Prevalence of *Borrelia burgdorferi* sensu lato in *Ixodes ricinus* ticks in Scandinavia

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SCANDTICK

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Summary

Lyme borreliosis (LB) is the most frequent human tick-borne disease in Scandinavia. This master thesis is focusing on the *Borrelia burgdorferi* sensu lato (s.l.) complex, which is the causative agent of LB. Today, the *B. burgdorferi* s.l. complex consists of 20 different genotypes, in which three (*B. afzelii, B. garinii* and *B. burgdorferi sensu stricto*) are commonly known as human pathogens. During the past decades, the abundance of the main tick vector, *Ixodes ricinus* in Scandinavia, seems to have increased due to factors as increased roe deer abundance, changes in habitat structure and climatic changes.

This master thesis describes the prevalence and genotype composition of *B. burgdorferi* s.l. in host-seeking *I. ricinus* ticks at different locations in Scandinavia. The locations in Norway were Hillevågen (n=100), Håøya (n=100), Tromøya (n=100), Brønnøya (n=98) and Spjærøya (n=70). One location was included from Denmark (Tokkekøb Hegn (n=100)), whereas ticks from two nearby locations in Sweden (Verkö (n=90) and Aspö (n=10)) were included. A total of 668 host-seeking nymphs were investigated for infection with *B. burgdorferi* s.l. by real-time polymerase chain reaction (PCR) amplification of the 16S rRNA gene. *Borrelia* spp. were genotyped by melting curve analysis after real-time PCR amplification of the *hbb* gene, as well as direct sequencing of the *rrs* (16S)-*rrlA* (23S) intergenic spacer. The *Borrelia* spp. prevalence in nymphal ticks were determined to be 16.5% in Norway, 24.0% in Tokkekøb Hegn, and 6.0% in Sweden. The most prevalent genospecies identified were *B. afzelii* (75.5%), followed by *B. garinii* (11.2%), *B. miyamotoi* (8.4%) and *B. burgdorferi sensu stricto* (3.7%). This is one of the first reports of *B. miyamotoi* in Norway and Denmark.

The second aim of this thesis was to compare tick DNA extraction methods, and study if these DNA extraction methods influence detection of *B. burgdorferi* s.l. In August of 2013, host-seeking *I. ricinus* nymphs were collected from Hummervika in Søgne, and DNA was extracted by four different methods (n=100 in each method); DNeasy Blood and Tissue Kit (Qiagen), phenol-chloroform, the Abbott m2000sp machine and the NukEx Kit (Orion Diagostica). DNA extracts from the DNeasy Kit had the highest purity, whereas DNA extracts by phenol-chloroform had the highest average concentration, measured by a spectrophotometer. In addition, DNA extracts were analyzed for *B. burgdorferi* s.l. infection as described above. The results indicate that the DNeasy Kit and phenol-chloroform give preferable DNA extracts when detecting the spirochete.

Abbreviations and explanations

16S rRNA (ribosomal ribonucleic acid) – A component of the 30S small subunit of prokaryotic ribosomes, which is highly conserved between different species of bacteria.

ACA (acrodermatitis chronica atrophicans) – A dermatological condition, shown as a skin rash, which is indicative of a late stage of Lyme borreliosis.

***B. burgdorferi* s.l. (*Borrelia burgdorferi* sensu lato) –** A diverse group of spirochete bacteria that, today, consist of 20 genospecies. The causative agent of Lyme borreliosis.

Ct-value (threshold cycle) – The intersection between an amplification curve and a threshold line, which reflects the number of cycles required before a given amount of PCR product (measured by fluorescence) has exceeded the background noise.

DNA (deoxyribonucleic acid) – The genetic material of living organisms, consisting of two complementary strands of nucleotides, that form an antiparallel double-helix.

EM (erythema migrans) – A red spreading annular skin lesion. Often an early symptom of Lyme borreliosis (LB).

***Hbb* gene –** Encodes the histone-like protein HBb (haemoglobin subunit beta). May be used for genotyping of *B. burgdorferi* s.l. species.

IGS (intergenic spacer) – A non-transcribed length of DNA, which in this study is the sequence between the 16S (*rrs*) and a 23S (*rrl*A) rRNA gene.

LB (Lyme borreliosis) – A tick-borne disease caused by *B. burgdorferi* s.l. spirochetes. Often referred to as Lyme disease (LD) in the USA.

MSIS (Norsk meldingssystem for smittsomme sykdommer, Norwegian Surveillance System for Communicable Diseases) – A national surveillance system for infectious diseases at The Norwegian Institute of Public Health.

Osps (outer surface proteins) – OspA-OspF are found in the outer membrane of the *B. burgdorferi* s.l. spirochetes. The different Osps are involved in various processes during *Borrelia* spp. transmission.

PCR (**polymerase chain reaction)** – A method to amplify a section of a DNA molecule by repeatedly denaturing this molecule by heat followed by hybridization to flanking primers which are elongated by a DNA polymerase.

Real-time PCR (**real-time polymerase chain reaction)** – A PCR method for the detection of an amplicon based on a probe labelled with a fluorescent dye.

Reservoir host – A vertebrate host animal that is capable of passing *B. burgdorferi* s.l. to a feeding tick vector. A good reservoir host of *B. burgdorferi* s.l. must be tolerant to infection, abundantly parasitized by ticks, abundant, and it must permit efficient transfer of the pathogen to the tick.

TBE (tick-borne encephalitis) – A viral infectious disease involving the central nervous system.

1 Introduction

Ticks and tick-borne diseases seem to have increased in Scandinavia, following the same trend as in Europe (Medlock et al. 2013). In this context, ScandTick, an EU funded Interreg IV transnational project in the Øresund, Kattegat and Skagerrak region (ØKS region, figure 1b) was created to strengthen the cooperation across borders. The increased collaboration between the three respective countries is central to the project, which may lead to an increased knowledge of ticks and tick-borne diseases in Scandinavia. The ScandTick project focuses on the *Borrelia burgdorferi* sensu lato (s.l.) bacteria (may cause Lyme borreliosis (LB) in humans) and the tick-borne encephalitis (TBE) virus (may cause meningitis in humans). The project consist of four work packages; (i) project management and communication, (ii) assessment and development of diagnostic methods, (iii) environmental assessment and survey, and (iv) regional collaboration. This thesis is a part of work package three.

The partners of ScandTick are Sahlgrenska University Hospital (lead partner), Sørlandet Hospital Health Enterprise (SSHF) (Norwegian project owner), Länshospital of Ryhov, the Rigshospital of Copenhagen and The Norwegian Institute of Public Health. The project period is from 1st of September 2012 to 31st of December 2014.

The ScandTick project provided the opportunity to study the prevalence and genotype composition of *B. burgdorferi* s.l. in host-seeking *Ixodes ricinus* ticks, which is important due to the increasing number of LB cases in Scandinavia. Simultaneously, a comparative study of tick DNA extraction methods was conducted. DNA extraction is often the first step in molecular detection of microorganisms in ticks, and such a comparative is therefore of great importance.

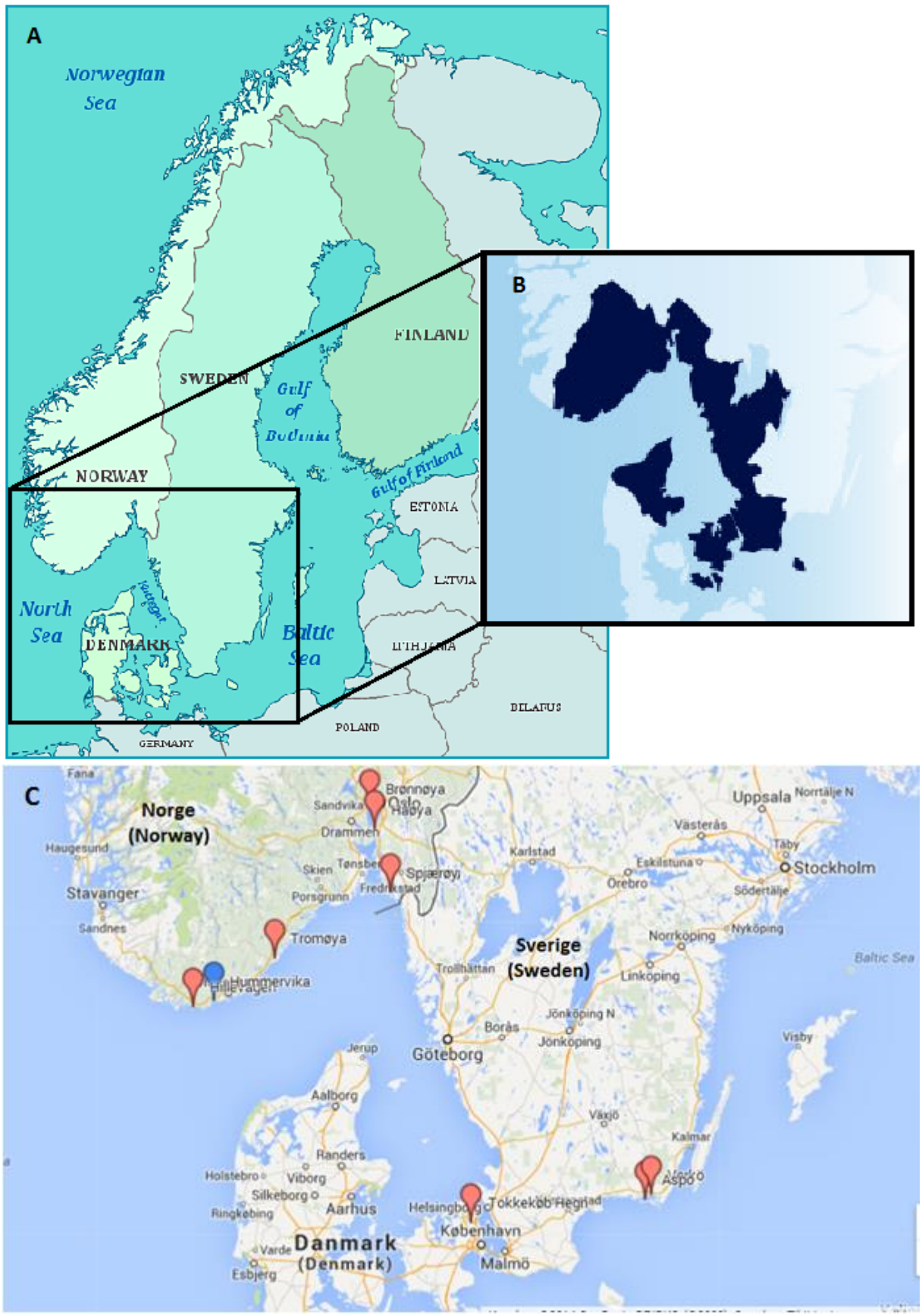

Figure 1: Scandinavian map (A). Dark blue represent the Øresund, Kattegat and Skagerrak, ØKS, region (B). Ticks used in the prevalence study were collected in locations represented with red markers, whereas the blue mark represent the collection site of nymphs used in comparison of tick DNA extraction methods (C).

1.1 Lyme borreliosis

Lyme borreliosis (LB), also known as Lyme disease (LD) in the USA, is the most frequent human tick-borne disease in the northern hemisphere (Rauter & Hartung 2005, Stanek et al. 2012). In 1982, Willy Burgdorfer and co-workers discovered that *Borrelia burgdorferi* was the causative agent of LB (Burgdorfer et al. 1982). Today, it is evident that *B. burgdorferi* occurs as several distinct genospecies collectively referred to as *B. burgdorferi* s.l., with apparently differing host preferences and pathological significance (Margos et al. 2011).

Three clinical stages of LB have been described where symptoms are commonly classified according to the infectious stage; early localized LB, disseminated LB, and late LB. The most frequent clinical manifestation of LB is the erythema migrans (EM) skin lesion, which is the typical sign of early localized LB (Stanek et al. 2012). EM often has a central clearing from the site of the tick bite, and the lesion expands until treatment or spontaneous healing. EM is often accompanied with influenza-like symptoms such as fever, headache, fatigue, nausea, arthralgia (joint pain) and myalgia (muscle pain) (Stanek et al. 2012). If EM is left untreated or the immune system fail to fight infection, it may disseminate to different organs, responsible for a range of clinical manifestations such as neurological symptoms (neuroborreliosis), arthritic symptoms (arthritis), cardiac symptoms, acrodermatitis chronica atrophicans (ACA), or lymphocytoma benigna cutis (Stanek et al. 2012). If the infection last for more than six months, it is known as late LB, and may be referred to as a chronic infection. Typical symptoms of late LB is ACA, arthritis or late neuroborreliosis (Stanek et al. 2012).

The number of LB patients in the Scandinavian countries are not directly comparable to each other, since the notifiable system varies. In Denmark, only neuroborreliosis is notifiable to Statens Serum Institut, and in Sweden no clinical stage of LB is notifiable (Hubalek 2009). In Norway, surveillance of LB was initiated in 1991, and from 1995 all disseminated clinical manifestations have been notifiable to the Norwegian Institute of Public Health. There is an increasing trend of reported cases of disseminated LB in Norway (Norwegian Surveillance System for Communicable Diseases [MSIS] 2014). However, this trend may be biased and caused by the improved notification system, greater awareness and better diagnostics for LB. Even though LB is intensively studied, an effective vaccine is still not available.

1.2 *Ixodes ricinus*

1.2.1 Developmental stages

The tick *I. ricinus* has a four-stage life cycle; egg, larva, nymph and adult. It is a three-host tick with each stage feeding on a different host. The tick climb the vegetation in order to attach to a passing host, which is a wide range of animal species. After feeding for several days (larva, 2-3 days; nymph, 4-5 days; adult female 7-9 days), the tick detach from the host, and subsequently develop into the next stage or, in the case of adult females, lay eggs (Gray 2002). The adult males do not engorge, however they may take several small blood meals and can remain on a host for a long period in search of females (Eisen & Lane 2002). The length of a tick's lifecycle varies between two and six years (Stanek et al. 2012).

1.2.2 The tick as a vector

Zoonoses are infectious pathogens that can be transmitted (often by a vector) to humans from animals and cause diseases. Ticks are general ectoparasites and versatile vectors capable of transmitting microorganisms to their vertebrate hosts. The ticks are infected when they feed of an infected animal, the bacteria persist in the tissues of the tick and the vector may transfer its pathogens while feeding on one of its many hosts (Eisen & Lane 2002). The main vectors for transmitting *B. burgdorferi* s.l. to humans are *I. ricinus* in Europe, *I. scapularis* and *I. pacificus* in the USA, and *I. persulcatus* in Asia (Stanek et al. 2012).

I. ricinus ticks may carry several infectious agents that may be transmitted separately or simultaneously with LB spirochetes, such as the following bacteria: *Babesia divergens* and *Babesia microti* (which can cause the disease babesiosis), *Anaplasma* spp. (anaplasmosis), *Rickettsia helvetica* (rickettsiosis), *Franciella tularensis* (tularaemia), *Neoehrlichia mukurensis* or *Coxiella burnetii* (Q fever) (Kazar 1999, Telford III & Goethert 2008, Jahfari et al. 2012). *I. ricinus* ticks may also transmit viruses, for instance *Flaviviruses* (TBE virus and louping ill virus) or *Coltivirus* (Eyach virus) (Charrel et al. 2004). LB and TBE are the two most prevalent tick-borne human diseases in Europe (Stanek et al. 2012).

1.2.3 Transmission

The *I. ricinus* tick may be infected with the LB spirochetes through several mechanisms. However, the main transmission pathway is through a blood meal on a reservoir host (Eisen & Lane 2002). The prevalence of *B. burgdorferi* s.l. is generally higher in adults compared to nymphs and larvae (Gray 1998), as host-seeking adult ticks have had two blood meals on different hosts, while nymphs have only had one blood meal. Still, some studies find a lower prevalence of the pathogen in adult ticks compared to nymphs (Skarphédinsson et al. 2007, Kjelland et al. 2010b). The discrepancy in the results may be explained by the nymphs feed on *Borrelia*-transmission-incompetent animals such as roe deer, or any other animals that are considered to be poor reservoir hosts and may have a diluting effect of the spirochete (Jaenson & Tälleklint 1992). It is assumed that complement and/or other factors derived from cervids during feeding have a borrelicidal effect on *B. burgdorferi* s.l. spirochetes, leading to lysis of the bacteria before they can be transmitted to the tick host (Kurtenbach et al. 2002a). Generally, no variation of *B. burgdorferi* s.l. prevalence is seen between tick gender (Rauter & Hartung 2005). However, local variations occur, in which the prevalence of *B. burgdorferi* s.l. may vary between adult female and adult male (Mysterud et al. 2013).

The spirochete may also be transmitted transovarially from the adult female to her larval offspring, however, a low detected prevalence in larvae suggest that this mode is of minor importance (Gern et al. 1998). Furthermore, ticks may be infected when feeding on an uninfected host by co-feeding. In this case, the spirochete is transmitted directly from an infected tick to an uninfected tick during feeding on the same host (Randolph, Gern & Nuttall 1996).

1.2.4 Distribution of *I. ricinus* in Scandinavia

The geographical distribution of *I. ricinus* ticks has increased in Scandinavia following the same trend as in Europe (Jore et al. 2011, Jaenson et al. 2012, Medlock et al. 2013). The increased tick abundance and distribution are possibly due to an elevated roe deer abundance and changes in both climate and habitat structure, which has been accompanied by an increase in notified disease incidence both of LB and other tick-borne diseases (Randolph 2004).

In Norway, *I. ricinus* ticks are found in a narrow belt along the coast from Østfold in the south to Nordland in the north (Tambs-Lyche 1943, Mehl 1983, Jore et al. 2011). A recent study from Hvidsten and co-workers found that the *I. ricinus* tick occurrence and *B. burgdorferi* s.l. prevalence are high in the Brønnøy district (Hvidsten et al. 2013). However, the occurrence are low north of the arctic Circle in Norway (Hvidsten et al. 2013).

