

Discussions of topics in this section are tended towards ticks and other parasites rather than mitochondria in general.

1.4.1 Mitochondria and their genome structure

Mitochondria are often called the “powerhouses” of the cell (Andersson *et al.*, 2003). They are 0.5-1.0µm in diameter and can be found in Eukaryotic cells. These organelles are responsible for creating adenosine tri-phosphate (ATP).

Mitochondria are made up of an inner and outer membrane, which are separated by an inter-membrane space. The inner membrane encloses the matrix, which is contained by the convolutions of the membrane, which are distinguished as cristae (singular crista). Cristae are studded with the enzyme ATP synthase forming part of the electron transport chain that manufactures ATP for use of all energy-dependent reactions both in and out of the cell (Hatefi, 1985). Apart from generating chemical energy (ATP) within the cell, mitochondria are also involved in the cell cycle, apoptosis, biosynthetic acid catabolic transformations and have a role in development.

The mitochondrial genomes of ticks are circular and contain 37 genes. Of these 37, 13 encode proteins, two are rRNAs (12S and 16S), and 22 encode tRNAs (as shown in Figure 8). Tick mitochondrial genomes like those of animals usually contain a single control region. In exception to this rule are two species of Australasian *Ixodes* and all metastriate ticks that have two control regions (Shao *et al.*, 2005). The control region (sometimes called the D-loop (Lorenzini & Lovari, 2006; Schneider *et al.*, 2010)), controls gene transcription and replication. The arrangement of these genes (Figure 8) has been determined to be ancestral for arthropods (Staton *et al.*, 1997) and has not changed for 400 million years (MY). Mitochondrial genomes have no introns and contains few regions which are non-coding other than the control region (Wolstenholme, 1992).

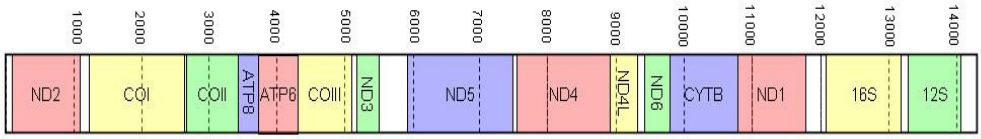

Figure 8: Mitochondrial genome of *Ixodes persulcatus*. Not all genes found in I. persulcatus are shown in this diagram. The shown genes include ND2: NADH dehydrogenase subunit 2, COI: cytochrome c oxidase subunit I, COII: cytochrome c oxidase II, ATP8: ATP synthase F0 subunit 8, ATP6: ATP synthase F0 subunit 6, COIII: cytochrome c oxidase subunit III, ND3: NADH dehydrogenase subunit 3, ND5: NADH dehydrogenase subunit 5, ND4: NADH dehydrogenase subunit 4, ND4L: NADH dehydrogenase subunit 4L, ND6: NADH dehydrogenase subunit 6, CYTB: Cytochrome B oxidase, ND1: NADH dehydrogenase subunit 1, 16S: 16S ribosomal RNA and 12S: 12S ribosomal RNA.

1.4.2 Origin of Mitochondria

The endosymbiosis of a bacterium into an ancestor of the Eukaryotic cell has long been accepted as the origin of mitochondria (Sagan, 1967). This is thought to have occurred approximately 2 billion years ago (Dimauro & Davidzon, 2005) consistent with the identity of the endosymbiont as a free-living α-proteobacteria (Andersson *et al.*, 1998; M. W. Gray, 1999; Rand *et al.*, 2004). Rickettsial bacteria are thought to be the closest living relatives of these ancient free-living bacteria (Lang *et al.*, 1999). More specifically, it has been found that a very high level of genetic similarity can be determined between mitochondria and *Rickettsia prowazekii* making this bacterium the most likely extant species most closely related to mitochondria (Andersson *et al.*, 1998).

1.4.3 Inheritance

Mitochondria can be found within almost all Eukaryotic cells including germ cells although a few highly specialised cell types lack them. Several mitochondria are found in each cell as they are able to replicate more than once per cell cycle (Birky, 1983). Some of these mitochondria are passed on to the next generation by fair meiosis (reviewed in Barr *et al.*, 2005).

Mitochondria are predominantly inherited maternally (Hayashi *et al.*, 1978). Challenges to this notion have begun to appear with some species showing that the male may pass on mitochondria to the next generation in a process called “paternal leakage”. For example, Kondo *et al.* (1990) reported evidence of paternal mtDNA persisting in

Drosophila into adulthood with an incidence of ~0.1%. Paternal leakage of mitochondria has also been shown to occur in mice (Gyllensten *et al.*, 1991). Studies on *Mytilus* marine mussels have also shown that recombination is possible for mitochondria owing to paternal leakage (Filipowicz *et al.*, 2008). Heteroplasmy of more than one mitochondrial genome, has also been associated with paternal leakage of mitochondria and has been shown in several cases (Hilsdorf & Krieger, 2004; Barr *et al.*, 2005; Pearl *et al.*, 2009) and more instances may be found in the future as paternal leakage may have been below detection limits, before modern techniques were used (Wolff & Gemmell, 2008).

Despite these cases of paternal mitochondrial inheritance, they remain very rare in animals and are likely to remain rare. Mitochondria from the father have been shown to be selectively degraded in mice (Kaneda *et al.*, 1995) even if mitochondrial numbers in the progeny are in high proportions compared to maternal mitochondria (Meusel & Moritz, 1993). It is therefore highly unlikely that paternal mtDNA would persist in later development, and such paternal inheritance of mitochondrial genes has not been detected in *I. ricinus*.

1.4.4 The uses of mitochondria in tick phylogenetics

Mitochondria have been utilised for many aspects in the study of ticks. Many of their mitochondrial genomes have been sequenced including the hard ticks *Ixodes hexagonus*, *Ixodes holocyclus*, *Ixodes persulcatus* and *Ixodes uriae* (Shao & Barker, 2007). Mitochondria were often use to resolve phylogenies of ticks and other organisms, as the rate of evolution is much faster than that of nuclear genes and could therefore be used to resolve low taxonomic levels such as genera and species. In addition, due to their abundance in most cells, simple amplification by PCR is often sufficient for analysis of the target gene.

In tick studies, most of the mitochondrial genes previously mentioned have been used in phylogenetic studies. The most common genes used are 12S and 16S (Black & Piesman, 1994; Norris *et al.*, 1996; Norris *et al.*, 1997; Mangold *et al.*, 1998b; Murrell *et al.*, 1999; Beati & Keirans, 2001) but other genes such as cytochrome oxidase I (*coi),* have also been used for taxonomic purposes e.g. *Ixodes philipi* (Mitani *et al.*, 2007).

Mitochondrial genes have also been utilised to study the phylogeography of ticks (Caporale *et al.*, 1995; Rich *et al.*, 1995; Norris *et al.*, 1996; Casati *et al.*, 2007; Burlini *et al.*, 2010). Casati *et al.* (2007) studied *I. ricinus* from various European countries using mitochondrial markers (*coi*, cytochrome oxidase ii (*coii)*, cytochrome B (*cytB)*,

12s and mitochondrial control region (CR)) but these authors reported a lack of geographic structure. In contrast, two studies investigating *Ixodes scapularis* in the United States reported evidence of geographical structuring. Rich *et al.* (1995) and Norris *et al*. (1996) used the *12s* and *16s* mitochondrial genes to study the *I. scapularis* populations on the eastern coast of the US. Both studies suggested that two distinct *I. scapularis* populations exist, one in the Northeast region and one in the Southeast region of the United States.

2 Methods and materials

2.1 Lab methods

2.1.1 Tick DNA extraction from environmental samples – questing ticks

Total genomic DNA from *I. ricinus* ticks was prepared using the alkaline hydrolysis method (Guy & Stanek, 1991). Various volumes depending on the size of the tick (200µl for nymphs and 300µl for adults) of aqueous ammonia at 1.25% was added to whole ticks in 1.5ml "safelock" eppendorf tubes, and the tick was partially crushed using a disposable sterile pipette tip. The homogenated samples were heated to 100°C for 20 minutes on a heated block with closed lids, removed, allowed to cool slightly and placed back onto the block with the lids open to allow 50% reduction in liquid volume to remove remaining ammonia. Samples were stored at -20°C until further use. Extracted DNA was used directly in polymerase chain reaction (PCR) applications. Controls were performed using the method shown above but omitting the tick tissue. These controls were used as template DNA in PCR reactions.

2.1.2 Tick DNA extraction from environmental samples – blood fed ticks

Ticks engorged, partially or fully with blood, were extracted using ammonia hydrolysis (2.1.1 above). The resulting tick lysate was then used in the Qiagen DNeasy Blood and Tissue extraction kit (Qiagen, Germany) according to the manufacturer's instructions apart from the first two initial steps in which 200µl of the animal tissue lysis (ATL) buffer was added to the tick lysate. 30µl of proteinase K was added to this mixture and incubated at 56°C for between 12-18 hours. Samples were eluted in the provided elution buffer with two elution steps of 100µl rather than the suggested single 200µl step. All solutions were pre-mixed and provided in the kit.

2.1.3 PCR methods for tick mtMLST

PCR was performed using Bioline Biomix™ (BIOTAQ™ DNA polymerase, 2mM dNTPs, 32mM $(NH_4)_2SO_4$, 125mM Tris HCl, 0.02% Tween 20, 4mM $MgCl_2$, stabiliser, inert dye) master-mix at 1X concentration (Bioline, UK). Various volumes of primer stock solution at 10pmol were added to PCR reactions according to optimisation

criteria (Appendix - Optimised conditions for PCR, p.183). Template DNA from questing (2.1.1 above) and blood fed (2.1.2 above) ticks was added to PCRs. The volume added to reactions was 2µl. Volumes were increased to 5µl if PCR reactions failed and required repeating. Reaction volumes were adjusted to 25µl with sterile distilled water. Negative controls were performed with sterile distilled water replacing template DNA. Controls were also performed using extraction control samples replacing template DNA. PCR samples were heated according to a variety of thermal cycles depending on the properties of the PCR and optimisation criteria as detailed in the appendices (Appendix - Thermal cycling conditions for PCR, p.181).

2.1.4 PCR amplification of the 5S-23S rRNA (rrf-rrl) intergenic spacer in ticks

Intergenic spacer (IGS) analysis was used to detect *Borrelia* in environmental tick samples to determine host association from host complement (Liveris *et al*., 1995; Bunikis *et al*., 2004). Host complement from certain groups of animals leads to the lysis of particular species of *Borrelia*, leading to distinct combinations of *Borrelia* in various types of animals. The intergenic spacer locus between the 5S-23S rRNA genes was amplified by PCR using two sets of primers (Appendix - Primers for amplification of 5S-23S rRNA (rrf-rrl) intergenic spacer, p.181). PCR reactions were performed using Bioline Biomix™ at a 1X concentration in 12.5µl and 25µl final volumes for the first and second reactions, respectively. Primers were stored at a 10pmol dilution. 1.5µl of primer stock solution was added to each reaction, which was made up to the correct volume using sterile distilled water. PCR samples were heated according to the protocol for the separate reactions (Appendix - Thermal cycling conditions for PCR, p.181).

2.1.5 Primer synthesis and DNA sequencing

All primers were supplied by Invitrogen and were supplied in desalted dehydrated form. Primers were rehydrated in sterile distilled water.

Forward and reverse nucleotide sequences of PCR amplicons were sequenced by Qiagen Genomics, Germany, and Agencourt, USA.

2.1.6 Tick identification

Ticks collected from all locations were identified using classification keys (Hillyard, 1996), an example of which is shown in Figure 9. These keys aided in identifying *Ixodes* ticks collected by blanket dragging. Since the number of ticks collected over several years was numerous, not all ticks were assigned to a species in this way. The majority of ticks collected by blanket dragging (2.3.1 Tick Collection) will be *I. ricinus* while other *Ixodes* species with a different ecology (for example *I. hexagonus*) are unlikely to be picked up by this method.

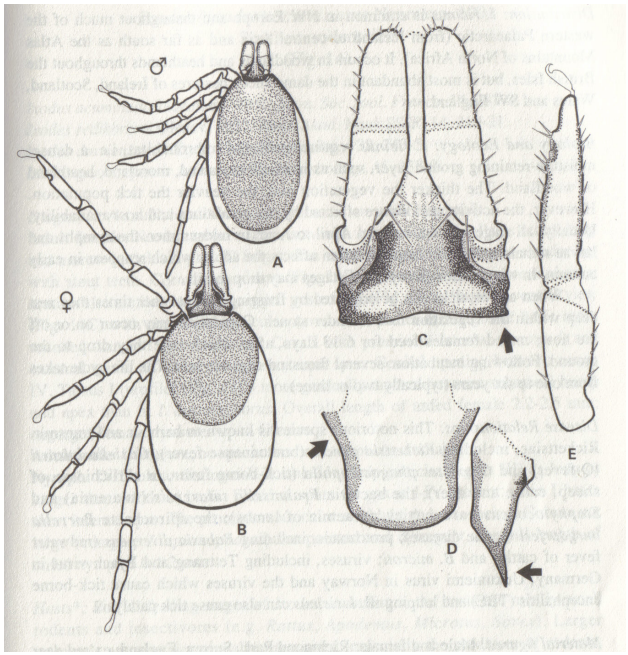

Figure 9: Anatomy of *I. ricinus*. **A. Adult male; B. Adult female; C. Dorsal view of female capitulum; D. Ventral view of female basis and coax I; E. Lateral view of female tarsus I. (Hillyard, 1996)**

Ticks were first identified as adults, nymphs or larvae. Adult males and females were also distinguished via the size of the scutum, which in males covers almost the entire podosoma and opisthosoma on the dorsal surface (Figure 9). Larvae were not classified as we rarely collected this stage and they are difficult to identify with a high degree of accuracy.

Nymphs constituted the majority of ticks classified. Nymphal stages of *I. ricinus* can be distinguished from other species by the following criteria: long palps and coxa I internal spur longer than external spur. Auriculae appear as dark divergent triangles. The most common alternative species, *I. hexagonus,* was identified by the hexagonal

shaped scutum and the presence of cornua on the capitulum with internal spurs on coxa I.

An elongated internal spur, a short external spur and lack of auriculae identified *I. ricinus* females. *I. ricinus* males were identified according to the internal spur of coxa I being three times the size of the external spur (Figure 9).

2.1.7 Agarose gel electrophoresis

Agarose gel electrophoresis was conducted using 1.5% Tris Acetate EDTA (TAE) buffer agarose gels stained with ethidium bromide. Gels were run in 1X TAE buffer solution at 100V for 30-40 minutes dependent on the gel size and viewed using a UV trans-illuminator.

2.1.8 Extraction of DNA from agarose gel slices

A minority of PCR reactions produced multiple bands. These PCR products could not be sent for sequencing directly. Other reactions produced poor products so bands were cut out and purified to decrease the probability of residual DNA fragments interfering with the sequencing reaction. Using a UV trans-illuminator, PCR products were visualised in agarose gels (2.1.7 above) and cut from the agarose gel using a sterile straight edged razor blade for each band in each PCR product range. DNA was extracted from gel slices using a Qiagen QIAquick kit according to the manufacturer's instructions, including all optional steps for maximum purity of samples (Qiagen, Germany). Purified samples were stored at -20°C until required for further use.

2.2 Computer based methods

2.2.1 Tick databases, data handling & mtMLST profiles

Ticks samples were assigned unique identifiers according to the year of collection and individual collection location. Origin identifiers were also used to distinguish ticks from a number of countries. All sequence types determined in the course of these studies are shown in Appendix - Sequence Type Profiles, p. 183.

Allele types were assigned to novel sequences using a non-redundant database (NRDB) (Gish, 2008) for each of the mitochondrial genes to build genetic profiles of individual ticks. Novel allelic profiles were assigned sequence types. Allelic profiles and sequence types (STs) were used in subsequent analyses to study population differentiation.

2.2.2 Alignment

Alignments were made using MEGA version 4.0 and 4.1 (Tamura *et al.*, 2007) using the default settings for ClustalW alignments. After alignment, some minor editing by hand was used for the adjustment of gaps that were created during the alignment process.

2.2.3 Model testing

Alignments of sequences were tested for suitable models in FindModel (Tao *et al.*, 2009). FindModel incorporated several processes to determine the correct model for the submitted data. Weighbour trees (Bruno *et al.*, 2000) based on Jukes-Cantor distances were used as starting trees. PAML (Yang, 1997, 2007) was used to calculate likelihood. Akaike information criterion (AIC) scores were calculated using MODELTEST (Posada & Crandall, 1998).

2.2.4 Neighbour-joining trees

Phylogenetic analyses were conducted in MEGA 4 (Tamura *et al.*, 2007) using the neighbour-joining method (Saitou & Nei, 1987) using the maximum composite likelihood method (Tamura *et al.*, 2004). The evolutionary distances are in the units of the number of base substitutions per site. Alignments of sequences were submitted to MEGA 4 in MEGA format files. The percentage of replicate trees in which the

associated taxa clustered together in the bootstrap test (10000) replicates are shown next to the branches (Felsenstein, 1985). The phylogenetic tree was linearised assuming equal evolutionary rates in all lineages (Takezaki *et al*., 1995). The trees were drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Codon positions included were 1^{st}+2^{nd}+3^{rd}+Noncoding. Pairwise comparisons of sequences to calculate distances had missing data and gaps deleted using the pairwise deletion option to retain as much information from all sequences. Complete deletion option was not used to ensure the comparison of neighbour-joining trees with maximum likelihood trees that account for gaps. Open slashes in branches indicate that the branch is not to scale.

2.2.5 Maximum likelihood trees

Maximum likelihood (ML) trees were constructed using PhyML 3.0 (Guindon & Gascuel, 2003) hosted in the ATGC Montpellier bioinformatics platform. The substitution model was determined as previously described (2.2.3 Model testing) for each dataset. Starting trees were set as BIONJ with tree improvement setting of Sub-tree Pruning and Regrafting (SPR) and Nearest Neighbour Interchange (NNI) with appropriate Likelihood Ratio Test (aLRT) Shimodaira-Hasegawa-like (SH-like) branch support parameters. All other parameters were set at default values. Open slashes in branches indicate that the branch is not to scale.

2.2.6 goeBURST

The program goeBURST (Francisco *et al*., 2009) is downloadable from the internet. The version 1.2.1 was used in all analyses of data. Datasets were constructed using allelic profiles and STs and were clustered according to BURST rules such as implemented in eBURST (Feil *et al*., 2004). Samples with the same STs form nodes, which are proportionally sized to reflect the frequency of the ST within the population i.e. larger nodes indicate STs that are more common. Nodes are related to each other with links. These links are of various colours determined by the clustering rule implemented (Francisco *et al*., 2009). These conditions are shown below:

Black - Link drawn without recourse to tiebreak rules, single locus variant (SLV)

Blue - Link drawn using tiebreak rule 1, SLV

Green - Link drawn using tiebreak rule 2, DLV

Red - Link drawn using tiebreak rule 3, TLV

Gray - Link drawn for higher levels (double locus variant (DLV) with darker gray or triple locus variant (TLV) with lighter gray)

Nodes differing from other nodes by more than three alleles are called singletons and are not linked to other nodes.

2.2.7 Arlequin

The program Arlequin (Excoffier *et al.*, 2005) is downloadable from the internet (http://cmpg.unibe.ch/software/arlequin3). The version 3.1 was used in all analyses of data. Populations were created using the grouping function according to the country of origin or other appropriate factor described for individual analyses. F_{ST} values were computed at significance level of 0.05 with 10,000 permutations. Values of zero for F_{ST} indicate that the populations are completely homogenous; values at one indicate disparate populations. Values between 0 and 0.05 indicate small levels of genetic differentiation, 0.05 to 0.25 indicate moderate levels and values greater than 0.25 indicate a large amount of genetic diversity (Freeland, 2005).

Mismatch distribution analyses of spatial and demographic expansion were computed using pairwise differences as the molecular distance and with 10000 bootstrap repeats. Mismatch distributions determined the number of pairwise differences between haplotypes from which parameters based upon demographic or spatial expansions of a particular population can be estimated. Outputs of this method are line graph showing the range of mismatches observed between individual samples. If the population from which the sample has been derived is in equilibrium, the distribution is usually multimodal. Whereas, if a population has recently passed through a demographic expansion, or through a range expansion, with reasonable levels of migration between neighbouring subpopulations, distributions seen in this graph are usually unimodal. Using a generalised least-squared approach, τ (time in generations since the modelled expansion), θ_0 (initial relative population size) and θ_1 (final population size) are estimated for the two models of expansion. These parameters are estimated using a parametric bootstrap approach.

In Arlequin, demographic expansions assume a stationary haploid population has gone from a population size of N_0 to N_1, τ generations ago. The method also assumes no recombination.

Spatial expansions are defined in Arlequin as population expansions that originate from a small area and over time expand to new ranges. Arlequin tests for trends that generally occur in these populations where populations tend to become genetically distinct, as individuals near each other will mate to produce offspring rather than individuals that are geographically remote.

Arlequin uses an “infinite-island” model which is equivalent to the continent island model: sampled sub-populations would exchange migrants at a define rate with a unique population of infinite size. The original population would have comprised of a single sub-population (N_0). This method assumes no recombination.

Some limitations of the spatial expansion model is that a large spatial expansion can produce the same distribution as a pure demographic expansion in a panmictic population if neighbouring sub-populations have exchanges of 50 migrants or more and must be considered when analysing results.

Fu’s Fs neutrality tests (Fu, 1997) were used to test sample datasets for evidence of population expansion. This tests selective neutrality of a random sample of DNA sequences under the infinite site model. More specifically, this test evaluates the probability of observing a random neutral sample with a number of alleles similar or smaller than the observed value given the observed number of pairwise distances. These analyses assume no recombination. Demographic expansions that are large scale lead to large negative Fs values. My results were calculated in Arlequin 3.1 using 1000 simulated samples. Significance of these values is considered by p-values of 0.02 or below, not below 0.05 as in many other statistical tests.

Further information regarding the mathematical derivations of these analyses can be found at http://cmpg.unibe.ch/software/arlequin3/arlequin31.pdf in the Arlequin manual.

2.2.8 DNAsp

Mismatch distributions using constant and changing population size models were computed in DNAsp v4.90.1 (Rozas *et al.*, 2003). All sequences were included in the analysis and all sites in the sample DNA length. Constant population analysis was initially performed to calculate values for theta initial (θ_0), theta final (θ_1) and τ. These computed values were used in mismatch distribution analysis of changing population size. Coalescent simulations using the observed values for θ_0, θ_1 and τ were used to calculate raggedness index (RI) values and p-values. The raggedness index is an estimation of the departure of the observed data from the derived model of expansion.

2.3 Field Methods

2.3.1 Tick Collection

Ticks were collected using blanket dragging from countries across Europe including Portugal, England, Scotland, Latvia, Switzerland and Germany (Appendix - Collection Site Maps, p.167). Ticks were collected from the area around Bath and a range of other sites across Europe for *Borrelia* analyses. Ticks were collected by the dragging method described in Hillyard (1996). Ticks from Latvia were also used in these analyses and were collected in a similar way by collaborators and stored in 70% ethanol until DNA extraction. A summary of the ticks collected in all geographic regions is shown in Appendix - Summary of sample numbers, Origin, Year and Sex, p. 196.

Strategies for sampling ticks from various regions were not restricted to a set pattern. The nature of the incidence of ticks results in, at best, patchy coverage and previous instances of searching for pockets of tick activity resulting in negative results. I have previously tried to determine a profile of tick habitat but this has lead to no ticks being found in seemingly ideal habitats.

3 A Multilocus Sequence Typing Scheme for Ticks: Rationale & Design

This chapter describes the design of a multilocus sequence typing-like (MLST) scheme for *Ixodes ricinus* ticks that is based on the use of six mitochondrial genes. Following primer design and optimisation, a sample-set was tested to determine the suitability of the method. We tested ticks from different geographical locations (i.e. Latvia and Britain) to obtain information whether these populations show genetic structuring that could indicate geographical origin, or homogeneity resulting from panmixis of populations in Europe. Both a homogenous and a heterogenous result have implications for our understanding of the movements of ticks and the hosts they utilise to migrate.

3.1 Introduction

Biogeographical studies, which aim to describe the genetic variation of populations within temporal and spatial frameworks, have been carried out on a range of organisms including birds (Morris-Pocock *et al*., 2008; Mayer *et al*., 2009), mammals (May-Collado & Agnarsson, 2006), and bacteria (reviewed in Spratt, 1999; Maiden, 2006). Arthropod vectors of disease have received special attention owing to their importance to human and animal health, and these studies have utilised various genetic markers.

Several groups have studied *I. ricinus* across Europe and *Ixodes scapularis* in N. America with varying results concerning the population structure. Delaye *et al.* (1997) used two enzyme markers to study the population structure of *I. ricinus* ticks in Switzerland to determine whether populations separated by significant geographical barriers formed genetically isolated populations. The allozymic data showed low variability at two loci suggesting a panmictic population. Casati *et al*. (2007) also studied the population of the European population of *I. ricinus* using mitochondrial markers but little population structure was observed. These results may have benefitted from a larger sample size as only 26 ticks were used in the analysis. Norris *et al*. (1996) used the *12s* and *16s* mitochondrial genes to study the *I. scapularis* populations on the eastern coast of the United States (US). Analyses of partial sequences from these loci suggested two distinct clades separated by geographical distance.

MLST has emerged as an invaluable tool to monitor the spread of virulent bacterial strains or fungal pathogens (Maiden *et al.*, 1998; Enright *et al.*, 2000; Meyer *et al.*, 2009). Advantages are the reproducibility of typing methods between laboratories, portability and data sharing via the internet and more robust analyses than single loci can offer (Maiden *et al.*, 1998; Urwin & Maiden, 2003). Several aspects need to be taken into consideration when developing MLST. The genes should be single copy genes, be nearly neutrally evolving and not prone to recombination. Traditionally, slowly evolving housekeeping genes which are under purifying selection were chosen (Maiden, 2006). Mitochondrial genes have all the required characteristics defined for MLST schemes. Nuclear genes from the Eukaryotic organism that causes fungal meningitis (Safdieh *et al.*, 2008) have been used successfully as part of an MLST scheme (Meyer *et al.*, 2009).

In this chapter, I document how the mtMLST for *I. ricinus* ticks was constructed using mitochondrial genes. This is the first use of a mitochondrial MLST scheme for an arthropod vector and a novel approach for the molecular discrimination of geographically distinct vector populations.

3.1.1 Aims and objectives

The aims of this chapter can be described as follows:

- to create primers and methods to successfully amplify *Ixodes ricinus* mitochondrial genes from environmental tick samples in a reproducible manner to create an mtMLST scheme
- confirm maternal clonal inheritance of mitochondria from female to larval offspring
- show the capabilities of any developed mtMLST scheme with sample-sets from discrete geographical areas

3.2 mtMLST scheme genes – Primer design and optimisations

MLST schemes traditionally use five to eight housekeeping genes that evolve nearly neutrally. The genes selected must be single copy and not prone to high levels of recombination. Mitochondria are prime candidates for MLST analysis as they fulfil all requirements.

There is no full genome for *I. ricinus* so primers could not be designed directly. Using *Ixodes persulcatus* and *Ixodes hexagonus*, genes from the mitochondrial genome (Figure 10) were used to design primers.

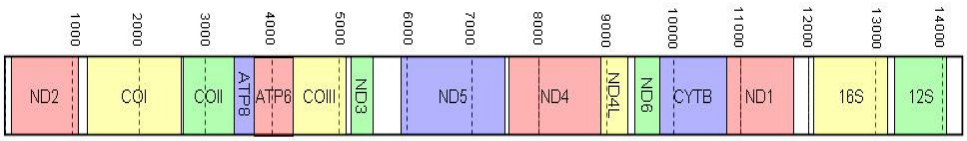

Figure 10: Mitochondrial genome of *Ixodes persulcatus*. Not all genes found in I. persulcatus are shown in this diagram. The shown genes include ND2: NADH dehydrogenase subunit 2, COI: cytochrome c oxidase subunit I, COII: cytochrome c oxidase II, ATP8: ATP synthase F0 subunit 8, ATP6: ATP synthase F0 subunit 6, COIII: cytochrome c oxidase subunit III, ND3: NADH dehydrogenase subunit 3, ND5: NADH dehydrogenase subunit 5, ND4: NADH dehydrogenase subunit 4, ND4L: NADH dehydrogenase subunit 4L, ND6: NADH dehydrogenase subunit 6, CYTB: Cytochrome B oxidase, ND1: NADH dehydrogenase subunit 1, 16S: 16S ribosomal RNA and 12S: 12S ribosomal RNA.

The *Ixodes persulcatus* genome is 14,539bp long, circular and contains 37 genes. Of these, 13 encode proteins, two are rRNAs (*12s* and *16s*), and 22 encode tRNAs (as shown in Figure 10) (Shao *et al.*, 2005). *I. persulcatus* has a single control region.

Primers were selected in accordance with the specifications used in Maiden *et al.*, (1998). The tRNA genes are 54-69bp long in *I. persulcatus,* and were therefore dismissed, as 400-500bp of sequence data needs to be available for each gene. Other genes were omitted from further analysis as they were not large enough to accommodate internal primers and produce enough legible sequence data from traces as often the first 20-50bp of trace data from each site is poor (e.g. ATP8 is only 156bp long).

Primers were designed (Appendix - Primers for amplification of mitochondrial genes, p. 173) based upon mitochondrial genomes of full mitochondrion genomes. The accession numbers of these genomes used in primer design for individual genes are

shown in Table 2. A summary of the characteristics of the genes are shown in the appendices (Appendix - Summary Statistics for Mitochondrial MLST Genes, p. 182). The following section of this chapter deals with the PCR analyses of genes that produced reliable and relevant PCR products.

3.2.1 Primer design for atp6, coi, coii and coiii

Primers were designed for ATPase subunit 6 (*atp6*), cytochrome c oxidase subunit I (*coi*), cytochrome c oxidase subunit II (*coii*), cytochrome c oxidase subunit III (*coiii*), cytochrome B (*cytB*) and 12S ribosomal RNA (*12s*) genes. Complete mitochondrion genome sequences for each gene (Table 2) were aligned with MEGA 4.1 (Tamura *et al*., 2007) using default settings. The accession number samples names are shown in Appendix - Accession Numbers used for primer design, p. 173. Some editing was done by hand to minimise gaps. Primers were designed by hand, unless otherwise stated, in conserved regions of the gene.

Table 2: Summary of primer positions and design.

Primer Name	Position of primer (NC_004370 as reference)	Accession numbers used in primer design alignment	Amplicon position (5'-3')
12S32F	13,104	NC_004370	13,350-13,834
12S821R	13,893		
12S002F	13,321		
12S601R	13,920		
ATP6004F	3,733	NC_002010 NC_004370 NC_006078	3,808-4,305
ATP6663F	4,392		
COI001F	1,222	NC_002010 NC_003470 NC_006078	1,297-1,932
COI786R	2,005		
COII071F	2,832	NC_002010 NC_004370 NC_006078	2,921-3,404
COII731R	3,492		
COIII001F	4,399	NC_002010 NC_004370 NC_006078	4,498-5,052
COIII780R	5,173		
CYTB222F	10,047	NC_004370 NC_006078	10,123-10,671
CYTB1004R	10,829		

Primers were tested using *I. ricinus* DNA template in a standard PCR amplification (2.1.3 PCR methods for tick mtMLST) to ensure clean single band amplification of target sequence. Amplification of a single product allows for direct sequencing of PCR products without separating and purifying samples on agarose gels. A small aliquot (5µl) of each PCR product was run on an agarose gel (2.1.7 Agarose gel electrophoresis) and visualised with UV-transillumination. Gels showing these PCR reactions are shown in the appendices (Appendix - Gel pictures showing tests of primers, p. 174).

PCRs for each gene were set up, and parallel settings modified using differing volumes of $MgCl_2$ (using a stock solution of 50mM). The total volume was 25µl. Following amplification as described, these reactions were run on an agarose gel. The PCR

reaction that gave the most efficient amplification of target DNA was used for all future reactions. Gels showing these PCR reactions are shown in the appendices (Appendix - Gel pictures showing $MgCl_2$ optimisations, p. 176).

Primer optimisations were also performed. Using the standard PCR, differing volumes of a 10pmol stock solution of each primer was added to PCR reactions (final volume of 25µl). PCR amplification products (5µl) were run on an agarose gel. The primer concentration of the reaction with the highest efficacy of PCR amplification was selected for further PCR reactions. Gels showing these PCR reactions are shown in the appendices (Appendix - Gel pictures showing primer concentration optimisations, p. 178).

All optimisations and tests of primers were performed using environmental tick samples. Different samples were used as DNA stocks were limited for each sample. The tick samples for each gene and the corresponding analysis is shown in Table 3. A summary of the optimised $MgCl_2$ and primer conditions for all genes is shown in Appendix - Optimised conditions for PCR, p. 183.

Table 3: Summary of tick samples used in optimisations and primer reaction tests.

Gene	Primer test	$MgCl_2$ optimisation	Primer optimisation
atp6	61204B	60228B	60228B
coi	64402B	60228B	61823B
coii	60404B	60126B	60228B
coiii	61204B	61823B	60228B

3.2.2 cytB *primer design and optimisation*

Primers for *cytB* (CYTB222F, CYTB1004R) were designed using an alignment of three tick species (Table 2 and Appendix - Accession Numbers used for primer design, p. 173).

Another researcher (Frederik Seelig) did primer and $MgCl_2$ optimisations according to the same protocols that I have previously mentioned. No further $MgCl_2$ was required for amplification of *cytB* from DNA of environmental samples. Primer concentrations were optimised for this PCR reaction. The optimal volume of primer to be added to the reaction was 1.5µl of both forward and reverse primers (stock solution of 10pmol).

3.2.3 12s primer design and optimisation

An alignment of the *12s* genes of *Ixodes uriae*, *I. persulcatus* and *I. hexagonus* showed high levels of variation. Primers (primers: 12S32F, 12S821R) were produced using FAST PCR primer design software (Kalendar *et al.*, 2009) using *I. persulcatus*, as a template. Primers were used in PCRs and for sequencing. The sequencing of samples produced adequate traces but was sometimes unreliable, showing shorter traces than required with poor quality peaks that were indecipherable. New primers (12S002F, 12S601R) were made by selecting alternative binding sites to complement the already existing selected region of analysis.

Another researcher (Frederik Seelig), according to the same protocols that I have detailed above, performed primer and $MgCl_2$ optimisations for the new primers for *12s*. No further $MgCl_2$ was required for amplification of environmental samples of *12s* PCR products. Primers were optimised for this reaction with 1µl of each stock solution of 10pmol primers giving the most efficient amplification of *12s* PCR products from environmental samples.

A summary of these optimised $MgCl_2$ and primer optimisations for the individual genes shown in this section can be found in the appendices (Appendix - Optimised conditions for PCR, p. 183).

3.3 Confirmation of methods

Differences in gene sequences occur due to natural variation in the population. It is important that differences detected in the genetic signal are true differences and not due to mistakes in DNA sequence editing. The editing of DNA sequences needs to be consistent, with errors easily identified and corrected. Single nucleotide changes due to natural variation are important indicators of population structure but errors in the sequence data could alter the population structure incorrectly. Although mitochondria are generally thought to be maternally inherited and clonal, this has never been shown for *I. ricinus*.

3.3.1 Sequence editing

The sequence identities of all PCR products were confirmed with forward and reverse sequencing using the primers for PCR amplification. Forward and reverse sequences were analysed using DNASTAR Lasergene SeqMan (version 7.1.0). DNA traces were aligned and trimmed according to the selected allele type region and error checked using automatic ambiguous base matching functions, and manually correcting at poor quality bases. Some sequences were too poor to determine reliable sequence data from and were therefore excluded from further analyses. No cases of double peaks (heteroplasmy) were observed in any of the data.

Alleles were assigned to sequences using the a Non-Redundant Data Base (NRDB) (Gish, 2008). New allele types were verified by aligning the new sequence with all existing allele types of that gene in MEGA 4.1 (Tamura *et al.*, 2007). Mismatches in the sequence alignment were identified using “Mark variable sites” function in the Data viewer in MEGA 4.1. Differing bases were verified by examining the original traces. New alleles were assigned if no other alleles match the new sequence.

After the allocation of sequence types according to the allelic profile, singleton sequence types were compared with the entire set of sequence types to find the closest match. New allele types, which differ from the more common sequence types, were reanalysed and differing bases checked once again in the sequence traces to confirm the sequence identity.

3.3.2 Repeating PCR sequencing through double blind testing

To confirm sequence-editing techniques used, double blind testing was conducted using two populations of ticks (Table 4 and Table 5). The samples were initially used in PCRs for all genes, sequenced, checked and edited. Edited sequences were assigned allele types. To double blind test these samples, another researcher (Frederik Seelig) performed the same PCR strategy without prior knowledge of the allele types already assigned. PCRs were performed according to the methods indicated previously and sequences were edited and assigned allele types. All allele sequences in all ticks were shown to be the same in both analyses. This has important implications for the use of such a scheme in the monitoring of *I. ricinus* populations from different regions, enabling comparisons of diverse populations using the same scheme.

Table 4: Samples from Britain 2006 used for method confirmation

Britain 2006				
64402B	60203B	60404B	61204B	61304B
61404B	61504B	60106B	60708B	60808B

Table 5: Samples from Latvia 2002 used for method confirmation

Latvia 2002				
20601L	21001L	21101L	20502L	20702L
23603L	24503L	20404L	20704L	20804L

3.3.3 Maternal inheritance of mitochondrial DNA through ovarian tissue

To confirm the clonality of mitochondrial DNA in ticks, three partially engorged female *I. ricinus* ticks were removed from a dog and placed in a petri dish with cotton wool moistened with sterile distilled water. After one month, two of the females had died. The remaining female had laid a small clutch of eggs of approximately 100+ and had subsequently died. The female tick and eggs were placed into separate eppendorf tubes and stored at -80°C until required.

The clutch of eggs and female were thawed at room temperature. The clutch of eggs had DNA extracted using the ammonia hydrolysis technique (2.1.1 Tick DNA extraction from environmental samples – questing) using 100µl of ammonia solution (Sample name = EGGS). The engorged female tick had DNA extracted using a combination of ammonia hydrolysis and DNA purification techniques (2.1.2 Tick DNA extraction from

environmental samples – blood fed) using 200µl of ammonia solution (Sample name = TICK). Purified DNA was stored at -20°C until further use.

The efficacy of 'EGGS' was tested using 18S primers (Appendix – Primers for positive controls of PCR, p. 180). Different volumes of purified DNA from the 'EGGS' sample was added to standard PCR solutions (Figure 11). The addition of 0.5µl of template DNA produced the most efficient PCR reaction and will be used in all further PCR reactions.

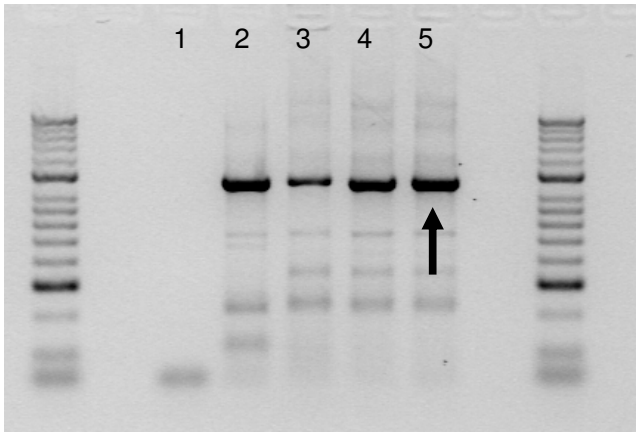

Figure 11: Agarose gel showing a DNA concentration gradient of 'EGGS' DNA using 18S primers. Different volumes of DNA were used in PCR reaction (final volume of 25µl). 1: negative control, 2: positive control 83222B, 3: 2µl, 4: 1µl, 5: 0.5µl. Arrow indicates the optimal volume of template DNA to be added to PCR reactions.

All six genes were amplified from both 'TICK' and 'EGGS' samples and had allele types assigned in the above-mentioned manner. Both samples showed identical genetic profiles which is a consistent with direct, sole maternal transfer of mitochondrial DNA in this example. These samples, when analysed were designated ST300 which is a relatively rare allele only found in the UK (only 4 other ticks were found to have this ST found in all analysed samples). As this allele is rare, the possibility that both parents of the juvenile ticks were ST300 is extremely unlikely.

3.4 Comparison of two populations of *Ixodes ricinus* from Latvia and Britain

The verification of the mtMLST scheme devised as a suitable method of distinction between two populations was tested using a selection of *I. ricinus* from sites across Britain (Figure 12) and from Latvia in the surrounding area of Riga (Figure 13). The scheme aims to distinguish the lineages of *I. ricinus* populations in geographically isolated areas. Samples were selected (Appendix - Tick Samples used for the Comparison of British and Latvian Populations in section 3.4, p. 198) from both the British sample-set (25 samples) and the Latvian sample-set (25 samples) at random from my complete sample-set (data not shown).

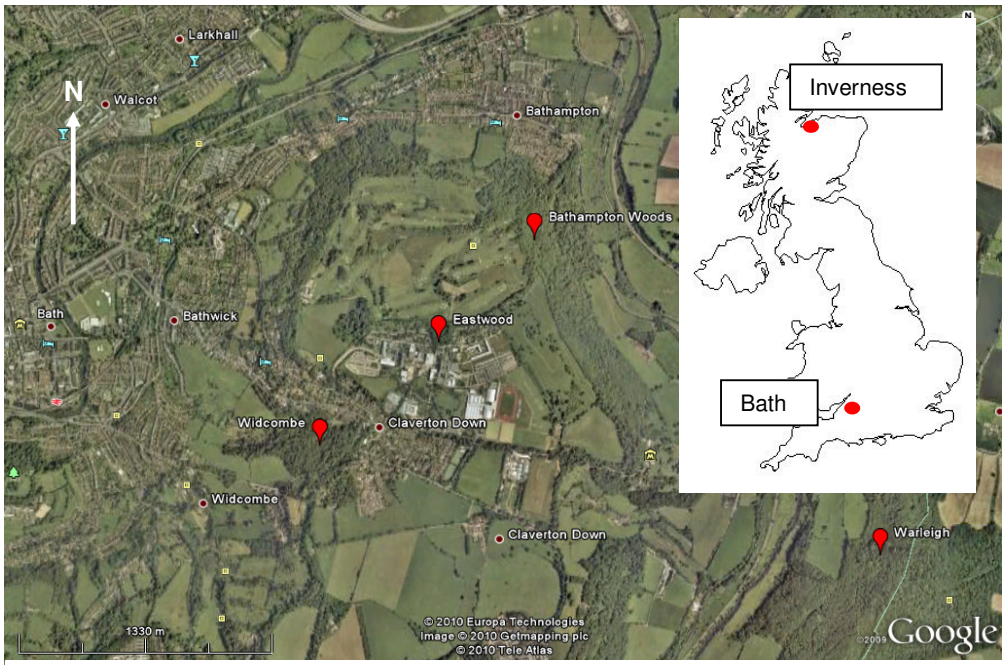

Figure 12: Tick collection locations in the UK. Pins show general locality of collections. Inset box indicates the collection regions in the UK. Map generated using Google Earth (Google, 2010).

Figure 13: Tick collection locations in Riga, Latvia. Pins show general locality of collections. Red inset box indicates the region of Latvia collections were made from. Map generated using Google Earth (Google, 2010).

3.4.1 Phylogenetic analyses of British and Latvian I. ricinus populations

Phylogenetic trees of 6 mitochondrial internal gene fragments (*atp6*, *coi*, *coii*, *coiii*, *cytB* and *12s*) were constructed using the neighbour-joining method (Saitou & Nei, 1987) in MEGA 4.1 (Tamura *et al.*, 2007) (Figure 14, Figure 15, Figure 16, Figure 17, Figure 18 and Figure 19). Trees were rooted with *I. persulcatus* sequences used as an outgroup. *I. persulcatus* sequences from GenBank sequence NC_002010 were trimmed according to the corresponding area of analysis for each gene.

Neighbour-joining analyses of the individual genes show a distinct difference between the two populations but many bootstrap values were poorly supported values of less than 50%. The geographical distinction between the two populations was not defined for each tick with a small number of Latvian ticks found within the British clade and vice versa.

Individual gene phylogenies: The topology of trees from each gene show differences in the population separation with some ticks moving between the two major

clades e.g. in the *atp6* analysis tick sample 7001105B clusters with the majority of British samples whereas in the remaining genes, is consistently clustered in the Latvian clade. Recombination in mitochondria is considered rare if occurring at all in animals and reports of recombination have been treated with a certain degree of scepticism (reviewed in Barr *et al.*, 2005). The incongruence that we see in the tick sample 7001105B derives from unique alleles in *cytB* and *12s* genes. The *cytB* and *12s* alleles differ by a single point mutation to allele type 14 for *cytB* and allele type 95 for *12s* and are unique to the dataset, an indication of mutation rather than recombination in the mitochondrial genes (Feil *et al.*, 2001). The clustering pattern of all genes indicates a relatively consistent geographical distinction between the two populations but with poor bootstrap values. The precise position in the trees of all terminal nodes varies between trees.

The individual genes all indicate that the geographical origin of *I. ricinus* ticks can be determined by examining the genetic profile of the population. In all individual gene analyses, the separation of the British and Latvian clade is clearly shown. The bootstrap values for the distinction of these sister clades are not as compelling as to form succinct conclusions but are indeed consistent across all genes selected for the scheme. The two clades exhibit a clear split according to geographic origin with a small number of ticks showing mixing across clades, which is consistent in all gene analyses.

Concatenated gene phylogenies: The concatenated sequences were analysed using the neighbour-joining method in MEGA 4.1 (Figure 20) and PhyML (Guindon et al., 2005) (Figure 21). The two analyses of these populations both indicate sister clades exhibiting clear geographic origins with the majority of samples in these clades from either Britain or Latvia. A small number of samples from Britain are shown to be within the Latvian clade and vice versa. However, this does not compromise the integrity of the conclusions of the genetic profile indicating geographic origin. The majority of the deep branches in the neighbour-joining analysis and the maximum likelihood analysis are well supported with good bootstrap and good aLRT on the internal branches with values greater than 75.

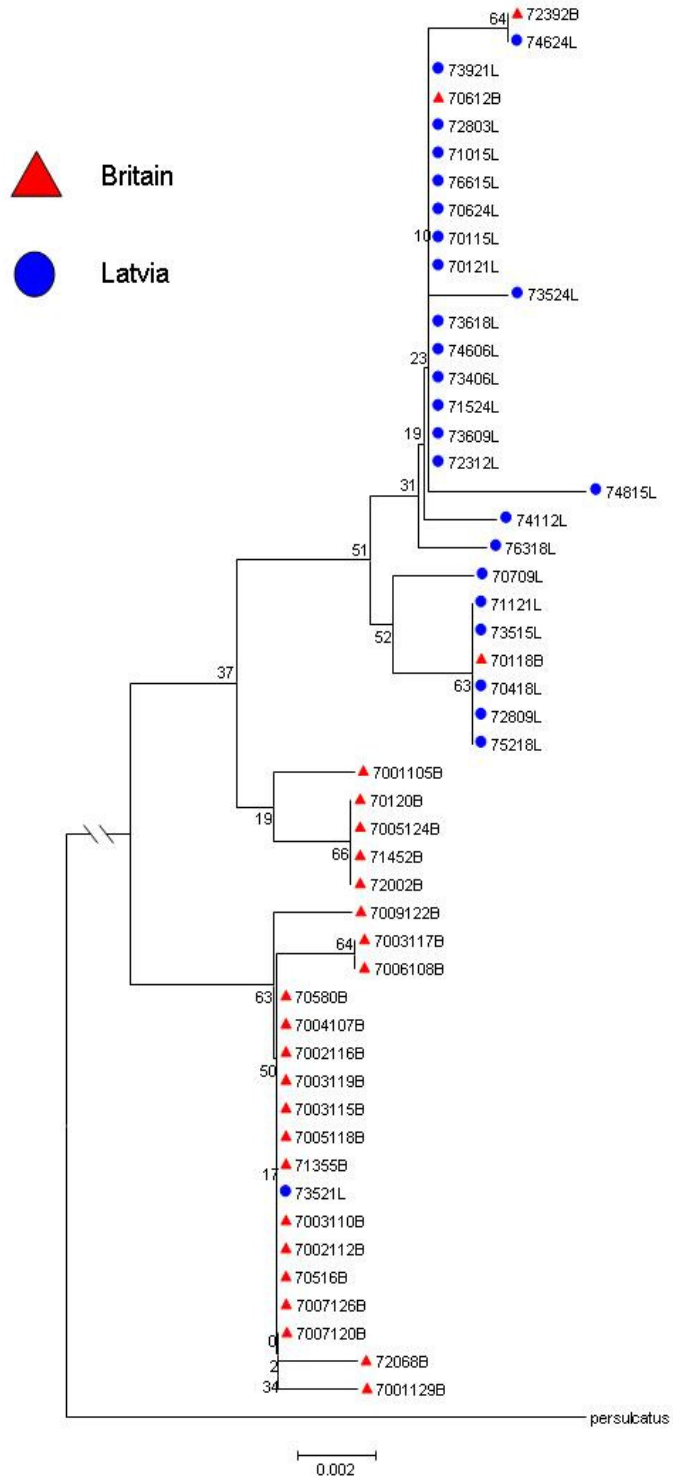

Figure 14: The evolutionary history of ATP6 from *I. ricinus* from Britain and Latvia from 2007 inferred using the neighbour-joining method. The optimal tree with sum of branch length = 0.19466153 is shown. The dataset was outgrouped using *I. persulcatus*. There were a total of 498 positions in the final dataset. The scale bar indicates 0.2% divergence. The branch for the outgroup is not to scale (indicated by slashes).

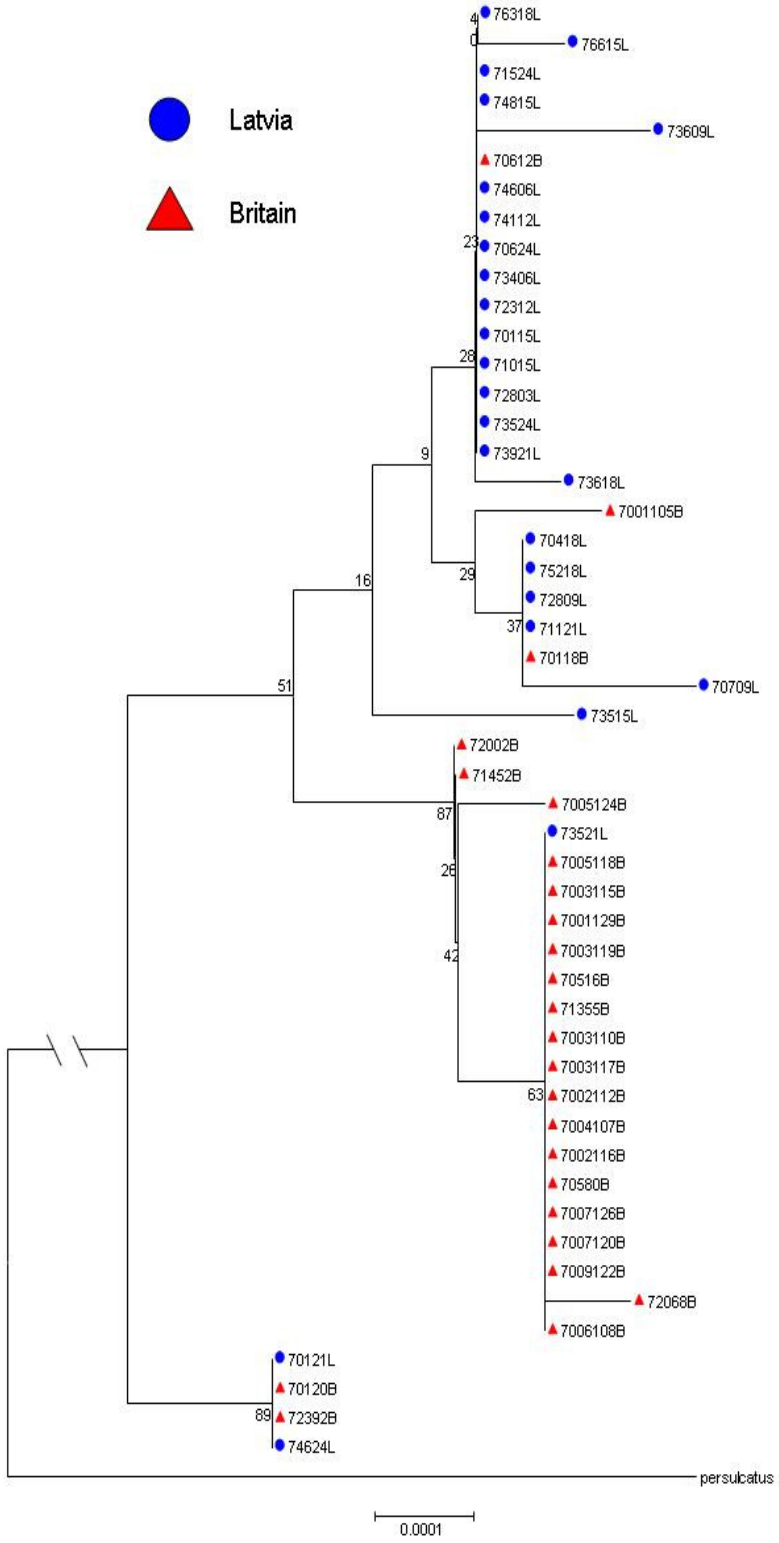

Figure 15: The evolutionary history of COI from *I. ricinus* from Britain and Latvia from 2007 inferred using the neighbour-joining method. The optimal tree with sum of branch length = 0.08897952 is shown. The dataset was out-grouped using *I. persulcatus*. There were a total of 636 positions in the final dataset. The scale bar indicates 0.01% divergence. The branch for the outgroup is not to scale (indicated by slashes).

