Longitudinal Study Regarding *Borrelia burgdorferi* sensu lato Populations in Defined Habitats in Latvia

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To my loving partner Stefan and adorable daughter Alice

Table of Contents

List of Abbreviations

µl	Microliter
12s rRNA	12s ribosomal RNA
ACA	Acrodermatitis Chronica Atrophicans
s.l.	sensu lato
s.s.	sensu stricto
COI	Cytochrome C Oxidase Subunit I
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetate acid
ELISA	Enzyme-linked Immunosorbent Assay
EM	Erythema Migrans
EtOH	Ethanol
LB	Lyme Borreliosis
MLST	Multilocus Sequence Typing
NH_4OH	Ammonium Hydroxide
NGS	Next Generation Sequencing
OSP	Outer Surface Protein
PCR	Polymerase Chain Reaction
QPCR	Quantitative Real Time PCR
RNA	Ribonucleic Acid
ST	Sequence Type
TAE	Tris Acetate EDTA Buffer

TBP	Tick Borne Pathogen
tHRF	Tick Histamine Release Factor
tRNA	transfer Ribonucleic Acid
TROSPA	Tick Receptor for Outer Surface Protein A
UV	Ultra Violet

Publications

Ticks Tick Borne Dis. 2019 Aug;10(5):1041-1045. doi: 10.1016/j.ttbdis.2019.05.002. Epub 2019 May 8.

Comparison of methods for economic and efficient tick and *Borrelia* DNA purification.

Okeyo M, Hartberger C, Margos G, Straubinger R. K, Sing A, Fingerle V.

Longitudinal study of prevalence and spatio-temporal distribution of *Borrelia burgdorferi* sensu lato in ticks from three defined habitats in Latvia, 1999-2010.

Okeyo, M., Hepner, S., Rollins, E. R., Hartberger, C., Straubinger, K. R., Marosevic, D., Bannister, A. S., Bormane, A., Donaghy, M., Sing, A., Fingerle, V., Margos, G.

Ticks Tick Borne Dis. 2019 Aug;10(5):1157-1161. doi: 10.1016/j.ttbdis.2019.06.013. Epub 2019 Jun 20.

First investigations on serum resistance and sensitivity of *Borrelia turcica*.

Hepner S, Fingerle V, Heylen D, Marosevic D, Ghaffari K, Okeyo M, Sing A, Margos G.

Infection, Genetics and Evolution. 2020 Jan; 10: 10.1016/j.meegid.2019.104050 Population structure of *Borrelia turcica* from Greece and Turkey

Hepner, S., Fingerle, V., Gerhard, D., Gerit, F., Durdica, M., Rollins, E. R., Okeyo, M., Sing, A., Margos, G.

Chapter 1 **Introduction**

Borrelia burgdorferi sensu lato (*B. burgdorferi* s.l.) is a species complex that currently comprise 22 named or proposed genospecies. In Europe five species are known to be the agents of the human disease - Lyme borreliosis (LB). With approximately 650,000-850,000 assumed new LB cases in Europe annually, LB is the most common human tick-borne disease in Europe (Lit EU). For control measures and eventual prevention of this tick-borne disease, it will be beneficial to study and interpret the *B. burgdorferi* s.l. population dynamics and structure.

The bacteria are maintained in a natural transmission cycle between reservoir hosts and ticks of the genus *Ixodes*. Keeping in mind that the tick vectors` life cycle may be up to more than five years, long term studies are required for a better understanding of such correlations. Hence this study is designed to cover the tick sampling periods between 1999 and 2010 in defined habitats in Latvia. As preliminary study the most economical and efficient method for DNA extraction was determined. Subsequently polymerase chain reaction (PCR) and Multilocus Sequence Typing (MLST) was used to obtain information about population structure, fluctuations and stability regarding *B. burgdorferi* s.l..

The average prevalence over all years was 18.9 %. From initial high infection prevalences of 25.5 %, 33.1 % and 31.8 %, from 2002 onwards the infection rates steadily decreased to 7.3 % in 2010. *Borrelia afzelii* and *B. garinii* were the most commonly found genospecies but striking local differences were obvious. In one habitat, a significant shift from rodent-associated to bird-associated *Borrelia* species was noted whilst in the other habitats, *Borrelia* species composition was relatively stable over time. Sequence types (STs) showed a random spatial and temporal distribution. These results demonstrated that there are temporal regional changes and extrapolations from one habitat to the next are not possible.

Chapter 2 **Causative Organism**

Borreliae (B.) are parasitic bacteria that are maintained in natural transmission cycles consisting of vectors (ticks and lice) and vertebrate reservoir hosts. The bacteria are helically shaped resembling a flat wave with a thin peptidoglycan layer between its double membrane wall (Merilainen et al., 2015). Seven to eleven periplasmic flagella for motility are inserted in the periplasmic space at the end of the cylindrical cell (Barbour and Hayes, 1986; Goldstein et al., 1994; Motaleb et al., 2000). Due to the double membrane, few proteins in the outer membrane and lack of lipopolysaccharides, *Borrelia* is considered diderm instead of gram negative (Samuels and Radolf, 2010). Human pathogenic *Borrelia* species can cause Lyme borreliosis (LB) or relapsing-fever (RF). A distinction between LB and RF species is that all *B. burgdorferi* s.l. that have been investigated so far have a duplication of the rRNA gene array as initially reported for *B. burgdorferi* s.s. by Schwartz et al. (1992). *B. burgdorferi* s.l. is a species complex comprising of both human pathogenic and nonpathogenic species and species of unknown pathogenicity (Margos et al., 2017). In Europe and North America, LB is considered the most frequently reported arthropode-borne disease (Margos et al., 2011; van den Wijngaard et al., 2017). A human vaccine is currently not available.

In the past decades much progress has been made in understanding the diversity and geographic distribution of *Borrelia* species (Kurtenbach et al., 2001; Schwarz et al., 2012). However, many studies investigating the distribution of *Borrelia* species and strains, accumulated data for short periods of time, i.e. perhaps one or two years. Due to the complexity of the ecology of both, pathogen and vector, long term studies are clearly required to understand changing epidemiological pattern relating to the structure and dynamics of species and populations. In the course of this thesis, population dynamic of *B. burgdorferi* s.l. in Latvian ticks was studied for a period of 11 years. This provides an insight into the migration and dissemination of individual species in this region.

2. 1 *Borrelia* Taxonomy

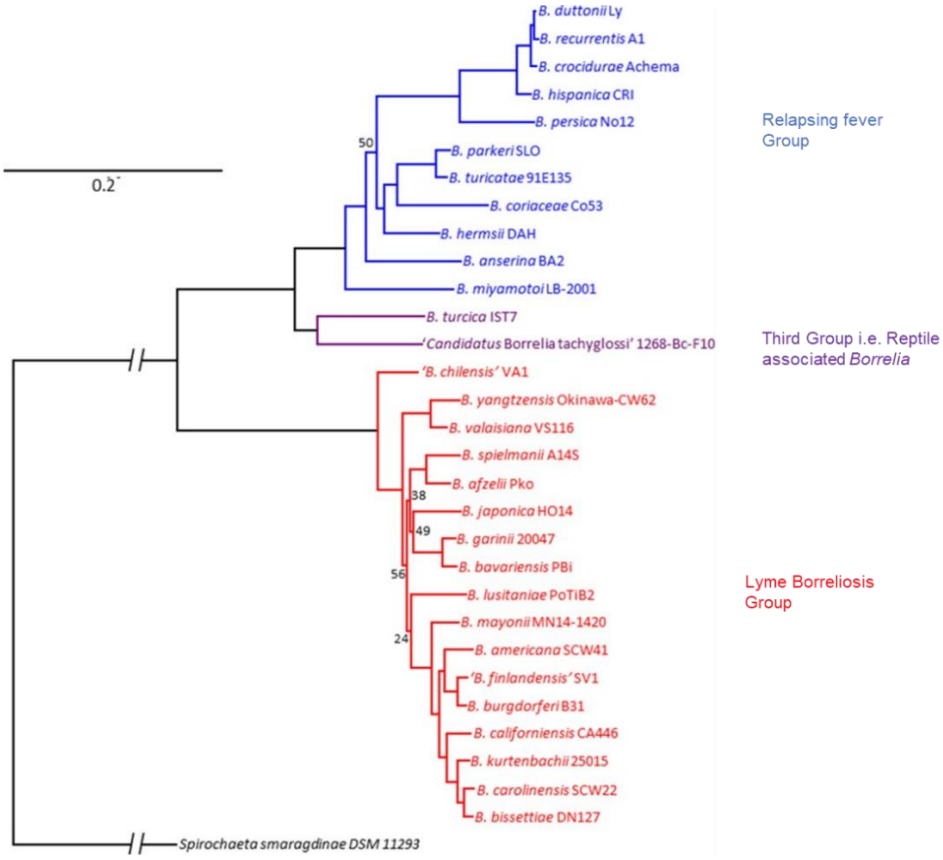


Figure 1: A phylogenetic tree of *Borrelia* species (Margos et al., 2018)

Borrelia spp*. can be divided into three main groups, relapsing fever *Borrelia* (RF), Lyme Borreliosis group (LB) and Reptile associated *Borrelia

The genus *Borrelia* belongs to the phylum Spirochaetes, order Spirochaetales and family Borreliaceae (Gupta et al., 2013). There are 53 known species in the genus *Borrelia.* Twenty two belong to the Lyme borreliosis group and 29 belong to the relapsing fever group. There is a third group, a reptile associated group to which for example *B. turcica*, *Candidatus* B. tachyglossi belongs to (Figure 1: A phylogenetic tree of *Borrelia* species (Margos et al., 2018)) (Takano et al., 2010; Loh et al., 2016; Cutler et al., 2017).

Genetically it was shown that this third group diverges from the Lyme borreliosis group and the relapsing fever group of spirochetes (Takano et al., 2010; Takano et al., 2011; Cutler et al., 2017; Gofton et al., 2018). *B. turcica* was isolated from a tortoise hosted tick - *Hyalomma aegyptium* - in Turkey (Güner et al., 2003) and other so far not characterized *Borrelia spp*. were found in exotic tortoises (Takano et al. 2010). *Candidatus* B. tachyglossi has been detected in Australia; it has been associated with echidna as reservoir hosts and *Bothriocroton concolor* ticks as vector (Loh et al. 2016, Gofton et al. 2018). The species complex *B. burgdorferi* s.l. contains the causative agents of human LB and henceforth will be focused on here.

Borrelia burgdorferi sensu lato is a species complex of currently 22 described *Borrelia* genospecies that form a sister clade to RF species (Barbour, 2014). These microorganisms circulate within tick vectors and are maintained in the environment by reservoir hosts (vector – host circulation) (see chapter Vectors and Hosts). Table 1 gives an account of currently known genospecies that belong to this complex (Table 1: List of the currently known 22 *B. burgdorferi s.*l. genospecies).

The name giving species of the complex, *B. burgdorferi* was first isolated from an *I. scapularis* tick in the early 1980s in the USA by W. Burgdorfer (Burgdorfer et al., 1982). Following his discovery of *B. burgdorferi* in ticks initially, DNA-DNA hybridization and ribosomal sequence typing was used to assign species and strains (Schmid et al., 1984; Postic et al., 1990; Bunikis et al., 2004; Wang et al., 2014). DNA-DNA hybridization, restriction fragment leng polymorphism (RFLP) and southern blotting revealed the diversity of LB group spirochaete *Borrelia* (LeFebvre et al., 1989; Masuzawa et al., 2001). DNA-DNA hybridization has now been replaced by Multilocus Sequence Typing/ Mulitlocus Sequence Analysis (MLST/ MLSA) (Postic et al., 2007; Margos et al., 2011).

Since the advent of MLST, it has become a valuable tool for bacterial epidemiological and taxonomic studies. In case of LB it enabled delineation of several *spp*. An important example is *B. bavariensis*;it revealed that the group of outer surface protein (Osp) A serotype 4 (rodent associated) spirochaetes, initially assigned to the bird associated *B. garinii*, were genetically distinct. This rodent-associated group was hence described as new genospecies and renamed as *B. bavariensis* (Margos et al., 2009; Margos et al., 2013). Other research groups suggested that this human pathogenic genospecies, with an apparent tropism for the central nervous system may possess higher pathogenicity (Wilske et al., 1993; Wilske et al., 1996; van Dam et al., 1997; Marconi et al., 1999). Interestingly, in field studies it was rarely found in questing ticks but contributes as much as other species e.g. *B. afzelii*, *B. garinii* to human infection (Fingerle et al., 2008; Margos et al., 2013).

Table 1: List of the currently known 22 *B. burgdorferi* s.l. genospecies

The geographical distribution, known vectors and reservoir hosts are listed. It also shows the pathogenic potential of individual genospecies to humans.

List of *Borrelia burgdorferi* sensu lato genospecies					Pathogenic potential to human	Reference for species description
	Genospecies	**Geographycal distribution**	**Vector**	**Host**		
1	***B. afzelii***	Europe, Asia	*I. ricinus, I. persulcatus*	Rodents	Yes	Canica et al. (1993)
2	***B. americana***	North America	*I. minor*	Birds	Not known	Rudenko et al. (2009)
3	***B. andersonii***	North America	*I. dentatus*	Cotton tail Rabbit	Not known	Marconi et al. (1995)
4	***B. bavariansis***	Europe, Asia	*I. ricinus, I. persulcatus*	Mice	Yes	Margos et al. (2013)
5	***B. bissettiae***	North America, Europe	*I. spinipalpis, I. pacificus*	Rodents	Potentially	Postic et al. (1998); Margos et al. (2016)
6	***B. burgdorferi* s.s.**	North America, Europe	*I. scapularis, I. ricinus, I. pacificus, I. affinis*	Birds, Rodents, Carnivores	Yes	Baranton et al. (1992)

List of *Borrelia burgdorferi* sensu lato genospecies					Pathogenic potential to human	Reference for species description
	Genospecies	**Geographycal distribution**	**Vector**	**Host**		
7	***B. californiensis***	North America	*Ixodes ticks*	Rodents	Not known	Postic et al. (2007); Margos et al. (2016)
8	***B. carolinensis***	North America	*I. minor*	Rodents	Not known	Rudenko et al. (2009)
9	***B. chilensis***	South America	*I. stilesi*	Rodents	Not known	Ivanova et al. (2014)
10	***B. finlandensis***	Europe	*I. ricinus*		Not known	Casjens et al. (2011)
11	***B. garinii***	Europe, Asia	*I. ricinus, I. persulcatus*	Birds	Yes	Baranton et al. (1992)
12	***B. japonica***	Japan	*I. ovatus*	Rodents	Not known	Kawabata et al. (1993)
13	***B. kurtenbachii***	North America	Unknown	Rodents	Potentially	Margos et al. (2010)
14	***B. lanei***	USA	*I. pacificus, I. spinipalpis*	Rodents, Rabbits	Not known	Margos et al. (2017)
15	***B. lusitaniae***	Europe	*I. ricinus*	Lizards	Potentially	Le Fleche et al. (1997)
16	***B. mayonii***	USA	*I. scapularis*		Yes	Pritt et al. (2016)
17	***B. sinica***	China	*I. ovatus*	Rodents	Not known	Masuzawa et al. (2001)
18	***B. spielmanii***	Europe	*I. ricinus*	Dormouse	Yes	Richter et al. (2006)
19	***B. tanuki***	Japan	*I. tanuki*		Not known	Fukunaga et al. (1996)
20	***B. turdi***	Japan, Europe	*I. turdi, I. frontalis*	Birds	Not known	Fukunaga et al. (1996)
21	***B. valaisiana***	Europe, Asia	*I. ricinus*	Birds	No	Wang et al. (1997)
22	***B. yangtzensis***	China	*I. granulatus*	Rodents	Potentially	Margos et al. (2015)

2. 2 Disease and Epidemiology

Lyme borreliosis (LB) is the most common tick borne disease in temperate regions of the northern hemisphere mainly between 40˚ and 60˚ northern latitude (Lindgren and Jaenson, 2006). LB is endemic in certain parts of the world, (Steere, 2001; Hubalek, 2009) including some regions in North America e.g. the Northeast, the Midwest and California (Lane and Lavoie, 1988; Fritz and Kjemtrup, 2003; Bacon et al., 2008; Hoen et al., 2009; Schwartz et al., 2017), in Central Europe (Lindgren and Jaenson, 2006) and Asia. Each year 300,000 cases and 200,000 cases are estimated to occur in the USA and Germany, respectively (Stevenson et al., 2019). According to the World Health Organization (WHO), the highest incidence of LB is reported in the Central European countries such as Czech Republic, Estonia, Lithuania, Slovenia, Austria, Germany and some Northern countries bordering the Baltic Sea (Rauter and Hartung, 2005; Estrada-Pena et al., 2011). With its increasing incidence, some countries have made it notifiable including some States in Germany (Bavaria, Thuringia, Saxony-Anhalt, Brandenburg amongst others) (Enkelmann et al., 2018), Czech Republic, Slovenia and the USA (Derdakova and Lencakova, 2005; Schwartz et al., 2017). While in USA only *B. burgdorferi* s.s. and *B. mayonii* are associated with human illness, in Europe known human pathogenic species include *B. burgdorferi* s.s., *B. afzelii*, *B. garinii*, *B. bavariensis* and *B. spielmanii*; with *B. afzelii* and *B. garinii* being the most common in questing ticks. Asia on the other hand, the most common species to cause human disease is *B. bavariensis* (Takano et al. 2011) although *B. afzelii*, *B. garinii* and perhaps *B. yangtzensis* (Ni et al., 2014; Margos et al., 2015) also contribute to human cases. Different LB species have been associated with different clinical symptoms (Table 1: List of the currently known 22 *B. burgdorferi* s.l. genospecies).

One of the most important attributes of LB is its difference in severity and the clinical manifestations which may primarily involve skin, nervous system, joints and heart (Steere et al., 1977). Erythema migrans (EM) is the pathognomonic clinical picture. It occurs at the tick bite site as an early symptom in approximately 80 % of cases. From the site of infection, the spirochaetes can

disseminate to the surrounding and eventually disseminate hematogenously to the whole organism (van Dam et al., 1997; Wormser et al., 2005).

There is an organotropic trend with some *Borrelia* species, i.e. *B. burgdorferi* s.s. is associated with arthritic and neurological symptoms, *B. garinii* with neuroboreliosis, *B. afzelii* with Acrodermitis chronica atrophicans (ACA) and *B. bavariensis* with neuroborreliosis (Balmelli and Piffaretti, 1995; Wang et al., 1999; Jungnick et al., 2015; Coipan et al., 2016).

Diagnostics are based first on characteristic clinical symptoms (Stanek et al., 2011; Dessau et al., 2018; Lohr et al., 2018). Except for EM all manifestations need confirmation by antibody detection which normally follow a two-step approach. Enzyme-linked immunosorbent assay (ELISA) is conducted, followed by confirmation in Western blot. Antibodies are detected normally within four to eight weeks after symptoms have been noticed (Stanek and Strle, 2009; Stanek et al., 2011; Fingerle et al., 2017). In unclear cases PCR and cultivation can also be employed (Karan et al., 2018; Lohr et al., 2018), from materials such as cerebrospinal fluid (CSF), synovia or skin biopsy. Culture from biopsies of patients is considered the gold standard; however it requires expertise and therefore should be perfomed only in specialized laboratoris (Fingerle et al., 2017; Lohr et al., 2018).

To every LB patient antibiotics should be administered. Recommended antibiotics include Doxycyclin, Amoxicillin, Cefuroxim, Ceftriaxone, Cefotaxime Penicillin. The type of antibiotic and duration of therapy – 10 to 30 days – depends on clinical signs and severity of the disease (Wormser et al., 2006; Hofmann et al., 2016; Rauer et al., 2018).

2. 3 Vectors and Hosts

Ticks and mites build the subclass Acari in the Class Arachnida. Ticks are further divided into three families: Ixodidae (hard ticks), Argasidae (soft ticks) and lastly Nuttalliellidae (Figure 2: Classification of ticks). To date, around 900 tick species have been described with 702 Ixodidae, 193 Argasidae and one species belonging to the Nuttalliellidae (Guglielmone et al., 2010). At least 42 tick species have been associated with natural infection of *B. burgdorferi* s.l., of which only 12 tick species had been experimentally confirmed to be competent vectors (Eisen and Lane, 2002). The four main vectors of human pathogenic LB genospecies include: *Ixodes* (*I.*) *ricinus* and *I. persulcatus* in Europe, *I. persulcatus* in Asia and *I. scapularis* and *I. pacificus* in North America (Rauter and Hartung, 2005; Geller et al., 2013; Schillberg et al., 2018; Gasmi et al., 2019). Besides these four *Ixodes* ticks, other *vector* competent *Ixodes* species contribute as well to the natural transmission cycle of LB (Margos et al. 2012). For example, even though *I. hexagonus* ticks (often associated with hedgehogs in Europe) are hardly associated with transmission of *Borrelia* to humans, they have been shown to be a competent vector for *Borrelia*, therefore contributing to the perpetuation of LB spirochetes in the environment (Mannelli et al., 2012).

Relapsing fever (RF) spirochetes are often vectored by soft bodied ticks belonging to the *Ornithododoros moubata* complex (Mitani et al., 2004). One species, *B. recurrentis* is transmitted by the human body louse (*Pediculus humanus humanus*). Hard-bodied ticks like *Amblyomma* or *Ixodes* may vector some species that belong to the RF group (e.g. *B. theileri*, *B. anserina*, and *B. miyamotoi*). *B. miyamotoi* which was first isolated from *I. persulcatus* in Japan in 1994 (Fukunaga et al., 1995) is particularly interesting, as it uses the same vector(s) as LB species. It occurs in sympatry with *B. burgdorferi* s.l. and is considered an emerging tick-borne pathogen (Platonov et al., 2011). This hard tick-vectored relapsing fever agent can cause human disease in immunocompetent (Platonov et al. 2011) and immunoincompetent individuals in several countries including Germany, the Netherlands, Japan and the United States (Krause et al., 2015; Boden et al., 2016; Hoornstra et al., 2018).

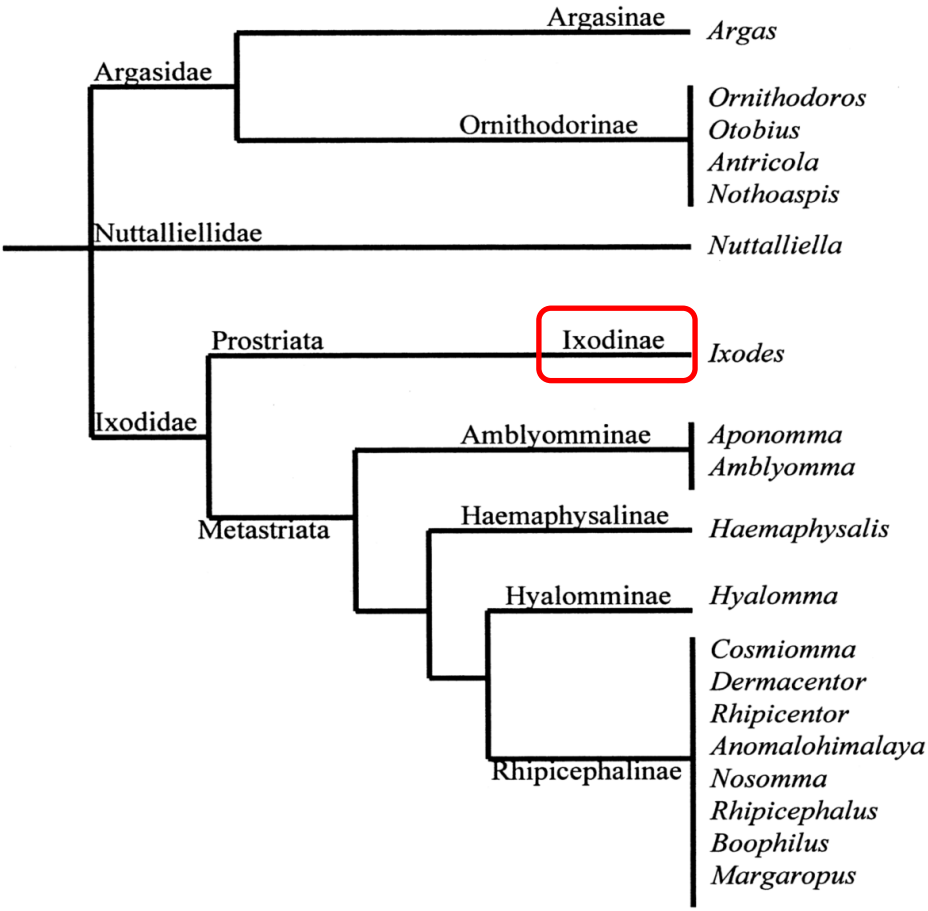


Figure 2: Classification of ticks

Ticks are classified into three main families (Parola and Raoult, 2001; Guglielmone et al., 2010). The hard-bodied ticks, i.e. Ixodidae, with approximately 700 species are the main vectors of the LB causing agents. The soft bodied ticks, i.e. Argasidae transmit mainly the relapsing fever group of spirochetes. The third family Nuttalliellidae with just one species has so far only been found in a few countries in Africa, for example in Tanzania and is clinically irrelevant.

Ticks have four developmental stages including egg, larvae (three pairs of legs), nymphs and adults (four pair of legs) (Figure 3: Tick life cycle). Depending on climatic conditions, periods of up to five years may be required for the completion of the cycle from egg to adult (Randolph et al., 2002). Vertical transmission of *B. burgdorferi* s.l. has been demonstrated for *I. ricinus* (experimentally) (Bellet-Edimo et al., 2005) and *I. persulcatus* (in field collected ticks) (Nefedova et al., 2004), although it may not occur in *I. scapularis* (Hoen et al., 2009). However, transovarial transmission may be fairly uncommon for LB spirochetes, as in the field only about 2 % of larvae are infected with *B. burgdorferi* s.l. (Gern and Humair, 2002; Nefedova et al., 2004; Bellet-Edimo et al., 2005; van Duijvendijk et al., 2016), nevertheless,it has been suggested that it may be of epidemiological consequence (Randolph, 1994). Trans-stadial transmission of *Borrelia* within tick populations is the common way of maintaining *Borrelia* infections in tick populations. Therefore, adults, nymphs and larvae play a major role in the maintenance of the enzoonotic life cycle of these bacteria (Patrican, 1997; Krause et al., 2015).

Infection rates of *Ixodes* ticks with *Borrelia* tend to vary in tick developmental stages, according to season, local ecological and environmental conditions (Rauter and Hartung, 2005; Cook, 2015). The infection rates within Europe varies widely. In adult ticks, the infection rate can be as high as 35 % whilst nymphal ticks have a prevalence of 13 % (Strle et al., 1995) . According to (Hubalek and Halouzka, 1998) the mean average infection rate for *Borrelia* within *I. ricinus* ticks range between 1.9 % for larvae, 10.8 % nymphs and 17.4 % in adults. These results are in agreement with a recent review on tick prevelance in Europe (Strnad et al., 2017). A long-term monitoring (2005, 2010 and 2015) carried out recently in Hannover (Germany) detected slightly higher prevalence of *Borrelia* in the ticks (Blazejak et al., 2018). In general, the prevalence of *B. burgdorferi* s.l. in Germany varies between 14 % and 21 %, similary as in other hotspots/ endemic regions of Europe (Baumgarten et al., 1999; Kampen et al., 2004).

Some ticks carry mixed infections, either they carry different tick-borne pathogens (*Borrelia*, *Anaplasma*, *Rickettsia*, *Babesia*) (Hoen et al., 2009) but also different *Borrelia* mixed infections. Using MLST it was shown that these can be mixed strain infections of a single *Borrelia* species or mixed infections of different genospecies (Vollmer et al., 2011; Vollmer et al., 2013; Mechai et al.,

2015; Mechai et al., 2016). Up to 45 % prevalence of mixed infection has been reported with the most common co-infection being *B. garinii* and *B. valasiana* in the same tick (Rauter and Hartung, 2005; Fingerle et al., 2008; Moutailler et al., 2016). It is worth mentioning that such mixed infections have also been reported in patients (Demaerschalck et al., 1995; Rijpkema et al., 1997).

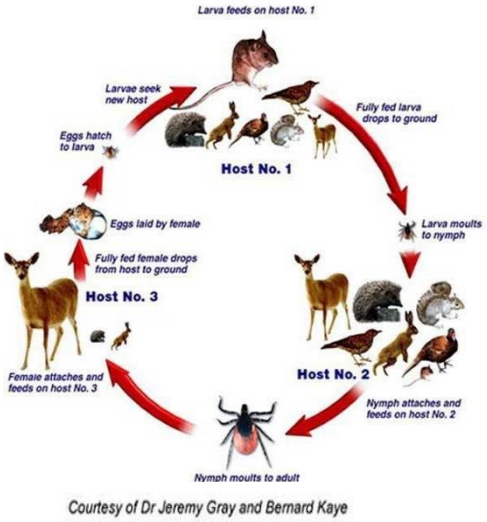
Ticks are obligate ectoparasites and need a blood meal at each developmental stage. Ticks can virtually feed on any vertebrate, up to 300 hosts have been reported (Gern and Humair, 2002; Randolph, 2008). However, the hosts they attack depend on their developmental stage. While larvae and nymphs (immature stages) tend to feed on smaller animals such as ground foraging birds, reptiles and smaller mammals, adult ticks prefer to feed on larger animals, for example deer (Keirans et al., 1996; Gray et al., 2009; Cook, 2015; Kocan et al., 2015).