I. ricinus ticks are common along the coast of southern Sweden (Jaenson et al. 2012). However, the tick has expanded its range in Northern Sweden and has become more abundant in Central Sweden during the past three decades, but it is still absent from the northern mountain region (Jaenson et al. 2012). The same study found the geographical presence of LB in Sweden to correspond to the distribution of *I. ricinus* ticks (Jaenson et al. 2012). *I. ricinus* ticks are found throughout Denmark (Medlock et al. 2013). However, an increased abundance of *I. ricinus* ticks has been detected in the northern parts of the country, in which the deer expansion appears to be a major driving force (Medlock et al. 2013).

1.3 *Borrelia burgdorferi* sensu lato

1.3.1 General characteristics

The genus *Borrelia* belong to the phylum spirochaetes and is a member of the family Spirochaetaceae. *Borrelia* contains different pathogenic groups; the LB *Borrelia* (*B. burgdorferi* s.l., principally transmitted by hard-bodied *Ixodidae* ticks), the relapsing fever (RF) *Borrelia* (principally transmitted by soft-bodied *Argasidae* ticks), and the aetiological agents of avian and bovine spirochetosis, *B. anserine* and *B. coriaceae* (both principally transmitted by soft-bodied *Argasidae* ticks), respectively.

The *Borrelia* spirochetes are elongated (10-30µm in length), thin (0,3-1µm in with) and helically curved (Burgdorfer et al. 1982). The spirochete have 7-11 bipolar flagella (composed of the outer sheet protein FlaA and the core protein FlaB) located in the periplasmic space, which make the bacteria able to orientate (Barbour & Hayes 1986).

The *Borrelia* spirochete sort with Gram-negative bacteria, as they have both an outer- and inner membrane, however, it lacks lipopolysaccharides (Takayama, Rothenberg & Barbour 1987). The inner membrane consists of a protoplasmic cylinder, and surrounding it is a fluid and labile outer membrane (Barbour & Hayes 1986). The outer membrane is covered with lipoproteins, such as outer surface proteins (Osps). To date, six Osps are well characterized, namely OspA-OspF. OspA and OspB are involved in adherence of the *Borrelia* bacteria to the tick midgut (Comstock et al. 1993), whereas OspC mediates the transmission of the spirochete from the tick midgut to the new host (Pal et al. 2004). Little is known about OspD, but it may be involved in pathogenesis (Norris et al. 1992). OspE and OspF are assumed to be of importance for the mammalian infection, where it may bind complement factor H (Hellwage et al. 2001), preventing the formation of the membrane-attack complex (MAC) by the alternative pathway in the complement system.

Borrelia has an obligate parasitic lifestyle, with no free-living stages known (Kurtenbach et al. 2002b). The *Borrelia* genome (based on the sequenced strain B31 Ml) consists of a linear chromosome approximately 0.9 Mb in size, and 21 plasmids (12 linear and 9 circular) comprising an additional 0.6 Mb (Fraser et al. 1997, Casjens et al. 2000). The *Borrelia* genome has a low guanine and cytosine content (28.6%) and encodes only limited biosynthetic genes (Fraser et al. 1997), suggesting that many of nutritional components are supplied by the host.

1.3.2 Genospecies

Today, the *B. burgdorferi* s.l. group consists of 20 genospecies of genetically diverse spirochetes (Table 1). The LB genospecies are not evenly distributed across the globe, where host specialization and vector compatibility of the spirochetes are likely to influence the global distribution (Margos et al. 2011). In North America, only *B. burgdorferi sensu stricto* is found to cause human disease, whereas in Europe at least five genospecies (*B. afzelii, B. garinii, B. burgdorferi sensu stricto, B. spielmanii* and *B. bavariensis*) are known to be pathogenic to humans (Stanek et al. 2012). Further three genospecies (*B. valaisiana, B. lusitaniae* and *B. bissettii*) have rare potential for human disease, as they have been detected in human patients in Europe (Stanek et al. 2012). Other genospecies of unknown pathogenicity are *B. americana, B. andersonii, B. californiensis, B. carolinensis, B. chilensis,*

B. finlandensis, B. japonica, B. kurtenbachii, B. sinica, B. tanukii, B. turdi, and *B. yangtze* (Margos et al. 2010, Casjens et al. 2011, Stanek & Reiter 2011, Ivanova et al. 2014).

It has been suggested that different genospecies correlates with various clinical symptoms of LB, in which *B. afzelii* is associated with skin manifestations (EM), *B. garinii* with neuroborreliosis and *B. burgdorferi sensu stricto* with arthritis (Stanek et al. 2012). Also, individual ticks may be infected with multiple genospecies of *B. burgdorferi* s.l. (Liebisch, Sohns & Bautsch 1998), and such co-infections have been detected in patients (Demaerschalck et al. 1995).

Various *B. burgdorferi* s.l. genospecies are associated with different reservoir hosts, in which *B. afzelii* and *B. bavariensis* are frequently isolated from rodents and ticks attached to rodents, while *B. garinii* and *B. valaisiana* are frequently isolated from birds and ticks attached to birds (Margos et al. 2011). *B. burgdorferi sensu stricto* does not show this specificity towards a reservoir host, and seems to be less specialized (Kurtenbach et al. 2002a). The various genospecies have different sensitivity towards complement proteins from the host animal species (Kurtenbach et al. 1998), and today it is well known that the complement system has a strong impact on the reservoir competence for different host animals and different strains of *Borrelia* (Kurtenbach et al. 2002a).

Table 1: Lyme *B. burgdorferi* s.l. genospecies, divided into the main tick vector, geographical distribution and main reservoir host.

***Borrelia* species**	**Reference**	**Main tick vector**	**Geographical distribution**	**Main reservoir animals**
B. afzelii	(Canica et al. 1993)	*I. ricinus*	Europe, Russia	Rodents
		I. persulcatus	Russia, China, Japan	Rodents
B. andersonii	(Marconi, Liveris & Schwartz 1995)	*I. dentatus*	North America	Rabbits
B. americana	(Rudenko et al. 2009b)	*I. pacificus* *I. minor*	Southern United States, California	Birds
B. bavariaensis	(Margos et al. 2009)	*I. ricinus*	Europe	
B. bissettii	(Postic et al. 1998)	*I. spinipalpis* *I. pacificus*	North America	Rodents
		I. ricinus	Europe	Rodents
B. burgdorferi sensu stricto	(Johnson et al. 1984)	*I. scapularis* *I. pacificus*	North America	Rodents, birds
		I. ricinus	Europe, Russia	Rodents, birds
B. californiensis	(Postic, Garnier & Baranton 2007)	*I. jellisonii* *I. spinipalpis* *I. pacificus*	California	
B. carolinensis	(Rudenko et al. 2009a)	*I. minor*	Southern United States	
B. chilensis	(Ivanova et al. 2014)	*I. stilensi*	South America	
B. finlandensis	(Casjens et al. 2011)	*I. ricinus*	Europe	
B. garinii	(Baranton et al. 1992)	*I. ricinus*	Europe, Russia	Rodents, birds
		I. uriae	Europe, Russia, North America, Arctic, Sub-Antarctic	Seabirds
		I.persulcatus	Russia, China, Japan	Rodents, birds
B. japonica	(Kawabata, Masuzawa & Yanagihara 1993)	*I. ovatus*	Japan	Rodents
B. kurtenbachii	(Margos et al. 2010)	*I. scapularis*	North America	
B. lusitaniae	(Le Fleche et al. 1997)	*I.ricinus*	Europe, North Africa, Russia	Birds, lizards
B. sinica	(Masuzawa et al. 2001)	*I. ovatus*	China, Nepal	Rodents
B. spielmanii	(Richter et al. 2006)	*I. ricinus*	Europe	Rodents
B. tanukii	(Fukunaga et al. 1996)	*I. tanuki*	Japan , Nepal	Rodents
B. turdi	(Fukunaga et al. 1996)	*I. turdus*	Japan	Birds
B. valaisiana	(Wang et al. 1997)	*I. ricinus*	Europe, Russia	Birds
		I. columnae	Japan	Birds
B. yangtze	(Chu et al. 2008)	*I. granulates* *I.nipponensis* *Haemophysalis longicomis*	Asia	

1.3.3 Genotyping

Earlier, most genotyping methods required cultivation of *Borrelia* spp. spirochetes, for instance ribotyping (Postic et al. 1996), pulsed-field gel electrophoresis (PFGE) (Belfaiza et al. 1993) and DNA-DNA reassociation (Johnson et al. 1984). However, cultivation of the spirochetes is time-consuming, has low efficiency (Gustafson, Gardulf & Svenungsson 1989), introduces a selection bias (as not all strains grow with equal efficiency in culture) (Cerar et al. 2008) and requires highly complex media (Barbour-Stoenner-Kelly medium supplemented with serum) to enhance growth (Barbour 1984, Barbour & Hayes 1986).

Today, genotyping methods by PCR amplified DNA are dominating. PCR is a sensitive and specific method for detecting the presence of tick-borne microorganisms in clinical specimen and infected ticks, without having to culture the microorganism. Several PCR-based methods are used, including species-specific PCR (Marconi & Garon 1992), randomly amplified polymorphic DNA (RAPD) fingerprinting (Wang et al. 1998), restriction fragment length polymorphism of PCR amplified fragments (PCR-RFLP) (Postic et al. 1994) and nucleotide sequencing (Bunikis et al. 2004). Genes commonly used for genotyping *B. burgdorferi* s.l. include 16S ribosomal RNA (rRNA), intergenic spacers (IGS), the plasmid located *ospA* and *ospC* genes, and the chromosomally located housekeeping genes *recA*, *groEL p*66, *kfla* or *hbb* (Margos et al. 2011).

In the present study, all samples were analyzed by real-time PCR targeting the 16S rRNA gene (Tsao et al. 2004). The 16S rRNA gene is highly conserved between different species of bacteria, and often used as a PCR target in an initial screening of samples. In the present study, specific 16S primers designed to target the LB *Borrelia* group were used. 16S real-time PCR positive samples were further analyzed by real-time PCR analysis of the *hbb* gene. The *hbb* gene encode a histone-like protein, which may be suitable to distinguish between the different genotypes of *B. burgdorferi* s.l. (Portnoï et al. 2006). Furthermore, nested PCR was used to amplify the *rrs* (16S)-*rrlA* (23S) intergenic spacer (from now on only referred to as IGS) in all 16S real-time PCR positive samples (Bunikis et al. 2004). Finally, all IGS products were sequenced.

Aims of the study

The main intention of this study is to determine the prevalence and genotype composition of *B. burgdorferi* s.l. genospecies in questing *I. ricinus* nymphs, contributing to the overall knowledge on tick-borne pathogens in Norway, Sweden and Denmark.

The second aim of this thesis is to compare tick DNA extraction methods, and study if the methods influence detection of *B. burgdorferi* s.l. spirochetes.

2 Materials and methods

2.1 Tick collection

To study the prevalence of *B. burgdorferi* s.l. in *I. ricinus* ticks in Scandinavia, questing nymphs were collected from the vegetation (by flagging) by The Norwegian Institute of Public Health in Norway, Renè Bødker in Denmark and Peter Wilhelmsson in Sweden. All the locations are shown in Figure 1C; the locations in Norway were Hillevågen (58º16'N; 07º22'E, collected 12.06.2012), Håøya (59º41'N; 10º34'E, collected 31.05.2013), Tromøya (58º28'N; 8º49'E, collected 13.06.2012), Brønnøya (59º51'N; 10º32'E, collected 06.06.2013) and Spjærøya (59º05'N; 10º55'E, collected 15.09.2012). The location in Denmark was Tokkekøb Hegn (55º53'N; 12º24'E, collected 16.10.2013), whereas the locations in Sweden were Verkö (56º10'N; 15º38'E, collected 15.10.2010) and Aspö (56º07'N; 15º31'E, collected 13.10.2010). Ten and ten nymphs were stored dry at -20°C. One hundred nymphs from each location were included in this study, with the following exceptions: 98 nymphs were collected from Brønnøya and 70 nymphs from Spjærøya in Norway. Ninety nymphs from Verkö and 10 nymphs from Aspö were included from Sweden, resulting in 100 nymphs from two nearby locations (approximately 6 km apart). Tick DNA were extracted by phenol-chloroform and stored at -20°C until analysis.

Questing *I. ricinus* nymphs were collected from the vegetation (by flagging) in Hummervika in Søgne (58°09'N; 07°76'E, blue mark in Figure 1C) in August of 2013 for comparison of tick DNA extraction methods. The nymphs were immersed in 100% ethanol and stored at 4ºC. DNA from one hundred nymphs were extracted by each method and stored at -20°C until analysis.

2.2 DNA extraction

2.2.1 DNeasy Blood and Tissue kit from Qiagen

The Kit contained all reagents used for the DNA extraction (Qiagen, Hilden, Germany). To remove ethanol, the nymphs were rinsed in 1x phosphate buffered saline (PBS) and MQ-water (Milli-Q, filtered and deionized water), before being lightly dried at a paper towel. Each nymph was cut using a sterile blade, and lysed in 20µl proteinase K and 180µl AL lysis buffer. The samples were vortexed, and incubated overnight at 56°C.

The samples were vortexed, and 200µl AL buffer was added before incubation at 70°C for 10 minutes. Then, 230µl ethanol (100%) was added before the samples were vortexed, transferred to DNeasy Mini Spin Column, and centrifuged for 1 minute at 8000 rpm. During centrifugation, DNA was selectively bound to the DNeasy membrane as contaminants pass through. Remaining contaminants and enzyme inhibitors were removed in two wash steps: first, (i) the column was transferred to a new collection tube, 500µl AW1 buffer was added (to denature proteins), and the samples were centrifuged at 8000 rpm for 1 minute, resulting in proteins being discarded in the flow through. Then, (ii) the column was transferred to a new collection tube, 500µl AW2 buffer was added, and the samples were centrifuged at 14000 rpm for 3 minutes to remove salts from the column. The supernatant was discarded and the collection tube was reused during a new centrifugation in 3 minutes at 14000 rpm.

The column was transferred to an eppendorf tube, and 30µl AE buffer was added, before being centrifuged at 8000 rpm for 1 minute, making the first DNA elution step. Another 25µl AE buffer was added, before being centrifuged at 8000 rpm for 1 minute, as a second elution step. The column was discarded, and the samples were stored at -20°C.

2.2.2 Phenol-chloroform

Each nymph was rinsed in 1xPBS and MQ-water before dried at a paper towel to remove ethanol. The nymphs were cut using a sterile blade, and lysed in a mixture of 20µl proteinase K (Roche Diagnostics GmbH, Roche Applied Science, Indianapolis, USA) and 180µl lysis buffer (Halos et al. 2004) by incubation at 56°C overnight.

All further work were conducted in a fume hood, since phenol-chloroform is toxic to inhale. 200µl phenol-chloroform-isoamyl alcohol (Sigma-Aldrich Chemie HmbH, Switzerland) was added to each sample. Then the samples were vortexed for 1 minute and centrifuged for 7 minutes at 14680 rpm, resulting in an upper aqueous phase and a lower organic phase. 180µl of the upper phase was transferred to a new eppendorf tube, and 200µl chloroform (Sigma-Aldrich) was added. The samples were vortexed for 1 minute and centrifuged for 7 minutes at 14680 rpm, resulting in nucleic acids in the upper aqueous phase, while proteins separated into the organic phase. 120µl of the upper phase was transferred to a new eppendorf tube. 12µl sodium acetate (3M, pH 4.8) and 300µl of -20°C ethanol (100%) were added before incubation at -20°C overnight to precipitate DNA.

After DNA precipitation, the samples were centrifuged at 14680 rpm for 10 minutes to create a DNA pellet, and the supernatants were carefully discarded. 500µl of 70% ethanol was added to each sample before centrifugation at 14680 rpm for 10 minutes to wash the DNA pellet. The supernatants were carefully discarded before the samples were incubated at 30°C, to let the ethanol evaporate. The samples were resuspended in 50µl AE buffer (included in the DNeasy Blood and Tissue Kit from Qiagen) before incubation for 4 minutes at 48°C. The samples were vortexed for 3 minutes to dissolve the DNA pellet, and finally stored at -20°C.

2.2.3 Abbott m2000sp

The kit contained all reagents used for the DNA extraction (Abbott Park, Illinois, USA). To remove ethanol, the nymphs were rinsed in 1xPBS and RO-water (reverse osmosis, purified water by filtration through a membrane). Each nymph was homogenized in 350µl nuclease-free water, by three steel spheres in a FastPrep®-24 Tissue and Cell Homogenizer (MP Biomedicals, Strasbourg, France) for 60 seconds. The samples were transferred to eppendorf tubes and centrifuged for 3 minutes at 14000 rpm to create cell-debris pellets. The supernatants (<300µl) were transferred to specific sampling tubes, and loaded to the Abbott m2000sp machine.

The DNA-Plasma-BA-200-050-v42809 program was used, which applies 200µl of the samples and finally elutes in 50µl elution buffer. First, the cells were lysed by incubation at 50ºC for 20 minutes in lysis buffer (containing 10% Tween; a detergent that destroys cell membranes). Then, the spherical microparticles captured the DNA, due to the silica based surface, the neutral pH and the high salt concentration. The microparticles were captured by a magnet during the two washes: (i) a chaotropic salt wash, and (ii) 70% ethanol, resulting in removal of cell debris and proteins. The ethanol evaporated before incubation at 65ºC for 20 minutes, resulting in detachment of DNA from the microparticles. A magnet captured the microparticles before the DNA was eluted in 50µl nuclease free water, transferred to collection tubes, and stored at -20ºC.

2.2.4 NukEx kit from Orion Diagnostica

The only reagent needed (Nucleic Acid Release Reagent; NukEx reagent) was included in the NukEx Kit from Orion Diagnostica (Gerbion GmbH & Co, Kornwestheim, Germany).