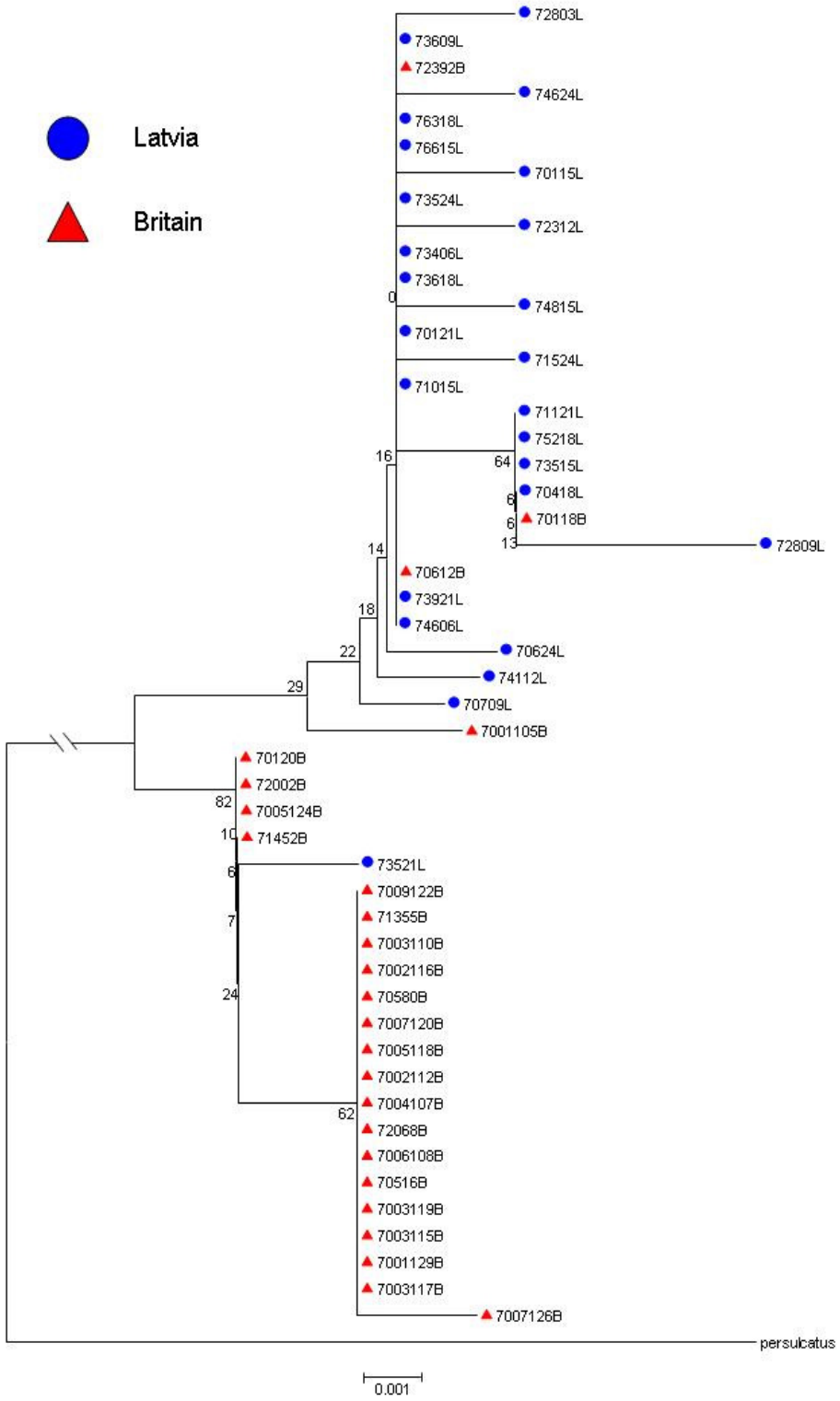

Figure 16: The evolutionary history of COII from *I. ricinus* from Britain and Latvia from 2007 inferred using the neighbour-joining method. The optimal tree with the sum of branch length = 0.18798353 is shown. The dataset was out-grouped using *I. persulcatus*. There were a total of 483 positions in the final dataset. The scale bar indicates 0.1% divergence. The branch of the outgroup is not to scale (indicated by slashes).

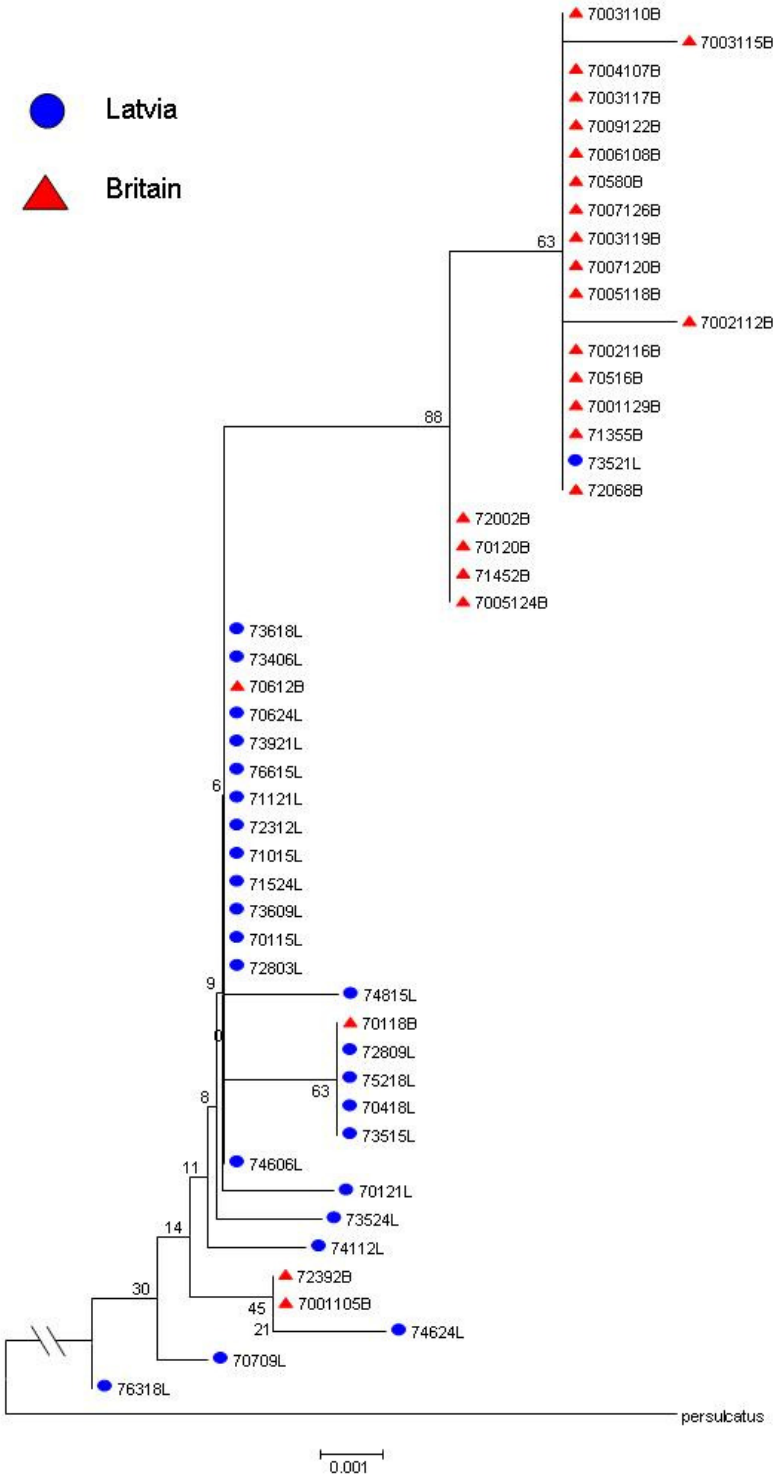

Figure 17: The evolutionary history of COIII from *I. ricinus* from Britain and Latvia from 2007 inferred using the neighbour-joining method. The optimal tree with the sum of branch length = 0.18020269 is shown. The dataset was out-grouped using *I. persulcatus*. There were a total of 555 positions in the final dataset. The scale bar indicates 0.1% divergence. The branch of the outgroup is not to scale (indicated by slashes).

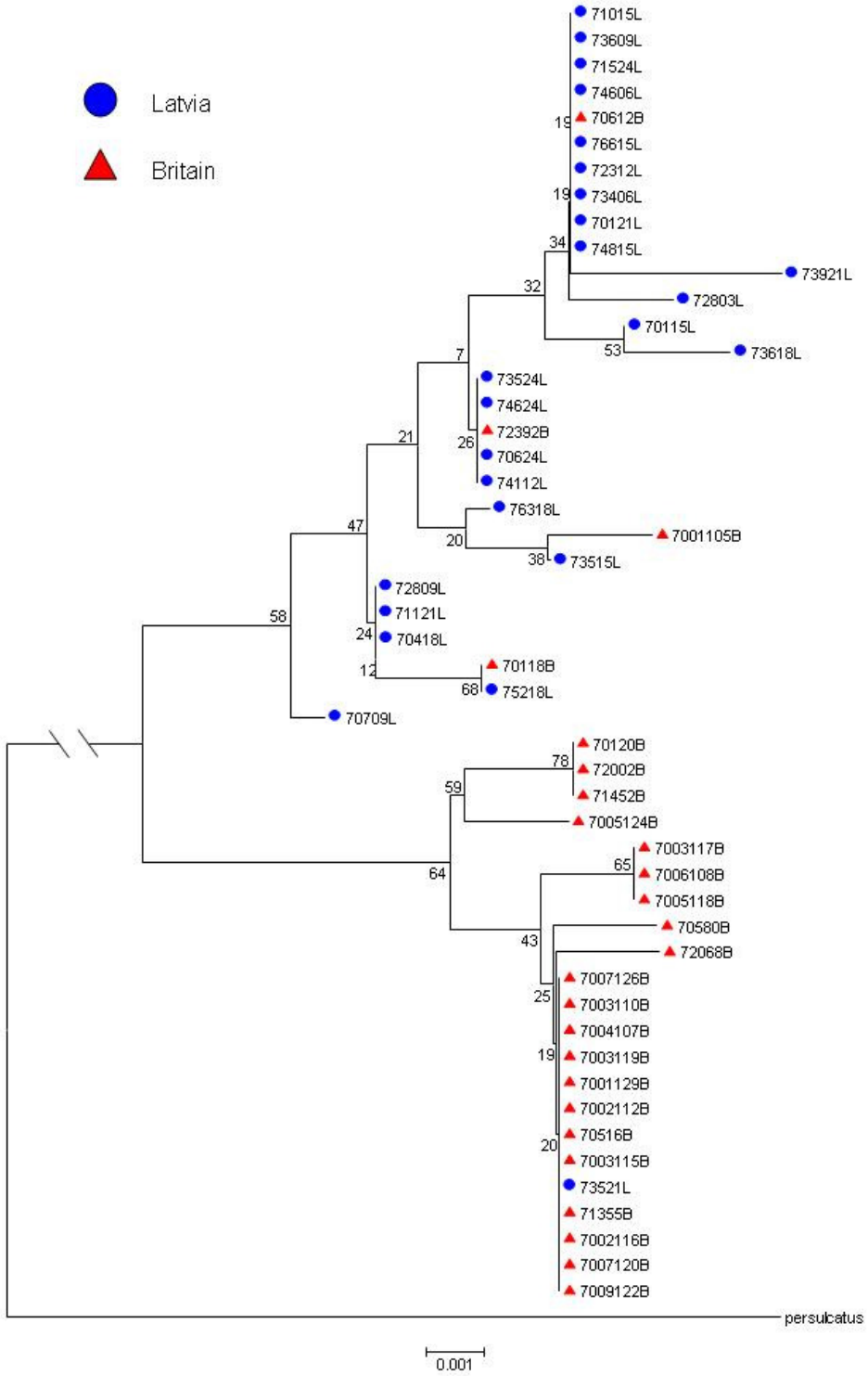

Figure 18: The evolutionary history of CYTB from *I. ricinus* from Britain and Latvia from 2007 inferred using the neighbour-joining method. The optimal tree with the sum of branch length = 0.17176504 is shown. The dataset was out-grouped using *I. persulcatus*. There were a total of 549 positions in the final dataset. The scale bar indicates 0.1% divergence. The branch for the outgroup is not to scale (indicated by slashes).

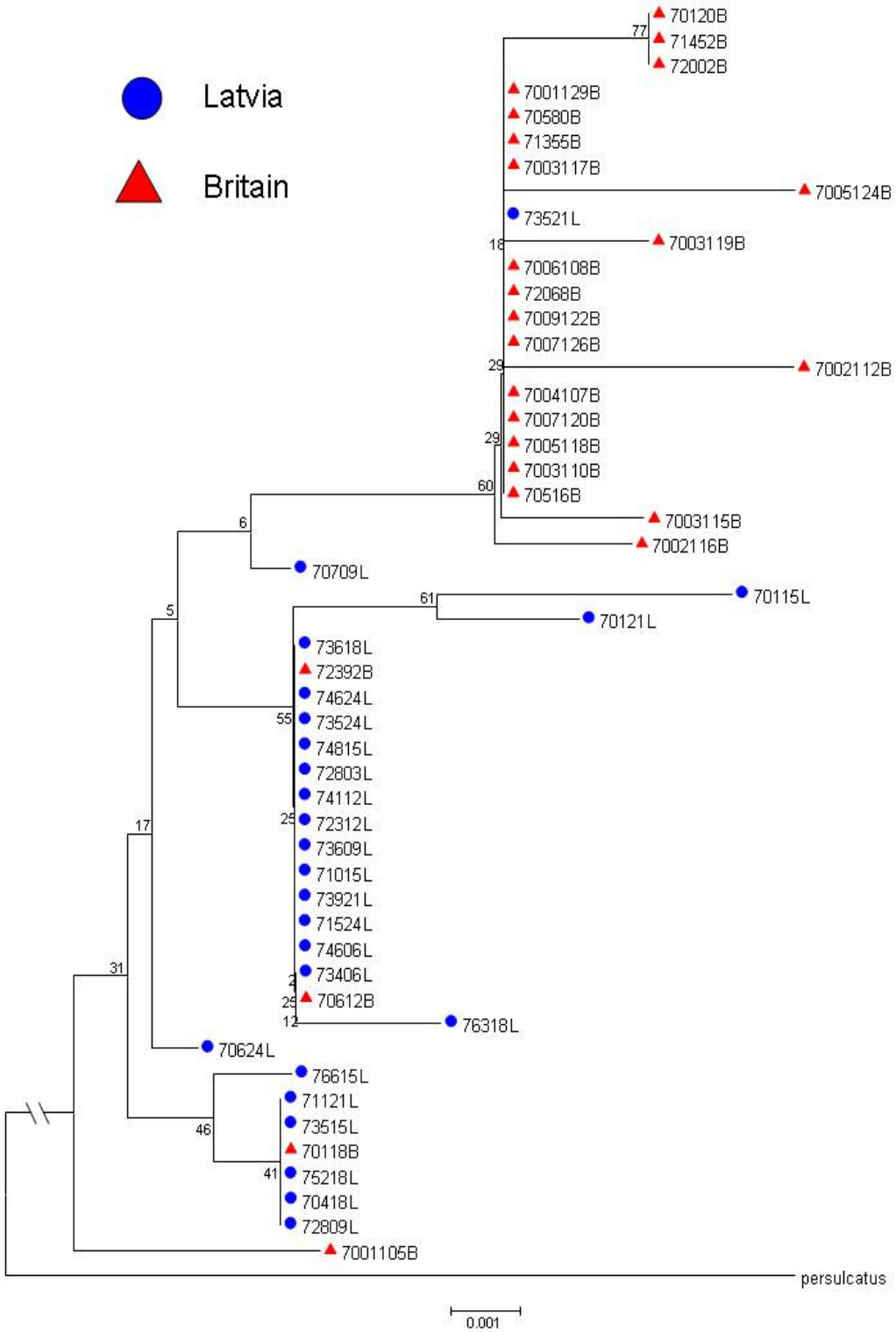

Figure 19: The evolutionary history of 12S from *I. ricinus* from Britain and Latvia in 2007 inferred using the neighbour-joining method. The optimal tree with the sum of branch length = 0.11874904 is shown. The dataset was out-grouped using *I. persulcatus*. There were a total of 472 positions in the final dataset. The scale bar indicates 0.1% divergence. The branch of the outgroup is not to scale (indicated by slashes).

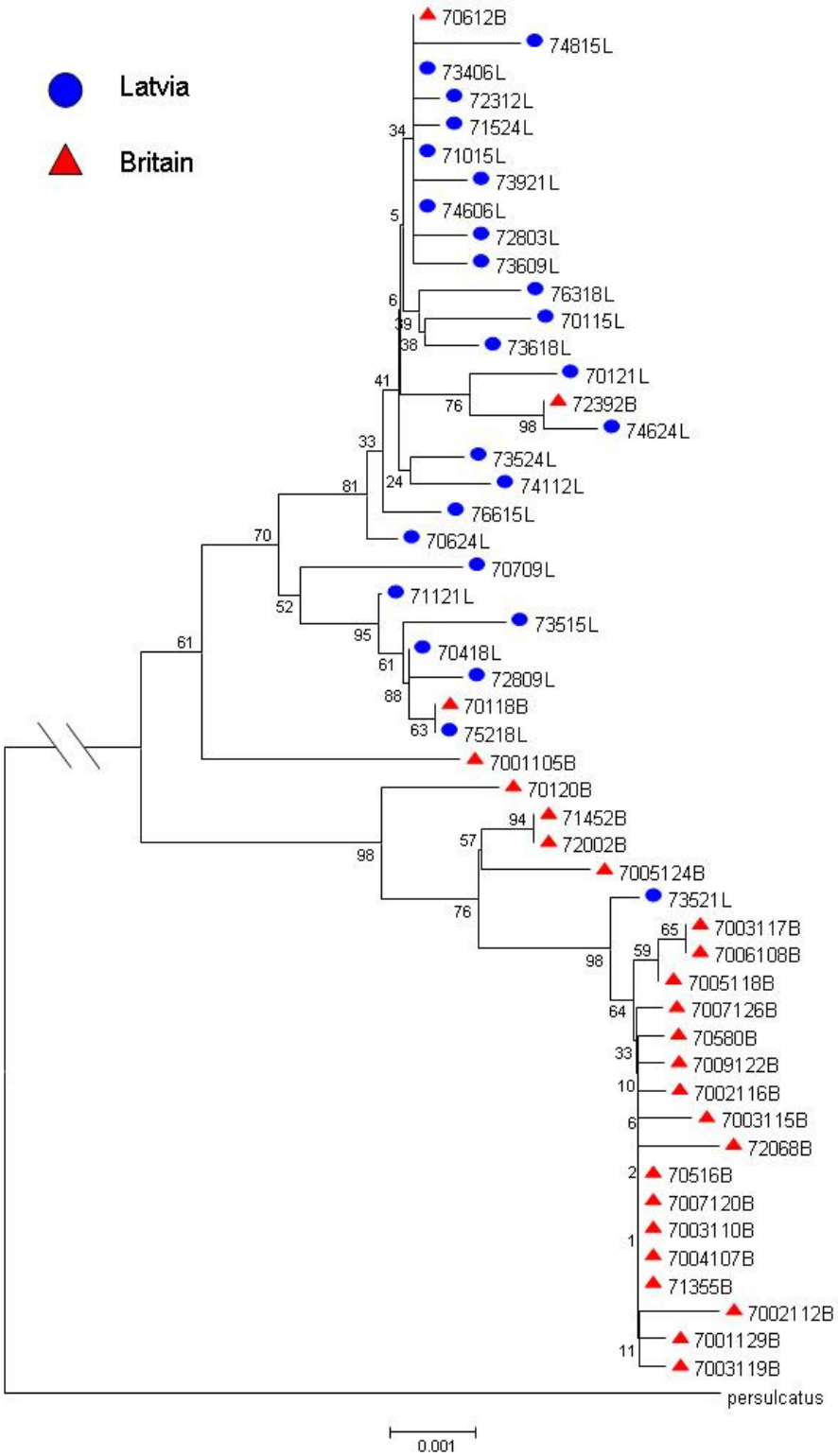

Figure 20: The evolutionary history of six mitochondrial genes (*atp6*, *coi*, *coii*, *coiii*, *12s* and *cytB*) concatenated from *I. ricinus* from Britain and Latvia in 2007 inferred using the neighbour-joining method. The optimal tree with the sum of branch length = 0.16758872 is shown. The dataset was out-grouped using *I. persulcatus*. There were a total of 3212 positions in the final dataset. The scale bar indicates 0.1% divergence. The branch of the outgroup is not to scale (indicated by slashes).

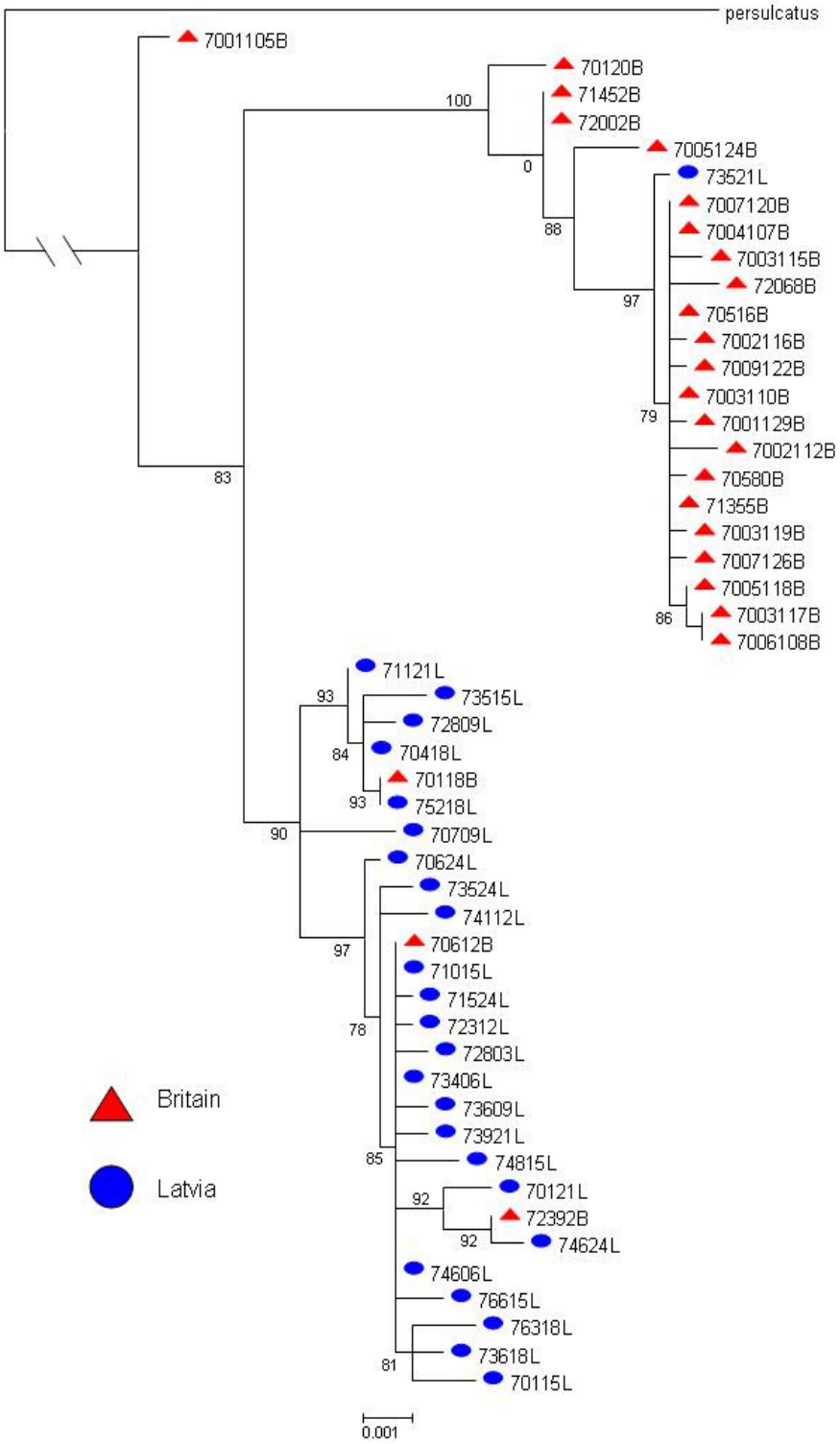

Figure 21: The evolutionary history of six mitochondrial genes (*atp6*, *coi*, *coii*, *coiii*, *12s* and *cytB*) concatenated from *I. ricinus* from Britain and Latvia in 2007 was inferred using PhyML maximum likelihood method. The optimal tree with the sum of branch length = 0.32023032 is shown. The dataset was out-grouped using *I. persulcatus*. There were a total of 3212 positions in the final dataset. The scale bar indicates 0.1% divergence. The branch of the outgroup is not to scale (indicated by slashes).

3.4.2 Population structure of British and Latvian I. ricinus

For the mtMLST analyses, 50 samples, 25 samples from various sites around Britain and 25 samples around Latvia were used (Appendix - Tick Samples used for the Comparison of British and Latvian Populations in section 3.4, p. 198) showing 43 unique sequence types (STs). Using these allelic profiles, a goeBURST analysis (Francisco et al., 2009) (Figure 22) shows the relationship of all the profiles up to a level of similarity of three loci.

The analysis shows two main clades (CC-B, CC-L) and three minor clades (CC-M1, CC-M2, CC-M3). The two major clades have samples from distinct locations, with each clade containing the majority of each separate population from Britain (CC-B) and Latvia (CC-L). Four samples from the dataset differed from the remaining samples by more than three loci, known as singletons. None of the singletons was represented by more than one sample. Three STs are made up of two or more samples and are all of solely British origin (ST88, ST144, and ST324) (Figure 22). All Latvian sequence types constitute only one sample. ST88 was the most common isolate with four samples contributing to this ST.

Only two STs are shared between the two populations (ST24 and ST106). The lack of shared STs between the two populations could have arisen through low migration rates between these two regions or through high migration rates of ticks with low survival to reproductive age. An F_{ST} value calculated in Arlequin 3.1 (Excoffier *et al.*, 2005) provided corroboration for this statement with a significant value of 0.57732 (p=0.0000), a value which indicates a high level of genetic differentiation (Freeland, 2005).

The clustering of the populations is distinct with a single large cluster for the British population and two clusters for the Latvian population. ST88 was found in four different ticks and is the founder of the British population with most of the other British STs related to this ST. The Latvian population has a greater number of STs and clusters, indicating a greater diversity in the population. The Latvian population forms two distinct clusters with most STs occurring in one or two individuals, which is in contrast to the British population where ST88 has four individuals with this ST, which is 16% of the 25 sample size and representative of the environmental population.

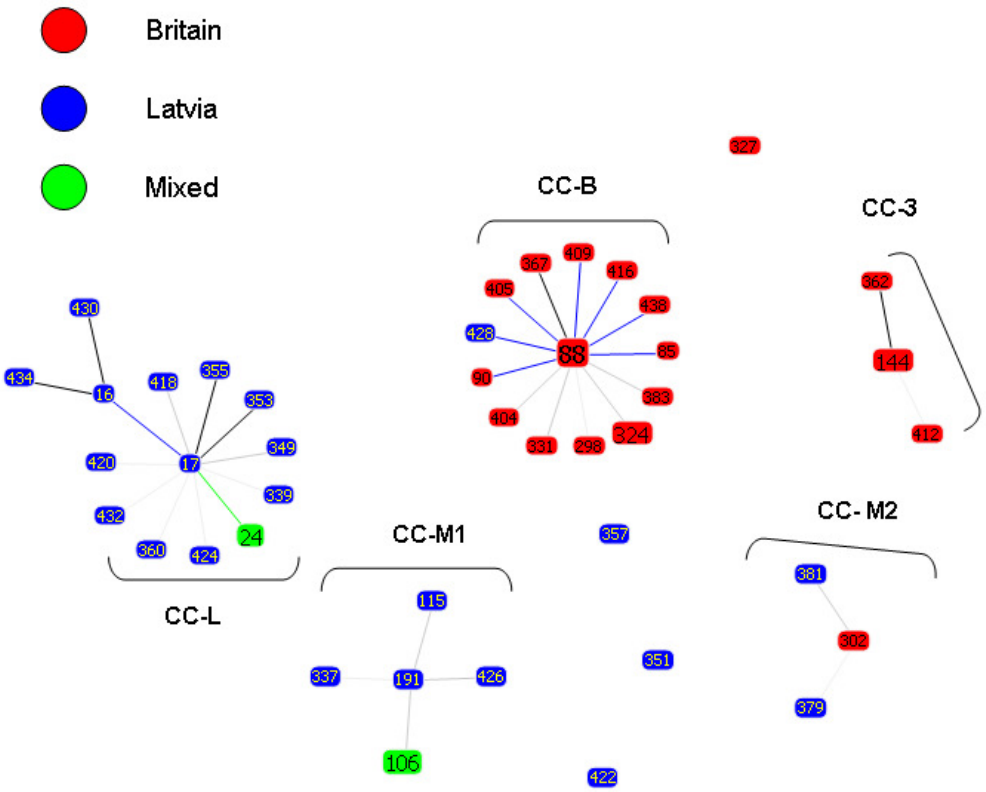

Figure 22: A goeBURST analysis for *I. ricinus* ticks collected in Britain (red) and Latvia (blue) in 2007, showing mixed STs (i.e. ST that were found both countries) in green.

3.5 Discussion

I have developed a novel system for the typing of *I. ricinus* based on MLST principles using mitochondrial genes to analyse the population structure of this disease vector. As proof-of-principle, *I. ricinus* ticks from Britain and Latvia were analysed using this scheme and the examined for signs of geographical clustering. Using this novel application of MLST, we have shown that two *I. ricinus* populations from discrete geographic locations can de distinguished as divergent lineages.

Ixodes ricinus is an important vector of bacterial and viral infections throughout Europe and is monitored closely (Pietzsch et al., 2008). Our understanding of where ticks have established populations may allow us to infer from which part of the distribution range of *I. ricinus* they have been introduced. A reproducible molecular method to delineate geographically distinct populations may help in identifying emerging populations and assess risks associated with the introduction of ticks from other countries.

The British and Latvian *I. ricinus* ticks differ from each other in more than three loci in most cases apart from only two STs, which were noted in both locations. The genetic differentiation of the British and Latvian populations reflects their physical separation, both in terms of geographical distance (approximately 1700 km) and physical barriers. There is evidence that immature tick stages can be carried long distances by migratory or part migratory birds (Ogden *et al*., 2008; Pietzsch *et al*., 2008), and these are probably the host species regularly able to introduce new ticks into Britain. However the limited number of putative migrants noted in the current study suggests that even when ticks are introduced, they rarely go on to establish new populations. This may be because the survival rate of each tick stage is poor, with 90% of a generation failing to reach the next developmental stage (Randolph *et al.*, 2002). Considering these survival rates, introductions of ticks from the continent to Britain would typically fail. Only in rare circumstances would an introduced tick survive to reproductive age. Randolph *et al.* (2002) estimate that the survival of only 10 adult ticks would on average require an introduction of 1,000 larvae.

The likelihood of successful transmission of a mitochondrial ST to a new region would also be halved as, in the majority of cases, only females are able to impart their genetic material to the next generation (Breton et al., 2007). Bi-parental inheritance of mitochondria is rare in many species and unproven in ticks, and unlikely to influence the result significantly (Dimauro & Davidzon, 2005). Therefore, a male tick surviving to reproductive age would not affect the mitochondrial genetic structure of the future

population. The study of nuclear genes could possibly allow for the study of this phenomenon but high levels of recombination of nuclear genes could make this challenging to reach meaningful conclusions about the descent of the genes studied.

Many studies have hypothesised that differences in the genetic structure of organisms from regions in Europe are due to effects of ice ages. Certainly, with ticks from across Europe it is convenient to assume that differences seen in the genetic patterns of our dataset is due to an ancestral population originating sometime during the Last Glacial Maximum approximately 12,000 years ago when Britain was joined to the continent (Mix *et al.*, 2001; Searle *et al.*, 2009; Ruiz-Fons & Gilbert, 2010). As deer are able to carry large numbers of ticks (Scharlemann *et al.*, 2008) it is possible that freely migrating individuals could have introduced a small population of ticks to the now isolated region called the British Isles.

Another possibility that could explain the patterns we have seen in our data are migratory birds. Many different bird species are known to carry *I. ricinus* ticks, many of whom are migratory passerines (Comstedt *et al.*, 2006). One of the most common migrants discussed by Comstedt *et al.* (2006) is the European Robin (*Erithacus rubecula*). Most of the Robins hatched in the UK and Ireland spend their lives within a kilometre of their natal site (Wernham *et al.*, 2002). Recovery exercises of this species in the UK from 1990 to 1997 showed 2.3% of the birds captured were from foreign countries. When the majority of bird migration from the UK is occurring in October (Taylor, 1984), nymphal numbers are declining (reviewed in Kurtenbach *et al.*, 2006) the impact of bird migration on the dispersal of *I. ricinus* may in fact be rather limited.

The phylogenetic analyses of concatenated mitochondrial genes showed markedly different clades for the Latvian and British ticks with some of the British specific ticks occurring in the Latvian clade and vice versa. The distinct differences between these two clades may be an indicator of distinct species of *I. ricinus* ticks that occur in these remote geographic locations. The ingression of the few British ticks in the Latvian clade and the Latvian in the British may be representative of rare occurrences of these species in the contrasting populations. The British Isles may represent a cryptic species of *I. ricinus* and would be a factor that required further investigation in the future in order to determine its validity.

We have tested the hypothesis that two geographically distinct populations of *I. ricinus* would have discrete allele frequencies and STs. The mtMLST scheme applied has provided evidence that tick populations from these two regions are distinct. It now needs to be applied to *I. ricinus* ticks across Europe to see whether it has the potential

to be typed according to origin. This finding has exciting applications, as it will now be possible to study the population dynamics of *I. ricinus* populations across Europe in a spatial and temporal manner. This scheme may also be able to discriminate tick populations with different ecotypes, e.g. ticks infected with different species of *Borrelia* to study the propensity of certain ticks to feed on particular animals depending on the resolution of the scheme. Temporal studies of tick populations within countries may also provide an insight into the level of mixing between populations and the rate of mutation within these populations.

3.6 Conclusions

In this chapter, we have provided evidence that the mtMLST scheme designed using mitochondrial genes for *I. ricinus* is able to determine geographically distinct lineages. Two hypotheses have emerged from the data. Firstly, deer could have introduced *I. ricinus* ticks to the region that was to become the British Isles during the last ice age, before the sea-levels rose and cut off Britain from the rest of Europe. Secondly, migratory birds could have introduced ticks from Continental Europe in large enough numbers in order for one individual to survive to reproductive age and create a new focus of an ST. Either could explain the isolation of STs seen in the British population although further analyses of Continental European ticks would be required. The British Isles may represent a niche that is occupied by a cryptic *I. ricinus* species. This use of mtMLST would be a useful tool to study the phylogeography of populations of *I. ricinus* and could be applied to other vector organisms.

4 Temporal studies of tick populations in Britain and Latvia

The temporal structure of *I. ricinus* populations from Latvia and Britain over a period of several years is examined in this chapter. These investigations aim to understand whether temporal fluctuations of genetic profiles occur in tick populations. In chapter 3 I have shown that *I. ricinus* ticks from Latvia form two genetically distinct populations. Since samples from one year were chosen, this may only be a 'snapshot' of a genetic diversity that may fluctuate temporally. The high degree of mortality between developmental stages of ticks may play a significant role in shaping the populations. The migration of ticks on hosts may also play a role in the movement of STs between the two populations. The migration of ticks between populations also has implications for disease dynamics of bacterial and viral diseases harboured by ticks.

4.1 Introduction

The *I. ricinus* species complex is found in most temperate zones of Europe in many parts of the Western Palaearctic (Hillyard, 1996), and as we have shown previously, within the *I. ricinus* species there is significant genetic variability, which could determine the geographic origin of a specific individual.

Ixodes ricinus ticks are considered mostly sedentary and able to move on their own over only short distances (Eisen & Lane, 2002) and therefore depend on vertebrate hosts for wide range dispersal (Ruiz-Fons & Gilbert, 2010). *I. ricinus* ticks as they mature tend to feed on larger animals, with larvae and nymphs tending to feed on small mammals and rodents and adults feeding on deer (Gilbert *et al.*, 2000; Ruiz-Fons *et al.*, 2006). The extent to which ticks migrate between geographic regions by feeding on these various hosts is difficult to estimate (Stone *et al.*, 1997; Pietzsch *et al.*, 2008).

The most effective method to determine the movement of tick populations is to study the population genetically. A temporal analysis of the genetic structure of the population can be performed assessing the change in the STs found year on year and relating this to other locations around Europe. We have previously shown that *I. ricinus* populations from Latvia and the UK can be distinguished from each other using an mtMLST method (Chapter 3: A Multilocus Sequence Typing Scheme for Ticks:

Rationale & Design) and could therefore help to determine the structure of a population within a specific geographic region e.g. a country.

Since 6,000BC, when a land bridge named Doggerland connected Continental Europe and what is now the British Isles (Stride, 1959), *I. ricinus* populations on the British Isles have been isolated from Continental European populations. With the English Channel acting as a significant barrier to the movement of land animals, the opportunities to move significant numbers of ticks from Continental Europe to Britain are much less than the probability of the movement of ticks one area of Continental Europe to other land linked regions. Migrant ticks in the British Isles would most likely come from birds as they can easily move great distances and are not restricted by the English Channel (Wernham *et al.*, 2002; Pietzsch *et al.*, 2008). *I. ricinus* populations in Continental Europe are isolated marginally from each other by distance, mountain ranges and bodies of water e.g. rivers and lakes depending on their ability to swim etc. Despite these isolating factors, we would expect that a population in the UK would be more isolated from populations within Continental Europe than these are from each other.

We have collected ticks from Latvia and from the UK to examine the genetic variation of the populations over several years. Looking at the population dynamics in one particular geographic area over several years enables the determination of population stability and the level of population change. Populations from two separate geographical locations may exchange genetic material through the migration of hosts that carry ticks. If these ticks reach maturity and survive the 90% death rate between each generation (Randolph et al., 2002) the genetic signal from their geographic region will be detected in the sample collected from the environment. Monitoring the populations from separate locations may allow us to develop hypotheses on factors important for range expansion and spread of *I. ricinus*.

The movement of ticks around Europe has important implications for disease dynamics (Pietzsch *et al.*, 2008). The British Isles are currently free from TBE, a serious encephalitic disease encountered in Continental Europe (Zeman & Bene, 2004). Monitoring the threat from migratory ticks would be an important epidemiological tool for health authorities for health initiatives (Health Protection Agency, 2010b). Tick populations could be monitored for Continental European-like STs as an early warning for the emergence of diseases in the British Isles.

4.1.1 Aims and Objectives

The aims of this chapter can be described as follows:

- assess the genetic variation within British and Latvian populations over several years
- determine the population dynamics of the British and Latvian populations in terms of tick and host migration

4.2 Temporal studies of *Ixodes ricinus* from Latvia

The variation in the Latvian population of *I. ricinus* ticks over several years is investigated in this section. Latvia is bordered by several countries and is therefore open to migration of ticks on all known hosts of ticks (e.g. birds, mice, deer etc). It is unknown how much variation there is in the Latvian population and how stable it is from one year to the next. Looking at the variation of several mitochondrial genes in successive years may enable us to detect population dynamics in this area, which is open to migration of both ticks and host. This could have important implications for the spread of tick borne diseases.

4.2.1 Phylogenetic analyses of three Latvian populations of I. ricinus *from 2002 to 2007*

I. ricinus nymphs from Latvia were collected from Babite, Jaunciems, Jurmala and Tireli (collection sites for this analysis shown in Figure 23 with all Latvian collection sites shown in Appendix - Collection Site Maps, p. 167) surrounding Riga, over several years, and were analysed according to the method indicated in the mtMLST scheme previously described (Chapter 3: A Multilocus Sequence Typing Scheme for Ticks: Rationale & Design). Tick samples from three years were used in these analyses with 35 samples from 2002, 104 from 2006 and 49 from 2007. These figures are summarised in Table 6. The unabridged version of this dataset is shown in an appendix (Appendix - Tick Samples used for the Comparison of Latvian populations in 2002, 2006 and 2007 in section 4.2.1, p. 201). Only nymphs were used in these analyses to ensure that ticks were from the same generation. Concatenated sequences of six mitochondrial genes (*atp6*, *coi*, *coii*, *coiii*, *cytB* and *12s*) were aligned using MEGA 4.1 and edited by hand to minimise gaps. Phylogenies were rooted with *I. persulcatus* sequences from GenBank sequence NC_002010. Sequences used as outgroup to root the phylogeny were trimmed according to the corresponding area of analysis for each gene.

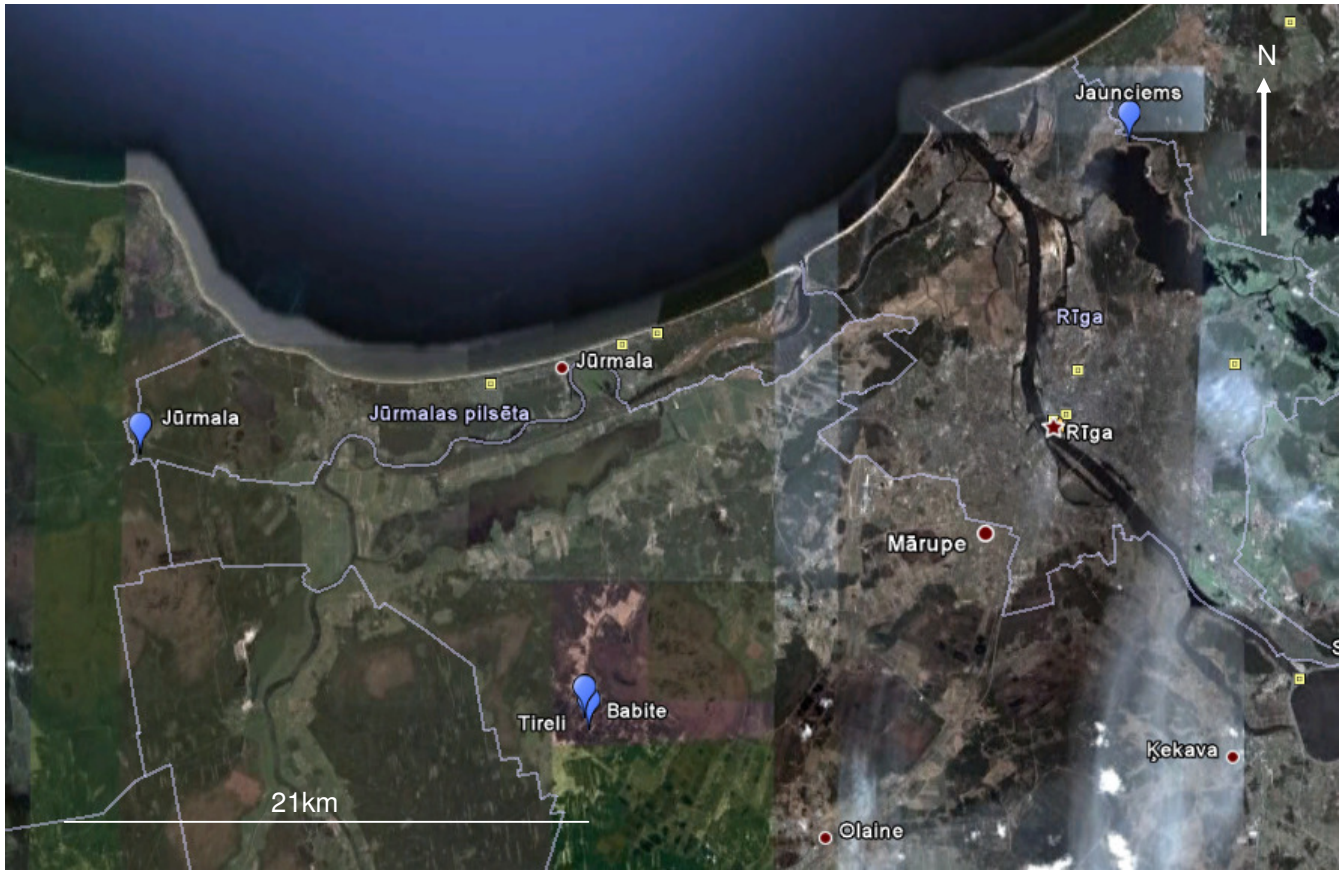

Figure 23: Collection locations around Riga, Latvia for *I. ricinus* ticks collected in 2002, 2006 and 2007. Map generated from Google Earth (Google, 2010).

Table 6: A summary of Latvian ticks collected in 2002, 2006 and 2007

Year	Collection Location	Number of ticks
2002	Babite	4
2006	Babite	34
2002	Jaunciems	17
2006	Jaunciems	20
2007	Jaunciems	14
2002	Jurmala	14
2006	Jurmala	26
2007	Jurmala	17

A maximum likelihood phylogeny was constructed using PhyML (Guindon et al., 2005) (Figure 24). The majority of the deep branches are well supported with good aLRT values above 75. Throughout the tree, there is no clear separation of the three year groups of ticks. There are no homogenous clades of ticks from one particular year. Many of the smaller clades show that the related STs were found at all three years. In no part of the tree is there evidence of a succession of years with a marked amount of genetic change from one year to the next.

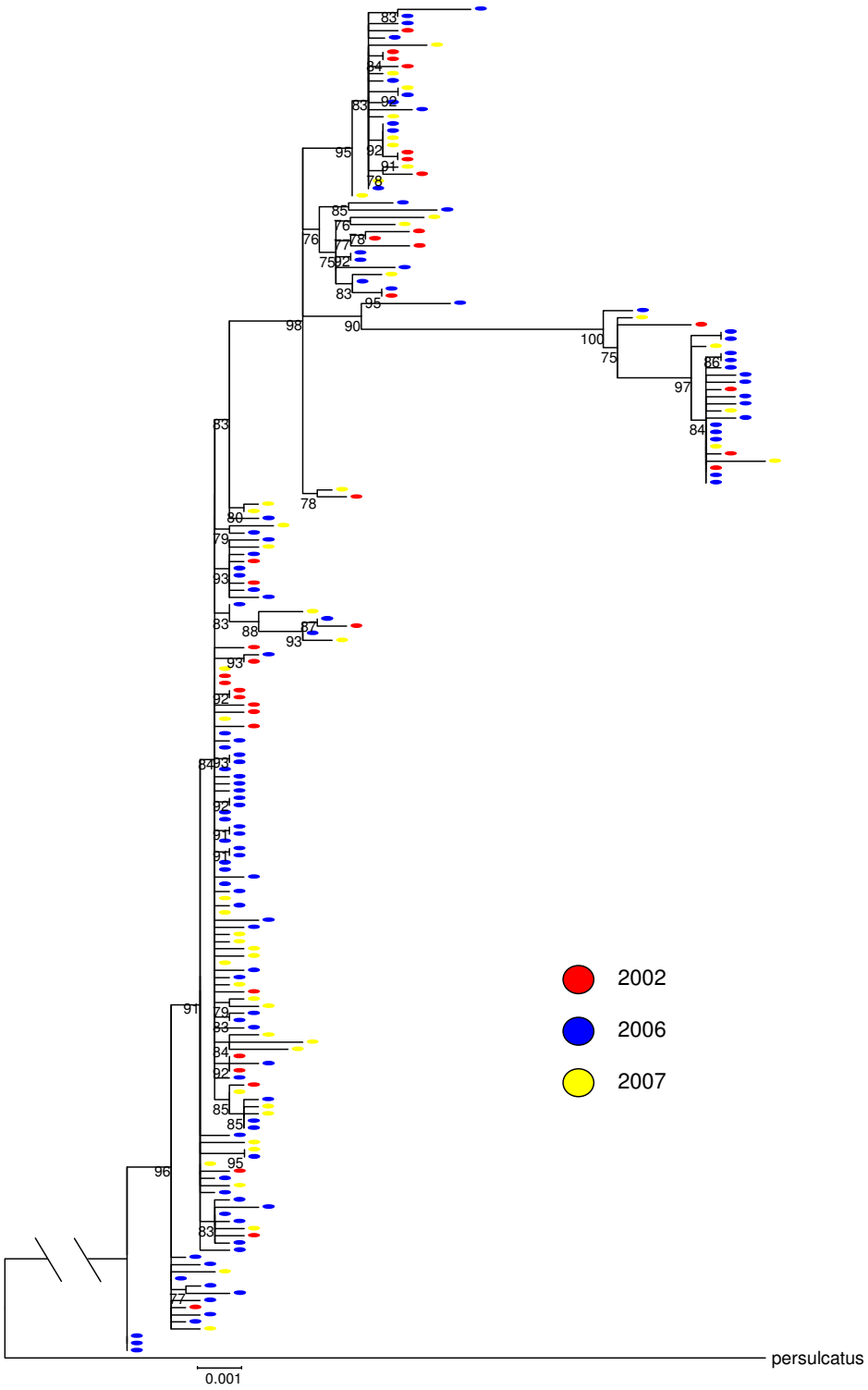

Figure 24: The evolutionary history of Latvian *I. ricinus* populations over three years (2002, 2006 and 2007) was inferred using a concatenation of six mitochondrial genes and using PhyML maximum likelihood method. The optimal tree with the sum of branch length = 0.32023032 is shown. The dataset was out-grouped using *I. persulcatus*. Taxon labels were deleted. There were a total of 3213 positions in the final dataset. The scale bar indicates 0.1% divergence.

4.2.2 Population structure

For the mtMLST analyses, ticks from (35 in 2002, 104 in 2006 and 49 in 2007) various sites in Latvia (Appendix - Tick Samples used for the Comparison of Latvian populations in 2002, 2006 and 2007 in section 4.2.1, p. 201) were studied. An analysis of the genetic variation within the samples showed 155 unique STs. Using these allelic profiles, a goeBURST analysis (Francisco *et al*., 2009)(Figure 25) shows the relationship of all the profiles up to a level of similarity of three loci.

The analysis shows one main clonal complex (CC-1) and four minor clonal complexes (CC-2, CC-3, CC-4 and CC-5). The main clonal complex consists of samples from all of the years sampled. The minor clonal complexes also showed a similar pattern with most showing samples from each year. CC-4 shows a mixed node, which contains a sample from both 2002 and 2006. CC-5 is a very small complex but still has a sample from two different years. Eleven STs from the dataset differed from the remaining STs by more than three loci, otherwise known as singletons. None of the singletons were represented by more than one sample. Nine STs are represented by more than one year (indicated as green in Figure 25). The most common ST was ST17 with seven samples representing this ST.

Table 7: F_{ST} values calculated in Arlequin for Latvian *I. ricinus* populations. P values for the statistics are shown in parentheses. Significant values are shown in bold.

	2002	**2006**	**2007**
2002	-	-	-
2006	0.00133 (0.30492)	-	-
2007	-0.01428 (0.94545)	-0.00006 (0.35561)	-

F_{ST} values were calculated in Arlequin 3.1 (Excoffier et al., 2005). Molecular distances were calculated with pairwise differences. 10,000 bootstrap replicates were performed. The results are shown in Table 7. F_{ST} values calculated between populations of *I. ricinus* from the three years indicated little genetic differentiation but no significant values.

These data suggests there is no distinct clustering of the three tick groups from the individual years. Even within the smaller clonal complexes, we see a mixture of STs from the individual years. The majority of STs in this analysis are represented by only one sample with a small minority of STs from several years. The samples found in several years are more common than homogenous STs from a single year are when

more than one sample is found for an ST. Around these common STs are large numbers of single, double and triple locus variants that are found in only one year. This structure would suggest a core population of common STs, from which each year variants arise and are removed from the population through the bottleneck of the death rate of ticks between generations (Randolph et al., 2002).