A synchronised activity of larva and nymph has been reported in Europe (Kubiak and Dziekonska-Rynko, 2006; Cayol et al., 2017). This synchrony in questing and host attachment may allow co-feeding transmission between ticks that are attached to the host in close proximity. The ability of co-feeding transmission first came to the focus when Jones and colleagues determined this mode of transmission in Arbovirusses, it was believed that transmission was only possible when systemic viramie in the host exists (Jones et al., 1987). Co-feeding facilitates or enhances transmission of tick-borne pathogens from one tick to the next without the host becoming systemically infected and without need for a competent host (Gern and Rais, 1996; Cayol et al., 2017). This co-feeding transmission has also been described for other vector borne pathogens (reviewed by (Voordouw, 2015). It does seem however, that co-feeding plays a minor role in the perpetuation of LB in nature (Richter et al., 2002)

At this point, it is worth mentioning that *I. ricinus*, *I. scapularis* and *I. persulcatus*, which are the principle vectors of *B. burgdorferi* s.l. in Europe, America and Asia, respectively, are not host specific but rather generalists. This generalist nature of vectors enables transmission between host species thereby potentially linking different ecological niches (Kurtenbach et al., 2006). Larger

mammals including deer or humans are assumed to be rather dead end hosts for *Borrelia* and not competent reservoir hosts (Mannelli et al., 2012).

In 1.9 % of females there may be transovarial transmission of spirochetes depending on tick and *Borrelia* species (Bellet-Edimo et al., 2005).



Transmission of *Borrelia* from infected larva/ nymph only occurs when ticks feed on competent hosts.

Hosts like deer act as reproductive objects hence are important in the enzoonotic life cycle of ticks.

Figure 3: Tick life cycle

Adult activities begin in spring; the female feeds on a host and lays 1,000 to 10,000 eggs (Hillyard, 1996). Eggs then hatch and the larvae remain inactive till the following spring. The larvae take a blood meal and moult into nymphs which quest for a host, take a blood meal on the host and eventually moult into adults and the cycle begins again. A wide range of vetebrates can serve as hosts to ticks, some of which are reproductive vessels (like deer) and some (like birds) are competent hosts.

Over 100 species are speculated to be reservoir competent hosts for *Borrelia*. Reservoir competence has been defined as the probability of an infected host to infect feeding vector (Schrauber and Ostfeld, 2002). In Europe, competent reservoir hosts include several species of rats, mice, voles, hedgehogs, shrews and several birds including passerines and seabirds (Gern et al., 1998). As reviewed by Piesman and Gern the yellow-necked mouse (*Apodemus flavicollis*), the wood mouse (*Apodemus sylvaticus*) and the bank

vole (*Myodes* (previously *Clethrionomy*) *glareolus*) have been identified in several European countries as potent competent hosts for *B. burgdorferi* (Piesman and Gern, 2004). In Germany, black rats (*Rattus rattus*) and Norway rats (*Rattus norvergicus*) were listed amongst others. For some *Borrelia* species, dormouse seemed to play a significant role; 95 % of larvae feeding on them were infected (Matuschka et al., 1994). Especially for *B. spielmanii*, the garden dormouse (*Eliomys quercinus*) appears to be the preferred host (Richter et al., 2011). Other rodents like grey and red squirrels were also reported in the UK, Switzerland and Germany, respectively, for being important hosts of *Borrelia*. The European hedghog also contributes to the enzoonotic transmission cycle of *Borrelia* in Germany, UK, Ireland and Switzerland (Piesman and Gern, 2004; Pfaffle et al., 2011). In Switzerland and the UK, *Borrelia* DNA has been isolated from badgers (*Meles meles*) and, although it may suggest their reservoir potential, it is no proof that they are indeed reservoir competent (Gern and Sell, 2009; Couper et al., 2010). *Borrelia* has also been identified directly (culture isolation) or indirectly (DNA detection, serologically) in a wide range of incompetent host species like humans, horses (Burgess, 1988; Cohen et al., 1992), small and large ruminants (Burgess et al., 1987; Ben Said et al., 2016) and cats (Krupka and Straubinger, 2010; Lappin et al., 2015). In North America several bird species, especially the American Robin (*Turdus migratorius*) are belived to be highly competent as a reservoir host (Ginsberg et al., 2005). White-footed mouse (*Peromyscus leucopus*) and other rodents also play an important role as host of *Borrelia* on this continent (Ginsberg et al., 2005; Hanincova et al., 2006; Vuong et al., 2014).

2. 4 Population Structure of *B. burgdorferi* s.l.

The population structure of *B. burgdorferi* s.l. is influenced by different extrinsic and intrinsic factors as depicted in figure 4 (Figure 4: Intrinsic and extrinsic factors shaping *Borrelia* population structure). Environmental factors such as temperature and rainfall are examples of extrinsic factors that affect the survival and geographical spread of the population of ticks, hence affecting also the *B. burgdorferi* s.l. population structure. For instance, high temperatures and low humidity can result in drying up of eggs and/ or larval stage. These climate parameters play direct roles in the seasonality/ yearly increase/ decrease of tick population in some parts of Europe,which may impact LB disease incidence (Estrada-Peña and Venzal, 2006).

The ecological niches that spirochaetes occupy are highly dependent on vector and host availability. The availability of the vectors and hosts is geographically demarcated and profoundly influenced by biotic and abiotic factors like temperature, humidity, plants, predators,climate and ecological conditions. Several genospecies of *B. burgdorferi* s.l. have been studied regarding their population structure in more detail. Amongst them are *B. burgdorferi* s.s., *B. garinii* and *B. afzelii* and findings about their population structure are outlined below.

Bird-associated LB species such as *B. garinii* and *B. valaisiana* show less population structure compared to rodent-associated LB species. Vollmer and colleagues reported spatial mixing of sequence types (STs) of *B. garinii* and *B. valaisiana* suggesting widespread dispersal of these species, hence reflecting the migratory behaviour of their avian hosts. In contrast, significant population differentiation was observed for *B. afzelii*; likewise reflecting the restricted movement of their rodent hosts (Vollmer et al., 2011; Vollmer et al., 2013).

As reviewed by (Margos et al., 2011) the population structure of *B. burgdorferi* s.l. in Europe is tightly correlated with their host association. Some host species are competent hosts only for specific LB species; i.e. even though the avian associated *B. garinii* can infect mice, a transmission of the

B. garinii from the infected mice to a competent feeding tick is not possible (Kurtenbach et al., 1998). *B. garinii* circulates within terrestrial and seabird transmission cycles (Olsen et al., 1995; Gylfe et al., 1999). It also appears to be one of the most heterogeneous species within the LB spirochete group. Whether this is because of the migratory behaviours of the hosts, which may provide the suitable prerequisite for genetic exchange and therefore variation of the *B. garinii*, is an open question. Furthermore, a *B. garinii* variant was found to be circulating within *I. ricinus* and *I. uriae*, a highly seabird-specific tick (Olsen et al., 1995; McCoy et al., 2001). This reinforces the role of birds as reservoirs and dissemination factor and that the two transmission cycles (terrestrial/seabird) overlap in Europe and allow exchange of *Borrelia* between them (Comstedt et al., 2006; Comstedt et al., 2009).

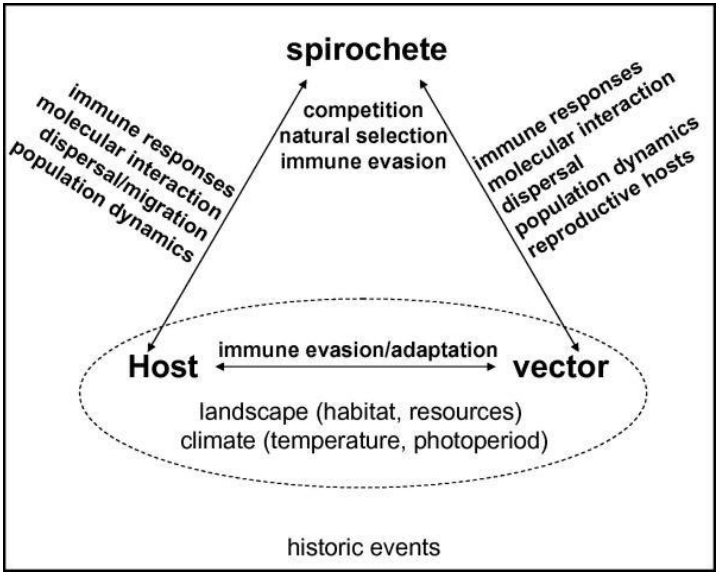


Figure 4: Intrinsic and extrinsic factors shaping *Borrelia* population structure

The figure demonstrates that spirochetes survival depends on the availability of hosts and vectors. It is obvious that many factors contributes to not just the distribution of *Borrelia* species worldwide but also to the current diversity within the species.(Margos et al., 2011).

Since ticks are only capable of moving short distances (Falco and Fish, 1991), their dispersal is strictly influenced by host movement; hence host migration significantly shapes the population structure of *Borrelia* (Kurtenbach et al., 2002; Mechai et al., 2018) (Figure 4: Intrinsic and extrinsic factors shaping *Borrelia* population structure).

Borrelia burgdorferi sensu stricto on the other hand is a special case as it is a generalist utilizing avian, rodent and insectivore species as reservoirs (Brisson and Dykhuizen, 2004; Hanincova et al., 2006) and its population structure remains todate unclear (Hoen et al., 2009; Ogden et al., 2011; Margos et al., 2012). Human LB cases in the USA have expanded in the recent past following reexpansion of the vector tick *I. scapularis*. This posed the question whether *B. burgdorferi* s.s. from the Northeast of the United States had expanded and caused a LB focus in the Midwestern States or whether the expansion of LB in the two regions, the Northeast and the Midwest, were independent events. Sequence mismatch distribution reflected the population expansion, which occurred at some point in the evolution thousands/ millions of years ago. Because no identical ST were collected in both regions and negative spatial dependence of allele frequencies was observed, it was hypothesized that the recent populations expansion of the two LB foci of *B. burgdorferi* s.s. in the USA must have occurred independently. Phylogenetic analyses suggested that the two foci of *B. burgdorferi* population; the Northeast and Midwest, must have belonged to an admixed population in the distant past (Hoen et al., 2009; Mechai et al., 2015). Thus, despite this geographical demarcation of certain STs in the hotspots of LB emergence in North America, it is believed that they have a shared ancestral background (Margos et al., 2012).

A study investigating *B. burgdorferi* s.s. STs from Canada hypothesized that the ancestry might not be defined geographically, but rather ecologically. Only one fifth of the analyzed STs were found in both Canada and USA (where the source population is believed to be) (Mechai et al., 2015), whilst the other four out of five STs were only found in Canada. It is estimated that 50-175 million *I. scpularis* are being dispersed in Canada annually by migrating birds providing opportunity for *B. burgdorferi* s.l. to be introduced (Ogden et al., 2008). Of course, it requires established *I. scapularis* populations for *B. burgdorferi* s.s. to be maintained in natural transmission cycles. Thus, the forces and dynamics

that shape *B. burgdorferi* s.s. populations in North America (and as a matter of fact in Europe) are still not clear (Walter et al., 2017).

Borrelia burgdorferi sensu stricto is one of the few species that occur on both sites of the Atlantic (Piesman and Gern, 2004). The origin and population dynamics of this genospecies is debated to present. While some studies hypothesize that this species must have been introduced from North America to Europe (Barbour and Fish, 1993; Ras et al., 1997), others hypothesized that it must have been introduced from Europe (Margos et al., 2008; Qiu et al., 2008). Nevertheless, data obtained by Hoen et al. 2009 suggested that *B. burgdorferi* s.s. has been in North America for thousands or millions of years. *B. burgdorferi* s.s. isolates from Europe formed the most diverged clade in the MLST tree generated by Margos and colleagues; henceforth they hypothesized the ancestry of this group originates from Europe (Margos et al., 2008).

Thus, there is still a lot to understand about population structure and dynamics in this highly interesting zoonotic system.

2. 5 Genome Organization and Protein Expression

The first whole genome of *B. burgdorferi* s.s. was sequenced in the late 1990s (Fraser et al., 1997). These data showed that, the *B. burgdorferi* genome is highly segmented and unusual for bacteria. Since then, more whole genome sequences have been analyzed, with the aim to further understand the genome structure, pathogenesis and the genetic basis for the ecological niche restrictions of different members of this complex (Schutzer et al., 2011; Becker et al., 2016). *B. burgdorferi* s.s. genospecies have a comparatively small linear chromosome (about 1 megabase) and diverse numbers of linear and circular plasmids (www.BorrliaBase.org). Strain B31 of *B. burgdorferi* s.s. possesses at least 21 plasmids that vary in size between 5 and 60 kb (Figure 5: Genome of *B. burgdorferi* s.s. strain B31) (Baril et al., 1989; Casjens and Huang, 1993; Casjens, 2000; Casjens et al., 2012; Casjens et al., 2017; Margos et al., 2017).

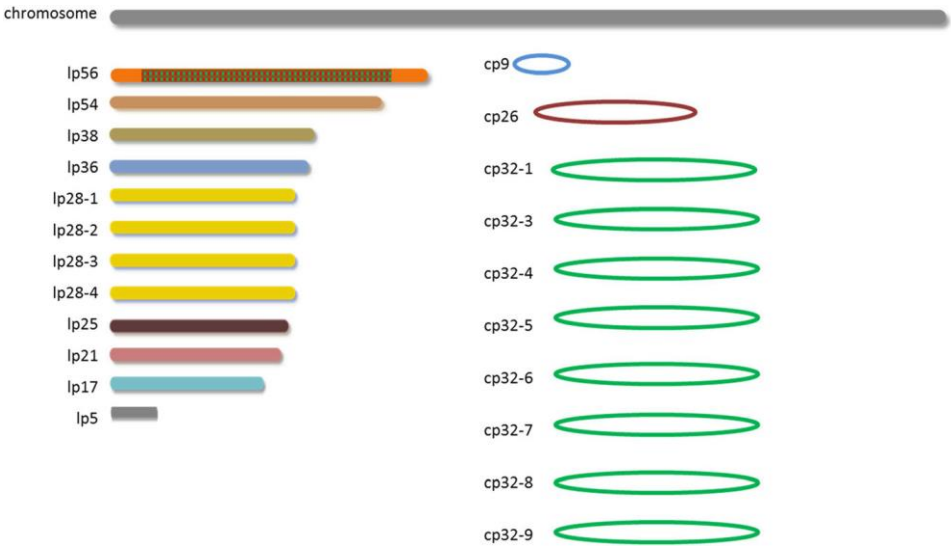


Figure 5: Genome of *B. burgdorferi* s.s. strain B31

There are 12 linear and 10 circular plasmids which are apparently located within this genome. Plasmids with similarity in their sequence have been displayed by the same colour (Margos et al., 2017).

Borrelia burgdorferi s.s. plasmids are particularly well investigated, while there is less information on other genospecies. It was shown that plasmids in different strains not only differ in content but also in structure within *B. burgdorferi* s.s. species (Casjens et al., 2012).

Borrelia burgdorferi s. l. has many lipoproteins as outer surface proteins, many of which are encoded by genes located on the plasmid. These proteins are important for interaction with host and vector. Amongst important Osp's are OspA (encoding gene located on linear plasmid lp54), OspB (encoding gene located on lp54) and OspC (encoding gene located on circular plasmid cp26). OspC and OspA are up/ down regulated depending on whether the bacteria are in the host or in the tick and whether the tick is feeding or not (Saint et al., 1994; Fingerle et al., 1995; Fingerle et al., 1998; Fingerle et al., 2000; Kumaran et al., 2001; Kenedy et al., 2012).

Outer surface protein A and OspB are being expressed during transmission from an infected animal host to the feeding tick (Woodman et al., 2008). In unfed ticks, *Borrelia* expresses the OspA gene. OspA and OspB play a major role in the persistence and maintenance of this pathogen in the environment (Yang et al., 2004). Neelakanta and colleagues found out that mutants lacking OspB were unable to adhere to the tick mid gut (Kenedy et al., 2012). It was ascertained that although OspA is not prerequisite for the persistence of the bacterium within the vector, it however shields the bacterium from the host immune attack during a blood meal (Neelakanta et al., 2007; Battisti et al., 2008). It was also shown that by blocking of TROSPA, a tick receptor to which OspA binds, reduced adherence of *B. burgdorferi* s.s. was noticed, resulting in inefficient colonization and pathogen transmission (Pal et al., 2004).

During a blood meal, the rising temperatures due to the feeding of the tick, induces expression of OspC in the spirochete (Schwan et al., 1995; Schwan and Piesman, 2000). Since the host immune system reacts against borrelial lipoproteins by producing antibodies, the bacterium downregulates some lipoproteins such as OspC, which is important during invasion of salivary glands and early infection of the host but not necessary for maintaining the infection (Radolf et al., 2012). Thus, OspC has been described as virulence factor needed for invasion of the salivary glands of the tick and/ or vertebrate infection (Fingerle et al., 2002; Grimm et al., 2004).

Another important attribute of OspC gene is its variability which also occurs within a single population (Wilske et al., 1993; Wang et al., 1999). Machanisms underlying this variability in OspC include immunological selection stimulated by the host, gene transfer, intergenomic recombination, environmental constrains and other factors (Lin et al., 2002). There is 10-20 % variation between OspC allele, which is comparatively high to other genes (1 %) (Dykhuizen and Baranton, 2001). This as analyzed by Dykhuizen can only be due to recombination and not rapid evolution, the recombination seem to occur by introducing small fragments of DNA into the central variable region of OspC gene (Gibbs et al., 1996; Dykhuizen and Baranton, 2001; Barbour and Travinsky, 2010). This attribute has been used to classify OspC into at least 22 major groups, further classification differentiates human pathogenic group, local infection causing group and the systemic infection causing group (Seinost et al., 1999; Brisson and Dykhuizen, 2004). Lateral gene transfer between species

has also been described (Wang et al., 1999), which is believed to increase heterogeneity within European *B. burgdorferi* s.l. strains (Ras et al., 1997). Jauris-Heipke and colleagues suggest that the recent intragenic recombination in conjunction with mechanisms to escape the host immune system may be the reason for the great heterogeneity within OspC (Jauris-Heipke et al., 1995).

2. 6 Molecular Typing of *B. burgdorferi* s.l. Species

As unambiguous typing systems are essential in understanding ecology, population structure and taxonomic position of microorganisms, current methods are set to incorporate a polyphasic approach using genotyping, phenotyping and phylogenetic properties in characterizing prokaryotic species (Vandamme et al., 1996; Stackebrandt et al., 2002; Gevers et al., 2005).

Genotyping methods have been used for decades in the species delineation of bacteria or as a measure of intraspecies relatedness. DNA-DNA hybridization has been the standard species delineation method since 1960s (Schildkraut et al., 1961; Rosselló-Mora, 2006). In 1987, a common criterion was summarized in order for organisms to be assigned to the same species by DNA-DNA hybridization. A cutoff point of ≥ 70 % and with ΔTm of 5°C or less was set for species delineation (Wayne, 1988). This technique has been used to delineate several *B. burgdorferi* s.l. species such as *B. burgdorferi* s.s. B31, *B. garinii*, *B. afzelii* (VS461) and *B. japonica* (Johnson et al., 1984; Baranton et al., 1992; Kawabata et al., 1993). However, species delineation by DNA-DNA hybridization has pitfalls; this technique is prone to fundamental errors. Expert laboratories are required and most variations are often not reproducible. Besides, evolutionary processes important for intraspecies relatedness, such as deletions, insertions or point mutations cannot be demonstrated by DNA-DNA hybridization (Stackebrandt, 2003). MLST is a widely used genotyping method in population and evolutionary studies of bacteria (Enright and Spratt, 1998; Urwin and Maiden, 2003) that has also been developed for *Borrelia*. The same method can be applied for both intra-species and inter-species studies, but it is then distinguished by name as MLST and MLSA, respectively.

2. 7 Multilocus Sequence Typing (MLST)

MLST was introduced as a general approach providing accurate, portable and reproducible data to characterize isolates of bacteria and other organisms via the internet for epidemiological purposes (Urwin and Maiden, 2003). MLST, as the name suggests, is a typing method utilizing multiple loci in the genome for the comparison of their sequence identity in order to investigate the genetic diversity among different bacterial isolates. Since the advent of MLST in 1998, it has become the “gold standard” for epidemiological studies involving bacterial microorganisms (Larsen et al., 2012).

MLST targets multiple (often seven) housekeeping genes that are located and scattered across the chromosome (not on plasmids), which are amplified by PCR and sequenced. Each unique sequence is assigned a unique allelic number, the combination of allelic numbers for one isolate are eventually grouped to build a sequence type(ST). MLST relies on distinguishing genetic variation amongst isolates. In comparison to its precursor, MLEE (multilocus enzyme electrophoresis) which compared different electrophoretic mobilities of multiple core metabolic enzymes, MLST is more sensitive as it also identifies synonymous differences. In addition, MLEE is laborious and results from different laboratories are not easily comparable (Maiden et al., 1998).

Target genes for MLST should be well conserved parts of the genomes, single copy genes, nearly neutrally evolving, not prone to recombination and diverse enough to identify variations within the target population (Maiden, 2006). Internal fragments of 400 to 600 base pairs (bp) of six to seven genes are normally used (Maiden, 2006). A nested PCR strategy is recommended, because such set up generally produces higher quality nucleotide sequence data and the possibility of sequencing spurious amplification products is eliminated. Consequently, less stringent PCR reaction conditions can be used at the amplification stage; this is an advantage for highly diverse bacteria where polymorphisms can occur in the gene sequences that are targeted by the primers. In addition, nesting provides a higher sensitivity that permits the determination of STs in clinical specimens from which bacteria cannot be cultivated (Urwin and Maiden, 2003).

A set of eight chromosomal genes i.e., *clpA*, *clpX*, *nifS*, *pepX*, *pyrG*, *recG*, *rplB*, and *uvrA* were developed for MLST for *B. burgdorferi* s.l. (Margos et al., 2008). Multilocus sequence analysis (MLSA) is based on the same eight chromosomal housekeeping genes that are used also for MLST, and follows the same principles. The only difference is that MLST is used intra-specific while MLSA is used at the inter-species level for taxonomic purposes. It has been shown to be a powerful tool able to distinguish *Borrelia spp*. and has of recent replaced DNA-DNA hybridization for the delineation of *Borrelia spp*. (Gevers et al., 2005; Margos et al., 2011).

2. 8 DNA Sequencing

DNA sequencing is the technique of determining the exact sequential arrangement of single nucleotides; adenine, guanine, cytosine and thymine within a DNA molecule. DNA sequencing is widely used in research, medical diagnosis and forensics amongst others.

Sanger sequencing was the first developed sequencing technique in the 1970 and was used to produce the first ever whole human genome in the year 2001 (Venter et al., 2001; Postic et al., 2007). Since mid 1990s, high throughput and scalable sequencing methods have been developed allowing sequencing of a whole genome at once and at a much cheaper price in comparison to the previously used Sanger method. These methods are today known as Next Generation Sequencing (NGS) (Ari and Arıkan, 2016).

2. 8. 1 Sanger Sequencing Method (Sanger et al., 1977)

In Sanger sequencing all four deoxnucleaotides which the polymerase uses to extend the free hydroxide end of the DNA and dideoxy nucleotides also 2',3' dideoxynucleotides (ddNTPs; ddGTP, ddATP, ddTTP and ddCTP) are used. These ddNTPs are also known as terminator nucleotides as they terminate a sequence prolongation whenever added.

2. 8. 2 Next Generation Sequencing (NGS)

The Illumina sequencing is based on sequencing by synthesis. This means that during controlled cycle of DNA synthesis, a modified DNA polymerase incorporates fluorescently marked dNTPs into a DNA template. These dNTPs are at the same time reversible terminators which allows addition of just a single base at a time. When this terminator dNTP is disjoined, the next dNTPs can be attached and at the same time the fluorophore at the disjoined dNTPs is excited by a laser beam. Each time an image is taken of the fluorescently labeled nucleotides.

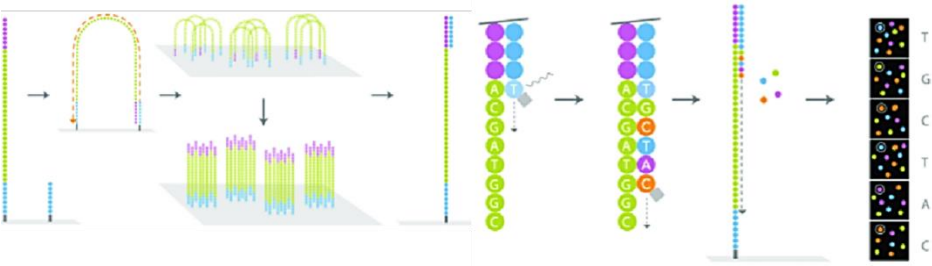


Figure 6: Cluster generation (left) and sequencing by revers terminator nucleotides

Single strand DNA with adapter is attached on the flow cell, by folding this single strand both ends attaches on the flow cell, a bridge like structure is formed, this is synthesized (sequencing by sythesis) to double strand DNA. The double strand is linearized and one strand is washed away, then follows clonal amplification of the single strands on the flow cell, thereby a cluster generation of identical single strands. The nucleotides to be used are fluorescently marked and they compete to be incorporated to the growing chain. After the incorporation, the clusters are excited by a light source and a fluorescent signal is emmited. Because the nucleotides are marked fluorescently, the incorporated ones can be detected. Through repetitions of nucleotide incorporation and detection the DNA sequence can be reconstructed (Westbury, 2018).

There are four basic steps involved: Sample preparation, cluster generation, sequencing and data analysis. During sample preparation, the DNA is tagmented and adapters are added to the single strand DNA that is used as a template. This process generates fragmented DNA, to which both ends are ligated with adaptors, what is called a library. In addition, through reduced cycle PCR, primer binding sites, indices and regions complementary to flow cell oligos are also added (Figure 6: Cluster generation (left) and sequencing by revers terminator nucleotides). Due to index, multiplexing is possible where several samples can be sequenced in one run.

After the libraries preparation, they run on a Bioanalyzer-Agilent Tapestation (Agilent Technology) or equivalent to determine their quality. The pooled libraries are added to a flow cell where clusters are generated. During clustering, the DNA strands are isothermally and clonally amplified through bridge amplification.

Chapter 3 **Aims and Objectives**

Since the population structure of *Borrelia spp*. is influenced by many factors including vectors, whose complete life cycle can take between two and six years depending on the availability of host, climate and other environmental factors, studying the population structure of *Borrelia* over short periods (e.g. one or two years) results only in a population snapshot. Thus, long term studies are required to get a better understanding of the population structure and it's dynamics. This may be of importance to other disciplinaries such as ecologists or epidemiologists when it comes down to models that can be used in the future to control such tick borne-pathogens.

MLST as an accurate and unambiguous typing method in the study of populations was employed to analyse the species distribution and its changes as well as the population structure of *Borrelia spp*. over a period of eleven years. In order to get more insight in the population fluctuation or stability of *Borrelia* over a longer period of time, the tick collection took place between 1999 and 2010 in three different habitats in Latvia: Babite, Jaunciems and Kemeri. Although OspC has been used as a population marker in several studies, there is an apparent recurrent balancing selection within the OspC gene leading to the genetic variation in *Borrelia*. MLST genes on the other hand are more stable and slowly evolving.

Since several thousand ticks were being processed in this study, initially tests were carried out on methods of DNA extraction (i) commercialy available DNA extraction kit and (ii) Ammonium-hydroxide (NH_4OH) DNA extraction to compare the DNA yield of both methods with ethanol preservation and without ethanol preservation.

The main goals of this study were to find out if (i) there is species variation of *Borrelia* within this tick population in the different locations in Latvia over the years, (ii) there are changes in the infection prevalence of ticks with *Borrelia*, (iii) intraspecies changes occur within this population i.e. do different sequence types dominate at different times among the same *Borrelia* sp., and (iv) lastly,

we wanted to compare if MLST-typing and OspC-type lineages would give consistent results and allow the same conclusion to be drawn.

Chapter 4 Materials and Methods

4. 1 Tick Samples

Table 2: Summary of total number of tick samples

Three regions were sampled in Latvia: Babite, Jaunciems and Kemeri.