The nymphs were rinsed in 1xPBS and MQ-water, before being dried at a paper towel. Each nymph was transferred to an eppendorf tube containing 300µl NukEx reagent, cut by a sterile blade, and incubated at 60ºC for one hour. The proteolytic enzymes were inactivated by incubation at 97ºC for 10 minutes, and the samples were further incubated at room temperature for 10 minutes to cool down. The samples were vortexed thoroughly and centrifuged briefly before being stored at -20ºC.

2.2.5 Purity and total concentration

The total concentration (ng/µl) and purity (260nm/280nm and 260nm/230nm) were determined using the NanoDrop™ spectrophotometer ND-1000 (Thermo Fischer Scientific, Wilmington, DE). Pure preparations of DNA are considered a ratio of 1.8 (260nm/280nm) and between 2.0-2.2 (260nm/230nm). Samples in the prevalence study were measured once, whereas samples in the comparison of extraction methods were measured three times. The spectrophotometer was blanked to the elution buffer used in each DNA extraction method.

2.3 PCR and sequencing

2.3.1 Detection of *B. burgdorferi* sensu lato

The DNA extracts were examined for *B. burgdorferi* s.l. spirochetes using a real-time PCR assay with probe and primer specific for a section of the 16S rRNA gene (see appendix 8.3 for primer and probe sequence). Real-time PCR was performed using an iCycler/MyIQ™ (Bio-Rad, California, USA). Briefly, the 20µl PCR mixture included 1x ready-to-use reaction mixture (TaqMan Universal PCR Master Mix, Applied Biosystems Inc., New Jersey, USA) containing reaction buffer, Taq DNA polymerase, deoxynucleoside thriphosphate and $MgCl_2$. The final concentration of the primers and probe were 1.125µM and 0.25µM, respectively. Finally, 5µl of template DNA was added. Positive and negative controls were used in each setup.

The PCR conditions are described in table 2. To avoid PCR contamination and amplicon carryover, pre- and post-PCR sample processing and PCR amplification were performed in separate rooms.

Table 2: 16S real-time PCR conditions.

Cycle (repeated)	Step	Temperature (ºC)	Time (min)
1 (1X)	1	50.0	02:00
2 (1X)	1	95.0	10:00
3 (50X)	1	95.0	00:15
	2	63.0	01:00
4 (1X)		4.0	∞

2.3.2 Genotyping *B. burgdorferi* sensu lato species

Samples considered positive by 16S real-time PCR were further analyzed by *hbb* real-time PCR. After amplification of the *hbb* template, a melting curve was generated for genotyping. The primers used in this study were unmarked, whereas the EasyBeacons™ probe was labelled with FAM (sequences listed in appendix 8.3).

Real-time PCR was performed using iCycler/MyIQ™ (Bio-Rad). The 20µl PCR mixture included 1x ready-to-use reaction mixture (TaqMan Universal PCR Master Mix). The final concentration of the primers and probe was 0.2µM each. Finally, 5µl of template DNA was added. The real-time PCR conditions are described in table 3. The melting points of the amplicons generated from the unknown samples and from known *B. burgdorferi* s.l. species (provided by Dr. Eva Ruzic-Sabljic and her team at the Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana, Slovenia) were compared for genotyping. Positive and negative controls were included in all runs.

Table 3: *hbb* gene real-time PCR conditions used in genotyping *Borrelia* species. Data collection and real-time analysis enabled after step 2 in cycle 2. Increase setpoint temperature after cycle 2 (in step 5) by 0.5°C. Melt curve data collection and analysis enabled after step 1 in cycle 5.

Cycle (repeated)	Step	Temperature (°C)	Time (min)
1 (1X)	1	95.0	10:00
2 (55X)	1	95.0	00:30
	2	50.0	00:45
	3	72.0	00:30
3 (1X)	1	95.0	01:00
4 (1X)	1	35.0	01:00
5 (100X)	1	35.0	01:00
6 (1X)	1	4.0	∞

2.3.3 Nested PCR and sequencing

All the samples analyzed by *hbb* real-time PCR were also genotyped by direct sequencing of the *rrs* (16S)-*rrlA* (23S) intergenic spacer (IGS). The IGS locus was amplified (GeneAmp® PCR System 9700, Applied Biosystems Inc.) by a nested PCR procedure, comprising of 35 cycles for the first reaction (IGS1) and 39 cycles for the second reaction (IGS2). The PCR reaction conditions are described in table 4.

The first PCR mix was made, consisting of 2µl IGS forward- and reverse primer (sequences listed in appendix 8.3), 2.5µl dNTP-mix (Applied Biosystems Inc.), 2.5µl 10x PCR Gold buffer (Applied Biosystems Inc.), 2.5µl $MgCl_2$ Solution (25mM) (Applied Biosystems Inc.), 0.2µl AmpliTaq Gold® (250 Units, 5U/µL) (Applied Biosystems Inc.) and 7.3µl MQ-water. Giving a final IGS1 primer concentration of 1.05µM. Finally, 6µl DNA was added to the nested PCR mix. After the first PCR reaction, 3µl of IGS1 products were used in the nested run (IGS2), resulting in a IGS2 primer concentration of 0.90µM, respectively. Positive and negative controls were included in all runs. All IGS2 PCR products were analyzed by gel electrophoreses (1.6% agar) to control the nested PCR setup.

Table 4: PCR conditions for nested PCR.

Cycle (repeats)	Step	Temperature (ºC)	Time (min)
1 (1X)	1	95.0	05:00
2 (35X / 39X)*	1	94.0	00:30
	2	52.0 / 57.0*	00:30
	3	74.0	03:00
3 (1X)	1	74.0	07:00
4 (1X)	1	4.0	∞

*First reaction (IGS 1) consists of 35 cycles with a temperature of 52ºC, whereas nested PCR (IGS 2) consists of 39 cycles with a temperature of 57ºC.

2.5µl IGS2 products were purified by 1µl ExoSAP-IT® (Affymetrix Inc., Cleveland, US) by incubation at 37ºC for 15 minutes to activate ExoSAP, followed by 15 minutes incubation at 80ºC to inactivate ExoSAP. ExoSAP consist of Exonuclease I (degrade single stranded DNA and primers), and Shrimp Alkaline Phosphatase (SAP) which removes the phosphate group on the nucleotides.

A BigDye mix (Applied Biosystems Inc., Austin, USA) was made consisting of 2µl Big Dye® Terminator v1.1 Ready Reaction mix, 3µl 5x Sequencing buffer, 1µl IGS2 reverse primer,

and 11µl MQ-water. 17µl of BigDye mix was added to each sample, giving a final IGS2 reverse primer concentration of 0.59µM. The BigDye program is described in table 5. One GEM positive control (pGEM® - 3Zf (+) Control Template, 0.2µg/µL and 21 M13 Control Forward Primer, 0.8pmol/µL) was included to check the sequencing procedure. The BigDye mix consists of dNTP and ddNTP which are added during amplification of the template, resulting in fragments with different lengths due to the lack of an OH group.

Table 5: BigDye PCR conditions.

Cycle (repeats)	Step	Temperature (ºC)	Time (min)
1 (1X)	1	96.0	01:00
2 (25X)	1	96.0	00:10
	2	50.0	00:05
	3	60.0	04:00
4 (1X)	1	4.0	∞

A mixture of 2µl sodium acetate (3M, pH 5), 50µl ethanol (100%) and 2µl 125mM ethylene diamine tetra acetic acid (EDTA) was added to each sample before 15 minutes incubation at room temperature to precipitate the templates. The samples were centrifuged at 2000 rpm in 45 minutes at 4ºC to form a pellet, and the supernatant was carefully discarded. 70µl of ethanol (70%) was added before centrifugation at 2000 rpm for 15 minutes at 4ºC, to wash the pellet. Any remaining ethanol was removed by incubation at 37ºC for three minutes. Then the samples were re-suspended in 20µl Formamide (Applied Biosystems Inc. Warrington, UK), which stabilizes single stranded template, by incubation at 95ºC for 5 minutes.

Finally, the Formamide treated samples were sequenced directly in reverse direction on a 3130 Genetic Analyzer automated capillary sequencer (Applied Biosystems Inc.). The sequences were compared to known sequences in the National Center for Biotechnology Information (NCBI) by Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.4 Statistical analyses

Logistic regression was used to examine differences between locations in the prevalence of *B. burgdorferi* s.l. in the nymphal *I. ricinus* ticks. A Tukey test was used to evaluate which, if any, location differ in prevalence.

The analysis of variance test (ANOVA) was used to examine differences in purity and total concentration of the DNA extracts from the different DNA extraction methods. The purity and concentration data were not Gaussian distributed, and were therefore log-transformed before used in ANOVA tests. Any difference in purity and concentration between the DNA extraction methods were evaluated by Tukey tests. Logistic regression and a Tukey test were used to evaluate which, if any, DNA extraction method differ in prevalence.

Calculations were performed using SPSS statistical software, version 21 (SPSS Inc., Chicago, IL, USA) and R version 3.02 (R Core Team, Vienna, Austria). In all cases a probability of $p<0.05$ was regarded as statistically significant.

3 Results

3.1 *Borrelia* spp. in Scandinavia

3.1.1 Prevalence of *Borrelia* spp. in ticks

Questing *I. ricinus* nymphs (n=668) were investigated for infection with *B. burgdorferi* s.l. One hundred and seven of 668 nymphs (16.0%) were determined to be *Borrelia* spp. positive (listed in appendix 8.1). The number of *Borrelia* spp. positives are summarized in table 6, and the mean prevalence was determined to be 16.5% in Norway (25.0% in Hillevågen, 16.0% in Håøya, 10.0% in Tromøya, 14.3% in Brønnøya, 17.1% in Spjærøya), 24.0% in Denmark (Tokkekøb Hegn), and 6.0% in Sweden (Verkö and Aspö), respectively. A significant difference in *Borrelia* spp. prevalence were detected between the locations (X^2=21.8, df=6, p=0.0013). The effect of this difference was caused by a significantly lower prevalence detected in the two locations in Sweden compared to Hillevågen in Norway (p=0.010) and Tokkekøb Hegn in Denmark (p=0.018).

Table 6: *Borrelia* spp. identified at different locations in Scandinavia.

Location		Number of positives	Number of negatives	Total
	Hillevågen	25	75	100
	Håøya	16	84	100
Norway	Tromøya	10	90	100
	Brønnøya	14	84	98
	Spjærøya	12	58	70
Denmark	Tokkekøb Hegn	24	76	100
Sweden	Verkö and Aspö	6	94	100
Total		107	561	668

3.1.2 Determination of *Borrelia* spp.

The following four genotypes of *Borrelia* spp. were detected in the present study (Table 7); *B. afzelii, B. garinii, B. burgdorferi sensu stricto* and *B. miyamotoi* (a relapsing fever *Borrelia*). Only *B. afzelii* was detected in all locations (with a mean prevalence of 75.7%), and was the most prevalent genotype detected in each location (with a range of 50%-100%). *B. garinii* was detected in 11.2% of the total infected nymphs, however, it was only detected in four of the locations (Hillevågen, Tromøya, Tokkekøb Hegn and Verkö), and its prevalence varied with a range of 4.2%-33.3% in these locations. *B. burgdorferi sensu stricto* was only detected in Tokkekøb Hegn (with a prevalence of 16.7% at this location), resulting in a total prevalence of 3.7% in the infected nymphs. *B. miyamotoi* was detected in 8.4% of the infected nymphs, and found in Brønnøya (28.5%), Tokkekøb Hegn (16.7%) and Verkö (16.7%), resulting in detection of *B. miyamotoi* in all three countries. This is one of the first studies to detect the relapsing fever *B. miyamotoi* in Norway and Denmark.

One sample (ST10, appendix 8.1) contained *Borrelia* DNA, however, the genotype was indeterminate, and was therefore categorized as "unknown". There were no confirmed mixed infections.

Table 7: Genotype distribution identified at 7 different locations in Scandinavia.

Location	n[a]	*B. afzelii*	*B. garinii*	*B. burgdorferi sensu stricto*	*B. miyamotoi*	Unknown
Hillevågen	25	17	7	0	0	1
Håøya	16	16	0	0	0	0
Tromøya	10	8	2	0	0	0
Brønnøya	14	10	0	0	4	0
Spjærøya	12	12	0	0	0	0
Tokkekøb Hegn	24	15	1	4	4	0
Verkö	6	3	2	0	1	0
Total	107	81	12	4	9	1

[a]Number of *Borrelia* spp. infected nymphs.

By *hbb* real-time PCR, *B. garinii* has an expected melting point of 50°C, whereas *B. afzelii* has a melting point of 66.5°C and *B. burgdorferi sensu stricto* of 68.5°C (Portnoï et al. 2006). In the present study, melting curves were generated by *hbb* real-time PCR, which resulted in the following melting points: *B. garinii* (49.5°C and 50.0°C), *B. afzelii* (66.0°C, 66.5°C, 67.0°C, and 67.5°C), *B. burgdorferi sensu stricto* (68.5°C) and *B. miyamotoi* (50.0°C). The

melting point of one *B. garinii* sample (ST26, genotyped by IGS sequencing, appendix 8.1) was determined to be 56.0°C. Analysis of two *B. miyamotoi* samples (ST585 and ST619, both genotyped by IGS sequencing) gave a melting point of 50°C.

All of the 107 positive samples were positive by 16S real-time PCR, with two exceptions (ST335 and ST345). Analysis of these two samples gave an atypical amplification curve with low fluorescence (they did not cross the threshold line, resulting in no C_T value). These two samples were further analysed due the detection of some fluorescence. Sample ST335 was negative by *hbb* real-time PCR, whereas three bands were visible on the agarose gel after nested PCR. Finally, the IGS sequence was determined to be *B. miyamotoi* by BLAST. Analysis of the other sample, ST345, had enough fluorescence to exceed the threshold line during amplification by *hbb* real-time PCR, however, no melting point. Two bands were visible on the agarose gel after amplification of IGS by nested PCR, and a BLAST search determined the sample to contain *B. miyamotoi* DNA.

Only 57 of the 107 positive samples were considered positive by *hbb* real-time PCR. Analysis of some of the samples gave enough fluorescence to exceed the threshold line (resulting in a C_T value), however, no melting points (T_m) were obtained, and vice versa.

By amplification of the IGS region in nested PCR, 95 of 107 samples were positive, which was determined by the presence of visible band(s) on an agarose gel.

3.2 Comparison of tick DNA extraction methods

3.2.1 Purity and concentration

The purity (260nm/280nm and 260nm/230nm) and total concentration (ng/µl) were measured by NanoDrop in DNA extracts from the following four methods; DNeasy Blood and Tissue Kit from Qiagen (from now on referred to as the DNeasy Kit), phenol-chloroform, the Abbott m2000sp machine (from now on referred to as Abbott) and the NukEx Kit.

The absorption ratio at 260nm/280nm and 260nm/230nm were measured in DNA extracts from all four methods (Figure 2). A significant difference ($F=79.72$, $df=3$, $p<0.0001$) was detected in the 260nm/280nm ratio between DNA extracts from the different methods, with the exception of phenol-chloroform and the NukEx Kit ($p=0.068$). A significant difference ($F=622,47$, $df=3$, $p<0.0001$) was detected in the 260nm/230nm ratio between the methods, with the exception of DNA extracts from phenol-chloroform and the NukEx Kit ($p=0.974$). DNA extracts by the DNeasy Kit have a significantly higher purity compared to the other tested DNA extraction methods, with a 260nm/280nm ratio of 1.91 and a 260nm/230nm of 2.25, respectively. DNA extracts from the Abbott machine had the lowest 260nm/230nm ratio of 0.10, however, the main measurement of the purity (260nm/280nm) was 1.80, indicating a good purity.

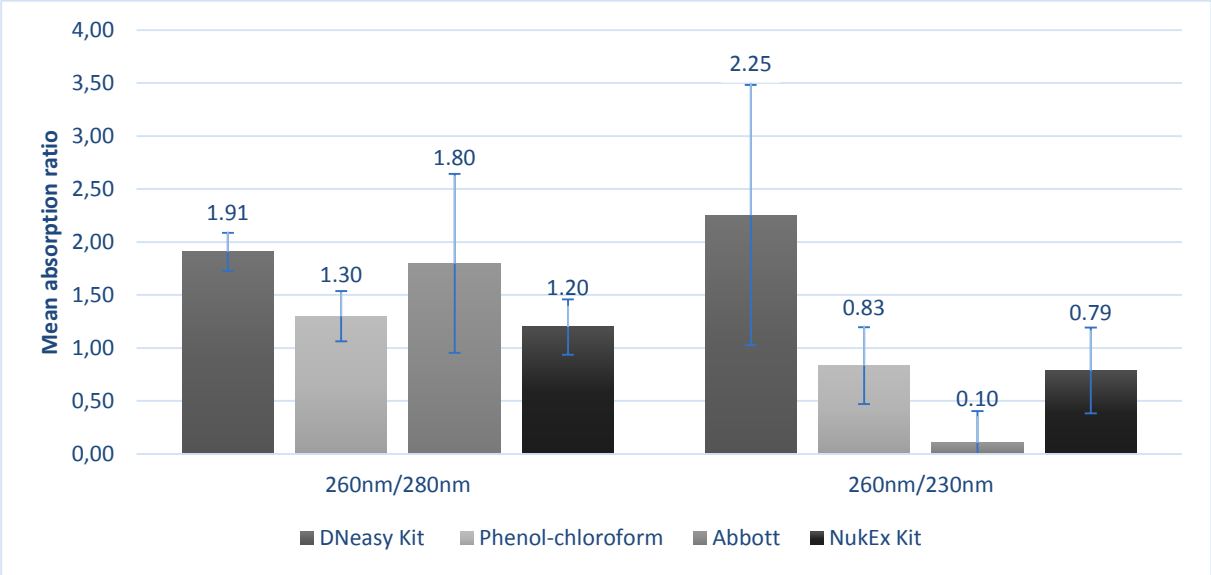

Figure 2: The mean purity (260nm/280nm, 260nm/230nm) and standard deviation of the DNA extracts by each DNA extraction method (n=100), measured by the NanoDrop spectrophotometer. A significant difference was detected in the purity of the DNA extracts between all methods, with the exception of phenol-chloroform and the NukEx Kit.