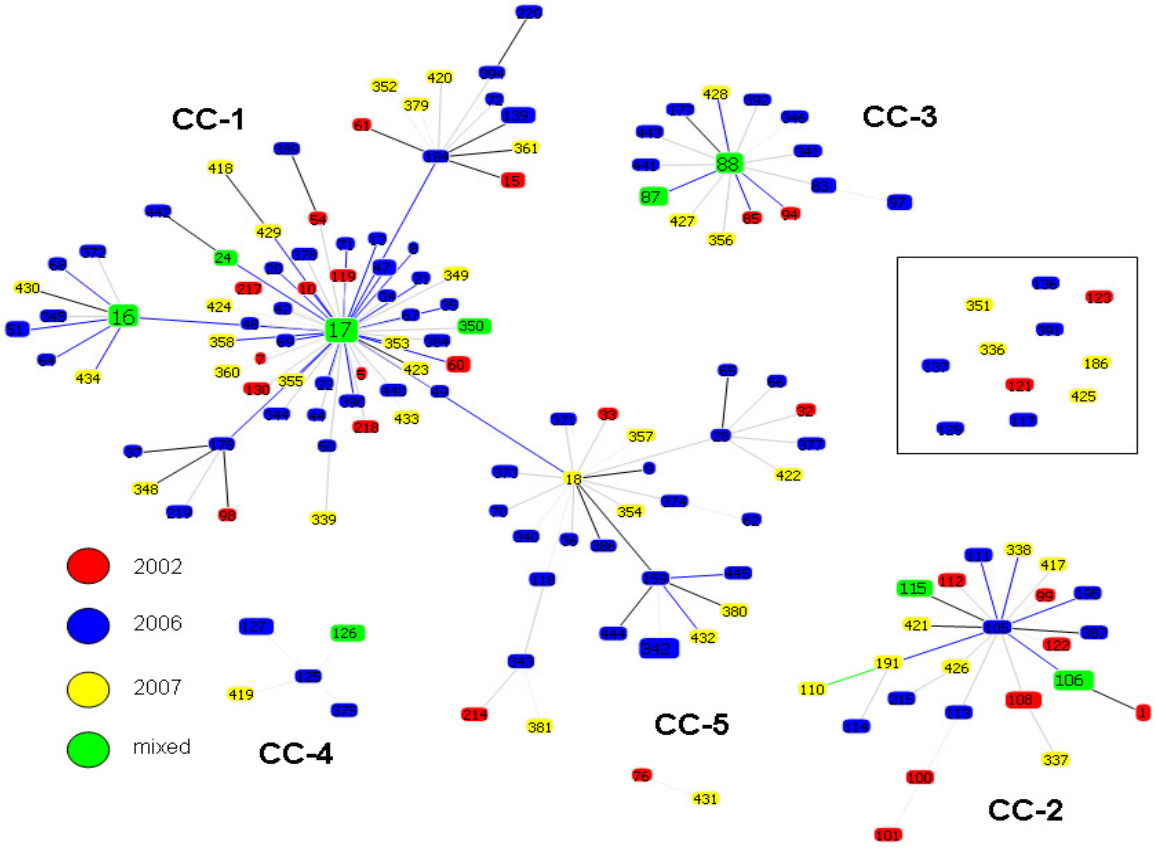

Figure 25: A goeBURST analysis for *I. ricinus* ticks collected in from three sites in Latvia. Mixed nodes are shown in green and indicate STs that were found in more than one year but can be mixes of any two or three years. Boxed STs show singletons.

4.2.3 Population statistics

Mismatch distribution statistics calculated for the Latvian populations were examined for evidence of population expansions. Mismatch distribution analyses in DNAsp computed distributions based on constant and expanding populations and compared to observed relative frequencies. Coalescent simulation analyses generated RI statistics and corresponding p-values. Mismatch distribution analyses in Arlequin compared observed distributions of pairwise differences with spatial and demographic models of population expansion. Fu's Fs statistics were also calculated for each population. Population analysis statistics are shown in Table 8. For a description of the methods used to compute these statistics and the theory behind the analyses, please refer to section 2.2.7, p. 26.

Mismatch distribution analyses were calculated for Latvian samples in Arlequin 3.1 from 2002 (Figure 28), 2006 (Figure 29) and 2007 (Figure 30) and also for combined Latvian populations in DNAsp (Figure 27) comparing constant and changing population models and Arlequin 3.1 (Figure 26) comparing spatial and demographic models of expansion. The distribution analyses data for the graph generation can be found in the following appendices: Mismatch distribution analyses data for Latvia combined populations (DNAsp), p. 216, Mismatch distribution analyses data for Latvia combined populations (Arlequin), p. 217, Mismatch distribution analyses data for 2002 Latvia population (Arlequin), p. 219, Mismatch distribution analyses data for 2006 Latvia population (Arlequin), p. 220, Mismatch distribution analyses data for 2007 Latvia population (Arlequin), p. 221.

For the combined populations from all years I computed a mismatch distribution analysis in DNAsp with a mean of 13 mismatches and the plot tends towards a decreasing proportion of mismatches with increasing number of pairwise differences (Figure 27). A significant p-value allows us to reject the null hypothesis of an expanding population and accept the stable nonexpanding population model. Looking at the plot of the distribution (Figure 27) shows a trend similar to that of the constant population model.

Mismatch analyses were performed in Arlequin 3.1 comparing the observed distribution to models of spatial and demographic expansion. The observed distributions produced non-significant RI statistics disallowing us to reject the null hypothesis of a stable and nonexpanding population although taken with the significant result in the DNAsp analysis it would seem that the data is not comprehensive enough to support the

models generated in the Arlequin analyses. A significant sum of squared deviations (SSD) value (0.151) for the demographic expansion model is several orders of magnitude higher than the other SSD values alluding to a poor fit of the observed data to the model.

The populations were examined individually according to the year of collection. Observed distributions were compared with models of distribution according to spatial and demographic expansions in Arlequin 3.1. Initial and final values of θ calculated in Arlequin for the compartmentalised populations produced similar values from approximately $\theta_0 = 13$ to $\theta_1 = \infty$ for demographic expansion model and similar initial theta values for the spatial expansion models. All RI statistics computed for the individual populations are non-significant suggesting that the populations are not stable and may be expanding but there is not enough data to infer the model to which the data is best fitted.

The Fs statistics for all populations, combined and split into yearly-designated populations, all showed significant negative values indicating that an expansion of the population in Latvia may have previously occurred.

Table 8: Mismatch analyses statistics for Latvian populations. P-values for SSD, RI and Fs are shown in parentheses with statistically significant values highlighted in red.

Population Name	Model	Mismatch observed mean	Mismatch observed variance	τ	θ_0	θ_1	SSD (p-value)	RI (p-value)	Fs (p-value)
Latvia	constant	13.276	112.785	3.300	9.9750	∞	na	0.004(0.0130)	-23.886 (0.0020)
Latvia	changing							0.004(0.0130)	
Latvia	demographic	14.734	131.892	2.363	0.0000	99999.0	0.151(0.0000)	0.003(1.0000)	
Latvia	spatial			2.362	12.239	constant	0.009(0.6441)	0.003(0.6392)	
Latvia 2002	demographic	15.197	116.923	2.309	15.516	99999.0	0.020(0.3372)	0.011(0.2331)	-11.077 (0.0040)
Latvia 2002	spatial			2.312	15.507	constant	0.020(0.2318)	0.011(0.2385)	
Latvia 2006	demographic	14.917	146.525	2.199	11.354	99999.0	0.011(0.5413)	0.004(0.6031)	-24.098 (0.0010)
Latvia 2006	spatial			2.199	11.354	constant	0.011(0.5385)	0.004(0.6002)	
Latvia 2007	demographic	14.076	111.125	3.111	11.241	99999.0	0.005(0.7535)	0.003(0.9575)	-24.330 (0.0000)
Latvia 2007	spatial			3.112	11.241	constant	0.005(0.8063)	0.003(0.9544)	

τ: time in mutational events since the modelled expansion event

RI: raggedness index

SSD: sum of squared deviations

Fs: Fu's Fs test

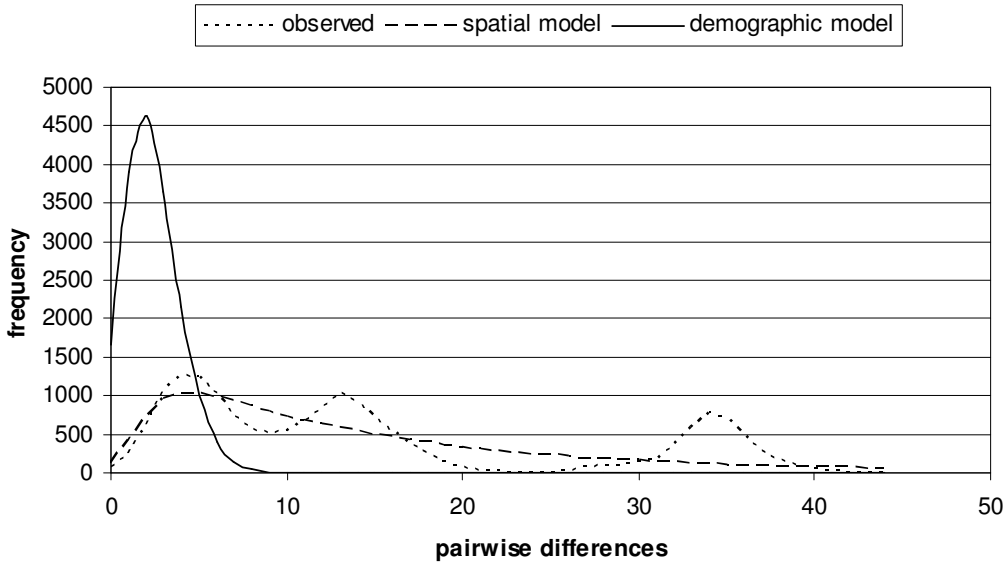

Figure 26: Mismatch analysis of Latvian samples in Arlequin

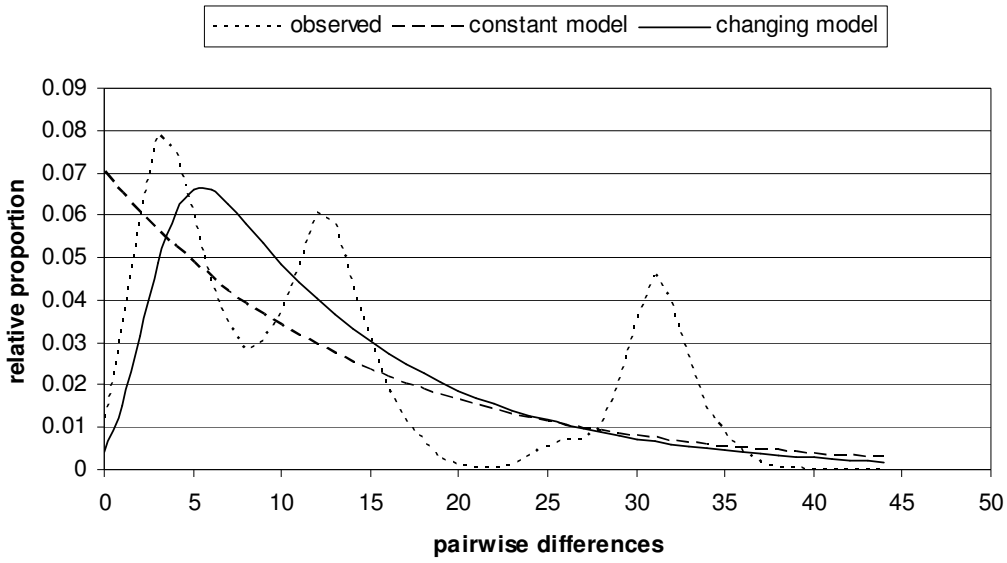

Figure 27: Mismatch analysis of Latvian samples in DNAsp

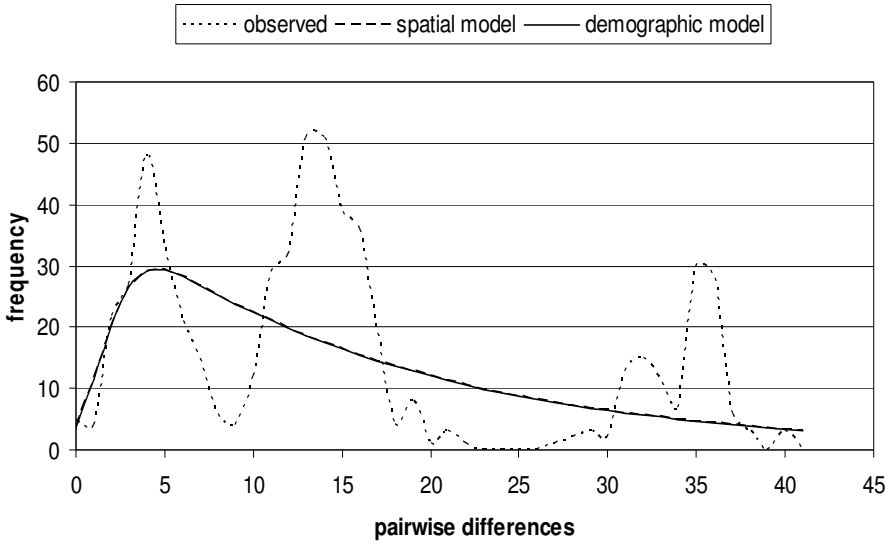

Figure 28: Mismatch analysis of 2002 Latvian samples in Arlequin

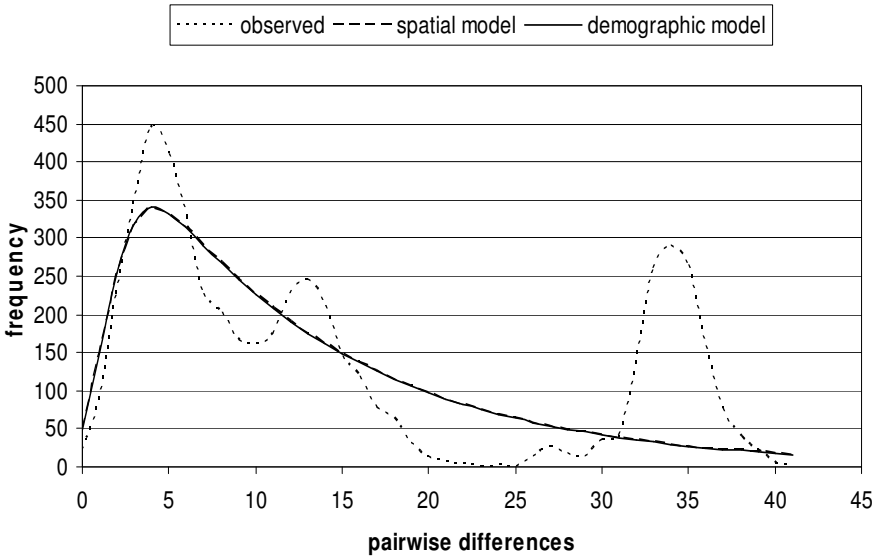

Figure 29: Mismatch analysis of 2006 Latvian samples in Arlequin

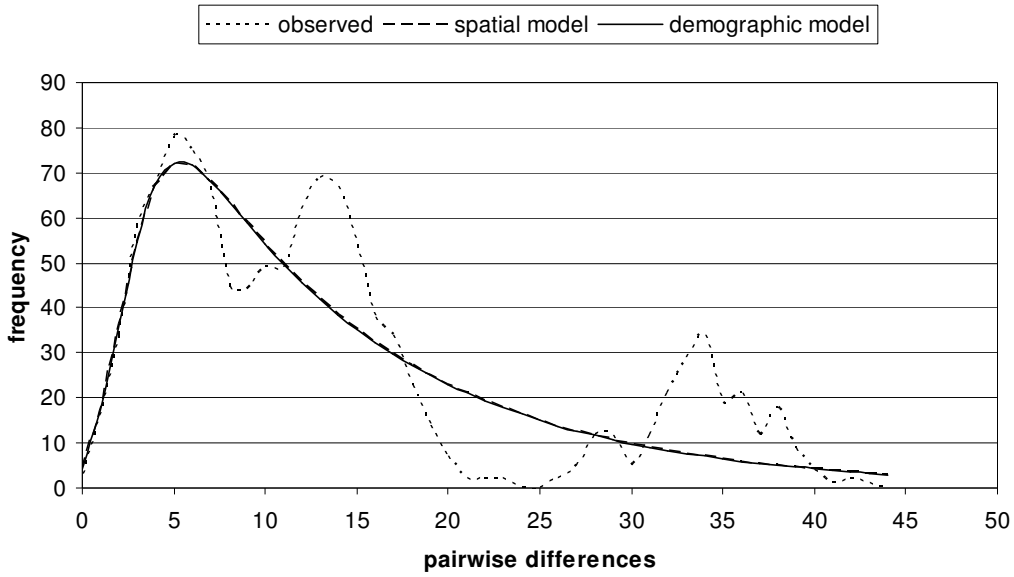

Figure 30: Mismatch analysis of 2007 Latvian samples in Arlequin

4.2.4 Latvian analyses summary

The Latvian populations from 2002 to 2007 have been shown to have no contiguous identical populations from one year to the next with different STs arising each year, but with only a small amount of variation. This could be a result of several possibilities. Firstly, there could be a high level of migration of ticks into and from the collection areas from other regions of Europe with a large number of STs not previously analysed. Secondly, the large number of different alleles could be a result of sampling bias though sampling ticks from different generations. *I. ricinus* ticks take on average two to three years to progress from egg to reproducing adult depending on environmental conditions (Hillyard, 1996). Therefore, ticks that are adults in one year will not breed with ticks from the next year. This may lead to several subpopulations within a geographical region resulting from non-interaction between ticks from different generations.

The goeBURST analysis confirmed the amorphous population in Latvia showing little or no crossover of STs found from one year to the next. The number of STs found was also very high suggesting that much larger samples sizes are needed to confirm the true diversity of the Latvian population (an additional analysis is shown in the discussion of this chapter referring to sample size). These findings are similar to findings that have been previously discussed (Delaye *et al*., 1997; Casati *et al*., 2007) with little structuring of the population in Continental Europe.

Analysing the structure of the population through mismatch distributions has suggested a currently stable population that had experienced an expansion in the past. Despite the estimation of τ, which estimates the time in mutational events since the modelled expansion event, it is difficult to determine the time in real terms since the mutational rate of mitochondrial DNA has not been determined for ticks. Mutation rates have been determined for *Caenorhabditis elegans* (Denver *et al*., 2000) at 8.9 mutations per site per million years but this may be significantly different for *Ixodes* species. Using this estimate of mutation for mitochondrial genes and using estimates of τ (the number of mutations events since expansion) we can estimate time since expansion using the relationship of $\tau=2\mu t$ where μ is the mutation rate per nucleotide per year and t is related to the time since expansion. Using this relationship, we can estimate time since expansion at approximately 130,000-400,000 years ago. These estimates indicate that the expansion event occurred before the LGM 20,000 years ago (Mix *et al*., 2001).

Until the true mutational rate of mitochondria in *Ixodes* ticks is resolved, we can only hypothesise about the population dynamics.

4.3 Temporal studies of *Ixodes ricinus* from Britain

Britain is surrounded by open water and is isolated from the rest of Continental Europe leading to significant problems for migration of ticks through ground habiting hosts of ticks. Looking at the variation of the STs of the mitochondrial genome in successive years may be able to show the population dynamics in the area, which could be more isolated from Continental Europe than land-linked countries in Europe. This section investigates the variation in the British population of *I. ricinus* ticks over several years.

4.3.1 Phylogenetic analyses of three British Ixodes ricinus *populations from 2006 to 2008*

I. ricinus nymphs from Britain were collected from the American museum, Bathampton Woods, Eastwood, Rainbow Woods, Thurlbear Woods, Warleigh, Widcombe and Winsley (collection sites for this analysis shown in Figure 31 and Figure 32 with all Latvian collection sites shown in Appendix - Collection Site Maps, p. 167).

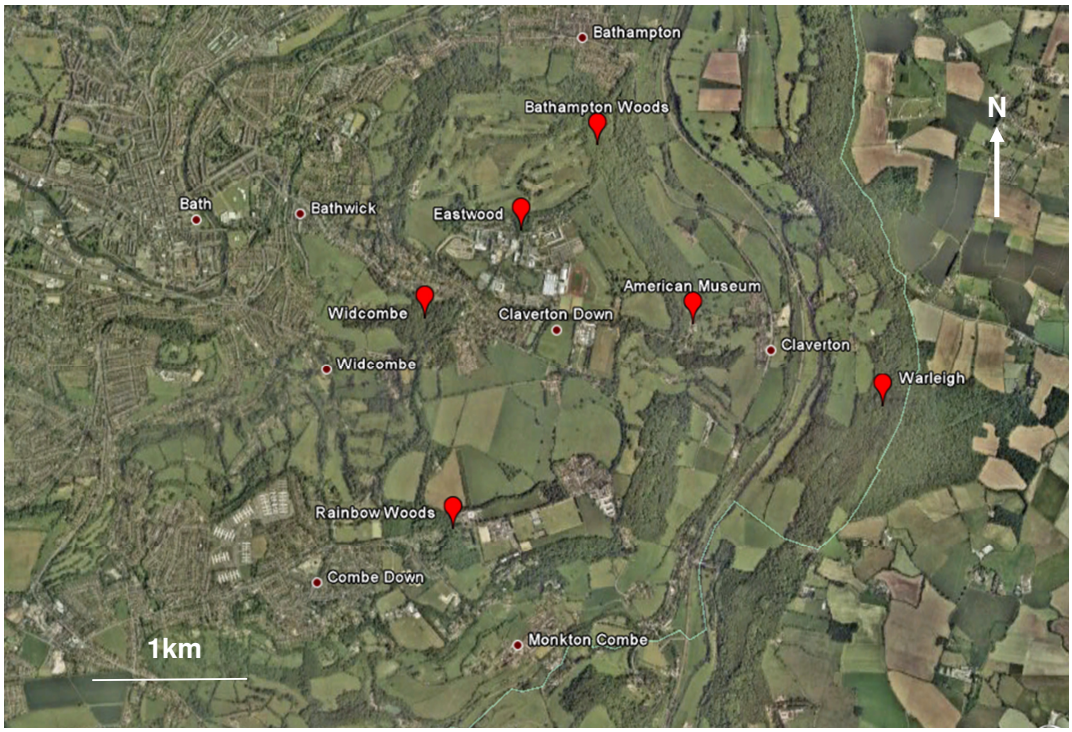

Figure 31: Collection locations around Bath, Somerset for *I. ricinus* ticks collected in 2006, 2007 and 2008. Map generated from Google Earth (Google, 2010).

Figure 32: Collection location of Thurlbear Woods for *I. ricinus* tick in 2006. Map generated from Google Earth (Google, 2010).

Tick samples were analysed according the method indicated in the mtMLST scheme previously described (Chapter 3: A Multilocus Sequence Typing Scheme for Ticks: Rationale & Design). Tick samples from three years (2006, 2007 and 2008) were used in these analyses with 83 samples from 2006, 41 from 2007 and 38 from 2008. These collections are summarised in Table 9 according to collection site and year with the full dataset information displayed in the appendices (Appendix - Tick Samples used for Comparison of British populations in 2006, 2007 and 2008 in section 4.3.1, p. 211). Only nymphs were used in these analyses. Concatenated sequences of six mitochondrial internal gene fragments (*atp6*, *coi*, *coii*, *coiii*, *cytB* and *12s*) were aligned using MEGA 4.1 and edited by hand to minimise gaps. Phylogenies were rooted with *I. persulcatus* sequences from GenBank (accession NC_002010). Sequences used as outgroup to root the phylogeny were trimmed according to the corresponding area of analysis for each gene.

Table 9: A summary of ticks collected from Britain in 2006, 2007 and 2008

Year	Collection Location	Number of ticks
2006	American Museum	6
2006	Bathampton Woods	6
2007	Bathampton Woods	19
2008	Bathampton Woods	15
2007	Eastwood	4
2008	Eastwood	6
2008	Rainbow Woods	7
2006	Thurlbear Woods	1
2006	Warleigh	1
2007	Warleigh	5
2006	Widcombe	69
2007	Widcombe	12
2008	Widcombe	10
2007	Winsley	1

A maximum likelihood phylogeny was constructed using PhyML (Guindon et al., 2005) (Figure 33). The majority of the deep branches are well supported with good aLRT values above 80. Within the tree, there is no clear separation of a tick population from

one year to the next. Genetic differentiation of mitochondrial genes within a period of less than ten years would be unexpected due previous estimates of mitochondrial mutation rates of 8.9 mutations per site per million years (Denver *et al.*, 2000).

Some clustering of ticks from 2006 onto individual branches was observed and this is indicated by the markers A, B and C in Figure 33. The majority of samples from these three clusters were found to be collected from a single location in Bath at different times throughout the collection season. All but one of the ticks found within these clusters were collected from Widcombe, an area of woodland near the University of Bath. This should not come as a surprise as the majority of ticks collected in 2006 were from this collection location (Figure 35). Many of the clades indicate the presence of a particular ST over the three years of analysis but no evidence of succession can be seen in the phylogenetic tree.

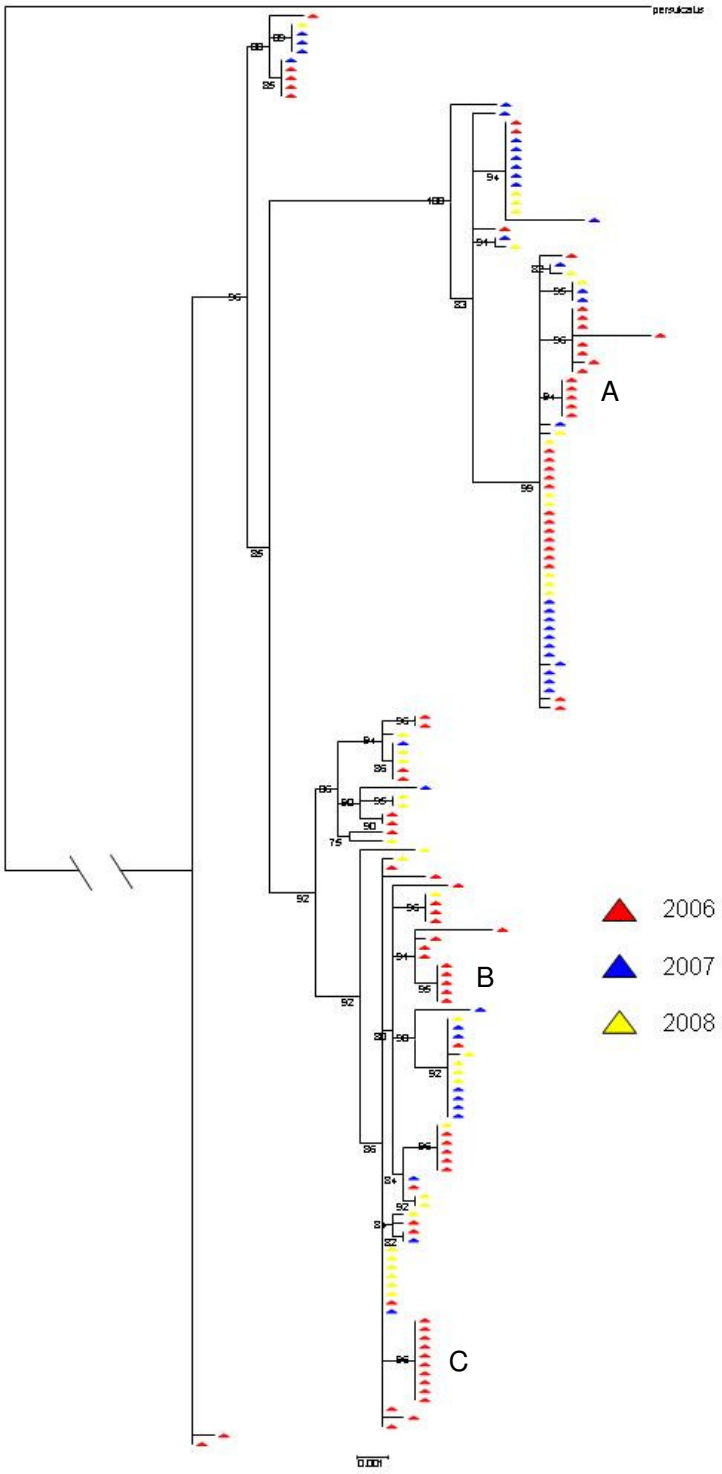

Figure 33: The evolutionary history of British *I. ricinus* populations over three years (2006-2008) was inferred using a concatenation of six mitochondrial genes and using PhyML maximum likelihood method. The optimal tree with the sum of branch length = 0.33173883 is shown. The dataset was outgrouped using *I. persulcatus*. Taxon labels were deleted. There were 3213 positions in the final dataset. Markers A, B and C indicate homogenous population clusters. The scale bar indicates 0.1% divergence.

4.3.2 Population structure

For the mtMLST analyses, ticks (83 in 2006, 41 in 2007 and 38 in 2008) from various sites in Britain (Table 9 and Appendix - Tick Samples used for Comparison of British populations in 2006, 2007 and 2008 in section 4.3.1, p. 198) were studied. An analysis of the genetic variation within the samples showed 59 unique STs. Using these allelic profiles, a goeBURST analysis (Figure 34) shows the relationship of all the profiles up to a level of similarity of three loci.

The analysis shows two main clonal complexes (CC-1 and CC-2) and seven minor clonal complexes (CC-3-9). The main clonal complexes consist of samples from all of the years samples. In CC-2, the samples from each year can be seen as individual clusters where in CC-1 the samples collected from the locations in 2007 are only seen in the mixed year clusters. The minor clonal complexes show an amalgamation of samples from all the years apart from CC-6 which is composed solely of samples collected in 2006 (ST137 and ST138). Only one tick represented each of these STs. Two STs from the dataset differed from the remaining STs by more than three loci, otherwise known as singletons. Neither of the singletons was represented by more than one sample. Eighteen STs were shown to be from more than one year (indicated in green in Figure 34). From the individual years, 23 STs were solely from collections in 2006 (red), 8 in 2007 (blue) and 10 in 2008 (yellow). The most common ST was ST88 with 19 samples representing this ST.

F_{ST} values were calculated in Arlequin 3.1. Molecular distances were calculated with pairwise differences. Ten-thousand bootstrap replicates were performed. The results are shown in Table 10. F_{ST} values calculated between populations of *I. ricinus* from the three years indicated moderate levels of genetic differentiation between 2006 and 2007 and 2007 and 2008 with significant p-values. The low level of genetic differentiation between 2008 and 2006 did not support a significant p-value, so larger sample sizes would be needed to determine the level of genetic differentiation between these two populations.

Table 10: F_{ST} values calculated in Arlequin of British *I. ricinus* populations. P values for the statistics are shown in parentheses. Significant values are shown in bold

	2006	**2007**	**2008**
2006	-	-	-
2007	**0.06360 (P=0.00950)**	-	-
2008	0.00037 (P=0.32333)	**0.06846 (P=0.01782)**	-

The clustering pattern of ticks in the goeBURST analysis shows a large number of STs that are found in more than one year. Figure 35 shows the proportion of ticks collected from each location over the three years. Due to collection restraints and issue with inclement weather, collections had not been made in equal proportions in the different locations. Two locations have ticks samples present for each year and were compared using F_{ST} values calculated in Arlequin 3.1. Samples sizes for the individual years are too small for individual year analysis with some years only having 10 samples. The F_{ST} value calculated between Widcombe (91 samples) and Bathampton (40 samples) showed a non-significant low level of population differentiation (0.013, p=0.14068). Between the two populations, 76 tick samples share the same STs. This would not be unexpected as the two locations are geographically close (1.56 km).

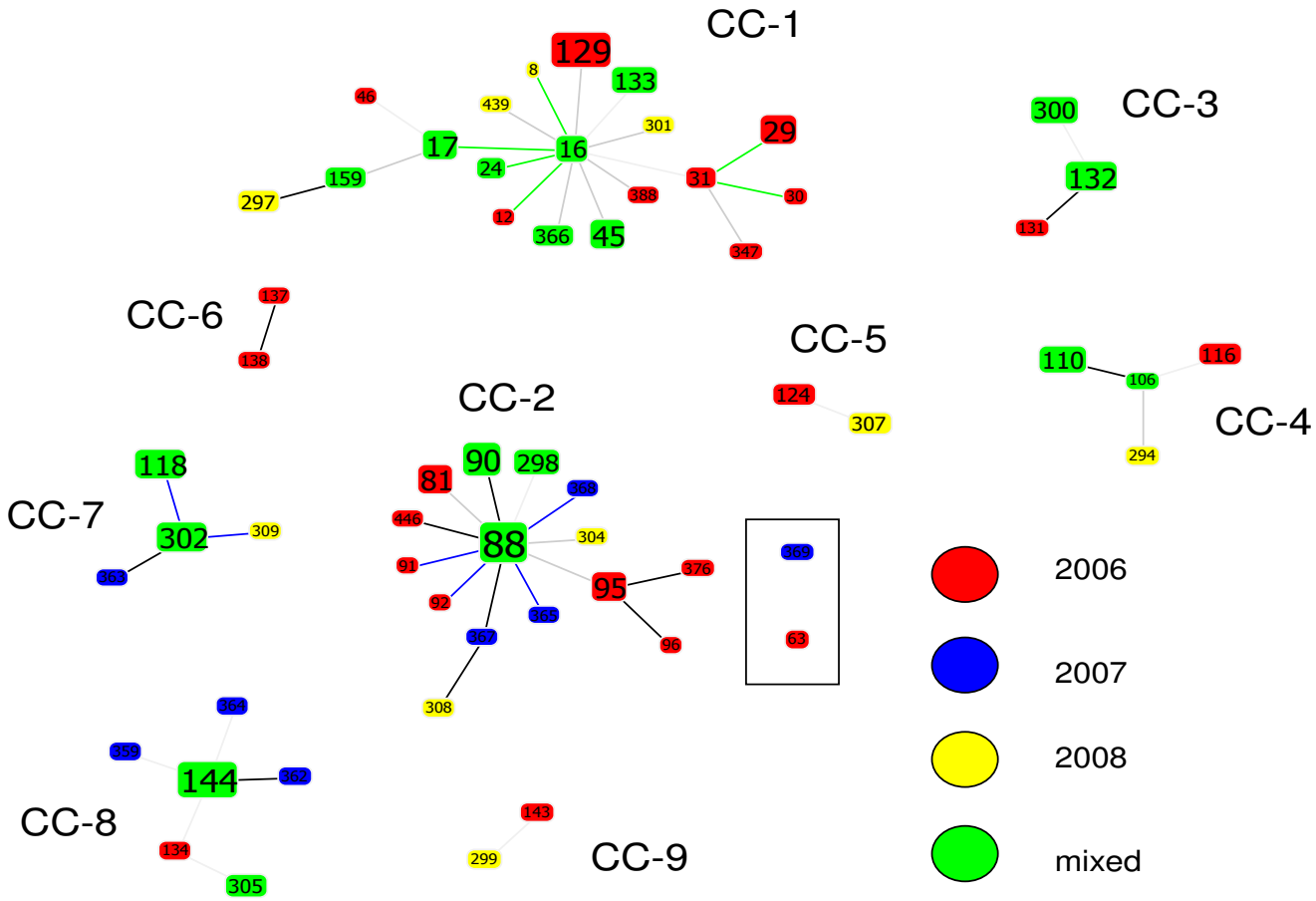

Figure 34: A goeBURST analysis for *I. ricinus* ticks collected from Britain from eight locations. Boxed STs show singletons. Mixed samples indicated in green show STs that were found in more than one year but not necessarily in all years

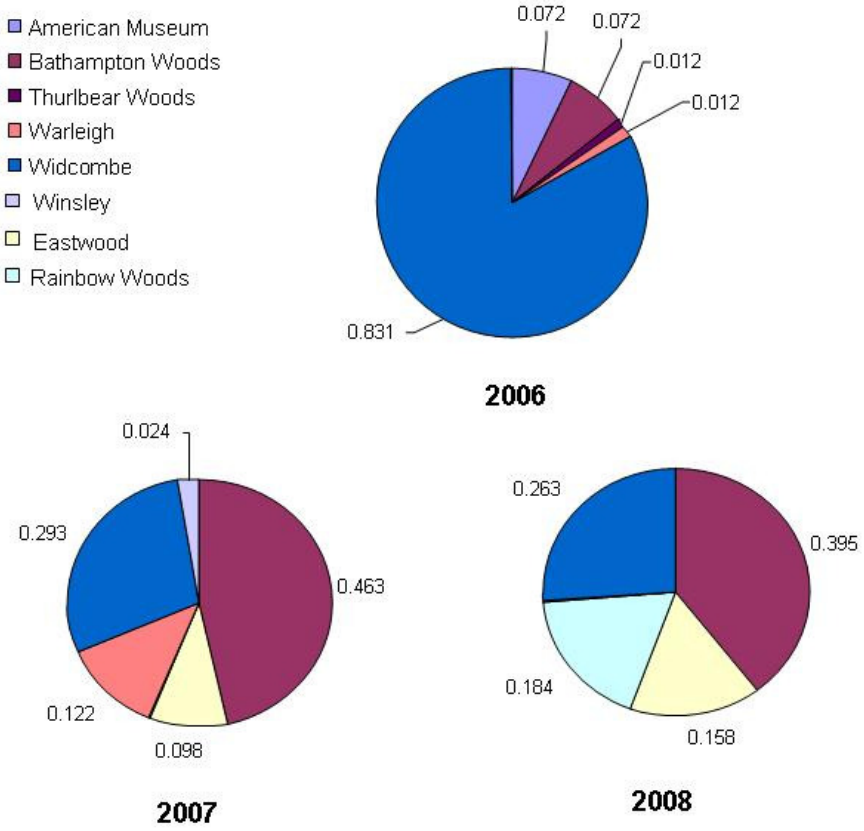

Figure 35: Pie charts showing the proportion of *I. ricinus* ticks from collection locations in Britain in 2006-8. Data labels indicate the proportion of total collection to 3 d.p.

4.3.3 Population statistics

I examined the mismatch distribution statistics calculated for the British populations for evidence of population expansions. Mismatch distribution analyses in DNAsp computed distances based on constant and expanding populations and compared to observed relative frequencies. Coalescent simulations analyses generated RI statistics and corresponding p-values. Mismatch distribution analyses in Arlequin 3.1 compared observed distributions of pairwise differences with spatial and demographic models of population expansion. Fu's Fs statistics were also calculated for each population. Population analysis statistics are shown in Table 11. For a description of the methods used to compute these statistics and the theory behind the analyses, please refer to section 2.2.7, p. 26.

Mismatch distribution analyses were calculated for the British samples in Arlequin from 2006 (Figure 38), 2007 (Figure 39) and 2008 (Figure 40) and also for combined British populations in DNAsp (Figure 36) comparing constant and changing population models and Arlequin (Figure 37) comparing spatial and demographic models of expansion. The distribution analyses data for the graph generation can be found in the following appendices: Mismatch distribution analyses data for Britain combined populations (DNAsp), p. 223, Mismatch distribution analyses data for Britain combined populations (Arlequin), p. 225, Mismatch distribution analyses data for 2006 British population (Arlequin), p. 226, Mismatch distribution analyses data for 2007 British population (Arlequin), p. 227, Mismatch distribution analyses data for 2008 British population (Arlequin), p. 229.

For the combined populations from all years I computed a mismatch distribution analysis in DNAsp with a mean of 19 mismatches. The plot is ragged with a large peak of mismatches at high numbers of pairwise differences (Figure 36). The RI statistic was non-significant ($p=0.3$). These figures indicate that the observed distribution does not differ from the constant and changing models but cannot differentiate between them. A visual inspection of the plots of constant and changing models confirms that neither model is a good fit for the observed data.

Mismatch analyses were performed in Arlequin 3.1 comparing the observed distribution to models of spatial and demographic expansion. The observed distributions in DNAsp produced non-significant RI statistics indicating an expanding population although when the observed data is compared to expanding populations in Arlequin the non-

significant RI statistics cannot reject the hypotheses of spatially and expanding populations, therefore requiring more data to determine the exact mode of expansion.

The populations were examined individually according to the year of collection. Observed distributions were compared with models of distribution according to spatial and demographic expansions in Arlequin 3.1 for 2006 (Figure 38), 2007 (Figure 39) and 2008 (Figure 40). Initial and final values of θ calculated in Arlequin 3.1 for the populations collected from each year produced similar values from approximately $\theta_0 =$ 34 to $\theta_1 = 38$ for demographic expansion model and $\theta_0 = 0$ to $\theta_1 =$ constant for spatial expansion models. No significant RI statistics were found not allowing us to reject the spatially or demographically expanding population models. Further investigation of these data and larger sample sizes may help resolve the mode of expansion.

The Fs statistics for all populations, combined and split into yearly-designated populations, all showed non-significant values. Further data would be required to determine any population expansion in Britain that may have previously occurred.

Table 11: Mismatch analyses statistics for British populations. P-values for SSD, RI and Fs are shown in parentheses with statistically significant values highlighted in red.

Population Name	Model	Mismatch observed mean	Mismatch observed variance	τ	θ_0	θ_1	SSD (p-value)	RI (p-value)	Fs (p-value)
Britain	constant	19.490	147.329	8.184	11.307	∞	NA	0.007(0.3320)	-4.988 (0.2180)
Britain	changing							0.007(0.3240)	
Britain	demographic	20.650	159.382	34.172	0.0000	41.9115	0.007(0.4871)	0.002(0.8977)	
Britain	spatial			26.500	18.799	constant	0.013(0.2486)	0.002(0.9992)	
British 2006	demographic	20.400	168.789	35.236	0.0018	43.7273	0.012(0.2017)	0.008(0.2486)	-0.795 (0.4780)
British 2006	spatial			27.800	17.609	constant	0.017(0.1549)	0.008(0.8948)	
British 2007	demographic	19.927	166.417	33.783	0.0000	36.1414	0.011(0.5274)	0.011(0.3636)	1.167 (0.6880)
British 2007	spatial			26.300	13.875	constant	0.013(0.4849)	0.011(0.8953)	
British 2008	demographic	19.687	157.608	33.992	0.0000	36.2719	0.009(0.6593)	0.006(0.8388)	-0.562 (0.4560)
British 2008	spatial			26.500	20.559	constant	0.014(0.3642)	0.006(0.9814)	

τ: time in mutational events since the modelled expansion event

RI: raggedness index

SSD: sum of squared deviations

Fs: Fu's Fs test

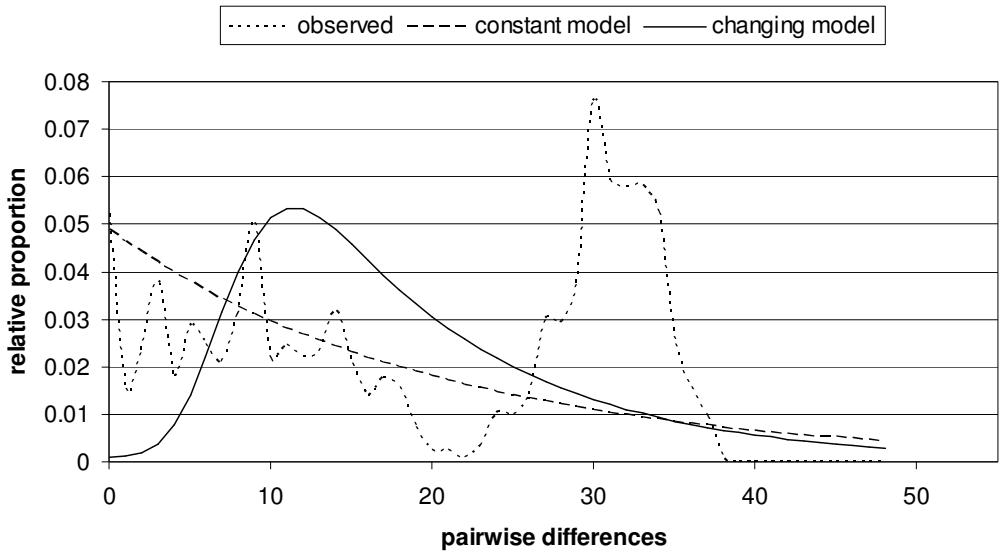

Figure 36: Mismatch analysis of British samples in DNAsp

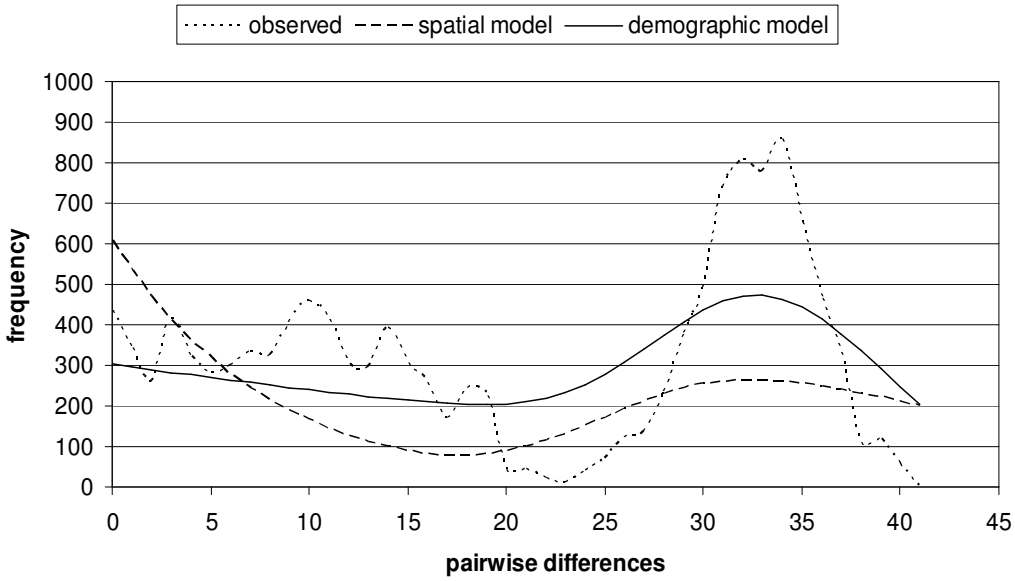

Figure 37: Mismatch analysis of British samples in Arlequin

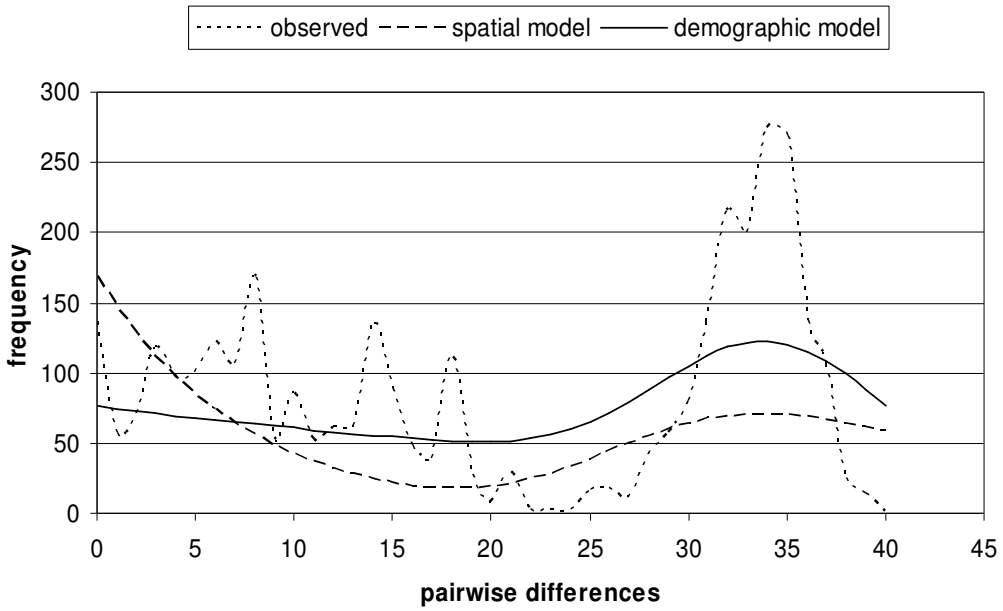

Figure 38: Mismatch analysis of British 2006 samples in Arlequin

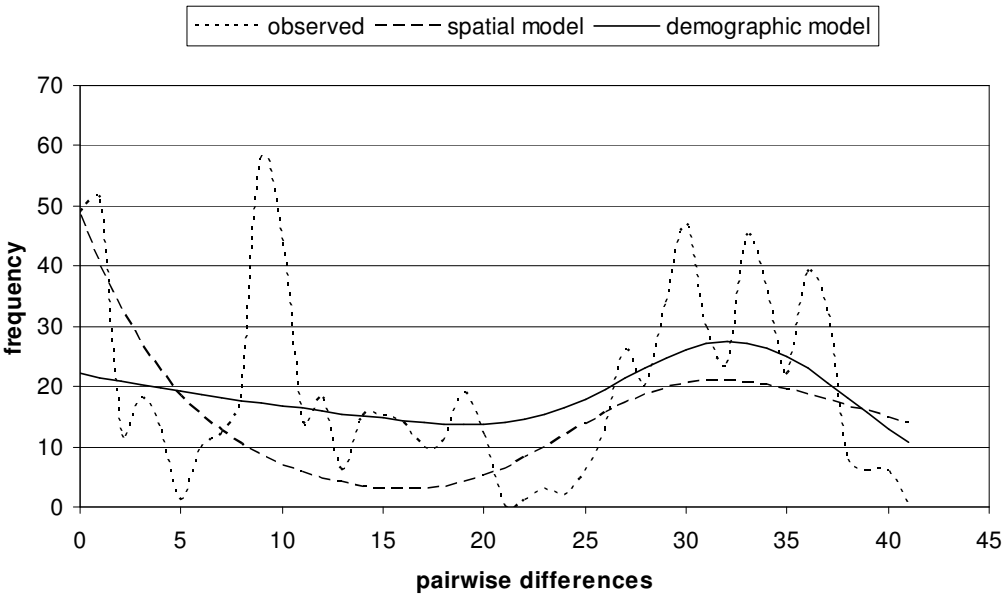

Figure 39: Mismatch analysis of British 2007 samples in Arlequin

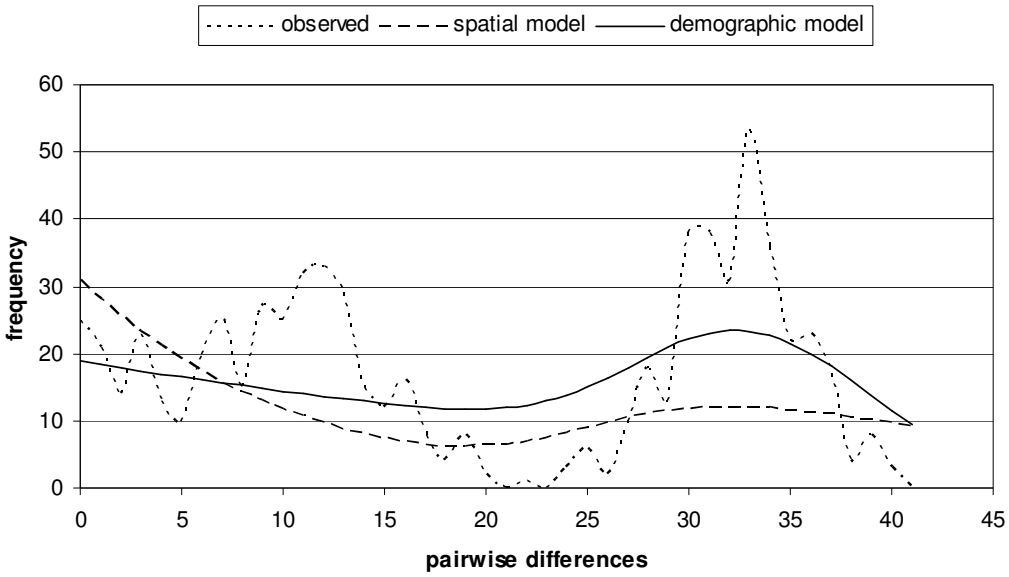

Figure 40: Mismatch analysis of British 2008 samples in Arlequin

4.3.4 British analyses summary

The British populations from 2006 to 2008 have been shown to have a population of *I. ricinus* ticks that share a large number of STs that span more than one year. A large proportion of STs found in the British population of ticks were often found in ticks collected in subsequent years. Analyses in goeBURST showed the sharing of STs across several years but did not indicate a succession of STs through the years due to gradual genetic change. Phylogenies have indicated that there was little separation between the ticks from the different years although ticks did sometimes cluster according to their year of collection on sole branches. Ticks on these branches were often collected from the same locations and may be artefacts of collecting ticks from the same egg batch but as the nymphs have already been dispersed by feeding on a host already during their larval stage it seems more likely that in some locations a single ST is dominant in that area.