Latvia							
Sampling year		**1999**	**2000**	**2001**	**2003**	**2010**	**Total Sum**
Total no. of samples per year		271	236	1133	883	642	3165
Stage							
	Adult	271	200	678	410	372	1931
	Nymph	0	36	418	473	270	1197
	Larvae	0	0	37	0	0	37
Region							
	Babite	180	85	640	438	352	1695
	Jaunciems	45	88	211	206	118	668
	Kemeri	46	63	282	239	172	802
Season							
	Spring	41	236	734	461	335	1807
	Summer	45	0	282	213	0	540
	Autumn	185	0	117	209	307	818
Region	**Stage**						
Babite	Adult	180	85	297	205	179	946
Babite	Nymph	0	0	306	233	173	712
Babite	Larvae	0	0	37	0	0	37
Jaunciems	Adult	45	88	169	102	61	465
Jaunciems	Nymph	0	0	42	104	57	203
Kemeri	Adult	46	27	212	103	132	520
Kemeri	Nymph	0	36	70	136	40	282
Sum		**271**	**236**	**1133**	**883**	**642**	**3165**

The highest numbers of ticks were acquired from Riga District Babite, (1,695 ticks), followed by Jurmala/Kemeri (802 ticks) and the least numbers of ticks (668) were collected from Riga Jaunciems (Table 2: Summary of total number of tick samples). Collected ticks were identified as *I. ricinus* and were stored in 70 % ethanol (EtOH) for preservation purposes.

Seventy-nine ticks from Oberschleissheim were collected in the year 2017. These ticks were also identified morphologically to be *I. ricinus*.

Additional to the sampling years mentioned above, also samples from the years 2002, 2006 and 2007 were analyzed in the course of this, however they were already processed in the previous studies so that only MLST sequences were available (Table 3: Summary of Latvian tick samples from which only MLST data was available for this study).

Table 3: Summary of Latvian tick samples from which only MLST data was available for this study

Sampling year	**2002**	**2006**	**2007**	**Total Sum**
Total no. of samples per year	368	492	459	1319
Region				
Babite	149	259	220	628
Jaunciems	127	106	100	333
Kemeri	92	127	139	358
Sum	**368**	**492**	**459**	**1319**

4. 2 DNA Extraction Methods

Whole ticks, which were initially placed in 70 % EtOH for preservation, were washed shortly using approximately 1 ml aqua destillata (Aqua dest). prior to DNA extraction which was done using two different methods:

- Alkaline hydrolysis using 1.25 % Ammonium Hydroxide Solution (NH_4OH)
- Commercially available DNA extraction kits

4. 2. 1 Handling of Ticks from Oberschleißheim

As part of the pre-study optimization in order to compare and measure DNA yield from different methods and commercially available kits, and storing conditions (with or without ethanol) 79 ticks from Oberschleissheim were analyzed (Okeyo et al., 2019). Twenty-eight of which were female, 13 were male and 38 nymphs. Different buffers and tick homogenization methods were also tested on some ticks from this group. In addition, cultures with defined cell (106, 105 and 104 cells) concentrations obtained from a cultivated *B. burgdorferi* s.s. B31 isolate were used for a quantitative comparison of DNA extraction via real-time PCR. This experiment was carried out to test the influence of the different extraction methods on the sensitivity of a real-time PCR targeting the *Borrelia* Flagellin B (FlaB) encoding gene. For all the methods, the same conditions and volumes were set.

From 34 ticks, total DNA was extracted using a commercial DNA extraction kit. 35 ticks were treated with 1.25 % NH_4OH. Six ticks from each batch were placed in 70 % EtOH for one week prior to DNA extraction to see the effect of EtOH preservation on total DNA yield. DNA yield was estimated using conventional PCR targeting the *Ixodes* Cytochrome c oxidase (*coi*) gene and quantitative real-time PCR (QPCR) targeting the FlaB encoding gene of *Borrelia*.

On the remaining ten ticks, different buffers and manual vs mechanical homogenization was tested as follows:

1. three ticks were homogenized in the SpeedMill (Analytikajena, Jena, Germany) + Lysis Buffer Qiagen (One for all vet kit Qiagen)
2. Three ticks were likewise homogenized by using the SpeedMill + Lysis buffer Analytikjena (BlackPrepTick DNA kit, Analytikjena, Germany)
3. Two ticks were manually crushed and processed with lysis Buffer by BlackPrepTick DNA kit
4. Two ticks were again homogenized in the SpeedMill and processed with Lysis Buffer Promega (Maxwell 16 Tissue DNA purification kit, Mannheim, Germany)

4. 2. 2 DNA Extraction by Ammonium Hydroxide Solution (NH_4OH)

DNA was extracted using 1.25 % aqueous ammonia (NH_4OH; Sigma Aldrich, Germany,) following the method described by Guy and Stanek in 1991 (Guy and Stanek, 1991). 27 % NH_4OH was diluted to 1.25 % by adding 3.75 ml NH_4OH to 7.25 ml aqua dest. A total volume of 11 ml was needed for approximately 100 samples. 1.25 % NH_4OH solution was freshly prepared each time and the rest discarded to minimize evaporation of ammonia, which may cause a change in concentration. Single washed ticks were transferred into a 1.5-ml safe lock Eppendorf tube and manually crushed using an individual sterile spatula one for each tick. For adults 200 µl of 1.25 % NH_4OH was added and for nymphs and larvae 120 µl. Closed tubes were put in heat block at 100°C for 20 minutes, then the tubes were opened for two minutes to alleviate the inner pressure and eventually centrifuged shortly. The tubes were then placed back on the heat block at 100°C with open lid until approximately 50 % of the volume evaporated (between 15-40 min). They were again shortly centrifuged and stored at 4°C until further use. Each time DNA was to be purified, 8 % of the total number of tubes were included but without ticks as negative controls. This procedure was done under a fume cupboard.

Used spatulas were placed into 50 % Microbac (Paul Hartmann, Heidenheim Germany) solution for 60 minutes, transferred into 12 % sodium hypochlorite solution (Roth, Karlsruhe Germany) for one hour, and finally autoclaved.

4. 2. 3 DNA Extraction Using Commercially Available Kits

DNA extraction for tick samples from Latvia year 1999 and 34 ticks from Oberschleissheim was perforemed using commercially available kits according to the manufacturers' instructions. The Following kits were used: One for all vet kit (Qiagen, Hilden Germany), BlackPrepTick DNA kit (Analytikjena, Jena, Germany), High Pure PCR Template Preparation Kit (Roche, Mannheim

Germany) and lastly Maxwell 16 Tissue DNA purification kit (Promega, Mannheim, Germany).

4. 3 Polymerase Chain Reaction (PCR)

Table 4: Pipetting scheme of PCRs carried out in this study

		coi gene PCR	*flaB* QPCR	Housekeeping genes		OspC	
				1st round	2nd round	1st round	2nd round
Component	**Working concentration**	**Volume [µl]**					
Reaction Mix	2 x conc.	10	12,5	10	15	10	15
Primer1 F	10 pmol/µl	2	0,75	2	3	2	3
Primer1 R	10 pmol/µl	2	2,25	2	3	2	3
Probe 1	10 pmol/µl	N/A	0,5	N/A	N/A	N/A	N/A
Primer2 F	10 pmol/µl	N/A	0,75	N/A	N/A	N/A	N/A
Primer2 R	10 pmol/µl	N/A	2,25	N/A	N/A	N/A	N/A
Probe 2	10 pmol/µl	N/A	0,5	N/A	N/A	N/A	N/A
Aq. Dest	N/A	3,5	0,5	3,75	6	3,75	6
$MgCl_2$	25 mM	0,5	N/A	0,25	N/A	0,25	N/A
Template	N/A	2	5	2	3	2	3
Total	**N/A**	**20**	**25**	**20**	**30**	**20**	**30**

Four different PCRs were employed in this study:

1. *coi* PCR to amplify the tick cytochrome oxidase subunit I,
2. A semi nested PCR to amplify the Outer Surface Protein C gene (OspC),

3. A duplex real time PCR for an initial screening of tick DNA for *Borrelia* before nested MLST PCR and lastly
4. MLST nested PCR for the housekeeping genes namely *clpA*, *clpX*, *nifS*, *pepX*, *pyrG*, *recG*, *rpLB* and *uvrA*.

All PCR reactions were carried out on either Mx3005P cycler (Agilent Technologies) or on Mx3000P cycler (Agilent Technologies) using NoROX Master Mix (Qiagen) PCR amplification Kit. All real-time PCR reactions were carried out on a Mastercycler® nexus gradient (Eppendorf) machine using appropriate PCR amplification Kit (see the respective PCRs and Appendix 1: List of primers used in this study). Table 4 shows the pipetting scheme for the respective PCRs carried out (Table 4: Pipetting scheme of PCRs carried out in this study).

4. 3. 1 Tick Cytochrome C Oxidase Subunit I PCR

A few samples were subjected to this PCR to amplify the tick *coi* as previously described (Dinnis et al., 2014) (Appendix 1: List of primers used in this study). MyTag Mix-Bioline was used containing 2x PCR buffer, dNTPs, MgCl2 and Taq polymerase (Bioline, Germany). The PCR reaction mix were pipetted as shown in table 4 (Table 4: Pipetting scheme of PCRs carried out in this study). Amplification was carried out in Qiagen cycler with the following conditions heating at 94°C for two minutes, followed by 35 cycles: at 94°C for 15 seconds, extension at 55°C for 15 seconds, elongation for one minute at 72°C. After 35 cycles it was held at 10°C for 10 minutes. This product was then visualized on a 1.5 % agarose gel containing 0.08 % gel red.

4. 3. 2 Real-time PCR to Screen for the Presence of *Borrelia*

After NH_4OH treatment/ DNA extraction, the samples were subjected to a duplex real-time PCR targeting the *flaB* gene encoding the flagellin B protein (P41) (Schwaiger et al., 2001). This PCR was further developed (Venczel et al., 2016) to simultaneously screen tick samples for both, *B. burgdorferi* s.l. and *B. miyamotoi*, a relapsing fever spirochete that occurs sympatrically with

B. burgdorferi s.l. in *Ixodes* ticks. QuantiTectMultilpex PCR (NoROX Master Mix, Qiagen) was used and the reaction mix pipetted as shown in table 4 (Table 4: Pipetting scheme of PCRs carried out in this study). The master mix was activated by heating at 95°C for 10 minutes, this was followed by 45 cycles of annealing, extension, and elongation at 95°C for 10 seconds, 56°C for 40 seconds and 72°C for 30 seconds respectively. Then held at 10°C. Samples with cycle threshold (Ct) values less than 35 were considered positive and used for MLST PCR.

4.3.3 Nested PCR for Multilocus Sequence Typing (MLST)

A MLST scheme was employed based on sequence fragments of eight housekeeping loci (Maiden et al., 1998; Urwin and Maiden, 2003; Margos et al., 2008) (i.e. *clpA*, *clpX*, *nifS*, *pepX*, *pyrG*, *recG*, *rplB*, *uvrA*). A two-step (nested) PCR was conducted for all eight genes, where a touchdown set up is applied in the first round for all genes except *recG* to minimize nonspecific binding of the primers. For the *recG* a regular PCR is used. For the genes the reaction mix was pipetted as shown in table 4 (Table 4: Pipetting scheme of PCRs carried out in this study).

For *clpA*, *clpX*, *nifS*, *pepX*, *pyrG*, *rplB* and *uvrA* in the first round an activation step was done by heating at 95°C for 15 minutes, this was followed by 9 cycles of denaturing at 95°C for 15 seconds, annealing at temperatures between 50°C and 58°C for 30 seconds and for clpA 55°C-48°C likewise for 30 seconds; the annealing temperatures were reduced by 1°C after every cycle until the desired temperature was reached. Lastly, elongation was done at 72°C for one minute. This step was followed by another 40 cycles of denaturing, annealing and elongation at 95°C for 15 seoconds, 48°C for 30 seconds and 72°C for one minute respectively. End elongation followed at 72°C for five mintes then held at 10°C.

Second round of PCR for *clpA*, *clpX*, *nifS*, *pepX*, *pyrG*, *rplB* and *uvrA* was no longer a touchdown PCR. The first step was activation by heating at 95°C for 15 minutes, then followed 40 cycles of denaturing at 95°C for 15 seconds,

annealing at 50°C for 30 seconds and elongation at 72°C for 1 minute. End elongation was then done at 72°C for 5 minutes and held at 10°C.

For *recG* both the first round and the second round were done with the same thermal profile and a normal PCR, not a touchdown. Activation was done first at 95°C for 15 minutes, then followed 40 cycles of denaturation at 95°C for 15 seconds, annealing at 55°C for 30 seconds and then elongation at 72°C for one minute. This step was followed by end elongation at 72°C for five minutes then held at 10°C after completion.

4. 3. 4 Outer Surface Protein C (OspC) PCR

A subset of samples positive by *Borrelia* screening real-time PCR were subjected to a seminested PCR to amplify the OspC gene. The first round was set to run at 95°C for 15 minutes for polymerase activation, this step was followed by 35 cycles of denaturating, annealing and elongating at 94°C for 30 second, 52°C for 30 seconds and 72°C for 30 seconds respectively. The second round was set to run using the same thermal profile and 40 cycles with a different reverse primer (Appendix 1: List of primers used in this study).

4. 4 Polyethylenglycol (PEG) DNA Precipitation

The PCR products were precipitated/purified using polyethylenglycol PEG 8000 (rotipuran, Roth, Germany). PEG and the PCR products were mixed 1:1 in a corning 96 well plate (Qiagen, Hilden Germany). The mixture was incubated at 37°C for 15 minutes and then centrifuged for 15 minutes at 2500 relative centrifugal force (rcf). The supernatant was discarded and 125 µl of 80 % cold EtOH was added to each well. The plate was further centrifuged for another 2 minutes at 1400 rcf. The supernatant was discarded and the plate centrifuged again for one minute at 140 rcf. All centrifugation steps were done at room temperature. The plate was left under a hood to dry for 45 minutes. The DNA was eluted with 30 µl Nuclease free water.

4. 5 Gel Electrophoresis

After PEG precipitation a 1.5 % agarose gel (Biozym Biotech Vienna, Austria) was prepared for visualizing DNA bands. 1XTris /Borat/ EDTA (TBE, Rothe, Germany) buffer was used to dissolve the agarose powder, by diluting 10x BE buffer solution 1:10 to achieve the required end concentration. Five µl orange Ruler 50-bp DNA marker (Thermo scientific, Karlsruhe, Germany) was loaded in one well, five µl of the PCR product mixed with two µl of 6xorange DNA loading dye (Biotium, Darmstadt, Germany) was loaded into each of the remaining pockets. Running time was as follows:

- Small gels (with up to 48 pockets) 60 minutes by 120 volts.
- Lager gels (with up to 120 pockets) 60-90 minutes by 185 volts.
- Visualizing with Gel Red end concentration 0.08 %.

4. 6 Sequencing

Twenty samples from this study were sent to GATC to be sequenced (Koblenz, Germany, a commercial sequencing company; https://www.mygatc.com/index.php?id=366&L=1). PCR products were mixed 1:1 with five pmol/ml appropriate primers prior to sending. This company uses first generation Sanger Sequencing Method (Sanger et al., 1977). The rest of the samples were sequenced inhouse by use of Illumina technique as per their protocol (Next Generation Sequencing (NGS)).

4. 7 Comparison of Nextera XT DNA Libraries and Nextera DNA Flex Libraries

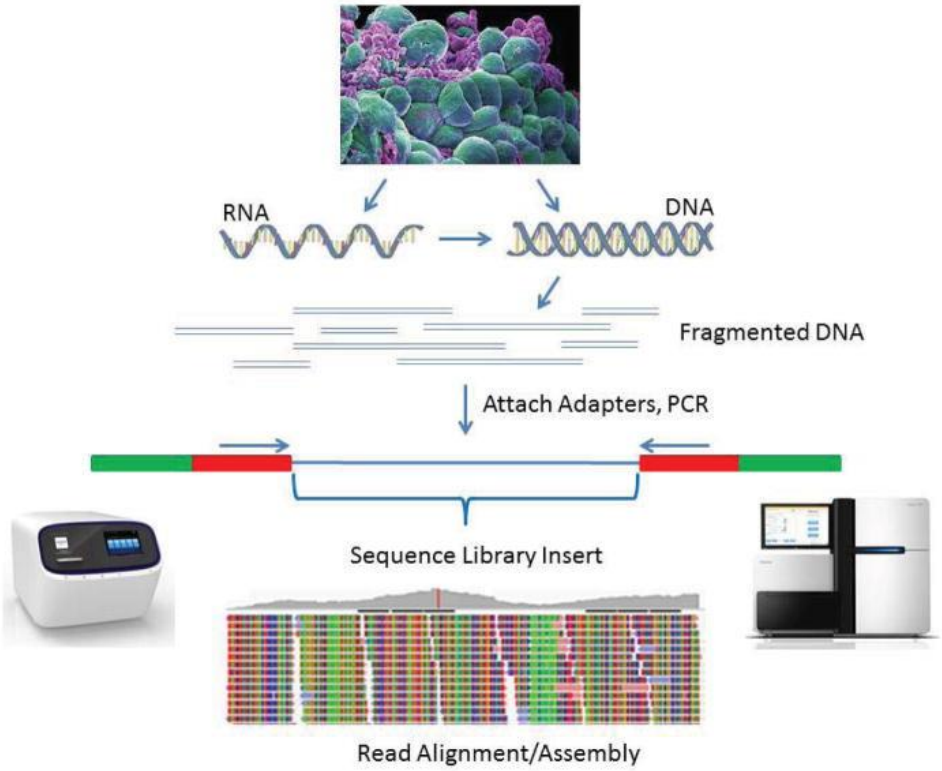


Figure 7: General procedure of library preparation for NGS (Head et al., 2014)

Genomic DNA or RNA is fragmented. Adapters are then attached to the ends

High-throughput sequencing / next generation sequencing (NGS) has in the recent past gained popularity, especially due to constant falling sequencing costs and rise in the application possibilities in life sciences (Head et al., 2014).

A critical step involved prior to NGS is the library preparation: at this step the targeted fragment of DNA/ RNA is prepared, so that it is in conform with the systems to be used. During library preparation the genomic DNA is fragmented and adapters are added to the template DNA (Figure 7: General procedure of library preparation for NGS (Head et al., 2014)).

For comparison purposes of the two kits provided by Illumina: Nextera XT DNA and Nextera DNA Flex were used in 10 samples to prepare libraries i.e. DNA of these 10 samples were divided in two parts so as to have the same DNA for every kit. The preparation was done according to the manufactures` protocol. Cleaned up libraries were run on Agilent technology 2100 Bioanalyzer using a high sensitivity DNA kit to check their quality.

4. 8 Computational Sequence Analysis

4. 8. 1 CLC Genomics Workbench

Readings generated by Illumina were analyzed using CLC workbench version 9.0.1. The readings were mapped to eight housekeeping genes; *clpA*, *clpX*, *nifS*, *pepX*, *pyrG*, *rplB* and *uvrA* of B31-*B. burgdorferi* s.s. as reference (GenBank accession number NC_001318.1) in order to generate a consensus sequence with the following mapping options: match score one, mismatch cost two, insertion and deletion cost three, similarity fraction 0.8 and length fraction 0.5. Minimum coverage was set to five and ambiguity codes were inserted at minimum threshold of 30 % base coverage in order to detect mixed infections. No further analysis was conducted on mixed sequences.

4. 8. 2 Alignments and Phylogenies Construction

If all MLST genes were detected and successfully sequenced, they were concatenated and used for phylogenetic tree generation. MEGA version 6 was used to align sequences from Sanger sequencing and consensus sequences exported from the CLC workbench too. ClustalW alignment was set as default. Phylogenetic trees for both MLST and OspC genes were generated in this

program by use of Maximum-Likelihood-Method. A bootstrap test with 1000 repetitions was set as a default. Phyloviz version 1.1 was used for goeBUSRT analysis and generation of minimum spanning tree. STs generated from MLST were entered in goeBURST which identifies founder STs and STs relationships though single and double locus variants.

4. 8. 3 Analysing GATC Sequences and Determining STs and Species

Sequences were analyzed using the software provided by Smartgene Inc., Lausanne, Switzerland, (https://apps.idns-smartgene.com/apps/IDNSPortal.po) or by using the Seqman Pro software (DNAStar, Lasergene, USA). Sequences were compared to available data at the pubmlst *Borrelia* website (https://pubmlst.org/bigsdb?db=pubmlst_borrelia_seqdef&page=sequenceQuery). Allele and sequence type numbers as well as *Borrelia* species were determined.

4. 9 Statistical Analysis

The probability of a tick to be *Borrelia* positive was modelled using a binomial generalized linear mixed effect model (GLMM). Site and life-stage were fitted as fixed effects and a random intercept for year was included. This model utilized all positive tick specimens (Ct value < 40) regardless of complete *Borrelia* species identification. No larvae were positive for *Borrelia* and therefore were excluded from this analysis. For all further statistical analyses, only positive tick specimens with *Borrelia* species identification were used.

Prevalence of host adaptation types per year were calculated and tested using Fisher's exact tests. For this all *Borrelia spp*. found in a site in a given year were combined into four categories: bird-adapted (*B. valaisiana*, *B. garinii*), rodent-adapted (*B. afzelii*, *B. bavariensis*), generalists (*B. burgdorferi* s.s.) or were identified as mixed infections (i.e. potentially more than one *Borrelia* species present).

To test potential carry over year effects on *Borrelia* genospecies prevalence, we modelled the absolute number of ticks infected with a specific *Borrelia* genospecies each year using a GLMM assuming a Poisson error distribution. For this, fixed effects were fitted for the prevalence of the given *Borrelia* genospecies in the previous sampling event and a binary factor if the genospecies was found (1) or not found (0) in the previous sampling event. Random effects were also included for site and year.

All analyses were done in R (Version 3.6.1) (R Core Team 2019). Fisher's exact tests were performed using the fisher.test command with a simulated p-value, based on 5,000 simulations, from the base R package (Team, 2019). All GLMMs were run with the glmer function from the "lme4" package (Bates et al., 2015). The posterior distributions of the model parameters were simulated using the sim function from the "arm" package (Gelman and Su, 2016). Mean estimates and their 95% credible intervals (CI), were extracted estimated based on 5,000 simulations. Residual errors were calculated according to (Nakagawa and Schielzeth, 2010).

Chapter 5 **Results**

5. 1 Comparison of Methods for Economic and Efficient Tick and *Borrelia* DNA Purification

Because several thousand ticks needed to be processed during this thesis, an efficient but also economic way of DNA extraction was needed to be established. Therefore, in the first part of the study factors that may have an influence on DNA extraction were compared. Commercially available DNA extraction kits (Qiagen) vs alkaline hydrolysis for DNA extraction were tested. The methods were applied to questing *I. ricinus* ticks collected for that purpose in the year 2017 in Oberschleissheim. In addition, cultures with defined cell concentrations obtained from a cultivated *B. burgdorferi* s.s. B31 isolate were used for a quantitative comparison of DNA extraction via real-time PCR.

Of the 69 ticks, from 34 ticks total DNA was extracted using a commercial DNA extraction kit and 35 ticks were treated with 1.25 % NH_4OH (Table 5: Number of ticks tested (A), results on the two-sided *t*-test conducted (B) and culture dilutions (C) processed using different DNA purification methods). A real-time PCR targeting the tick *coi* gene was used to assess the success and yield of DNA extraction.

In total 57 samples were used fresh (i.e. without EtOH preservation): 28 tick samples for DNA extraction via a commercial kit and 29 were used for NH_4OH DNA extraction. Column DNA extraction yielded slightly better results than NH_4OH treatment when tested in a PCR targeting a tick-specific *coi* gene ($p = 4.77E-05$, Table 5: Number of ticks tested (A), results on the two-sided *t*-test conducted (B) and culture dilutions (C) processed using different DNA purification methods). In the first set (commercial kit) the *coi* gene fragment was successfully amplified by conventional PCR in 27/28 tick samples (96 %). Samples in which DNA was extracted using NH_4OH, only 25/29 (86 %) were positive in a conventional PCR targeting the *coi* gene (Figure 8: Agarose gels of *coi* gene PCR products from samples after DNA extraction by different

methods). These gel images also showed that DNA extraction was similar, regardless whether adult or nymphal ticks were processed.

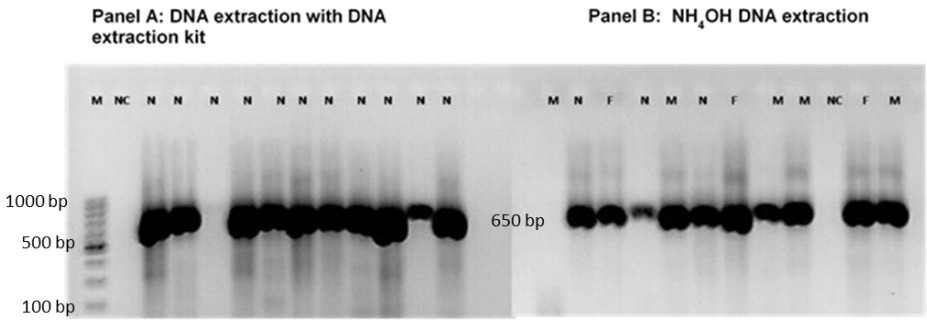


Figure 8: Agarose gels of *coi* gene PCR products from samples after DNA extraction by different methods

Panels A and B show a subset of samples from freshly handled ticks (i.e. without preservation in EtOH). Panel A: PCR products (*coi* gene) obtained from tick samples purified with commercial DNA extraction kits, Panel B: DNA extracted with alkaline hydrolysis. In both groups at least in one sample the *coi* gene was not successfully amplified, i.e. the PCR failed for unknown reasons. However, it is apparent that PCR amplification on DNA purified using a commercial kit produced stronger bands in comparison to NH_4OH extracted DNA. Comparable results were obtained for all 57 samples included in this experiment (data not shown). MR = DNA low molecular marker (Orange Ruler 100-bp DNA marker, Thermo scientific, Karlsruhe, Germany); NC = negative control; N = nymph; F = female; M = male, 1 = undiluted, 2 = 10-1, 3 = 10-2, 4 = 10-3, 5 = 10-4, 6 = 10-5

To obtain some information about the influence of EtOH on the performance of DNA extraction, 12 samples were stored in 70 % EtOH for one-week prior to DNA extraction (six for commercial kit extraction group and six for NH_4OH-treatment group). For all samples, DNA extraction was successful as in all of them the tick *coi* gene locus was successfully PCR amplified, irrespective of the extraction method (Figure 9: Ticks samples stored in 70 % EtOH for one week prior to handling). EtOH preservation had a slightly negative effect on DNA yield and – again – slightly stronger PCR products were observed by

commercial kit extraction (Figure 9: Ticks samples stored in 70 % EtOH for one week prior to handling).

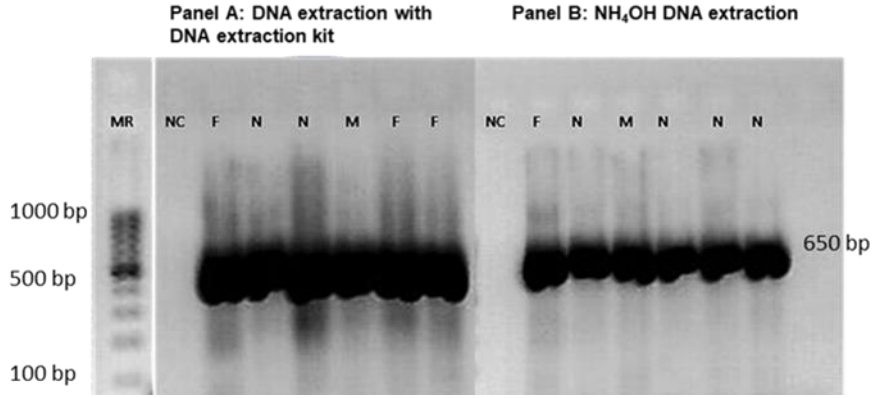


Figure 9: Ticks samples stored in 70 % EtOH for one week prior to handling

Agarose gel of PCR products (*coi* gene) obtained from tick samples purified with commercial DNA extraction kits (Panel A) vs alkaline hydrolysis (Panel B) after storage in 70 % EtOH for one week. Panel A) DNA extracted from six ticks, three females, one male and two nymphs, all of which gave very strong bands. Panel B) DNA extraction of one male, one female and four nymphs using alkaline hydrolysis also resulted in strong bands, the bands were less pronounced than using commercial kit extraction. MR = DNA low molecular marker (Orange Ruler 100-bp DNA marker, Thermo scientific, Karlsruhe, Germany); NC = negative control; N = nymph; F = female; M = male

Thus, for five ticks (two females, two males and one nymph; one included in commercial DNA extraction kit, four in alkaline hydrolysis) the amplification was not successful. Since it was possible that inhibitory factors present in the four samples extracted by alkaline hydrolysis might have resulted in failure of PCR amplification of the *coi* gene fragment, DNA was re-extracted using a commercial DNA purification kit.

In all four re-purified samples, a PCR product was amplified, with three samples producing prominent bands on the gel electrophoresis (Figure 10: Re-extracted DNA using a commercial kit after the first PCR failed). These results support the notion that inhibitory factors were removed by column-mediated DNA extraction.