The total concentration (ng/µl) was measured in DNA extracts from the four methods (Figure 3). A significant difference ($F=195.82$, $df=3$, $p<0.0001$) was detected in the total concentration of the DNA extracts from the different methods. DNA extracts from the DNeasy Kit and phenol-chloroform had a higher average concentration than DNA extracts from the Abbott machine and the NukEx Kit. Although the average concentration in DNA extracts from the Abbott machine and the NukEx Kit was very similar, a significant difference ($p=0.048$) were detected, due to small variations within the two groups.

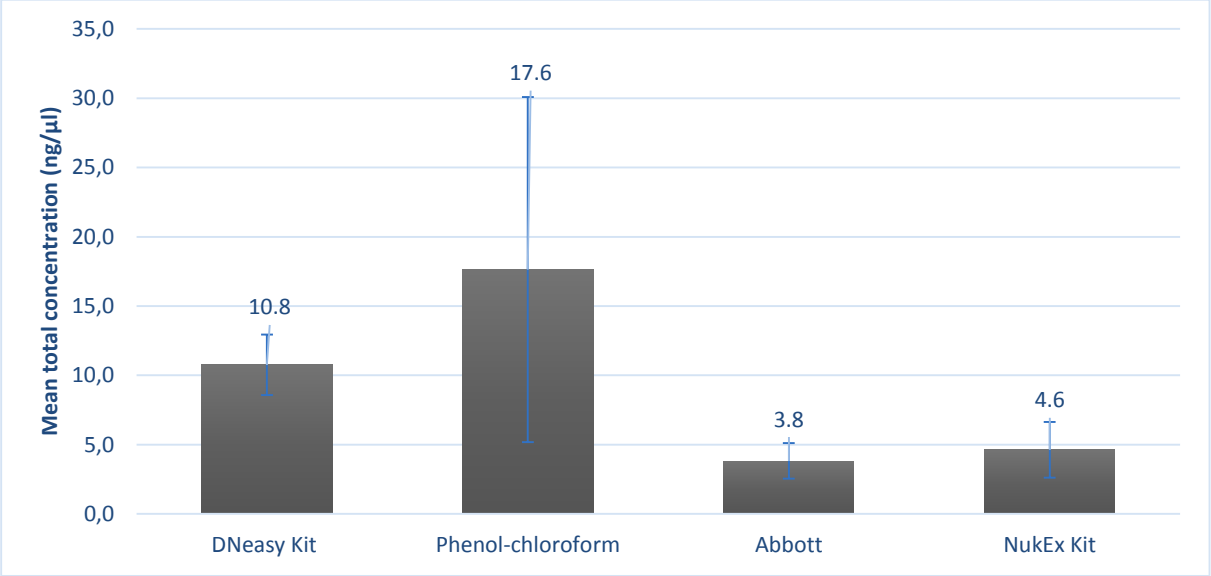

Figure 3: The mean total concentration (ng/µl) and standard deviation of the samples identified by the four different DNA extraction methods. A significant difference was detected between all methods.

3.2.2 PCR and sequencing

The present study tested if the four DNA extraction methods influenced the detection of *Borrelia* spp. (results are presented in appendix 8.2). The *Borrelia* spp. prevalence was determined to vary between 16% and 27% (Table 8), depending on the DNA extraction method. There was no significant difference (X^2=7.32, df=3, p=0.06) in the number of *Borrelia* spp. positive samples by the different DNA extraction methods. However, the *Borrelia* spp. prevalence was lower in DNA extracts from the Abbott machine and the NukEx Kit, compared to DNA extracts from the DNeasy Kit and phenol-chloroform.

Table 8: *Borrelia* spp. positive samples identified by the DNA extraction method (n=100 in each method).

	Number of nymphs	DNeasy Kit	Phenol-chloroform	Abbott m2000sp	NukEx Kit
Positive	87	27	27	17	16
Negative	313	73	73	83	84

The present study tested how the DNA extraction methods could influence the results when detecting *B. burgdorferi* s.l. Therefore, the mean C_T value (number of cycles required before the amount of PCR product has exceeded the background noise) of 16S real-time PCR positive DNA extracts was calculated (Table 9). A low C_T value indicates a high concentration of starting template, and vice versa. There was a significant difference (F=21.18, df=3, p<0.0001) in the C_T values of the DNA extracts from all methods, with the exception of phenol-chloroform and the NukEx Kit (p=0.120) and the DNeasy Kit and phenol-chloroform (p=0.134).

Table 9: Mean C_T value and range in 16S real-time PCR

Method	Mean C_T value	Range
DNeasy Kit	30.54	26.31-39.85
Phenol-chloroform	32.24	25.11-38.67
Abbott	38.54	35.92-41.54
NukEx	35.42	25.17-43.18

B. afzelii was the most prevalent genotype identified by all four methods (86.2%), followed by *B. garinii* (9.2%) and *B. miyamotoi* (2.3%), respectively (Table 10). Two samples (R198 and R337, appendix 8.2) contained *Borrelia spp.* DNA, however, the genospecies were indeterminate and they were therefore categorized as "unknown" (see short discussion under Table 8.2 in appendix 8.2).

Table 10: *Borrelia* spp. genospecies identified by the DNA extraction method (n=100 in each method).

Method	n[a]	*B. afzelii*	*B. garinii*	*B. miyamotoi*	Unknown
DNeasy Kit	27	24	3	0	0
Phenol-chloroform	27	22	3	1	1
Abbott m2000sp	17	17	0	0	0
NukEx Kit	16	12	2	1	1
Total	87	75	8	2	2

[a]Number of *Borrelia* spp. infected nymphs.

Melting curves were generated by *hbb* real-time PCR, and the following melting points were obtained; *B. afzelii* (65°C, 66°C, 67.5°C, 68°C and 68.5°C), *B. garinii* (50°C, 50.5°C and 66.5°C) and *B. miyamotoi* (66.5°C) respectively.

4 Discussion

4.1 *Borrelia* spp. in Scandinavia

4.1.1 Prevalence of *Borrelia* spp. in ticks

In the present study, a total of 668 questing *I. ricinus* nymphs were collected by flagging from five locations in Norway (Hillevågen, Tromøya, Brønnøya, Håøya and Spjærøya), one location in Denmark (Tokkekøb Hegn) and two locations in Sweden (Verkö and Aspö). Since Verkö and Aspö are two nearby locations, as well as only a low number of nymphs were examined from Aspö (n=10), the samples from these locations were combined. The collection sites were mainly chosen due to the location within the ØKS-region, also, a high frequency of LB cases are recorded in the respective counties in Norway (Norwegian Surveillance System for Communicable Diseases [MSIS] 2014) and southern Sweden (Wilhelmsson et al. 2010). Previous studies have shown that the *B. burgdorferi* s.l. prevalence tend to be low in larvae (Gern et al. 1998, Rauter & Hartung 2005, Kjelland et al. 2010b), whereas the *B. burgdorferi* s.l prevalence in adult *I. ricinus* ticks may be biased to *Borrelia*-transmission-incompetent animals (see section 1.2.3 Transmission). For this reason, as well as being overrepresented by flagging, only *I. ricinus* nymphs were included in the present study.

The detected prevalence of *Borrelia* spp. in questing *I. ricinus* nymphs varied greatly between the respective locations in Scandinavia. The mean *Borrelia* spp. prevalence in Norway was determined to be 16.5%, 24.0% in Tokkekøb Hegn and a combined prevalence of 6.0% in Verkö and Aspö, respectively. These findings are in accordance to what have previously been described by a metaanlysis from 2005 (Rauter & Hartung 2005), which estimate the prevalence of *B. burgdorferi* s.l. to be 15.8% in Norway, varying from 2.4%-18.6% in Denmark and 2.0%-29.6% in Sweden. In Norway, previous studies have reported *B. burgdorferi* s.l. prevalence in nymphs to be 4% (Mehl, Sandven & Braathen 1987), 6% (Paulauskas et al. 2008), 15% (Jenkins et al. 2001), and 24.5% (Kjelland et al. 2010b).

There highest prevalence in Norway was detected in Hillevågen (25.0%), which is an island outside Mandal in Southern Norway. The present study detected a higher prevalence of *B. burgdorferi* s.l. in questing *I. ricinus* nymphs than previously described in Mandal (13.7%) (Kjelland et al. 2010b). A positive correlation between tick density and prevalence of *Borrelia*

spp. in ticks have been found (Tälleklint & Jaenson 1996), and the high prevalence in Hillevågen correspond to most LB cases being reported from the southern counties of Norway (Norwegian Surveillance System for Communicable Diseases [MSIS] 2014), as well as a high tick density at the island (Å. Andreassen (FHI) personal communication). The *B. burgdorferi* s.l. prevalence differences between the present and the previous study (Kjelland et al. 2010b) could be explained by differences in both microclimate and the animal population (Randolph 2008) at the Island (Hillevågen) compared to the mainland (Mandal). The detected prevalence in Tromøya (10.0%) was lower than previously described (21.4%) from the same location (Kjelland et al. 2010b), which might be explained by seasonal variance, as the *B. burgdorferi* s.l. prevalence tend to vary greatly from year to year (Mejlon & Jaenson 1993, Hubalek 2009, Margos et al. 2011). The other Eastern locations in Norway had a somewhat equal prevalence (Håøya: 16.0%, Brønnøya: 14.3% and Spjærøya: 17.1%). The tick abundance and number of reported LB cases from these areas are lower than in the southern counties of Norway they have, however, both increased during the last decade (Jore et al. 2011, Norwegian Surveillance System for Communicable Diseases [MSIS] 2014).

The *Borrelia* spp. prevalence in Tokkekøb Hegn was determined to be 24.0% in the present study, which is higher than previously described from other locations in Denmark; for instance 11.0% were detected in Jutland, Funen and Bornholm (Skarphédinsson et al. 2007), 5.0% in Grib Forest (North Zealand) (Jensen & Frandsen 2000) and between 7.0%-22.0% in Eastern Jutland (Landbo & Flöng 1992). The prevalence differences between the present and previous studies might be explained by the detection methods used, as indirect fluorescent antibody staining was used to detect the presence of *B. burgdorferi* s.l. in some of the earlier studies (Landbo & Flöng 1992, Jensen & Frandsen 2000). However, a metanalysis concluded that no effect of detection methods was seen (Rauter & Hartung 2005). Skarphèdinsson et al. used amplification of the 16S rRNA gene (by PCR) to determine the *B. burgdorferi* s.l. prevalence (Skarphédinsson et al. 2007). The prevalence differences between the present study and Skarphèdinsson et al. could be due to local variations in for instance tick abundance and animal population (Randolph 2008). The high prevalence detected in the present study could be explained by a high abundance of good reservoir hosts for *B. burgdorferi* s.l. There might also be a local high tick density in Tokkekøb Hegn, possibly increasing the prevalence at that particular location (Tälleklint & Jaenson 1996). Precautions were taken at all steps of analysis to avoid contamination of samples (sample processing and analysis were for instance performed in separate rooms), and the high rates are believed to be true estimates of infection.

The two locations investigated from Sweden had an unexpected low *Borrelia* spp. prevalence (6.0%), considering the high tick abundance and the high number of LB cases reported from Southern Sweden earlier (Hubalek 2009, Wilhelmsson et al. 2010). A previous study examined 15 different locations on the South and East coasts of Sweden and determined the *B. burgdorferi* s.l. prevalence to be 11.0% (Fraenkel, Garpmo & Berglund 2002). Also, an earlier study detected the *B. burgdorferi* s.l. prevalence to vary between 0.0%-15.0% by the use of indirect immunofluorescence (Gustafson et al. 1995), whereas another study determined the prevalence to vary between 6.8%-12.9% by the use of phase-contrast microscopy (Mejlon & Jaenson 1993). The variations between the reported prevalences may be affected by natural factors such as climate, habitat, animal population and tick density, and may account for some of the spatial and seasonal variations (Randolph 2008). Furthermore, the animal population in the investigated locations may be dominated with poor reservoir hosts of *B. burgdorferi* s.l. and thereby have a diluting effect on the *B. burgdorferi* s.l. prevalence at these locations (see section 1.2.3 Transmission). The low prevalence detected in the present study is believed to be a true estimate of infection in the nymphs investigated, as the detection methods used are the same throughout the study.

Knowledge of the geographic distribution of the different genospecies of *B. burgdorferi* s.l. within their tick vector may have important clinical relevance, as some genospecies are connected to various clinical symptoms (see section 1.3.2 Genospecies). Due to the different notification systems in the three respective countries (section 1.1 Lyme borreliosis), the number of LB incidences in Scandinavia are not directly comparable. The number of *B. afzelii* infections to humans (typically EM) may have been underestimated as EM is not notifiable in any of the respective countries. Furthermore, it is also possible that *B. afzelii* infections more often than other genospecies starts with an EM (Strle et al. 2002) that is detected and treated, thereby preventing a "successful" dissemination of *Borrelia* infection. Neuroborreliosis is notifiable in Norway and Denmark, which might have lead to a false impression that *B. garinii* is the most prevalent genospecies infecting humans. However, it may be possible that *B. garinii* is more invasive, and more frequently establishes an infection. Future research may provide more knowledge on this topic.

B. afzelii was the most predominant genotype of *B. burgdorferi* s.l. (75.7%), followed by *B. garinii* (11.2%), and *B. burgdorferi sensu stricto* (3.7%), respectively. These results are in accordance with the metaanalysis from Europe, based on 112579 host-seeking ticks, covering

24 countries (Rauter & Hartung 2005). The infection prevalence and genotype composition varied between the different locations, which might be particular influenced by which tick hosts are found in an area. For instance, rodents have a high capacity to infect larvae, and high population densities of these hosts in an area may lead to high prevalence of *B. burgdorferi* s.l. infection in ticks (Kurtenbach et al. 2002b). Rodents are assumed to be the main reservoir hosts for *B. afzelii* (Table 1), which was detected in all locations, and might therefore explain the high prevalence of this genotype as well as its wide distribution. Some species of ground-feeding birds are assumed to be important reservoir hosts for *B. garinii* (Table 1). However, as this genotype was detected in only four of the locations, it may indicate that the locations without *B. garinii* lack good reservoir hosts for this genotype. *B. burgdorferi sensu stricto* was only detected in one location (Tokkekøb Hegn) in the present study. *B. burgdorferi sensu stricto* has been detected in Norway and Sweden earlier (Rauter & Hartung 2005), however, the prevalence of this genotype tend to be low (Rauter & Hartung 2005). The different genotype composition may have evolved randomly, independent of the presence or absence of specific hosts, or different host reservoir capacity may have had an influence (Rauter & Hartung 2005). The uneven distribution of *B. burgdorferi* s.l. genospecies might be explained by differences in *I. ricinus`* susceptibility to different genospecies. For instance, a comparative study of the three main clusters of European *I. ricinus* indicated significant differences in their susceptibility to *B. afzelii* (Estrada-Peña et al. 1998).

B. valaisiana has previously shown a widespread distribution in Southern Norway (Kjelland et al. 2010b, Hasle et al. 2011), as well as being detected in Denmark (Vennestrøm, Egholm & Jensen 2008) and Sweden (Fraenkel et al. 2002). However, *B. valaisiana* was not detected in the present study, and as most of the locations are from the Eastern counties in Norway, it could explain why *B. valaisiana* was not detected. *B. valaisiana* was not detected in the locations from Denmark and Sweden, even though it has been detected in nearby locations earlier (Fraenkel et al. 2002, Vennestrøm et al. 2008), which indicate high local variations in the genotype composition. A previous study detected a low prevalence of *B. valaisiana* in nymphs (1.1%) and a higher prevalence of this genotype in adult ticks (11.6%) (Kjelland et al. 2010b). The low detected prevalence in nymphs could be an explanation for why the present study did not detect *B. valaisiana.* The detection methods used in the present study have previously shown to detect *B. valaisiana* (Kjelland et al. 2010b), therefore, it is most likely that this genotype was not present in the investigated nymphs.

No confirmed mixed infections was observed in the present study. The metaanalysis found 13% of *Borrelia* spp. positive *I. ricinus* ticks to be infected with multiple genospecies (Rauter & Hartung 2005). The animal population at the different locations might participate to the non-existing mixed infections, as for instance animals with a possible borrelicidal effect may dominate the areas. As mixed infections would be detected by the methods used in the present study (giving two or more melting points by *hbb* real-time PCR, V. Kjelland personal communication) there were most likely no mixed infections present in the investigated nymphs.

Determination of genospecies in one sample (ST10, Table 8.1) was not possible by the methods used in the present study, and finally characterized as "unknown". The sample was positive throughout the analysis and the final IGS sequence was of high quality (with approximately 400 base pairs of high quality with low background noise). However, only a short part of the IGS sequence (~74 base pairs) gave alignments to various genospecies of *B. burgdorferi* s.l (*e.g; B. carolinensis, B. afzelii, B. burgdorferi sensu stricto, B. garinii, B. bissettii, B. spielmanii* and *B. valaisiana*), when compared by discontiguous megaBLAST. The genotype *B. carolinensis* had the highest similarity to the sample sequence (74 base pairs aligned). Furthermore, the melting point by *hbb* real-time PCR was determined to be 60°C, which could correspond to *B. spielmanii* (Kjelland 2011). However, only 73 base pairs were aligned with *B. spielmanii* by BLAST. *B spielmanii* has been detected from a skin biopsy from a Danish patient (Richter et al. 2006), and it could therefore be possible to discover this genotype in Norway, as for instance migrating birds could spread ticks and tick-borne pathogens between countries (Kjelland et al. 2010a, Hasle et al. 2011). The sample could contain *B. valaisiana* DNA, since an earlier study found the *hbb* melting point to be 60.5°C (Kjelland 2011), however, only 71 base pairs were aligned to this genotype. It is clear that there are *Borrelia* spp. DNA within the sample, however, since the aligned sequence is short it is not sufficient to conclude the genospecies. The spirochetal infection might have been confirmed by the use of different PCR target genes, cultivation, dark-field microscopy or immunofluorescence assays.