The *I. ricinus* population in Britain was shown to be expanding although there was insufficient data to confirm whether the population expansion was due to a spatial or demographic expansion. The described population structure seen in ticks from the UK could be explained in many ways. If the ticks we see in the UK were a result of introduction through bird migration, the lower levels of variation in the number of STs would suggest that the same STs from Continental Europe are introduced. These introduced STs could originate from one particular region of Continental Europe or could represent a subpopulation of ticks that are better suited to the British climate and are more likely to survive to reproduce. More collections over an extended period are needed to explore how the British population changes, and using these data, extrapolate an estimate for mitochondrial mutation rate in *Ixodes ricinus* ticks.

4.4 Discussion

Populations of *I. ricinus* ticks throughout Europe are often documented for public health purposes (Health Protection Agency, 2010b). In recent times, tick numbers have seen a dramatic increase in certain areas, including the UK (Pietzsch *et al.*, 2005; Scharlemann *et al.*, 2008), possibly through human intervention. In the past 30 years we have seen evidence of an increase in many different species of deer (Ward, 2005) which is the reproductive host of *I. ricinus* ticks (J. S. Gray *et al.*, 1992; J. S. Gray, 1998). Some investigations have suggested that climate change has lead to increases in tick incidence, and therefore in manifestations of disease in humans e.g. Tick Borne Encephalitis (Randolph, 2001, 2008), which is of great concern to the medical profession, and therefore makes *I. ricinus* and related species that are able transmit disease to humans, of great scientific interest.

The surge of ticks in the UK and possibly in other areas of Europe requires monitoring and with these analyses, I aimed to monitor two separate tick populations and investigate temporally how the population structure was affected. I hypothesised that, in a stable population with no migration, there would be little or no change in the mitochondrial allelic profiles of the ticks sampled. Even though mitochondrial genes are considered to evolve at a significantly faster rate than nuclear genes (Brown *et al.*, 1979), these changes may not be seen in such a brief period. Significant differences in the populations compared from one year to the next may indicate several different processes, which we will discuss here in light of my analyses.

We observed that the Latvian populations of *I. ricinus* did not have a genetic signature of homology from year to the next. Some STs were found in more than one year, but not as frequently as ticks in the British population (e.g. ST17, ST129). The diversity of STs in Latvia is twice that of Britain. From 188 samples analysed in the Latvian population we found 155 unique STs compared to 162 samples and 59 STs in Britain. This translates to 2.75 samples per ST discovered in Britain and 1.21 samples per ST discovered in Latvia. It is possible that we could have seen only a small proportion of the true diversity seen in Latvia. In Figure 41 a line graph is shown that represents the sequence type diversity discovered according to the number of samples used in this analysis. If the true diversity of the population had been sampled from the population, a plateau would be observed in the line for each of the countries from which samples were collected. This data plot serves to highlight that the true diversity of the Latvian

population was not observed. The line associated with the British population indicates that new sequence types were discovered at an increasingly reducing rate and the data appears to be near a plateau, indicating a saturation of sampling. This indicates that the sampling of the British population is more representative of the diversity seen in Britain.

If Latvian tick populations have a larger effective population size, we would require more sampling to capture the full spectrum of genetic variability. This high level of diversity found in Latvia may be a 'snapshot' of the diversity in Latvia and indeed in the rest of Europe. Having shown the difference in British and Latvian ticks, probably by the geographic distance (approximately 1700km) and significant bodies of water (the English Channel) it is probable that neighbouring countries of Latvia will share a high number of STs and this deserves further investigation.

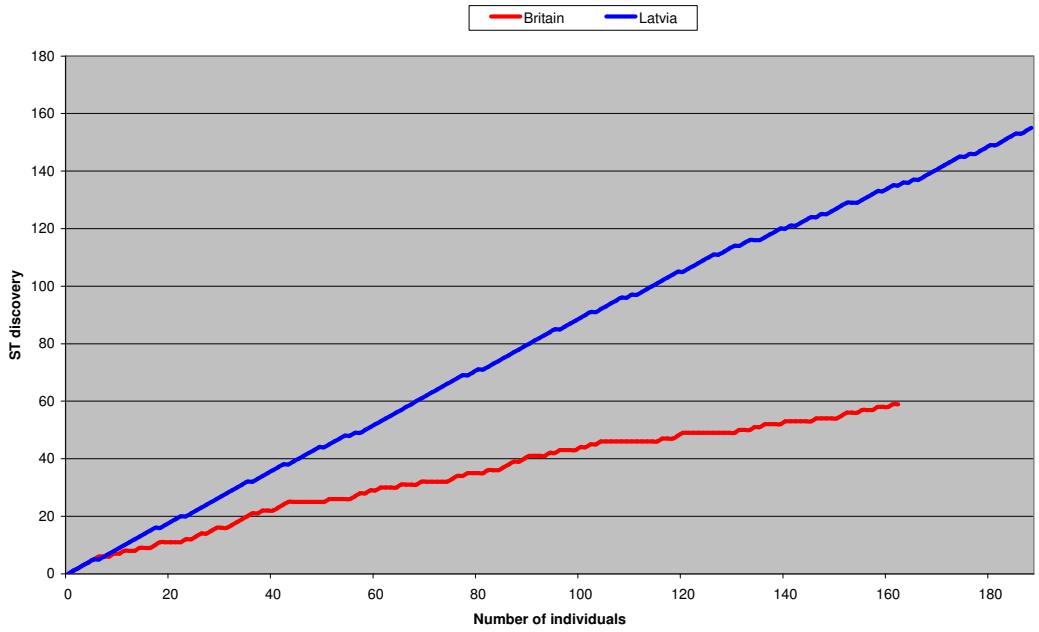

Figure 41 A line graph showing the sequence type discovery according to the number of individual samples. A plateau of the data indicates that sampling has reached saturation according to the population. This graph was generated using excel equations for the cumulative addition of sequence types.

The phylogeny of the British ticks has shown clustering to a small degree. By clustering the STs of the British population and comparing derived F_{ST} values, we observe a distinct genetic differentiation between 2006 and 2007, and 2007 and 2008 but not between 2006 and 2008. This pattern of genetic association across the years of British ticks may seem in conflict with traditional views but when these data are combined with the knowledge of tick reproductive lifecycles, a rational explanation of

these associations can be deduced. The typical length of a tick lifecycle is 2-3 years (Hillyard, 1996). Nymphs collected and analysed in 2006 form part of a population that could conceivably have moulted and reproduced to provide nymphs for collection in 2008. The usually clonally maternally inherited nature of mitochondria (Barr *et al.*, 2005; Breton *et al.*, 2007; White *et al.*, 2008) may therefore lead to a genetic profile that is repeated every two or three years and may be isolated from other genetic profiles due to the length of the tick lifecycle.

The Latvian population of *I. ricinus* ticks was a complete contrast to the ticks in Britain. Latvian *I. ricinus* ticks analysed were suggested to be a panmictic population with all years sampled showing no evidence of genetic differentiation. All samples from each year showed no evidence of clustering either in the phylogenetic studies or in the F_{ST} statistical analyses. This clustering pattern showing a panmictic population of ticks from one year to the next may suggest that the migration of ticks from and into Latvia is much greater than previously estimated. We would not expect to see a change in the genetic profile of mitochondrial genes in a population due to mutation in a period of only a few years. The changes in the STs seen in these analyses must have originated from the migration of ticks from different areas of Latvia or Europe. Another possibility is that the sampling of the Latvian population is not sufficient to capture all the STs in the geographic region.

With such contrasting population structures found between British and Latvian populations of *I. ricinus* ticks, it is clear that different ecological pressures are acting on this population. We hypothesised that the migration of ticks would have a marked effect on the genetic profiles of the population within a geographic region. The British profile evidence suggests that the ticks from this geographical area do not vary much at the mitochondrial genes from one year to the next, which would suggest little migration of ticks from other geographic regions. Land based hosts in Continental Europe are isolated from Britain through the English Channel, and therefore, birds must be assumed the main source of *I. ricinus* tick introductions. Despite studies of common migratory passerine birds harbouring ticks (Comstedt *et al.*, 2006) the effect of migration of these birds on tick introductions is likely to be limited. One of the most common species to harbour ticks studied by Comstedt *et al.* (2006) was the European Robin and has been shown to rarely live very far from their natal site (Wernham *et al.*, 2002). In contrast, the Latvian population of *I. ricinus* ticks has been found to be panmictic. Without barriers to land based hosts of *I. ricinus* such as the English Channel, *I. ricinus* populations of ticks in Latvia would be able to be carried by land-

based animals (Hillyard, 1996; Kierans *et al.*, 1999). With a greater number of possible hosts on which ticks could potentially travel to new areas, we would expect to see a greater level of heterogeneity within the population based on the assumption that we have not yet found all STs found in Continental Europe.

The population structure of the ticks in Britain and Latvia are very different and require further study to create records of populations in order to detect fluctuations that could have been caused by immigrant ticks and the possible introductions of diseases into previously unknown ranges.

4.5 Conclusions

The *I. ricinus* populations in Britain and in Latvia have been shown to be fundamentally different using the mtMLST scheme. Latvian and British populations have been shown to vary in their level of genetic variability within the populations and the succession of STs from one year to the next. Latvian ticks were shown to be a stable population that was likely to be a small part of a larger picture of variation that inhabits Europe as few STs were discovered in more than one sample. The British populations analysed over three successive years indicated an expanding population of ticks with multiple STs that were common in many areas. These patterns of genetic association are likely to have been shaped by the limitations of hosts upon which *I. ricinus* ticks feed.

5 A study of *Ixodes ricinus* populations from locations in Southern England

This chapter studies the *Ixodes ricinus* populations in Southern England as a means to study the regional variations that could be determined by using our mitochondrial mtMLST scheme developed in chapter 3. Populations from three locations in Southern England were analysed for genetic differentiation to determine whether *I. ricinus* populations within a small geographic region can be differentiated.

5.1 Introduction

Ixodes ricinus is arguably the most important vector of disease in Northern Europe. These blood sucking ectoparasites are responsible for a variety of microbial diseases across Europe in both humans (e.g. *Borrelia burgdorferi* s.l. (Burgdorfer *et al*., 1982)) and animals (e.g. Louping ill virus (Gilbert *et al*., 2000) and *Anaplasma phagocytophilum* (Dumler *et al*., 2001)) making it of veterinary importance as well as of concern to human health. Owing to these significant risks to human and animal health the phenology of tick populations has been investigated (Randolph *et al*., 2002; Estrada-Pena *et al*., 2006) leading to further questions regarding the population structure of *I. ricinus* ticks in Europe. Despite intensive investigation of populations from various geographical locations across Europe there has been little consensus of whether there is structure in the tick populations in Europe at either large geographical distances (Caporale *et al*., 1995; Casati *et al*., 2007) or at much smaller geographic distances (Delaye *et al*., 1997).

Having used the *I. ricinus* mtMLST scheme to distinguish populations at low resolution (over large geographical regions e.g. in different countries) we aim to determine the maximum useful resolution of the devised scheme. If the resolution of the scheme can delineate ticks from geographical regions from a small geographic region (i.e. within a country), we could feasibly use this scheme to make detailed maps of tick populations and in the medium to long term to track the movements of the populations.

The British Isles are isolated from Continental Europe by the English Channel. Using an mtMLST scheme we have shown that the *I. ricinus* populations from Latvia and Britain (approximately 1,800 km apart) are genetically distinct (Chapter 3: A Multilocus Sequence Typing Scheme for Ticks: Rationale & Design).

The incidence of *I. ricinus* ticks in Britain is highly dependent on microclimate (Randolph & Storey, 1999) and has been shown to be heterogeneous in part due to these specific requirements. Some studies have been done in the UK examining the abundance of *I. ricinus* ticks spatially (Pietzsch *et al.*, 2005; Medlock *et al.*, 2008; Scharlemann *et al.*, 2008). These heterogenous populations in the British Isles have not been previously examined for genetic heterogeneity in concurrence with the differences in abundance and developmental rates previously reported (Randolph *et al.*, 2002).

Using tick samples collected in 2008 from three locations in Southern England we are aiming to delineate tick samples within England according to geographical location. Ticks from Exmoor, the New Forest and Richmond Park in London were analysed using the devised scheme to look for differences between these populations. These findings will be the first genetic analysis of the British population of *I. ricinus* ticks.

5.1.1 Aims and objectives

The aims of this chapter can be described as follows:

- examine the population structure of *I. ricinus* ticks from Southern England
- determine whether mtMLST using mitochondrial genes has enough resolution to delineate populations within a small geographic area

5.2 Analyses of *Ixodes ricinus* populations from the South of England

In this section, I describe populations of *I. ricinus* ticks collected in Exmoor, Richmond Park in London and the New Forest. Using the devised mtMLST scheme, I will examine the resolution of the scheme to determine whether it can be used to examine populations of *I. ricinus* at a high resolution (i.e. within a country). This scheme could be a useful tool in tracking tick populations in the medium to long term. Analysis of populations from a small geographic area may also be able to assess the effect that different barriers i.e. roads and major rivers have on the phenology of ticks.

5.2.1 Phylogenetic analyses of ticks from three localities in Southern England

Questing *I. ricinus* nymphs from Southern England were collected 2008 by blanket dragging from Exmoor, the New Forest and Richmond Park (Figure 42 and also Appendix - Collection Site Maps, p. 167) and were analysed according to the method indicated in the mtMLST scheme previously described (Chapter 3: A Multilocus Sequence Typing Scheme for Ticks: Rationale & Design). Similar sample sizes were used from each of the locations constituting 39 samples from Richmond Park, 28 samples from Exmoor and 28 samples from the New Forest (Appendix - Tick samples used for comparison of Southern England populations in section 5.2, p. 231). Only nymphs were used in these analyses to ensure that ticks were from the same generation. Concatenated sequences of six mitochondrial internal gene fragments (*atp6*, *coi*, *coii*, *coiii*, *cytB* and *12s*) were aligned using MEGA 4.1 and edited by hand to minimise gaps. Phylogenies were rooted with *I. persulcatus* sequences from GenBank (accession NC_002010). Sequences used as outgroup to root the phylogeny were trimmed according to the corresponding area of analysis for each gene.

Figure 42: Collection locations of ticks from Southern England. Map generated by Google Earth (Google, 2010).

A ML phylogeny was constructed using PhyML (Guindon *et al.*, 2005) (Figure 43). The majority of the deep branches are well supported with good aLRT values above 80. No branches had an aLRT value below 70. Throughout the phylogeny, there are no large clusters of samples from a single location and no clear separation of the three locations in Southern England. Some clusters found for Richmond Park are indicated in Figure 43 as A, B and C. Despite this small amount of clustering, many of the clades show that the related STs were found in all three locations. There is no evidence in the phylogenetic analysis that can indicate a separation of genetic profiles and populations between these three locations in Southern England.

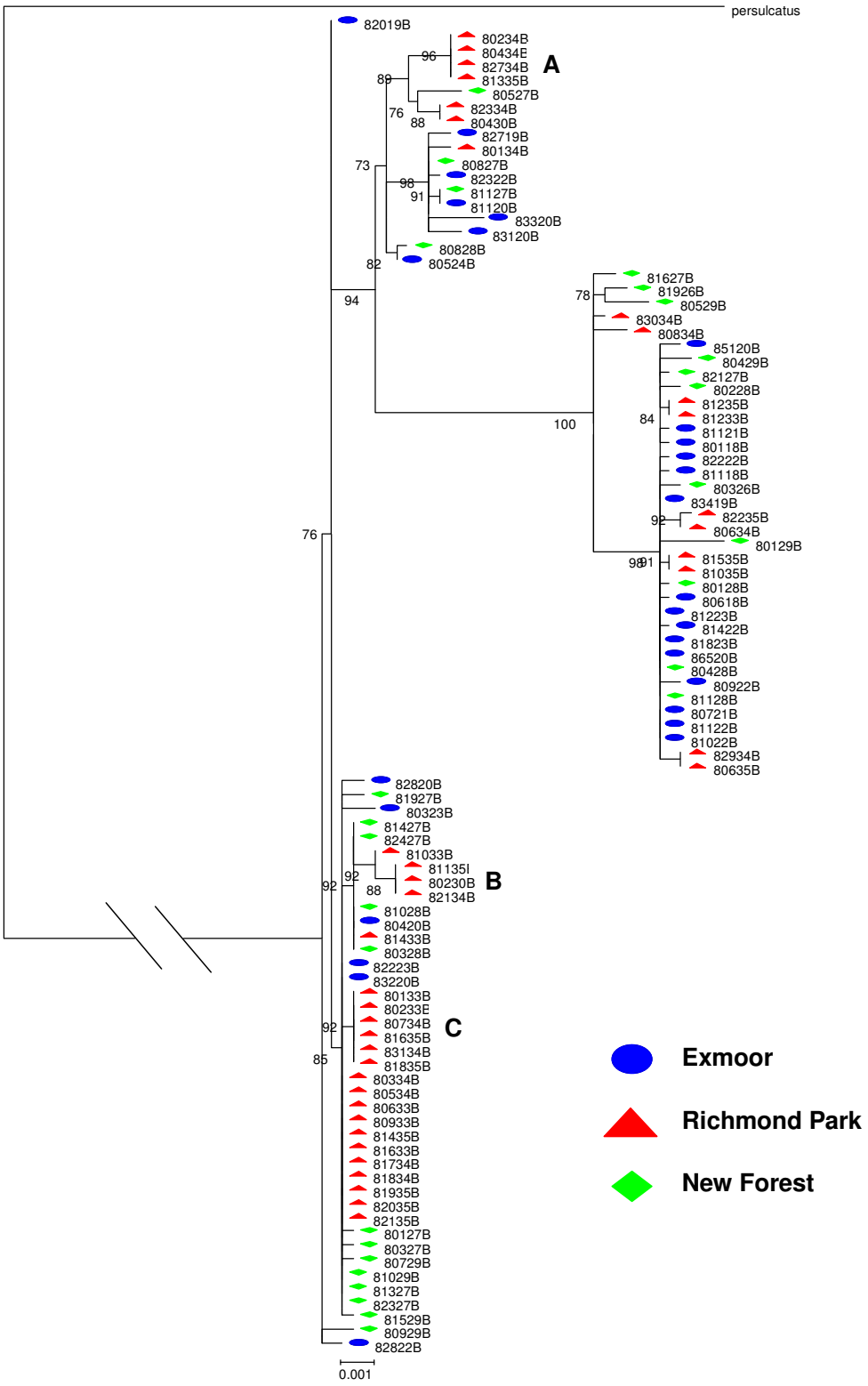

Figure 43: The evolutionary history of Southern England *I. ricinus* populations collected in 2008 in three locations (Exmoor, the New Forest and Richmond Park) was inferred using a concatenation of six mitochondrial genes and using PhyML maximum likelihood method. The optimal tree with the sum of branch length = 0.25102919 is shown. The dataset was outgrouped using *Ixodes persulcatus*. There were a total of 3216 positions in the final dataset. The scale bar indicates 0.1% divergence

5.2.2 Population structure

Tick samples from the three locations in Southern England (39 in Richmond Park, 28 in Exmoor and 28 in the New Forest) (Appendix - Tick samples used for comparison of Southern England populations in section 5.2, p. 231). An analysis of the genetic variation within the samples showed 58 unique STs. Using their allelic profiles, a goeBURST analysis (Francisco *et al*., 2009) (Figure 44) shows the relationship of all the profiles up to a level of similarity of three loci.

The analysis shows two large main clonal complexes (CC-1 and CC-2) and three small clonal complexes (CC-3, CC-4 and CC-5). The larger clonal complexes consist of STs from all of the locations sampled. The minor clonal complexes also show samples from more than one location. The main clonal complexes both show a central node that was found in more than one location. ST88, the predicted founder of CC-1 was found in Exmoor and New Forest and ST17, the predicted founder for CC-2, in all three localities. Four STs from the dataset differed from the remaining STs by more than three loci, otherwise known as singletons. ST317 and ST285 only comprise of one sample. ST175 and ST183 comprise of six and three samples respectively. The mixed samples (indicated as yellow in Figure 44) are represented by STs from more than one location but not necessarily from all three locations. The most common ST was ST17 with 22 samples representing this ST.

Table 12: F_{ST} values calculated in Arlequin for Southern England populations. P values for the statistics are shown in parentheses. Significant values are in bold.

	Exmoor	**New Forest**	**Richmond Park**
Exmoor	-	-	-
New Forest	0.02339 (0.18632)	-	-
Richmond Park	**0.11883 (0.01039)**	0.00832 (0.23245)	-

F_{ST} values were calculated in Arlequin 3.1 (Excoffier *et al*., 2005). Molecular distances were calculated with pairwise differences. 10,000 bootstrap replicates were performed. The results are shown in Table 12. F_{ST} values calculated between populations of *I. ricinus* from the three locations indicate that only the pairwise distances for populations from Exmoor and Richmond Park gave a significant value of genetic differentiation. Between these two populations an F_{ST} value of 0.11883 indicates a moderate level of genetic diversity (Freeland, 2005).

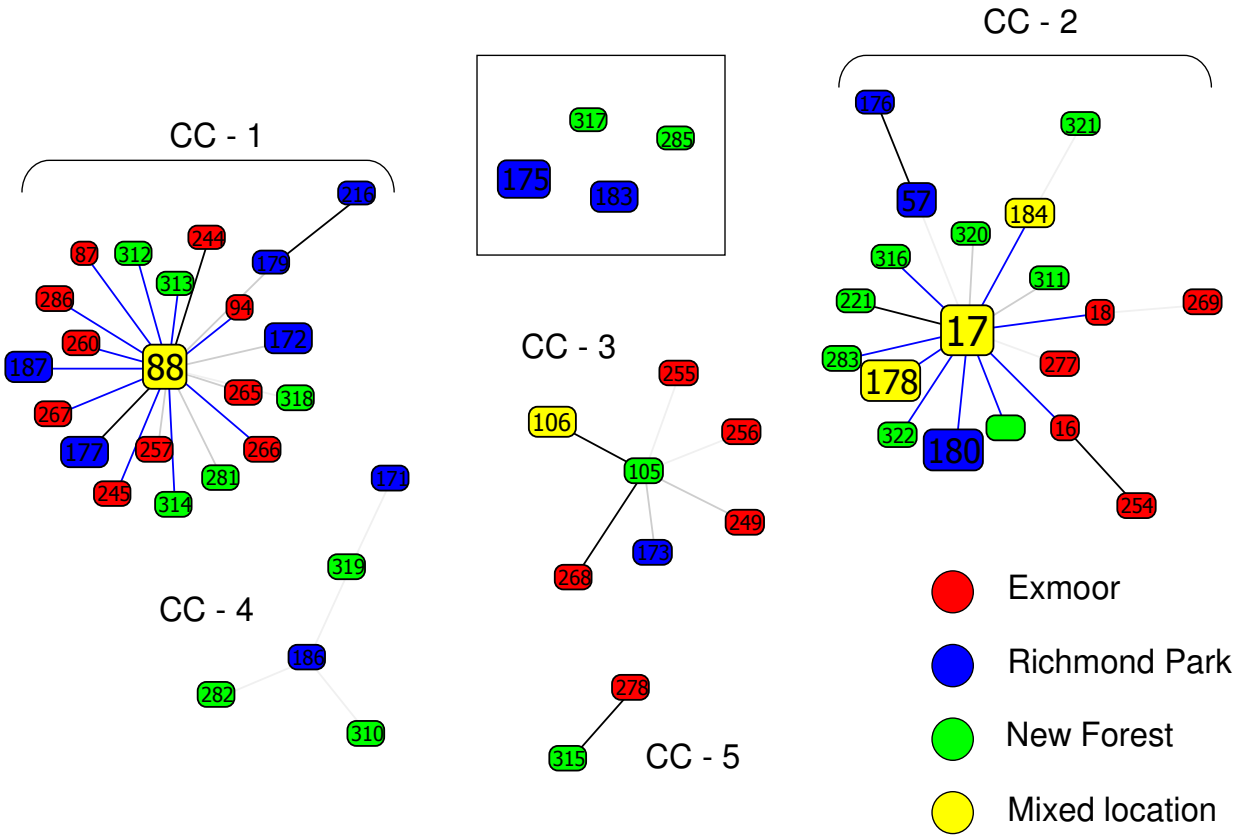

Figure 44: A goeBURST analysis for *I. ricinus* ticks collected from the New Forest, Richmond Park and Exmoor in 2008. Boxed STs show singletons. Mixed location indicated in yellow shows STs that were found in more than one location but not necessarily all locations

5.2.3 Population statistics

Mismatch distribution statistics calculated for the Southern England populations were examined for evidence of population expansions. Mismatch distribution analyses in DNAsp computed distributions based on constant and expanding populations and compared to observed relative frequencies. Coalescent simulation analyses generated RI statistics and corresponding p-values. Mismatch distribution analyses in Arlequin 3.1 compared observed distributions of pairwise differences with spatial and demographic models of population expansion. Fu's Fs statistics were also calculated for each population. Population analysis statistics are shown in Table 13. For a description of the methods used to compute these statistics and the theory behind the analyses, please refer to section 2.2.7, p. 26.

Mismatch distribution analyses were calculated for Southern England samples in Arlequin 3.1 from Exmoor (Figure 45), the New Forest (Figure 46) and Richmond Park (Figure 47) and also for combined Southern England populations in DNAsp (Figure 48) comparing constant and changing population models and Arlequin 3.1 (Figure 49) comparing spatial and demographic models of expansion. The mismatch distribution analyses data for the graph generation can be found in the following appendices: Mismatch distribution analyses data for Exmoor samples from a Southern England population (Arlequin), p. 237, Mismatch distribution analyses data for New Forest samples from a Southern England population (Arlequin), p. 238, Mismatch distribution analyses data for Richmond Park samples from a Southern England population (Arlequin), p. 239, Mismatch distribution analyses data for a Southern England population (Arlequin), p. 241, Mismatch distribution analyses data for a Southern England population (DNAsp), p. 242.

For the combined populations from all locations in Southern England I computed a mismatch distribution analysis in DNAsp with a mean of 18 mismatches. The plot is ragged with large peaks at both the low number of pairwise differences and the high number of differences (Figure 48). The RI statistics calculated to compare the observed mismatches to the changing and constant model were non-significant (P=0.3520 and P=0.3480 respectively). These figures indicate that the observed distribution does not differ from the constant and changing models. A visual inspection of the plot of the observed mismatch distribution compared to the models of constant and changing population size indicates that neither of the models represents the data sufficiently.

Mismatch analyses were performed in Arlequin 3.1 comparing the observed distribution to models of spatial and demographic expansion. The observed distributions in DNAsp produced non-significant RI statistics in both the expanding and constant models showing uncertainty in the data. When the observed data was compared to models of spatial and demographic expansion in Arlequin the non-significant RI statistics indicates that the observed data did not differ significantly from the expected models of spatial and demographic expansion. With the current dataset, we have not been able to associate the data with a model of population expansion or constant population size.

The populations were examined individually, according to their collection location. Observed distributions were compared with models of distribution according to spatial and demographic expansions in Arlequin 3.1 for Exmoor (Figure 45), New Forest (Figure 46) and Richmond Park (Figure 47). Initial and final values of $\theta_0 = 0$ to $\theta_1 = 26$-40 for the demographic expansion model and $\theta_0 = 8$-15 to $\theta_1 =$ constant for spatial expansion models. No significant RI statistics were calculated from the data therefore indicating that the data does not differ from the expected models of spatially and demographically expanding populations. The only significant value was a SSD value that was found in the analysis of the demographic model in the Exmoor population. The SSD value for the spatial analysis is lower that the value for the demographic analysis indicating that the spatial model is a better fit to the observed data but the RI statistics does not allow us to reject the models of expansion statistically. A visual inspection of the observed distribution compared to the models certainly supports the SSD statistics with the curve of the spatial model following the observed data more closely for the Exmoor population. Visual inspections of the other observed data for Richmond Park and the New Forest show that the data fit the spatial and demographic models of expansion respectively. Further investigation of these data and larger sample sizes may help resolve the mode of expansion.

The Fs statistics for all populations, combined and split into individual locations, all showed significant values apart from Richmond Park. The significant Fs values were all negative indicating that an expansion of these populations may have previously occurred. The statistic describing Richmond Park was non-significant so more data is needed to determine whether this population has been previously shaped by expansion.

Table 13: Mismatch analyses statistics for Southern England populations. P-values for SSD, RI and Fs are shown in parentheses with statistically significant values highlighted in bold.

Population Name	Model	Mismatch observed mean	Mismatch observed variance	τ	θ_0	θ_1	SSD (p-value)	RI (p-value)	Fs (p-value)
S. England	constant	18.296	180.096	5.576	12.720	∞	na	0.009(0.3480)	-15.398 (0.007)
S. England	changing							0.009(0.3520)	
S. England	demographic	19.713	199.076	34.951	0.000	36.105	0.0204(0.080)	0.008(0.2379)	
S. England	spatial			28.591	12.813	constant	0.0216(0.240)	0.008(0.9361)	
Exmoor	demographic	19.987	230.183	35.562	0.000	40.781	0.0294(0.049)	0.017(0.2924)	-5.049 (0.040)
Exmoor	spatial			30.215	8.523	constant	0.0219(0.501)	0.017(0.9260)	
New Forest	demographic	20.204	192.385	35.457	0.000	41.559	0.0183(0.212)	0.0107(0.654)	-6.452 (0.017)
New Forest	spatial			28.570	15.071	constant	0.0213(0.273)	0.0107(0.969)	
Richmond Park	demographic	17.398	197.353	36.064	0.004	26.191	0.0293(0.123)	0.0308(0.065)	3.413 (0.881)
Richmond Park	spatial			30.833	8.780	constant	0.0248(0.323)	0.0308(0.603)	

T: time in mutational events since the modelled expansion event

RI: raggedness index

SSD: sum of squared deviations

Fs: Fu's Fs test

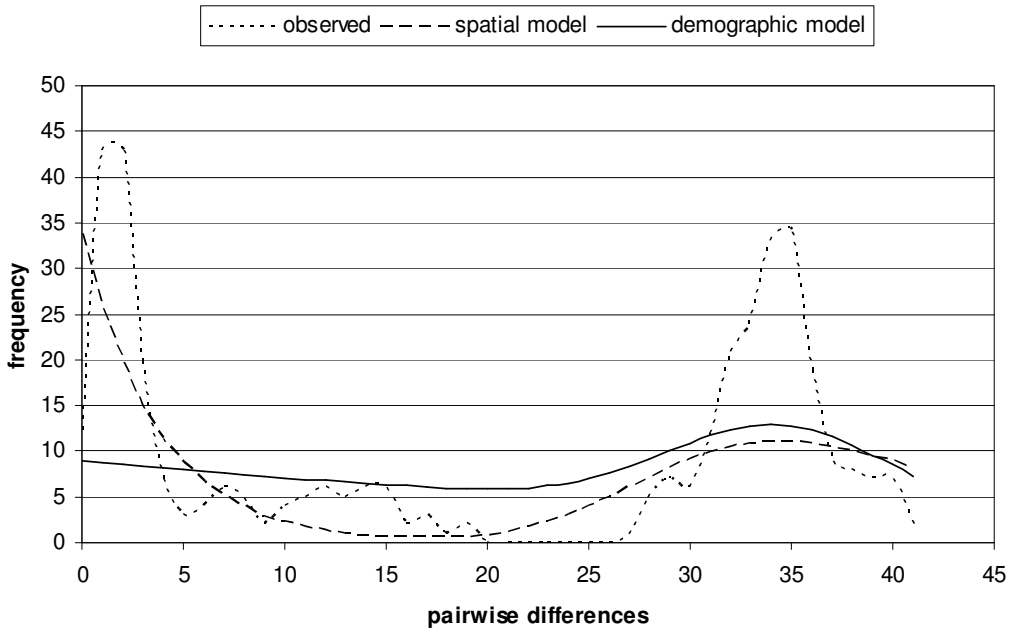

Figure 45: Mismatch analysis of Exmoor samples in Arlequin

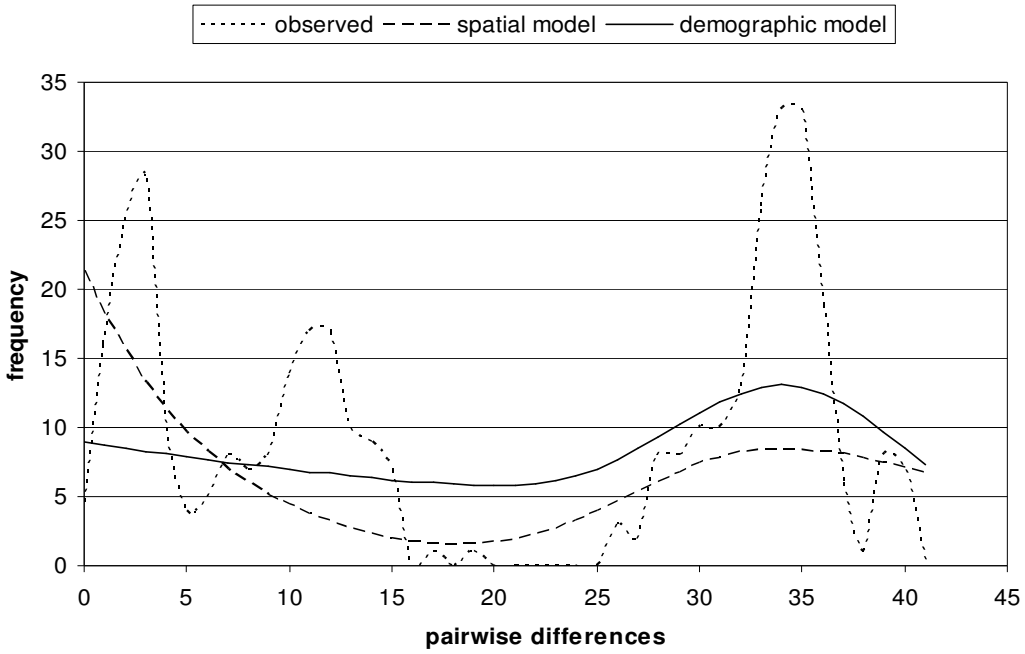

Figure 46: Mismatch analysis of New Forest samples in Arlequin

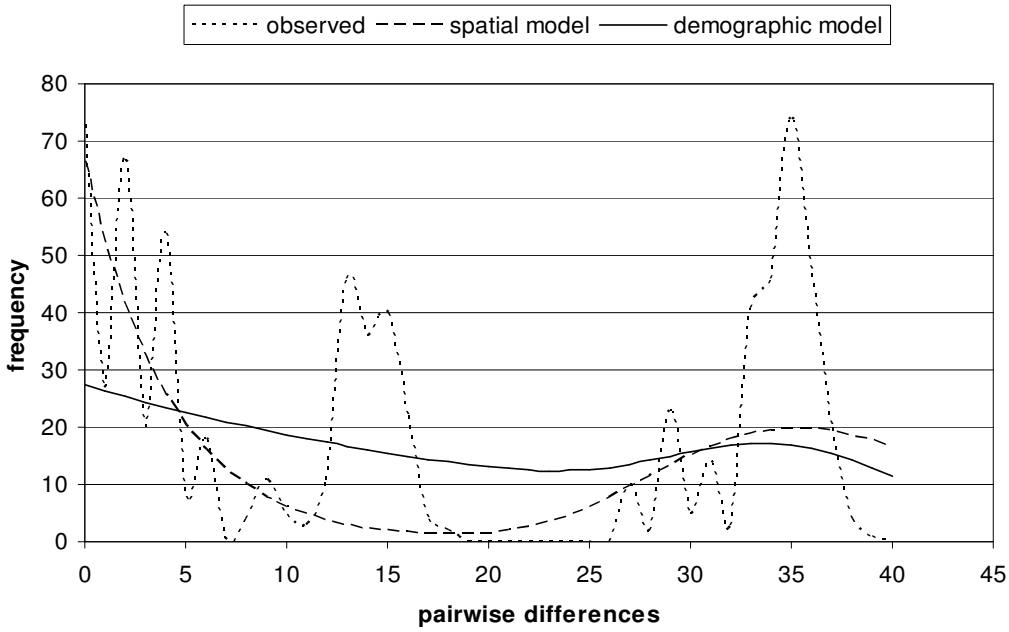

Figure 47: Mismatch analysis of Richmond Park samples in Arlequin

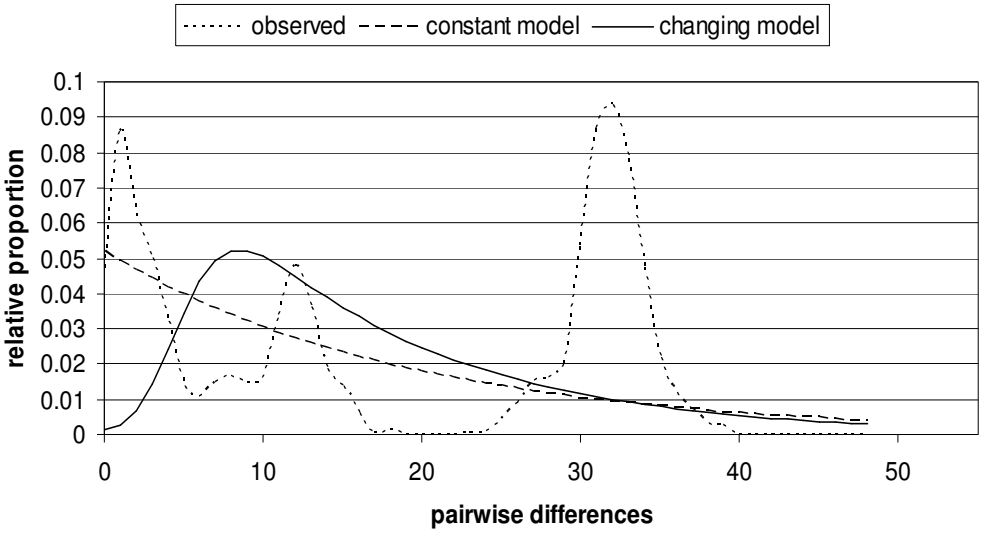

Figure 48: Mismatch analysis of Southern England samples in DNAsp

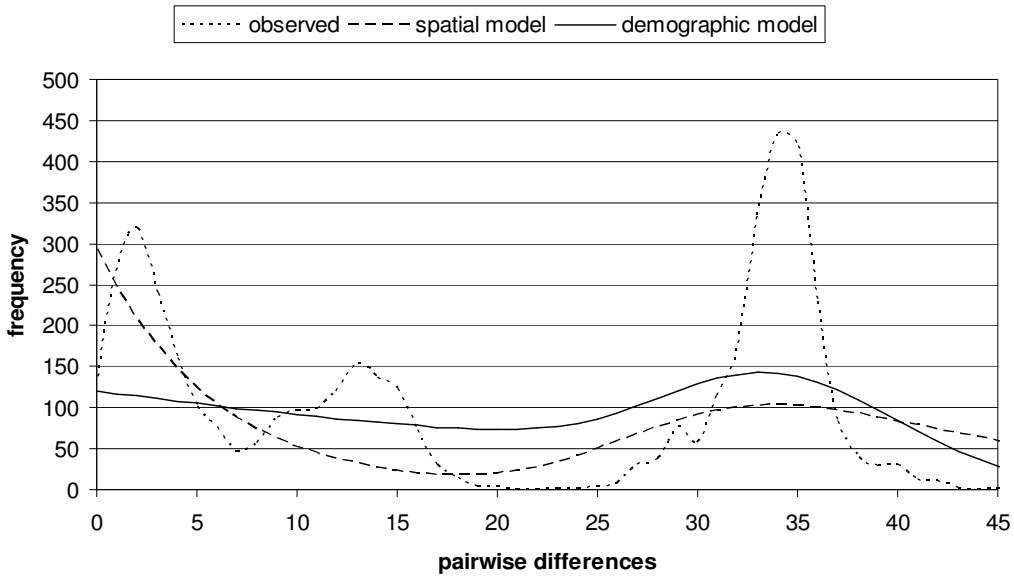

Figure 49: Mismatch analysis of Southern England samples in Arlequin

5.3 Discussion

In this study I have used the previously described mtMLST scheme (Chapter 3: A Multilocus Sequence Typing Scheme for Ticks: Rationale & Design) to examine ticks that were collected from three locations in Southern England (Exmoor, the New Forest and Richmond Park in London). These ticks were analysed to determine how the populations of *I. ricinus* ticks in these locations were related. From these locations, I hoped to determine whether these populations formed discrete clusters within the UK or whether the population within the UK is homogenous.

In previous investigations of *I. ricinus* populations, we devised an mtMLST scheme that could differentiate between populations from different geographical regions. The populations from the UK and Latvia were approximately 1800km apart and the genetic differentiation between these two populations was highly significant. The resolution of this scheme has not been tested on samples from smaller geographic regions (i.e. within a country).

Investigations using the mtMLST scheme have shown that there is little to no clustering of the ticks at the three locations in Southern England. Phylogenetic analyses have shown that the ticks from the New Forest, Richmond Park and Exmoor cannot be distinguished according to the current mtMLST scheme. The population of ticks in the UK, although genetically distinct from *I. ricinus* populations in Latvia, could be homogenous when compared at such a high resolution and barriers such as the English Channel are the only effective means by which populations can be suitably separated thus leading to no genetic drift and no spread of common alleles. Deer are the reproductive hosts of *I. ricinus* ticks and are known to be able to move ticks over large distances (Ruiz-Fons & Gilbert, 2010) including over bodies of water. The movement of ticks across the English Channel in this way would be highly unlikely due to distance, high levels of human activity and proximity of woodland areas to the coast at Dover and Calais. Another scenario is that the *I. ricinus* in the UK has been recently introduced to the British Isles through introduction by migratory birds, expanded rapidly throughout the UK and the introduced alleles have not yet been isolated by geographical distance within the British Isles long enough for mutation of mitochondrial genes to be detected. The mismatch analyses of these populations were inconclusive and cannot support or disprove these hypotheses.

Previous studies of *I. ricinus* ticks within a small geographic region have failed to discern any differences (Delaye *et al.*, 1997) and parts of my data supports this finding. Despite the phylogenetic analysis of the three Southern England populations not indicating any clear differentiation, F_{ST} pairwise analysis of the three populations indicated a moderate level of differentiation between Exmoor and Richmond Park ticks. These two collection points are 231km apart and despite no clear division of samples in the phylogeny, statistically the majority of alleles from Exmoor and Richmond Park are found in diverse clades in the phylogeny. This analysis has shown that there are differences that can be found within countries but only with statistical inference. One of the most significant divisions that occur between all of these geographic locations are motorways which could represent significant barriers to maintenance and reproductive hosts of ticks whom are land-based e.g. deer, rodents. The most significant are the M25, which cuts Richmond Park off from the other two sites, the M5, which isolates Exmoor. Thus, between Richmond Park and Exmoor are two barriers and only one between the New Forest.

I have seen that my mtMLST scheme does not have enough resolution to distinguish *I. ricinus* tick populations within very small geographic locations but at a distance of 231km, differences were noted within the dataset using statistical inference. This is the first indication of intra-country differentiation of tick populations and could have useful applications in public health and vector monitoring schemes.

5.4 Conclusion

Populations of *Ixodes ricinus* ticks in the UK were analysed using my mtMLST scheme and were shown to be differentiated at a resolution of 231km. There were no discernable differences, either through phylogenetic or statistical tests, between *I. ricinus* ticks collected from locations nearer to each other than this threshold distance.

6 Comparison of *Ixodes ricinus* populations from Britain and Latvia infected with *Borrelia burgdorferi* s.l. spirochaetes

In this chapter, I will explore how infection of *Borrelia burgdorferi* s.l. in *I. ricinus* ticks is linked to population structure of these ticks. Populations of ticks from Britain and Latvia that are infected with *Borrelia burgdorferi* s.l. spirochaetes were used in these analyses. These spirochaetes occupy distinct ecological niches with the different genospecies adapted to transmission by a limited host range due to a reaction with host complement. Using the mtMLST scheme devised in chapter 3, *I. ricinus* ticks from Britain and Latvia will be examined, and the relationship that *Borrelia burgdorferi* s.l. infection has with the genetic structure of the tick population.

6.1 Introduction

The spirochaete *Borrelia burgdorferi* sensu lato (s.l.) is the most common vector-borne pathogen in temperate climates, causing Lyme borreliosis (LB) in humans (J. S. Gray *et al.*, 2002). Named after the town of Old Lyme in Connecticut, USA, where it was discovered in the mid-1970s (Steere *et al.*, 1977a), this zoonotic disease system is becoming a paradigm model for studying the emergence of vector-borne diseases.

B. burgdorferi s.l. forms a species complex of 17 named species (Postic *et al.*, 1998; Masuzawa *et al.*, 2005; Chu *et al.*, 2008; Margos *et al.*, 2009; Rudenko, Golovchenko, Grubhoffer *et al.*, 2009; Rudenko, Golovchenko, Lin *et al.*, 2009). A large number of different vertebrate hosts species are known to be involved in the maintenance cycles of the microparasites. The different species of *B. burgdorferi* s.l. in Eurasia are specialized to different hosts, such as small rodents or certain bird species (Kurtenbach *et al.*, 1998c; Kurtenbach *et al.*, 2002). The relative abundance of these microparasite species is therefore a result of the structure of the vertebrate host community (Etti *et al.*, 2003). *Borrelia* spirochaetes are associated with rodents and avian species (Kurtenbach *et al.*, 2002; Klara Hanincova *et al.*, 2003a; Hanincova *et al.*, 2003b) more specifically, *Borrelia afzelii* with rodents and *Borrelia valaisiana* and *Borrelia garinii* with birds.

Bacterial infection of a host has a cost to the host through supply of nutrients for the amplification of bacterial numbers. The association of *Borrelia* with ticks has prompted surprising tick adaptations specifically associated with *Borrelia* infection. Tick receptor of outer surface protein A (TROSPA) is a tick expressed receptor, exclusively for a role in *Borrelia* transmission, which interacts with a surface expressed antigen of *Borrelia* and has been shown to be important in the survival of the spirochaete in the tick gut (Pal *et al*., 2004; reviewed in Hovius *et al*., 2007). Salivary proteins (e.g. Salp15) are also expressed by the ticks and have been shown to aid survival of *Borrelia* once inside the host (Ramamoorthi *et al*., 2005; Paveglio *et al*., 2007). *Borrelia* spirochaetes are only associated with ticks in the British Isles in approximately 5% of questing nymphs (Mitchell, unpub) and are therefore not an obligate bacterial infection essential for nutrient production and survival. The association of *Borrelia* with ticks may be due to a genetic component in ticks, not previously detected.

Blood meal analysis of questing ticks will allow an association of *Borrelia* species with specific hosts. I investigate whether *I. ricinus* ticks infected with *Borrelia burgdorferi* s.l. and ticks uninfected with this spirochaete form genetically distinct groups within the population. Using *Borrelia* infected ticks combined with the use of the tick mtMLST scheme I also aim to investigate whether there is any evidence of host preference in *I. ricinus* that can be deduced from analysis of mitochondrial genes

6.1.1 Aims and objectives

The aims of this chapter can be described as follows:

- examine *I. ricinus* ticks infected with *Borrelia* in the UK to determine whether there are differences in the infected and uninfected populations
- compare the different species of *Borrelia* found in *I. ricinus* populations in the UK and Latvia to determine whether host preference of ticks can be shown through the analysis of mitochondrial genes

6.2 Analyses of *Ixodes ricinus* populations infected with rodent and avian specialised species of *Borrelia burgdorferi* s.l.

In this section, I investigate *I. ricinus* ticks infected with different species of *Borrelia burgdorferi* s.l. and whether these differences in the pattern of infection can be explained by a genetic determinant. Using the mtMLST scheme, I analyse infected ticks from Britain and Latvia in the year 2006.

6.2.1 Phylogenetic analyses of Borrelia *infected* I. ricinus *populations from the UK and Latvia*

I. ricinus nymphs from the UK and Latvia were tested for *Borrelia burgdorferi* s.l. using PCR (2.1.4 PCR amplification of the 5S-23S rRNA (rrf-rrl) intergenic spacer in ticks) and sequenced to determine the specific species (2.1.5 Primer synthesis and DNA sequencing). Ticks positive for *Borrelia* sp. were used in this analysis (Appendix - Tick samples used for comparison of infected ticks in section 6.2 and section 6.3, p. 245). *Borrelia* positive adult and nymphs collected in Britain (n=19) and Latvia (n=43) were included in this analysis. I would have preferred to include only one developmental stage of tick but due to a small number of identified *Borrelia* infections in ticks from the two locations, this was not possible. Concatenated sequences of six mitochondrial internal gene fragments (*atp6*, *coi*, *coii*, *coiii*. *cytB* and *12s*) were aligned using MEGA 4.1 and edited by hand to minimise gaps. Phylogenies were rooted with *I. persulcatus* sequences from GenBank (accession NC_002010). Sequences used as outgroup to root the phylogeny were trimmed according to the corresponding area of analysis for each gene.

A maximum likelihood phylogeny was constructed using PhyML (Guindon *et al*., 2005) (Figure 50). The majority of the deep branches are well supported with good aLRT values above 80. There is no clear separation of the ticks according to the *Borrelia* species identified by PCR analysis. Using the same topology as in Figure 50, we have assigned the taxa to the country of origin (Figure 51). Interestingly, in this phylogeny, there is no clear structure according to the country of origin that can be determined.

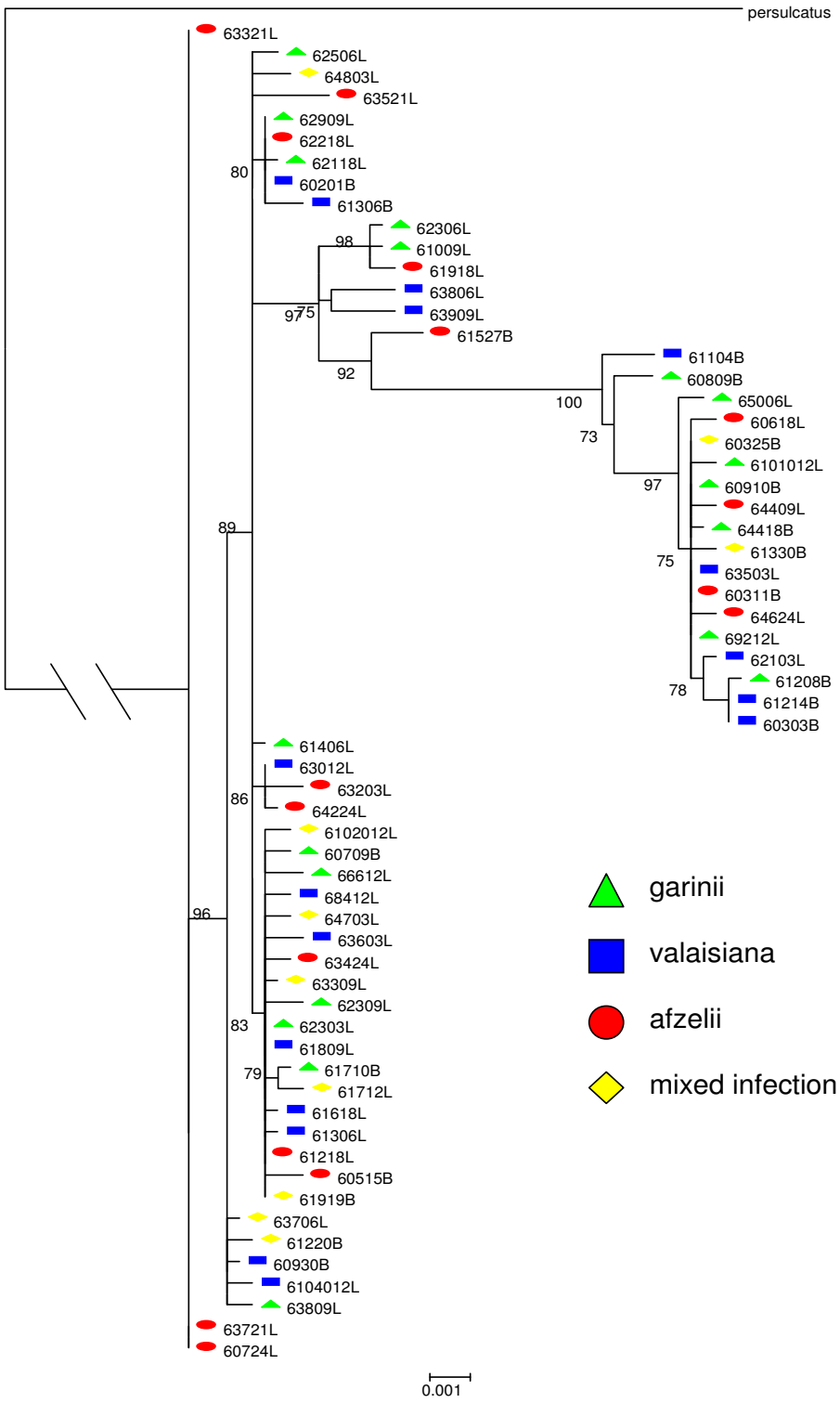

Figure 50: The evolutionary history of *Borrelia burgdorferi* s.l. infected ticks from the UK and Latvia was inferred using a concatenation of 6 mitochondrial genes and using PhyML maximum likelihood method. The optimal tree with the sum of branch length = 0.24226123 is shown. The dataset was outgrouped using *I. persulcatus*. There were a total of 3203 posititons in the final dataset. The scale bar indicate 0.1% divergence.