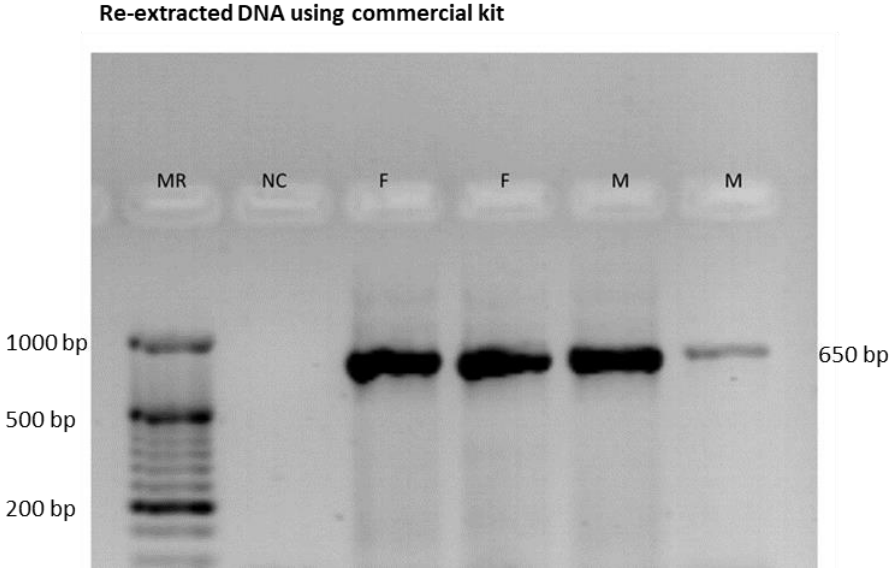


Figure 10: Re-extracted DNA using a commercial kit after the first PCR failed

After DNA re-extraction using a commercial kit after the initial *coi* PCR failed when DNA was extracted by NH_4OH method. MR = DNA low molecular marker (Orange Ruler 50-bp DNA marker, Thermo scientific, Karlsruhe, Germany); NC = negative control; N = nymph; F = female; M = male

To quantify the PCR results and to exclude the possibility of inhibitory factors being present in NH_4OH ticks, a serial dilution of eight samples was conducted from 10^{-1} to 10^{-6}; two samples from each group (Figure 11: Agarose gels of *coi* gene PCR products from samples after DNA extraction by different methods and serial dilutions; panels C to F). These data indicate that *coi* gene

amplification was successful in freshly prepared samples in dilutions up to 10^{-2} in NH_4OH DNA (Panel D) and up to 10^{-3} in kit extracted DNA (tick 14, Panel C). Following EtOH preservation and DNA purification by NH_4OH (Panel F), only in the dilution of 10^{-1} a PCR product was obtained.

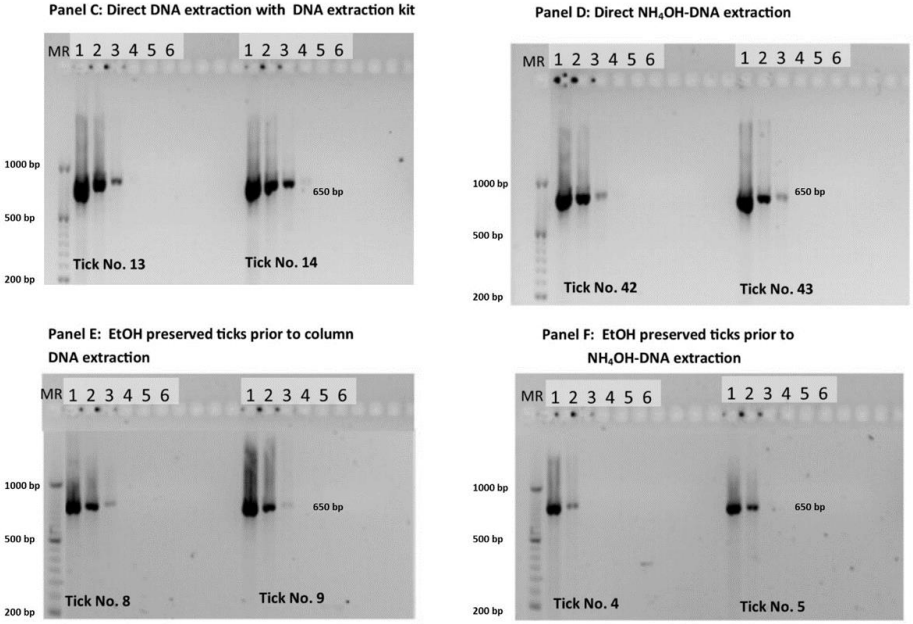


Figure 11: Agarose gels of *coi* gene PCR products from samples after DNA extraction by different methods and serial dilutions

Panels C-F show the PCR amplification in samples of eight nymphs after a serial dilution from 10^{-6} to 10^{-1}. Panel C and D are ticks which were handled freshly (i.e. without preservation in EtOH). Panel E and F are ticks, which were preserved in 70 % EtOH for one week prior to DNA extraction. These data indicate that *coi* gene amplification was successful in freshly prepared samples extracted using the NH_4OH DNA method in dilutions up to 10-2 (Panel D) and in kit extracted DNA also freshly prepared in dilutions up to 10^{-3} (tick 14, Panel C). Following EtOH preservation and DNA purification by NH_4OH (Panel F), only in the dilution of 10^{-1} a PCR product was obtained. MR = DNA low molecular marker (Orange Ruler

50-bp DNA marker, Thermo scientific, Karlsruhe, Germany); NC = negative control; N = nymph; F = female; M = male, 1 = undiluted, 2 = 10^{-1}, 3 = 10^{-2}, 4 = 10^{-3}, 5 = 10^{-4}, 6 = 10^{-5}

To additionally quantify the differences in DNA extraction between commercial kit and NH_4OH, cultures of *B. burgdorferi* s.s. with known cell numbers were used for DNA extraction and real-time PCR targeting a *Borrelia flaB* gene was employed. Ct-values of column-based DNA vs NH_4OH extraction performed with three different dilutions in three different runs are shown in table 5 section (C) (Table 5: Number of ticks tested (A), results on the two-sided *t*-test conducted (B) and culture dilutions (C) processed using different DNA purification methods). A difference of three to five Ct-values was observed between dilution steps (i.e. from 10^6 to 10^5 to 10^4). A difference of two to three Ct-values was observed between the different DNA extraction methods, i.e. commercial kit vs alkaline hydrolysis, with higher discrepancies in lower cell concentrations. Thus, results from this study indicated that the detection limit is reduced by two to three Ct-values which corresponds to a 10-fold dilution in samples when alkaline hydrolysis is used for DNA extraction. A one-sided *t*-test showed a significant difference between the methods at lower concentration of *Borrelia,* i.e. better extraction with a commercial kit at lower borrelial DNA concentration in contrast to a higher concentration (106 cells per ml) (Table 5: Number of ticks tested (A), results on the two-sided *t*-test conducted (B) and culture dilutions (C) processed using different DNA purification methods).

For statistical analysis BioNumerics version 7.6 was used to quantify the PCR product bands in agarose gels. The values were subsequently analyzed employing the Shapiro-Wilk test (Wilk and Shapiro, 1965) and a two-sided *t*-test (Pillemer, 1991; Ned, 2015).

A Shapiro-Wilk test conducted revealed a significance-level of 90 % for both the methods, indicating a normal distribution of the values generated by BioNumerics quantification. A two-sided *t*-test was conducted. A significant ($p<0.01$) mean difference was determined to be 4.40, which is greater than the critical *t*-value for the two-sided *t*-test in this study, which was determined to be 2.00 (Table 5: Number of ticks tested (A), results on the two-sided *t*-test conducted (B) and culture dilutions (C) processed using different DNA purification methods).

Table 5: Number of ticks tested (A), results on the two-sided *t*-test conducted (B) and culture dilutions (C) processed using different DNA purification methods

Methods	**Commercial kit (Qiagen)**		**NH_4OH**	
A: Number of ticks processed using different purification methods				
EtOH-preservation	without	with	without	with
Total ticks	34		35	
Ticks	28	6	29	6
Male, n / positive	2 / 2	1 / 1	9 / 7	4 / 4
Female, n / positive	3 / 2	3 / 3	11 / 9	1 / 1
Nymph, n / positive	23 / 22	2 / 2	9 / 9	1 / 1
B: Two-sided *t*-test on the different methods assuming equal variance				
Average	65615		49808	
Variance	227195838		151761455	
T-statistic	4.40			
P(T<=t) two-sided	4.77E-05			
Critical t-value for two-sided *t*-test	2.00			

C: Ct-values of real-time PCR on serial dilutions of *B. burgdorferi* s.s. DNA						
Dilution	**10^4**		**10^5**		**10^6**	
Method	**Roche***	**NH_4OH**	**Roche**	**NH_4OH**	**Roche**	**NH_4OH**
1st run	34.09	38.28	31.11	33.4	26.7	28.72
2nd run	33.13	36.81	31.03	32.15	26.93	29.16
3rd run	35.25	36.93	30.22	34.58	25.87	26.12
Mean Ct-value	34.16	37.34	30.79	33.38	26.5	28
Standard deviation	0.867	0.666	0.402	0.992	0.455	1.341
***t*-test p-value**	0.0267		0.0559		0.0697	

*DNA from cultured specimen purified with different extraction methods (commercial DNA extraction kit vs alkaline hydrolysis).

n = number of ticks; positive = number of positives

DNA was purified from cultured *B. burgdorferi* s.s. with known number of cells: 10^4, 10^5 and 10^6 per ml and DNA eluted in 60 µl RNA and nuclease free water (Table 5: Number of ticks tested (A), results on the two-sided *t*-test conducted (B) and culture dilutions (C) processed using different DNA purification methods). Five µl were used as template DNA for PCR, corresponding to 833, 8.3 and 83.3 cells/ PCR, respectively. Three experiments were run (first run, second run, and third run) to show the reproducibility of the results. A difference of three to five Ct-values was observed between dilution steps (i.e. from 10^6 to 10^5 to 10^4). A difference of two to three Ct-values was observed between the different DNA extraction methods, i.e. commercial kit vs alkaline hydrolysis, with higher discrepancies in lower cell concentrations.

Summing up it can be said that DNA extraction using a commercial kit generated slightly better results than NH_4OH DNA extraction. DNA extraction was equally good on both nymphal and adult ticks and lastly all EtOH stored ticks prior to DNA extraction generated PCR product regardless of the extraction method.

5. 2 Comparison of Sequencing and Library Production Methods

Sanger sequencing was the first developed sequencing technique in the 1970 and is still widely used, however more modern sequencing methods i. e. NGS have been developed. At the beginning of this study Sanger sequencing was used, nevertheless there were some difficulties on achieving sequences with non specific background on PCR products. For this reason, (i) Sanger sequencing vs Illumina sequencing were compared. Besides Illumina offers different library production kits, (ii) these two kits; Nextera XT libraries vs Nextera DNA flex libraries were also compared as they are slightly different according to the manufacture (Table 7: Differences between Illumina Nextera XT and DNA Flex kits as described by the company). Twenty samples were chosen randomly (10 for each group).

For comparison of Sanger sequencing vs Illumina sequencing, ten samples were randomly chosen that had already been sequenced by Sanger method,

and had not yielded evaluable sequneces for all the eight genes, these were then resequenced by NGS method (Table 6: Ten samples which were resequenced by NGS after Sanger sequencing).

Table 6: Ten samples which were resequenced by NGS after Sanger sequencing

Sample ID	*clpA*	*clpX*	*nifS*	*pepX*	*pyrG*	*recG*	*rplB*	*uvrA*
1-14	repeat	repeat	repeat	√	√	repeat	repeat	repeat
1-14-10	repeat	repeat	repeat	√	√	√	repeat	√
1-14-43	repeat	repeat	repeat	√	√	√	repeat	√
1-14-44	√	repeat	repeat	√	repeat	√	repeat	√
1-14-48	√	repeat	repeat	√	repeat	√	repeat	√
1-18	repeat	repeat	repeat	repeat	repeat	√	repeat	√
1-18-20	repeat	repeat	repeat	repeat	√	√	repeat	√
1-18-21	repeat	repeat	repeat	repeat	repeat	repeat	repeat	√
1-5-10	√	√	repeat	√	repeat	repeat	repeat	√
1-5-15	repeat	√	repeat	√	√	√	repeat	repeat

Randomly chosen samples which were resequenced by Illumina method. In table 6 √ means the sequence were good otherwise they had to be repeated. As can be seen several samples had to be repeated after the first sequencing round by Sanger method. After resequencing with Illumina, nine samples had readable sequences for all the eight genes except for one sample, *clpA* gene failed.

There are two available kits for libraries preparations provided by Illumina which differ slightly from one another (Table 7: Differences between Illumina Nextera XT and DNA Flex kits as described by the company). Nextera XT is slightly inexpensive though there is one additional step in the library preparation which is not required when using DNA Flex kit. Besides DNA flex kit has a flexible workflow with broad DNA input range (1-500 ng). These two kits were compared here, in order to find out whether the adverted difference in terms of total library generation and optimal distribution of single-isolates DNA were realistic. Ten

isolates were prepared using Nextera XT DNA and Nextera DNA Flex. Figure 12 shows the libraries distribution of single isolates as measured on Agilent tape station after library preparation with Nextera XT DNA and Nextera DNA Flex (Figure 12: Quantity and size distribution of DNA libraries prepared using different kits).

Table 7: Differences between Illumina Nextera XT and DNA Flex kits as described by the company

Nextera XT DNA library prep		Nextera DNA Flex library prep	
Advantages	**Disadvantages**	**Advantages**	**Disadvantages**
-	Normalization required	No normalization required	-
-	More time consuming	First library prep workflow	-
-	-	Intergrated sample input	-
-	-	Flexible workflow with broad DNA input range (1-500 ng)	-
-	-	Wide range of amplifications	-
-	-	Optimised library prep performance	-
Cheaper in comparison to Nextera DNA Flex	-	-	More expensive in comparion to Nextera DNA XT

The Nextera XT DNA kit and Nextera Flex DNA kit library preparations differ slightly from one another.

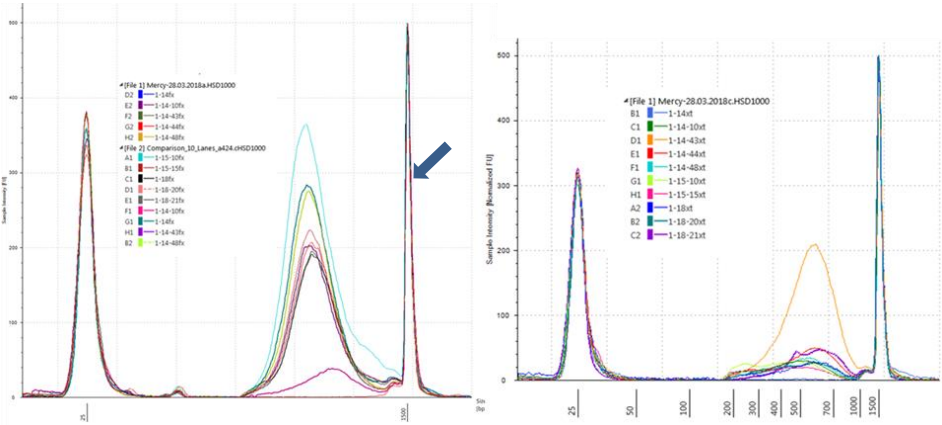


Figure 12: Quantity and size distribution of DNA libraries prepared using different kits

Left diagram shows Nextera DNA Flex libraries and right diagram shows Nextera XT DNA libraries as measured on an Agilent Tapestation. The arrows show the single library peaks and the letters shows numbering of the single isolates and their colour coding. More reproducibility and fragment size uniformity was noted for DNA Flex (left).

The main conclusion which was drawn from this comparison was that, with the Nextera DNA flex, libraries showed a sharper size peak and better distribution of the single isolates-DNA than libraries prepared by Nextera XT DNA kit. Despite the better size distribution with Nextera DNA flex, Nextera XT DNA kit was used in this study as it was more economical, the difference in time required for library production was not much longer and the results obtained were sufficient.

5. 3 Infection Prevalence of *B. burgdorferi* s.l. in Questing Ticks from Latvia

Figure 13: Google map showing tick collection sites in Latvia

This study focused on three collection sites in Latvia, Riga-Babite District, Jurmala Kemeri and lastly Riga Jaunciems.

A total of 3,165 questing ticks were collected by drag sampling in three defined habitats in Latvia: Babite, Kemeri and Jaunciems (Figure 13: Google map showing tick collection sites in Latvia). These ticks were sampled in the years 1999, 2000, 2001, 2003 and 2010. Additionally sequences were available for the ticks which had been collected in the same regions in the years 2002, 2006 and 2007 (Table 3: Summary of Latvian tick samples from which only MLST data was available for this study) and had been processed by Stephanie Vollmer (Vollmer et al., 2011; Vollmer et al., 2013). There were no samples available for the years 2004, 2005, 2008 and 2009.

The three collection regions constitute different ecological habitats. Babite, (longitude, 23° 48′; latitude, 56° 50′) is a sylvatic habitat (forested habitation). Jaunciems (longitude, 24° 09′; latitude, 57° 03′) is peridomestic (around human habitation) and close to the suburbs of Riga. Kemeri (longitude, 23° 29′; latitude, 56° 56′) represents a peridomestic habitat with mixed forest habitats along marshes (Etti et al., 2003).

The tick collection site Babite is far from residential areas with a rural road sectioning it in two parts. The most common tree found in this region is *Picea abies,* also known as *Alnus glutinosa*. In both sections, there is heavy growth of *Picea abies* saplings. Other trees found in this site included: *Populus tremula, Alnus* sp*., Frangula alnus, Padus avium, Betula* sp*., Salix* sp*.* and *Rubus idaeus. Oxalis acetosella* mainly covers the ground, but there are also areas with dense growth of different common grass species.

Jaunciems is near Riga, the capital city of Latvia. There are two heavily used main roads, private houses and a lake near the tick collection site. The most common tree found in this region was *Pinus sylvestris.* Other tree species found in this region included: *Acer plantanoides, Sorbus aucuparia, Padus avium, Ameanchier spicata, Tilia cordata* amongst others. In contrast to Babite, the underwood and ground are not densely covered. Low plants and grass are found in these regions.

Kemeri is also far away from residential areas and has mixed vegetation. There is a small pedestrians' road. Nearby is woodland and marshes, along which tick collection was conducted. Frequently found trees were *Pinus sylvestris* and *Picea abies,* whilst *Alnus glutinosa* was infrequent at this site. The ground was covered either with *Oxalis acetosella or Vaccinium myrtillus* or with moss. Different underwood tree species like *Alnus* sp. or *Acer plantanoides* are found. In Kemeri the ground is mostly densely covered.

Out of a total of 4,484 screened ticks, 1,931 were adults, 2,516 were nymphs and 37 larvae (Appendix 2: Total number of ticks analyzed in the whole study). In total, 848/4,484 ticks (18.9 %) were identified as *Borrelia* positive in the screening real-time PCR targeting the *flaB* locus. Of the 1,931 screened adults, 522 (27 %) were positive and of 2,516 nymphs, 326 (13.0 %) were positive.

None of the 37 screened larvae were positive. Babite, Jaunciems and Kemeri had mean prevalences for *B. burgdorferi* s.l. in ticks of 19.5 %, 16.7 % and 19.8 %, respectively (Table 8: Total number of positive ticks per year). Ticks coming from the three sites were equally likely to be *Borrelia* positive (Table 9: Model outputs of *Borrelia spp.* presence analysis). However, ticks coming from different years did differ in how likely they were to be *Borrelia* positive (Table 10: Results of GLMM exploring impacts to the absolute number of ticks infected with specific *Borrelia* genospecies in a given year). Remarkably, infection prevalences declined with time in all habitats.

In the years 1999, 2000, and 2001 *Borrelia* prevalences in tick populations collected in Latvia were 25.5 %, 33.1 % and 31.8 % respectively. In the years 2002, 2003, 2006, 2007 and 2010 prevalence of between16.0 % and 7.3 % in *Borrelia* infected ticks was observed.

In Babite and in Jaunciems there was a comparable rise of infection prevalence followed by decreasing prevalences after the year 2001, both in nymphs and adults. In Babite there was a slight rise in 2010 after the lowest prevalence of *Borrelia* infection was reached in 2003. In Kemeri, on the other hand the prevalence of *Borrelia* infection in adults was different from the other regions, there was a substantial fall between 1999 and 2001, an increase in 2003 and a further decrease thereafter. Data for nymphs were comparable to the other regions; an increase and a steep fall after the year 2001 was observed.

Table 8: Total number of positive ticks per year

Sampling years	1999	2000	2001	2002 *	2003	2006 *	2007 *	2010	Total
Total number of collected ticks									
Per year	271	236	1133	368	883	492	459	642	4,484
Adults	271	200	678	nd	410	nd	nd	372	1,931
Nymphs		36	418	368	473	492	459	270	2,516
Larvae			37						37
Number of positives in total and (%)									
Per year (% of all)	69 (25.5)	78 (33.1)	360 (31.8)	53 (14.4)	141 (16.0)	48 (9.8)	52 (11.3)	47 (7.3)	848 (18.9)
Adults (% of total adults)	69 (25.5)	60 (30.0)	280 (41.3)	nd	77 (18.8)	nd	nd	36 (9.7)	522 (27)
Nymphs (% of total nymphs)	0	18 (50.0)	80 (19.1)	53 (14.4)	64 (13.5)	48 (9.8)	52 (11.3)	11 (4.1)	326 (13)

* Data of 2002, 2006 and 2007 are taken from a former study (Vollmer et al., 2011; Vollmer et al., 2013), for these years only nymphal stage was screened. There were no data of for the years 2005, 2008 and 2009 available for this study.

Sampling years	1999	2000	2001	2002 *	2003	2006 *	2007 *	2010	Total
Number of positives in total and (%) per Region									
Babite	46 (25.6)	19 (22.4)	169 (26.9)	31 (20.8)	83 (18.9)	35 (13.5)	36 (16.4)	32 (9.1)	453 (19.5)
Babite adults	46 (25.6)	18 (22.4)	127 (42.8)	nd	35 (17.1)	nd	nd	23 (12.8)	250 (26.4)
Babite nymphs	0	0	42 (13.7)	31 (20.8)	48 (20.6)	35 (13.5)	36 (16.4)	9 (5.2)	201 (15.0)
Jaunciems	7 (15.6)	23 (26.1)	73 (34.6)	16 (12.6)	30 (14.6)	7 (6.6)	4 (4.0)	7 (5.9)	167 (16.7)
Jaunciems adults	7 (15.6)	23 (26.1)	62 (36.7)	nd	15 (14.7)	nd	nd	6 (9.8)	113 (24.3)
Jaunciems nymphs	0	0	11 (26.2)	16 (12.6)	15 (14.4)	7 (6.6)	4 (4.0)	1 (1.8)	54 (10.1)
Kemeri	16 (34.8)	36 (57.1)	118 (41.8)	6 (6.5)	28 (11.7)	6 (4.7)	12 (8.6)	8 (4.7)	230 (19.8)
Kemeri adults	16 (34.8)	18 (66.7)	91 (42.9)	nd	27 (26.2)	nd	nd	7 (5.3)	159 (30.6)
Kemeri nymphs	0	18 (50.0)	27 (38.6)	6 (6.5)	1 (0.7)	6 (4.7)	12 (8.6)	1 (2.5)	71 (11.1)

Table 9: Model outputs of *Borrelia spp.* presence analysis

***Borrelia* presence models a binary factor of a tick being either infected (1) or non-infected (0) with *Borrelia spp.*.**

Fixed Effects	**β (95% CI)**
Intercept	-1.17 (-1.59, -0.75)
Riga District Babite[a]	0.00 (-0.19, 0.18)
Riga Jaunciems[a]	-0.15 (-0.37, 0.08)
Life stage[b]	-0.75 (-0.95, -0.54)
Random Effects	**σ^2 (95% CI)**
Year	0.34 (0.24, 0.46)
Residual	$0.32\pi^2$ ($0.32\pi^2$, $0.29\pi^2$)

[a] Reference category: Kemeri

[b] Reference category: Adult

Table 10: Results of GLMM exploring impacts to the absolute number of ticks infected with specific *Borrelia* genospecies in a given year

Fixed Effects	**β (95% CI)**
Intercept	-0.64 (-1.44, 0.15)
Genospecies prevalence in previous sampling[a]	1.75 (1.16, 2.33)
Genospecies found in previous sampling[b]	1.22 (0.80, 1.64)
Random Effects	**σ^2 (95% CI)**
Year	0.64 (0.47, 0.84)
Site	0.14 (0.07, 0.24)
Residual	2.63 (3.20, 2.20)

[a] Calculated per genospecies per year

[b] Binary factor given per genospecies per year

The highest infection rate in adults was determined for the year 2001, where 41.3 % of adult ticks were infected while the highest infection in nymphs was in 2000 where 50 % of nymphs were infected, this was followed by a drastic fall in the following years with the lowest prevalence rate in nymphs being 4.1 % in the year 2010 (Table 8: Total number of positive ticks per year and Figure 14: Prevalence of *Borrelia spp.* infection in adult and nymphal ticks from 1999 to 2010).

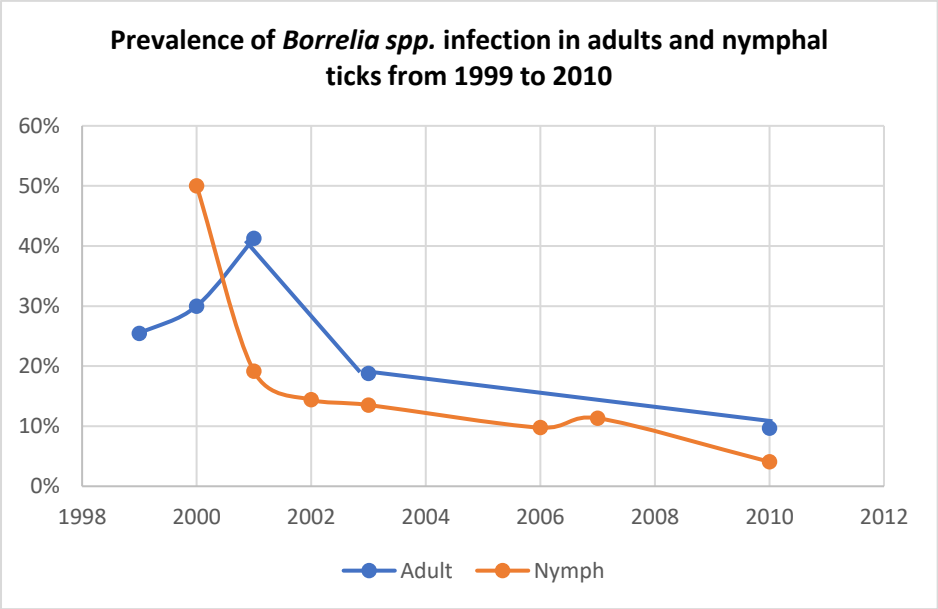
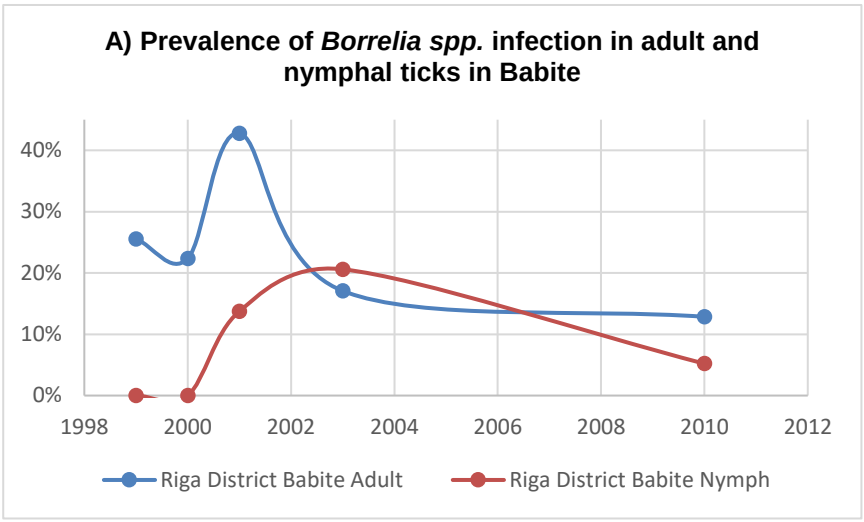
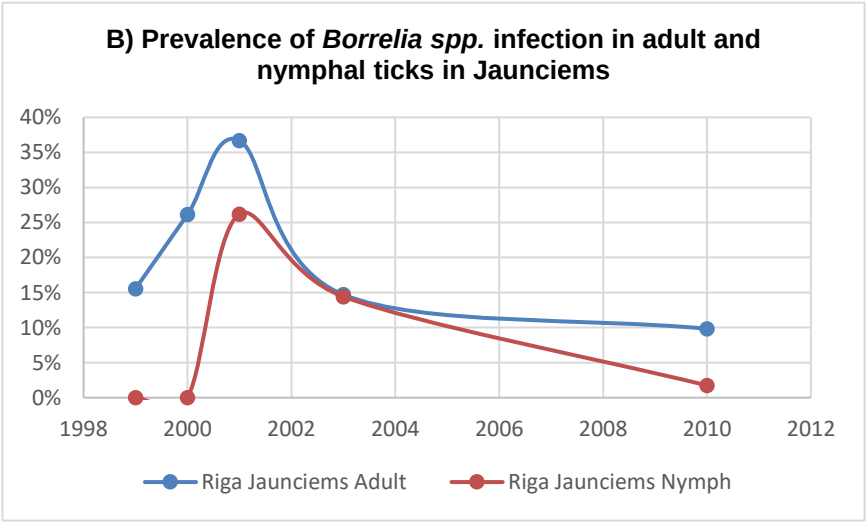


Figure 14: Prevalence of *Borrelia spp.* infection in adult and nymphal ticks from 1999 to 2010

Y axis shows the infection percentage and X axis the years in ascending order. The peak *Borrelia spp.* prevalence infection years were 2000 and 2001 for nymphs and adults respectively. From the year 2003 to 2010, the infection rate plummeted.