Different factors may affect both the abundance and distribution of ticks and the prevalence of *B. burgdorferi* s.l. in an area, such as local variations in animal population densities and microclimate (Randolph 2008). The roe deer abundance has, for instance, previously been shown to have a significant impact on tick population densities (as they can feed a large

number of adult ticks, leading to high tick reproduction rates), and reduction of deer in a tick habitat has been suggested as a method to control the LB vector (Jaenson et al. 1991). This method is, however, not practical in most locations, as it would require prevention of immigration of new deer to the habitat. Furthermore, reducing the roe deer population in a habitat may lead to an increased prevalence of *B. burgdorferi* s.l. infection in the remaining tick population, as the number of animals that have a diluting effect on borrelial infections in ticks is reduced. By interfering with the animal population in an area, one might change the genotype composition, as different animal species might have different impact on the various genospecies (Kurtenbach et al. 2002b).

The *I. ricinus* nymphs used in the present study were collected at different times of the year, as well as different years, which may be a contributing factor to the variation in the detected *Borrelia* spp. prevalence. The nymphs from Norway were collected between June 2012 and July 2013, whereas the nymphs from Sweden were collected in October 2010, and the nymphs from Denmark were collected in October 2013. This decreases the possibility of comparing the prevalence and genotype composition at the different locations, as the proportion and prevalence of *B. burgdorferi* s.l. tends to vary greatly from year to year (Mejlon & Jaenson 1993, Hubalek 2009, Margos et al. 2011), as well as throughout the season (Kjelland et al. 2010b). Furthermore, due to the low number of locations from Sweden (Verkö and Aspö) and Denmark (Tokkekøb Hegn) included in the present study, a direct comparison between the three Scandinavian countries is not possible. A spot study, such as this thesis, has therefore only local and temporary applicability.

Future studies should aim to observe and elucidate the reservoir hosts role (such as mice and birds) in spreading the *Borrelia* spp., as well as their ecology to other tick-borne pathogens. Such a study should be conducted over time, and include multiple locations in all three Scandinavian countries, as the *Borrelia* spp. prevalence tend to vary greatly (Mejlon & Jaenson 1993, Hubalek 2009, Margos et al. 2011). The prevalence of the genospecies detected in the present study did not correlate to reported clinical manifestations of LB patients in Scandinavia, which might be explained by the notification systems used in the different countries, or the possibility that some strains of *Borrelia* spp. could be more invasive than others (Stanek et al. 2012). A future study should therefore aim to characterize the *Borrelia* spp. in all LB patients, to gain more knowledge on the genospecies infecting humans. Also, *I.*

ricinus ticks are able to transmit multiple pathogens, therefore, a study involving multiple bacteria and viruses and their possible endemic consequence should be conducted.

4.1.2 Detection of *B. miyamotoi*

The main intention of this study was to determine the prevalence of genospecies belonging to the *B. burgdorferi* s.l. complex. However, the relapsing fever *B. miyamotoi* were detected in all the respective countries (8.4%), indicating a widespread distribution in Scandinavia. The *B. miyamotoi* distribution in Norway seems to be local, as it was only detected in one location. Four ticks from Norway (Brønnøya), four ticks from Denmark (Tokkekøb Hegn) and one tick from Sweden (Verkö) were infected by *B. miyamotoi*. So far, no studies from Norway and Denmark have reported the detection of *B. miyamotoi,* however, a *B. miyamotoi*-like *Borrelia* has been detected in Sweden (Fraenkel et al. 2002).

Fukunaga and co-workers were the first to describe *B. miyamotoi* (Fukunaga et al. 1995), which is now known to infect hard bodied *Ixodes* ticks world-wide (Margos et al. 2011). *B. miyamotoi* differs in the clinical spectra to *B. burgdorferi* s.l., in which periodic fever is the main symptom (Platonov et al. 2011). In contrast, LB *Borrelia* causes various inflammatory reactions involving the skin, joints, heart, and central nervous system (see section 1.1 Lyme borreliosis). The *B. miyamotoi* spirochete multiplies in the bloodstream (Fukunaga et al. 1995), whereas the LB *Borrelia* rarely emerges into the bloodstream. The relapsing fever like spirochetes (*B. miyamotoi*) are transmitted transovarilly in *Ixodes* ticks and occur sympatrically with LB group spirochetes, which may explain some or perhaps all observations of transovarial transmission (Scoles et al. 2001, Piesman 2002). The two related pathogens are therefore quite different from one another in their adaptations to their arthropod and vertebrate hosts. The detection of *B. miyamotoi* in Scandinavia has great clinical value, as the manifestations are different to LB *Borrelia.*

The relapsing fever *B. miyamotoi* has been detected in central Europe (Richter, Schlee & Matuschka 2003) and Sweden (Fraenkel et al. 2002, Wilhelmsson et al. 2013) during the past decade. These studies used PCR screening towards the 16S rRNA gene (Fraenkel et al. 2002, Richter et al. 2003), 5S-23S and 16S-23S intergenic spacers (Wilhelmsson et al. 2013), and they were able to detect both *B. burgdorferi* s.l and *B. miyamotoi.* A new study from Canada

found *B. miyamotoi* to have a widespread distribution, and they concluded that infection due to this agent should be considered in patients who have been exposed to *I. scapularis* ticks (Dibernardo et al. 2014).

Two *B. miyamotoi* positive samples (ST335 and ST345) were included in the study, even though analysis did not give enough fluorescence to exceed the threshold line during amplification (resulting in no C_T value) of the 16S rRNA gene. The primers were not designed to detect RF *Borrelia*, which explain why they were negative by the 16S real-time PCR. Therefore, by applying low stringency to inclusion criteria in the first screening, one might find quite interesting results. During the nested PCR and sequencing, *B. miyamotoi* was most likely detected due to a high similarity to the IGS sequence of *B. burgdorferi* s.l., making the primers able to anneal to the template.

4.1.3 Evaluation of the detection methods

In the present study, the sensitivity of the 16S real-time PCR was high (as all confirmed positive samples were detected by 16S real-time PCR), thereby increasing the possibility of detecting all the *B. burgdorferi* s.l. positive samples. However, the 16S real-time PCR has the possibility to yield false positive results, as a BLAST search revealed tick genes in some samples, which indicates a somewhat lower specificity of the method. The 16S real-time PCR results should therefore be confirmed by another method to exclude possible false positives.

The melting points of the amplicons generated from the unknown samples and from known *Borrelia* spp. species were compared for genotyping. Melting point analysis clearly allowed the distinction between *B. garinii* (50ºC), *B. afzelii* (66.5ºC) and *B. burgdorferi sensu stricto* (68.5ºC), resulting in a high specificity of the method. Mixed infections were not observed in the present study, however, mixed infections would be observed as two (or more) melting points by the *hbb* real-time PCR. Even though there was a clear distinction between the genospecies, only 57 of the 107 positive samples, were positive by *hbb* real-time PCR, resulting in a somewhat low sensitivity of the *hbb* real-time PCR. For this reason, all samples were further analysed by nested PCR, and the final IGS products were sequenced.

Analysis of all *B. afzelii* positive samples in the present study had melting points in a range between 66.0°C and 67.5°C, which are in accordance with previous studies (Portnoï et al. 2006, Kjelland et al. 2010b). The different genospecies have sequence variations within the *hbb* gene, and it is the probes ability to align that set the melting point (as it is defined as the temperature leading to 50% dissociation of the amplicon-probe adducts (Portnoï et al. 2006)). Therefore, the variations within the melting points of *B. afzelii* (66.0°C-67.5°C) could be explained by small differences in the *hbb* sequence. The analysis of *B. burgdorferi sensu stricto* positive samples gave a melting point of 68.5°C, which are in accordance with earlier studies (Portnoï et al. 2006, Kjelland et al. 2010b)

Most analysis of *B. garinii* samples gave a melting point of either 49.5°C or 50.0°C, which are in accordance with earlier studies (Portnoï et al. 2006, Kjelland et al. 2010b). However, analysis of one sample (ST26) gave a clearly higher melting point of 56.0°C. Portnoï et al. found that a mutation in the region covered by the probe was responsible for the shift in the melting point (Portnoï et al. 2006). They discovered that this *B. garinii* strain had a G in position 25 instead of an A, resulting in an increased melting temperature of 56.5°C. In the present study, the IGS region was sequenced, therefore, one can only speculate that there is a point mutation in the *hbb* sequence resulting in the high melting point.

Analysis of two *B. miyamotoi* positive samples (ST580 and ST619) gave melting points of 50°C. These two samples had 99% identity with *B. miyamotoi* and 96% identity with *B. garinii* by BLAST, indicating a possibility of mixed infections of the two. Such mixed infections have previously been detected in Canada (Dibernardo et al. 2014). Since the present study aimed to detect genospecies within the *B. burgdorferi* s.l. complex, the primers and probe were not designed to amplify the *hbb* sequence of *B. miyamotoi* (RF *Borrelia*). The possibility of mixed infections was not confirmed in the present study, since the genetic analyser available is not able to detect co-infections.

The *hbb* real-time PCR was used to detect the presence of *B. burgdorferi* s.l. as well as distinguish between the genotypes. Analysis of some samples gave a melting point (T_m) whereas not enough fluorescence to exceed the threshold line (resulting in no C_T value), and vice versa. These discrepancies could be explained by a low concentration of the staring template, as a certain amount of template is required to get enough amplicons for the fluorescence to cross the threshold line, whereas the starting template will have minor influence on the melting point analysis. Furthermore, the primers could influence the

possibility to create an amplification curve. The primers must be able to anneal and not bind to each other (primer dimers), or a low amount of amplicons could be generated, resulting in low fluorescence (since the EasyBeacon probe is quenched when the probe is unbound, and fluoresce at hybridization to the *hbb* sequence). On the other hand, the melting point analysis does not depend on the primers, as only the probe anneal to the sequence. One could speculate that many factors contribute to the discrepancy between an amplification curve and melting point, for instance temperature and concentrations, indicating the complexity of the issue.

Ninety five of the samples were positive by amplification of the IGS sequence during nested PCR, which was confirmed by visual bands on an agarose gel. The IGS products were sequenced, and a BLAST search revealed mostly *Borrelia* spp. DNA, however, tick genes were also detected. The nested PCR does have the advantage of reducing the number of unspecific amplicons (due to the two primer sets being used in two separate runs), increasing the specificity of the method. Detection of tick genes may be due to unspecific binding of primers. However, one advantage of the method was the possibility of detecting *B. miyamotoi.* The number of visible bands on the agarose gel varied, as analysis of some samples gave one band whereas analysis of other samples gave two or three bands. The IGS sequence amplified in the nested PCR is proned to mutations, insertions and/or deletions since the sequence does not encode a specific gene, resulting in varied fragment size of the IGS amplicons. In the present study, the different genospecies tended to have different lengths in the IGS sequence, which *B. afzelii* often had an IGS fragment size of approximately 500 base pairs, whereas *B. garinii* had a fragment size of approximately 1000 base pairs.

The combination of real-time PCR, nested PCR and sequencing is valid to detect *B. burgdorferi* s.l. DNA, however, doing all the analysis are both time consuming and expensive. The *hbb* real-time PCR step could be eliminated, if all 16S positive samples were sequenced. On the other hand, the sequence procedure is quite expensive and labor intensive, and since the *hbb* real-time PCR is efficient to distinguish between the three commonly detected genospecies (*B. afzelii*, *B. garinii* and *B. burgdorferi sensu stricto*), it is often used to reduce the number of samples to be sequenced. By generating melting points, the *hbb* real-time PCR could also be used as a control of the sequencing results. However, as described earlier, some genospecies may have a shift in the melting point, also, some genospecies have melting points close to each other (*B. valaisiana* (60.5°C), *B. spielmanii* (60.0°C) and *B. lusitaniae* (62°C)) and must therefore be confirmed by sequencing. The present study found all the detection

methods necessary to be able to conclude the genospecies of *Borrelia* spp., and due to the low sensitivity and specificity of some of the detecting methods, one should always sequence to confirm the results. A future study could aim to design a multiplex PCR with probes detecting all known genospecies of *Borrelia* spp. within one sample in a single run. This would reduce the hands on time and the number of analysis needed. However, a multiplex PCR could have a reduced sensitivity and specificity, and the cost of using multiple probes is high.

4.2 Comparison of tick DNA extraction methods

4.2.1 Study design

A total of 400 questing *I. ricinus* nymphs from Hummervika in Søgne were included in a comparison study of tick DNA extraction methods. The collection site was chosen due to the high abundance of nymphs (V. Kjelland, personal communication) as well as the high prevalence of *B. burgdorferi* s.l. (26.0%) previously observed in questing nymphs in the region (Kjelland et al. 2010b). The comparison was done on host-seeking nymphs since earlier studies strongly suggest that blood meal components in engorged ticks may inhibit the PCR, resulting in an underestimation of the prevalence of the respective pathogen (Sparagano et al. 1999).

Four different tick DNA extraction methods (the DNeasy Kit, phenol-chloroform, the Abbott machine and the NukEx Kit) were included in the present comparison study. These extraction methods were chosen for various reasons; the DNeasy Kit is frequently used in many laboratories, and is considered efficient for tick DNA extraction (Ullmann et al. 2007). However, phenol-chloroform has previously been recommended as the method of choice when extracting DNA from ticks (Sparagano et al. 1999). The Abbott machine may be considered state of the art when extracting DNA; it requires minimal hands on time, it is an efficient DNA extraction method, and there is a reduced human bias and risk of contamination (as the samples are in an enclosed compartment). The NukEx Kit is an enzyme digestion based DNA extraction method, and were included in this study due to the minimal hands on time as well as the short time used to prepare the DNA extracts for further analysis.

The methods included in this study varies in the total time used to extract the DNA, as well as the cost per sample (not considering labor cost). DNA extraction by the DNeasy Kit takes two days and costs approximately 56NOK per sample, whereas DNA extraction by phenol-chloroform takes three days and costs approximately 7NOK per sample. DNA extraction by the Abbott machine takes approximately 4 hours, of which one hour is needed to homogenize the ticks in a solution. Neither Abbott Inc. nor The Norwegian Institute of Public Health would comment on the cost of the machine, nor the kits needed to extract DNA. Therefore, the costs per sample is not included in this study. The NukEx Kit requires minimal hands on time, the DNA extraction takes only 1.5 hours, and the cost per sample is approximately 14NOK.

Ideally, one nymph should be cut into four equal pieces, and a portion tested by each method. However, the nymphs are very small in size, and the number of *B. burgdorferi* s.l. spirochetes within the tick may be low, making it hard to do so. Also, an earlier study suggested that there may not be an equal distribution of the *Borrelia* spirochete when cutting the tick, resulting in different *Borrelia* DNA concentrations in each part (Kampen et al. 2004). Future studies may therefore aim to use adult ticks (due to the size), and infect the ticks with *Borrelia* spp. spirochetes by xenodiagnosis (expose infected tissue (for instance a mouse) to the tick vector, and then study the presence of the spirochetes in these ticks) (Gern et al. 1998). However, even though the ticks have been feeding of an infected animal, they might not be infected with *Borrelia* spp. (V. Kjelland personal communication), indicating the complexity of this procedure. If the ticks are infected with *Borrelia* spp., the influence of the DNA extraction methods tested could be accounted for. However, there might be PCR inhibitors in DNA extracts from engorged ticks which must be taken into account, and may be reduced by the use of a tick housekeeping gene.

4.2.2 Purity and concentration

The NanoDrop spectrophotometer provides the possibility of measuring the purity and total concentration in a minimal volume of a sample with quite high accuracy. The method requires minimal hands on time, there is no contamination of the DNA extracts and the method is cost efficient. Therefore, the NanoDrop spectrophotometer was used to measure the purity (260nm/280nm and 260nm/230nm) and the total concentration (ng/µl) of DNA extracts from the four different methods tested in the present study.

The DNeasy Kit gave DNA extracts with the highest purity, followed by DNA extracts from the Abbott machine. There was no significant difference between the purity of DNA extracts from phenol-chloroform and the NukEx Kit. The ratio at 260nm/280nm provides an estimate of the purity of the DNA extracts. A ratio of 1.8 between 260nm/280nm is generally accepted as pure for DNA (Thermo Fisher Scientific Inc. 2014), which was detected in DNA extracts from the DNeasy Kit (1.91) and the Abbott machine (1.80). However, a lower 260nm/280nm ratio were detected in DNA extracts from phenol-chloroform (1.30) and the NukEx Kit (1.20). These findings are most likely due to the presence of phenol and/or proteins in the DNA extracts from phenol-chloroform, as there is a risk of transferring some of the lower organic phase during extraction (see section 2.2.2 Phenol-chloroform). All constituents within the tick cells were still present in DNA extracts by the NukEx Kit, containing cell debris, proteins and other molecules that strongly absorb at or near 280nm, resulting in the low 260nm/280nm ratio of 1.20. Also, the low 260nm/280nm ratio may be due to low DNA concentrations in DNA extracts from the NukEx Kit.

The 260nm/280nm ratio of 1.8 is only considered a “rule of thumb”. Nucleic acids absorb so strongly at 260nm that only a significant level of protein contamination will cause a significant change in the ratio of absorbance at the two wavelengths (Wilfinger, Mackey & Chomczynski 1997). A small change in sample pH will also cause the 260nm/280nm ratio to vary, in which acidic solutions will under-represent the 260nm/280nm ratio by 0.2-0.3 (resulting in reduced sensitivity to protein contamination), while a basic solution will over-represent the ratio by 0.2-0.3 (Wilfinger et al. 1997). It is therefore important to calibrate the spectrophotometer with the same solution used to elute the samples. This was taken into account in the present study, in which the NanoDrop spectrophotometer was blanked to AE buffer when measuring DNA extracts from the DNeasy Kit and phenol-chloroform. The spectrophotometer was blanked to the elution buffer included in the kit when measuring the

DNA extracts from the Abbott machine, and blanked to the NukEx reagent when measuring the DNA extracts from the NukEx Kit.