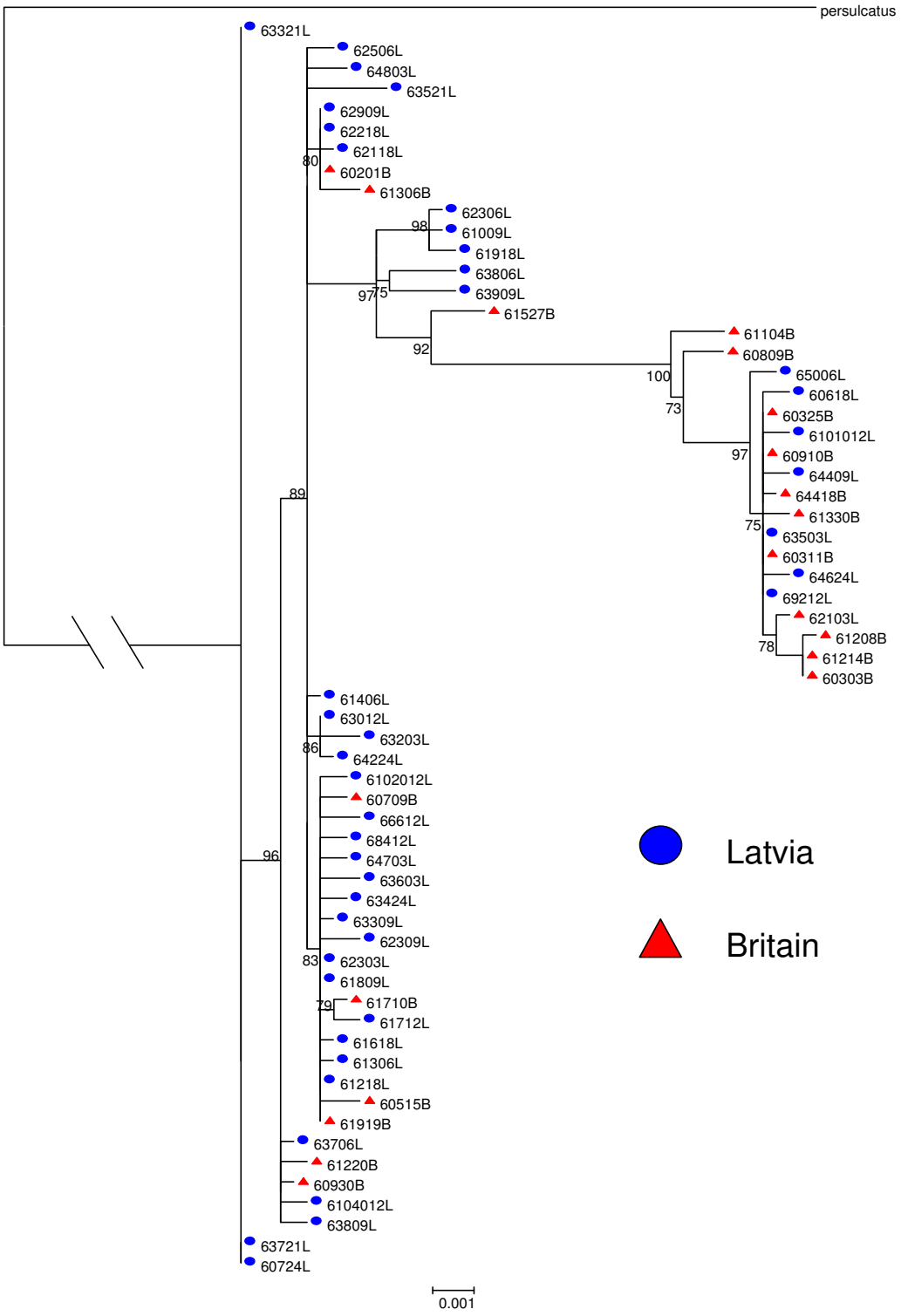

Figure 51: The evolutionary history of *Borrelia burgdorferi* s.l. infected ticks from the UK and Latvia was inferred using a concatenation of 6 mitochondrial genes and using PhyML maximum likelihood method. The optimal tree with the sum of branch length = 0.24226123 is shown. The dataset was outgrouped using *I. persulcatus*. There were a total of 3203 posititons in the final dataset. The scale bar indicates 0.1% divergence.

6.2.2 Population structure

For the mtMLST analyses, ticks from Latvia and Britain (n=43 and n=19 respectively) infected with *Borrelia burgdorferi* s.l. (Appendix - Tick samples not infected from Britain in 2006 used in section 6.3, p. 249) were studied. An analysis of the genetic variation within the samples showed 54 unique STs. Using these allelic profiles, goeBURST analyses (Francisco *et al*., 2009) were done, one was labelled according to the *Borrelia* species harboured by the tick (Figure 52) and another analysis according to the country of origin (Figure 53). The figures show the relationship of all the profiles up to a level of similarity of three loci.

The analysis shows a large main clonal complex (CC-1) and two smaller clonal complexes (CC-2 and CC-3). Six STs from the dataset differed from the remaining STs by more than three loci, otherwise known as singletons. None of the singletons was represented by more than one sample. The most common ST was ST342 with three samples representing this ST.

In Figure 52, all clonal complexes are represented by all *Borrelia* species, showing no clustering. In Figure 53, the same result was observed with all the clonal complexes showing infected ticks from both countries contributing to the topology.

Table 14: F_{ST} values calculated in Arlequin for *Borrelia burgdorferi* s.l. infected *I. ricinus* ticks from the UK. P values are shown in parentheses. Significant values are shown in bold

	afzelii	**garinii**	**valaisiana**	**mixed**
afzelii	-	-	-	-
garinii	-0.02609 (0.64865)	-	-	-
valaisiana	-0.03158 (0.70676)	-0.02945 (0.64241)	-	-
mixed	-0.03937 (0.64211)	-0.00223 (0.28631)	-0.03573 (0.54232)	-

F_{ST} values were calculated in Arlequin 3.1 (Excoffier *et al*., 2005). Molecular distances were calculated with pairwise distances. 10,000 bootstrap replicates were performed. The results are shown in Table 14. F_{ST} values calculated between the *Borrelia* infected ticks were not significant in any combination of comparison.

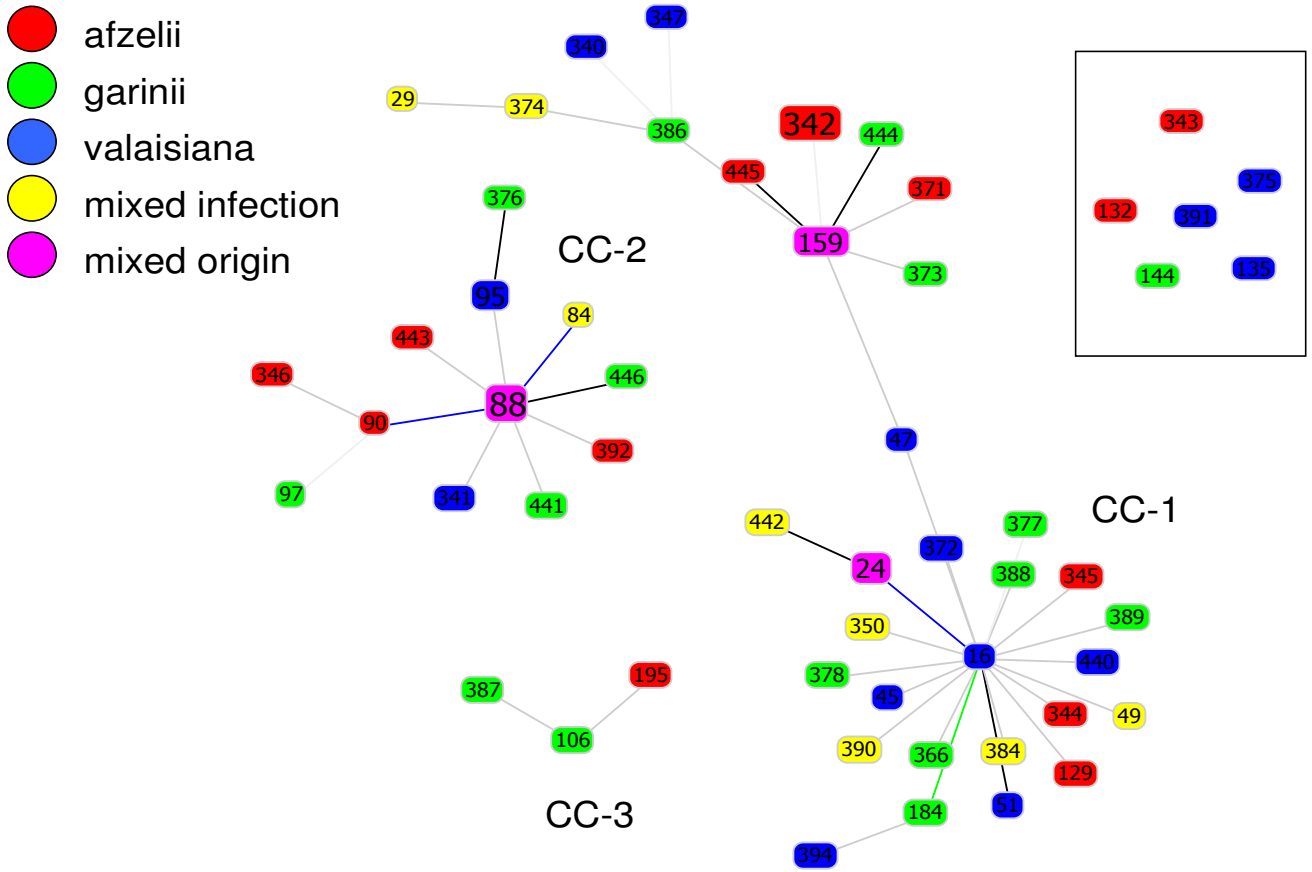

Figure 52: A goeburst analysis for *I. ricinus* in Britain and Latvia infected with Borrelia in 2006 according to Borrelia species. Mixed infections shown in yellow indicates that more than one Borrelia species was found in an individual tick. Mixed origin nodes shown in purple indicate this node has tick STs with individuals carrying two or more *Borrelia* genospecies but not necessarily from all three.

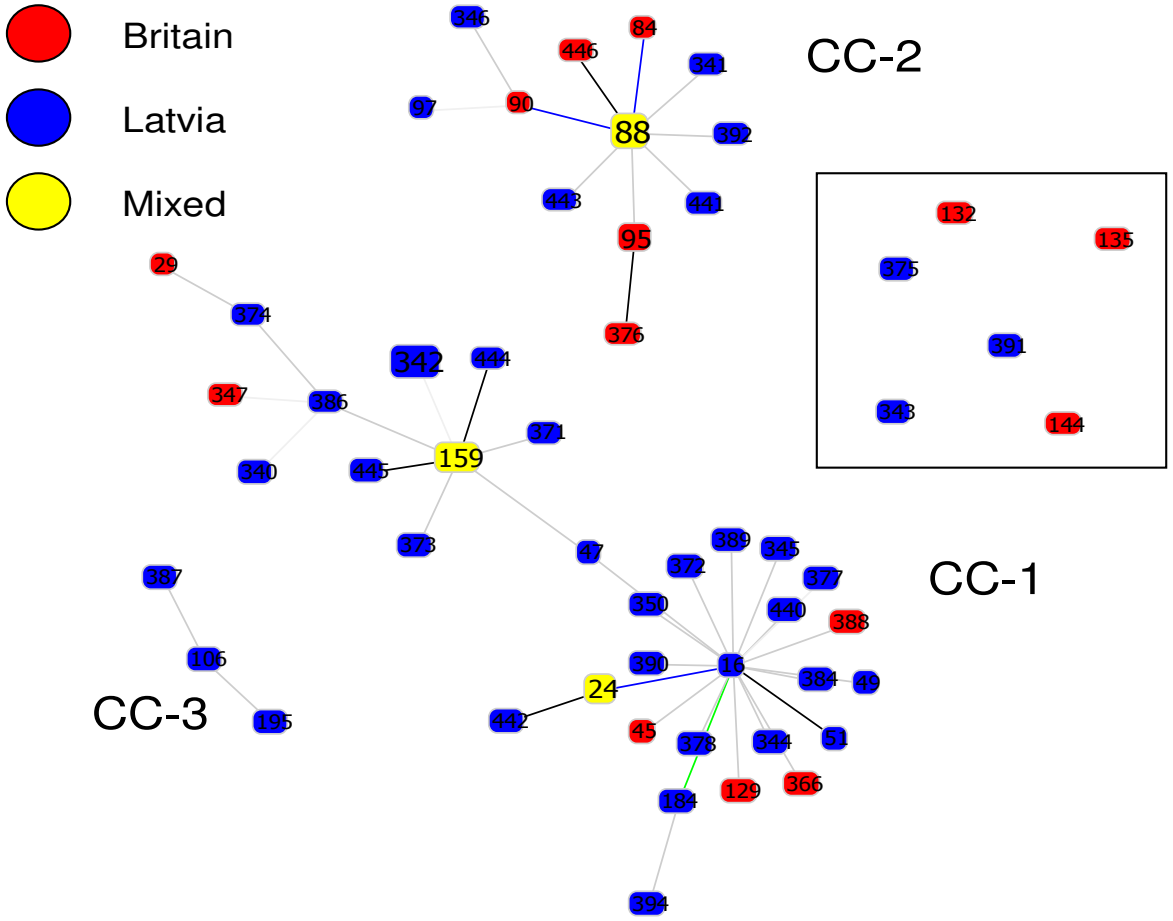

Figure 53: A goeburst analysis for *I. ricinus* in Britain and Latvia infected with Borrelia in 2006. Mixed samples were STs that were found in both Britain and Latvia.

6.3 Analyses of *Ixodes ricinus* populations infected with *B. burgdorferi* s.l. vs. un-infected ticks from Britain

This section of the chapter investigates whether *I. ricinus* infected with *Borrelia burgdorferi* s.l. are genetically distinct from *I. ricinus* ticks that do not carry the bacteria. Using my mtMLST scheme, I analyse the mitochondrial genomes of these tick populations to determine whether genetics has an effect on the proportion of the population of ticks that carry these bacterial pathogens.

6.3.1 Phylogenetic analyses of I. ricinus populations from Britain which are infected with B. burgdorferi s.l. vs. uninfected ticks

I. ricinus nymphs collected from Britain were tested for the presence of *Borrelia burgdorferi* s.l. according to the protocol in section 2.1.4 (PCR amplification of the 5S-23S rRNA (rrf-rrl) intergenic spacer in ticks). Samples were selected and divided into two categories of either negative (19 samples) for *Borrelia* or positive (19 samples) (Appendix - Tick samples used for comparison of infected ticks in section 6.2 and section 6.3, p. 245 and Appendix - Tick samples not infected from Britain in 2006 used in section 6.3, p. 249). These samples were analysed according to the previously described mtMLST protocol (Chapter 3: A Multilocus Sequence Typing Scheme for Ticks: Rationale & Design). Concatenated sequences of six mitochondrial internal gene fragments (*atp6*, *coi*, *coii*, *coiii*, *cytB* and *12s*) were aligned using MEGA 4.1 and edited by hand to minimise gaps. Phylogenies were rooted with *I. persulcatus* sequences from GenBank (accession NC_002010). Sequences used as outgroup to root the phylogeny were trimmed according to the corresponding area of analysis for each gene.

A maximum likelihood phylogeny was constructed using PhyML (Guindon *et al*., 2005) (Figure 54). The majority of the deep branches are well supported with aLRT values above 70. Throughout the tree, there is no definitive separation of the infected and uninfected groups of ticks with little clustering of ticks that share uninfected or infected status. These small clustering events may be due to low samples sizes and more sampling may resolve this.

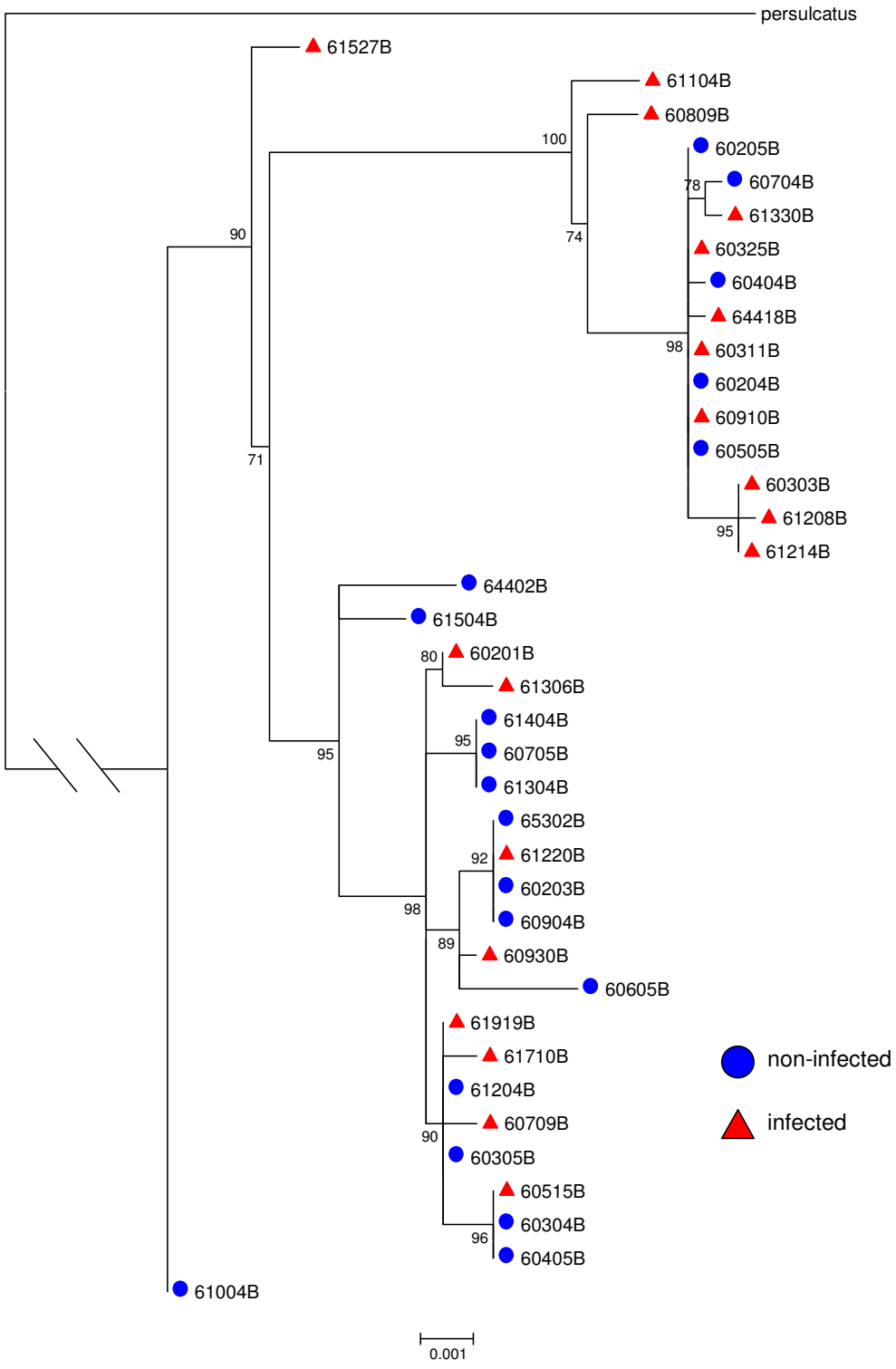

Figure 54: The evolutionary history of ticks from Britain in 2006 infected with *Borrelia burgdorferi* s.l. and ticks not infected was inferred using a concatenation of six mitochondrial genes using PhyML maximum likelihood method. The optimal tree with the sum of branch length = 0.21999503 is shown. The dataset was outgrouped using *I. persulcatus*. There were a total of 3209 positions in the final dataset. The scale bar indicates 0.1% divergence.

6.3.2 Population structure

For the mtMLST analyses, ticks from Britain infected and uninfected with *Borrelia burgdorferi* s.l. (Appendix - Tick samples used for comparison of infected ticks in section 6.2 and section 6.3, p. 245 and Appendix - Tick samples not infected from Britain in 2006 used in section 6.3, p. 249). An analysis of the genetic variation within the samples showed 25 unique STs. Using these allelic profiles, a goeBURST analysis (Francisco *et al*., 2009) (Figure 55) shows the relationship of all the profiles up to a level of similarity of three loci.

The analysis shows two large clonal complexes (CC-1 and CC-2). The clonal complexes consist of samples from both the infected and uninfected pool of *I. ricinus* ticks. Six STs represented singletons. None of the singletons was represented by more than one sample. Five STs were found to be present in both the infected and uninfected population of *I. ricinus.* The most common STs were ST129 and ST133 both with three samples representing these STs.

F_{ST} values were calculated in Arlequin 3.1 (Excoffier *et al*., 2005). Molecular distances were calculated with pairwise distances. 10,000 bootstrap replicates were performed. A value of 0.03373 (p-value = 0.17513) was determined. This is non-significant for this analysis.

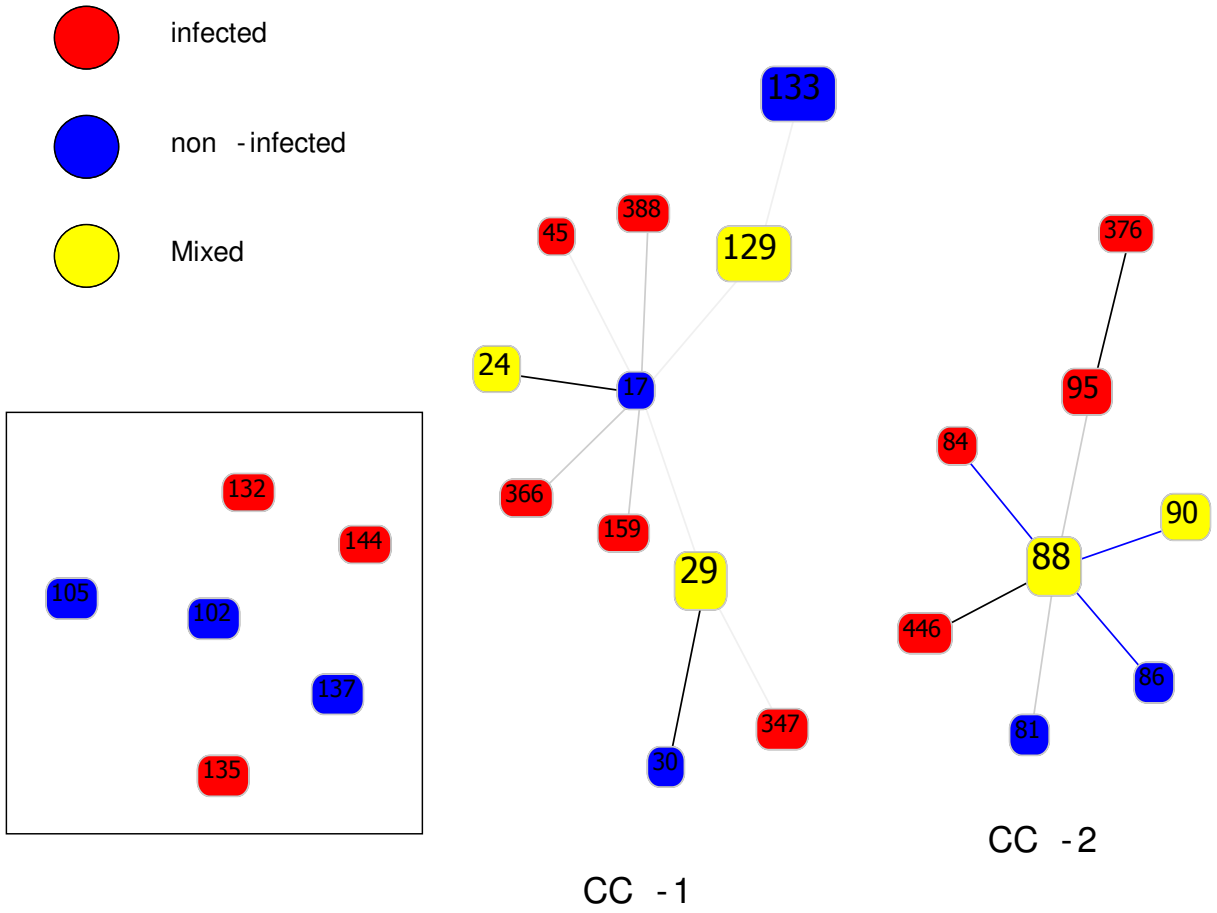

Figure 55: A goeBURST analysis for *I. ricinus* ticks infected with *Borrelia burgdorferi* s.l. and uninfected from Britain in 2006. Boxed STs show singletons

6.4 Discussion

Borrelia burgdorferi s.l. is the causative agent of Lyme Borreliosis, which is the most commonly reported tick transmitted disease in Europe. Many attempts have been made to control the spirochaetal infection through vaccines and the control of reproductive hosts for ticks and the spirochaete (reviewed in Piesman & Eisen, 2008). A genetic component of the vector ticks may lead to a differentiation of a subpopulation of ticks with increased vector competence that are more prone to contract, amplify and infect new hosts. In this chapter, I investigated whether such a diversification of ticks would be evident across Europe possibly using the mtMLST scheme devised.

The analysis has shown that the hypotheses concerning the separation of *I. ricinus* population infected with *Borrelia* and those not infected have to be rejected, with little or no genetic differentiation between the two population subtypes. It seems that *Borrelia* infection of ticks has no relationship to the efficacy of contraction of this bacterial infection. The control of tick numbers is indeed key to the incidence of *Borrelia* infection prevalence (Piesman, 2006) but the infection of *I. ricinus* with *B. burgdorferi* s.l. cannot be predicted using the mtMLST scheme.

Borrelia burgdorferi s.l. infections in ticks are highly complex and are shaped by host complement interactions with different *Borrelia* species (Kurtenbach *et al*., 2002). These interactions with host complement have lead to diversification of the *Borrelia* clade, species with separate specialised to rodent and avian species (Kurtenbach *et al*., 1998c; Kurtenbach *et al*., 2001). Ticks are known to have host preference according to their developmental stage (Hillyard, 1996), which may contribute to the isolation of species of *Borrelia.* However, in this data analysis no separation of species according to the structure of the *I. ricinus* population was found. When analysed according to the different genospecies of *Borrelia,* no differentiation of populations was found in either the evolutionary history of the concatenated genes or the statistical inference of alleles according to the pairwise comparisons of F_{ST} values.

6.5 Conclusions

Use of an mtMLST scheme to genotype I. ricinus ticks fails to provide any evidence for a relationship between tick genotype and the incidence of B. burgdorferi s.l. infection. The null hypothesis that there is no relationship between tick genotype and the presence of the spirochaete could not be rejected.

7 The differentiation of *Ixodes ricinus* ticks across Europe

7.1 Introduction

Ixodes ricinus ticks are commonly found in many areas in Northwest Europe and in most of the western Palaearctic (Hillyard, 1996). The movement of individual ticks from one geographic location to another within Europe is generally associated with deer (Ruiz-Fons & Gilbert, 2010) but the migration of birds is also known to distribute a variety of tick species (Pietzsch *et al*., 2008). Questing *I. ricinus* ticks rarely move more than a few metres horizontally (Eisen & Lane, 2002) and are therefore totally reliant on hosts they feed (i.e. deer, rodents and birds) to move them from one geographical location to another.

It has been estimated that 13 million birds migrate to Britain from Africa each year which carry an estimate of approximately 1.01 million ticks of various species (Stone *et al*., 1997). Other studies have found the proportion of *I. ricinus* ticks brought into the UK on migratory birds is 15.79% (Pietzsch *et al*., 2005). Combined with the estimate of 1.01 million ticks this translates to 159,479 *I. ricinus* ticks brought to the UK each year from Africa alone. Deer are able to feed large numbers of adult ticks (Gilbert *et al*., 2000; Ruiz-Fons *et al*., 2006) and over the last 30 years have expanded across the UK (Ward, 2005). The impact of this large number of immigrants on the genetic make-up of UK tick species is probably very limited. Half of the ticks introduced will be male and will probably play no part in the mitochondrial inheritance to offspring, and therefore will not be considered in our investigations, but will have an impact on nuclear inheritance. Mortality rates of 90% between generation and reproduction (Randolph *et al*., 2002) translate to approximately 800 female individuals surviving to reproduce. Ticks used my analyses are possible immigrant larvae and are subjected to a 99% rate of mortality before they reach reproductive age.

Ticks from Europe have been the subject of many investigations to determine the phylogeography of the populations and thus determine the effects of hosts on the distribution of the populations. Delaye *et al.* (1997) investigated the population structure of *I. ricinus* ticks in Switzerland to determine whether populations separated by significant geographical barriers formed genetically isolated populations. The populations in Switzerland were considered panmictic after the analysis of allozymic

data. Mitochondrial markers have also been used in similar investigations for the study of ticks and recently, many studies have been performed using these genes. Casati *et al.* (2007) studied *I. ricinus* from various European countries using mitochondrial markers (*coi*, *coii*, *cytB*, *12s* and the mitochondrial control region (CR)). These analyses found no evidence of population structuring, but due to the small sample size (n=26) the true population structure may not have been fully resolved.

The phylogeny of tick populations (*Ixodes scapularis*) in the United States (US) have also been scrutinised using molecular methods. Norris *et al.* (1996) used the 12S and 16S mitochondrial genes to study the *I. scapularis* populations on the eastern coast of the United States (US) as have Rich *et al.* (1995). Analyses of partial sequences from these two loci suggested that two distinct *I. scapularis* populations exist, one in the Northeast region and one in the Southeast region of the US.

This chapter performs a pilot study of *I. ricinus* ticks from several locations in Europe and the possible effects of host migration on the introduction of ticks from one geographic area to another. *I. ricinus* ticks were collected from Europe as far west as Portugal and East, Latvia, as far north as Inverness and as Southern as Switzerland.

7.1.1 Aims and objectives

The aims of this chapter can be described as follows:

- assess the genetic variation of populations of ticks from distinct geographic regions in Europe
- assign probable host migration patterns from data derived from phylogeography of ticks populations

7.2 *Ixodes ricinus* populations across Europe

The variation in *I. ricinus* populations from several locations across Europe is examined in this section. These locations are separated by significant geographical barriers (e.g. the English Channel), which may lead to the formation of distinct genetic differences between these populations that can be revealed by the analysis of mtMLST of mitochondrial genes. The migration of ticks, and whether they migrate at all in large enough numbers to create an impact on the genetic profile of geographically distant populations, is debated here.

7.2.1 Phylogenetic analyses of I. ricinus *populations from across Europe*

I. ricinus nymphs from Europe were collected from Britain, Latvia, Germany, Portugal and Switzerland (Appendix - Collection Site Maps, p. 167) from 2002, 2003, 2007 and 2008 (Appendix - Tick samples from Europe used in section 7.2, p. 251) and were analysed according to the method indicated in the mtMLST scheme previously described (Chapter 3: A Multilocus Sequence Typing Scheme for Ticks: Rationale & Design). Ticks samples were split into two analysis categories: 2002 and 2003 collections (n = 54) and 2007 and 2008 collections (n = 170). In this analysis, only nymphs were used to ensure that the ticks were from the same generation. Using six concatenated mitochondrial gene fragments (*atp6*, *coi*, *coii*, *coiii*, *12s*, *cytB*) sequences were aligned using MEGA 4.1 and edited by hand to minimise gaps. Phylogenies were rooted with *I. persulcatus* sequences from GenBank sequence NC_002010. Sequences used for outgrouping were trimmed according to the corresponding area of analysis for each gene.

ML phylogenies were constructed using PhyML (Guindon *et al.*, 2005) for 2002/2003 (Figure 57) and 2007/2008 (Figure 56). The majority of deep branches are well supported with good aLRT values above 75. No branches were observed to have an aLRT value below 70 in either analysis. The 2007-2008 analysis of ticks from around Europe indicates a largely British clade distinct from the other clade, which comprises mostly of Continental European *I. ricinus* samples. Within the Continental European clade there was no distinct clustering of ticks from geographical regions.

The 2002-2003 analysis of samples from Portugal and Latvia showed no clustering of ticks from these two disparate geographical locations. Despite these samples coming

from locations that are separated by a distance of approximately 3,150km, no genetic differentiation of samples can be seen in the phylogeny, whereas samples compared in the 2007-2008 analysis e.g. Britain and Germany, are separated by 665km and have been differentiated according to the phylogenetic analysis.

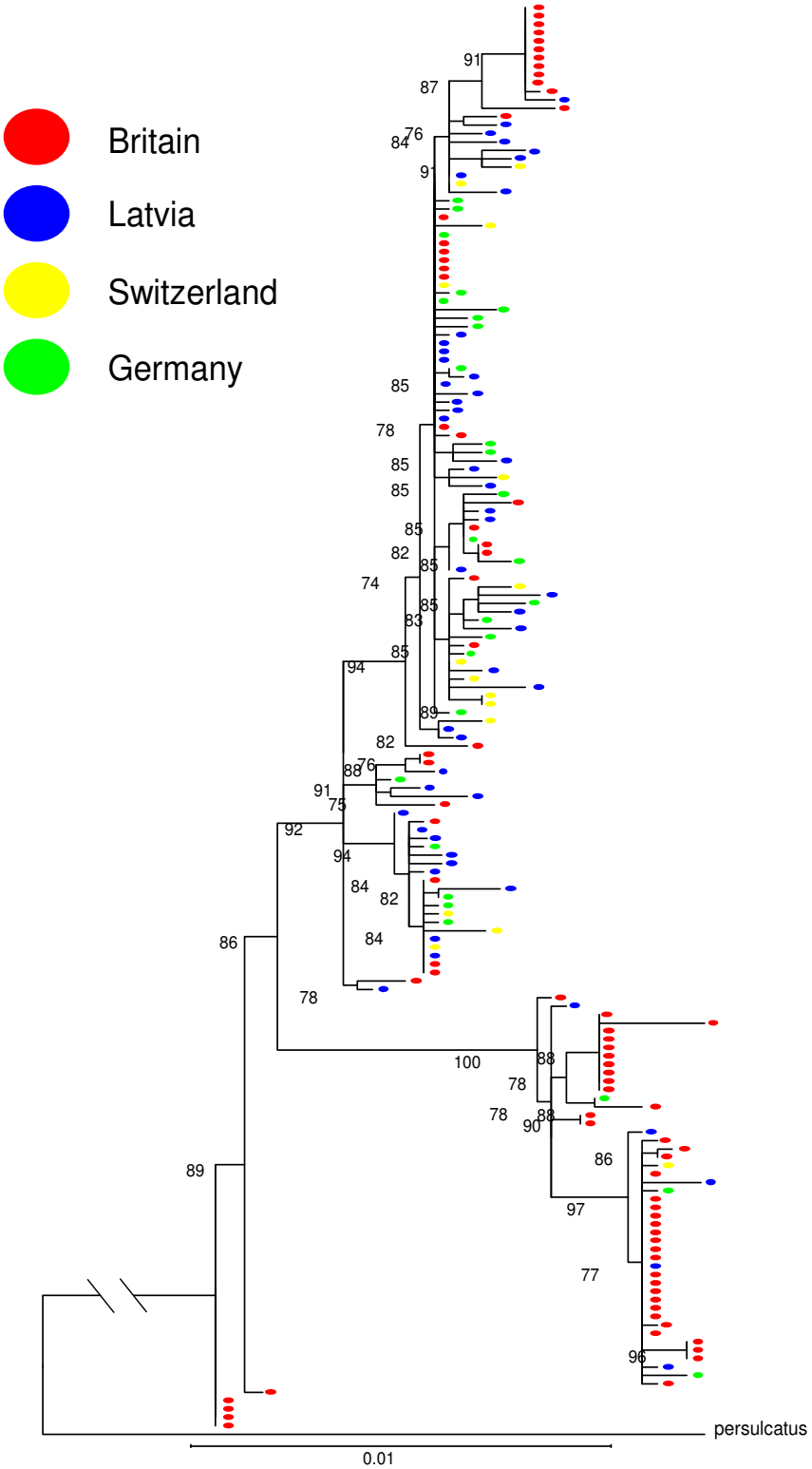

Figure 56: The evolutionary history of *I. ricinus* ticks from Europe collected from 2007 and 2008 from Britain, Latvia, Switzerland and Germany. The phylogeny was inferred using a concatenation of 6 mitochondrial genes and using PhyML maximum likelihood method. The optimal tree with sum of branch length = 0.42884468 is shown. The dataset was outgrouped using *I. persulcatus*. Taxon labels were deleted. There were a total of 3218 positions in the final dataset. The scale bar indicates 1% divergence.

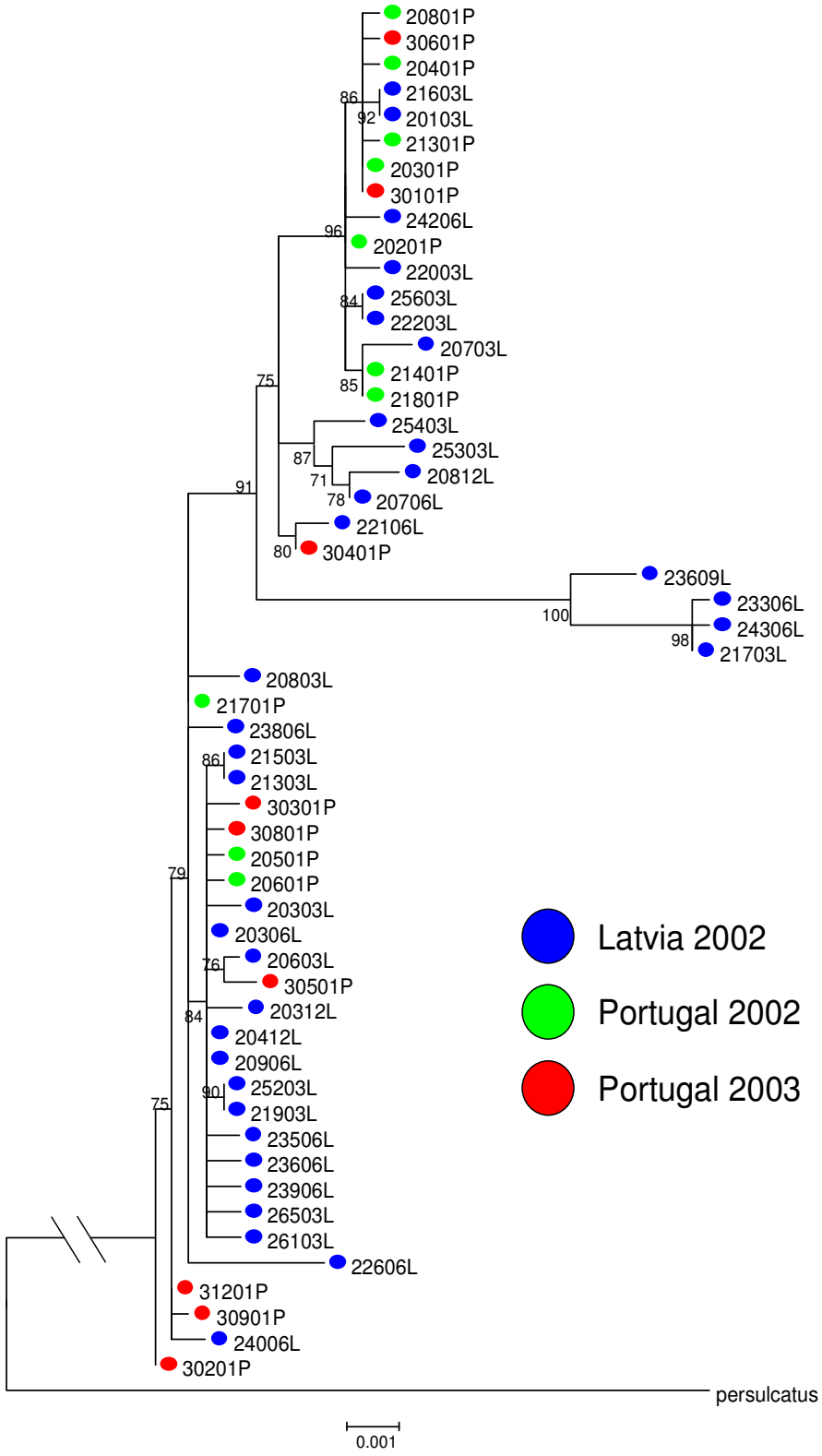

Figure 57: The evolutionary history of a European *I. ricinus* population collected in 2002 and 2003 from two locations (Portugal and Latvia) was inferred using a concatenation of six mitochondrial genes using PhyML maximum likelihood method. The optimal tree with the sum of branch length = 0.22801980 is shown. The dataset was outgrouped using *I. persulcatus*. There were a total of 3221 positions in the final dataset. The scale bar indicates 0.1% divergence.

7.2.2 mtMLST analysis of I. ricinus *populations from across Europe*

For the mtMLST analyses, ticks from Europe were split into two groups according to their year of collection. For the first analysis, ticks from (79 from Britain, 49 from Latvia, 27 from Germany and 15 from Switzerland) four locations in Europe (Appendix - Tick samples from Europe used in section 7.2, p. 251) were studied. For the second analysis, ticks from (35 from Latvia in 2002, 10 from Portugal in 2002 and 9 from Portugal in 2003) two locations (Appendix - Tick samples from Europe used in section 7.2, p. 251) were studied. Using the allelic profiles derived from the analysis of six mitochondrial genes (*atp6*, *coi*, *coii*, *coiii*, *12s* and *cytB*) goeBURST analyses (Francisco *et al*., 2009) were performed for 2007-2008 samples (Figure 58) and 2002-2003 samples (Figure 59) showing the relationships of all the profiles up to a level of similarity of three loci.

The 2007-2008 analysis shows one large main clonal complex (CC-1), three other main clonal complexes (CC-2, CC-3 and CC-4) and three minor clonal complexes (CC-5, CC-6 and CC-7). Four STs from this dataset differed from the remaining STs by more than three loci, and were labelled as singletons. The STs were grouped according to their country of origin. STs that were found in more than one country were also distinguished as originating from Continental Europe (no samples from Britain) or European samples (STs found in Britain and any other country from Continental Europe). CC-1 forms the majority of the population structure in this European population sample. ST17, which forms the founder of this clonal complex, was found in both the UK and in Continental counties. All clonal complexes in this analysis were represented by more than one geographic location. Some clonal complexes are dominated by a majority of samples from one country, i.e. CC-2 is dominated by samples from Continental Europe, and the ST17 cluster is dominated by samples not from Britain. The network of nodes indicates that ST18 is a SLV of ST17 and has formed a succession of related STs with the terminal node ST302 originating in Britain. The majority of British samples in this clonal complex were found in relation to this terminal node ST302. The majority of STs in this analysis were represented by a single sample. The most common ST was ST88 with 13 samples representing this ST.

The 2002-2003 analysis shows two main clonal complexes (CC-1 and CC-2) and two minor clonal complexes (CC-3 and CC-4). Four STs differed from the other samples in the dataset by more than three loci, and were labelled as singletons. The majority of

STs in this analysis were represented by only one sample. Seven STs were represented by two samples (ST1, ST15, ST17, ST60, ST106, ST108 and ST293). Clonal complexes 1-3 are represented by more than one geographic location. CC-4 is comprised solely of samples from Latvian samples collected in 2002. The clonal complexes show no signs of succession from one year to another in the Portuguese samples.

Table 15: F_{ST} values calculated in Arlequin for *I. ricinus* populations collected in 2007-2008 in different geographic locations in Europe. P values for the statistics are shown in parentheses. Significant values are indicated in bold.

	Britain	**Latvia**	**Switzerland**	**Germany**
Britain	-	-	-	-
Latvia	**0.17311 (0.0000)**	-	-	-
Switzerland	**0.18969 (0.0002)**	-0.02149 (0.86318)	-	-
Germany	**0.16857 (0.0003)**	-0.01423 (0.86021)	-0.02510 (0.83160)	-

F_{ST} statistics were calculated in Arlequin 3.1 (Excoffier *et al.*, 2005) for both sets of analyses. Molecular distances were calculated with pairwise differences. 10,000 bootstrap replicates were performed. The results are shown in Table 15 (2007-2008) and Table 16 (2002-2003). F_{ST} values calculated between populations. Values that were not significant were not considered further. The significant values that were observed were found between the British population and all other populations from Continental Europe. All F_{ST} values considered between Britain and the rest of the locations in Continental Europe (Table 15) indicate moderate levels of genetic differentiation.

Table 16: F_{ST} values calculated in Arlequin for *I. ricinus* populations in from Portugal and Latvia collected in 2002-2003. P values for the statistics are shown in parentheses. Significant values are indicated in bold.

	Latvia	**Portugal**
Latvia	-	-
Portugal	0.03912 (0.06207)	-

F_{ST} values for the 2002-2003 analysis (Table 16) were not significant and no inference of genetic differentiation can be derived from this data.

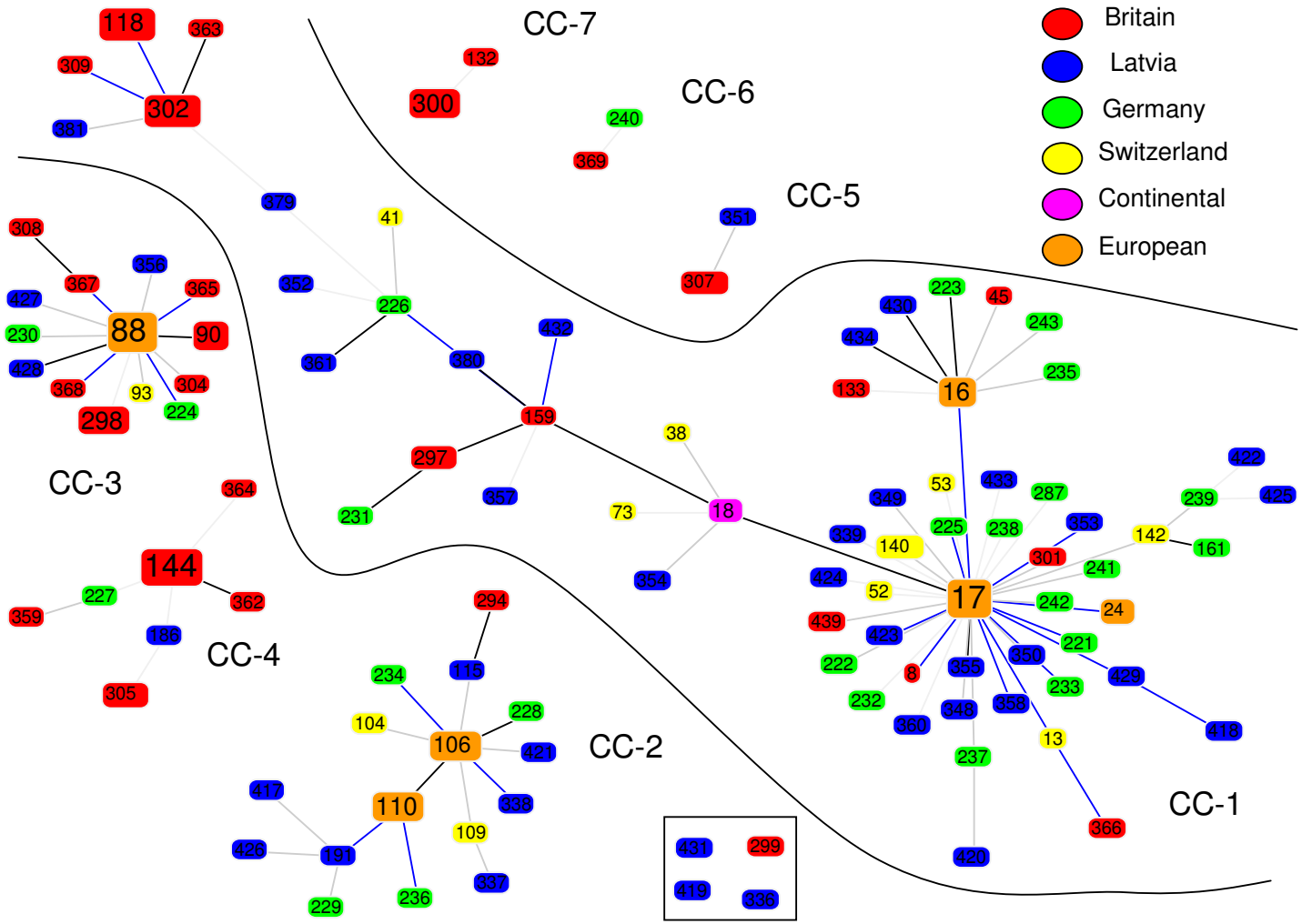

Figure 58: A goeBURST analysis for *I. ricinus* ticks collected from Britain, Latvia, Germany and Switzerland from 2007 and 2008. Boxed STs show singletons. Orange nodes indicate STs found in ticks from Continental Europe. Pink nodes indicate STs from Britain and at least one other Continental European country.

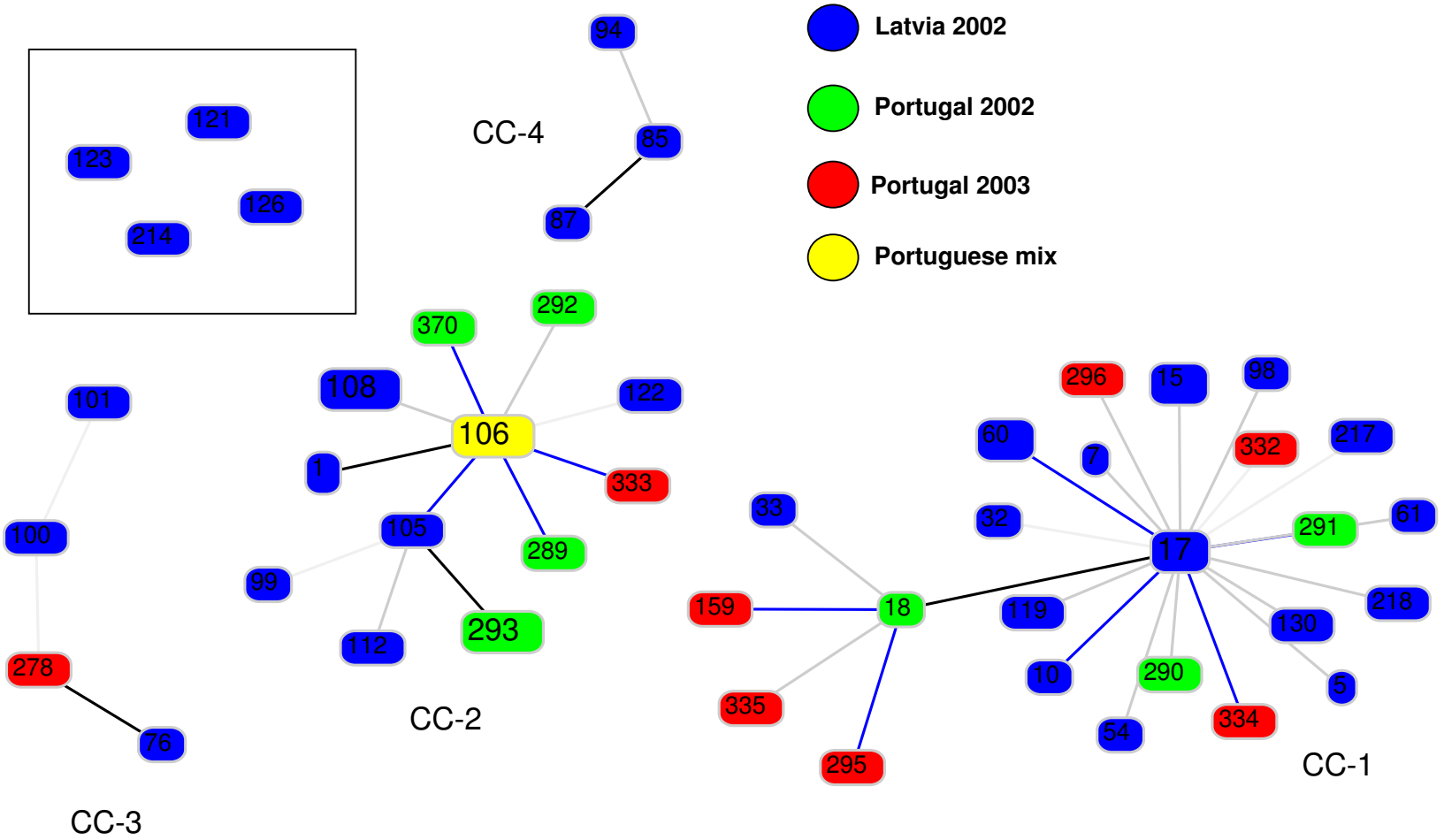

Figure 59: A goeBURST analysis for *I. ricinus* ticks collected from Latvia and Portugal in 2002 and 2003. Boxed STs show singletons. Yellow nodes show a mixture of STs from both 2002 and 2003 from Portugal. No STs were found in both Latvia and Portugal.