A) Prevalence of Borrelia spp. infection in adult and nymphal ticks in Babite
40%
30%
20%
10%
0%
1998
2000
2002
2004
2006
2008
2010
2012
Riga District Babite Adult
Riga District Babite Nymph


B) Prevalence of Borrelia spp. infection in adult and nymphal ticks in Jaunciems
40%
35%
30%
25%
20%
15%
10%
5%
0%
1998
2000
2002
2004
2006
2008
2010
2012
Riga Jaunciems Adult
Riga Jaunciems Nymph

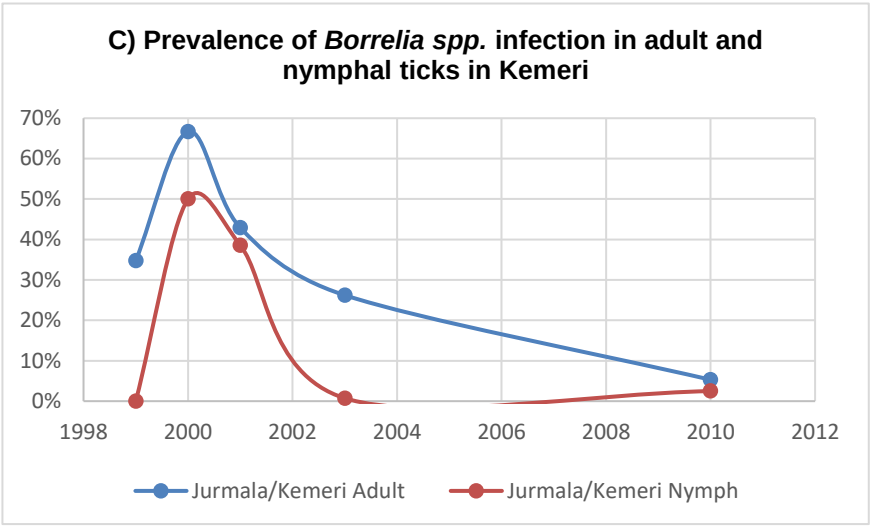


Figure 15: Illustrates infection trends in adults and nymphs in Babite (A), Jaunciems (B) and Kemeri (C)

The Y axis shows the infection percentage and X axis the years in ascending oder. The infection rates in adults was as expected higher than in nypmhs in Babite, Jaunciems and Kemeri. Strikingly in Babite, more nymphs were infected than adults in the year 2003.

The tick collection was conducted in regions with different habitation; (i) Babite has a sylvatic habitation (forested), (ii) Jauncimes has a peridometic habitation (around human habitation) and lastly (iii) Kemeri has both sylvatic and peridomestic habitation (mixed habitat). Comparison of these regions revealed that mixed habitation (with humans and forest) has the highest prevalence (Figure 16: Prevalence of *Borrelia spp.* infection in adult and nymphal ticks sampled in Babite, Jauncimes and Kemeri). This was particularly clear in the years 1999 - 2001. In Kemeri the highest infection rate was in the year 2000 (57.1 %), then sank steadily to 4.7 % in the year 2010 (Table 8: Total number of positive ticks per year). The infection rates in Babite and Jaunciems had the same patterns, in both regions the infection rates were highest in the year 2001

(26.9 % and 34.6 % respectively), then sunk steadily like in Kemeri to infection rates of 9.1 % and 5.9 % in Babite and Jauncimes. (Table 8: Total number of positive ticks per year).

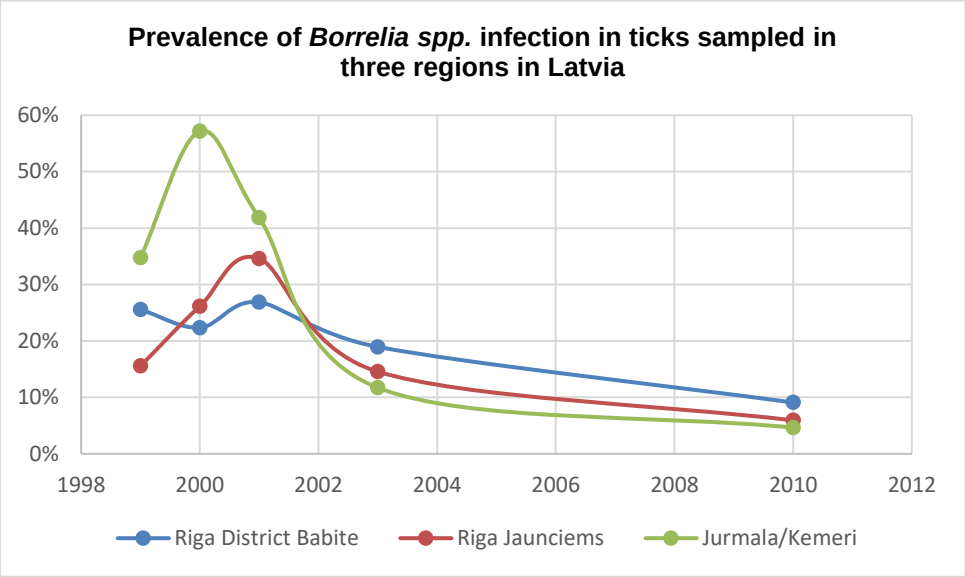


Figure 16: Prevalence of *Borrelia spp.* infection in adult and nymphal ticks sampled in Babite, Jauncimes and Kemeri

This diagram illustrates the prevalence of *Borrelia spp.* infection in the three samples regions. The Y axis shows the infection percentage and X axis the years in ascending order. Peak prevalences were found in the years 1999, 2000 and 2001 for Kemeri, Babite and Jauncimes, respectively.

For the year 2000 there were no samples available for the seasons autumn and summer. In addition, the year 2010 there were no samples available from summer. For this reason, the years 2000 and 2010 were omitted when determining the prevalence of *Borrelia spp.* Per season and year. The years 2002, 2006 and 2007 there were only nymphal ticks available hence were also omitted from this analysis. Summer 2001 had the highest infection rates,

followed by spring 1999 and 2003 (Figure 17: Prevalence of *Borrelia spp.* in sampled ticks per season and year).

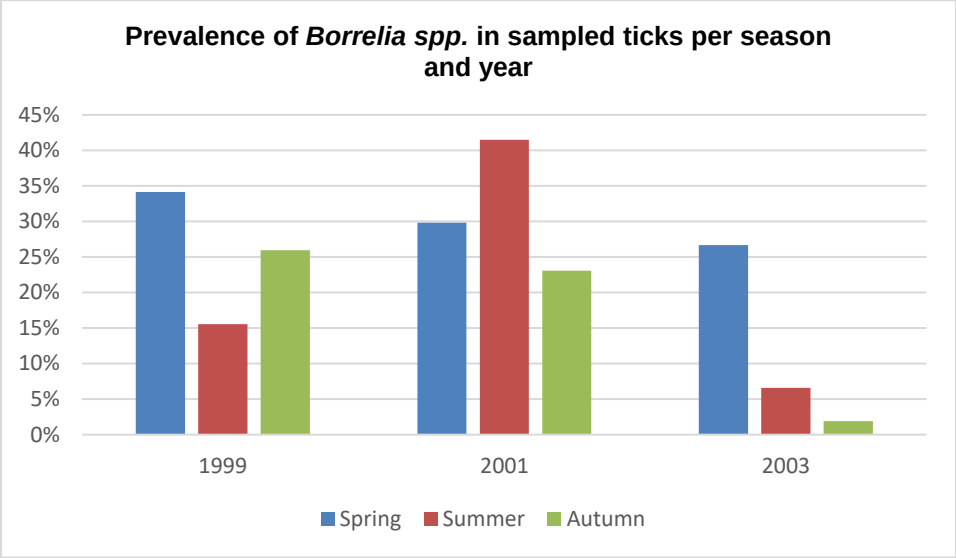


Figure 17: Prevalence of *Borrelia spp.* in sampled ticks per season and year

The Y axis shows the infection percentage and X axis the years in ascending order. In 1999 the infection rates were high in spring and autumn, in 2001 on the other hand infection rates were high in spring and summer but low in autumn. In 2003 the infection rates in summer and autumn were below 10%.

5. 4 Population Structure of *B. burgdorferi* s.l. in Latvia

5. 4. 1 Spatial Distribution of *Borrelia spp.* in the Sampling Sites

In order to gain insight in the geographical distribution of *Borrelia* species in different habitats in Latvia, a spatial distribution analysis was carried out (Figure 18: Spatial distribution of *B. burgdorferi* s.l. in Babite, Figure 19: Spatial distribution of *B. burgdorferi* s.l. in Jaunciems, Figure 20: Spatial distribution of *B. burgdorferi* s.l. in Kemeri and Appendix 4: Total number of samples used for spatio-temporal distribution analysis).

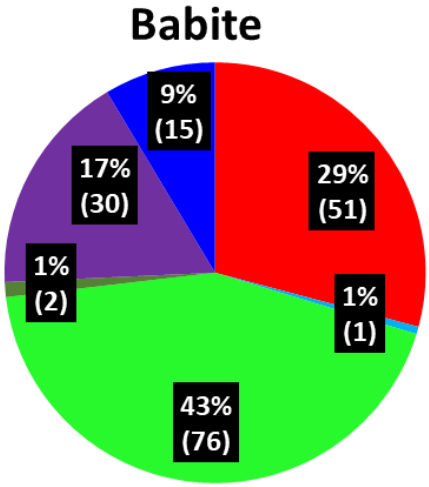


Figure 18: Spatial distribution of *B. burgdorferi* s.l. in Babite

The colors were assigned to the respective species as follows:

B. afzelii ***B. bavariensis*** ***B. burgdorferi* s.s.**

B. garinii ***B. lusitaniae*** ***B. valaisiana***

***B. garinii* is the dominating species in this region, this is followed by *B. afzelii*, then *B. valaisiana*, *B. burgdorferi* s.s., and finally *B. bavariensis* and *B. lusitaniae* which are almost equally represented.**

From Babite, 175 isolates were available. *B. garinii* dominated this habitat, making up 51 % of infected ticks. *B. afzelii* followed with 29 %, *B. valaisiana* 17 %, *B. burgdorferi* s.s. 15 %, and *B lusitaniae* and *B. bavariensis* at 1 % each. Thus, the bird associated species *B. garinii* and *B. valaisiana* accounted for nearly 70 % of infected ticks.

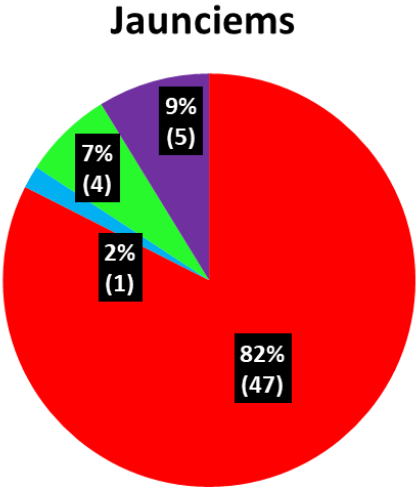


Figure 19: Spatial distribution of *B. burgdorferi* s.l. in Jaunciems

Color-coding as in fig. 18. *B. afzelii* accounts for 82 % of infected ticks in this region, whilst the remaining species are represented at 5 % or lower.

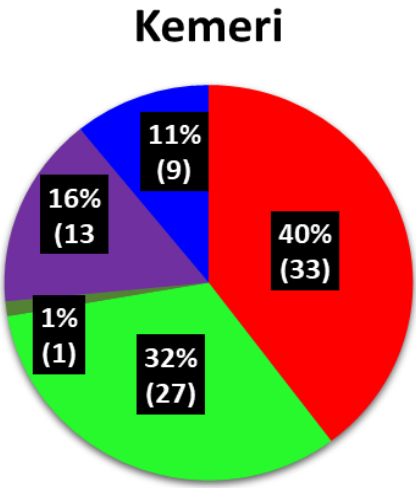


Figure 20: Spatial distribution of *B. burgdorferi* s.l. in Kemeri

Color-coding as in fig. 18. Even though *B. afzelii* dominated also in Kemeri with 33/83 number of assigned species (40 %), *B. garinii, B. valaisiana* and *B. burgdorferi* s.s. were also well represented at 32 %, 13 %, 9 %, respectively. *B. lusitaniae* was also identified in a single tick in this habitat

In summary, *B. afzelii* was found to be dominanting in two regions, Jaunciems and Kemeri. *Borrelia garinii* was found to be dominating in Babite. Other common species in Europe were also well represented in Babite and Kemeri. Babite was the most species rich region, six species were observed. Kemeri and Jaunciems had five and four species respectively.

5. 4. 2 Temporal Distribution of *B. burgdorferi* s.l. in Latvia

To better understand the composition variation of *B. burgdorferi* s.l. over the years in Latvia, a temporal distribution of species was conducted. A total of 315 isolates were included in this study from the year 1999 to 2010 (Figure 21: Temporal distribution of *B. burgdorferi* s.l. species in Babite; Figure 22:

Temporal distribution of *B. burgdorferi* s.l. species in Jaunciems; Figure 23: Temporal distribution of *B. burgdorferi* s.l. species in Kemeri). For some periods i.e. from Kemeri 2002, Kemeri 2010 and Jaunciems 2010, there were not enough data to make a comparison hence such periods were excluded from this comparison.

Babite was the most species rich region; in this region six species were observed: *B. afzelii*, *B. bavariensis*, *B. burgdorferi* s.s., *B. garinii*, *B. lusitaniae* and lastly *B. valaisiana* (Figure 21: Temporal distribution of *B. burgdorferi* s.l. species in Babite). In Kemeri all these species were identified with exception of *B. bavariensis* which was not found in this habitat (Figure 23: Temporal distribution of *B. burgdorferi* s.l. species in Kemeri). Jaunciems on the other hand had only four species: *B. afzelii*, *B. bavariensis*, *B. garinii* and *B. valaisiana* (Figure 22: Temporal distribution of *B. burgdorferi* s.l. species in Jaunciems).

In Babite *B. afzelii* dominated in the years 1999, 2000 and 2002. In the last sampling years; 2006, 2007 and 2010 however *B. garinii* dominated. *B. valaisiana* was also well represented in the years 2002, 2006, 2007 and 2010. *B. burgdorferi* s.s. was observed in the years 1999, 2000, 2003 and 2007.

The apparent domination of *B. afzelii* in Jaunciems was once again striking. Whereby in 2002, 2003, 2006 and 2007 this was the only species identified in this region. *B. lusitaniae*, *B. valaisiana* and *B. garinii* were also identified but in small percentages in the years 1999, 2000 and 2001.

Kemeri seem to have a constant species fluctuation. Even though *B. afzelii* dominated in the years 2000, 2001, 2006 and 2007, other species like *B. valaisina*, *B. garinii* and *B. burgdorferi* s.s. were well represented. *B. lusitaniae* was also identified in the year 2001.

In conclusion, it can be said that Babite and Kemeri seem to undergo constant species fluctuations whereby species domination interchange between *B. afzelli* and *B. garinii*. Jaunciems on the other hand seem to be majorly dominated by *B. afzelii*.

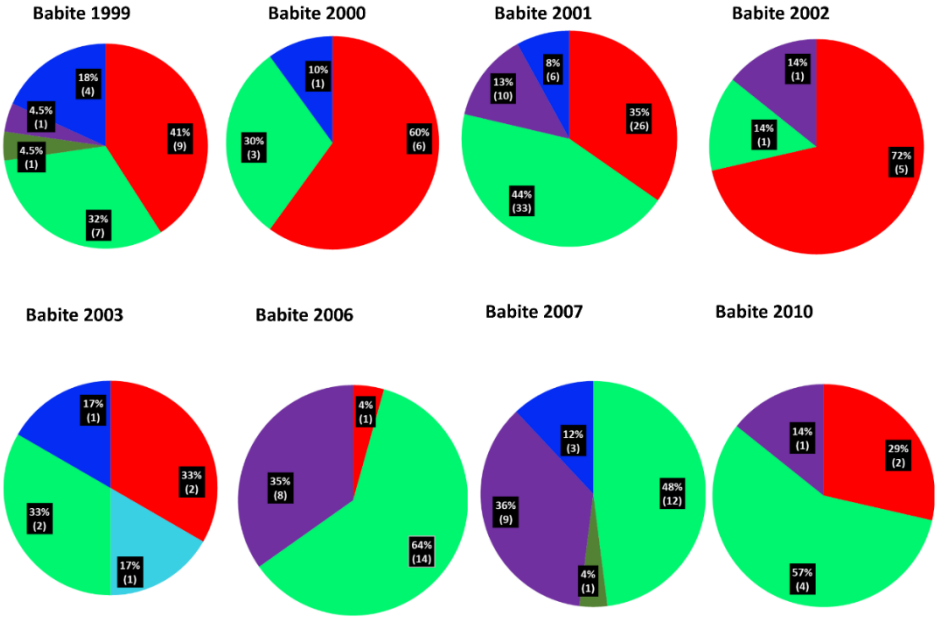


Figure 21: Temporal distribution of *B. burgdorferi* s.l. species in Babite

The colour-coding was as follows;

B. afzelii ***B. bavariensis*****,** ***B. burgdorferi* s.s.**

B. garinii ***B. lusitaniae*** ***B. valaisiana***

As can be seen *B. afzelii* dominated in the years 1999, 2000 and 2002, and the rest of the years *B. garinii* dominated, with exception of 2003 where the prevalence of *B. afzelii* and *B. garinii* were equal.

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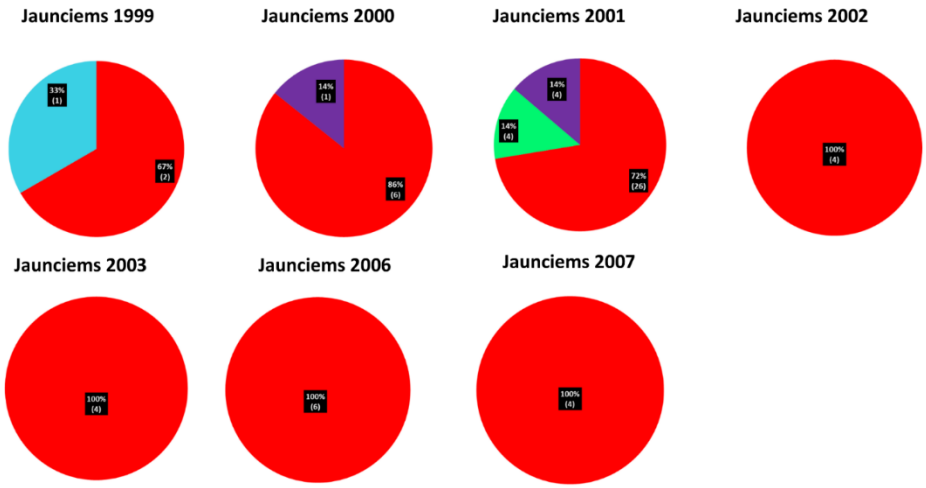


Figure 22: Temporal distribution of *B. burgdorferi* s.l. species in Jaunciems

Color- coding as in figure 21. *B. garinii* was the only species identified in the years 2002-2007. In the years 1999 and 2001, it was the dominating species but othe species like *B. bavariensis*, *B. garinii* and *B. valaisiana* were also identified

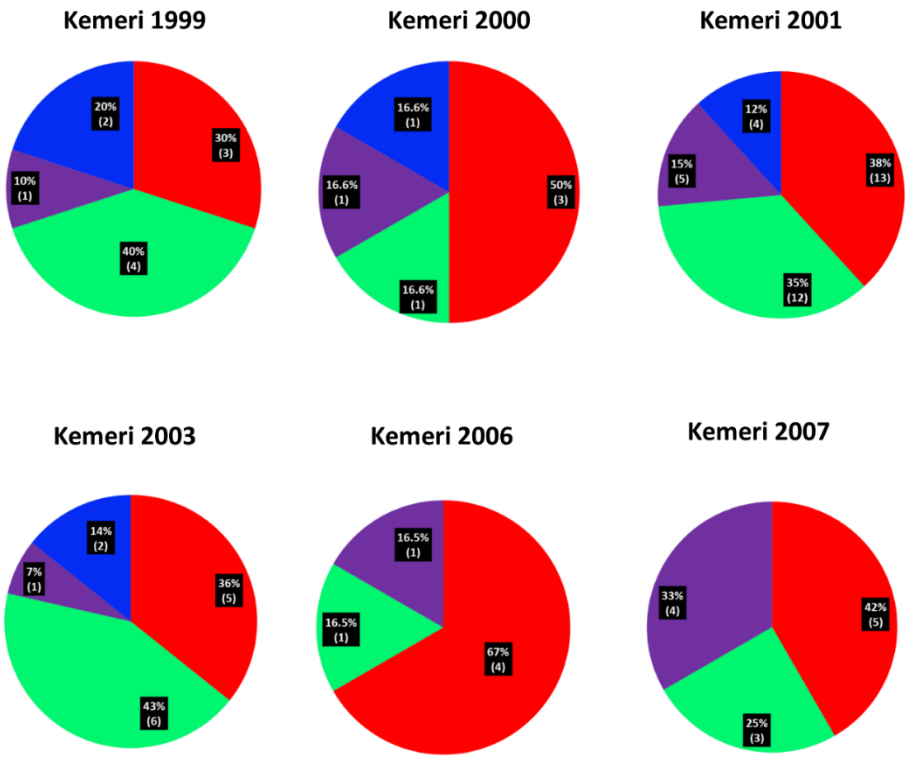


Figure 23: Temporal distribution of *B. burgdorferi* s.l. species in Kemeri

Color- coding as in figure 21. In this habitat all the species identified were well represented, even though *B. afzelii* and *B. garinii* dominated in some years.

In summary, Babite and Kemeri exhibits greater fluctuations of species over the entire period of sampling. *B. afzelii* is the most common species in Jaunciems, particularly for the years 2002, 2006 and 2007 it was the only species in this region. In 1999, 2000 and 2001 a small percentages of other species were also detected.

5. 4. 3 Species Diversity

In order to assess the *Borrelia* species diversity at the three sites in Latvia, MLST PCR was conducted on the 364 tick samples positive by screening PCR. Eighty-two of these 364 samples were untypeable, i.e. no species determination was possible, perhaps due to low total amout of *Borrelia* DNA. Mixed infection, i.e. sequences in which mixed bases were found at the same position in the fragment amounted to 55 samples, those could not be assigned to species level or included in further analyses. Only 227 samples generated MLST PCR products; i.e at least one out of eight housekeeping genes were successfully amplified and sequenced. In addition to this we had 88 MLST sequences which had been processed in the years 2002, 2006 and 2007.

Combined data over all years and all habitats showed that the most prevalent species detected in Latvian ticks was *B. afzelii* (40.95 %), followed by *B. garinii* (34.29 %), *B. valaisiana* (15.87 %), *B. burgdorferi* s.s. (7.30 %), *B. bavariensis* and *B. lusitaniae* but at low prevalences of about 1 % (Table 11: Species diversity in Latvia per year).

Table 11: Species diversity in Latvia per year

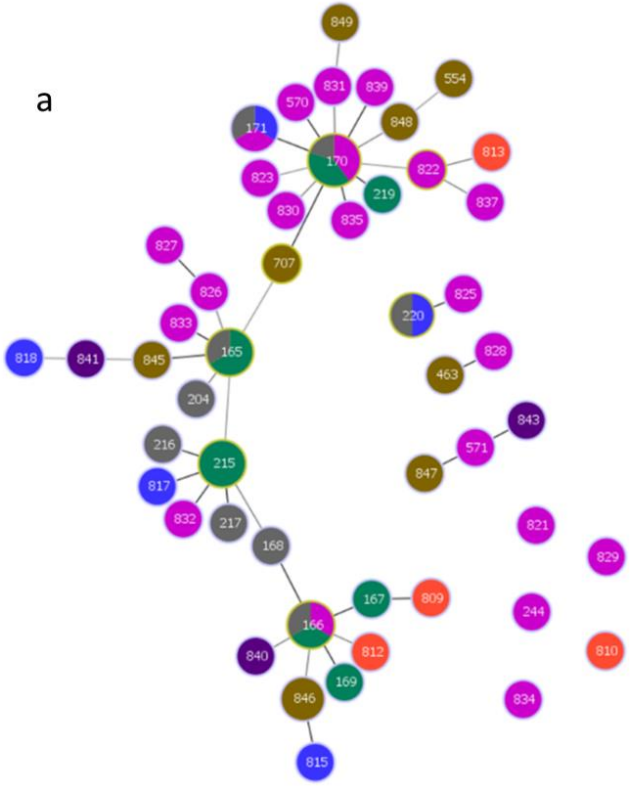
Species	1999	2000	2001	2002	2003	2006	2007	2010	Total	Total %
B. afzelii	13	15	61	9	10	11	9	1	129	40.95
B. bavariensis	1				1				2	0.63
B. burgdorferi s.s.	6	2	9		3		3		23	7.30
B. garinii	10	5	50	1	8	15	15	4	108	34.29
B. lusitaniae	1			1			1		3	0.95
B. valaisiana	2	3	20	1	1	9	13	1	50	15.87
Total	**33**	**25**	**140**	**12**	**23**	**35**	**41**	**6**	**315**	**100**

Genospecies *B. afzelii*, *B. garinii*, *B. valaisiana* and *B. burgdorferi* s.s. were present in all of the three collection sites in Latvia. The three common genospecies in Europe: *B. afzelii*, *B. garinii* and *B. valaisiana* accounted for more than 90 % of the total genospecies counted.

5. 4. 4 Temporal Distribution of Sequence Types in the Sampling Sites

One of the aims of this study was to determine the interspecies changes within the population, i.e to see whether different STs dominate at different times. For this reason, available STs were grouped into species analyzed by help of goeBURST software. In goeBURST, allelic profiles generated from MLST sequences are used. It gives information on how STs of a given species are distributed and whether certain STs of a given species dominates at one given time point or in a certain region. goeBURST identifies founder and subfounder STs and STs relationship through single (SLV), double (DLV), and triple locus variant (TLV). Following figures show the temporal distribution of *B. burgdorferi* s.l. MLST STs which were available from eight different years (Figure 24: Temporal distribution of *B. afzelii* STs in Lativa; a) STs distribution and b) the quantity of STs per year; Figure 25: Temporal distribution of *B. burgdorferi* s.s. STs in Lativa; a) STs distribution and b) the quantity of STs per year; Figure 26: Temporal distribution of *B. garinii* STs in Lativa: a) STs distribution and b) quantity of STs per year; Figure 27: Temporal distribution of *B. valaisiana* STs in Lativa; a) STs distribution and b) the quantity of STs per year). It should be noted that in the year 2003 infection rates were particularly low in comparison to other years (16 %) hence it is difficult to draw a conclusion for this year.

In conclusion, there was no evidence of specific STs dominating at specific times; rather there was arbitrary distribution of STs regardless of the sampling year for the four analyzed species.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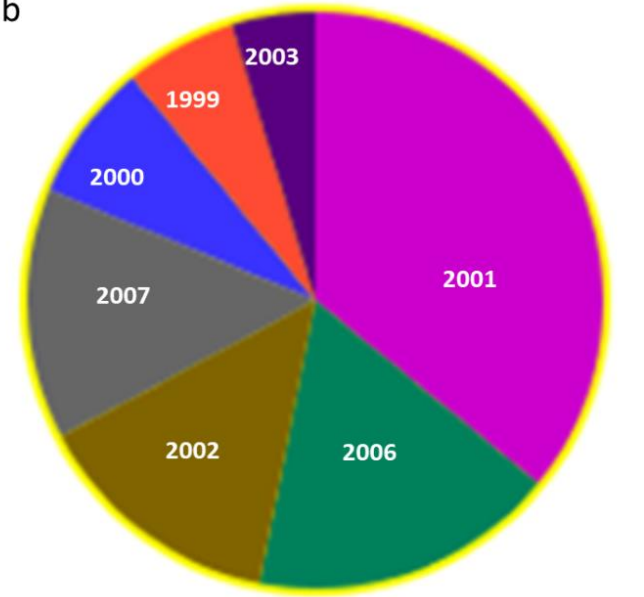
b
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1999
2000
2001
2007
2002
2006

Figure 24: Temporal distribution of *B. afzelii* STs in Lativa; a) STs distribution and b) the quantity of STs per year

The diagram shows the relationship of *B. afzelii* STs based on TLV. The distribution of STs from 1999-2007 in Latvia is indicated with different colors as follows:

1999 2000 2001 2002 2003 2006 2007

The size of the circles corresponds to the number of STs. Many STs occurred only once, while a few appeared more than once; e.g. ST166 and 170, which were onserved in the years 2001, 2003 and 2006, and ST165 which was observed in the years 2006 and 2007. As can be seen the greatest number of samples were from the year 2001 (pink) and the least number of samples were from the year 2003 (purple).