There was a significant difference in the 260nm/280nm ratio between DNA extracts from the different methods, which could be explained by the nucleotide composition within the solutions (Thermo Fisher Scientific Inc. 2014). The five nucleotides that comprise DNA exhibit widely varying 260nm/280nm ratios (Guanine: 1.15, Adenine: 4.50, Cytosine: 1.51, Thymine: 1.47). The resultant 260nm/280nm ratio for the nucleic acid will be approximately equal to the weighted average of the 260nm/280nm ratios for the four nucleotides present. Therefore, the actual ratio will depend on the composition of the nucleic acid. However, this is probably of minor importance, as DNA extracts from all methods should contain a predominance of tick DNA.

The 260nm/230nm ratio is used as a secondary measure of nucleic acid purity, and pure preparations of DNA are commonly in the range of 2.0-2.2. DNA extracts from the DNeasy Kit are considered pure, with a 260nm/230nm ratio of 2.25. However, the 260nm/230nm ratio was significantly lower in DNA extracts from phenol-chloroform (0.83), Abbott (0.10) and the NukEx Kit (0.79), which may indicate the presence of contaminants which absorb at 230nm (*e.g.* EDTA, carbohydrates or phenol) (Stulnig & Amberger 1994). The presence of phenol is likely to influence the DNA extracts from phenol-chloroform, whereas carbohydrates and other cell constituents are likely to decrease the 260nm/230nm ratio of DNA extracts from the NukEx Kit. The pipettes were reused in the two washes during DNA extraction by the Abbott machine, and there is a possibility that liquid containing cell constituents could be present on the pipettes during the washes, decreasing the ability to remove all contaminants, which again could explain the low 260nm/230nm ratio.

DNA extracts from phenol-chloroform had the highest average concentration (17.6ng/µl), followed by DNA extracts from the DNeasy Kit (10.8ng/µl), the NukEx Kit (4.6ng/µl) and the Abbott machine (3.8ng/µl), respectively. When extracting DNA by phenol-chloroform, a relatively small volume (compared to the other methods tested) is obtained (180µl and 120µl of the upper phase), and little is lost during the procedure, which might explain the high average concentration. In DNA extraction by the DNeasy Kit, a membrane bind the DNA, while other cell constituents were removed by two washes. The DNA were then eluted in two steps to ensure a high concentration of DNA (see section 2.2.1 DNeasy Blood and Tissue Kit from Qiagen). DNA extracts from the NukEx Kit had a surprisingly low average

concentration, considering that cell constituents were not removed from the samples. However, the total volume of DNA extracts from the NukEx Kit was 300µl, which compared to the other DNA extracts of 50µl may result in a diluted solution and thereby a low DNA concentration. The average concentration of DNA extracts from the Abbott machine was low (3.8ng/µl), which may be explained by the DNA extracts being eluted in nuclease free water. The low pH of deionized water from some water purifiers may reduce DNA yield. To avoid the eluting water from influencing the total concentration, the pH of the water should be at least 7.0 (this was not taken into account in the present study). The low average concentration of DNA extracts from the Abbott machine may also be due to the nymphs being homogenised in a large volume of nuclease free water (300µl), in which some of the volume was lost during the procedure. Furthermore, when the samples (<300µl) entered the machine, only 200µl were used during the extraction, to prevent the pipettes to suck up air. This procedure may have caused some of the DNA to be lost, and may explain the low average concentration of the DNA extracts from the Abbott machine.

4.2.3 PCR and sequencing

The likelihood of detecting *B. burgdorferi* s.l. was considered equal in each method tested, as the nymphs were collected from the same location within the same time period. The present study determined the *Borrelia* spp. prevalence to vary between 16 – 27 %, which is in accordance with earlier findings from the same area (26%) (Kjelland et al. 2010b).

When detecting *B. burgdorferi* s.l. by the PCR and sequencing methods used in the present study, it seems that DNA extraction by DNeasy Kit or phenol-chloroform is preferable. The detected *Borrelia* spp. prevalence was considerably higher in DNA extracts from the DNeasy Kit (27%) and phenol-chloroform (27%) compared to the Abbott machine (17%) and the NukEx Kit (16%) (Table 8). The present study found DNA extracts from the DNeasy Kit and phenol-chloroform to have a significant lower mean C_T value in 16S real-time PCR compared to DNA extracts from the Abbott machine and the NukEx Kit (Table 9). These findings indicate that DNA extracts from the DNeasy Kit and phenol-chloroform had a higher concentration of *Borrelia* spp. starting template, which might have led to the higher detected prevalence by these methods. The lower C_T value in DNA extracts from the DNeasy Kit and phenol-chloroform could be due to the significantly higher purity (DNeasy Kit) and average

concentration (phenol-chloroform), compared to DNA extracts from the other two methods. On the other hand, there is a possibility that only 16 and 17 of the nymphs investigated by the Abbott machine and the NukEx Kit were infected with *Borrelia* spp., and the results could therefore be true estimates. However, the low average concentration (3.8ng/µl) of the DNA extracts by the Abbott machine could influence the ability to detect *Borrelia* spp. Also, DNA extracts from the NukEx Kit had the lowest purity, which could result in a low detected prevalence of *Borrelia* spp.

The NukEx Kit generated DNA extracts with a lower quality compared the other methods tested, which were visualized by the presence of weak smears on the agarose gel after nested PCR as well as a some background noise in the sequencing results (data not shown). Five of the DNA extracts from the NukEx Kit had a weak smear on the gel after nested PCR, which is most likely due to the presence of cell constituents. A spin column (similar to the DNeasy Kit) could be used to remove possible inhibitors (cell constituents) in DNA extracts from the NukEx Kit. However, the aim of this study was to compare the DNA extraction methods as described by the manufacturer. Furthermore, a strong advantage of the NukEx Kit is the minimal hands on time as well as a low cost per sample (14NOK), both of which will be considerably increased by purification using additional kits.

The most frequent genotype detected was *B. afzelii* (86.2%), followed by *B. garinii* (9.2%) and *B. miyamotoi* (2.3%), which is accordance with findings in the prevalence study. *B. afzelii* was detected in DNA extracts from all the methods tested (Table 10).

Analysis of three *B. afzelii* positive samples (determined by sequencing of the IGS sequence) from the Abbot machine gave atypical melting points (R207 and R286: 68.0°C and R243: 68.5°C). Furthermore, analysis of one *B. afzelii* positive sample (determined by the IGS sequence) from the DNeasy Kit (R100: 68°C) and one sample from the NukEx Kit (R313: 66.5°C) did also give atypical melting points. No atypical melting points of *B. afzelii* were detected in DNA extracts from phenol-chloroform. One could speculate that these discrepancies are a result of point mutations causing a shift in the melting point, as described earlier for *B. garinii* (section 4.1.3 Evaluation of the detection methods), and since the *hbb* gene was not sequenced in the present study, this was not confirmed.

A previous study from Halos et al. (Halos et al. 2004) compared three different DNA extraction methods; (i) enzymatic digestion by proteinase K followed by DNA extraction by a

commercial Kit (Qiamp, Qiagen), (ii) mortar crushing, proteinase K digesting and phenol-chloroform and (iii) fine crushing with a bead beater, proteinase K digestion and DNA extraction using a commercial kit (Qiamp, Qiagen). They concluded that enzymatic digestion before DNA extraction is not sufficient for maximum isolation of DNA, and must be associated with an initial step of fine crushing (due to the polysaccharide chains of the chitin of the tick exoskeleton). The present study compared three methods based on enzyme digestion and manual cutting of the ticks (DNeasy Kit, phenol-chloroform and the NukEx Kit), and mechanical crushing by a homogenizer before entering the Abbott machine. This study strongly suggest that manual cutting and enzymatic digestion is efficient to detect *Borrelia* spp. DNA. The combination of enzymatic digestion and mechanical crushing was not tested it seems, however, that mechanical crushing (the Abbott machine) is not preferable (due to the low total concentration) and could be optimized considering detection of pathogens within a tick.

In the present study, the efficiency of the DNA extractions was not confirmed by a positive control (a tick gene), which could be considered a disadvantage since potentially inhibitory factors were not accounted for. Previous studies suggest the use of the tick mitochondrial 16S rRNA gene as a positive control to confirm the efficiency of the DNA extractions (Sparagano et al. 1999, Halos et al. 2004).

When selecting a tick DNA extraction method to be used in a study different factors should be considered; for instance the work load, hands on time, cost per sample, chemical hazards, the number of samples to be analyzed, as well as the accuracy needed in the study. In the present study, both the DNeasy Kit and phenol-chloroform were considered efficient and preferable when detecting *Borrelia* spp. DNA. However, if one consider consumable cost, chemical cost, researcher's time, and chemical hazards, phenol-chloroform extractions are not cost efficient. On the other hand, if many samples are to be analyzed, the DNeasy Kit will not be cost efficient, due to the high cost per sample. DNA extraction by the Abbott machine seems to need optimization towards ticks (due to the low DNA concentrations). DNA extraction by the NukEx Kit seems to be the least preferable when detecting *Borrelia* spp. DNA by the PCR and sequencing methods used in the present study. This is probably due to the low purity of the DNA extracts resulting in lower detection sensitivity by PCR and high background noise by sequencing. A future study could therefore aim to find a cost efficient DNA extraction method that ensures high quality DNA extracts, requiring a minimum of the researchers' time.

5 Conclusions

The main intention of this thesis was to determine the prevalence and genotype composition of *B. burgdorferi* s.l. at different locations in Scandinavia. The present study detected a mean *Borrelia* spp. prevalence of 16.5% in Norway, 24% in Denmark and 6% in Sweden, respectively. *B. afzelii* was the predominant genotype (75.5%), followed by *B. garinii* (11.2%), *B. miyamotoi* (8.4%) and *B. burgdorferi sensu stricto* (3.7%). This is one of the first reports of *B. miyamotoi* in Norway and Denmark. Knowledge of the prevalence and genotype composition of *Borrelia* spp. in different locations may have high clinical value, as various clinical symptoms are connected to some genospecies.

The second aim of this thesis was to compare tick DNA extraction methods and study if these DNA extraction methods influenced detection of *B. burgdorferi* s.l. DNA. A comparative study such as this is important since DNA extraction is often the first step of molecular detection of microorganisms in ticks. The results of this study indicate that the DNeasy Kit and phenol-chloroform gives DNA extracts that are preferable when detecting *Borrelia* spp. by the methods used in the present study. DNA extracts from the DNeasy Kit had the highest purity, whereas DNA extracts from phenol-chloroform had the highest average concentration. The Abbott machine may need some optimization towards DNA extraction of ticks, as the average DNA concentration was low. The NukEx Kit seem to be the least preferable when extracting tick DNA that were analyzed for *Borrelia* spp. DNA, as the DNA extracts had a low purity which seem to interfere with both PCR and sequencing.

6 Future perspectives

There are still many unsolved questions and much more knowledge to be gained on ticks and tick-borne pathogens. This master thesis is a small contribution to the big picture of tick-borne pathogens in Scandinavia, however, future studies are needed to fully understand the complexity of this topic.

Future studies should aim to:

- Characterize *Borrelia* spp. in all LB patients, to gain more knowledge of the genospecies infecting humans, as the prevalence of *Borrelia* spp. in questing nymphs in the present study did not correlate to reported clinical manifestations of LB patients in Scandinavia.

- Elucidate the possible clinical consequences involving multiple bacteria and viruses that could be transmitted by *I. ricinus* ticks.

- Observe and elucidate the reservoir hosts' roles in spreading the *Borrelia* spp. spirochete, as well as their ecology to other tick-borne pathogens.

- Find a cost efficient DNA extraction method that ensures high quality DNA extracts, requiring a minimal of the researchers' time.

- Design a sensitive and specific multiplex PCR that detects all known tick-borne pathogens within one sample in a single run. This would reduce the hands on time and the number of analysis needed.

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8 Appendices

8.1 Summary results - prevalence of *Borrelia* spp.

Table 8.1: *Borrelia* spp. positive samples.

Sample	Collection site	Purity		Total concentration (ng/µl)	16S real-time PCR (C_T value)	*Hbb* real-time PCR (C_T value, T_m value in °C)	IGS Nested PCR (size and number of bands)	Sequencing (genotype, length (bp*))
		260/280 ratio	260/230 ratio					
ST6	Hillevågen	1.49	0.35	4.8	33.28	C_T = 51.77 T_m = 66.0	~500 bp*	*B. afzelii*, 426
ST9	Hillevågen	1.57	0.60	9.6	36.97	C_T =51.74 T_m = 66.0	~500 bp	*B. afzeliik*, 426
ST10	Hillevågen	2.58	0.40	2.4	39.58	C_T = 48.25 T_m = 60.0	~500 bp Weak band	Unknown
ST16	Hillevågen	1.57	0.23	35.9	30.94	Atypical amp. curve T_m = 66.5	~500 bp	*B. afzelii*, 108
ST17	Hillevågen	1.44	0.64	32.5	40.84	Negative	~500 bp ~1000 bp	*B. afzelii*, 422
ST18	Hillevågen	1.47	0.76	40.6	30.05	C_T = 51.96 T_m = 66.5	~500 bp ~700 bp ~1000 bp	*B. afzelii*, 423
ST23	Hillevågen	1.38	0.94	40.1	33.90	Negative	~700 bp	*B. garinii*, 512
ST26	Hillevågen	1.40	1.01	47.6	33.93	No amp. curve T_m = 56.0	~700 bp Weak band	*B. garinii*, 646
ST28	Hillevågen	1.39	0.82	22.7	35.62	C_T = 52.60 T_m = 66.5	~500 bp	*B. afzelii*, 414
ST40	Hillevågen	1.38	1.16	41.5	38.78	Negative	~500 bp	*B. afzelii*, 404
ST45	Hillevågen	1.37	1.31	43.9	41.08	Negative	~500 bp	*B. afzelii*, 421
ST50	Hillevågen	1.38	0.90	21.4	30.77	No amp. curve T_m = 50.0	~500 bp	*B. garinii*, 348
ST51	Hillevågen	1.51	0.50	24.7	31.66	No amp. curve T_m = 49.5	~700 bp	*B. garinii*, 600
ST57	Hillevågen	1.42	1.03	24.6	31.26	No amp. curve T_m = 49.5	~1600 bp	*B. garinii*, 506
ST59	Hillevågen	1.38	1.08	24.4	38.89	C_T = 52.92 T_m = 66.0	Negative	*B. afzelii*, 412
ST64	Hillevågen	1.43	1.13	36.3	39.12	Negative	~1000 bp	*B. afzelii*, 395
ST66	Hillevågen	1.43	1.02	46.6	31.82	No amp. curve T_m = 50.0	Negative	*B. garinii*, 621
ST71	Hillevågen	1.38	1.14	39.1	35.80	Negative	~500 bp	*B. afzelii*, 426
ST72	Hillevågen	1.44	0.93	25.7	40.78	Negative	~500 bp	*B. afzelii*, 428

Sample	Collection site	Purity		Total concentration (ng/µl)	16S real-time PCR (C_T value)	*Hbb* real-time PCR (C_T value, T_m value in °C)	IGS Nested PCR (size and number of bands)	Sequencing (genotype, length (bp))
		260/280 ratio	260/230 ratio					
ST74	Hillevågen	1.39	1.39	47.6	32.33	No amp. curve T_m = 66.5	~500 bp	*B. afzelii*, 420
ST79	Hillevågen	1.33	1.80	53.1	30.30	Negative	~500 bp	*B. garinii*, 387
ST82	Hillevågen	1.35	1.18	34.1	36.35	Negative	~500 bp	*B. afzelii*, 422
ST83	Hillevågen	1.38	1.09	30.9	35.04	C_T = 44.51 T_m = 66.5	~500 bp	*B. afzelii*, 429
ST84	Hillevågen	1.43	1.11	29.4	33.68	C_T = 47.87 No melting point	~500 bp Weak band	*B. afzelii*, 364
ST92	Hillevågen	1.40	1.32	32.9	31.52	C_T = 54.02 T_m = 66.0	~500 bp	*B. afzelii*, 420
ST101	Håøya	2.35	0.29	7.0	40.58	C_T = 54.60 No melting point	~500 bp	*B. afzelii*, 413
ST102	Håøya	1.67	0.55	28.2	38.32	C_T = 54.07 T_m = 67.0	~500 bp	*B. afzelii*, 138
ST107	Håøya	1.56	1.11	38.9	35.86	Negative	~500 bp	*B. afzelii*, 407
ST108	Håøya	2.46	0.39	7.5	35.05	Negative	~500 bp	*B. afzelii*, 337
ST110	Håøya	1.60	0.92	33.8	40.64	C_T = 54.05 T_m = 66.5	~500 bp	*B. afzelii*, 414
ST114	Håøya	1.61	0.93	38.0	35.33	C_T = 50.54 T_m = 66.5	~500 bp	*B. afzelii*, 421
ST116	Håøya	1.60	1.22	51.4	33.71	C_T = 54.08 T_m = 67.0	~500 bp ~1000bp	*B. afzelii*, 419
ST121	Håøya	1.74	0.90	23.8	35.19	C_T =50.01 T_m = 67.0	~500 bp ~1000bp	*B. afzelii*, 415
ST122	Håøya	1.71	0.82	25.4	35.21	C_T = 50.90 T_m = 66.0	~500 bp ~1000bp	*B. afzelii*, 430
ST130	Håøya	1.57	1.18	35.0	39.62	Negative	~500 bp ~1000bp	*B. afzelii*, 398
ST132	Håøya	1.54	1.14	41.1	40.14	Negative	~500 bp ~1000bp	*B. afzelii*, 429
ST135	Håøya	1.52	1.26	53.4	37.48	No amp. curve T_m = 66.5	~400 bp	*B. afzelii*, 419
ST152	Håøya	1.52	1.23	59.3	46.77	Negative	~500 bp	*B. afzelii*, 426
ST159	Håøya	1.62	1.21	25.7	39.37	Negative	~500 bp	*B. afzelii*, 421
ST163	Håøya	1.60	1.07	25.6	37.05	C_T = 54.02 T_m = 66.5	~500 bp ~1000 bp	*B. afzelii*, 413
ST172	Håøya	1.55	1.09	50.4	35.30	Negative	~500 bp	*B. afzelii*, 422
ST204	Tromøy	1.49	1.21	33.3	33.27	Negative	~400 bp	*B. garinii*, 355
ST242	Tromøy	1.42	1.45	31.6	36.70	C_T = 30.03 No melting point	~500 bp	*B. afzelii*, 393
ST251	Tromøy	1.43	1.10	33.6	41.95	C_T = 33.26 No melting point	~500 bp ~700 bp ~1000 bp	*B. afzelii*, 427
ST254	Tromøy	1.42	0.93	29.6	41.62	Negative	~500 bp	*B. afzelii*, 422