7.3 Discussion

Using the novel typing scheme based on MLST principles (Maiden *et al.*, 1998; Enright & Spratt, 1999; Spratt, 1999) we have typed *Ixodes ricinus* ticks from several locations across Europe to understand the population structure of this ectoparasite. *I. ricinus* ticks from Britain, Germany, Latvia, Switzerland and Portugal were analysed using mitochondrial genes to examine whether these geographically disparate populations showed signs of geographic clustering. These clustering patterns of ticks are highly dependent on the hosts upon which these ticks feed as ticks themselves do not move significant distances (Eisen & Lane, 2002), therefore, any geographic structuring of these ticks is solely due to the movements of birds, rodents and deer. The British Isles are separated from the rest of Continental Europe by a significant body of water, the English Channel. Despite migratory birds possibly introducing large number ticks from other parts of Europe, bird migration does not lead to panmixis throughout Europe of *I. ricinus*. My investigations have shown that *I. ricinus* ticks from the British Isles are distinct from the rest of *I. ricinus* ticks, found in Continental Europe.

While ticks from the British Isles were found to be genetically distinct from *I. ricinus* ticks from Continental Europe, ticks from geographically isolated populations (e.g. Portugal and Latvia) within Continental Europe could not be distinguished with the methods used in our analyses, probably due to low samples numbers. Casati *et al*. (2007) also used mitochondrial genes to perform a study of *I. ricinus* ticks from locations across Europe (Switzerland, Italy, Austria, Denmark, Sweden and Finland) and found no evidence of geographic clustering. Another study by Delaye *et al*. (1997) used allozymes and also found no evidence of geographic structuring, although these sites were within a 3000km^2 area in a small region of Western Switzerland and genetic differentiation would be difficult to determine in such a small region due to local migration of hosts such as rodents and deer.

Using the results found in our analyses we can only hypothesise their meaning and possible interactions that have occurred in order for these results to transpire. One possibility could be that land based animals acting as carriers for ticks (e.g. deer, see Ruiz-Fons & Gilbert, 2010) are responsible for the spread of ticks to all geographic regions that are linked via land. The extent of the movement of ticks through these means, results in a panmictic population. Bird migration has been previously earmarked as a method of introducing ticks from other regions in Europe (Pietzsch *et*

al., 2008). Bird migration to and from the UK occurs at many different times during the year depending on different factors, but mostly involving the availability of food. Insectivorous birds (especially young birds that require extra nutrients for growth) alight in the UK during the summer months to take advantage of the brief window of insect abundance (Wernham *et al*., 2002). Also driven by food but in different conditions, waterfowl (e.g. Bewick's Swan (*Cygnus colombianus*)) migrate to the UK from arctic regions as the lakes and ground they inhabit have frozen, restricting access to food (Wernham *et al*., 2002). The seasonal dynamics of ticks in the UK (discussed in Kurtenbach *et al*., 2006) make the most likely export of ticks form the UK to occur during spring and early summer when tick numbers are at their peak. One of the most widespread birds known to harbour ticks is the common Blackbird (*Turdus merula*) (Comstedt *et al*., 2006), which migrates predominantly through the UK in mid-October (Taylor, 1984; Wernham *et al*., 2002). At this time of year tick numbers in the UK are declining rapidly, and therefore the impact of ticks entering Continental Europe from the UK, would be very small. At the peak of tick questing activity in Continental Europe, (late spring/early summer) Blackbird populations are sedentary. Most documented evidence of the movements of Blackbirds has been recovered in the months October to December (Wernham *et al*., 2002). The impact of birds in the migration of ticks to and from the UK seems to be limited. In the rare instances that ticks do alight in the UK, the chances of survival to reproductive maturity is slight (Randolph *et al*., 2002).

The findings in this chapter have shed light on why previous studies such as those performed by Casati *et al*. (2007) and Delaye *et al*. (1997) have not previously found any evidence of geographic clustering. Further research into this phenomenon needs to be addressed with studies concentrating on the relationship of ticks from the UK and *I. ricinus* populations in common migratory destinations of passerine birds. These species are the most frequent carriers of ticks (Anderson *et al*., 1986; Comstedt *et al*., 2009; Dubska *et al*., 2009) and most likely to be able to move populations from one geographic location to another.

7.4 Conclusions

British populations of *I. ricinus* ticks are genetically distinct when compared to *I. ricinus* populations collected from Continental Europe. No evidence of geographic clustering was found in geographically distinct populations within Continental Europe suggesting a panmictic population of all land linked countries. The UK formed the only distinct population, which could be due to no land-based animals capable of crossing the English Channel and the limited impact of birds to distribute ticks from one geographic location to another. More research is needed on the impact that birds and land-based animals have in the distribution of *I. ricinus* ticks.

8 Overall discussion and summary

8.1 Summary of findings

The most important elements and findings of my work can be summarised as follows:

- primers were designed for six mitochondrial genes (*atp6*, *coi*, *coii*, *coiii*, *cytB* and *12s*) for *Ixodes ricinus* ticks and using MLST principles, an mtMLST scheme was devised
- evidence of clonal mitochondrial inheritance from mother to offspring in *I. ricinus*
- studies of mitochondrial genes provided evidence that *I. ricinus* populations in Britain were genetically distinct from ticks in Continental Europe
- *I. ricinus* ticks analysed from three years in Britain showed moderate levels of genetic differentiation between ticks from 2006 and 2008; the nymphs from 2008 were likely to be progeny of nymphs in 2006
- statistical analysis of ticks from Richmond Park and Exmoor showed moderate levels of genetic differentiation possibly due to land based hosts of ticks isolated by major roads and therefore, hosts such as deer, are unable to transport ticks to new regions
- no evidence of host association of *Borrelia burgdorferi* s.l. spirochaetes with *I. ricinus* ticks was identified using the mtMLST scheme
- *Borrelia* infected *I. ricinus* ticks seem not to have a link to the mitochondrial population structure
- the patterns found in mitochondrial genes suggest that bird assisted migration of ticks does not have a profound effect on the genetic structure of *Ixodes ricinus*

8.2 Population structure of *Ixodes ricinus* ticks in Europe

In this thesis, I have documented the development and implementation of a novel system for typing *Ixodes ricinus* ticks based on the principles of MLST (Maiden *et al.*, 1998; Enright & Spratt, 1999; Spratt, 1999) using mitochondrial genes. As proof-of-principle, *I. ricinus* populations from Britain and Latvia were examined using this scheme in order to ascertain the degree of population differentiation and geographical clustering. My data demonstrate that *I. ricinus* ticks from Britain and Latvia form discrete populations, which challenges the view that the entire range of *I. ricinus* are panmictic (Delaye *et al.*, 1997; Casati *et al.*, 2007).

The majority of samples have been collected in the UK (more specifically in Southern England) and Latvia. In the proof-of-principle study, these collections were sufficient to provide evidence that *I. ricinus* populations from Latvia and Britain were genetically diverse. Ticks are collected from habitats with specific conditions, due to their requirements for hosts and microclimatic conditions (Eisen & Lane, 2002). For my work, I relied on samples that were provided from collaborators from Latvia and other European countries and, therefore, for the interpretation of my results, I have to take into account that these may not represent the entire population. This presents challenges when assessing whether a sample-set is representative of the entire population. The implementation of sampling across a large geographic area in all areas that ticks are found would require a huge research effort as has been previously performed in the USA (Hoen *et al.*, 2009). This was a concerted effort of the Centre for Disease Control and several other laboratories. It would be worth making such an effort for the area that would represent the entire range of *I. ricinus*, but this could be done only through combined efforts of many research institutions, perhaps as represented by the EDEN project (EDEN, 2010). My studies have been limited by the availability of ticks collected from collaborators, and in the future, a more concerted effort in different regions of Europe may identify *I. ricinus* sub-species associated with specific ecological niches.

Using the devised scheme, other sample-sets were studied. I collected ticks from Britain and Latvia over several years and using this scheme, I examined the population for signs of change in the genetic profile. Significant levels of mutation in mitochondrial genes are unlikely to occur in three to five years (Brown *et al.*, 1979; Denver *et al.*, 2000) and therefore the changes in the sequence types seen in the temporal data

would be due to migration of sequence types from one geographical region to another. I also studied the population structure of ticks collected from Southern England and ticks collected from Latvia and Britain infected with *Borrelia burgdorferi* s.l. spirochaetes. No significant levels of genetic differentiation were found in either the temporal analyses or the study of infections associated with these ticks. My investigations have focussed on mitochondrial genes and therefore the associations that I have tried to draw from these bacterial infections may only be linked with nuclear genes, such as those that encode antigens.

Ticks are considered generalist feeders as they will parasitize all land vertebrates apart from amphibians (Kierans *et al.*, 1999). In the UK since 1000AD, sheep have formed an integral part of the economy and lifestyle of British people and Britain has been recognised as one of two of the most important producers of sheep in the Western world (Ensminger & Parker, 1986). Sheep have been indicated as important hosts of *I. ricinus* on moorland and upland pastures (Ogden *et al.*, 1997) but during recent years the densities of sheep in the UK have decreased due to imported meat from other major producers of lamb, such as New Zealand. It has been suggested that nymphal *Ixodes ricinus* in Britain do not feed readily on rodents (Randolph & Storey, 1999; Seelig, unpublished), despite high levels of exposure and opportunity. Ticks in the UK may have developed a host feeding preference for sheep resulting from ancient farming practices. These host preferences have possibly translated into divergent mitochondrial DNA sequences that I have now compared.

The pattern of association of *Borrelia* species and hosts, due to the host complement system (Kurtenbach *et al.*, 1998b; Kurtenbach *et al.*, 1998c; Kurtenbach *et al.*, 2002), allowed investigation of the pattern of tick feeding in comparison to the genetic profile of these ticks. Unfortunately, sheep that have been previously exposed to *B. burgdorferi* s.l. infections have been shown not to sustain systemic *Borrelia* infections (Ogden *et al.*, 1997) and therefore could not be determined as the last blood meal using these methods. Analysing the association of *I. ricinus* ticks in context of mitochondrial DNA did not discriminate between feeding behaviour, which reinforced the notion of ticks as generalist feeders.

The results from our sample-sets showed that the British Isles were isolated from Continental Europe but the different geographic regions analysed from Continental Europe were shown to be panmictic. The hosts that *Ixodes* feed on are responsible for the migration of ticks as questing is a sedentary process relying on ambush and therefore do not need to, or are able to, travel large distances (Eisen & Lane, 2002).

These hosts therefore, have a pivotal role in the tick population structure. *I. ricinus* ticks are generalists and feed on a variety of animals (J. S. Gray, 1998) which is likely to result in many different distributions at different rates.

Another consideration that requires further research is whether the *I. ricinus* ticks from the British Isles have formed a cryptic species. Clades of *I. ricinus* ticks seen in chapter three analyses may represent different species. In the mismatch distribution analyses of Latvian and British samples two distinct peaks were seen. A large proportion of the ticks analysed were designated as having few mismatches when compared with all other ticks. A second peak of mismatches was also seen showing a high proportion of mismatches. Few mismatches were seen bridging these two peaks. Assuming breeding between these two geographically distant clades one would expect hybrid ticks with intermediate levels of mismatch to be seen in these distributions. A hypothesis that could be considered would be that these two clades could be distinct species. Further analyses of ticks from Continental Europe and Britain could help to determine whether these assumptions are likely.

Ticks are very sensitive to small changes in humidity and temperature (Eisen & Lane, 2002) and moving from one location to another in a small amount of time might make them more prone to desiccation and therefore death. The gradual movement of ticks by rodents and deer may be advantageous for ticks to adapt to local microclimates. Ticks in continental Europe would be able to move from one geographic location to another on land-based animals unrestricted but unable to migrate across any body of water such as the English Channel. Deer are the reproductive hosts of *I. ricinus* ticks and are known to be able to move ticks over large distances (Ruiz-Fons & Gilbert, 2010) and combined with their ability to harbour large numbers of ticks (Gilbert *et al.*, 2000; Ruiz-Fons *et al.*, 2006) make these ungulates pivotal in the distribution of *Ixodes*.

It is likely that the English Channel presents a substantial barrier to tick movement. There is evidence that immature tick stages can be carried long distances by migratory or part migratory birds (Ogden *et al.*, 2008; Pietzsch *et al.*, 2008), and these are probably the host species regularly able to introduce ticks into Britain. However, the limited number of putative migrants noted in these studies suggests that even when ticks are introduced, they rarely establish new populations. Certainly, if the migration of birds had a significant contribution to the migration of ticks then all *I. ricinus* ticks, from both Britain and the rest of Europe would have the same set of allelic profiles, shown in these studies to be false.

A large number of birds are known to carry *I. ricinus* ticks, many of which are migratory passerine birds (Comstedt *et al*., 2006; Taragel'ova *et al*., 2008; Dubska *et al*., 2009). One of the most common migrants discussed by Comstedt *et al*., (2006) is the European Robin (*Erithacus rubecula*). Most of the Robins which are hatched in Britain spend their lives within a kilometre of their natal site (Wernham *et al*., 2002). Recovery exercises of this species in Britain from 1990 to 1997 showed 2.3% of the birds captured were from foreign countries. Other species of migrant birds studied in Comstedt et al., (2006) showed low levels of migration to and from Britain with only 2.8% of the common blackbirds (*Turdus merula*) captured were from Continental Europe. With the majority of blackbird migration occurring in October from Britain (Taylor, 1984) when nymphal numbers are declining (reviewed in Kurtenbach *et al*., 2006) the impact of bird migration on the dispersal of *I. ricinus* may in fact be rather limited. Nevertheless, considering the large numbers of migrants that visit Britain each year it is likely that some ticks survive the journey to Britain.

The survival rate of each tick stage is poor, with 90% of a generation failing to reach the next developmental stage (Randolph *et al*., 2002). Considering these survival rates, introductions of ticks from the continent to Britain would typically fail. Only in rare circumstances would an introduced tick survive to reproductive age. Randolph *et al*., (2002) estimate that the survival of only 10 adult ticks would on average require an introduction of 1,000 larvae.

An investigation by de Meeus *et al*., (2002) agrees that birds do not have a significant effect on the dissemination of ticks over large distances as was previously thought. Their analyses of ticks from Switzerland and Tunisia showed they were genetically isolated, but ticks within Switzerland were homogenous. However, when analysing the males and females independently, the males were more homogenous and were therefore more prone to dispersal. This could be due to host preferences exhibited by males and females. Although this would seem unlikely due to the mating behaviour of *I. ricinus* (mating occurs on the host during feeding).

Generally, only females are able to impart their mitochondrial genetic material to the next generation (Breton *et al*., 2007). Bi-parental inheritance of mitochondria is rare in many species and unproven in ticks and unlikely to influence the result significantly (Dimauro & Davidzon, 2005). Indeed, our investigations of the genetic profile of the progeny of ticks have provided some evidence suggesting clonal maternal inheritance. Male ticks surviving to reproductive age would not influence the mitochondrial genetic structure of the future population. The study of nuclear genes could possibly allow for

the study of this phenomenon but high levels of recombination of nuclear genes could make this challenging to reach meaningful conclusions about the descent of the genes studied.

My preliminary studies of the inheritance of mitochondria from mother to offspring showed no signs at paternal leakage that has been indicated in many other organisms (Kondo *et al.*, 1990; Meusel & Moritz, 1993; Eyre-Walker, 2000; White *et al.*, 2008; Wolff & Gemmell, 2008; Pearl *et al.*, 2009). Paternal leakage is acknowledged as rare so I would not dismiss this mechanism of mitochondrial gene transfer from *I. ricinus* until a focussed study had been performed. Incidences of heteroplasmy in ticks could well be found with different genes indicating different passages of descent.

The differences of the British ticks from the rest of Continental Europe raises a lot of questions about how the ticks in Britain diverged from the rest of ticks in Europe. Many different circumstances could have arisen to form the British population. One could consider 12,000 years ago, during the last glacial maximum when the UK was joined to the rest of Europe via a land bridge called Doggerland (Mix *et al.*, 2001; Searle *et al.*, 2009) *Ixodes ricinus* populations were likely to move freely between locations. When sea levels rose, the ticks still in the British Isles were cut off and from that time to the present day diverged creating two discrete populations.

The distributions of many species have been described in the context of climatic fluctuations in order to account for the current distribution and range (Webb & Bartlein, 1992). During the LGM approximately 20,000 years ago (Mix *et al.*, 2001) the temperate regions in Europe were thought to be restricted to the Iberian, Italian and Balkan peninsulas (Bennett *et al.*, 1991; Taberlet & Bouvet, 1994; Hewitt, 1996). The contraction of these populations into peninsulas would be expected to have reduced the level of genetic variation within the peninsulas but increased genetic variation between populations (Hewitt, 1996; Rowe *et al.*, 2004). As glacial conditions and tundra habitats were overtaken by temperate habitats as temperatures increased, populations of animals, such as those that harbour ticks and ticks, would have expanded into central Europe. Studies using mitochondrial DNA have found conflicting evidence denoting either homogenous populations (Valdiosera *et al.*, 2007) or differentiation of current populations (Brito, 2005). In my data, I have seen support for the hypothesis of a population of ticks on mainland Europe that is panmictic with a refuge in England that has shown evidence of genetic heterogeneity. British ticks and Latvian ticks were found in both regions implying that movement occurs between these regions, albeit at a very low rate. Considering the large migration of birds to and from

the UK each year (Taylor, 1984), this low level of migration between Latvia and Britain is unrealistic unless other factors affecting the survival rate are acting upon the ticks that find their way to new regions through feeding on migratory hosts.

MLST has been shown to be a powerful technique to study bacterial populations (Enright & Spratt, 1998; Maiden *et al.*, 1998; Enright *et al.*, 2000; Feil *et al.*, 2000b; Maiden, 2006; Margos *et al.*, 2008; Margos *et al.*, 2009). The sharing of data between laboratories according to set schemes has allowed for global initiative of pathogenic microorganisms. My scheme in particular uses simply processed environmental samples, which makes it accessible to most laboratories in terms of equipment and cost of processing. Additionally, the devised primers amplify products to such a degree that allows direct sequencing from the PCR sample. However, there is a possibility of contamination of samples if handling large numbers but this can be controlled using rigorous cleaning routines.

Mitochondrial genomes are generally considered to accumulate mutations approximately ten times faster that nuclear genes (Brown *et al.*, 1979; Taanman, 1999) which limits their resolution when considering deep phylogenies. Housekeeping genes traditionally used in MLST schemes are typically slower evolving than the genes selected for this scheme therefore changing the focus of analysis from ancient clustering patterns of samples to more recent clustering events. Both methods have their merit and need to be applied accordingly dependent upon the era of study.

Wolbachia have been found in both *I. scapularis* (Benson *et al.*, 2004) and *I. ricinus* (van Overbeek *et al.*, 2008) at a low prevalence but as many studies have found no evidence of Wolbachia (Niebylski *et al.*, 1997; Noda *et al.*, 1997; Hirunkanokpun *et al.*, 2003) the effects on the population may be negligible. These bacteria may have a profound effect on the sex ratio of tick populations due to reproductive changes that lead to skewed sex ratios by cytoplasmic incompatibility (Stouthamer *et al.*, 1999). *Wolbachia* infection in tick populations in Europe may lead to one ST sweeping across through the population but more research is needed to determine the effects if any.

Overall, my investigations suggest that the probability of ticks arriving in the UK from migratory birds is improbable, but not impossible. Conditions in the region, that was to become the British Isles may not have been suitable for ticks and were only introduced to this region by rare influxes of small numbers of ticks. In addition, the mtMLST scheme developed represents a portable and universal method that can be used in all laboratories to front a global initiative for the study of *Ixodes ricinus* ticks.

8.3 Future work

This proof-of-principle scheme has shown that mitochondrial genes are suitable for use in MLST schemes. The first MLST scheme devised by Maiden *et al.* (1998) used six loci to resolve *Neisseria meningitidis* serogroups as this produced congruent results compared to results using greater than six loci. Other schemes have used eight loci to great effect (Margos *et al.*, 2008) and therefore, increasing the number of loci may increase the resolution of the scheme at the lower taxonomic levels.

This thesis has used preliminary sample-sets for analysis and the expansion of the scheme could easily be implemented to form a tick-monitoring scheme. I have hypothesised that deer and other land-based hosts for *I. ricinus* contribute mostly to dispersal of ticks, unlike birds. If this assumption is correct, *I. ricinus* ticks from Ireland should be genetically distinct from ticks from Britain and other parts of Europe. Other islands such as the Isle of Wight would similarly be isolated and should show similar patterns of association. Hypotheses based on the temporal data considered that ticks collected in 2008 from Britain were the progeny of the ticks collected and analysed in 2006. Extension of this sampling over several years could determine whether this hypothesis is correct.

The extension of this scheme to other organisms represents an exciting opportunity to create standard schemes for the study of phylogeography, particularly in parasitic arthropods. The materials and equipment required for the creation of a sample-set is widely available in most laboratories and could be part of a global initiative (Urwin & Maiden, 2003) for the study of medically and veterinary important organisms.

8.4 Final summary

In this thesis, I have shown the development of a multilocus sequence typing scheme for *Ixodes ricinus* ticks using six mitochondrial genes (*atp6*, *coi*, *coii*, *coiii*, *12s* and *cytB*). As a proof-of-principle test of this scheme, *I. ricinus* ticks from Latvia and Britain were analysed and evidence was found that ticks from Britain were genetically isolated from ticks in Continental Europe. The application of this scheme to other sample sets provided support that Continental European ticks form a panmictic population whereas the ticks from Britain are isolated from other tick populations in Europe. This had lead to an interpretation of the data that birds do not have a significant role in the migration of ticks. The infection of ticks with *B. burgdorferi* s.l. was also investigated and no evidence was found of population structure within the *I. ricinus* population that could be linked to host association with *B. burgdorferi* s.l. spirochaetes. This scheme is the first example of an mtMLST initiative for an arthropod vector. The use of mitochondrial genes for mtMLST schemes could be extended to include other organisms, especially those of veterinary and medical importance.

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Appendices

Collection Site Maps

Figure 60: Collection locations of ticks in the Bath area. Inset shows general locality of collections. Map generated by Google Earth (Google, 2010).

Figure 62: Collection locations of ticks from the Taunton area. Inset shows general location of collection sites. Map generated by Google Earth (Google, 2010).

Figure 61: Collection locations of ticks from Southern England. Map generated by Google Earth (Google, 2010).

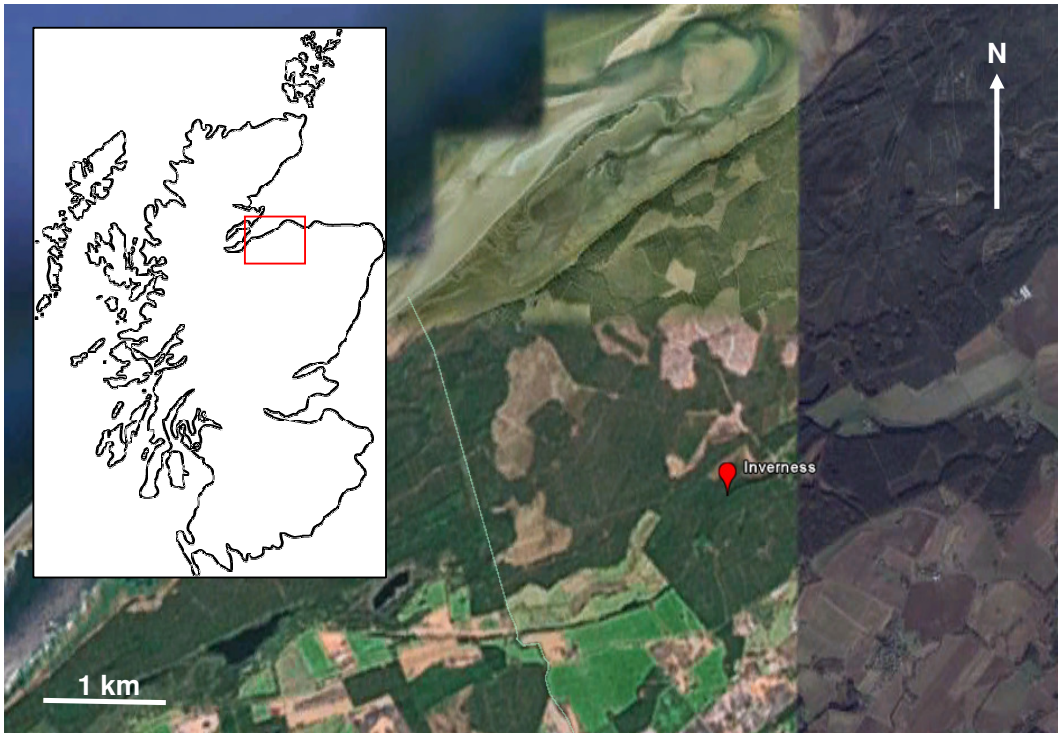

Figure 63: Collection location of ticks from Inverness. Inset indicates shows general location in Scotland. Map generated by Google Earth (Google, 2010).

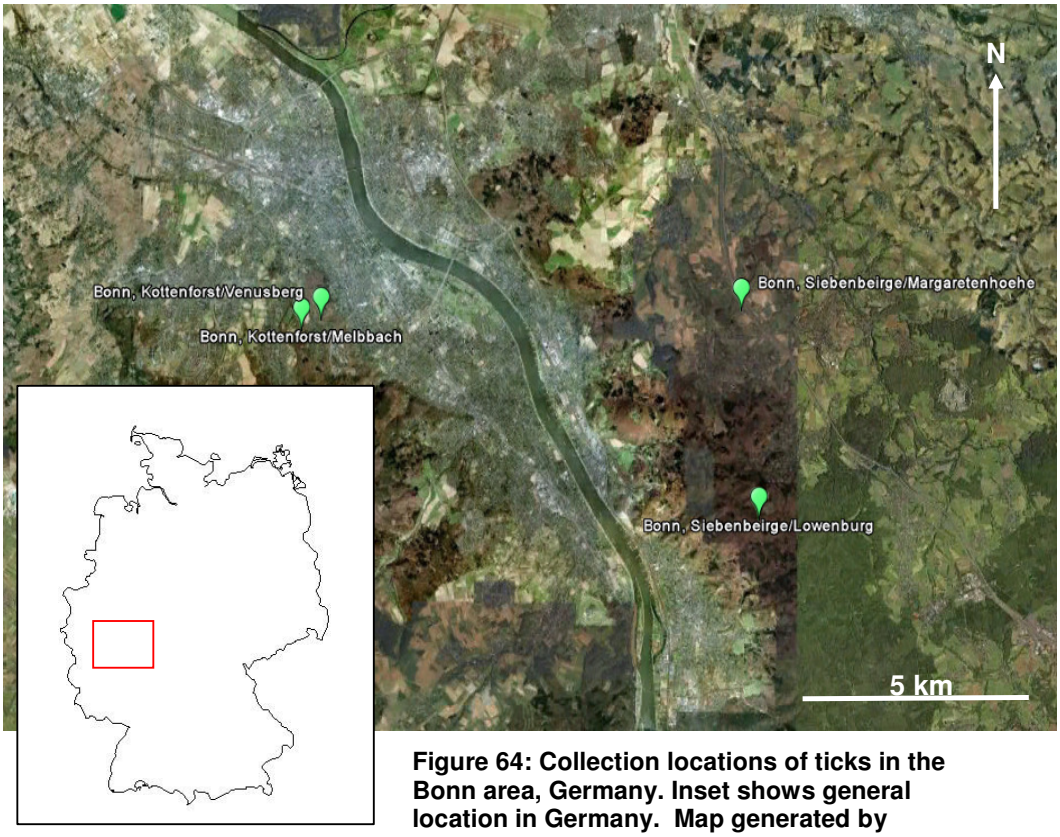

Figure 64: Collection locations of ticks in the Bonn area, Germany. Inset shows general location in Germany. Map generated by Google Earth (Google, 2010).

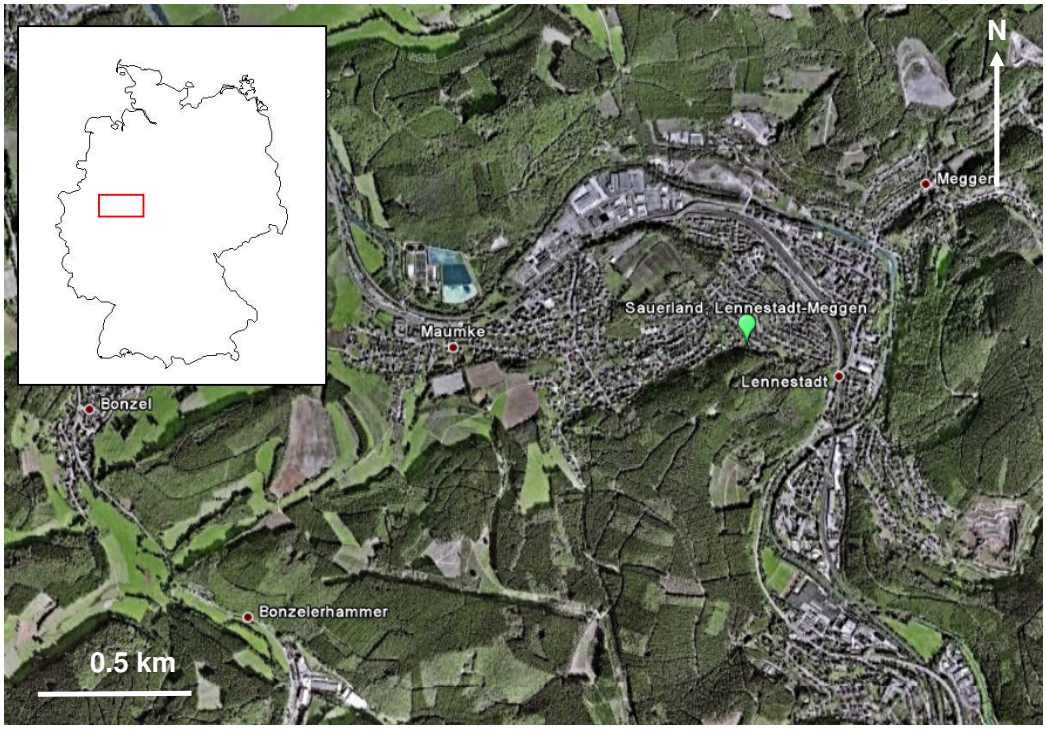

Figure 65: Collection locations of ticks from the Lennestadt area, Germany. Inset shows general location in Germany. Map generated by Google Earth (Google, 2010).

Figure 66: Collection location of ticks from Switzerland. Map generated by Google Earth (Google, 2010).

Figure 67: Collection locations of ticks from Latvia. Inset shows general location in Latvia. Map generated by Google Earth (Google, 2010).

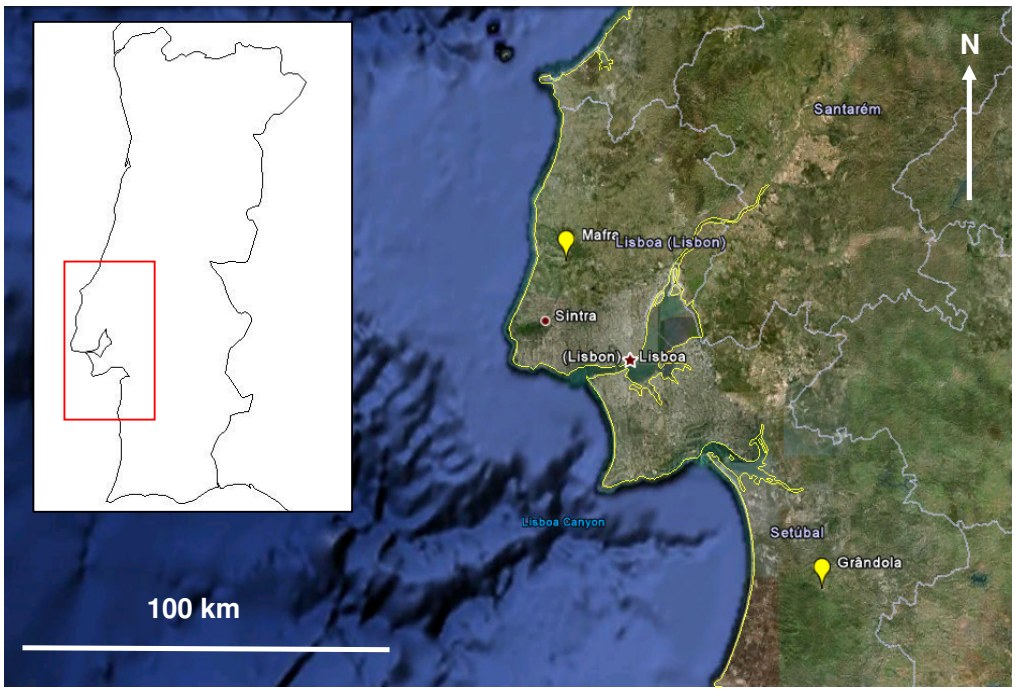

Figure 68: Collection locations of ticks from Portugal. Inset shows the general location in Portugal. Map generated by Google Earth (Google, 2010).

Accession Numbers used for primer design

Accession number	Name of sample
NC_002010	*Ixodes hexagonus* mitochondrion, complete genome
NC_004370	*Ixodes persulcatus* mitochondrion, complete genome
NC_006078	*Ixodes uriae* mitochondrion, complete genome

Primers for amplification of mitochondrial genes

Primer Name	Primer Sequence 5'-3'
12S32F	CCCTAATGCAAAAGGTACCCTAA
12S821R	GCCGCGGTTATACAAGTGAA
12S002F	AAAACACTTTCCAGTATTTTTACTTTG
12S601R	GATGATTTGGCTAAACTTGTGC
ATP6004F	AYAAAYYTWTTTTCWATTTTTGATCC
ATP663R	TTAAATTTCRTTWGTRTAWARDGA
COI001F	ATTTTACCGCGATGAYTWTWCTC
COI786R	TCCTGTGRAAACARATRATATGGGA
COII071R	TTTTTCCATGACCATTCAATAATAA
COII731R	ATAAAGTGGTTTAAGAGACCAATGC
COIII001F	ATGATATTYCAYCCWTTTCAYATAG
COIII780R	AWAYTCATCATTATATRAAWGTAAATA
CYTB222F	CCATTCAAATGGAGCATCAA
CYTB1004R	ACAGGGCAAGCTCCTAAGAA

Gel pictures showing tests of primers

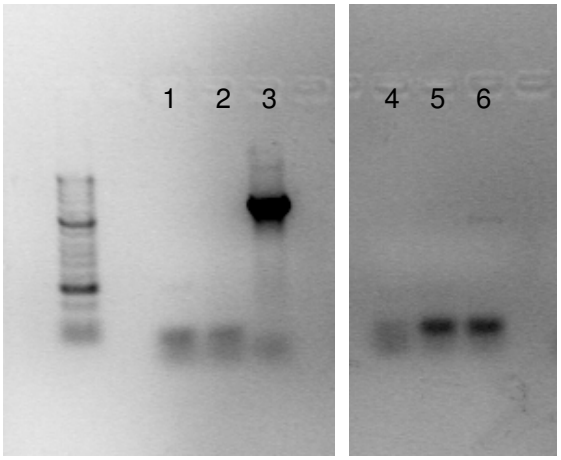

Figure 69: Agarose gel showing test of *ATP6* primers. Additional $MgCl_2$ was added to samples. 1: negative control, 2: negative control, 3: positive control 18S, 4: 0µl, 5: 1µl, 6: 2µl. Other areas of gel have been omitted.

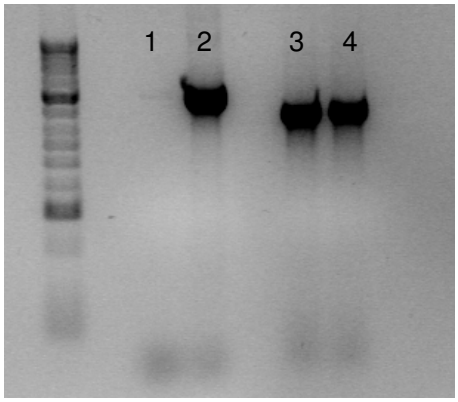

Figure 70: Agarose gel showing test of *COI* primers. 1: negative control, 2: positive control, 3: 64402B, 4: 64402B.

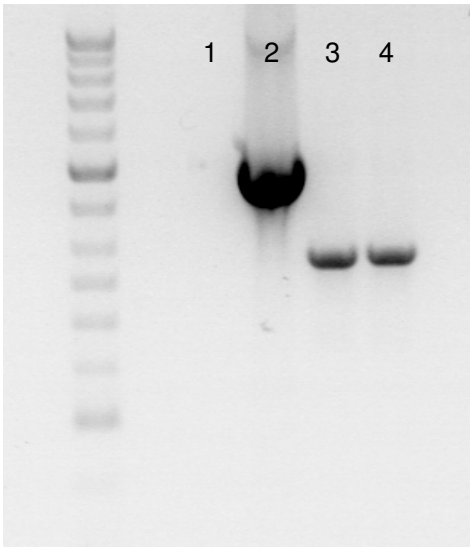

Figure 71: Agarose gel showing test of *COII* primers. 1: negative control 18S, 2: positive control 18S, 3: 60404B, 4: 20204L.

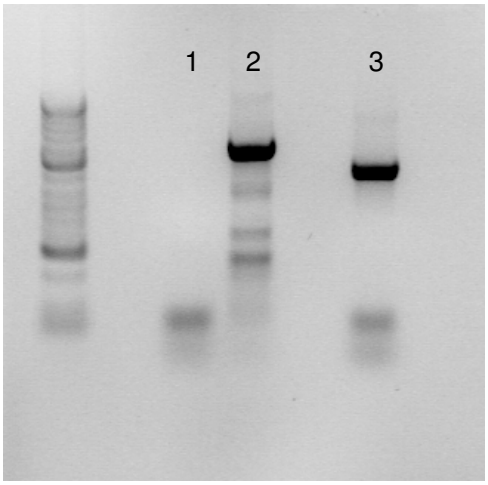

Figure 72: Agarose gel showing test of *COII* primers. 1: negative control 18S, 2: positive control 18S, 3: 61204B

Gel pictures showing $MgCl_2$ optimisations

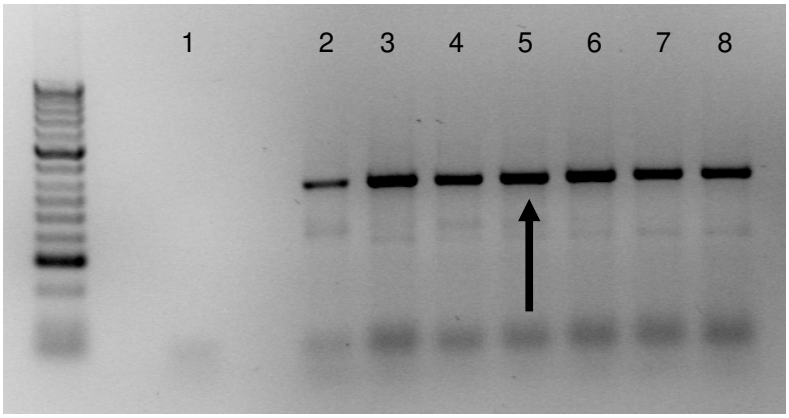

Figure 73: Agarose gel showing $MgCl_2$ gradient for *ATP6*. Samples shown indicate additional volume of $MgCl_2$ added to PCR reaction. 1: negative control, 2: 0µl, 3: 0.5µl, 4: 1µl, 5: 1.5µl 6: 2µl, 7: 2.5µl, 8: 3µl. The arrow indicates the optimal volume of additional $MgCl_2$.

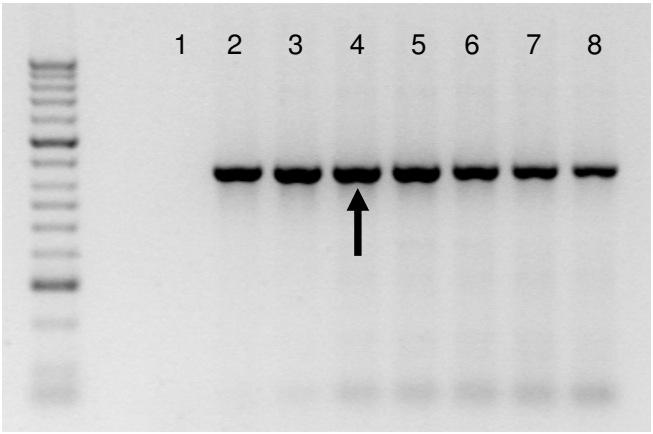

Figure 74: Agarose gel showing $MgCl_2$ gradient for *COI*. Samples shown indicate additional volume of $MgCl_2$ added to PCR reaction. 1: negative control, 2: 0µl, 3: 0.5µl, 4: 1µl, 5: 1.5µl 6: 2µl, 7: 2.5µl, 8: 3µl. The arrow indicates the optimal volume of additional $MgCl_2$.

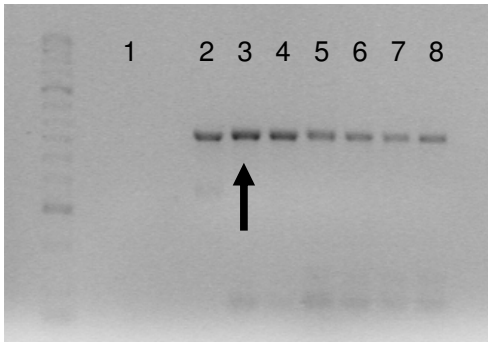

Figure 75: Agarose gel showing $MgCl_2$ gradient for *COII*. Samples shown indicate additional volume of $MgCl_2$ added to PCR reaction. 1: negative control, 2: 0µl, 3: 0.5µl, 4: 1µl, 5: 1.5µl 6: 2µl, 7: 2.5µl, 8: 3µl. The arrow indicates the optimal volume of additional $MgCl_2$.

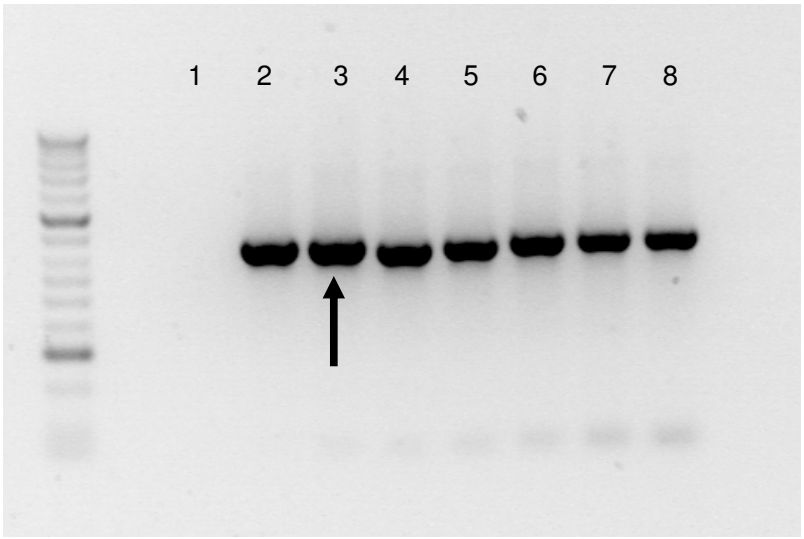

Figure 76: Agarose gel showing magnesium chloride gradient for *COIII* primers. Numbers indicate volume of additional 50mM stock solution $MgCl_2$ added to PCR reactions. 1: negative control, 2: 0µl, 3: 0.5µl, 4: 1µl, 5: 1.5µl, 6: 2µl, 7:2.5µl, 8: 3µl. The arrow indicates the optimal volumes of additional $MgCl_2$.

Gel pictures showing primer concentration optimisations

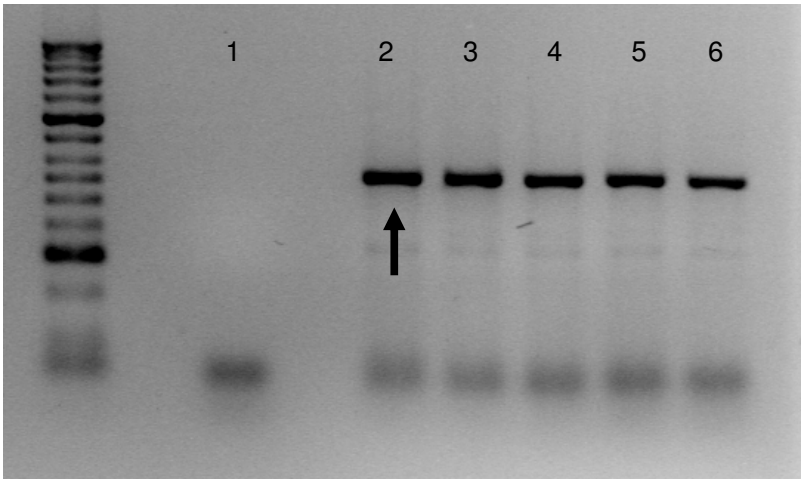

Figure 77: Agarose gel showing primer concentration gradient of *ATP6* primers. Samples shown indicate volume of each forward and reverse primers added to PCR reaction. 1: negative control, 2: 1.5µl, 3: 2µl, 4: 2.5µl, 5: 3µl, 6: 3.5µl. Arrow indicates optimal volume of additional primers.

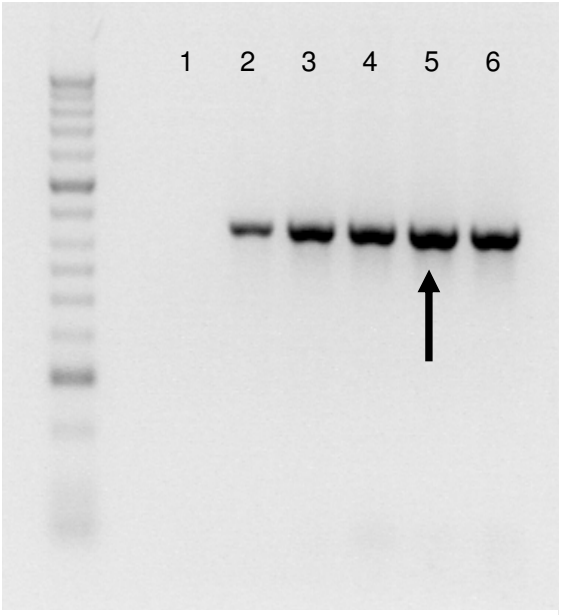

Figure 78: Agarose gel showing primer concentration gradient of *COI* primers. Samples shown indicate volume of each forward and reverse primers added to PCR reaction. 1: negative control, 2: 1.5µl, 3: 2µl, 4: 2.5µl, 5: 3µl, 6: 3.5µl. Arrow indicates optimal volume of additional primers.

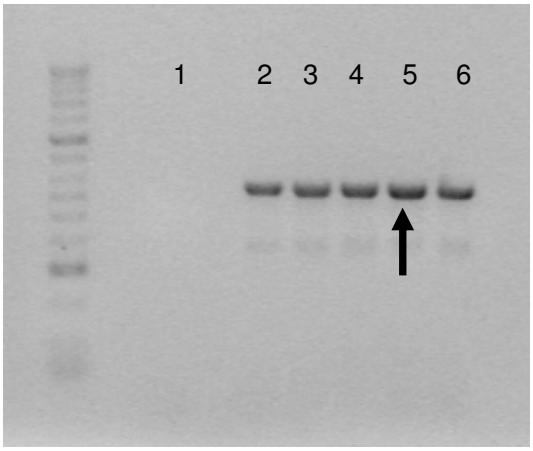

Figure 79: Agarose gel showing primer optimisation gradient for *COII*. Numbers indicate volume of primer added to PCR reaction of each primer stock solution (10pmol). 1: negative control, 2: 1.5µl, 3: 2µl, 4: 2.5µl, 5: 3µl, 5: 3.5µl. Arrow indicates the optimal volume of primers.

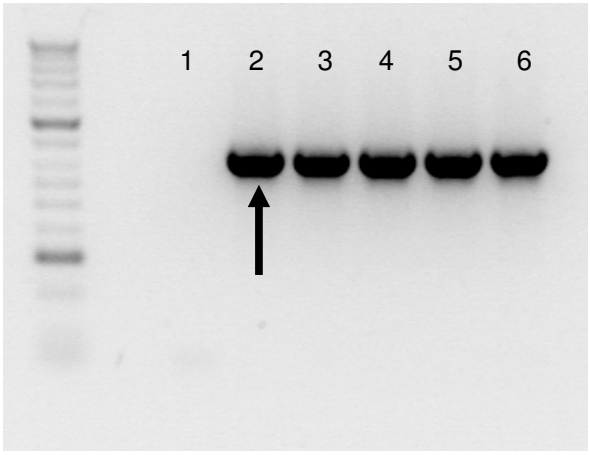

Figure 80 Agarose gel showing primer optimisation gradient for *COIII*. Numbers indicate volume of primer added to PCR reaction of each primer stock solution (10pmol). 1: negative control, 2: 1.5µl, 3: 2µl, 4: 2.5µl, 5: 3µl, 5: 3.5µl. Arrow indicates the optimal volume of primers.