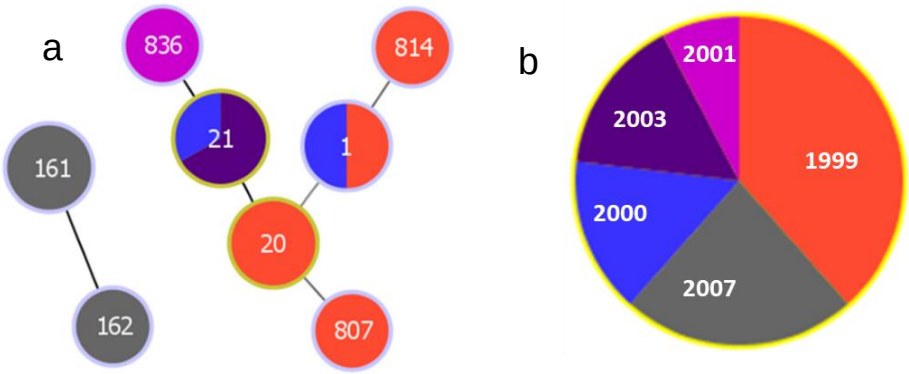

Figure 25: Temporal distribution of *B. burgdorferi* s.s. STs in Lativa; a) STs distribution and b) the quantity of STs per year

Population structure of *B. burgdorferi* s.s. and temporal distribution of STs in Lativa based on TVL Color-coding as in fig. 24. There was a small number of positive *B. burgdorferi* s.s. for this analysis. Figure 25 shows that there were four STs from the year 1999 (red), two STs from the year 2000 (blue), one ST from the year 2001, one ST from the year 2003 (purple) and two STs from the year 2007 (dark grey). The greatest number of samples were from the year 1999 (red) and the least number of samples from the year 2001 (pink).

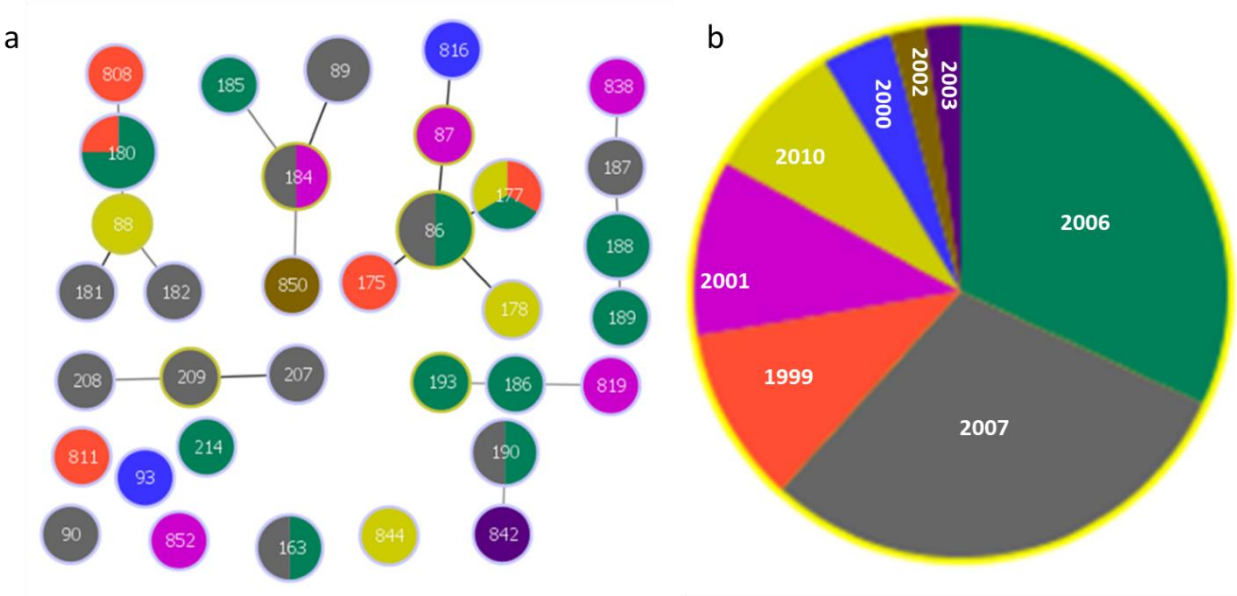

Figure 26: Temporal distribution of *B. garinii* STs in Lativa: a) STs distribution and b) quantity of STs per year

Population structure of *B. garinii* and temporal distribution of STs in Lativa. The diagram shows the relationship of *B. gariniii* STs based on TLV. The colour-coding was as follows:

1999 **2000** **2001** **2002** **2003** **2006** **2007** **2010**

Again, here random distribution of STs is apparent. Shared STs were observed in the years 1999, 2006 and 2010 (ST177), 1999 and 2006 (ST180), 2001 and 2007 (ST184) and lastly 2006 and 2007 (ST86, ST163 and ST190). There was just one single ST of *B. garinii* from the years 2002 and 2003.

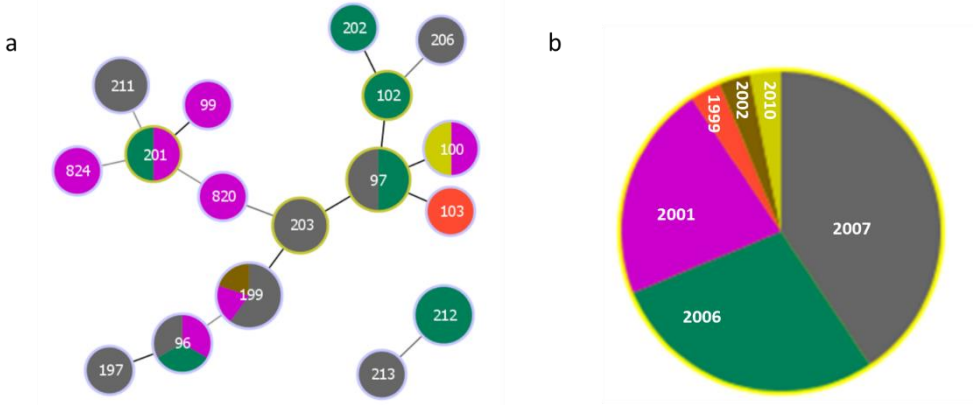

Figure 27: Temporal distribution of *B. valaisiana* STs in Lativa; a) STs distribution and b) the quantity of STs per year

Population structure of *B. valaisiana* and temporal distribution of STs in Lativa. Color-coding as in fig. 26. There was a a single ST of *B. valaisiana* from the year 1999 (red), 2002 (olive green) and 2010 (lemon yellow). Shared STs were found in the years 2001, 2006 and 2007 (ST96), 2001, 2002 and 2007 (ST199), 2001 and 2006 (ST201) and 2006 and 2007 (ST97). The highest number of different STs was found in the year 2007.

Generally it was demonstrated that there was no clear pattern when it come to STs distribution over the years. This raises the question of gene stability of *Borrelia* species.

5. 4. 5 Phylogenetic Analysis of *B. burgdorferi* s.l. based on MLST Housekeeping Genes

Out of 227 samples of which at least one MLST gene was successfully amplified and sequenced, 71 samples were used for further analysis. All the eight housekeeping genes from these 71 samples did not contain any mixed sequences and had good coverage when analyzed on the CLC workbench hence were used for phylogenetic analysis. The following species were noted, 34 *B. afzelii*, one *B. bavariensis*, ten *B. burgdorferi* s.s., 17 *B. garinii* and nine *B. valaisiana*. A total number of 56 different STs were identified. Some STs were found multiple times: ST177, ST171, ST170, ST100, ST21, ST20 (were all found twice with exception of ST21 which was found three times) and lastly ST1 which was also found twice in the dataset. In such cases, one sample was choosen randomly to represent the others, such that every ST was represented just once in the phylogenetic tree. In addition, for each species a type strain was included to get a better understanding how the isolates from this study clustered with the type strains of species. Sequences of type strains were downloaded from the MLST database (https://pubmlst.org/borrelia/).

The molecular evolutionary history of these isolates was inferred using the Maximum-Likelihood method based on the General Time Reversible model. The analysis involved 68 nucleotide sequences (63 from our study and five type strains).

The bootstrapping value varied widely, some branches were well supported while others were not, and this was regardless of the species. Interspecies nodes were well supported with values of > 90 %. However terminal nodes of *B. afzelii* were not so well supported. The species types which were included in the generation of phylogenetic tree clustered closely with the respective species isolates in this study.

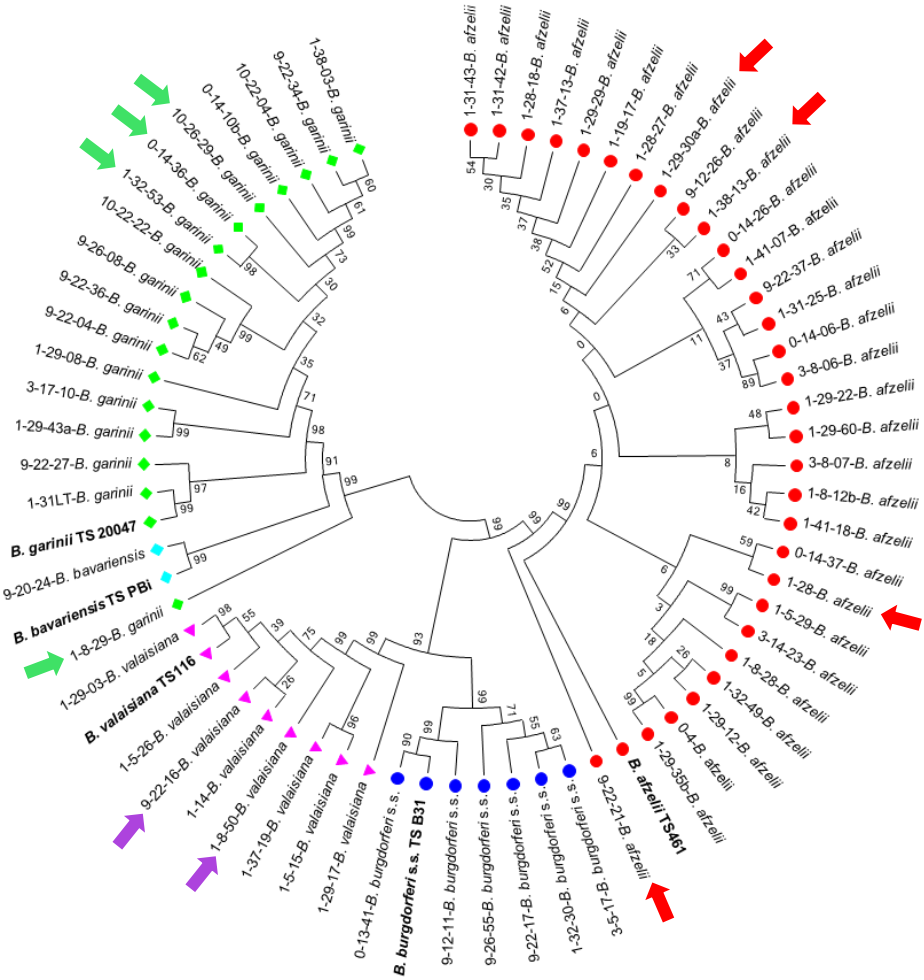

Figure 28: Pyholgenetic tree of concatenated MLST genes of *Borrelia spp.*

Specific colours are designated to species as follows:

B. afzelii ***B. bavariensis*** ***B. burgdorferi* s.s.**

B. garinii ***B. valaisiana*.**

Possible recombinations are shown in colour coded arrows as follows: red arrows for *B. afzelii*, green arrow for *B. garinii* and purple arrows for

***B. valaisiana*. The tree is well supported at the root level, at species level *B. garinii*, B. valaisiana and *B. burgdorferi* s.s. are also well supported at the terminal nodes.**

One *B. garinii* strain (1-8-29) clustered outside the *B. garinii*-clade. This is because seven of the eight housekeeping genes were classified as B. *garinii* on MLST database and one gene (*pyrG*) as *B. afzelii*. This may be probably due to recombination.

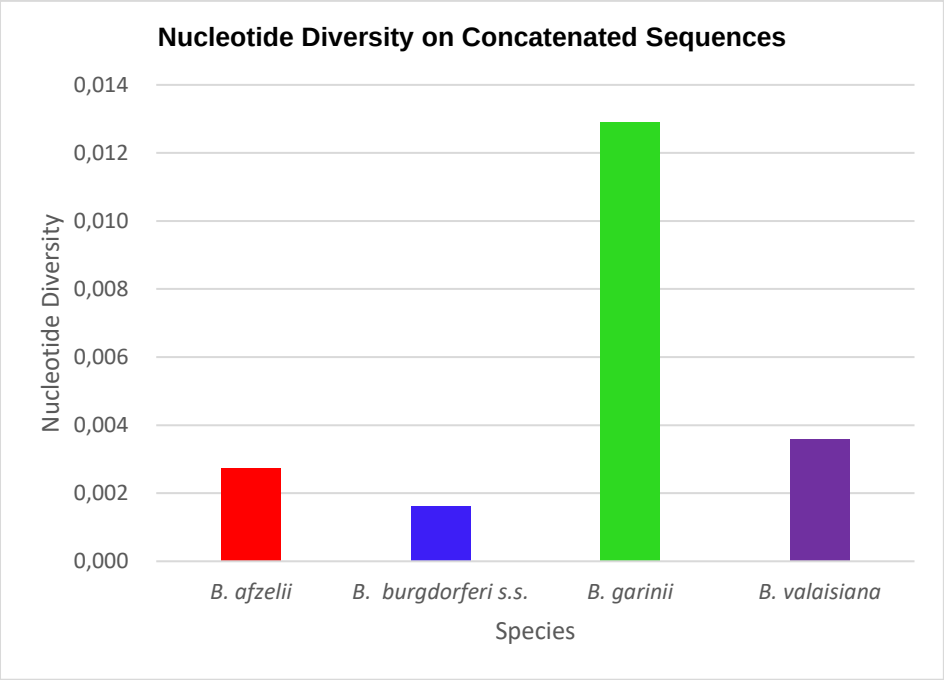

Figure 29: Tajima's test of neutrality on concatenated sequences

As can be seen the bird associated *B. burgdorferi* s.l. species (*B. garinii* and *B. valaisiana*) showed the most diversity, whereby the difference between *B. valaisiana*, *B. afzelii* and *B. burgdorferi* s.s. is probably negligible.

Tajima`s test of neutrality was carried out to provide statistical data for the sequences. It was conducted with concatenated sequences (Figure 29: Tajima's test of neutrality on concatenated sequences) and also on non concatenated isolates at single gene level per species (Figure 30: Tajima's test of neutrality at single gene level). The nucleotide diversity (π) values obtained were between 0.012 and 0.002 on concatenated sequences and 0.000 and 0.039 at single gene level. Generally *B. garinii* shows the greatest diversity both at single gene level and at concatenated sequences level. *B. garinii* had the highest value with 0.039 (*clpX*). *B. burgdorferi* s.s. (*clpX, pepX*) and *B. valaisiana* (*uvrA*) showed the least diversity at single gene levels.

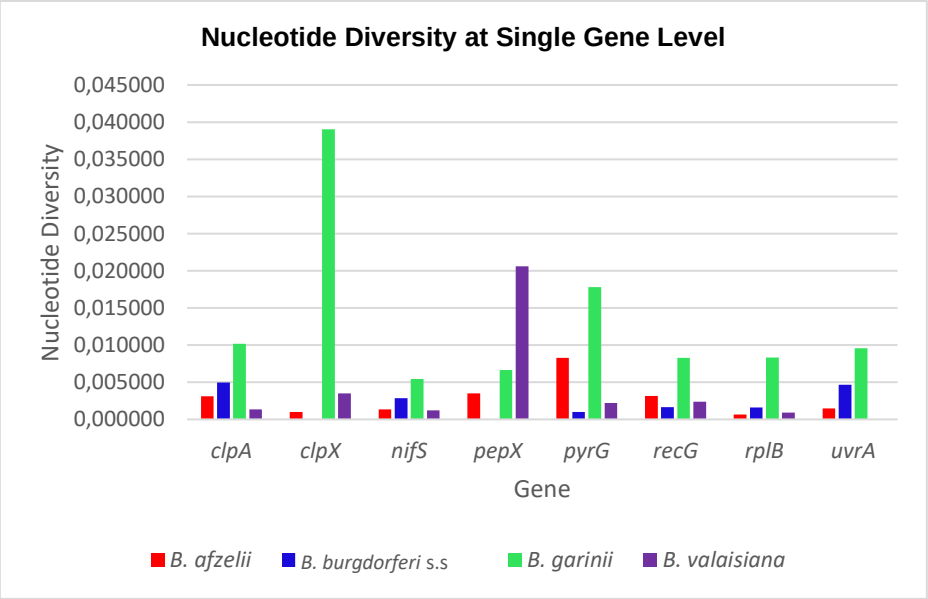

Figure 30: Tajima's test of neutrality at single gene level

Again *B. garinii* shows the most diversity, however it is apparent that *clpX* and *pyrG* possesses by far the highest variation. Also *pepX* of *B. valaisiana* shows a higher variation. It is also apparent that *rplB* (*B. afzelii* and *B. valaisiana*), *pyrG* (*B. burgdorferi* s.s.), *clpX* (*B. afzelii* and *B. burgdorferi* s.s.), *pepX* (*B. burgdorferi s.s.*) and lastly *uvrA* (*B. valaisiana*) have little or no variation at all.

5. 4. 6 Analysis of Relationships Among Sequence Types by goeBURST

In order to get a different view of how the isolates from this study are related to one another a goeBURST analysis was conducted. goeBURST replaced the software eBUSRT which groups closely related MLST dataset together and creates a network of clonal complex and predicts the ancestral founder of each clonal complex (Feil et al., 2004).The analysis is based on allelic profiles determined for each ST and therefore gives a different view of the relationship in comparison to phylogenetic sequence based analysis. It is assumed that a large percentage of a population belong to clusters of closely related genotypes, which build up a clonal complex (CC). A clonal complex comprises of a single founder genotype linked with other closely related genotypes which diversifies from it over time (Feil and Spratt, 2001).

In the case of MLST the progenies of the founder genotype remain unchanged in their allelic profile at the beginning, but new variants arise as in one of the eight housekeeping loci changes occur either by point mutation or by recombination (Feil et al., 2004) . If for instance the allelic profile of an isolate differs from its founder in only one of the eight loci, a single locus variant (SLV) is generated = distance level one. If they differ in two loci = distance level two, a double locus variant (DLV) is generated, if they differ at three loci = distance level three, triple locus variants (TLV) are generated and so on. Since there are eight housekeeping genes the highest possible distance level is eight.

A total of 159 isolates were used here for phylogenetic inferences. These isolates were all from the three regions in Latvia as already mentioned in chapter two: Babite, Jaunciems and Kemeri. *B. bavariensis* and *B. lusitaniae* were excluded from this analysis as there were too few isolates to draw a meaningful conclusion. Samples were available from the years 1999, 2000, 2002, 2003, 2006, 2007 and 2010. For the purpose of this study, a default of TLV was set, meaning only isolates whose allelic profile differs in maximum three loci are connected. Thereby, a cc diagram is created.

The 159 isolates were grouped into 109 STs, meaning identical STs were identified (see Appendix 16: STs Analysed in this study.

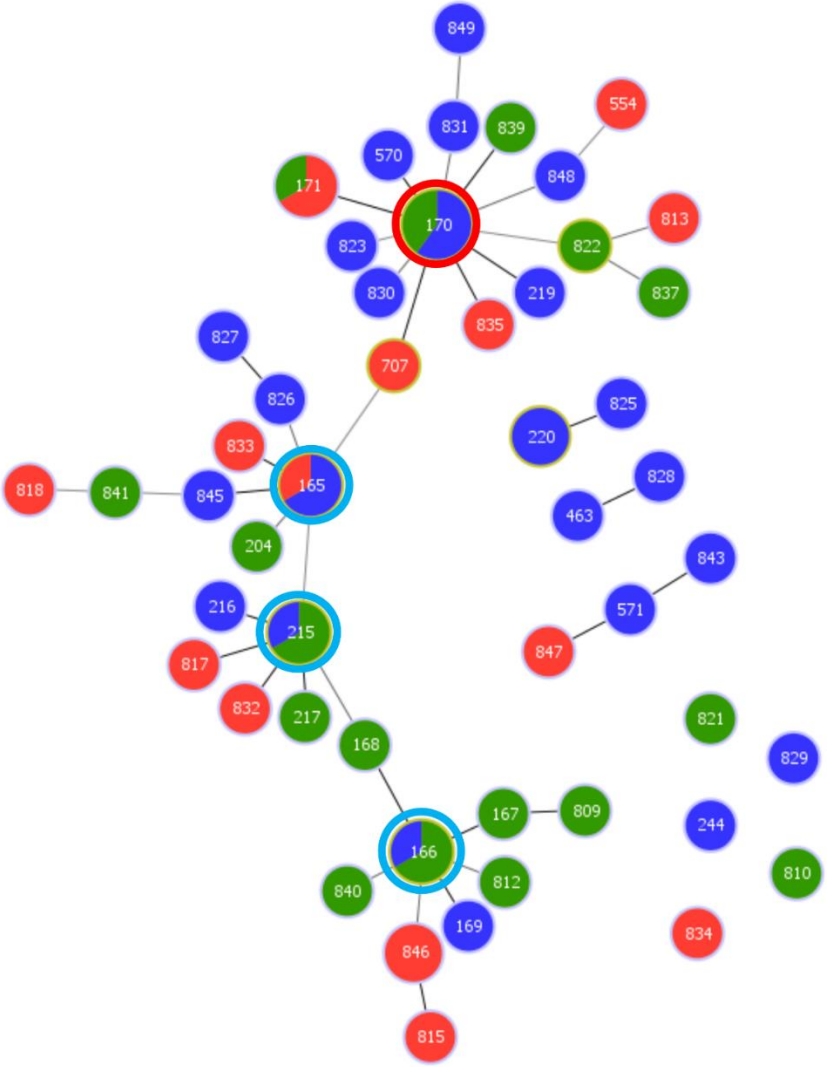

Figure 31: Spatial distribution of B. afzelii STs in Babite, Jaunciems and Kemeri

The color-coding was as follows:

Babite **Jaunciems** **Kemeri**

The greatest number of *B. afzelii* STs were from Jaunciems, then Kemeri and lastly Babite. Shared STs were identified between Jaunciems and Kemeri (ST166, ST170 and ST215) and also between Babite and Jaunciems (ST165). ST170 was also identified to be the possinle founder of this complex (circled red), the other shared STs were identified to be subfounders.

There were 64 STs in *B. afzelii* which were combined/ joint into two major clonal complexes, two minor (consisting of only two STs) clonal complexes and five singletons (Figure 31: Spatial distribution of *B. afzelii* STs in Babite, Jaunciems and Kemeri) One large clonal complex consisted of a founder (ST170) which, according to eBURST bootstrapping, had 99 % probability of being the founder of this complex (Table 12: Clonal complexes formed based on TVL settings and their founders / subfounders per species). ST165, ST166 and ST215 were classified as subfounders in the clonal complex (red circle showing the founder and clue circles the subfounders). The second clonal complex consisted of three STs with ST571, ST843 and ST847, the latter likely to be the founder.

More shared STs occurred in Jaunciems and Kemeri than in Jaunciems and Babite or Babite and Kemeri.

Thirteen isolates were used for *B. burgdorferi* s.s. goeBURST. STs from only two regions were available. One clonal complex was found and one minor clonal complex. There was no group founder identified, ST21 (see blue circle) was identified to be subfounder.

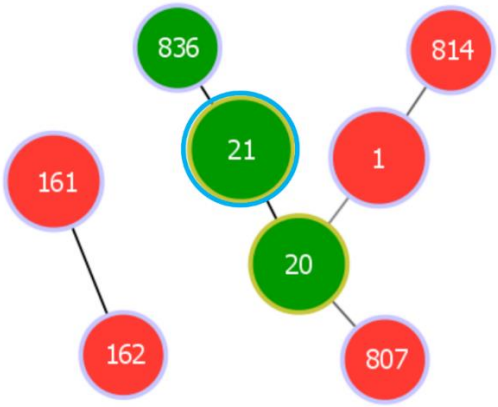

Figure 32: Spatial distribution of *B. burgdorferi* s.s. STs in Babite, Jaunciems and Kemeri

The color-coding as in fig. 31. Founder ST was not identified for *B. burgdorferi* s.s. but a subfounder was identified to be ST21 (circled blue). STs available were only from Babite (red) and Kemeri (green). No mutual *B. burgdorferi* s.s. ST were found for these regions. There was no ST available for this analysis from Jaunciems.

Forty-seven STs were used in goeBUSRT analysis of *B. garinii* (Figure 33: Spatial distribution of *B. garinii* STs in Babite, Jaunciems and Kemeri). Six clonal complexes were built (consisting of at least 3-STs) and one minor clonal complex with just 2-STs. A group subfounder was only determinable for one clonal complex (circled ST in blue).

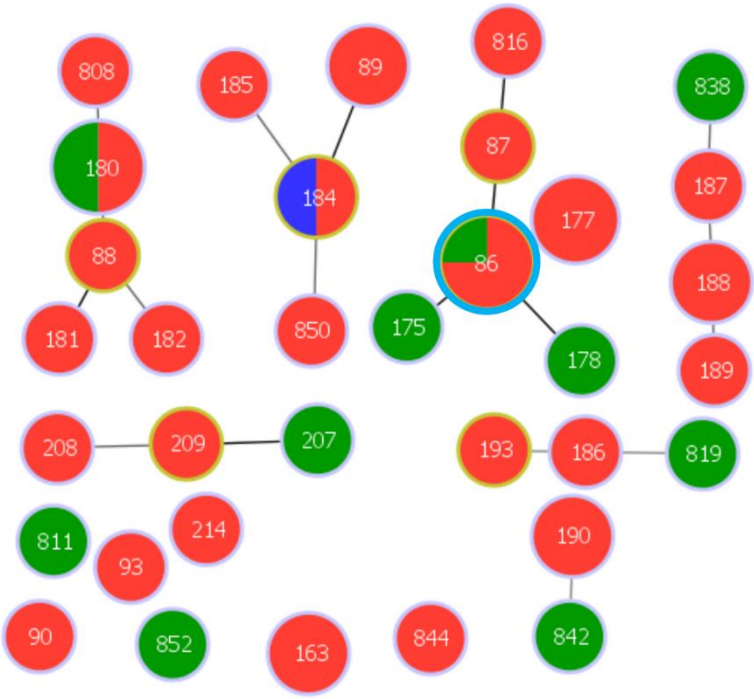

Figure 33: Spatial distribution of *B. garinii* STs in Babite, Jaunciems and Kemeri

The greatest number of *B. garinii* were from Babite (red), followed by Kemeri (green) and lastly Jaunciems with just a single ST (blue). Six clonal complexes were found, one minor clonal complex and seven singletons. ST180 and ST86 were found in Babite and Kemeri, and ST184 was found in Babite and Jaunciems.

Thirty-two *B. valaisiana* STs were available for goeBUSRT analysis in this group (Figure 34: Spatial distribution of B. valaisiana STs in Babite, Jaunciems and Kemeri). One clonal complex was created and one minor clonal complex. ST97 was identified as the group founder (in red circle) and three subfounders were also indentified (showed in blue circles).

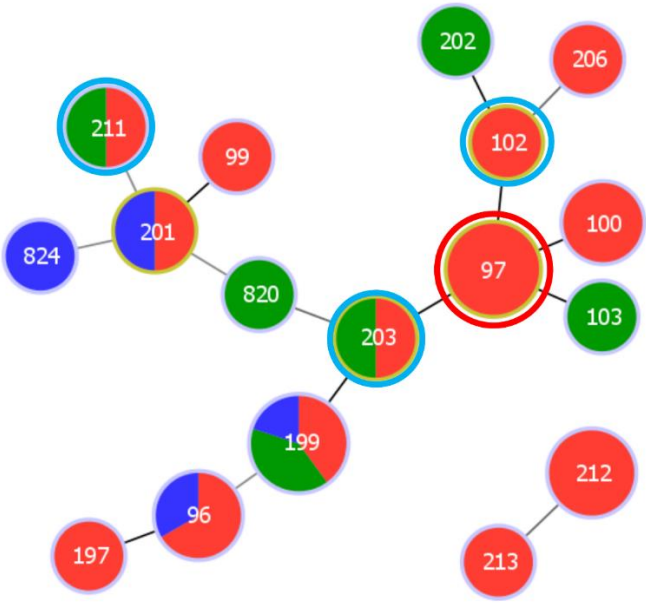

Figure 34: Spatial distribution of B. valaisiana STs in Babite, Jaunciems and Kemeri

Babite was the locality where the greatest number of *B. valaisiana* were found (red), followed by Kemeri (green) and lastly Jaunciems (blue). ST211 and ST203 were found in Babite und Kemeri, ST201 was found in Babite and Jaunciems and lastly ST199 was found in all the three regions.