Sample	Collection site	Purity		Total concentration (ng/µl)	16S real-time PCR (C_T value)	*Hbb* real-time PCR (C_T value, T_m value in °C)	IGS Nested PCR (size and number of bands)	Sequencing (genotype, length (bp))
		260/280 ratio	260/230 ratio					
ST260	Tromøy	1.47	1.09	26.2	40.43	Negative	~500 bp	*B. afzelii*, 416
ST266	Tromøy	1.39	0.89	14.1	38.67	Negative	~500 bp	*B. afzelii*, 424
ST269	Tromøy	1.43	1.07	39.5	43.71	Negative	~500 bp	*B. afzelii*, 425
ST272	Tromøy	1.42	1.13	36.8	38.77	Negative	~500 bp	*B. afzelii*, 422
ST292	Tromøy	1.43	1.30	36.4	36.53	Negative	~500 bp	*B. afzelii*, 421
ST300	Tromøy	1.51	0.39	6.3	33.91	No amp. curve T_m = 50.0	~400 bp ~500 bp ~1000 bp	*B. garinii*, 420
ST306	Brønnøya	1.43	0.88	63.6	38.55	C_T = 43.49 T_m = 67.0	~500 bp ~700 bp	*B. afzelii*, 406
ST308	Brønnøya	1.43	1.03	40.1	37.06	No amp. curve T_m = 66.5	~500 bp ~1000 bp	*B. afzelii*, 420
ST310	Brønnøya	1.44	0.93	47.5	39.95	Negative	~500 bp	*B. afzelii*, 423
ST325	Brønnøya	1.46	1.10	35.9	39.86	C_T = 47.02 T_m = 66.5	~500 bp	*B. afzelii*, 407
ST330	Brønnøya	1.56	1.08	40.4	45.87	Negative	~500 bp ~700 bp ~1000 bp	*B. miyamotoi*, 376
ST334	Brønnøya	1.40	0.79	58.2	41.82	Negative	~500 bp	*B. afzelii*, 419
ST335	Brønnøya	1.42	1.04	48.7	Possibly positive**	Negative	~500 bp ~700 bp ~1000 bp	*B. miyamotoi*, 367
ST336	Brønnøya	1.40	1.25	52.0	46.08	Negative	~500 bp	*B. afzelii*, 413
ST337	Brønnøya	1.40	0.96	32.1	37.27	C_T = 53.09 T_m = 66.5	~500 bp	*B. afzelii*, 425
ST338	Brønnøya	1.46	1.01	66.7	38.66	C_T = 31.95 No melting point	~500 bp	*B. miyamotoi*, 375
ST345	Brønnøya	1.43	1.12	43.5	Possibly positive**	C_T = 38.46 No melting point	~500 bp ~1000 bp	*B. miyamotoi*, 366
ST351	Brønnøya	1.49	1.06	40.9	39.99	Negative	~500 bp	*B. afzelii*, 424
ST352	Brønnøya	1.47	0.62	47.5	37.85	Negative	~500 bp ~700 bp	*B. afzelii*, 405
ST369	Brønnøya	1.44	1.17	69.3	34.27	C_T = 44.15 T_m = 66.5	~500 bp	*B. afzelii*, 427
ST403	Spjærøya	1.43	0.69	30.6	32.48	C_T = 43.54 T_m = 67.0	~500 bp	*B. afzelii*, 426
ST405	Spjærøya	1.38	0.81	29.5	37.43	C_T =48.56 T_m = 67.0	~500 bp	*B. afzelii*, 428
ST406	Spjærøya	1.47	0.49	33.8	34.51	C_T = 46.98 T_m = 67.0	~500 bp	*B. afzelii*, 424
ST408	Spjærøya	1.39	1.03	45.9	41.25	Negative	Negative	*B. afzelii*, 428
ST415	Spjærøya	1.38	0.68	21.4	40.70	Negative	~500 bp	*B. afzelii*, 416
ST422	Spjærøya	1.28	1.57	34.9	35.95	Negative	~500 bp	*B. afzelii*, 427
ST436	Spjærøya	1.48	1.00	60.3	42.53	C_T = 54.07 T_m = 67.0	Negative	*B. afzelii*, 399

Sample	Collection site	Purity		Total concentration (ng/µl)	16S real-time PCR (C_T value)	*Hbb* real-time PCR (C_T value, T_m value in °C)	IGS Nested PCR (size and number of bands)	Sequencing (genotype, length (bp))
		260/280 ratio	260/230 ratio					
ST443	Spjærøya	1.43	1.08	46.6	33.46	C_T = 33.88 No melting point	~500 bp	*B. afzelii*, 422
ST446	Spjærøya	1.42	1.02	54.7	36.03	C_T = 54.05 No melting point	~500 bp	*B. afzelii*, 414
ST456	Spjærøya	1.46	1.05	31.4	36.68	Negative	~500 bp	*B. afzelii*, 424
ST458	Spjærøya	1.43	1.08	48.4	39.36	C_T = 36.94 No melting point	Negative	*B. afzelii*, 152
ST465	Spjærøya	1.43	1.13	15.4	39.52	C_T = 54.05 T_m = 67.5	~500 bp	*B. afzelii*, 422
ST504	Tokkekøb Hegn	1.43	1.11	85.7	29.80	Negative	Negative	*B. afzelii*, 255
ST513	Tokkekøb Hegn	1.34	1.25	89.8	17.86	C_T = 30.19 No melting point	Negative	*B. afzelii*, 77
ST521	Tokkekøb Hegn	1.36	1.17	67.3	19.36	C_T = 26.81 No melting point	Negative	*B. afzelii*, 271
ST522	Tokkekøb Hegn	1.41	1.05	48.0	36.44	C_T = 45.13 T_m = 68.5	~1000 bp	*B. burgdorferi sensu stricto*, 528
ST535	Tokkekøb Hegn	1.41	1.27	24.0	37.18	Negative	~1000 bp	*B. burgdorferi sensu stricto*, 285
ST538	Tokkekøb Hegn	1.33	1.00	34.3	32.72	Negative	~500 bp Weak band	*B. garinii*, 520
ST542	Tokkekøb Hegn	1.37	1.40	24.2	36.09	C_T = 51.87 T_m = 68.5	~1000 bp	*B. burgdorferi sensu stricto*, 452
ST550	Tokkekøb Hegn	1.40	1.04	61.4	32.71	Negative	~500 bp	*B. afzelii*, 394
ST557	Tokkekøb Hegn	1.44	0.73	53.7	38.22	Negative	~500 bp	*B. afzelii*, 392
ST564	Tokkekøb Hegn	1.40	1.19	62.0	35.60	Negative	~500 bp	*B. miyamotoi*, 350
ST569	Tokkekøb Hegn	1.42	1.02	44.7	34.15	C_T = 56.05 No melting point	~500 bp	*B. afzelii*, 392
ST570	Tokkekøb Hegn	1.41	1.11	67.3	32.15	C_T = 43.48 T_m = 67.5	~500 bp	*B. afzelii*, 398
ST577	Tokkekøb Hegn	1.42	1.84	23.7	32.60	C_T = 47.02 T_m = 67.0	~500 bp	*B. afzelii*, 399
ST580	Tokkekøb Hegn	1.40	0.73	37.5	33.39	C_T = 54.05 T_m = 68.5	~500 bp	*B. burgdorferi sensu stricto*, 105
ST583	Tokkekøb Hegn	1.41	1.55	27.6	39.59	C_T = 52.53 No melting point	~500 bp	*B. miyamotoi*, 332

Sample	Collection site	Purity		Total concentration (ng/µl)	16S real-time PCR (C_T value)	*Hbb* real-time PCR (C_T value, T_m value in °C)	IGS Nested PCR (size and number of bands)	Sequencing (genotype, length (bp))
		260/280 ratio	260/230 ratio					
ST585	Tokkekøb Hegn	1.37	3.53	6.2	31.09	No amp. curve T_m = 50.0	~500 bp	*B. miyamotoi*, 325
ST587	Tokkekøb Hegn	0.94	0.74	5.9	40.44	Negative	~500 bp	*B. afzelii*, 356
ST588	Tokkekøb Hegn	0.22	0.86	0.1	39.93	Negative	Negative	*B. afzelii*, 164
ST591	Tokkekøb Hegn	1.33	0.95	23.5	39.13	Negative	Negative	*B. afzelii*, 363
ST592	Tokkekøb Hegn	1.36	1.22	17.9	38.85	Negative	~500 bp	*B. miyamotoi*, 342
ST597	Tokkekøb Hegn	1.30	1.23	71.7	11.47	C_T = 33.05 No melting point	~500 bp	*B. afzelii*, 395
ST598	Tokkekøb Hegn	1.35	0.97	87.4	18.80	C_T = 25.06 No melting point	~500 bp	*B. afzelii*, 349
ST599	Tokkekøb Hegn	1.29	1.25	88.4	16.73	Negative	~500 bp	*B. afzelii*, 371
ST600	Tokkekøb Hegn	1.26	1.43	79.5	22.42	Negative	Negative	*B. afzelii*, 368
ST601	Verkö	1.41	1.16	29.1	42.73	Negative	~500 bp	*B. afzelii*, 311
ST619	Verkö	1.40	0.49	33.6	36.96	No amp. curve T_m = 50.0	~500 bp	*B. miyamotoi*, 345
ST625	Verkö	1.36	1.16	62.0	38.52	Negative	~500 bp	*B. afzelii*, 402
ST648	Verkö	1.52	0.23	4.1	38.37	C_T = 54.05 No melting point	Weak smear	*B. garinii*, 326
ST675	Verkö	1.41	0.87	37.9	36.22	Negative	Negative	*B. garinii*, 602
ST680	Verkö	1.35	0.88	19.3	37.71	Negative	~500 bp	*B. afzelii*, 427

*bp: base pairs

**No enough fluorescence to exceed the threshold line, resulting in no C_T value

8.2 Summary results - tick DNA extraction methods

Table 8.2: *Borrelia* spp. positive samples.

Sample	Method	Purity		Total concentration (ng/µl)	16S real-time PCR (C_T value)	*Hbb* real-time PCR (C_T value, T_m in °C)	IGS Nested PCR (size and number of bands)	Sequencing (Genotype, length (bp*))
		260/280 ratio	260/230 ratio					
R3	DNeasy kit	1.76	1.11	10.8	29.72	Negative	~500 bp*	*B. afzelii*, 413
R6	DNeasy kit	2.18	1.10	7.1	26.31	C_T = 50.00 T_m = 50.5	~1000 bp Weak band	*B. garinii*, 182
R16	DNeasy kit	1.79	2.70	9.7	27.97	C_T = 53.00 T_m = 67.0	~500 bp	*B. afzelii*, 433
R25	DNeasy kit	1.99	9.28	7.1	34.93	Negative	~500 bp	*B. afzelii*, 425
R31	DNeasy kit	2.19	1.50	10.4	29.00	Negative	~500 bp	*B. afzelii*, 401
R37	DNeasy kit	2.06	1.75	11.9	30.76	Negative	~500 bp	*B. afzelii*, 409
R38	DNeasy kit	1.91	1.58	12.0	27.95	No amp. curve T_m = 50.5	~1200 bp	*B. garinii*, 537
R39	DNeasy kit	2.06	1.67	12.2	31.43	No amp. curve T_m = 67.0	~500 bp	*B. afzelii*, 414
R40	DNeasy kit	1.88	4.54	6.7	30.32	C_T = 53.00 T_m = 67.0	~500 bp	*B. afzelii*, 421
R42	DNeasy kit	1.88	3.15	9.4	28.29	C_T = 47.00 No melting point	~500 bp	*B. afzelii*, 413
R43	DNeasy kit	1.33	0.53	12.6	30.73	No amp. curve T_m = 50.0	~1000 bp Weak band	*B. garinii*, 605
R44	DNeasy kit	1.86	3.31	13.7	32.49	No amp. curve T_m = 66.0	~500 bp	*B. afzelii*, 410
R45	DNeasy kit	1.99	3.74	10.3	29.08	C_T = 53.00 T_m = 66.5	~500 bp	*B. afzelii*, 427
R52	DNeasy kit	2.00	2.09	13.5	28.13	C_T = 53.50 T_m = 66.0	~500 bp	*B. afzelii*, 228
R65	DNeasy kit	1.82	2.66	13.7	36.41	Negative	~500 bp	*B. afzelii*, 420
R67	DNeasy kit	1.89	3.71	11.9	29.67	C_T = 48.00 T_m = 67.0	~500 bp	*B .afzelii*, 406
R70	DNeasy kit	1.67	3.78	8.5	29.69	C_T = 48.90 T_m = 67.5	~500 bp	*B. afzelii*, 402
R73	DNeasy kit	1.98	2.13	8.1	31.36	C_T = 51.87 T_m = 67.0	~500 bp	*B. afzelii*, 428
R79	DNeasy kit	1.89	1.92	9.4	29.97	C_T = 52.01 T_m = 67.0	~500 bp	*B. afzelii*, 421
R83	DNeasy kit	1.82	-3.32	7.9	31.17	C_T = 36.72 T_m = 66.5	~500 bp	*B. afzelii*, 402

Sample	Method	Purity		Total concentration (ng/µl)	16S real-time PCR (C_T value)	*Hbb* real-time PCR (C_T value, T_m in °C)	IGS Nested PCR (size and number of bands)	Sequencing (Genotype, length (bp))
		260/280 ratio	260/230 ratio					
R87	DNeasy kit	1.68	2.06	11.0	27.96	C_T =52.80 T_m = 66.5	~500 bp	*B. afzelii*, 423
R88	DNeasy kit	1.66	1.54	10.0	26.88	C_T = 50.74 T_m = 66.0	~500 bp	*B. afzelii*, 408
R89	DNeasy kit	1.68	2.20	12.5	32.43	C_T = 38.18 No melting point	~500 bp	*B. afzelii*, 424
R92	DNeasy kit	1.73	1.76	11.9	32.68	Negative	~500 bp	*B. afzelii*, 411
R94	DNeasy kit	1.85	0.84	11.2	39.85	Negative	~500 bp	*B. afzelii*, 396
R96	DNeasy kit	1.67	1.59	11.4	28.86	C_T = 46.90 T_m = 67.0	~500 bp	*B. afzelii*, 423
R100	DNeasy kit	1.54	0.27	9.1	31.93	C_T = 47.82 T_m = 68.0	~500 bp	*B. afzelii*, 351
R102	Phenol-chloroform	1.49	0.67	18.8	37.42	C_T = 51.31 T_m = 66.5	~500 bp	*B. miyamotoi*, 361
R109	Phenol-chloroform	1.47	0.38	8.0	36.29	C_T = 54.75 T_m = 66.5	~500 bp	*B. afzelii*, 419
R112	Phenol-chloroform	1.46	0.79	35.1	31.52	C_T = 44.67 T_m = 66.5	~500 bp	*B. afzelii*, 415
R114	Phenol-chloroform	1.45	0.86	50.2	31.71	C_T = 50.24 T_m = 67.0	~500 bp	*B. afzelii*, 414
R117	Phenol-chloroform	1.49	0.81	24.1	33.69	C_T = 40.84 T_m = 67.0	~500 bp	*B. afzelii*, 417
R118	Phenol-chloroform	1.58	0.44	16.5	33.52	C_T = 48.66 T_m = 67.0	~500 bp	*B. afzelii*, 422
R121	Phenol-chloroform	1.28	1.25	27.7	30.84	C_T = 43.76 T_m = 66.0	~500 bp	*B. afzelii*, 385
R123	Phenol-chloroform	1.18	0.85	17.6	32.25	C_T = 47.01 T_m = 65.5	~500 bp	*B. afzelii*, 290
R128	Phenol-chloroform	1.16	0.91	11.7	33.53	Negative	~500 bp	*B. afzelii*, 411
R129	Phenol-chloroform	1.11	1.00	10.3	25.11	C_T = 45.68 T_m = 67.0	~500 bp	*B. afzelii*, 419
R130	Phenol-chloroform	0.79	0.38	3.6	34.82	C_T = 45.32 T_m = 67.0	~500 bp	*B. afzelii*, 420
R133	Phenol-chloroform	1.23	0.86	18.2	33.64	No amp. curve T_m = 50.5	~500 bp ~1000 bp	*B. garinii*, 594
R135	Phenol-chloroform	1.21	0.78	12.7	31.28	C_T = 46.55 T_m = 67.0	~500 bp	*B. afzelii*, 403
R146	Phenol-chloroform	0.85	0.61	2.8	35.66	C_T = 45.63 T_m = 67.0	~500 bp	*B. afzelii*, 403
R148	Phenol-chloroform	1.25	0.66	11.4	33.69	C_T = 42.43 T_m = 66.5	~500 bp	*B. afzelii*, 410
R150	Phenol-chloroform	1.37	0.61	17.8	36.61	C_T = 48.89 T_m = 66.5	~500 bp	*B. afzelii*, 428
R153	Phenol-chloroform	0.89	0.69	3.0	27.93	No amp. curve, T_m = 50.5	~1000 bp ~1600 bp	*B. garinii*, 616
R164	Phenol-chloroform	1.24	0.94	20.8	30.11	C_T =49.99 T_m = 67.0	~500 bp	*B. afzelii*, 421