Primers for amplification of 5S-23S rRNA (rrf-rrl) intergenic spacer

Primer Name	Primer Sequence 5'-3'	Position
23SN1	ACCATAGACTCTTATTACTTTGAC	469-446
23SC1	TAAGCTGACTAATACTAATTACCC	92-115
23SN2	ACCATAGACTCTTATTACTTTGACCA	469-444
5SCB	GAGAGTAGGTTATTGCCAGGG	243-363

Primers for positive controls of PCR

Primer Name	Primer sequence 5'-3'
18SF	TACCTGGTTGATCCTGCCAGTAG
18SR	CTTGGCAAATGCTTTCGC

Thermal cycling conditions for PCR

Protocol Name	Initial heating	DNA melting	Primer annealing	Extension	Cycles	Final extension	Expected band size (bp)
12s	94°,2:00	94°, 0:30	46°, 1:00	72°, 1:30	40	72°, 10:00	600
COI	94°,2:00	94°, 1:00	55°, 1:00	72°, 2:00	35	72°, 10:00	785
COII	94°,2:00	94°, 1:00	57°, 1:30	72°, 2:00	40	72°, 10:00	661
COIII	94°,2:00	94°, 1:00	45°, 1:30	72°, 2:00	40	72°, 10:00	779
CYTB	94°,2:00	94°, 0:30	52°, 1:30	72°, 1:00	40	72°, 10:00	780
ATP6	94°, 2:00	94°, 1:00	47°, 1:30	72°, 2:00	40	72°, 10:00	659
IGS1	95°, 1:00	94°, 0:20	52°, 0:20	72°, 0:45	29	72°, 5:00	380
IGS2	95°, 1:00	94°, 0:20	55°, 0:20	72°, 0:45	40	72°, 5:00	225-270

Summary Statistics for Mitochondrial MLST Genes

Gene Name	Number of Alleles/ STs	Length (bp)	Gene Product	GC content (%)	% poly-morphic sites	% parsimony informative sites	dN/dS	mean nucleotide p-distance
ATP6	15	498	ATPase 6	20.9	3.82	1.81	0.161	0.0075
COI	13	636	Cytochrome oxidase I	29.6	2.99	1.42	0.005	0.0057
COII	17	483	Cytochrome oxidase II	28.7	3.73	1.04	0.026	0.0057
COIII	14	555	Cytochrome oxidase III	26.5	2.52	0.90	0.032	0.0038
12S	23	476-480	Small rRNA subunit	21.2	3.73	-	-	0.0055
CYTB	18	549	Cytochrome B	25.4	3.28	2.00	0.388	0.0085
Concatenated genes – all samples	43	3198-3200	-	25.6	3.31	1.44	0.130	0.0061
Concatenated British samples	20	3198-3200	-	25.5	2.09	1.16	0.162	0.0039
Concatenated Latvian samples	25	3198-3200	-	25.7	2.56	0.53	0.134	0.0033

Optimised conditions for PCR

Protocol name	$MgCl_2$ per 50µl reaction†	Primer per 50µl reaction*
12s	0	2
COI	2	6
COII	0	3
COIII	1	3
CYTB	0	3
ATP6	3	3

† $MgCl_2$ at 50mM concentration

* Primer solutions at 10pmol concentration

Sequence Type Profiles

ST	ATP6	COI	COII	COIII	12S	CYTB
1	1	1	1	1	63	2
2	2	2	2	2	34	10
3	3	2	3	2	9	13
4	4	2	2	2	7	13
5	4	2	2	2	13	35
6	21	1	3	2	42	13
7	4	2	2	2	23	19
8	4	2	2	2	27	3
9	4	2	2	2	28	13
10	4	2	2	2	30	3
11	4	2	2	2	31	3
12	4	2	2	2	32	3
13	4	2	2	2	37	3
14	4	2	2	2	39	3
15	4	2	2	2	40	30
16	4	2	2	2	41	3
17	4	2	2	2	42	3
18	4	2	2	2	42	13
19	4	2	2	2	42	23
20	4	2	2	2	42	47

21	4	2	2	2	42	48
22	4	2	2	2	42	51
23	4	2	2	2	43	24
24	4	2	2	2	44	3
25	4	2	2	3	42	3
26	4	2	2	8	42	3
27	4	2	2	10	22	3
28	4	2	2	12	33	13
29	4	2	2	12	34	4
30	4	2	2	12	34	5
31	4	2	2	12	34	13
32	4	2	2	12	35	25
33	4	2	2	18	25	13
34	4	2	2	26	36	3
35	4	2	2	34	42	3
36	4	2	2	35	20	13
37	4	2	2	40	38	3
38	4	2	2	45	57	13
39	4	2	3	2	42	28
40	4	2	4	4	43	3
41	4	2	6	2	40	57
42	4	2	6	2	45	3
43	4	2	7	7	19	3
44	4	2	18	2	15	3
45	4	2	19	2	41	6
46	4	2	23	24	42	11
47	4	2	29	2	42	3
48	4	2	31	2	11	3
49	4	2	32	2	8	3
50	4	2	33	39	42	3
51	4	2	35	2	41	3
52	4	2	37	44	42	29
53	4	2	38	2	16	58
54	4	3	2	2	14	3

55	4	5	2	2	10	3
56	4	8	6	2	42	13
57	39	46	2	2	34	3
58	4	11	2	2	42	3
59	4	12	2	2	42	29
60	4	13	2	2	42	3
61	4	16	2	2	40	3
62	4	1	2	19	34	52
63	4	26	2	26	21	15
64	4	29	2	2	41	3
65	4	30	2	12	33	13
66	4	31	2	32	33	13
67	4	33	2	2	42	44
68	4	35	2	2	41	3
69	4	35	2	2	42	3
70	4	36	36	2	42	13
71	4	37	2	2	42	3
72	4	38	2	2	40	53
73	4	40	2	42	33	13
74	4	47	2	2	42	13
75	5	1	2	6	4	22
76	5	1	13	2	70	22
77	5	4	2	2	70	20
78	5	7	5	2	68	1
79	6	6	2	5	46	1
80	7	2	2	2	6	3
81	8	9	8	9	49	5
82	8	9	8	9	55	5
83	8	9	8	9	56	5
84	8	9	9	9	17	5
85	8	9	9	9	48	5
86	8	9	9	9	50	5
87	8	9	9	9	54	5
88	8	9	9	9	55	5

89	8	9	9	9	55	27
90	8	9	9	9	56	5
91	8	9	9	29	55	5
92	8	9	9	31	55	5
93	8	9	9	43	54	5
94	8	9	16	9	55	5
95	8	21	21	9	55	5
96	8	21	21	9	55	9
97	8	34	26	9	56	46
98	9	2	2	2	38	3
99	10	10	1	1	60	1
100	11	1	2	11	70	1
101	11	1	11	14	3	1
102	11	18	18	20	67	1
103	12	2	10	13	42	13
104	13	1	1	1	2	56
105	13	1	1	1	63	1
106	13	1	1	1	63	2
107	13	1	1	1	63	26
108	13	1	1	1	64	32
109	13	1	1	1	64	55
110	13	1	1	1	65	2
111	13	1	1	1	72	1
112	13	1	1	15	61	1
113	13	1	2	1	59	1
114	13	1	30	1	65	49
115	13	1	34	1	63	1
116	13	2	20	1	63	7
117	13	39	2	41	12	54
118	14	15	2	16	42	13
119	15	2	2	2	42	33
120	16	45	43	21	1	21
121	17	17	15	17	55	34
122	19	1	1	1	58	36

123	20	18	2	20	66	37
124	21	1	2	20	71	8
125	21	1	2	20	73	41
126	21	19	2	20	71	38
127	21	27	2	20	72	1
128	21	32	27	33	70	42
129	22	2	2	22	41	3
130	22	2	17	2	42	3
131	23	22	22	2	75	4
132	23	22	22	2	75	9
133	24	2	2	23	41	10
134	25	17	26	28	51	17
135	25	24	24	25	52	12
136	25	24	26	25	53	12
137	26	25	25	16	76	14
138	27	25	25	16	76	14
139	28	2	2	2	40	3
140	30	2	2	2	29	3
141	31	2	2	54	38	3
142	32	2	2	2	38	3
143	5	27	2	27	70	16
144	25	17	26	25	47	18
145	4	1	2	1	18	1
146	5	2	2	2	43	3
147	4	36	2	2	42	13
148	35	51	1	1	64	1
149	36	9	9	46	56	5
150	4	2	2	36	41	3
151	37	15	2	16	24	39
152	4	2	2	48	42	3
153	38	27	2	49	72	43
154	4	52	39	50	42	3
155	4	53	2	2	62	29
156	39	2	2	51	42	31

157	25	54	26	25	69	17
158	4	55	2	47	41	3
159	4	2	3	2	42	13
160	4	2	23	20	70	7
161	49	2	2	2	38	3
162	4	2	1	1	63	2
163	13	1	2	2	42	29
164	4	2	40	52	42	3
165	5	1	41	2	70	22
166	13	1	42	1	63	1
170	21	1	2	37	81	62
171	46	17	26	25	49	18
172	8	9	9	29	78	5
173	45	1	1	1	63	50
175	11	1	44	11	70	1
176	39	46	2	2	37	3
177	8	9	9	9	55	59
178	4	2	2	2	38	3
179	47	48	9	9	55	5
180	4	2	2	2	74	3
181	8	50	9	9	55	5
182	8	9	45	9	56	5
183	21	1	46	20	80	60
184	4	2	2	2	40	3
186	25	17	26	28	55	17
187	8	9	9	30	55	5
188	33	23	2	55	76	41
189	42	9	9	57	82	5
190	4	41	2	59	42	3
191	13	1	1	1	65	1
192	4	44	2	2	38	3
193	13	28	1	1	64	1
194	4	42	2	2	83	3
195	13	1	1	1	84	1

196	34	2	48	12	33	63
197	43	29	2	2	42	64
198	13	1	1	1	63	65
199	4	2	2	2	41	13
200	41	2	2	2	88	66
201	4	2	2	58	25	68
202	44	2	49	2	41	69
203	4	2	2	45	42	3
204	13	1	1	1	85	1
205	13	43	1	1	86	1
206	4	2	2	20	44	28
207	8	9	9	60	87	70
208	4	2	50	2	42	3
210	4	2	52	61	41	3
211	14	15	2	16	41	13
212	4	2	51	56	42	67
213	8	9	2	9	55	5
214	14	15	14	16	5	28
215	13	2	1	38	64	1
216	47	48	9	9	55	59
217	4	20	2	2	43	40
218	18	2	3	2	42	3
219	29	2	2	36	38	3
220	4	36	2	2	90	13
221	4	72	2	2	42	3
222	4	2	52	65	42	3
223	56	2	2	2	41	3
224	8	9	9	9	55	78
225	4	69	2	2	42	3
226	4	2	3	2	40	13
227	25	24	26	25	91	12
228	13	1	54	1	63	2
229	13	2	1	1	64	1
230	8	9	9	25	55	74

231	4	61	3	64	42	13
232	39	2	55	2	94	3
233	64	2	2	2	42	3
234	13	1	1	1	63	80
235	50	2	2	62	41	3
236	13	1	1	1	65	73
237	4	29	2	2	40	3
238	54	68	11	2	42	3
239	34	2	2	2	38	29
240	58	1	2	20	72	1
241	4	70	2	2	42	76
242	4	2	57	2	42	77
243	4	29	58	2	41	3
244	8	21	9	9	55	5
245	55	9	9	9	55	5
246	25	54	26	67	55	75
247	4	2	2	2	40	13
248	57	58	56	55	72	1
249	65	1	1	1	63	81
250	8	9	60	9	55	5
251	4	2	2	2	97	3
252	25	66	26	25	55	17
253	8	56	9	9	56	71
254	66	2	2	2	41	3
255	13	59	1	71	98	1
256	13	60	61	1	100	1
257	8	9	9	73	55	83
258	25	63	26	25	103	12
259	8	9	9	9	89	5
260	60	9	9	9	55	5
261	8	9	9	9	96	5
262	4	2	2	69	42	3
263	63	62	26	25	55	18
264	5	1	2	2	70	79

265	59	9	9	68	55	5
266	8	9	9	9	95	5
267	61	9	9	9	55	5
268	13	1	59	1	63	1
269	67	2	3	2	44	13
270	8	9	9	9	99	5
271	4	2	2	2	42	82
272	4	2	62	2	42	3
273	68	2	2	2	101	3
274	8	9	9	9	102	5
275	25	64	63	28	55	17
276	8	9	9	72	55	5
277	51	71	2	63	42	3
278	5	1	2	2	70	22
279	42	9	9	9	48	5
280	25	65	26	25	55	18
281	52	9	9	9	92	5
282	25	54	24	70	55	17
283	4	2	2	2	42	72
284	4	2	2	2	93	3
285	53	57	2	66	72	7
286	69	9	9	9	55	5
287	4	67	2	6	36	3
288	21	1	2	20	72	90
289	13	1	1	1	63	85
290	4	73	2	2	41	3
291	4	74	2	2	42	3
292	13	75	1	1	64	2
293	40	1	1	1	63	1
294	13	1	71	1	63	1
295	4	2	64	2	42	13
296	73	2	65	2	42	3
297	4	2	3	64	42	13
298	72	77	9	9	55	86

299	5	1	2	76	70	87
300	74	22	69	2	75	1
301	75	2	2	2	62	3
302	14	15	2	16	40	13
303	77	2	3	2	44	13
304	78	9	9	9	54	5
305	62	17	26	17	108	17
306	21	1	6	2	70	91
307	21	1	23	20	106	7
308	8	9	9	9	89	84
309	14	15	1	16	110	13
310	62	17	26	17	56	17
311	4	2	2	51	107	3
312	8	9	72	9	55	5
313	8	9	9	9	49	5
314	8	9	9	77	55	5
315	5	79	2	2	70	22
316	4	2	2	2	62	3
317	71	78	67	9	105	5
318	76	9	9	9	48	88
319	63	17	26	70	55	18
320	4	2	2	78	44	3
321	70	2	70	2	40	89
322	4	2	2	2	43	3
323	8	9	68	9	54	5
324	84	9	9	9	55	88
325	82	9	9	83	55	5
326	8	48	9	9	111	5
327	26	25	25	16	112	95
328	48	46	47	53	55	61
329	8	9	9	9	113	5
330	8	9	9	9	114	5
331	8	9	9	84	115	5
332	4	76	2	2	38	29

333	13	1	1	75	63	2
334	4	2	2	2	104	3
335	4	2	66	2	40	13
336	21	99	80	20	121	60
337	13	100	1	1	64	101
338	13	1	1	1	63	41
339	96	2	65	39	42	3
340	4	29	2	19	138	13
341	8	21	9	9	55	86
342	89	2	3	102	126	13
343	14	15	2	16	40	28
344	4	2	2	103	127	3
345	4	88	2	2	41	13
346	8	89	9	9	56	106
347	4	87	2	12	33	13
348	4	2	2	2	38	102
349	4	2	89	2	42	105
350	4	2	2	2	135	104
351	21	91	23	20	70	7
352	4	94	88	2	40	103
353	4	96	2	2	42	3
354	4	92	2	2	134	13
355	4	2	40	2	42	3
356	93	9	9	104	55	5
357	89	2	6	105	42	13
358	4	2	2	2	88	3
359	25	24	26	25	131	107
360	4	2	87	2	132	29
361	4	2	3	2	40	3
362	25	15	26	25	47	18
363	14	9	2	16	40	13
364	25	54	26	25	133	17
365	90	9	9	9	55	5
366	4	80	2	2	37	3

367	8	9	9	9	55	84
368	91	9	9	9	55	5
369	92	95	2	20	136	1
370	13	1	1	74	63	2
371	4	82	74	2	42	13
372	80	2	2	2	41	92
373	79	2	73	2	42	13
374	4	2	2	93	34	13
375	81	1	2	20	72	93
376	8	21	21	94	55	5
377	4	2	2	80	33	94
378	4	2	2	82	128	3
379	43	2	2	100	40	13
380	4	102	3	2	42	13
381	14	15	83	101	40	13
382	8	9	9	9	55	97
383	8	9	9	87	49	5
384	4	2	2	81	141	3
386	4	2	2	64	42	13
387	13	103	1	1	63	1
388	4	2	2	106	139	3
389	4	81	2	2	14	3
390	4	2	2	107	42	3
391	6	83	2	5	140	1
392	8	9	9	108	55	108
393	8	85	9	9	152	5
394	4	36	2	2	40	13
395	8	9	9	9	55	96
396	8	9	77	85	55	98
397	86	9	9	9	54	5
398	8	9	9	86	143	5
399	8	9	9	25	55	5
400	8	9	9	79	151	5
401	83	9	77	89	146	5

402	8	9	9	92	48	5
403	8	9	26	9	144	5
404	8	9	9	9	95	88
405	8	9	9	9	145	5
406	25	24	26	25	147	12
407	87	9	9	9	55	5
408	47	9	9	91	150	5
409	47	9	9	9	55	5
410	88	9	9	88	55	5
411	25	54	26	25	55	17
412	25	24	26	25	148	12
413	59	9	9	9	55	5
414	8	9	76	9	120	88
415	8	84	79	9	149	99
416	8	9	75	9	55	5
417	13	1	82	1	124	1
418	4	80	2	2	122	3
419	21	1	2	97	118	109
420	4	101	2	2	40	110
421	97	1	1	1	63	1
422	34	2	2	12	33	28
423	98	2	2	2	42	3
424	4	15	2	96	116	3
425	95	98	2	2	117	29
426	13	1	1	2	64	1
427	8	9	9	98	120	5
428	8	9	81	9	55	5
429	4	2	2	2	122	3
430	4	2	2	2	41	111
431	5	27	2	2	123	22
432	4	2	3	2	6	13
433	4	2	2	99	119	39
434	4	2	84	2	41	3
435	8	9	78	9	55	5

436	8	9	9	90	55	5
438	85	9	9	9	55	5
439	4	2	2	2	55	82
440	4	2	90	2	129	3
441	8	97	9	9	137	5
442	94	2	2	2	44	3
443	8	9	85	9	125	5
444	4	2	3	2	42	89
445	4	2	3	2	43	13
446	8	9	86	9	55	5

Summary of sample numbers, Origin, Year and Sex

Origin	Year	Sex	Origin Total	Year Total	Sex Total
Britain	-	-	406	-	-
Britain	2006	-	-	100	-
Britain	2006	adult	-	-	17
Britain	2006	nymph	-	-	83
Britain	2007	-	-	102	-
Britain	2007	adult	-	-	0
Britain	2007	nymph	-	-	102
Britain	2008	-	-	202	-
Britain	2008	adult	-	-	69
Britain	2008	nymph	-	-	133
Britain	2009	-	-	2	-
Britain	2009	adult	-	-	2
Britain	2009	nymph	-	-	0
Latvia	-	-	254	-	-
Latvia	2002	-	-	69	-
Latvia	2002	adult	-	-	34
Latvia	2002	nymph	-	-	35
Latvia	2006	-	-	136	-
Latvia	2006	adult	-	-	32
Latvia	2006	nymph	-	-	104
Latvia	2007	-	-	49	-

Latvia	2007	adult	-	-	0
Latvia	2007	nymph	-	-	49
Germany	-	-	52	-	-
Germany	2008	-	-	52	-
Germany	2008	adult	-	-	25
Germany	2008	nymph	-	-	27
Portugal	-	-	19	-	-
Portugal	2002	-	-	10	-
Portugal	2002	adult	-	-	0
Portugal	2002	nymph	-	-	10
Portugal	2003	-	-	9	-
Portugal	2003	adult	-	-	0
Portugal	2003	nymph	-	-	9
Switzerland	-	-	15	-	-
Switzerland	2008	-	-	15	-
Switzerland	2008	adult	-	-	0
Switzerland	2008	nymph	-	-	15
Totals	**-**	**-**	**746**	**746**	**746**

Tick Samples used for the Comparison of British and Latvian Populations in section 3.4

Tick ID	ST	Year	Stage	Origin	Collection Location	ATP6	COI	COII	COIII	12S	CYTB
70612B	24	2007	nymph	Britain	Bathampton Woods	4	2	2	2	44	3
72068B	298	2007	nymph	Britain	Eastwood	72	77	9	9	55	86
7006108B	324	2007	nymph	Britain	Inverness	84	9	9	9	55	88
7005118B	404	2007	nymph	Britain	Inverness	8	9	9	9	95	88
7001129B	438	2007	nymph	Britain	Inverness	85	9	9	9	55	5
71355B	88	2007	nymph	Britain	Warleigh	8	9	9	9	55	5
72392B	302	2007	nymph	Britain	Warleigh	14	15	2	16	40	13
7003110B	88	2007	nymph	Britain	Inverness	8	9	9	9	55	5
7003119B	405	2007	nymph	Britain	Inverness	8	9	9	9	145	5
70516B	90	2007	nymph	Britain	Bathampton Woods	8	9	9	9	56	5
70120B	362	2007	nymph	Britain	Bathampton Woods	25	15	26	25	47	18
7002112B	331	2007	nymph	Britain	Inverness	8	9	9	84	115	5
7007120B	88	2007	nymph	Britain	Inverness	8	9	9	9	55	5
70118B	106	2007	nymph	Britain	Bathampton Woods	13	1	1	1	63	2
70580B	367	2007	nymph	Britain	Widcombe	8	9	9	9	55	84
7003115B	383	2007	nymph	Britain	Inverness	8	9	9	87	49	5

7009122B	409	2007	nymph	Britain	Inverness	47	9	9	9	55	5
72002B	144	2007	nymph	Britain	Widcombe	25	17	26	25	47	18
7001105B	327	2007	nymph	Britain	Inverness	26	25	25	16	112	95
7002116B	85	2007	nymph	Britain	Inverness	8	9	9	9	48	5
7005124B	412	2007	nymph	Britain	Inverness	25	24	26	25	148	12
71452B	144	2007	nymph	Britain	Bathampton Woods	25	17	26	26	47	18
7004107B	88	2007	nymph	Britain	Inverness	8	9	9	9	55	5
7003117B	324	2007	nymph	Britain	Inverness	84	9	9	9	55	88
7007126B	416	2007	nymph	Britain	Inverness	8	9	75	9	55	5
71015L	16	2007	nymph	Latvia	Jurmala	4	2	2	2	41	3
73515L	337	2007	nymph	Latvia	Jurmala	13	100	1	1	64	101
74112L	357	2007	nymph	Latvia	Tireli	89	2	6	105	42	13
76318L	422	2007	nymph	Latvia	Jurmala	34	2	2	12	33	28
71524L	434	2007	nymph	Latvia	Jaunciems	4	2	84	2	41	3
74606L	17	2007	nymph	Latvia	Tireli	4	2	2	2	42	3
74815L	339	2007	nymph	Latvia	Jurmala	96	2	65	39	42	3
70115L	360	2007	nymph	Latvia	Jurmala	4	2	87	2	132	29
70121L	424	2007	nymph	Latvia	Jaunciems	4	15	2	96	116	3
73406L	24	2007	nymph	Latvia	Jurmala	4	2	2	2	44	3

72803L	349	2007	nymph	Latvia	Tireli	4	2	89	2	42	105
73524L	379	2007	nymph	Latvia	Jaunciems	43	2	2	100	40	13
71121L	426	2007	nymph	Latvia	Jaunciems	13	1	1	2	64	1
75218L	106	2007	nymph	Latvia	Jurmala	13	1	1	1	63	2
70709L	351	2007	nymph	Latvia	Tireli	21	91	23	20	70	7
74624L	381	2007	nymph	Latvia	Jaunciems	14	15	83	101	40	13
73521L	428	2007	nymph	Latvia	Jaunciems	8	9	81	9	55	5
72809L	115	2007	nymph	Latvia	Tireli	13	1	34	1	63	1
73609L	353	2007	nymph	Latvia	Tireli	4	96	2	2	42	3
76615L	418	2007	nymph	Latvia	Jurmala	4	80	2	2	122	3
73921L	430	2007	nymph	Latvia	Jaunciems	4	2	2	2	41	111
70418L	191	2007	nymph	Latvia	Jurmala	13	1	1	1	65	1
72312L	355	2007	nymph	Latvia	Tireli	4	2	40	2	42	3
73618L	420	2007	nymph	Latvia	Jurmala	4	101	2	2	40	110
70624L	432	2007	nymph	Latvia	Jaunciems	4	2	3	2	6	13

Tick Samples used for the Comparison of Latvian populations in 2002, 2006 and 2007 in section 4.2.1

Tick ID	ST	Year	Stage	Collection Location	*ATP6*	*COI*	*COII*	*COIII*	*12s*	*CYTB*
20103L	1	2002	nymph	Jaunciems	1	1	1	1	63	2
20303L	7	2002	nymph	Jurmala	5	4	2	2	70	20
20306L	10	2002	nymph	Jurmala	4	2	2	2	30	3
20312L	54	2002	nymph	Babite	4	3	2	2	14	3
20412L	17	2002	nymph	Babite	4	2	2	2	42	3
20603L	98	2002	nymph	Jaunciems	9	2	2	2	38	3
20703L	99	2002	nymph	Jaunciems	10	10	1	1	60	1
20706L	100	2002	nymph	Jurmala	11	1	2	11	70	1
20803L	32	2002	nymph	Jaunciems	4	2	2	12	35	25
20812L	101	2002	nymph	Babite	11	1	11	14	3	1
20906L	17	2002	nymph	Jurmala	4	2	2	2	42	3
21303L	60	2002	nymph	Jaunciems	4	13	2	2	42	3
21503L	60	2002	nymph	Jaunciems	4	13	2	2	42	3
21603L	1	2002	nymph	Jaunciems	1	1	1	1	63	2
21703L	87	2002	nymph	Jaunciems	8	9	9	9	54	5
21903L	15	2002	nymph	Jaunciems	4	2	2	2	40	30
22003L	112	2002	nymph	Jaunciems	13	1	1	15	61	1

22106L	76	2002	nymph	Jurmala	5	1	13	2	70	22
22203L	108	2002	nymph	Jaunciems	13	1	1	1	64	32
22606L	214	2002	nymph	Jurmala	14	15	14	16	5	28
23306L	85	2002	nymph	Jurmala	8	9	9	9	48	5
23506L	119	2002	nymph	Jurmala	15	2	2	2	42	33
23606L	61	2002	nymph	Jurmala	4	16	2	2	40	3
23609L	121	2002	nymph	Babite	17	17	15	17	55	34
23806L	33	2002	nymph	Jurmala	4	2	2	18	25	13
23906L	5	2002	nymph	Jurmala	4	2	2	2	13	35
24006L	218	2002	nymph	Jurmala	18	2	3	2	42	3
24206L	122	2002	nymph	Jurmala	19	1	1	1	58	36
24306L	94	2002	nymph	Jurmala	8	9	16	9	55	5
25203L	15	2002	nymph	Jaunciems	4	2	2	2	40	30
25303L	123	2002	nymph	Jaunciems	20	18	2	20	66	37
25403L	126	2002	nymph	Jaunciems	21	19	2	20	71	38
25603L	108	2002	nymph	Jaunciems	13	1	1	1	64	32
26103L	217	2002	nymph	Jaunciems	4	20	2	2	43	40
26503L	130	2002	nymph	Jaunciems	22	2	17	2	42	3
60103L	125	2006	nymph	Tireli	21	1	2	20	73	41
60112L	17	2006	nymph	Tireli	4	2	2	2	42	3

60115L	64	2006	nymph	Babite	4	29	2	2	41	3
60121L	16	2006	nymph	Jurmala	4	2	2	2	41	3
60203L	65	2006	nymph	Jaunciems	4	30	2	12	33	13
60212L	66	2006	nymph	Tireli	4	31	2	32	33	13
60215L	128	2006	nymph	Babite	21	32	27	33	70	42
60221L	8	2006	nymph	Jurmala	4	2	2	2	27	3
60303L	178	2006	nymph	Jaunciems	4	2	2	2	38	3
60312L	105	2006	nymph	Tireli	13	1	1	1	63	1
60315L	118	2006	nymph	Babite	14	15	2	16	42	13
60403L	113	2006	nymph	Jurmala	13	1	2	1	59	1
60412L	88	2006	nymph	Tireli	8	9	9	9	55	5
60415L	47	2006	nymph	Babite	4	2	29	2	42	3
60421L	44	2006	nymph	Jurmala	4	2	18	2	15	3
60503L	67	2006	nymph	Jaunciems	4	33	2	2	42	44
60512L	16	2006	nymph	Tireli	4	2	2	2	41	3
60515L	13	2006	nymph	Babite	4	2	2	2	37	3
60603L	97	2006	nymph	Jurmala	8	34	26	9	56	46
60612L	177	2006	nymph	Tireli	8	9	9	9	55	59
60615L	20	2006	nymph	Babite	4	2	2	2	42	47
60618L	443	2006	nymph	Jurmala	8	9	85	9	125	5

60703L	21	2006	nymph	Jurmala	4	2	2	2	42	48
60712L	35	2006	nymph	Tireli	4	2	2	34	42	3
60715L	69	2006	nymph	Babite	4	35	2	2	42	3
60721L	114	2006	nymph	Jurmala	13	1	30	1	65	49
60724L	342	2006	nymph	Jaunciems	89	2	3	102	126	13
60803L	48	2006	nymph	Jaunciems	4	2	31	2	11	3
60812L	68	2006	nymph	Tireli	4	35	2	2	41	3
60815L	17	2006	nymph	Babite	4	2	2	2	42	3
60821L	137	2006	nymph	Jurmala	26	25	25	16	76	14
60912L	36	2006	nymph	Jaunciems	4	2	2	35	20	13
60915L	127	2006	nymph	Babite	21	27	2	20	72	1
61003L	16	2006	nymph	Jurmala	4	2	2	2	41	3
61009L	106	2006	nymph	Tireli	13	1	1	1	63	2
61012L	136	2006	nymph	Babite	25	24	26	25	53	12
61015L	87	2006	nymph	Jurmala	8	9	9	9	54	5
61021L	139	2006	nymph	Jaunciems	28	2	2	2	40	3
61112L	219	2006	nymph	Babite	29	2	2	36	38	3
61115L	127	2006	nymph	Jurmala	21	27	2	20	72	1
61121L	220	2006	nymph	Jaunciems	4	36	2	2	90	13
61212L	106	2006	nymph	Babite	13	1	1	1	63	2

61215L	22	2006	nymph	Jurmala	4	2	2	2	42	51
61218L	24	2006	nymph	Jurmala	4	2	2	2	44	3
61221L	34	2006	nymph	Jaunciems	4	2	2	26	36	3
61306L	51	2006	nymph	Babite	4	2	35	2	41	3
61312L	126	2006	nymph	Babite	21	19	2	20	71	38
61315L	71	2006	nymph	Jurmala	4	37	2	2	42	3
61321L	62	2006	nymph	Jaunciems	4	1	2	19	34	52
61406L	386	2006	nymph	Babite	4	2	2	64	42	13
61412L	72	2006	nymph	Babite	4	38	2	2	40	53
61415L	28	2006	nymph	Jurmala	4	2	2	12	33	13
61421L	42	2006	nymph	Jaunciems	4	2	6	2	45	3
61512L	215	2006	nymph	Babite	13	2	1	38	64	1
61515L	83	2006	nymph	Jurmala	8	9	8	9	56	5
61612L	17	2006	nymph	Babite	4	2	2	2	42	3
61615L	87	2006	nymph	Jurmala	8	9	9	9	54	5
61618L	47	2006	nymph	Jurmala	4	2	29	2	42	3
61712L	49	2006	nymph	Babite	4	2	32	2	8	3
61715L	50	2006	nymph	Jurmala	4	2	33	39	42	3
61721L	37	2006	nymph	Jaunciems	4	2	2	40	38	3
61809L	16	2006	nymph	Tireli	4	2	2	2	41	3

61812L	111	2006	nymph	Babite	13	1	1	1	72	1
61815L	83	2006	nymph	Jurmala	8	9	8	9	56	5
61821L	115	2006	nymph	Jaunciems	13	1	34	1	63	1
61912L	51	2006	nymph	Babite	4	2	35	2	41	3
61915L	9	2006	nymph	Jurmala	4	2	2	2	28	13
61918L	195	2006	nymph	Jurmala	13	1	1	1	84	1
61921L	139	2006	nymph	Jaunciems	28	2	2	2	40	3
62012L	117	2006	nymph	Babite	13	39	2	41	12	54
62015L	70	2006	nymph	Jurmala	4	36	36	2	42	13
62103L	341	2006	nymph	Tireli	8	21	9	9	55	86
62118L	444	2006	nymph	Jurmala	4	2	3	2	42	89
62218L	445	2006	nymph	Jurmala	4	2	3	2	43	13
62303L	184	2006	nymph	Tireli	4	2	2	2	40	3
62306L	387	2006	nymph	Babite	13	103	1	1	63	1
62309L	389	2006	nymph	Tireli	4	81	2	2	14	3
62506L	373	2006	nymph	Babite	79	2	73	2	42	13
62909L	159	2006	nymph	Tireli	4	2	3	2	42	13
63012L	394	2006	nymph	Babite	4	36	2	2	40	13
63203L	371	2006	nymph	Tireli	4	82	74	2	42	13
63309L	390	2006	nymph	Tireli	4	2	2	107	42	3

63321L	342	2006	nymph	Jaunciems	89	2	3	102	126	13
63424L	344	2006	nymph	Jaunciems	4	2	2	103	127	3
63503L	88	2006	nymph	Tireli	8	9	9	9	55	5
63521L	343	2006	nymph	Jaunciems	14	15	2	16	40	28
63603L	372	2006	nymph	Tireli	80	2	2	2	41	92
63706L	374	2006	nymph	Babite	4	2	2	93	34	13
63721L	342	2006	nymph	Jaunciems	89	2	3	102	126	13
63806L	375	2006	nymph	Babite	81	1	2	20	72	93
63809L	377	2006	nymph	Tireli	4	2	2	80	33	94
63909L	391	2006	nymph	Tireli	6	83	2	5	140	1
64224L	345	2006	nymph	Jaunciems	4	88	2	2	41	13
64409L	392	2006	nymph	Tireli	8	9	9	108	55	108
64624L	346	2006	nymph	Jaunciems	8	89	9	9	56	106
64703L	384	2006	nymph	Tireli	4	2	2	81	141	3
64803L	350	2006	nymph	Tireli	4	2	2	2	135	104
65006L	97	2006	nymph	Babite	8	34	26	9	56	46
66612L	378	2006	nymph	Babite	4	2	2	82	128	3
68412L	440	2006	nymph	Babite	4	2	90	2	129	3
69212L	88	2006	nymph	Babite	8	9	9	9	55	5
6101012L	441	2006	nymph	Babite	8	97	9	9	137	5

6102012L	442	2006	nymph	Babite	94	2	2	2	44	3
6104012L	340	2006	nymph	Babite	4	29	2	19	138	13
70115L	360	2007	nymph	Jurmala	4	2	87	2	132	29
70121L	424	2007	nymph	Jaunciems	4	15	2	96	116	3
70412L	18	2007	nymph	Tireli	4	2	2	2	42	13
70415L	361	2007	nymph	Jurmala	4	2	3	2	40	3
70418L	191	2007	nymph	Jurmala	13	1	1	1	65	1
70621L	425	2007	nymph	Jaunciems	95	98	2	2	117	29
70624L	432	2007	nymph	Jaunciems	4	2	3	2	6	13
70709L	351	2007	nymph	Tireli	21	91	23	20	70	7
70718L	419	2007	nymph	Jurmala	21	1	2	97	118	109
70806L	88	2007	nymph	Tireli	8	9	9	9	55	5
71015L	16	2007	nymph	Jurmala	4	2	2	2	41	3
71121L	426	2007	nymph	Jaunciems	13	1	1	2	64	1
71212L	354	2007	nymph	Tireli	4	92	2	2	134	13
71324L	433	2007	nymph	Jaunciems	4	2	2	99	119	39
71403L	348	2007	nymph	Tireli	4	2	2	2	38	102
71524L	434	2007	nymph	Jaunciems	4	2	84	2	41	3
71709L	352	2007	nymph	Tireli	4	94	88	2	40	103
71821L	427	2007	nymph	Jaunciems	8	9	9	98	120	5

72306L	350	2007	nymph	Tireli	4	2	2	2	135	104
72312L	355	2007	nymph	Tireli	4	2	40	2	42	3
72415L	110	2007	nymph	Jurmala	13	1	1	1	65	2
72803L	349	2007	nymph	Tireli	4	2	89	2	42	105
72809L	115	2007	nymph	Tireli	13	1	34	1	63	1
72815L	336	2007	nymph	Jurmala	21	99	80	20	121	60
73212L	356	2007	nymph	Tireli	93	9	9	104	55	5
73406L	24	2007	nymph	Tireli	4	2	2	2	44	3
73515L	337	2007	nymph	Jurmala	13	100	1	1	64	101
73521L	428	2007	nymph	Jaunciems	8	9	81	9	55	5
73524L	379	2007	nymph	Jaunciems	43	2	2	100	40	13
73609L	353	2007	nymph	Tireli	4	96	2	2	42	3
73618L	420	2007	nymph	Jurmala	4	101	2	2	40	110
73821L	429	2007	nymph	Jaunciems	4	2	2	2	122	3
73824L	380	2007	nymph	Jaunciems	4	102	3	2	42	13
73921L	430	2007	nymph	Jaunciems	4	2	2	2	41	111
74112L	357	2007	nymph	Tireli	89	2	6	105	42	13
74606L	17	2007	nymph	Tireli	4	2	2	2	42	3
74612L	358	2007	nymph	Tireli	4	2	2	2	88	3
74624L	381	2007	nymph	Jaunciems	14	15	83	101	40	13

74715L	338	2007	nymph	Jurmala	13	1	1	1	63	41
74721L	431	2007	nymph	Jaunciems	5	27	2	2	123	22
74809L	186	2007	nymph	Tireli	25	17	26	28	55	17
74815L	339	2007	nymph	Jurmala	96	2	65	39	42	3
75218L	106	2007	nymph	Jurmala	13	1	1	1	63	2
75403L	17	2007	nymph	Tireli	4	2	2	2	42	3
75818L	421	2007	nymph	Jurmala	97	1	1	1	63	1
76015L	417	2007	nymph	Jurmala	13	1	82	1	124	1
76318L	422	2007	nymph	Jurmala	34	2	2	12	33	28
76615L	418	2007	nymph	Jurmala	4	80	2	2	122	3
76918L	423	2007	nymph	Jurmala	98	2	2	2	42	3

Tick Samples used for Comparison of British populations in 2006, 2007 and 2008 in section 4.3.1

Tick ID	ST	Year	Stage	Collection Location	*ATP6*	*COI*	*COII*	*COIII*	*12s*	*CYTB*
60107B	110	2006	nymph	Widcombe	13	1	1	1	65	2
60108B	129	2006	nymph	Widcombe	22	2	2	22	41	3
60201B	159	2006	nymph	Thurlbear Woods	4	2	3	2	42	13
60204B	90	2006	nymph	Widcombe	8	9	9	9	56	5
60205B	88	2006	nymph	American Museum	8	9	9	9	55	5
60206B	45	2006	nymph	Widcombe	4	2	19	2	41	6
60207B	88	2006	nymph	Widcombe	8	9	9	9	55	5
60208B	129	2006	nymph	Widcombe	22	2	2	22	41	3
60303B	95	2006	nymph	Widcombe	8	21	21	9	55	5
60304B	129	2006	nymph	Widcombe	22	2	2	22	41	3
60305B	17	2006	nymph	American Museum	4	2	2	2	42	3
60306B	129	2006	nymph	Widcombe	22	2	2	22	41	3
60307B	90	2006	nymph	Widcombe	8	9	9	9	56	5
60308B	116	2006	nymph	Widcombe	13	2	20	1	63	7
60325B	88	2006	nymph	Bathampton Woods	8	9	9	9	55	5
60405B	129	2006	nymph	American Museum	22	2	2	22	41	3
60406B	118	2006	nymph	Widcombe	14	15	2	16	42	13
60407B	124	2006	nymph	Widcombe	21	1	2	20	71	8
60408B	45	2006	nymph	Widcombe	4	2	19	2	41	6
60505B	88	2006	nymph	American Museum	8	9	9	9	55	5
60506B	129	2006	nymph	Widcombe	22	2	2	22	41	3
60507B	129	2006	nymph	Widcombe	22	2	2	22	41	3
60508B	96	2006	nymph	Widcombe	8	21	21	9	55	9
60515B	129	2006	nymph	Widcombe	22	2	2	22	41	3
60605B	30	2006	nymph	American Museum	4	2	2	12	34	5
60606B	131	2006	nymph	Widcombe	23	22	22	2	75	4
60607B	95	2006	nymph	Widcombe	8	21	21	9	55	5
60704B	81	2006	nymph	Widcombe	8	9	8	9	49	5
60705B	133	2006	nymph	American Museum	24	2	2	23	41	10
60706B	95	2006	nymph	Widcombe	8	21	21	9	55	5

60707B	95	2006	nymph	Widcombe	8	21	21	9	55	5
60709B	388	2006	nymph	Bathampton Woods	4	2	2	106	139	3
60806B	16	2006	nymph	Widcombe	4	2	2	2	41	3
60807B	46	2006	nymph	Widcombe	4	2	23	24	42	11
60809B	144	2006	nymph	Bathampton Woods	25	17	26	25	47	18
60904B	29	2006	nymph	Widcombe	4	2	2	12	34	4
60906B	124	2006	nymph	Widcombe	21	1	2	20	71	8
60907B	31	2006	nymph	Widcombe	4	2	2	12	34	13
60908B	133	2006	nymph	Widcombe	24	2	2	23	41	10
60910B	88	2006	nymph	Bathampton Woods	8	9	9	9	55	5
60930B	347	2006	nymph	Warleigh	4	87	2	12	33	13
61004B	137	2006	nymph	Widcombe	26	25	25	16	76	14
61006B	12	2006	nymph	Widcombe	4	2	2	2	32	3
61007B	90	2006	nymph	Widcombe	8	9	9	9	56	5
61008B	129	2006	nymph	Widcombe	22	2	2	22	41	3
61106B	31	2006	nymph	Widcombe	4	2	2	12	34	13
61107B	90	2006	nymph	Widcombe	8	9	9	9	56	5
61108B	81	2006	nymph	Widcombe	8	9	8	9	49	5
61206B	81	2006	nymph	Widcombe	8	9	8	9	49	5
61207B	29	2006	nymph	Widcombe	4	2	2	12	34	4
61208B	376	2006	nymph	Widcombe	8	21	21	94	55	5
61214B	95	2006	nymph	Widcombe	8	21	21	9	55	5
61220B	29	2006	nymph	Widcombe	4	2	2	12	34	4
61306B	45	2006	nymph	Widcombe	4	2	19	2	41	6
61307B	45	2006	nymph	Widcombe	4	2	19	2	41	6
61308B	63	2006	nymph	Widcombe	4	26	2	26	21	15
61406B	132	2006	nymph	Widcombe	23	22	22	2	75	9
61407B	133	2006	nymph	Widcombe	24	2	2	23	41	10
61408B	143	2006	nymph	Widcombe	5	27	2	27	70	16
61506B	88	2006	nymph	Widcombe	8	9	9	9	55	5
61507B	134	2006	nymph	Widcombe	25	17	26	28	51	17
61508B	144	2006	nymph	Widcombe	25	17	26	25	47	18
61527B	132	2006	nymph	Widcombe	23	22	22	2	75	9
61606B	116	2006	nymph	Widcombe	13	2	20	1	63	7

61607B	91	2006	nymph	Widcombe	8	9	9	29	55	5
61608B	132	2006	nymph	Widcombe	23	22	22	2	75	9
61706B	88	2006	nymph	Widcombe	8	9	9	9	55	5
61708B	90	2006	nymph	Widcombe	8	9	9	9	56	5
61710B	366	2006	nymph	Bathampton Woods	4	80	2	2	37	3
61806B	110	2006	nymph	Widcombe	13	1	1	1	65	2
61807B	16	2006	nymph	Widcombe	4	2	2	2	41	3
61808B	45	2006	nymph	Widcombe	4	2	19	2	41	6
61906B	29	2006	nymph	Widcombe	4	2	2	12	34	4
61907B	95	2006	nymph	Widcombe	8	21	21	9	55	5
61908B	138	2006	nymph	Widcombe	27	25	25	16	76	14
61919B	24	2006	nymph	Widcombe	4	2	2	2	44	3
62007B	81	2006	nymph	Widcombe	8	9	8	9	49	5
62107B	92	2006	nymph	Widcombe	8	9	9	31	55	5
62108B	129	2006	nymph	Widcombe	22	2	2	22	41	3
62207B	81	2006	nymph	Widcombe	8	9	8	9	49	5
62307B	132	2006	nymph	Widcombe	23	22	22	2	75	9
64418B	446	2006	nymph	Bathampton Woods	8	9	86	9	55	5
65302B	29	2006	nymph	Widcombe	4	2	2	12	34	4
70102B	90	2007	nymph	Widcombe	8	9	9	9	56	5
70115B	359	2007	nymph	Bathampton Woods	25	24	26	25	131	107
70118B	106	2007	nymph	Bathampton Woods	13	1	1	1	63	2
70120B	362	2007	nymph	Bathampton Woods	25	15	26	25	47	18
70136B	88	2007	nymph	Bathampton Woods	8	9	9	9	55	5
70142B	300	2007	nymph	Winsley	74	22	69	2	75	1
70222B	364	2007	nymph	Bathampton Woods	25	54	26	25	133	17
70246B	88	2007	nymph	Warleigh	8	9	9	9	55	5
70307B	159	2007	nymph	Widcombe	4	2	3	2	42	13
70316B	144	2007	nymph	Bathampton Woods	25	17	26	25	47	18
70320B	363	2007	nymph	Bathampton Woods	14	9	2	16	40	13
70329B	300	2007	nymph	Bathampton Woods	74	22	69	2	75	1
70334B	302	2007	nymph	Bathampton Woods	14	15	2	16	40	13
70408B	144	2007	nymph	Widcombe	25	17	26	25	47	18
70457B	88	2007	nymph	Warleigh	8	9	9	9	55	5

70516B	90	2007	nymph	Bathampton Woods	8	9	9	9	56	5
70580B	367	2007	nymph	Widcombe	8	9	9	9	55	84
70612B	24	2007	nymph	Bathampton Woods	4	2	2	2	44	3
70636B	365	2007	nymph	Bathampton Woods	90	9	9	9	55	5
70679B	88	2007	nymph	Bathampton Woods	8	9	9	9	55	5
70738B	305	2007	nymph	Bathampton Woods	62	17	26	17	108	17
70793B	302	2007	nymph	Eastwood	14	15	2	16	40	13
71053B	118	2007	nymph	Bathampton Woods	14	15	2	16	42	13
71152B	118	2007	nymph	Bathampton Woods	14	15	2	16	42	13
71268B	300	2007	nymph	Eastwood	74	22	69	2	75	1
71355B	88	2007	nymph	Warleigh	8	9	9	9	55	5
71380B	90	2007	nymph	Widcombe	8	9	9	9	56	5
71393B	144	2007	nymph	Eastwood	25	17	26	25	47	18
71452B	144	2007	nymph	Bathampton Woods	25	17	26	25	47	18
71457B	366	2007	nymph	Warleigh	4	80	2	2	37	3
71690B	118	2007	nymph	Bathampton Woods	14	15	2	16	42	13
72002B	144	2007	nymph	Widcombe	25	17	26	25	47	18
72068B	298	2007	nymph	Eastwood	72	77	9	9	55	86
72392B	302	2007	nymph	Warleigh	14	15	2	16	40	13
72402B	88	2007	nymph	Widcombe	8	9	9	9	55	5
72688B	368	2007	nymph	Widcombe	91	9	9	9	55	5
72790B	369	2007	nymph	Bathampton Woods	92	95	2	20	136	1
74002B	144	2007	nymph	Widcombe	25	17	26	25	47	18
74402B	132	2007	nymph	Widcombe	23	22	22	2	75	9
74902B	88	2007	nymph	Widcombe	8	9	9	9	55	5
75702B	298	2007	nymph	Widcombe	72	77	9	9	55	86
80104B	298	2008	nymph	Eastwood	72	77	9	9	55	86
80109B	302	2008	nymph	Rainbow Woods	14	15	2	16	40	13
80204B	88	2008	nymph	Eastwood	8	9	9	9	55	5
80210B	88	2008	nymph	Rainbow Woods	8	9	9	9	55	5
80405B	300	2008	nymph	Bathampton Woods	74	22	69	2	75	1
80701B	88	2008	nymph	Eastwood	8	9	9	9	55	5
80809B	307	2008	nymph	Rainbow Woods	21	1	23	20	106	7
80839B	110	2008	nymph	Widcombe	13	1	1	1	65	2

80905B	144	2008	nymph	Bathampton Woods	25	17	26	25	47	18
80906B	304	2008	nymph	Widcombe	78	9	9	9	54	5
80910B	17	2008	nymph	Rainbow Woods	4	2	2	2	42	3
81001B	297	2008	nymph	Eastwood	4	2	3	64	42	13
81005B	144	2008	nymph	Bathampton Woods	25	17	26	25	47	18
81010B	17	2008	nymph	Rainbow Woods	4	2	2	2	42	3
81039B	144	2008	nymph	Widcombe	25	17	26	25	47	18
81101B	294	2008	nymph	Eastwood	13	1	71	1	63	1
81206B	17	2008	nymph	Widcombe	4	2	2	2	42	3
81301B	17	2008	nymph	Eastwood	4	2	2	2	42	3
81305B	118	2008	nymph	Bathampton Woods	14	15	2	16	42	13
81406B	45	2008	nymph	Widcombe	4	2	19	2	41	6
81409B	16	2008	nymph	Rainbow Woods	4	2	2	2	41	3
81439B	8	2008	nymph	Widcombe	4	2	2	2	27	3
81509B	110	2008	nymph	Rainbow Woods	13	1	1	1	65	2
81539B	16	2008	nymph	Widcombe	4	2	2	2	41	3
81639B	307	2008	nymph	Widcombe	21	1	23	20	106	7
82214B	88	2008	nymph	Bathampton Woods	8	9	9	9	55	5
82705B	301	2008	nymph	Bathampton Woods	75	2	2	2	62	3
82805B	299	2008	nymph	Bathampton Woods	5	1	2	76	70	87
82806B	305	2008	nymph	Widcombe	62	17	26	17	108	17
83105B	88	2008	nymph	Bathampton Woods	8	9	9	9	55	5
83205B	439	2008	nymph	Bathampton Woods	4	2	2	2	55	82
83206B	133	2008	nymph	Widcombe	24	2	2	23	41	10
83305B	302	2008	nymph	Bathampton Woods	14	15	2	16	40	13
83714B	308	2008	nymph	Bathampton Woods	8	9	9	9	89	84
84114B	297	2008	nymph	Bathampton Woods	4	2	3	64	42	13
85314B	90	2008	nymph	Bathampton Woods	8	9	9	9	56	5
85714B	309	2008	nymph	Bathampton Woods	14	15	2	16	110	13
85814B	118	2008	nymph	Bathampton Woods	14	15	2	16	42	13

Mismatch distribution analyses data for Latvia combined populations (DNAsp)

Differences	observed	constant	changing
0	0.00956	0.07005	0.00431
1	0.03248	0.06514	0.01494
2	0.05825	0.06058	0.03169
3	0.07902	0.05634	0.0487
4	0.07498	0.05239	0.06067
5	0.05951	0.04872	0.06597
6	0.04386	0.04531	0.06591
7	0.03442	0.04213	0.06271
8	0.02799	0.03918	0.05816
9	0.03032	0.03644	0.05328
10	0.03686	0.03388	0.04857
11	0.04745	0.03151	0.04418
12	0.06064	0.0293	0.04017
13	0.05831	0.02725	0.03651
14	0.04295	0.02534	0.03319
15	0.03055	0.02357	0.03016
16	0.01849	0.02192	0.02741
17	0.01132	0.02038	0.02492
18	0.00666	0.01895	0.02265
19	0.00273	0.01763	0.02058
20	0.00125	0.01639	0.01871
21	0.00051	0.01524	0.017
22	0.00051	0.01417	0.01545
23	0.00091	0.01318	0.01405
24	0.00341	0.01226	0.01277
25	0.00558	0.0114	0.0116
26	0.00711	0.0106	0.01055
27	0.00694	0.00986	0.00959
28	0.01024	0.00917	0.00871
29	0.02048	0.00853	0.00792

30	0.03476	0.00793	0.0072
31	0.04597	0.00737	0.00654
32	0.03954	0.00686	0.00594
33	0.026	0.00638	0.0054
34	0.01445	0.00593	0.00491
35	0.00882	0.00551	0.00446
36	0.00432	0.00513	0.00406
37	0.00188	0.00477	0.00369
38	0.00063	0.00443	0.00335
39	0.00028	0.00412	0.00305
40	0.00006	0.00384	0.00277
41	0	0.00357	0.00252
42	0	0.00332	0.00229
43	0	0.00308	0.00208
44	0	0.00287	0.00189

Mismatch distribution analyses data for Latvia combined populations (Arlequin)

Differences	observed	spatial	demographic
0	60	125.2	1654.4
1	261	411.1	3909.6
2	640	729	4619.6
3	1071	948.8	3639.1
4	1262	1039.4	2150.1
5	1245	1037.6	1016.2
6	1030	989.4	400.3
7	749	924.9	135.1
8	558	858	39.9
9	522	794	10.5
10	529	734.2	2.5
11	646	678.8	0.5
12	847	627.5	0.1
13	1019	580.1	0

14	915	536.3	0
15	732	495.8	0
16	540	458.3	0
17	375	423.7	0
18	252	391.7	0
19	140	362.1	0
20	64	334.8	0
21	24	309.5	0
22	16	286.1	0
23	7	264.5	0
24	3	244.5	0
25	10	226	0
26	28	209	0
27	67	193.2	0
28	94	178.6	0
29	98	165.1	0
30	135	152.6	0
31	170	141.1	0
32	339	130.4	0
33	587	120.6	0
34	770	111.5	0
35	712	103.1	0
36	484	95.3	0
37	274	88.1	0
38	157	81.4	0
39	92	75.3	0
40	36	69.6	0
41	13	64.3	0
42	3	59.5	0
43	2	55	0
44	0	50.8	0

Mismatch distribution analyses data for 2002 Latvia population (Arlequin)

Differences	observed	spatial	demographic
0	5	3.9	3.6
1	4	11.6	11.6
2	22	20.5	20.5
3	27	26.6	26.6
4	48	29.2	29.2
5	33	29.4	29.4
6	21	28.4	28.4
7	15	26.9	26.9
8	6	25.3	25.3
9	4	23.8	23.8
10	12	22.4	22.4
11	29	21	21
12	32	19.8	19.8
13	51	18.6	18.6
14	51	17.4	17.4
15	39	16.4	16.4
16	36	15.4	15.4
17	19	14.5	14.5
18	4	13.6	13.6
19	8	12.8	12.8
20	1	12	12
21	3	11.3	11.3
22	1	10.6	10.6
23	0	9.9	9.9
24	0	9.3	9.3
25	0	8.8	8.8
26	0	8.2	8.2
27	1	7.7	7.7
28	2	7.3	7.3
29	3	6.8	6.8

30	2	6.4	6.4
31	13	6	6
32	15	5.7	5.7
33	11	5.3	5.3
34	7	5	5
35	30	4.7	4.7
36	28	4.4	4.4
37	6	4.1	4.1
38	3	3.9	3.9
39	0	3.7	3.7
40	3	3.4	3.4
41	0	3.2	3.2

Mismatch distribution analyses data for 2006 Latvia population (Arlequin)

Differences	observed	spatial	demographic
0	23	48.1	48.1
1	88	149.9	149.9
2	227	254	254.1
3	345	318.7	318.7
4	444	339.7	339.8
5	411	332.9	332.9
6	329	313.5	313.5
7	227	290.5	290.5
8	204	267.6	267.6
9	165	246.1	246.1
10	161	226.2	226.2
11	175	207.9	207.9
12	228	191.1	191.1
13	246	175.6	175.6
14	213	161.4	161.4
15	146	148.3	148.3
16	120	136.3	136.3