5. 4. 7 Detection of Potential Recombination Events

In this study a clonal complex (CC) was defined based on a TLV setting, STs which differed in no more than three alleles were connected by the goeBURST alogarithm to form a clonal complex. Following this definition, ten clonal complexes were formed and five minor clonal complexes (in which only two STs were connected) (Table 12: Clonal complexes formed based on TVL settings and their founders / subfounders per species). The formed clonal complexes were assigned with a number according to the founder.

Table 12: Clonal complexes formed based on TVL settings and their founders / subfounders per species

Species	Major clonal complex	Minor clonal complex	Founder ST
B. afzelii	CC170		ST170
	CC571		Not found
		CC220	Not found
		CC463	Not found
B. burgdorferi s.s.	CC21		ST21 (subfounder)
		CC161	Not found
B. garinii	CC86		ST86
	CC180		Not found
	CC185		Not found
	CC187		Not found
	CC207		Not found
	CC193		Not found
		CC190	Not found
B. valaisiana	CC97		ST97
		CC203	Not found
		CC102	Not found

Borrelia afzelii showed one major clonal complex (CC170), in which at least 38 STs were involved with one group founder (ST170) and three subfounders (ST65, ST166 and ST215). This complex shows how *B. afzelii* isolates are closely related. *B. garinii* formed the greatest number of clonal complexes (six), which in turn mirrors the diversity of this genospecies. Clonal complexes of *B. valaisiana* and *B. burgdorferi* s.s. were comparable to that of *B. afzelii*. Several singletons were also identified for *B. afzelii* and *B. garinii*. There was no recognizable tendency that STs from a specific year or region formed a clonal complex. The comparison of clustering of STs in goeBURST diagrams and phylogenetic trees is one way of recognizing putative recombination events within species. If there is no recombination the position of different STs in

goeBURST diagrams should be mirrored in the phylogenetic tree. Using this method, we compared the goeBURST diagrams of STs of the species with their positioning in phylogenies and found that there were very few differences. However, some STs clustered with completely different neighbors in goeBURST diagrams and phylogenies, suggesting possible recombination events. For *B. afzelii*, *B. garinii* and *B. valaisiana* four, four and two putative recombinations were observed respectively (indicated with arrows in). No potential recombination was recognized within the *B. burgdorferi* s.s. isolates analyzed here.

5. 4. 8 Phylogenetic Analysis of OspC Gene Sequences

One of the aims of this study was to find out whether MLST-types correlated with OspC-type lineages. To this end samples were chosen from which at least three MLST genes had already been amplified and sequenced successfully. Following this a total of 121 samples were subjected to OspC PCR to amplify this gene. Of these 108 samples generated good PCR products which could be sequenced but only 52 samples had good sequences with no ambiguities and hence could be used for further analysis. The remaining 69 sequences had either mixed base that would indicate mixed strains or were too short (<450 bp) to be used and were excluded from phylogenetic analysis.

The phylogenetic tree in figure 35 depicts the relationship of OspC gene fragments of *Borrelia* species which were available for this analysis (Figure 35: Phylogenetic tree of OspC gene fragment as constructed for *Borrelia spp.*). The tree with the highest log likelihood (-8333, 1220) is shown. An unrooted tree was created. Some terminals (*B. afzelii*, *B. burgdorferi* s.s. and *B. valaisiana*) were well supported at the terminal clade. *B. garinii* seemed to have originated from a common ancestor with exception of one sample, which clustered with *B. afzelii*. Also one *B. burgdorferi* s.s. and one *B. valaisiana* did clade outside their respective groups.

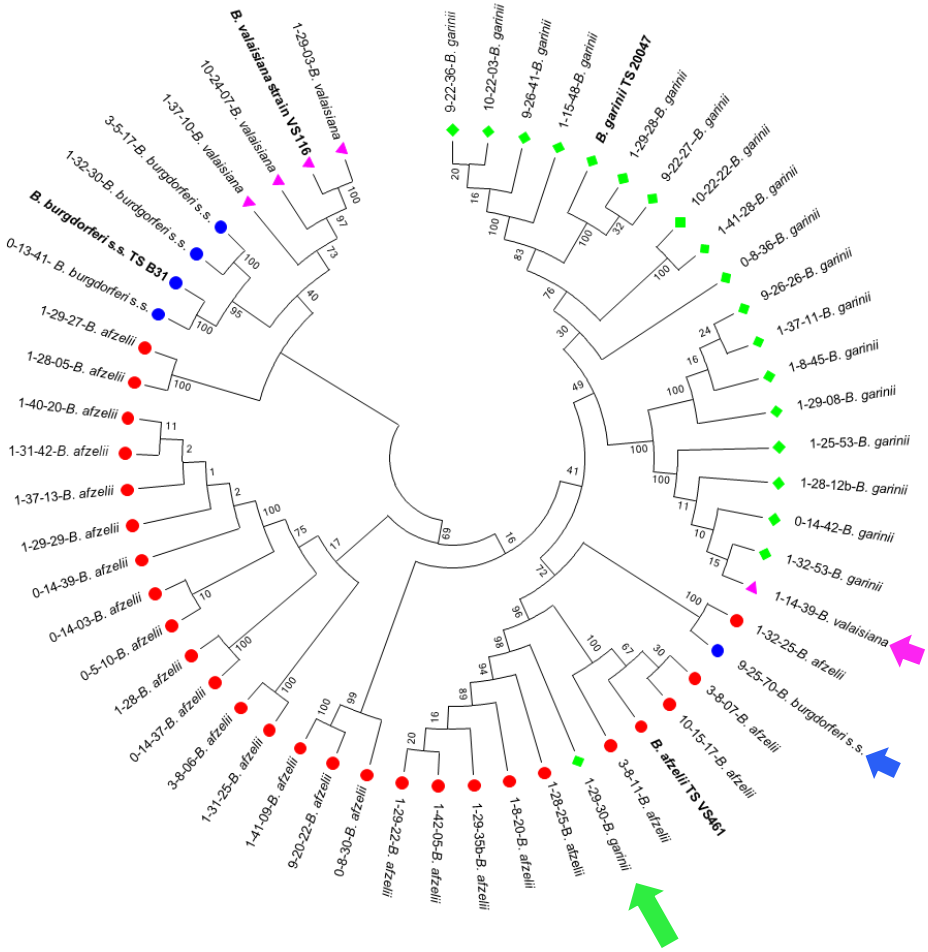

Figure 35: Phylogenetic tree of OspC gene fragment as constructed for *Borrelia spp.*

The tree with the highest log likelihood is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 56 nucleotide sequences. Colour red represents *B. afzelii*, coulor blue *B. burgdorferi* s.s., green for *B. garinii*,

colour pink *B. valaisiana*. The arrows are pointing to the isolates which have possible recombinations.

In conclusion it can be said that, OspC strains of the same species do not always cluster together as can be seen above. This is an indication of possible recombination of these gene fragments in the past.

5. 4. 9 Determination of OspC Major Groups

The pairwise distance of OspC gene fragments was calculated in MEGA6 (Tamura et al., 2013). This provides a value for the evolutionary distance between pairs of sequences. Sequences either belong to the same OspC major group, when the p-distance ≤ 0.02 or to different OspC major groups, when p-distance ≥ 0.08. Analysis were conducted using the Kimura 2-parameter model (Kimura, 1980). All positions containing gaps and missing data were eliminated. For a full list of the calculated distances, *B. burgdorferi* s.s. OspC types were named according to the Barbour`s described system (Barbour and Travinsky, 2010) (Appendix 8: OspC major groups of *B. afzelii*; Appendix 9: OspC major groups of *B. burgdorferi* s.s.; Appendix 10: OspC major groups of *B. garinii*; Appendix 11: OspC major groups of *B. valaisiana*). Other *B. burgdorferi* s.l. species OspC types were named according to the species and country i. e. afzeLA1 for *B. afzelii* from Latvian ticks.

There were 25 *B. afzelii* OspC sequences, these were resolved into 10 OspC major groups, the first two major groups consisted of at least five isolates. From group 7-10 there was just a single isolate for each group.

There were four *B. burgdorferi* s.s., the first major group consisted of two isolates, and the second major group consisted of one isolate. The third unclassified group seem to have undergone recombination in the recent past hence could not be classified, as the MLST sequences shows it is a *B. burgdorferi* s.s. strain and according to the OspC sequences-blast on the pubmed (https://www.ncbi.nlm.nih.gov/pubmed/) it is an *B. afzelii* strain. In the OspC phylogenetic tree it did cluster with *B. afzelii* (Figure 35: Phylogenetic tree of OspC gene fragment as constructed for *Borrelia spp.*).

Table 13: *Borrelia afzelii* groups, assigned isolates numbers and types

Group	Isolate no.							Type
Group 1	1	4	6	12	15	18	21	afzeLA1
Group 2	3	7	9	10		14		afzeLA2
Group 3	5	8						afzeLA3
Group 4	16	19	20					afzeLA4
Group 5	22	23						afzeLA5
Group 6	24	25						afzeLA6
Group 7	2							afzeLA7
Group 8	13							afzeLA8
Group 9	17							afzeLA9
Group 10	26							afzeLA10

Table 14: *B. burgdorferi s.s.* groups, assigned isolate numbers and types

Group	Isolate No.		Type
Group 1	3	4	OspC MT Q
Group 2	1		OspC MT A
Group 3	2		

MT = Major Type

There were 18 *B. garinii* samples which were resolved into seven OspC major groups, groups 1, 3 and 4 consist of four isolates each, groups 2 and 5 have two representatives each and groups 6 and 7 have just a single isolate each.

Table 15: *Borrelia garinii* groups, assigned isolate numbers and types

Group	Isolate No.				Type
Group 1	1	2	3	12	garLG1
Group 2	4	5			garLG2
Group 3	6	7	8	9	garLG3
Group 4	10	13	16	17	garLG4
Group 5	11	14			garLG5
Group 6	15				garLG6
Group 7	18				garLG7

There were four *B. valaisiana* isolate, each of them belonged to a different OspC major group hence four major groups consisting of one isolate for each group.

Table 16: *Borrelia valaisiana* groups, assigned isolate number and types

Group	Isolate No.	Type
Group 1	1	valLV1
Group 2	2	valLV2
Group 3	3	valLV3
Group 4	4	valLV4

5. 4. 10 Comparison of Sequence Types and OspC type Strains

To determine whether an OspC major type is always associated with the same ST, both OspC types and ST were compared. There was not always a ST for each existing OspC strain, this is because only in cases where all the eight housekeeping genes were successfully sequenced a ST type could be generated. For OspC major types where this was not the case are marked with ND (= no data). Following this, only 20 isolates had both OspC and ST: eleven *B. afzelii*, three *B. burgdorferi* s.s., four *B. garinii* and two *B. valaisiana* (Table 17: Showing the comparison of ST and OspC type strains). Direct comparison was however still not possible as there was no ST which was represented at least 2x for which also an OspC sequence was available.

Nevertheless, it was noted that there was not always congruency between the OspC major type and the respective ST, for instance isolates 0-14-39, 1-29-29, 1-37-13 and 1-32-42 belonged to the same OspC major group but all have different STs. This trend was noted for all the 20 isolates.

Table 17: Showing the comparison of ST and OspC type strains

OspC strains belonging to the same major OspC group are highlighten with the same colouring. Unhighlighted strains do not belong in any group rather are single isolates of their own groups.

Strain	Group	ST	OspC type
0-5-10-*B. afzelii*		ND	afzeLA1
0-14-03-*B. afzelii*		ND	afzeLA1
0-14-39-*B._afzelii*		171	afzeLA1
1-29-29-*B. afzelii*		570	afzeLA1
1-37-13-*B. afzelii*		835	afzeLA1
1-40-20-*B. afzelii*		ND	afzeLA1
1-31-42-*B. afzelii*	1	170	afzeLA1
1-8-20-*B. afzelii*		ND	afzeLA2
1-28-25-*B. afzelii*		ND	afzeLA2
1-29-35b-*B. afzelii*		825	afzeLA2
1-42-05-*B. afzelii*		ND	afzeLA2
1-29-22-*B. afzelii*	2	827	afzeLA2
0-14-37-*B. afzelii*		818	afzeLA3
1-28-*B. afzelii*	3	829	afzeLA3
3-8-11-*B. afzelii*		ND	afzeLA4
3-8-07-*B. afzelii*		841	afzeLA4
10-15-17-*B. afzelii*	4	ND	afzeLA4
1-28-05-*B. afzelii*		ND	afzeLA5
1-29-27-*B. afzelii*	5	ND	afzeLA5
9-20-22-*B. afzelii*		ND	afzeLA6
1-41-09-*B. afzelii*	6	ND	afzeLA6
1-32-25-*B. afzelii*	7	ND	afzeLA7
0-8-30-*B. afzelii*	8	ND	afzeLA8
1-31-25-*B. afzelii*	9	166	afzeLA9
3-8-06-*B. afzelii*	10	840	afzeLA10

Strain	Group	ST	OspC type
9-25-70-*B. burgdorferi* s.s.		ND	
1-32-30-*B. burgdorferi* s.s.		836	
3-5-17-*B. burgdorferi* s.s.	1	21	OspC MT Q
0-13-41-*B. burgdorferi* s.s.	2	1	OspC MT A
1-25-53-*B._garinii*		ND	garLG1
1-28-12b-*B. garinii*		ND	garLG1
0-14-42-*B. garinii*		ND	garLG1
1-32-53-*B. garinii*	1	838	garLG1
10-22-22-*B. garinii*		88	garLG2
1-41-28-*B. garinii*	2	ND	garLG2
1-29-08-*B. garinii*		184	garLG2
1-8-45-*B. garinii*		ND	garLG2
9-26-26-*B. garinii*		ND	garLG2
1-37-11-*B. garinii*	3	ND	garLG3
1-15-48-*B. garinii*		ND	garLG4
10-22-03-*B. garinii*		ND	garLG4
9-26-41-*B. garinii*		ND	garLG4
9-22-36-*B. garinii*	4	ND	garLG4
1-29-28-*B. garinii*		ND	garLG5
9-22-27-*B. garinii*	5	811	garLG5
0-8-36-*B. garinii*	6	ND	garLG6
1-29-30-*B. garinii*	8	ND	garLG7
10-24-07-*B. valaisiana*	1	100	valLVaa
1-29-03-*B. valaisiana*	2	96	valLVab
1-14-39-*B. valaisiana*	3	ND	valLVac
1-37-10-*B. valaisiana*	4	ND	valLVad

In table 17, isolates of the same OspC major groups are shown with the same colouring. No data (ND) shows isolates with existing OspC but no available ST. In 20 isolates both OspC and ST were available hence could be compared directly. There was not always congruency between the OspC major type and the respective ST.

Chapter 6 Discussion

The aim of this study was to investigate *Borrelia* species and populations with regard to their structure and temporal fluctuations in defined habitats in Latvia over several years. Ticks have a long-life cycle that may take up to seven years to complete. This means that pathogens that infect ticks will remain in the environment for extended periods of time (or as long as the tick stays alive). Thus, in order to understand changes in pathogen species distribution and fluctuations in habitats, questing ticks were collected regularly over a period of eleven years. MLST as unambiguous strain typing sytem was used to characterize *B. burgdorferi* s.l. isolates sequenced from Latvian ticks and to investigate temporal and spatial species and population structures, fluctuations and/ stability of populations. As thousands of ticks needed to be processed, in initial investigations two methods of DNA extraction (i) commercialy available DNA extraction kit and (ii) NH_4OH DNA extraction with and without EtOH preservation, different library production methods and different sequencing methods, i.e. Sanger sequencing vs NGS were compared.

6. 1 Methods for *Borrelia* DNA Extraction and MLST Sequencing

A pre requisite for molecular investigatings or typing of microbial pathogens is to ensure the most economic and efficient purification of high-quality DNA. In this study two methods that are frequently used to extract DNA from questing ticks, namely NH_4OH and DNA purification with a commercial kit were directly compared. NH_4OH DNA treatment has been described and used previously (Guy and Stanek, 1991; Rijpkema and Bruinink, 1996; Vollmer et al., 2011; Szekeres et al., 2017).

Amplification of the target locus tick *coi* gene was successful in 86 % of NH_4OH extracted DNA samples and 96 % of kit extracted DNA. Thus, results from this

study indicated that a higher number of samples (4/35; 11.4 %) in the group of NH_4OH the PCR was unsuccessful suggesting the presence of some PCR inhibitory factors in the extracted DNA.

However, when DNA was re-extracted in PCR negative NH_4OH extracted samples using a commercial kit, amplification of the tick *coi* gene by PCR was successful in all of them suggesting presence of an inhibitor. PCR inhibition in samples extracted with NH_4OH is not uncommon as this method does not include protein digestion and/ or filtration as in commercial kits, thus the presence of inhibitory factors was assumed. One cannot exclude that the ticks that were collected – although questing – had taken a small interrupted blood meal that may have caused the PCR inhibition in these samples (Schwartz et al., 1997; Richter et al., 2012; Apanaskevich and Oliver, 2014). Other inhibiting components present, for example proteins interacting with the DNA or chelating agents in the biological samples may have contributed to the failure of PCR. In conclusion, the NH_4OH DNA extraction method may lead to false negative results.

Although some fluctuations in the amount of extracted DNA was observed, in the majority of samples PCR amplification of the tick *coi* gene was successful. In contrast to previous reports suggesting that alkaline hydrolysis does not work on nymphal ticks (Ammazzalorso et al., 2015), in this study, DNA using this method also from nymphs was successfully amplified as demonstrated by the presence of PCR products. The efficiency of NH_4OH was about one magnitute lower in extracting DNA from samples than commercial kits. This was shown by dilution series on extracted tick DNA and real-time PCR on *Borrelia* cultures. Therefore, samples containing low numbers of *Borrelia* could be missed when DNA is extracted using NH_4OH and this needs to be considered when evaluating results. It is also worth mentioning that NH_4OH extraction is only suited for (questing) ticks that have not taken (even a small) blood meal; for engorged ticks one should use either commercially available DNA extraction kits or potassium acetate (Rodríguez et al., 2014).

Considering the fact that DNA purification with a commercial kit is approximately 90 times more expensive per sample than with NH_4OH, the latter method is an inexpensive alternative to commercially available DNA extraction kits. Thus, despite the lower efficiency of alkaline hydrolysis for DNA extraction from ticks and from cultured *Borrelia* organisms, which was ascertained in this study, it

appears that NH_4OH DNA hydrolysis in conjunction with manual/ fine crushing may still be the method of choice especially in low resourced laboratories. Albeit bead beating as a DNA preparation method could be expensive, it generates the most reliable results (Halos et al., 2004).

In conclusion we can say that even though DNA-purification by help of NH_4OH single tubes are used which may be open at times, the risk of contamination is low and 90 samples can be processed at one time. Using a commercial kit contamination risk may be as high as for NH_4OH, depending on the kit. Columns pose risk due to handling them and only 24 samples can be processed at any one time. All-for-one kit high-throughput allows processing of 90 samples in one go, but the 96 well plates are also open hence same contamination risk as in NH_4OH and more expensive.

In conclusion, this study has found that alkaline hydrolysis is an economical and inexpensive method for DNA extraction from vectors of pathogens that may be used especially in low resourced laboratories. In this study at least 800 bacteria cells from cultured *Borrelia* were detected. Thus, using it on field samples, it may detect the bulk of infected ticks as it has been shown that the average infection burden of *I. scapularis* was around 800 organisms per tick (Barbour et al., 2009). Inhibition of PCR did occur in a number of samples. As it is known that alkaline hydrolysis is not suitable for blood fed vectors, it was considered likely that the observed inhibition was related to uptake of small amounts of blood and re-questing of ticks. The occurrence of false negative samples needs to be considered when conducting field work and using ammonia hydrolysis as DNA extraction method.

6. 2 Sequence Acquisition for MLST/MLSA

Due to problems with Sanger sequencing during the course of this study, the sequencing method for MLST PCR products was changed to Illumina sequencing (Kingry et al., 2018). Kingry et al. had published a method employing Illumina sequencing for MLST PCR products that seemed to be better suited for samples with non-specific background in PCR products. To this end Sanger sequencing was (i) directly compared to Illumina sequencing and (ii) two methods of library preparation namely Nextera XT (the most commonly method used in our lab) and Nextera DNA flex both by Illumina were directly compared.

For direct comparison of Sanger sequencing and Illumina sequencing, ten samples were chosen randomly which had been sequenced by Sanger method. After Sanger sequencing none of these ten samples had readable sequences for all the eight housekeeping genes, hence they were re-sequenced by the Illumina method. After re-sequencing with Illumina, all the eight housekeeping genes were good for nine samples; for one sample the *clpA* gene failed even in Illumina sequencing. This means that Illumina sequencing at least in this study proved to be better suitable for sequencing PCR product from difficult DNA template samples than the Sanger method. Since the reads acquired by Illumina sequencing are mapped to the reference, background is reduced. Besides multiple reads are generated for one single isolate in both directions when NGS is employed, hence higher sensitivity is achieved (Illumina, 2019). Thus, the rest of the samples were sequenced using the Illumina method.

Besides analyzing Illimina sequences on CLC genomics workbench allows easy identification and filtering out of mixed sequence. The sequence are mapped to a reference and a critical value (this was set at 30 % in our case) and ambiguity codes (Y, R, W, S, K or M) are set. This means that unsecured bases on the consencus sequence exceeding the 30 % limit are marked with any of the ambiguity codes above and shown as mixed sequences.

In our hands, the method of Illimina sequencing appeared to increase the output of readable sequences. However Illimina has different kits in the market for library production, so we opted to compare out the two kits (Nextera XT kit and

DNA flex) before exclusively deciding for one. These kits differ in input DNA and processing of samples. For example, the Nextera XT kit requires a DNA input as low as 0.2 ng/ul. A recently introduced kit (DNA flex kit) requires although more DNA but with a broader range (1-500 ng). This is one of its supposed advantages described by the company. Other advantages include first library preparation workflow and optimized library prepation performance (Illumina, 2017).

Ten samples were again chosen randomly and libraries were prepared for all samples using both kits. The results showed that DNA flex produced libraries with a much more defined size range although at a higher cost. DNA flex also required a higher DNA input. There was no handling time difference between kits. Sequencing output i.e reads obtained and sequencing quality were equally good whether Nextera XT or DNA flex was used. Thus, Nextera XT was chosen over DNA flex kit as it was the more economical.

6. 3 Infection Prevalence of *B. burgdorferi spp*. in Questing Ticks from Latvia

To understand the population diversity of *B. burgdorferi* s.l. in Latvia and fluctuations that occur over this time period between 1999 and 2010, *B. burgdorferi* s.l. DNA was extracted from Latvian ticks. Tick sampling took place between 1999 and 2010 in Babite, Jaunciems and Kemeri. For three years 2002, 2006 and 2007 only MLST sequences were available as screening and sequencing had been perfomed in the previous study (Vollmer et al., 2011). Just over 3000 ticks from the years 1999, 2000, 2001, 2003 and 2010 were processed in the course of this study.

In the past many studies have been conducted, often as short term survey involving a year or two of tick collection. As already mentioned a tick life cycle can be as long as seven years. Short term studies may therefore, give just a snap short of the prevalence or population structure and not the actual diversity of *B. burgdorferi* s.l. or its fluctuations over the years. A few extensive studies have been conducted involving up to six years (Pawelczyk and Sinski, 2004; Takken et al., 2017; Sormunen et al., 2018; Galfsky et al., 2019; Lejal et al.,

2019). This is the first longitudinal study covering a period of 11 years of tick collection in Latvia in defined habitats and using MLST/MLSA as instrument for strain typing.

For the presence of *Borrelia* a duplex real-time PCR targeting the flagellin B (*flaB*) gene encoding the flagellin B Protein (P41) was used to screen the samples (Schwaiger et al., 2001). The overall prevalence of *Borrelia* in Latvian ticks was in line with previously published tick prevalences in Europe. The mean average tick prevalence over all years was determined to be 27 %, and 13 % in adults and nymphs respectively (with mean average for both adults and nymphs being 18.9 %). Whereby reviews of Rauter and Hartung, and Strnad et al. observed prevelances of 13.5 % and 15.6 % respectively (Rauter and Hartung, 2005; Strnad et al., 2017). Bormane et al. determined a prevalence of 10.6 % in nymphal ticks and 18 % - 38 % in adults in Latvia (Bormane et al., 2004). However a mean prevalences of as low as 6.9 % and 9.4 % have been registered in the neighboring countries such as Lithuania and Belarus respectively (Ambrasiene et al., 2004; Reye et al., 2013).

Over the years mean prevalence fluctuations varying from 33.3 % to 7.3 % was observed. The years 1999, 2000, 2001, 2003 and 2010 showed prevalences of 25.5 %, 33.1 %, 31.8 %, 16 % and 7.3 % respectively. As can be seen lowest prevalences were noted in the years 2003 and 2010. In these two years (for reasons unknown), the mean prevalence in ticks investigated dropped to values of 16 % and 7.3 % respecitively. In the Netherlands a substantially lower prevalence of infection rates was registered in the years 2001 and 2003 in comparison to the rest of the analyzed years (2000, 2002 and 2004) in the same study (Wielinga et al., 2006; Hoen et al., 2009; Herrmann et al., 2013). Thus, prevalence fluctuations seem to be a general trend and this needs to be taken into consideration when predicting future trend of tick-borne pathogens (TBP).

Prevalence fluctuations over the years could be for instance due to favorable weather conditions or abundancy of competent host at specific years (Wielinga et al., 2006; Marchant et al., 2017). Without doubt underlying biotic and abiotic conditions directly influencing ticks and (tick and *Borrelia*) reservoir hosts in their natural habitats are bound to eventually contribute to the fluctuations of TBP (Randolph, 2004; Pfaffle et al., 2013). Ostfeld and colleagues illustrated in their study of determining risk variation of LB disease that, the abundance of suitable

hosts (mice and chipmunks) in a particular year leads to high risk in the following year (Ostfeld et al., 2006).

Copian and colleagues performed a longitudinal study of TBP for a period of ten years in the Netherlands between 2000 and 2009. During this period the prevalence of *B. burgdorferi* s.l. amongst other studied TBP was relatively stable (prevalence of 7 % for *B. burgdorferi* s.l.). Except for the years 2004 and 2005 where the highest nymphal tick densities and peak prevalences of *B. burgdorferi* s.l. were observed. The authors speculate that the variation in the tick density may be due to the annual fluctuation in the reservoir host availability (Coipan et al., 2013). Prevalence variation in the areas was also observed, which agrees with observations in this study (Coipan et al., 2013).

In this study, high tick densities did not necessarily mean high prevalence as observed by Copian et al. (2013) in the Netherlands. For example, taking the years 1999 and 2010, total collected ticks were 271 and 642 respectively, prevalence were 25.5 % for the year 1999 and 7.3 % for the year 2010. Correlation of tick density and the infection prevalence has also been observed in Switzerland, even though this was only observed in adult ticks (Jouda et al., 2004). It was also observed in a longitudinal study conducted between 2006 and 2011 that even though there was variation in prevalence between the years, the tick density did not correspond to the infection prevalence (Takken et al., 2017). Both biotic and abiotic factors and the population dynamics determine the seasonal availability of questing ticks and thus the infection risk, therefore, just a mere abundancy of suitable host does not automatically mean high infection rates (Randolph, 2004).

As reviewed by Estrada-Peña et al. (2012), tick stress response (TSR), questing behavior and pathogen transmission are all connected. TSR is as a response to different stress factors such as temperatures, blood feeding and pathogen transmission. Extreme dryness for example may force ticks to undertake quiescence so as to prevent extensive energy loss which would be especially high when they carry pathogen (Estrada-Peña et al., 2012). Therefore, extreme weathers in 2003 and 2010 in Latvia might have led to collection of non infected ticks. Unfortunately, in this study there was no weather records hence it can only speculate (Reye et al., 2010).

Herrmann and Gern reviewed that *Borrelia spp.* infected ticks have better chances of survival during extreme weather conditions as the pathogen alters the arthropod's perception of the environment or the behaviour in order to complete its transmission cycle. Besides these ticks supposedly have increased questing behaviour in order to find host and eventually transmit the pathogen (Herrmann and Gern, 2015). This is in contrast to what Estrada-Peña et al. (2012) proposed. Would there have been extreme weather conditions, according to Herrmann and Gern, we would have captured more infected ticks than non infected. Since we do not have weather data for these periods, we cannot say which theory better fits our findings.

But such fluctuations have been noted in other regions, i. e. in France for instance it was observed that prevalence of *B. burgdorferi* s.l. and other tick borne pathogens vary significantly to both years and season, a monthly prevalence of between 0.8 % (in May) to 23. 5 % (in October) (Lejal et al., 2019) was observed.

Overall, the infection rate in adults was higher than in nymphs. This is consistent with previous studies of prevalence of *Borrelia* in ticks in Europe (Rauter and Hartung, 2005; Strnad et al., 2017). This has been attributed by the fact that adults have fed on at least two hosts assuming that both were infected and that the feeding was not interrupted, they had a chance of acquiring *Borrelia* twice. In contrast nymphs have only fed on one host. Also, an efficient trans-stadial transmission of the spirochaete purportedly contributes to this (Mejlon and Jaenson, 1993).