Sample	Method	Purity		Total concentration (ng/µl)	16S real-time PCR (C_T value)	*Hbb* real-time PCR (C_T value, T_m in °C)	IGS Nested PCR (size and number of bands)	Sequencing (Genotype, length (bp))
		260/280 ratio	260/230 ratio					
R166	Phenol-chloroform	1.35	0.79	37.3	30.09	C_T = 40.54 T_m = 67.0	~500 bp	*B. afzelii*, 410
R170	Phenol-chloroform	1.17	0.62	10.1	32.13	C_T = 32.53 T_m = 67.5	~500 bp	*B. afzelii*, 423
R172	Phenol-chloroform	1.27	0.98	26.1	31.32	C_T = 40.67 T_m = 67.0	~500 bp	*B. afzelii*, 425
R173	Phenol-chloroform	1.44	1.21	19.4	29.79	C_T = 35.57 T_m = 67.5	~500 bp	*B. afzelii*, 425
R179	Phenol-chloroform	1.41	1.17	29.6	28.12	C_T = 44.33 T_m = 65.0	~500 bp	*B. afzelii*, 419
R182	Phenol-chloroform	1.47	0.99	22.0	30.16	C_T = 52.71 T_m = 67.0	~500 bp	*B. afzelii*, 429
R193	Phenol-chloroform	1.50	1.47	12.1	30.87	No amp. curve T_m = 50.0	~1000 bp	*B. garinii*, 607
R197	Phenol-chloroform	2.07	0.96	4.0	30.06	C_T =41.36 T_m = 67.5	~500 bp	*B. afzelii*, 422
R198	Phenol-chloroform	1.56	1.83	20.0	38.67	C_T =44.30 T_m = 62.0	~1000 bp Weak	Unknown**
R206	Abbott m2000sp	2.83	0.04	4.3	36.30	C_T = 53.00 T_m = 67.5	~500 bp	*B. afzelii*, 428
R207	Abbott m2000sp	3.04	0.07	5.5	38.31	C_T = 50.43 T_m = 68.0	~500 bp	*B. afzelii*, 421
R231	Abbott m2000sp	3.16	0.03	4.7	36.48	C_T = 50.53 T_m = 66.5	~500 bp	*B. afzelii*, 414
R233	Abbott m2000sp	2.29	0.02	2.5	39.21	No amp. curve T_m = 66.5	~500 bp	*B. afzelii*, 422
R238	Abbott m2000sp	2.75	0.07	4.3	36.41	C_T = 54.00 No melting point	~500 bp	*B. afzelii*, 438
R239	Abbott m2000sp	2.14	0.06	7.3	36.72	C_T = 52.32 T_m = 67.5	~500 bp	*B. afzelii*, 427
R243	Abbott m2000sp	1.90	0.04	6.1	37.29	C_T = 51.29 T_m = 68.5	~500 bp	*B. afzelii*, 409
R247	Abbott m2000sp	1.74	0.05	3.8	38.22	C_T = 51.95 T_m = 67.5	~500 bp	*B. afzelii*, 421
R253	Abbott m2000sp	1.45	0.05	3.7	38.82	C_T = 50.60 No melting point	~500 bp	*B. afzelii*, 407
R255	Abbott m2000sp	1.75	0.02	4.3	41.07	C_T = 36.80 No melting point	~500 bp	*B. afzelii*, 421
R258	Abbott m2000sp	1.19	0.06	2.7	39.67	C_T = 42.97 No melting point	~500 bp	*B. afzelii*, 421
R261	Abbott m2000sp	1.34	0.16	3.6	40.40	Negative	~500 bp	*B. afzelii*, 415
R274	Abbott m2000sp	1.09	0.14	3.0	39.33	C_T = 53.02 No melting point	~500 bp	*B. afzelii*, 404
R280	Abbott m2000sp	1.02	0.12	2.5	39.17	Negative	~500 bp	*B. afzelii*, 400

Sample	Method	Purity		Total concentration (ng/µl)	16S real-time PCR (C_T value)	*Hbb* real-time PCR (C_T value, T_m in °C)	IGS Nested PCR (size and number of bands)	Sequencing (Genotype, length (bp))
		260/280 ratio	260/230 ratio					
R284	Abbott m2000sp	1.45	0.07	3.4	41.53	C_T = 49.92 T_m = 67.5	~500 bp	*B. afzelii*, 416
R286	Abbott m2000sp	0.93	0.15	3.0	35.92	C_T = 30.02 T_m = 68.0	~500 bp	*B. afzelii*, 416
R299	Abbott m2000sp	1.19	0.05	2.9	40.38	C_T = 52.04 T_m = 67.0	~500 bp	*B. afzelii*, 425
R313	NukEx kit	0.89	0.39	3.0	37.00	No amp. curve Tm = 66.5	Weak smear	*B. garinii*, 351
R318	NukEx kit	1.17	0.62	7.7	34.01	C_T = 54.03 T_m = 67.0	~500 bp	*B. afzelii*, 421
R325	NukEx kit	1.05	0.58	4.0	37.89	Negative	Weak smear	*B. garinii*, 370
R329	NukEx kit	0.91	0.41	3.8	25.17	C_T = 54.23 T_m = 66.5	~500 bp	*B. afzelii*, 415
R330	NukEx kit	1.26	0.59	3.0	34.46	Negative	~500 bp	*B. afzelii*, 415
R331	NukEx kit	1.29	0.67	4.5	32.22	Negative	~500 bp	*B.miyamotoi*, 363
R337	NukEx kit	1.03	0.94	4.5	34.98	C_T = 51.42 T_m = 67.0	Weak smear	Unknown**
R351	NukEx kit	1.89	0.77	2.7	33.94	C_T = 53.54 T_m = 67.0	~500 bp	*B. afzelii*, 413
R355	NukEx kit	1.09	0.83	5.3	33.10	C_T = 49.18 T_m = 67.5	~500 bp	*B. afzelii*, 422
R360	NukEx kit	1.13	0.54	4.3	32.99	Negative	~500 bp	*B. afzelii*, 414
R366	NukEx kit	1.25	0.61	4.7	39.95	C_T = 49.65 T_m = 67.0	~500 bp	*B. afzelii*, 416
R368	NukEx kit	1.14	0.56	5.2	32.18	C_T = 49.70 T_m = 67.0	~500 bp	*B. afzelii*, 414
R374	NukEx kit	1.05	0.57	1.9	43.18	C_T = 50.59 T_m = 67.5	Negative	*B. afzelii*, 399
R383	NukEx kit	0.70	2.83	2.2	38.04	C_T = 48.59 T_m = 68.0	Weak smear	*B. afzelii*, 379
R394	NukEx kit	1.59	2.00	3.3	35.14	C_T = 53.41 No melting point	~500 bp	*B. afzelii*, 426
R398	NukEx kit	1.36	1.01	5.8	35.59	Negative	Weak smear	*B. afzelii*, 363

**bp: base pairs

**"Unknown" samples; sample R198 had a melting point of 62°C, which could correspond to *B. lusitaniae* (Portnoï et al. 2006). *B. lusitaniae* is not reported from Norway, however it has been detected in Sweden (Wilhelmsson et al. 2010). A discontiguous megaBLAST search shows high similarity (~270bp) to multiple genospecies. Sample R337 had a melting point of 67°C, typical of *B. afzelii*. However, a somewhat similar BLAST search reveal similarity (22bp) to *B. hermsii*, *B. miyamotoi* and *B. parkeri*, which are all RF *Borrelia*. It is evident that there are *Borrelia* spp. DNA within the two samples, however, it is not possible to conclude the genospecies.

8.3 Sequences of primers and probes

Table 8.3: Primer and probe sequences used in 16S real-time PCR, *hbb* real-time PCR and nested PCR (IGS; intergenic spacer).

	Sequence	**Reference**
LB forward primer	5'GCTGTAAACGATGCACACTTGGT	(Tsao et al. 2004)
LB reverse primer	5'GGCGGCACACTTAACACGTTAG	(Tsao et al. 2004)
LB Probe	6FAM-TTCGGTACTAACTTTTAGTTAA-MGBNFQ	(Tsao et al. 2004)
Hbb forward primer	5'GTAAGGAAATTAGTTTATGTCTTT	(Kjelland et al. 2010b)
Hbb reverse primer	5'TAAGCTCTTCAAAAAAAGCATCTA	(Kjelland et al. 2010b)
Hbb probe	5'FAM-CAATGTCTGACTTAGTAACCTTTGGTCTTCTTGA-BHQ1	(Kjelland et al. 2010b)
IGS1 forward primer	5'GTATGTTTAGTGAGGGGGGTG	(Bunikis et al. 2004)
IGS1 reverse primer	5'GGATCATAGCTCAGGTGGTTAG	(Bunikis et al. 2004)
IGS2 forward primer	5'AGGGGGGTGAAGTCGTAACAAG	(Bunikis et al. 2004)
IGS2 reverse primer	5'GTCTGATAAACCTGAGGTCGGA	(Bunikis et al. 2004)

8.4 Physical maps of the genes

Figure 8.4: The 16S rRNA gene and the *rrs* (16S)-*rrlA* (23S) intergenic spacer found on the linear chromosome. The 16S gene is situated 2kb upstream from the 23S-5S duplication. The individual copies of the *rrlA* (23S)-*rrf* (5S) duplication are separated by a 182bp spacer. Edited from Bunikis et al. (Bunikis et al. 2004).

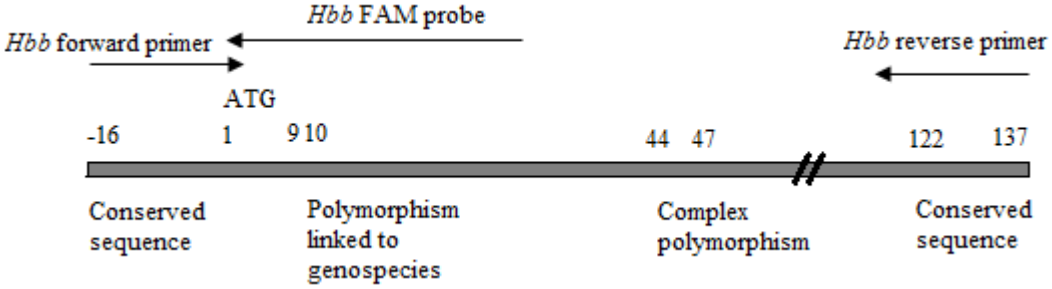

Figure 8.5: The *Hbb* gene located on the linear chromosome. The primers are unmarked, whereas the probe is marked with FAM. The probe is quenched when the probe is unbound, but fluoresce at hybridization to a target sequence. Edited from Portnoï et al. (Portnoï et al. 2006).

8.5 Chemicals and reagents

Table 8.5: Hazards and notifications of the chemicals and reagents used in the master thesis.

Chemical/reagent	Hazards/notifications
AE buffer (Qiagen, Hilden, Germany)	None
Agarose powder (Lonza, Rockland, ME USA)	None
AL buffer (Qiagen, Hilden, Germany)	Harmful if swallowed, irritation to eyes and skin
AW1 wash (Qiagen, Hilden, Germany)	Harmful if swallowed, irritation to eyes and skin
AW2 wash (Qiagen, Hilden, Germany)	None
Big Dye® Terminator v1.1 Ready Reaction mix (Applied Biosystems Inc., Austin, USA)	None
BigDye sequencing buffer (5X) (Applied Biosystems Inc., Austin, USA)	None
Buffer (10X) with EDTA (Applied Biosystems Inc., Austin, USA)	Sequencing buffer; light sensitive and must be kept cold
Chloroform (Sigma-Aldrich Chemie HmbH, Switzerland)	Toxic to inhale, harmful if swallowed and may cause irritation to eyes and skin
Deoxynucleoside triphosphate, dNTP mix (Applied Biosystems Inc., New Jersey, USA)	None
Ethylenediaminetetraacetic acid (EDTA, 0.5M, pH 8.0)	None
Ethanol (EtOH) (Kemetyl Norge AS, Vestby)	Harmful to swallow. Flammable liquid and vapor
Ethidium bromide (Pharmacia, Biotech AB, Uppsala, Sweden)	Toxic, a potent mutagen to humans
ExoSAP-IT® (Affymetrix Inc., Cleveland, US)	Store at -20ºC. Might cause skin and eye irritations
External Well Factor solution (BioRad Laboratories Inc., Oslo, Norway)	Light sensitive
Formamide (Applied Biosystems Inc. Warrington, UK)	Toxic; causes eye, skin and respiratory tract irritation. Possible developmental and birth defect hazards. Avoid breathing vapor.
Hydrochloric acid (HCl, 6M)	Acidic; can cause chemical burns
Ladder (Qiagen, Hilden, Germany)	None
Lysisbuffer (Abbott Molecular Inc., Illinois, USA)	Contains Guanidinium thiocyanate and detergent; harmful in contact with skin, inhale and swallow
Lysisbuffer (Halos et al. 2004)	Harmful in contact with skin, inhale and swallow
Magnesium Chloride ($MgCl_2$) (Applied Biosystems Inc., New Jersey, USA)	None
$Microparticles_{DNA}$ (Abbott Molecular Inc., Illinois, USA)	Contains Guanidinium hydrochloride; harmful to swallow, and in contact with skin.
Nucleic Acid Release Reagent (Gerbion GmbH & Co, Kornwestheim, Germany)	Must be stored at -18ºC and in the dark.
Phosphate buffered saline (PBS, 1X)	May cause eye and skin irritation. Harmful to swallow.
PCR reaction buffer (Applied Biosystems Inc., New Jersey, USA)	None
Phenol-chloroform (Sigma-Aldrich Chemie HmbH, Switzerland)	Air and light sensitive. Toxic to inhale, swallow and corrosive in contact with skin.
Proteinase K (Roche Diagnostics, Roche Applied Science, Indianapolis, USA)	May cause sensitization by inhalation and skin contact.

Chemical/reagent	Hazards/notifications
Sodium acetate (NaAc, 3M, pH 5)	Harmful to inhale and swallow. Irritant if in contact with skin or eyes
Sodium chloride (NaCl, 5M)	None
Sodium hydroxide (NaOH, 1M)	Corrosive acid; can cause chemical burns
Tris-acetate-EDTA, TAE buffer (50X)	Can cause eyes and skin irritation. Harmful if swallowed, inhaled or absorbed through skin
Taq DNA polymerase (Applied Biosystems Inc., New Jersey, USA)	None
Tris(hydroxymethyl)amino methane (Tris-HCl, 1M, pH 8.0)	Prolonged skin contact may cause irritation.
Universal Master Mix (Applied Biosystems Inc., New Jersey, USA)	Hazardous, cause eye and skin irritation, or discomfort if swallowed
Wash 1_{DNA} (Abbott Molecular Inc., Illinois, USA)	Contains Guanidinium thiocyanate and detergent; harmful in contact with skin, inhale and swallow
Wash 2_{DNA} (Abbott Molecular Inc., Illinois, USA)	None

8.6 Solutions

Ethylene diamine tetra acetic acid, EDTA (0,5 M, pH 8.0)

Add 186,1 grams of disodium EDTA to 800ml of MQ-water. Adjust to pH 8.0 with NaOH (Sodium hydroxide) pellets. Aliquot the solution to 1000ml with MQ-water, and autoclave at program 2.

Note: disodium EDTA will not be resolved until the pH is approximately 8.0. NaOH is toxic, and gloves must be worn when handling it.

Loading dye

Blend 2.0ml glycerol (85%), 0.01 grams Bromphenol Blue and 150µl 1XTAE buffer. Add MQ-water until 5 ml. Vortex and transfer to Eppendorf tubes. Store at -20ºC.

Lysis buffer (Halos et al. 2004)

Fill two Erlenmayer flasks (500mL) half full with MQ-water. Add 2.922 grams NaCl, 105ml Tris-HCl (1M), 50ml EDTA (0.5M) and 2.5 grams SDS to each flask. Aliquot to 500mL with MQ-water, and mix gently until it is a homogenous liquid. Autoclave at program 2.

Phosphate buffered saline, PBS (1X)

Dissolve the following salts in 800ml of MQ-water: 8.2 grams NaCl, 0.2 grams KCl, 1.44 grams Na_2HPO_4 and 0.24 grams KH_2PO_4. Adjust to pH 7.4 with 1M NaOH solution. Aliquot the solution to 1000ml with MQ-water, and autoclave at program 2.

Sodium acetate, NaAc (3M, pH 5.0)

Dissolve 102,08 grams of $NaAc \cdot 3H_2O$ into 100ml of MQ-water. Adjust to pH 5.0 with glycial acetic acid. Aliquot the solution to 250ml with MW-water, and autoclave at program 2.

Sodium chloride, NaCl (5M)

Dissolve 292,2 grams of NaCl in 800ml of MQ-water. Aliquot the solution to 1000ml with MQ-water, and autoclave at program 2.

Sodium dodecyl sulfate, SDS

Add 5 grams of SDS to 1000ml MQ-water.

Tris-acetate-EDTA, TAE buffer (50X)

Add 24.2 grams of Tris base to 57.1ml of glacial acetic acid and 100 ml of 0.5M EDTA (pH 8.0). Aliquot to 1000ml with MQ-water, and autoclave at program 2.

Tris(hydroxymethyl)amino methane, Tris-HCl (1M, pH 8.0)

Dissolve 121,1 grams of Tris-base in 800 MQ-water. Adjust to pH 8.0 with 6M HCl solution. Aliquot the solution to 1000ml with MQ-water, and autoclave at program 2.

Note: Throw the solution away if it is yellow.