17	78	125.3	125.3
18	64	115.2	115.2
19	30	105.8	105.8
20	14	97.3	97.3
21	6	89.4	89.4
22	4	82.2	82.2
23	0	75.5	75.5
24	2	69.4	69.4
25	1	63.8	63.8
26	13	58.6	58.6
27	26	53.9	53.9
28	18	49.5	49.5
29	14	45.5	45.5
30	36	41.8	41.8
31	41	38.4	38.4
32	135	35.3	35.3
33	262	32.5	32.5
34	289	29.8	29.8
35	266	27.4	27.4
36	160	25.2	25.2
37	77	23.2	23.2
38	40	21.3	21.3
39	23	19.6	19.6
40	5	18	18
41	0	16.5	16.5

Mismatch distribution analyses data for 2007 Latvia population (Arlequin)

Differences	observed	spatial	demographic
0	1	4.3	4.3
1	16	17.2	17.3
2	34	36.5	36.6
3	58	55	55

4	67	67.2	67.3
5	78	72.2	72.2
6	75	71.7	71.7
7	67	68.2	68.2
8	45	63.6	63.6
9	44	58.7	58.7
10	49	54	54
11	49	49.6	49.6
12	62	45.6	45.6
13	69	41.8	41.8
14	67	38.4	38.4
15	54	35.3	35.3
16	38	32.4	32.4
17	34	29.8	29.8
18	23	27.3	27.3
19	14	25.1	25.1
20	7	23	23
21	2	21.2	21.2
22	2	19.4	19.4
23	2	17.8	17.8
24	0	16.4	16.4
25	0	15	15
26	2	13.8	13.8
27	5	12.7	12.7
28	12	11.7	11.7
29	12	10.7	10.7
30	5	9.8	9.8
31	12	9	9
32	21	8.3	8.3
33	29	7.6	7.6
34	34	7	7
35	19	6.4	6.4
36	21	5.9	5.9
37	12	5.4	5.4

38	18	5	5
39	9	4.6	4.6
40	4	4.2	4.2
41	1	3.8	3.8
42	2	3.5	3.5
43	1	3.2	3.2
44	0	3	3

Mismatch distribution analyses data for Britain combined populations (DNAsp)

Differences	observed	constant	changing
0	0.05222	0.0488	0.00102
1	0.01557	0.04642	0.0012
2	0.02369	0.04416	0.00193
3	0.03796	0.042	0.00388
4	0.01802	0.03995	0.00779
5	0.0286	0.038	0.01403
6	0.025	0.03615	0.02223
7	0.02109	0.03438	0.03132
8	0.03106	0.0327	0.03991
9	0.05038	0.03111	0.04678
10	0.02193	0.02959	0.05126
11	0.02446	0.02815	0.05325
12	0.02208	0.02677	0.05312
13	0.02339	0.02547	0.05144
14	0.03175	0.02422	0.04881
15	0.02078	0.02304	0.04568
16	0.01388	0.02192	0.0424
17	0.01764	0.02085	0.03916
18	0.01595	0.01983	0.03608
19	0.00744	0.01886	0.03318
20	0.00261	0.01794	0.0305
21	0.00253	0.01707	0.02803

22	0.00092	0.01623	0.02576
23	0.00345	0.01544	0.02366
24	0.01035	0.01469	0.02174
25	0.00989	0.01397	0.01998
26	0.01426	0.01329	0.01835
27	0.03014	0.01264	0.01686
28	0.02922	0.01202	0.01549
29	0.03765	0.01144	0.01423
30	0.07591	0.01088	0.01308
31	0.05958	0.01035	0.01201
32	0.05805	0.00984	0.01104
33	0.05835	0.00936	0.01014
34	0.05276	0.00891	0.00932
35	0.02492	0.00847	0.00856
36	0.01603	0.00806	0.00786
37	0.00974	0.00766	0.00722
38	0.00077	0.00729	0.00664
39	0	0.00693	0.0061
40	0	0.0066	0.0056
41	0	0.00627	0.00515
42	0	0.00597	0.00473
43	0	0.00568	0.00434
44	0	0.0054	0.00399
45	0	0.00514	0.00367
46	0	0.00489	0.00337
47	0	0.00465	0.0031
48	0	0.00442	0.00284

Mismatch distribution analyses data for Britain combined populations (Arlequin)

Differences	observed	spatial	demographic
0	438	608.8	303.9
1	346	534.2	296.8
2	259	468.8	289.9
3	416	411.4	283.1
4	324	361	276.6
5	283	316.8	270.1
6	300	278.1	263.8
7	333	244	257.7
8	325	214.1	251.7
9	406	187.9	245.8
10	461	165	240.1
11	415	144.9	234.5
12	304	127.5	229.1
13	298	112.5	223.8
14	394	100	218.7
15	309	90	214
16	258	82.7	209.7
17	170	78.5	206.2
18	245	77.7	203.8
19	226	80.7	203.2
20	46	87.7	205
21	44	98.6	210.1
22	24	113.3	219.4
23	11	131.1	233.6
24	42	151	253.2
25	69	172	278.3
26	121	192.9	308
27	137	212.5	341
28	237	229.7	375.3

29	382	243.8	408.2
30	492	254.3	437.1
31	742	261	459
32	809	264.1	471.8
33	776	263.9	473.9
34	859	260.9	464.8
35	654	255.5	444.9
36	466	248.3	415.4
37	332	239.9	378.5
38	110	230.7	336.5
39	118	221	292
40	60	211.1	247.5
41	0	201.3	204.8

Mismatch distribution analyses data for 2006 British population (Arlequin)

differences	observed	spatial	demographic
0	136	168.4	76.1
1	58	146.7	74.4
2	71	127.8	72.7
3	119	111.3	71.1
4	97	97	69.5
5	101	84.5	67.9
6	122	73.6	66.4
7	107	64.1	64.9
8	169	55.8	63.5
9	54	48.6	62.1
10	86	42.4	60.7
11	52	36.9	59.3
12	61	32.2	58
13	63	28.2	56.7
14	135	24.7	55.5
15	90	21.9	54.3

16	48	19.7	53.2
17	40	18.2	52.2
18	111	17.5	51.4
19	28	17.6	51
20	8	18.7	51
21	29	20.8	51.6
22	3	23.9	53.2
23	3	28	55.7
24	3	32.9	59.6
25	16	38.3	64.8
26	18	44.1	71.4
27	11	49.9	79.1
28	42	55.3	87.6
29	58	60.1	96.5
30	81	64.1	105
31	136	67.2	112.4
32	216	69.2	118.1
33	201	70.3	121.6
34	275	70.4	122.3
35	270	69.7	120.3
36	138	68.4	115.5
37	107	66.5	108.2
38	26	64.3	99.1
39	14	61.7	88.5
40	0	59.1	77.2

Mismatch distribution analyses data for 2007 British population (Arlequin)

Differences	observed	spatial	demographic
0	49	48.7	22.1
1	51	40.1	21.5
2	12	33	20.9
3	18	27.2	20.3

4	13	22.4	19.8
5	1	18.4	19.3
6	10	15.2	18.7
7	12	12.5	18.2
8	18	10.3	17.7
9	58	8.5	17.3
10	44	7	16.8
11	14	5.8	16.4
12	18	4.8	15.9
13	6	4	15.5
14	15	3.4	15.1
15	15	3.1	14.7
16	14	2.9	14.4
17	10	3	14.1
18	11	3.4	13.8
19	19	4.1	13.7
20	12	5.1	13.8
21	0	6.4	14
22	1	8.1	14.5
23	3	9.9	15.3
24	2	11.8	16.5
25	6	13.8	17.9
26	13	15.7	19.5
27	26	17.4	21.3
28	20	18.8	23.1
29	34	19.9	24.8
30	47	20.6	26.2
31	30	20.9	27.1
32	24	21	27.5
33	45	20.7	27.2
34	36	20.2	26.4
35	22	19.5	24.9
36	39	18.7	23
37	32	17.8	20.7

38	8	16.8	18.1
39	6	15.8	15.6
40	6	14.9	13
41	0	13.9	10.6

Mismatch distribution analyses data for 2008 British population (Arlequin)

Differences	observed	spatial	demographic
0	25	31	18.9
1	21	28.2	18.4
2	14	25.6	17.9
3	23	23.2	17.4
4	13	21.1	16.9
5	10	19.1	16.5
6	20	17.3	16
7	25	15.7	15.6
8	15	14.3	15.2
9	27	13	14.8
10	25	11.8	14.4
11	32	10.7	14
12	33	9.7	13.6
13	29	8.8	13.3
14	15	8.1	12.9
15	12	7.4	12.6
16	16	6.9	12.3
17	8	6.5	12
18	4	6.2	11.8
19	8	6.2	11.7
20	2	6.3	11.7
21	0	6.5	11.9
22	1	7	12.3
23	0	7.5	12.9
24	3	8.2	13.8

25	6	8.9	15
26	2	9.7	16.3
27	10	10.4	17.9
28	18	11	19.4
29	13	11.4	20.9
30	38	11.8	22.1
31	38	12	23
32	31	12.1	23.4
33	53	12	23.3
34	36	11.9	22.7
35	22	11.6	21.5
36	23	11.3	20
37	17	10.9	18.1
38	4	10.5	16
39	8	10.1	13.8
40	3	9.7	11.6
41	0	9.3	9.5

Tick samples used for comparison of Southern England populations in section 5.2

This table shows ticks collected from Exmoor.

Tick ID	ST	Year	Stage	Collection Location	*ATP6*	*COI*	*COII*	*COIII*	*12s*	*CYTB*
80118B	244	2008	nymph	Exmoor	8	21	9	9	55	5
80323B	277	2008	nymph	Exmoor	51	71	2	63	42	3
80420B	178	2008	nymph	Exmoor	4	2	2	2	38	3
80524B	278	2008	nymph	Exmoor	5	1	2	2	70	22
80618B	245	2008	nymph	Exmoor	55	9	9	9	55	5
80721B	88	2008	nymph	Exmoor	8	9	9	9	55	5
80922B	265	2008	nymph	Exmoor	59	9	9	68	55	5
81022B	88	2008	nymph	Exmoor	8	9	9	9	55	5
81118B	94	2008	nymph	Exmoor	8	9	16	9	55	5
81120B	106	2008	nymph	Exmoor	13	1	1	1	63	2
81121B	260	2008	nymph	Exmoor	60	9	9	9	55	5
81122B	266	2008	nymph	Exmoor	8	9	9	9	95	5
81223B	88	2008	nymph	Exmoor	8	9	9	9	55	5
81422B	267	2008	nymph	Exmoor	61	9	9	9	55	5
81823B	88	2008	nymph	Exmoor	8	9	9	9	55	5

82019B	18	2008	nymph	Exmoor	4	2	2	2	42	13
82222B	286	2008	nymph	Exmoor	69	9	9	9	55	5
82223B	17	2008	nymph	Exmoor	4	2	2	2	42	3
82322B	268	2008	nymph	Exmoor	13	1	59	1	63	1
82719B	249	2008	nymph	Exmoor	65	1	1	1	63	81
82820B	254	2008	nymph	Exmoor	66	2	2	2	41	3
82822B	269	2008	nymph	Exmoor	67	2	3	2	44	13
83120B	255	2008	nymph	Exmoor	13	59	1	71	98	1
83220B	16	2008	nymph	Exmoor	4	2	2	2	41	3
83320B	256	2008	nymph	Exmoor	13	60	61	1	100	1
83419B	87	2008	nymph	Exmoor	8	9	9	9	54	5
85120B	257	2008	nymph	Exmoor	8	9	9	73	55	83
86520B	88	2008	nymph	Exmoor	8	9	9	9	55	5

This table shows ticks collected from the New Forest.

Tick ID	**ST**	**Year**	**Stage**	**Collection Location**	***ATP6***	***COI***	***COII***	***COIII***	***12s***	***CYTB***
80127B	283	2008	nymph	New Forest	4	2	2	2	42	72
80128B	313	2008	nymph	New Forest	8	9	9	9	49	5
80129B	317	2008	nymph	New Forest	71	78	67	9	105	5

80228B	314	2008	nymph	New Forest	8	9	9	77	55	5
80326B	281	2008	nymph	New Forest	52	9	9	9	92	5
80327B	284	2008	nymph	New Forest	4	2	2	2	93	3
80328B	178	2008	nymph	New Forest	4	2	2	2	38	3
80428B	88	2008	nymph	New Forest	8	9	9	9	55	5
80429B	318	2008	nymph	New Forest	76	9	9	9	48	88
80527B	285	2008	nymph	New Forest	53	57	2	66	72	7
80529B	319	2008	nymph	New Forest	63	17	26	70	55	18
80729B	320	2008	nymph	New Forest	4	2	2	78	44	3
80827B	105	2008	nymph	New Forest	13	1	1	1	63	1
80828B	315	2008	nymph	New Forest	5	79	2	2	70	22
80929B	321	2008	nymph	New Forest	70	2	70	2	40	89
81028B	316	2008	nymph	New Forest	4	2	2	2	62	3
81029B	322	2008	nymph	New Forest	4	2	2	2	43	3
81127B	106	2008	nymph	New Forest	13	1	1	1	63	2
81128B	88	2008	nymph	New Forest	8	9	9	9	55	5
81327B	17	2008	nymph	New Forest	4	2	2	2	42	3
81427B	178	2008	nymph	New Forest	4	2	2	2	38	3
81529B	221	2008	nymph	New Forest	4	72	2	2	42	3
81627B	310	2008	nymph	New Forest	62	17	26	17	56	17

81926B	282	2008	nymph	New Forest	25	54	24	70	55	17
81927B	311	2008	nymph	New Forest	4	2	2	51	107	3
82127B	312	2008	nymph	New Forest	8	9	72	9	55	5
82327B	184	2008	nymph	New Forest	4	2	2	2	40	3
82427B	178	2008	nymph	New Forest	4	2	2	2	38	3

This table shows ticks collected from Richmond Park

Tick ID	**ST**	**Year**	**Stage**	**Collection Location**	***ATP6***	***COI***	***COII***	***COIII***	***12s***	***CYTB***
80133B	180	2008	nymph	Richmond Park	4	2	2	2	74	3
80134B	173	2008	nymph	Richmond Park	45	1	1	1	63	50
80230B	57	2008	nymph	Richmond Park	39	46	2	2	34	3
80233B	180	2008	nymph	Richmond Park	4	2	2	2	74	3
80234B	175	2008	nymph	Richmond Park	11	1	44	11	70	1
80334B	17	2008	nymph	Richmond Park	4	2	2	2	42	3
80430B	183	2008	nymph	Richmond Park	21	1	46	20	80	60
80434B	175	2008	nymph	Richmond Park	11	1	44	11	70	1
80534B	17	2008	nymph	Richmond Park	4	2	2	2	42	3
80633B	17	2008	nymph	Richmond Park	4	2	2	2	42	3
80634B	179	2008	nymph	Richmond Park	47	48	9	9	55	5

80635B	172	2008	nymph	Richmond Park	8	9	9	29	78	5
80734B	180	2008	nymph	Richmond Park	4	2	2	2	74	3
80834B	171	2008	nymph	Richmond Park	46	17	26	25	49	18
80933B	17	2008	nymph	Richmond Park	4	2	2	2	42	3
81033B	176	2008	nymph	Richmond Park	39	46	2	2	37	3
81035B	187	2008	nymph	Richmond Park	8	9	9	30	55	5
81135B	57	2008	nymph	Richmond Park	39	46	2	2	34	3
81233B	177	2008	nymph	Richmond Park	8	9	9	9	55	59
81235B	177	2008	nymph	Richmond Park	8	9	9	9	55	59
81335B	175	2008	nymph	Richmond Park	11	1	44	11	70	1
81433B	178	2008	nymph	Richmond Park	4	2	2	2	38	3
81435B	17	2008	nymph	Richmond Park	4	2	2	2	42	3
81535B	187	2008	nymph	Richmond Park	8	9	9	30	55	5
81633B	17	2008	nymph	Richmond Park	4	2	2	2	42	3
81635B	180	2008	nymph	Richmond Park	4	2	2	2	74	3
81734B	17	2008	nymph	Richmond Park	4	2	2	2	42	3
81834B	184	2008	nymph	Richmond Park	4	2	2	2	40	3
81835B	180	2008	nymph	Richmond Park	4	2	2	2	74	3
81935B	17	2008	nymph	Richmond Park	4	2	2	2	42	3
82035B	17	2008	nymph	Richmond Park	4	2	2	2	42	3

82134B	57	2008	nymph	Richmond Park	39	46	2	2	34	3
82135B	17	2008	nymph	Richmond Park	4	2	2	2	42	3
82235B	216	2008	nymph	Richmond Park	47	48	9	9	55	59
82334B	183	2008	nymph	Richmond Park	21	1	46	20	80	60
82734B	175	2008	nymph	Richmond Park	11	1	44	11	70	1
82934B	172	2008	nymph	Richmond Park	8	9	9	29	78	5
83034B	186	2008	nymph	Richmond Park	25	17	26	28	55	17
83134B	180	2008	nymph	Richmond Park	4	2	2	2	74	3

Mismatch distribution analyses data for Exmoor samples from a Southern England population (Arlequin)

differences	observed	spatial	demographic
0	10	33.8	9
1	43	25.8	8.8
2	43	19.7	8.6
3	20	15	8.4
4	7	11.4	8.2
5	3	8.7	8
6	4	6.6	7.8
7	6	5.1	7.6
8	5	3.9	7.5
9	2	2.9	7.3
10	4	2.2	7.1
11	5	1.7	6.9
12	6	1.3	6.8
13	5	1	6.6
14	6	0.8	6.4
15	6	0.6	6.3
16	2	0.5	6.2
17	3	0.5	6
18	1	0.5	5.9
19	2	0.6	5.8
20	0	0.8	5.8
21	0	1.2	5.8
22	0	1.7	5.9
23	0	2.3	6.2
24	0	3	6.5
25	0	3.9	7
26	0	4.9	7.6
27	1	6	8.3
28	5	7.1	9.2
29	7	8.1	10

30	6	9.1	10.9
31	12	9.9	11.7
32	21	10.5	12.3
33	24	10.9	12.7
34	33	11.1	12.9
35	34	11.1	12.7
36	19	10.9	12.3
37	9	10.5	11.6
38	8	10.1	10.7
39	7	9.5	9.6
40	7	8.9	8.5
41	2	8.2	7.3

Mismatch distribution analyses data for New Forest samples from a Southern England population (Arlequin)

differences	observed	spatial	demographic
0	4	21.4	8.9
1	16	18.3	8.7
2	25	15.6	8.5
3	28	13.3	8.3
4	10	11.3	8.1
5	4	9.7	7.9
6	5	8.3	7.7
7	8	7	7.5
8	7	6	7.3
9	8	5.1	7.2
10	14	4.4	7
11	17	3.7	6.8
12	17	3.2	6.7
13	10	2.7	6.5
14	9	2.3	6.4
15	7	2	6.2
16	0	1.8	6.1

17	1	1.6	6
18	0	1.5	5.9
19	1	1.6	5.8
20	0	1.7	5.8
21	0	1.9	5.8
22	0	2.3	5.9
23	0	2.7	6.2
24	0	3.3	6.5
25	0	4	7
26	3	4.7	7.7
27	2	5.4	8.5
28	8	6.1	9.3
29	8	6.8	10.2
30	10	7.4	11.1
31	10	7.8	11.9
32	13	8.2	12.5
33	26	8.4	12.9
34	33	8.4	13.1
35	33	8.4	12.9
36	19	8.3	12.4
37	6	8.1	11.7
38	1	7.8	10.8
39	8	7.5	9.7
40	7	7.1	8.5
41	0	6.8	7.3

Mismatch distribution analyses data for Richmond Park samples from a Southern England population (Arlequin)

differences	observed	spatial	demographic
0	73	66.5	27.3
1	27	52.4	26.2
2	67	41.3	25.3

3	20	32.5	24.4
4	54	25.6	23.5
5	8	20.2	22.6
6	18	15.9	21.8
7	0	12.5	21
8	4	9.9	20.2
9	11	7.8	19.5
10	5	6.1	18.7
11	3	4.8	18
12	11	3.8	17.4
13	46	3	16.7
14	36	2.4	16.1
15	40	1.9	15.5
16	22	1.6	15
17	4	1.4	14.4
18	2	1.3	13.9
19	0	1.3	13.5
20	0	1.5	13.1
21	0	2	12.8
22	0	2.6	12.5
23	0	3.5	12.4
24	0	4.7	12.5
25	0	6.1	12.7
26	0	7.8	13
27	10	9.6	13.5
28	2	11.4	14.2
29	23	13.3	14.9
30	5	15.1	15.6
31	14	16.7	16.3
32	3	18	16.8
33	41	18.9	17.1
34	46	19.5	17.2
35	74	19.8	16.9
36	47	19.7	16.3

37	20	19.3	15.4
38	4	18.6	14.2
39	1	17.7	12.9
40	0	16.7	11.4

Mismatch distribution analyses data for a Southern England population (Arlequin)

differences	observed	spatial	demographic
0	127	293.4	120.3
1	267	247.1	117.1
2	319	208.1	113.9
3	240	175.2	110.9
4	162	147.6	107.9
5	103	124.3	105
6	77	104.7	102.1
7	46	88.1	99.4
8	57	74.2	96.7
9	87	62.5	94.1
10	97	52.6	91.6
11	98	44.4	89.1
12	120	37.4	86.7
13	154	31.6	84.4
14	137	26.8	82.1
15	124	23	80
16	74	20.1	78
17	31	18.2	76.1
18	15	17.4	74.6
19	3	17.7	73.5
20	3	19.4	72.9
21	0	22.6	73.1
22	0	27.3	74.4
23	1	33.4	77
24	1	40.8	81

25	3	49.3	86.6
26	7	58.4	93.6
27	31	67.7	101.8
28	37	76.6	110.8
29	77	84.7	120
30	58	91.6	128.5
31	115	97.1	135.6
32	168	100.8	140.5
33	335	102.9	142.8
34	434	103.3	142
35	420	102.3	138
36	230	100	131
37	84	96.7	121.5
38	40	92.7	110.1
39	28	88.2	97.4
40	31	83.3	84.2
41	12	78.4	71.1
42	10	73.5	58.7
43	1	68.6	47.4
44	0	64	37.4
45	1	59.6	28.9
46	0	55.4	21.8

Mismatch distribution analyses data for a Southern England population (DNAsp)

differences	observed	constant	changing
0	0.04479	0.05182	0.00127
1	0.08623	0.04914	0.00276
2	0.06271	0.04659	0.00684
3	0.05039	0.04418	0.01423
4	0.03247	0.04189	0.02416
5	0.01389	0.03972	0.03461
6	0.01075	0.03766	0.04342

7	0.01501	0.03571	0.04928
8	0.0168	0.03386	0.05198
9	0.01501	0.0321	0.05209
10	0.01635	0.03044	0.05046
11	0.03427	0.02886	0.04789
12	0.04793	0.02737	0.04491
13	0.03628	0.02595	0.04185
14	0.01904	0.0246	0.03889
15	0.01389	0.02333	0.03609
16	0.00649	0.02212	0.03347
17	0.00067	0.02097	0.03103
18	0.00134	0.01989	0.02877
19	0	0.01885	0.02668
20	0	0.01788	0.02473
21	0	0.01695	0.02293
22	0.00022	0.01607	0.02126
23	0.00045	0.01524	0.01971
24	0.0009	0.01445	0.01827
25	0.00448	0.0137	0.01694
26	0.00896	0.01299	0.01571
27	0.01545	0.01232	0.01456
28	0.01635	0.01168	0.0135
29	0.02016	0.01107	0.01252
30	0.05375	0.0105	0.0116
31	0.08712	0.00996	0.01076
32	0.09384	0.00944	0.00997
33	0.07839	0.00895	0.00925
34	0.0477	0.00849	0.00857
35	0.02217	0.00805	0.00795
36	0.01232	0.00763	0.00737
37	0.00717	0.00723	0.00683
38	0.00314	0.00686	0.00633
39	0.00269	0.0065	0.00587
40	0.00022	0.00617	0.00544

41	0	0.00585	0.00505
42	0.00022	0.00554	0.00468
43	0	0.00526	0.00434
44	0	0.00498	0.00402
45	0	0.00473	0.00373
46	0	0.00448	0.00346
47	0	0.00425	0.00321
48	0	0.00403	0.00297

Tick samples used for comparison of infected ticks in section 6.2 and section 6.3

This table shows ticks collected from Latvia in 2006

Tick ID	ST	Year	Stage	Collection Location	Borrelia sp	*ATP6*	*COI*	*COII*	*COIII*	*12s*	*CYTB*
60618L	443	2006	nymph	Jurmala	B. afzelii	8	9	85	9	125	5
60724L	342	2006	nymph	Jaunciems	*B. afzelii*	89	2	3	102	126	13
61009L	106	2006	nymph	Tireli	*B. garinii*	13	1	1	1	63	2
61218L	24	2006	nymph	Jurmala	*B. afzelii*	4	2	2	2	44	3
61306L	51	2006	nymph	Babite	*B. valaisiana*	4	2	35	2	41	3
61406L	386	2006	nymph	Babite	*B. garinii*	4	2	2	64	42	13
61618L	47	2006	nymph	Jurmala	*B. valaisiana*	4	2	29	2	42	3
61712L	49	2006	nymph	Babite	mixed	4	2	32	2	8	3
61809L	16	2006	nymph	Tireli	*B. valaisiana*	4	2	2	2	41	3
61918L	195	2006	nymph	Jurmala	*B. afzelii*	13	1	1	1	84	1
62103L	341	2006	nymph	Tireli	*B. valaisiana*	8	21	9	9	55	86
62118L	444	2006	nymph	Jurmala	*B. garinii*	4	2	3	2	42	89
62218L	445	2006	nymph	Jurmala	*B. afzelii*	4	2	3	2	43	13
62303L	184	2006	nymph	Tireli	*B. garinii*	4	2	2	2	40	3
62306L	387	2006	nymph	Babite	*B. garinii*	13	103	1	1	63	1

62309L	389	2006	nymph	Tireli	*B. garinii*	4	81	2	2	14	3
62506L	373	2006	nymph	Babite	*B. garinii*	79	2	73	2	42	13
62909L	159	2006	nymph	Tireli	*B. garinii*	4	2	3	2	42	13
63012L	394	2006	nymph	Babite	*B. valaisiana*	4	36	2	2	40	13
63203L	371	2006	nymph	Tireli	*B. afzelii*	4	82	74	2	42	13
63309L	390	2006	nymph	Tireli	mixed	4	2	2	107	42	3
63321L	342	2006	nymph	Jaunciems	*B. afzelii*	89	2	3	102	126	13
63424L	344	2006	nymph	Jaunciems	*B. afzelii*	4	2	2	103	127	3
63503L	88	2006	nymph	Tireli	*B. valaisiana*	8	9	9	9	55	5
63521L	343	2006	nymph	Jaunciems	*B. afzelii*	14	15	2	16	40	28
63603L	372	2006	nymph	Tireli	*B. valaisiana*	80	2	2	2	41	92
63706L	374	2006	nymph	Babite	mixed	4	2	2	93	34	13
63721L	342	2006	nymph	Jaunciems	*B. afzelii*	89	2	3	102	126	13
63806L	375	2006	nymph	Babite	*B. valaisiana*	81	1	2	20	72	93
63809L	377	2006	nymph	Tireli	*B. garinii*	4	2	2	80	33	94
63909L	391	2006	nymph	Tireli	*B. valaisiana*	6	83	2	5	140	1
64224L	345	2006	nymph	Jaunciems	*B. afzelii*	4	88	2	2	41	13
64409L	392	2006	nymph	Tireli	*B. afzelii*	8	9	9	108	55	108
64624L	346	2006	nymph	Jaunciems	*B. afzelii*	8	89	9	9	56	106
64703L	384	2006	nymph	Tireli	mixed	4	2	2	81	141	3

64803L	350	2006	nymph	Tireli	mixed	4	2	2	2	135	104
65006L	97	2006	nymph	Babite	*B. garinii*	8	34	26	9	56	46
66612L	378	2006	nymph	Babite	*B. garinii*	4	2	2	82	128	3
68412L	440	2006	nymph	Babite	*B. valaisiana*	4	2	90	2	129	3
69212L	88	2006	nymph	Babite	*B. garinii*	8	9	9	9	55	5
6101012L	441	2006	nymph	Babite	*B. garinii*	8	97	9	9	137	5
6102012L	442	2006	nymph	Babite	mixed	94	2	2	2	44	3
6104012L	340	2006	nymph	Babite	*B. valaisiana*	4	29	2	19	138	13

This table shows samples collected from Britain in 2006

Tick ID	**ST**	**Year**	**Stage**	**Collection Location**	***Borrelia* sp**	***ATP6***	***COI***	***COII***	***COIII***	***12s***	***CYTB***
60201B	159	2006	nymph	Thurlbear Woods	*B. valaisiana*	4	2	3	2	42	13
60303B	95	2006	nymph	Widcombe	*B. valaisiana*	8	21	21	9	55	5
60311B	90	2006	adult	Widcombe	*B. afzelii*	8	9	9	9	56	5
60325B	88	2006	nymph	Bathampton Woods	mixed	8	9	9	9	55	5
60515B	129	2006	nymph	Widcombe	*B. afzelii*	22	2	2	22	41	3
60709B	388	2006	nymph	Bathampton Woods	*B. garinii*	4	2	2	106	139	3
60809B	144	2006	nymph	Bathampton Woods	*B. garinii*	25	17	26	25	47	18
60910B	88	2006	nymph	Bathampton Woods	*B. garinii*	8	9	9	9	55	5

60930B	347	2006	nymph	Warleigh	*B. valaisiana*	4	87	2	12	33	13
61104B	135	2006	adult	Widcombe	*B. valaisiana*	25	24	24	25	52	12
61208B	376	2006	nymph	Widcombe	*B. garinii*	8	21	21	94	55	5
61214B	95	2006	nymph	Widcombe	*B. valaisiana*	8	21	21	9	55	5
61220B	29	2006	nymph	Widcombe	mixed	4	2	2	12	34	4
61306B	45	2006	nymph	Widcombe	*B. valaisiana*	4	2	19	2	41	6
61330B	84	2006	adult	Warleigh	mixed	8	9	9	9	17	5
61527B	132	2006	nymph	Widcombe	*B. afzelii*	23	22	22	2	75	9
61710B	366	2006	nymph	Bathampton Woods	*B. garinii*	4	80	2	2	37	3
61919B	24	2006	nymph	Widcombe	mixed	4	2	2	2	44	3
64418B	446	2006	nymph	Bathampton Woods	*B. garinii*	8	9	86	9	55	5

Tick samples not infected from Britain in 2006 used in section 6.3

Tick ID	ST	Year	Origin	Stage	Collection Location	*ATP6*	*COI*	*COII*	*COIII*	*12s*	*CYTB*
64402B	102	2006	Britain	adult	Widcombe	11	18	18	20	67	1
60404B	86	2006	Britain	adult	Widcombe	8	9	9	9	50	5
61304B	133	2006	Britain	adult	Widcombe	24	2	2	23	41	10
60405B	129	2006	Britain	nymph	American Museum	22	2	2	22	41	3
65302B	29	2006	Britain	nymph	Widcombe	4	2	2	12	34	4
60704B	81	2006	Britain	nymph	Widcombe	8	9	8	9	49	5
61404B	133	2006	Britain	adult	Widcombe	24	2	2	23	41	10
60505B	88	2006	Britain	nymph	American Museum	8	9	9	9	55	5
60203B	29	2006	Britain	adult	Widcombe	4	2	2	12	34	4
60904B	29	2006	Britain	nymph	Widcombe	4	2	2	12	34	4
61504B	105	2006	Britain	adult	Widcombe	13	1	1	1	63	1
60605B	30	2006	Britain	nymph	American Museum	4	2	2	12	34	5
60204B	90	2006	Britain	nymph	Widcombe	8	9	9	9	56	5
61004B	137	2006	Britain	nymph	Widcombe	26	25	25	16	76	14
60205B	88	2006	Britain	nymph	American Museum	8	9	9	9	55	5
60705B	133	2006	Britain	nymph	American Museum	24	2	2	23	41	10
60304B	129	2006	Britain	nymph	Widcombe	22	2	2	22	41	3

61204B	24	2006	Britain	adult	Widcombe	4	2	2	2	44	3
60305B	17	2006	Britain	nymph	American Museum	4	2	2	2	42	3

Tick samples from Europe used in section 7.2

This table shows tick samples used in analyses in 2007 and 2008

Tick ID	ST	Year	Origin	Stage	Collection Location	*ATP6*	*COI*	*COII*	*COIII*	*12s*	*CYTB*
70102B	90	2007	Britain	nymph	Widcombe	8	9	9	9	56	5
70115B	359	2007	Britain	nymph	Bathampton Woods	25	24	26	25	131	107
70118B	106	2007	Britain	nymph	Bathampton Woods	13	1	1	1	63	2
70120B	362	2007	Britain	nymph	Bathampton Woods	25	15	26	25	47	18
70136B	88	2007	Britain	nymph	Bathampton Woods	8	9	9	9	55	5
70142B	300	2007	Britain	nymph	Winsley	74	22	69	2	75	1
70222B	364	2007	Britain	nymph	Bathampton Woods	25	54	26	25	133	17
70115L	360	2007	Latvia	nymph	Jurmala	4	2	87	2	132	29
70121L	424	2007	Latvia	nymph	Jaunciems	4	15	2	96	116	3
70412L	18	2007	Latvia	nymph	Tireli	4	2	2	2	42	13
70415L	361	2007	Latvia	nymph	Jurmala	4	2	3	2	40	3
70418L	191	2007	Latvia	nymph	Jurmala	13	1	1	1	65	1
70621L	425	2007	Latvia	nymph	Jaunciems	95	98	2	2	117	29
70624L	432	2007	Latvia	nymph	Jaunciems	4	2	3	2	6	13
70246B	88	2007	Britain	nymph	Warleigh	8	9	9	9	55	5
70307B	159	2007	Britain	nymph	Widcombe	4	2	3	2	42	13
70709L	351	2007	Latvia	nymph	Tireli	21	91	23	20	70	7

70718L	419	2007	Latvia	nymph	Jurmala	21	1	2	97	118	109
70806L	88	2007	Latvia	nymph	Tireli	8	9	9	9	55	5
71015L	16	2007	Latvia	nymph	Jurmala	4	2	2	2	41	3
71121L	426	2007	Latvia	nymph	Jaunciems	13	1	1	2	64	1
71212L	354	2007	Latvia	nymph	Tireli	4	92	2	2	134	13
70316B	144	2007	Britain	nymph	Bathampton Woods	25	17	26	25	47	18
71324L	433	2007	Latvia	nymph	Jaunciems	4	2	2	99	119	39
71403L	348	2007	Latvia	nymph	Tireli	4	2	2	2	38	102
71524L	434	2007	Latvia	nymph	Jaunciems	4	2	84	2	41	3
71709L	352	2007	Latvia	nymph	Tireli	4	94	88	2	40	103
71821L	427	2007	Latvia	nymph	Jaunciems	8	9	9	98	120	5
72306L	350	2007	Latvia	nymph	Tireli	4	2	2	2	135	104
70320B	363	2007	Britain	nymph	Bathampton Woods	14	9	2	16	40	13
72312L	355	2007	Latvia	nymph	Tireli	4	2	40	2	42	3
72415L	110	2007	Latvia	nymph	Jurmala	13	1	1	1	65	2
72803L	349	2007	Latvia	nymph	Tireli	4	2	89	2	42	105
72809L	115	2007	Latvia	nymph	Tireli	13	1	34	1	63	1
72815L	336	2007	Latvia	nymph	Jurmala	21	99	80	20	121	60
73212L	356	2007	Latvia	nymph	Tireli	93	9	9	104	55	5
73406L	24	2007	Latvia	nymph	Tireli	4	2	2	2	44	3
73515L	337	2007	Latvia	nymph	Jurmala	13	100	1	1	64	101
73521L	428	2007	Latvia	nymph	Jaunciems	8	9	81	9	55	5

70329B	300	2007	Britain	nymph	Bathampton Woods	74	22	69	2	75	1
70334B	302	2007	Britain	nymph	Bathampton Woods	14	15	2	16	40	13
70408B	144	2007	Britain	nymph	Widcombe	25	17	26	25	47	18
73524L	379	2007	Latvia	nymph	Jaunciems	43	2	2	100	40	13
73609L	353	2007	Latvia	nymph	Tireli	4	96	2	2	42	3
73618L	420	2007	Latvia	nymph	Jurmala	4	101	2	2	40	110
73821L	429	2007	Latvia	nymph	Jaunciems	4	2	2	2	122	3
73824L	380	2007	Latvia	nymph	Jaunciems	4	102	3	2	42	13
73921L	430	2007	Latvia	nymph	Jaunciems	4	2	2	2	41	111
74112L	357	2007	Latvia	nymph	Tireli	89	2	6	105	42	13
70457B	88	2007	Britain	nymph	Warleigh	8	9	9	9	55	5
70516B	90	2007	Britain	nymph	Bathampton Woods	8	9	9	9	56	5
74606L	17	2007	Latvia	nymph	Tireli	4	2	2	2	42	3
74612L	358	2007	Latvia	nymph	Tireli	4	2	2	2	88	3
74624L	381	2007	Latvia	nymph	Jaunciems	14	15	83	101	40	13
74715L	338	2007	Latvia	nymph	Jurmala	13	1	1	1	63	41
74721L	431	2007	Latvia	nymph	Jaunciems	5	27	2	2	123	22
74809L	186	2007	Latvia	nymph	Tireli	25	17	26	28	55	17
74815L	339	2007	Latvia	nymph	Jurmala	96	2	65	39	42	3
75218L	106	2007	Latvia	nymph	Jurmala	13	1	1	1	63	2
70580B	367	2007	Britain	nymph	Widcombe	8	9	9	9	55	84
75403L	17	2007	Latvia	nymph	Tireli	4	2	2	2	42	3

75818L	421	2007	Latvia	nymph	Jurmala	97	1	1	1	63	1
76015L	417	2007	Latvia	nymph	Jurmala	13	1	82	1	124	1
76318L	422	2007	Latvia	nymph	Jurmala	34	2	2	12	33	28
76615L	418	2007	Latvia	nymph	Jurmala	4	80	2	2	122	3
76918L	423	2007	Latvia	nymph	Jurmala	98	2	2	2	42	3
70612B	24	2007	Britain	nymph	Bathampton Woods	4	2	2	2	44	3
70636B	365	2007	Britain	nymph	Bathampton Woods	90	9	9	9	55	5
70679B	88	2007	Britain	nymph	Bathampton Woods	8	9	9	9	55	5
70738B	305	2007	Britain	nymph	Bathampton Woods	62	17	26	17	108	17
70793B	302	2007	Britain	nymph	Eastwood	14	15	2	16	40	13
71053B	118	2007	Britain	nymph	Bathampton Woods	14	15	2	16	42	13
71152B	118	2007	Britain	nymph	Bathampton Woods	14	15	2	16	42	13
71268B	300	2007	Britain	nymph	Eastwood	74	22	69	2	75	1
71355B	88	2007	Britain	nymph	Warleigh	8	9	9	9	55	5
71380B	90	2007	Britain	nymph	Widcombe	8	9	9	9	56	5
71393B	144	2007	Britain	nymph	Eastwood	25	17	26	25	47	18
71452B	144	2007	Britain	nymph	Bathampton Woods	25	17	26	25	47	18
71457B	366	2007	Britain	nymph	Warleigh	4	80	2	2	37	3
71690B	118	2007	Britain	nymph	Bathampton Woods	14	15	2	16	42	13
72002B	144	2007	Britain	nymph	Widcombe	25	17	26	25	47	18
72068B	298	2007	Britain	nymph	Eastwood	72	77	9	9	55	86
72392B	302	2007	Britain	nymph	Warleigh	14	15	2	16	40	13

72402B	88	2007	Britain	nymph	Widcombe	8	9	9	9	55	5
72688B	368	2007	Britain	nymph	Widcombe	91	9	9	9	55	5
72790B	369	2007	Britain	nymph	Bathampton Woods	92	95	2	20	136	1
74002B	144	2007	Britain	nymph	Widcombe	25	17	26	25	47	18
74402B	132	2007	Britain	nymph	Widcombe	23	22	22	2	75	9
74902B	88	2007	Britain	nymph	Widcombe	8	9	9	9	55	5
75702B	298	2007	Britain	nymph	Widcombe	72	77	9	9	55	86
80101S	73	2008	Switzerland	nymph	Susten	4	40	2	42	33	13
80201S	106	2008	Switzerland	nymph	Susten	13	1	1	1	63	2
80201G	221	2008	Germany	nymph	Bonn, Siebenbeirge/Margaretenhoehe	4	72	2	2	42	3
80301S	140	2008	Switzerland	nymph	Susten	30	2	2	2	29	3
80401S	140	2008	Switzerland	nymph	Susten	30	2	2	2	29	3
80501G	222	2008	Germany	nymph	Bonn, Siebenbeirge/Margaretenhoehe	4	2	52	65	42	3
80501S	109	2008	Switzerland	nymph	Susten	13	1	1	1	64	55
80601S	13	2008	Switzerland	nymph	Susten	4	2	2	2	37	3
80701S	16	2008	Switzerland	nymph	Susten	4	2	2	2	41	3
80701G	223	2008	Germany	nymph	Bonn, Siebenbeirge/Margaretenhoehe	56	2	2	2	41	3
80104B	298	2008	Britain	nymph	Eastwood	72	77	9	9	55	86
80801S	93	2008	Switzerland	nymph	Susten	8	9	9	43	54	5
80901S	52	2008	Switzerland	nymph	Susten	4	2	37	44	42	29
81001S	104	2008	Switzerland	nymph	Susten	13	1	1	1	2	56
80109B	302	2008	Britain	nymph	Rainbow Woods	14	15	2	16	40	13

81101S	41	2008	Switzerland	nymph	Susten	4	2	6	2	40	57
80204B	88	2008	Britain	nymph	Eastwood	8	9	9	9	55	5
81201S	38	2008	Switzerland	nymph	Susten	4	2	2	45	57	13
81301S	142	2008	Switzerland	nymph	Susten	32	2	2	2	38	3
80210B	88	2008	Britain	nymph	Rainbow Woods	8	9	9	9	55	5
81401S	18	2008	Switzerland	nymph	Susten	4	2	2	2	42	13
81401G	224	2008	Germany	nymph	Bonn, Siebenbeirge/Margaretenhoehe	8	9	9	9	55	78
81501G	17	2008	Germany	nymph	Bonn, Siebenbeirge/Margaretenhoehe	4	2	2	2	42	3
81501S	53	2008	Switzerland	nymph	Susten	4	2	38	2	16	58
81701G	17	2008	Germany	nymph	Bonn, Siebenbeirge/Margaretenhoehe	4	2	2	2	42	3
81901G	225	2008	Germany	nymph	Bonn, Siebenbeirge/Margaretenhoehe	4	69	2	2	42	3
82001G	226	2008	Germany	nymph	Bonn, Siebenbeirge/Margaretenhoehe	4	2	3	2	40	13
80102G	161	2008	Germany	nymph	Bonn, Siebengibierge/Lowenburg	49	2	2	2	38	3
80202G	227	2008	Germany	nymph	Bonn, Siebengibierge/Lowenburg	25	24	26	25	91	12
80302G	228	2008	Germany	nymph	Bonn, Siebengibierge/Lowenburg	13	1	54	1	63	2
80103G	229	2008	Germany	nymph	Bonn, Kottenforst/Melbbach	13	2	1	1	64	1
80405B	300	2008	Britain	nymph	Bathampton Woods	74	22	69	2	75	1
80701B	88	2008	Britain	nymph	Eastwood	8	9	9	9	55	5
80304G	230	2008	Germany	nymph	Bonn, Kottenforst/Venusberg	8	9	9	25	55	74
80404G	231	2008	Germany	nymph	Bonn, Kottenforst/Venusberg	4	61	3	64	42	13
80504G	232	2008	Germany	nymph	Bonn, Kottenforst/Venusberg	39	2	55	2	94	3
80809B	307	2008	Britain	nymph	Rainbow Woods	21	1	23	20	106	7

80839B	110	2008	Britain	nymph	Widcombe	13	1	1	1	65	2
80905B	144	2008	Britain	nymph	Bathampton Woods	25	17	26	25	47	18
81305G	287	2008	Germany	nymph	Bonn, Kottenforst/Venusberg	4	67	2	6	36	3
80906B	304	2008	Britain	nymph	Widcombe	78	9	9	9	54	5
82005G	233	2008	Germany	nymph	Bonn, Kottenforst/Venusberg	64	2	2	2	42	3
82105G	234	2008	Germany	nymph	Bonn, Kottenforst/Venusberg	13	1	1	1	63	80
80910B	17	2008	Britain	nymph	Rainbow Woods	4	2	2	2	42	3
81001B	297	2008	Britain	nymph	Eastwood	4	2	3	64	42	13
81005B	144	2008	Britain	nymph	Bathampton Woods	25	17	26	25	47	18
81010B	17	2008	Britain	nymph	Rainbow Woods	4	2	2	2	42	3
81039B	144	2008	Britain	nymph	Widcombe	25	17	26	25	47	18
80106G	235	2008	Germany	nymph	Sauerland, Lennestadt-Meggen	50	2	2	62	41	3
80206G	236	2008	Germany	nymph	Sauerland, Lennestadt-Meggen	13	1	1	1	65	73
80306G	237	2008	Germany	nymph	Sauerland, Lennestadt-Meggen	4	29	2	2	40	3
80606G	238	2008	Germany	nymph	Sauerland, Lennestadt-Meggen	54	68	11	2	42	3
80706G	239	2008	Germany	nymph	Sauerland, Lennestadt-Meggen	34	2	2	2	38	29
81101B	294	2008	Britain	nymph	Eastwood	13	1	71	1	63	1
80906G	240	2008	Germany	nymph	Sauerland, Lennestadt-Meggen	58	1	2	20	72	1
81006G	241	2008	Germany	nymph	Sauerland, Lennestadt-Meggen	4	70	2	2	42	76
81106G	242	2008	Germany	nymph	Sauerland, Lennestadt-Meggen	4	2	57	2	42	77
81206B	17	2008	Britain	nymph	Widcombe	4	2	2	2	42	3
81306G	243	2008	Germany	nymph	Sauerland, Lennestadt-Meggen	4	29	58	2	41	3

81301B	17	2008	Britain	nymph	Eastwood	4	2	2	2	42	3
81305B	118	2008	Britain	nymph	Bathampton Woods	14	15	2	16	42	13
81406B	45	2008	Britain	nymph	Widcombe	4	2	19	2	41	6
81409B	16	2008	Britain	nymph	Rainbow Woods	4	2	2	2	41	3
81439B	8	2008	Britain	nymph	Widcombe	4	2	2	2	27	3
81509B	110	2008	Britain	nymph	Rainbow Woods	13	1	1	1	65	2
81539B	16	2008	Britain	nymph	Widcombe	4	2	2	2	41	3
81639B	307	2008	Britain	nymph	Widcombe	21	1	23	20	106	7
82214B	88	2008	Britain	nymph	Bathampton Woods	8	9	9	9	55	5
82705B	301	2008	Britain	nymph	Bathampton Woods	75	2	2	2	62	3
82805B	299	2008	Britain	nymph	Bathampton Woods	5	1	2	76	70	87
82806B	305	2008	Britain	nymph	Widcombe	62	17	26	17	108	17
83105B	88	2008	Britain	nymph	Bathampton Woods	8	9	9	9	55	5
83205B	439	2008	Britain	nymph	Bathampton Woods	4	2	2	2	55	82
83206B	133	2008	Britain	nymph	Widcombe	24	2	2	23	41	10
83305B	302	2008	Britain	nymph	Bathampton Woods	14	15	2	16	40	13
83714B	308	2008	Britain	nymph	Bathampton Woods	8	9	9	9	89	84
84114B	297	2008	Britain	nymph	Bathampton Woods	4	2	3	64	42	13
85314B	90	2008	Britain	nymph	Bathampton Woods	8	9	9	9	56	5
85714B	309	2008	Britain	nymph	Bathampton Woods	14	15	2	16	110	13
85814B	118	2008	Britain	nymph	Bathampton Woods	14	15	2	16	42	13

This table shows the tick samples used in analyses in 2002 and 2003

Tick ID	ST	Year	Origin	Stage	Collection Location	*ATP6*	*COI*	*COII*	*COIII*	*12s*	*CYTB*
20103L	1	2002	Latvia	nymph	Jaunciems	1	1	1	1	63	2
20303L	7	2002	Latvia	nymph	Jaunciems	4	2	2	2	23	19
20603L	98	2002	Latvia	nymph	Jaunciems	9	2	2	2	38	3
20703L	99	2002	Latvia	nymph	Jaunciems	10	10	1	1	60	1
20803L	32	2002	Latvia	nymph	Jaunciems	4	2	2	12	35	25
21303L	60	2002	Latvia	nymph	Jaunciems	4	13	2	2	42	3
21503L	60	2002	Latvia	nymph	Jaunciems	4	13	2	2	42	3
21603L	1	2002	Latvia	nymph	Jaunciems	1	1	1	1	63	2
21703L	87	2002	Latvia	nymph	Jaunciems	8	9	9	9	54	5
21903L	15	2002	Latvia	nymph	Jaunciems	4	2	2	2	40	30
22003L	112	2002	Latvia	nymph	Jaunciems	13	1	1	15	61	1
22203L	108	2002	Latvia	nymph	Jaunciems	13	1	1	1	64	32
25203L	15	2002	Latvia	nymph	Jaunciems	4	2	2	2	40	30
25303L	123	2002	Latvia	nymph	Jaunciems	20	18	2	20	66	37
25403L	126	2002	Latvia	nymph	Jaunciems	21	19	2	20	71	38
25603L	108	2002	Latvia	nymph	Jaunciems	13	1	1	1	64	32
26103L	217	2002	Latvia	nymph	Jaunciems	4	20	2	2	43	40
26503L	130	2002	Latvia	nymph	Jaunciems	22	2	17	2	42	3
20306L	10	2002	Latvia	nymph	Jurmala	4	2	2	2	30	3

20706L	100	2002	Latvia	nymph	Jurmala	11	1	2	11	70	1
20906L	17	2002	Latvia	nymph	Jurmala	4	2	2	2	42	3
22106L	76	2002	Latvia	nymph	Jurmala	5	1	13	2	70	22
22606L	214	2002	Latvia	nymph	Jurmala	14	15	14	16	5	28
23306L	85	2002	Latvia	nymph	Jurmala	8	9	9	9	48	5
23506L	119	2002	Latvia	nymph	Jurmala	15	2	2	2	42	33
23606L	61	2002	Latvia	nymph	Jurmala	4	16	2	2	40	3
23806L	33	2002	Latvia	nymph	Jurmala	4	2	2	18	25	13
23906L	5	2002	Latvia	nymph	Jurmala	4	2	2	2	13	35
24006L	218	2002	Latvia	nymph	Jurmala	18	2	3	2	42	3
24206L	122	2002	Latvia	nymph	Jurmala	19	1	1	1	58	36
24306L	94	2002	Latvia	nymph	Jurmala	8	9	16	9	55	5
23609L	121	2002	Latvia	nymph	Babite	17	17	15	17	55	34
20312L	54	2002	Latvia	nymph	Babite	4	3	2	2	14	3
20412L	17	2002	Latvia	nymph	Babite	4	2	2	2	42	3
20812L	101	2002	Latvia	nymph	Babite	11	1	11	14	3	1
20201P	105	2002	Portugal	nymph	Mafra	13	1	1	1	63	1
20301P	106	2002	Portugal	nymph	Mafra	13	1	1	1	63	2
20401P	289	2002	Portugal	nymph	Mafra	13	1	1	1	63	85
20501P	290	2002	Portugal	nymph	Mafra	4	73	2	2	41	3
20601P	291	2002	Portugal	nymph	Mafra	4	74	2	2	42	3
20801P	370	2002	Portugal	nymph	Mafra	13	1	1	74	63	2

21301P	292	2002	Portugal	nymph	Mafra	13	75	1	1	64	2
21401P	293	2002	Portugal	nymph	Mafra	40	1	1	1	63	1
21701P	18	2002	Portugal	nymph	Mafra	4	2	2	2	42	13
21801P	293	2002	Portugal	nymph	Mafra	40	1	1	1	63	1
30101P	106	2003	Portugal	nymph	Grandola	13	1	1	1	63	2
30201P	295	2003	Portugal	nymph	Grandola	4	2	64	2	42	13
30301P	296	2003	Portugal	nymph	Grandola	73	2	65	2	42	3
30401P	278	2003	Portugal	nymph	Grandola	5	1	2	2	70	22
30501P	332	2003	Portugal	nymph	Grandola	4	76	2	2	38	29
30601P	333	2003	Portugal	nymph	Grandola	13	1	1	75	63	2
30801P	334	2003	Portugal	nymph	Grandola	4	2	2	2	104	3
30901P	335	2003	Portugal	nymph	Grandola	4	2	66	2	40	13
31201P	159	2003	Portugal	nymph	Grandola	4	2	3	2	42	13