In the year 2000 a different trend was observed, more nymphs than adults were found to be *Borrelia* infected. Similar observations have been made in Italy, France, Slovakia, and Sweden where higher infection rates in nymphs than in adults were described (Aureli et al., 2015; Chvostáč et al., 2018; Ehrmann et al., 2018; Akl et al., 2019). It could be that for some reasons transovarial transmission was particularly high, hence high infection rates in the subsequent generation were observed. However, this reasoning is probably far-fetched as transovarial transmission is known to be extremely low (Matuschka et al., 1992; Rijpkema et al., 1994).

Further findings showed that the average infection rates in summer and spring was higher than that in autumn, this has been reported previously in England

and Sweden (TäLleklint and Jaenson, 1996; Hansford et al., 2017). In Europe, both unimodal and bimodal pattern of tick activities have been described (TäLleklint and Jaenson, 1996; Estrada-Pena et al., 2004; Reye et al., 2010; Buczek et al., 2014; Cayol et al., 2017; Remesar et al., 2019).

Another possible reason could be that some hatched nymphs (possibly infected) do not quest immediately rather go through the behavioral diapause first and start questing activity the following year (Cayol et al., 2017). Or if there was a large number of incompetent hosts to which the nymphs attached to and fed on, this may lead to an overall reduction in prevalence in the adult population (Jouda et al., 2004; Nahimana et al., 2004). Also, abundance of non permissive hosts for the nymphal stage would lead to general low infection rates in adults. Coipan and colleagues speculate a mast year for small mammals leading to an upsurge of nymphal ticks in the subsequent year (Coipan et al., 2013) as another possible explanation.

Tick samples which were sampled in the same regions in Latvia and data published in the year 2003 showed that, prevalence of *Borrelia* in nymphal ticks was higher than that in adults in Jaunciems (Etti et al., 2003). So, it is likely that this is due to environmental/ weather-related factors contributing to higher infection rates in nymphs than adults.

In this study 22.5 % of samples positive in screening PCR could not be analyzed further. This is because attempts to get PCR products in the subsequent nested PCR failed even after several repetitions. This may perhaps be due to extremely low *Borrelia spp*. load in these samples or they were false positive. Untypeable positive *Borrelia* samples have been reported (Rauter et al., 2002; Wielinga et al., 2006). James et al. suggested that this is probably due to sequence polymorphism in the oligos primers used (James et al., 2014).

It was also shown that the infection rate in sylvatic (Babite) habitat was slightly higher than that of peridomestic (Jaunciems) region. This may be because sylvatic regions offer more heterogeneity in terms of vegetation hence is more likely to be inhabited by a wider range of hosts which are potential carriers which may augment the chances of vector acquiring infection (Kirstein et al., 1997). It has been speculated that it is the ecotones that offer the suitable environment for the hosts hence higher prevalence is expected than in the forests. In our

study however, forested habitat (Babite) exhibited higher prevalence than the ecotone habitat in Jaunciems (Morellet et al., 2011; Ehrmann et al., 2018).

Ticks may carry mixed infections; using MLST it was shown that these can be mixed strain infections of a single *Borrelia* species and mixed infections of different genospecies (Hoen et al., 2009; Vollmer et al., 2011; Vollmer et al., 2013; Mechai et al., 2016). Up to 45 % prevalence of mixed infection has been reported with the most common co-infection found being between *B. garinii* and *B. valasiana* (Rauter and Hartung, 2005; Fingerle et al., 2008; Moutailler et al., 2016).

In this study mixed infection was observed in 85 (30 samples on screening PCR and 55 on MLST PCR) samples (12.18 %). Sequences of such samples show double peaks at least at one point in different genes belonging to the same sample (Ogden et al., 2011; Vollmer et al., 2011; Mechai et al., 2015; Coipan et al., 2016). Such samples were excluded from further analysis. A simple explanation for mixed infection would be that in every development stage ticks feed on different hosts, which are potential carriers of different *Borrelia* strains or species. In addition, it has been shown that individual hosts and vectors can be infected with several *Borrelia* strains or species (Seinost et al., 1999; Durand et al., 2017). Co-feeding transmission may be also another route of acquiring several *Borrelia* strain (Gern and Rais, 1996; Cayol et al., 2017).

6. 4 Species Diversity – Spatial and Temporal

The *B. burgdorferi* s.l. species complex currently consists of 22 named genospecies, which occur in a belt across the globe in the northern latitudes between 40 and 60 degree. Coipan and colleagues suggest the intraspecies variation within *Borrelia* could be explained by

(i) Balancing selection of the bacteria to adapt to the wide range of verterbrate hosts,

(ii) Geographical differenciation amongst sampling sites as they observed for their studied regions and lastly

(iii) The interaction between the vector and the bacterium as some *Ixodes* species are able to transmit multiple *B. burgdorferi* s.l. while others seem to be less competent (Coipan et al., 2018).

In Europe, the most commonly identified genospecies are *B. afzelii*, *B. garinii* and *B. valaisiana* which are unequally distributed on this continent. (Kurtenbach et al., 2001; Kurtenbach et al., 2006; Strnad et al., 2017; Estrada-Pena et al., 2018; Mysterud et al., 2019).

Expectedly, they were also the most common species identified in the studied habitats in Latvia, with *B. afzelii* being the most dominant though not in all the three investigated habitats and years. A meta-study conducted in 2005 also suggested that this is the dominant species in Europe (Rauter and Hartung, 2005).

The species composition differed markedly between the investigated habitats and varied from year to year in the sampled regions. In Babite five species were detected: *B. afzelii*, *B. garinii*, *B. valaisiana*, *B. burgdorferi* s.s. and *B. lusitaniae*. *B. garinii* was detected in all the years and dominated particularly the last three years of sampling (2006, 2007 and 2010). Noticeably the infection rates with *B. afzelii* in Babite seemed to be diminishing in the course of the years. Etii and colleagues also observed this trend in the years 1999 and 2000 (Etti et al., 2003). In our study *B. afzelii* was dominant in the years 1999, 2000 and 2002. In 2003 the prevalent of *B. afzelii* was equal to the prevalence of *B. garinii*.

Between 2006 and 2010 it seems like the bird associated *B. garinii* and *B. valaisiana* (with *B. garinii* dominating in these three last sampling years) were in the rise as the prevalence of *B. afzelii* went down. Etti and colleaques suggests a gradual change in the host composition in Babite leading to the gradual reduction of *B. afzelii* prevalence. In deed it seems like to some extent the ground foraging competent hosts of *B. afzelii* are diminishing in Babite as birds increase. The reasons to this supposed gradual host change in this habitat would require further studies to investigate.

In Jaunciems *B. afzelii* was strikingly dominant, in fact in the years 2002, 2006 and 2007 exclusively this species was detected in this region, this is also conform with what was observed in this region (Etti et al., 2003). In Kemeri, four species were existent: *B. afzelii*, *B. garinii*, *B. valaisiana* and *B. burgdorferi* s.s.. All these species were more or less almost equally distributed in the years analyzed, except in 2006 and 2007 where *B. burgdorferi* s.s. was not detected.

In summary, it was interesting to note the seeming shift in the host composition in Babite which has been speculated in the previous study. Species composition in Jaunciems was stable over the years. In Kemeri there was a constant fluctuation of species from year to year. Host species and populations maintained in a specific region is the determining factor for species diversity. It seems Babite and Kemeri have diversity when it comes to host inhabouring resulting in being the genospecies rich regions in Latvia.

6. 5 Population Structure of *B. burgdorferi* s.l. in Latvia

For population analysis studies only samples to which sequences of all the eight housekeeping genes were available could be used. A total of 159 strains from Latvia were available from the year 1999 to 2010, which were grouped into 109 STs. A complete list of the strains can be seen in the appendix section (Appendix 16: STs Analysed in this study. There were 64 *B. afzelii*, 47 *B. garinii*, 32 *B. valaisiana*, 13 *B. burgdorferi* s.s., two *B. lusitaniae* and one *B. bavariensis*. This general pattern of species abundance is consistent with what has been reported for Europe (Rauter and Hartung, 2005). The 64 *B. afzelii* strains were resolved into 51 STs, 47 *B. garinii* strains into 34 STs, 13 *B. burgdorferi* s.s. strains into 8 STs and 32 *B. valaisiana* strains into 17 STs. These STs were

analyzed in goeBURST which shows the relationships of single STs to each other. This analysis revealed a close relationship between *B. afzelii* STs, as one major clonal complex consisting of 38/ 64 STs was formed.

Since the samples had been acquired from a wide range of different times and regions, it was expected to see some kind of pattern. One hypothesis was that there would be clustering of closely related STs according to the collection time or region. However, no recongnizable trend concerning collection year or site was observed.

The distribution and diversity of *Borrelia* species in the respective habitats is largely influenced by the host movement since ticks cannot make long distance movement (Falco and Fish, 1991). So, in the case of *B. garinii* a lack of pattern could be explained by the fact that birds move randomly, hence are more likely to distribute the vectors carrying this species arbitrarily. This supports the hypothesis of Vollmer et al. which suggests free movement of *B. garinii* strains (Vollmer et al., 2011).

Borrelia garinii was found to be most divers species in this study. This is consistent with previous studies which have shown in the previous more heterogeneity within *B. garinii* strain than within the other strains (Wilske et al., 1993; Will et al., 1995; Busch et al., 1996; Comstedt et al., 2009). Heterogeneity within *B. garinii* strain is hypothesized to be due to the seasonal migrating birds which not only facilitate the spread but also fosters the diversity (Comstedt et al., 2011). Another factor which has been hypothesized to contributing to this is that birds as hosts show much more species diversity, than small mammals which hosts *B. afzelii* and other *B. burgdorferi* s.l. species (James et al., 2014).

6. 5. 1 Temporal and Spatial Distribution of STs in the Collection Regions

Generally, it can be said that there were many STs-specific to respective regions. This shows that distribution of *B. burgdorferi* s.l. can vary significantly even within a geographic region (Kirstein et al., 1997; Milutinovic et al., 2006). There were however shared STs in the regions, for instance it was shown that there were several *B. afzelii* STs shared between Jaunciems and Kemeri and just a few between Babite and Jaunciems or between Babite and Kemeri. This

is in conform with previous findings on these regions (Vollmer, 2010). Study of Hoen et al. also noticed shared STs within sites of the same region (Hoen et al., 2009). It was proposed that possibly there are more migrations between Jaunciems and Kemeri in comparison to Babite, even though this theory seemed far-fetched, as Kemeri and Babite are geographically closer than Kemeri to Jaunciems (Etti et al., 2003).

It was also observed that even though *B. afzelii* in Babite was not low in all the sampling years, it was gradually going down, this findings agree with finding from 2003 (Etti et al., 2003). There was no association of this trend to the general prevalence as even the years with comparatively low prevances (2003 and 2010) this trend was observed as in the other years. Vollmer et al. also analyzed temporal distribution of *B. afzelii* and *B. garinii* and in her work. *B. afzelii* STs did not show any trend for a particular time period. *B. garinii* however showed domination for some years, no meaningful conclusion was drawn from these findings as the dominating STs for specific years were only ascertained once hence conclusion would have been too specific (Vollmer, 2010).

Another finding was that, STs were either shared in two regions or specific to a region, this was however not the case with *B. valaisiana* ST199, which was found in all the three regions. It can be argued that since *B. valaisiana* is bird related species, there are chances that birds migrate back and forth within these three regions. However, then it would be expected to find *B. garinii* specific to all the three regions which was not the case. Or perhaps this strain is a result of population differentiation in these regions, and not “solely”-host dependent, signs of populations differentiation with *B. valaisiana* has been reported (Vollmer et al., 2011). Or perhaps it is the ancestral strain for these regions.

Borrelia is a vector obligate bacterium, and the vectors inturn are host-reliant. Generally, host abundance is the determining factor when it comes to vector survival hence spirochete survival. Therefore, one can say distribution of *B. burgdorferi* s.l. species is dependent on the host distribution and movement (Kurtenbach et al., 2002; Comstedt et al., 2011; James et al., 2014). Another possible explanation of the species variation would be as a result of strong bottleneck within these sites (Bruyndonckx et al., 2009; Lemoine et al., 2018).

6. 6 Comparison of MLST and OspC Phylogenetic trees of *B. burgdorferi* s.l. from Latvia

Both MLST and OspC sequences were used to generate a phylogenetic tree on MEGA version 6. Phylogenetic analysis on *B. burgdorferi* s.l. population structure studies is a widely used method (Margos et al., 2008; Hoen et al., 2009; Hanincova et al., 2013; Mechai et al., 2016) There were 71 MLST sequences and 52 OspC sequences available for phylogeny (Figure 37 and 42). For both MLST and OspC phylogenetic tree generation a type strain for each species was included.

Of the 71 MLST sequences only 63 samples were used in the generation of MLST phylogenetic tree as from double represented STs just one was chosen randomly to represent the group. Unrooted tree was generated with well supported internal and terminal nodes in most cases except for *B. afzelii* at the terminal nodes.

From the unrooted tree of MLST genes went out two major branches. One main branch built the *B. garinii* and *B. bavariensis* clade. The second main branch was divided into two further branches; from which *B. afzelii* group arose and further subdivision of its second branch formed *B. burgdorferi* s.s. and *B. avalaisiana* clades. This phylogenetic tree had well supported internal clades for MLST genes except for *B. afzelii*, this is probably due to the limited heterogeneity within *B. afzelii* (Wang et al., 1998).

One *B. garinii* isolate clustered outside the *B. garinii* clade. It was noted that of the eight MLST genes of this specific isolate one gene (*pyrG*) was found to be *B. afzelii* in MLST database. This may be one due to recombination which occurred in the recent past. Recombinations at loci level has been described. It was suggested that some diversity within the *B. burgdorferi* species is probably due to recombination at the loci level (Hanincova et al., 2013), in fact Jacquot and colleagues hypothesized that recombination within species happen 50 times higher than within different species (Jacquot et al., 2014). Even though all possible measures were undertaken to ensure no contamination occurred, it

cannot be ruled out completely. Thus, another possible reason of one out of the eight genes being *B. afzelii* would be contamination.

Likewise phylogenetic tree of the OspC was formed. This showed the diversity and the heterogeneity within the OspC gene. OspC phylogeny was not well supported at the internal clades as the MLST phylogeny, however external clades were supported for most of *B. burgdorferi* s.s., *B. garinii* and *B. valaisiana* strains. Even though there were also two major branches arising from the unrooted tree, further subdivision generated several brances for the respective species which were not as well structured as the MLST phylogeny indicating undergone recombination (Dykhuizen and Baranton, 2001; Margos et al., 2008).

Pairwise distance was calculated for OspC sequences in MEGA. Isolates of the same species with a pairwise distance of ≤ 0.02 are said to belong to the major OspC group and once with a pairwise distance of ≥ 0.08 belong to different OspC major group (Wang et al., 1999). Based on this, from the 52 OspC sequences 24 major groups were built. Ten, three, seven and four major OspC groups were identified for *B. afzelii*, *B. burgdorferi* s.s., *B. garinii* and *B. valaisiana* respectively, this is inconsistent with what has been observed. Apparently there is an upper limit of possible major OspC groups in a particular population, so as to maintain its fuctionality despite its short length of only 600 bp (Durand et al., 2015). In our study, more than 50 % of the total sequenced OspC strains could not be used in further analysis as they had mixed infections (Durand et al., 2015).

On the OspC gene, three possible recombinations were observed; (i) *B. burgdorferi s.s.* with *B. afzelii*. This *B. burgdorferi* s.s. strain is according to MLST housekeeping genes a *B. burgdorferi* s.s. strain and *B. afzelii* according to pubmed, (ii) one *B. garinii* clustered as well with *B. afzelii* and lastly (iii) One *B. valaisiana* clustered between *B. garinii* and *B. afzelii* and not with the rest *B. valaisiana* strains, given the close relatedness of *B. valaisiana* and *B. garinii* clustering with this group is not a surprise. However, intraspecific gene transfer is also not uncommon within OspC gene and has been described before (Livey et al., 1995; Bunikis et al., 2004). Wang and colleagues speculated a frequent gene transfer between *B. valaisiana* and *B. garinii* and also between *B. valaisiana* and *B. afzelii* (Wang et al., 1999). According to the authors simultaneous infections with different *B. burgdorferi* s.l. enhances the

high gene transfer they observed. Indeed multiple species infections has been observed severally and and also in avian hosts, in fact some do say it is the rule and not the exception (Humair et al., 1998; Kurtenbach et al., 1998; Liebisch et al., 1998). An experiment carried out on sumultanious feeding larvae and nymphal tick stages observed that not only was the prevalence higher in these larval ticks, but also the diversity within OspC groups was higher, this suggests that co feeding also contributes to the intraspecific diversity within OspC gene (Pérez et al., 2011).

Todate up to 300 different host have been described for *B. burgdorferi* (Gern and Humair, 2002; Randolph, 2008). This according to Haven and colleagues is due to the pervasive recombination ability of it`s genome. OspC genome showed the highest recombination rates in comparison to the main chromosome and plasmids investigated (Dykhuizen and Baranton, 2001; Haven et al., 2011). Nonetheless from evolutionary point of view, physical selection is important for the survival of all living organisms (Pearson et al., 2009).

We noted that OspC major groups did not always have the same STs, rather an OspC major group consisted of multiple STs, this is in conform with what has already been published (Hanincova et al., 2013).

Whereas MLST housekeeping genes are under purifying selection and evolves almost neutrally (Enright and Spratt, 1999; Margos et al., 2008). OspC gene of *Borrelia* is a highly variable protein and under balancing selection (Brisson and Dykhuizen, 2004; Bunikis et al., 2004; Ogden et al., 2011). Margos and colleagues observed a different evolutionary of housekeeping genes from the plasmid located OspA and OspC genes.(Margos et al., 2009) demonstrated a different evolutionary pathway. This has in the passed raised the question how suitable OspC is for population studies (Hanincova et al., 2013).

Chapter 7 Summary - Zusammenfassung

7. 1 English version

The average prevalence of *Borrelia* infection on Latvian ticks was 18.9 %. From high infection prevalences of 25.5 % (1999), 33.1 % (2000) and 33.8 % (2001), from 2002 onwards the infection rates steadily decreased to 7.3 % in 2010. Adult ticks had generally higher infection rates than nymphs although in the year 2000 there were more nymphs infected than adults. This is not what is expected as it is normally the other way round, due to the fact that, adults have had theoretically the opportunity for two infected blood meals (assuming the blood meals were not interrupted) hence higher chances of being infected than the nymphs. Ticks from peridomestic regions seems to be more highly infected than the ticks from sylvatic habitat. Spring and summer had the highest infection prevalence.

DNA hydrolysis in conjunction with manual crushing may be the method of choice especially in low resourced laboratories. NH_4OH works only on questing ticks and not engorged ticks, this should be put into consideration before extracting DNA and when evaluating the results. Besides, it does not involve any sort of “foreign protein purification” in the process of DNA extraction, so PCR may be comparatively inhibited leading to reduced efficiency. Therefore, low positive samples might be missed out while using alkaline hydrolysis method.

Pooled Nextera DNA flex libraries seemed to have better distribution on Agilent Bioanalyzer. However both kits, Nextera XT and DNA flex were in our hands comparable. Generally, Illumina sequencing showed higher sensitivity than Sanger sequencing.

Over the eleven years period *B. afzelii* was the most common species identified. Herein the prevalence of *B. afzelii* went down gradually with time in Babite. It was also noted that Babite is the most species rich of the studied regions. It would be interesting to investigate the circumstances underlying this apparent

gradual change in the host/species composition in this region. Generally Jaunciems seem to have a stable species composition, while Babite and Kemeri experiences constant fluctuations from year to year. *B. afzelii* was the dominating species in Jaunciems and Kemeri while in Babite *B. garinii* dominated. In addition, *B. garinii* was found to be most diverse species when Tajima`s test of neutrality was conducted.

No recognizable distribution pattern of *B. burgdorferi* s.l. species was observed in terms of collection site. A negligible temporal distribution of *B. burgdorferi* s.l. STs was noted, this agrees with what was ascertained in the previous studies in this region. Further studies would be needed to verify the random temporal distribution of STs which were ascertained in this study. For this a longitudianal study over years is recommended.

There is possible migrations between Kemeri and Jaunciems as there were more shared STs within these two regions. Perhaps a tracking study on the competent reservoir hosts in these two regions would shade some light on this.

Due to the limited number of sequences achieved a direct comparison of OspC sequence types and MLST type strains was not possible, this may be an interesting part which can be conducted in the future work to gain more insight into the relationships between housekeeping genes and their counterparts OspC.

Using MLST and OspC sequencing we have gained insights into the population structure of *B. burgdorferi* s.l. in Latvia. Spatio-temporal fluctuation was observed, probably as a result of ecological changes evocating variable responses from different habitats over time. This long term study has therefore shown that extrapolation of data in regard to prevalence of TBP or fluctuation or stability of population structure is not possible.

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7. 2 Deutsche Version

Die durchschnittliche Prävalenz der *Borrelia spp.*-Infektion bei lettischen Zecken betrug 18,9 %. Von ursprünglich hohen Infektionsprävalenzen von 25,5 % (1999), 33,1 % (2000), und 33,8 % (2001) sanken die Infektionsraten ab dem Jahr 2002 stetig auf 7,3 % im Jahr 2010. Adulte Zecken hatten im allgemeinen höhere Infektionsraten als Nymphen, obwohl im Jahr 2000 mehr Nymphen infiziert waren als Adulte. Dies ist nicht zu erwarten, da es normalerweise umgekehrt ist, weil Adulten theoretisch die Möglichkeit hatten, zwei *Borrelia spp*. infizierte Blutmahlzeiten zu sich zu nehmen (wenn man davon ausgeht, dass die Mahlzeiten nicht unterbrochen wurden), wodurch die Wahrscheinlichkeit einer Infektion bei Adulten höher ist als bei den Nymphen. Zecken aus peridomestischen Regionen scheinen stärker infiziert zu sein als Zecken aus sylvatischem Lebensraum. Frühling und Sommer zeigten die höchste Infektionprävalenz.

Die DNA-Hydrolyse in Verbindung mit manuellem Zerkleinern kann die Methode der Wahl sein für Zecken zerkleinern, insbesondere in Laboratorien mit geringen Ressourcen. NH_4OH funktioniert nur bei „questing“ Zecken und nicht bei Zecken, die bereits gesaugt haben. Dies sollte vor der DNA-Extraktion und bei der Auswertung der Ergebnisse berücksichtigt werden. Außerdem beinhaltet es keine Art von „Fremdprotein-Reinigung“ im Prozess der Dann-Extraktion, so dass das PCR inhibiert werden kann, was zu einer verringerten Effizienz führt. Daher können bei der Verwendung der alkalischen Hydrolyse-Methode niedrig positive Proben übersehen werden.

Gepoolte Nextera-DNA-Flex-Bibliotheken schienen auf dem Agilent Bioanalyzer besser verteilt zu sein. Beide Kits, Nextera XT und DNA Flex waren jedoch nach unserer Anwendung vergleichbar. Im allgemeinen zeigte die Illumina-Sequenzierung eine höhere Empfindlichkeit als die Sanger-Sequenzierung.

In den 11 Jahren des Beobachtungszeitraums war *B. afzelii* die am häufigste identifizierte Art Darin nahm die Prävalenz von *B. afzelii* in der Region Babite mit der Zeit allmählich ab. Es wurde auch festgestellt, dass Babite die artenreichste der untersuchten Regionen ist. Es wäre interessant zu

untersuchen, welche Umstände der Zusammensetzung von Wirt und Art in dieser Region dieser offensichtlich allmählichen Änderung zugrunde liegen. Im Allgemeinen scheint Jaunciems eine stabile Zusammensetzung der Arten zu haben, während in Babite und in Kemeri von Jahr zu Jahr konstant Schwankungen auftreten. *B. afzelii* war die dominierende Art in Jaunciems und Kemeri, währende in Babite *B. garinii* dominierte. Außerdem erwies sich *B. garinii* bei der Durchführung des Tajima-Neutralitätstests als die vielfältigste Art.

In Bezug auf die untersuchten Orten wurde kein erkennbares Verteilungsmuster von *B. burgdorferi* s.l. Spezies beobachtet. Eine vernachlässigbare zeitliche Verteilung von *B. burgdorferi* s.l. STs wurde festgestellt, was mit Feststellungen in den vorherigen Studien über diese Region übereinstimmt. Weitere Studien wären erforderlich, um die zufällige zeitliche Verteilung der STs zu überprüfen, die in diese Studie ermittelt wurde. Hierzu wird eine Langzeitstudie über Jahre empfohlen.

Es gibt mögliche Migrationen zwischen Kemeri und Jaunciems, da es in diesen beiden Regionen gemeinsame STs gab. Vielleicht würde eine Verfolgungsstudie über die dazu fähigen Reservoir-Wirte von diesen beiden Regionen etwas Licht ins Dunkel bringen.

Aufgrund der begrenzten Anzahl von Sequenzen, die verfügbar waren, war ein direkter Vergleich von OspC-ST und MLST-ST nicht möglich. Dies könnte eine interessante Studie für die Zukunft sein, um mehr Einblicke in die Beziehungen zwischen Housekeeping-Genen und ihren OspC-Gegenstücken erhalten.

Mit Hilfe der MLST- und OspC Sequenzierungen haben wir Einblicke in die Populationsstruktur von *B. burgdorferi* s.l. in Lettland erhalten. Es wurden räumliche und zeitliche Schwankungen beobachtet, wahrscheinlich als Folge ökologischer Veränderungen, die im Laufe der Zeit unterschiedliche Reaktionen aus den verschiedenen Lebensräumen hervorgerufen haben. Diese Langzeitstudie hat daher gezeigt, dass eine Extrapolation von Daten hinsichtlich der Prävalenz von TBP oder der Fluktuationen oder Stabilität der Bevölkerungsstruktur nicht möglich ist.

Table of Figures

List of Tables

Bibliography

Akl, T., Bourgoin, G., Souq, M.L., Appolinaire, J., Poirel, M.T., Gibert, P., et al. (2019) Detection of tick-borne pathogens in questing *Ixodes ricinus* in the French Pyrenees and first identification of *Rickettsia monacensis* in France. Parasite (Paris, France), 26: 20-20.

Ambrasiene, D., Turcinaviciene, J. and Vaščilo, I. (2004) The Prevalence of *Borrelia burgdorferi* in *Ixodes ricinus* ticks detected by PCR in Lithunia.

Ammazzalorso, A.D., Zolnik, C.P., Daniels, T.J. and Kolokotronis, S.O. (2015) To beat or not to beat a tick: Comparison of DNA extraction methods for ticks (*Ixodes scapularis*). PeerJ, 3: e1147.

Apanaskevich, D.A. and Oliver, J.H., Jr. (2014) Life cycles and natural history of ticks. In Biology of Ticks. Sonenshine, D.E. and Roe, R.M. (eds). Oxford University Press. 59-73.

Ari, S. and Arıkan, M. (2016) Next-Generation Sequencing: Advantages, disadvantages, and future. In. 109-35.

Aureli, S., Galuppi, R., Ostanello, F., Foley, J.E., Bonoli, C., Rejmanek, D., et al. (2015) Abundance of questing ticks and molecular evidence for pathogens in ticks in three parks of Emilia-Romagna region of northern Italy. Ann Agric Environ Med, 22: 459-66.

Bacon, R.M., Kugeler, K.J. and Mead, P.S. (2008) Surveillance for Lyme disease United States, 1992-2006. MMWR Surveill Summ, 57: 1-9.

Balmelli, T. and Piffaretti, J.C. (1995) Association between different clinical manifestations of Lyme disease and different species of *Borrelia burgdorferi* sensu lato. Res Microbiol, 146: 329-40.

Baranton, G., Postic, D., Saint, G.I., Boerlin, P., Piffaretti, J.C., Assous, M. and Grimont, P.A. (1992) Delineation of *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* sp. nov., and group VS461 associated with Lyme borreliosis. Int J Syst Bacteriol, 42: 378-83.

Barbour, A.G. (2014) Phylogeny of a relapsing fever *Borrelia* species transmitted by the hard tick *Ixodes scapularis*. Infect Genet Evol, 27: 551-8.

Barbour, A.G., Bunikis, J., Travinsky, B., Hoen, A.G., Diuk-Wasser, M.A., Fish, D. and Tsao, J.I. (2009) Niche partitioning of *Borrelia burgdorferi* and *Borrelia miyamotoi* in the same tick vector and mammalian reservoir species. Am J Trop Med Hyg, 81: 1120-31.

Barbour, A.G. and Fish, D. (1993) The biological and social phenomenon of Lyme disease. Science, 260: 1610-6.

Barbour, A.G. and Hayes, S.F. (1986) Biology of *Borrelia* species. Microbiol Rev, 50: 381-400.

Barbour, A.G. and Travinsky, B. (2010) Evolution and distribution of the ospC gene, a transferable serotype determinant of *Borrelia burgdorferi*. mBio, 1: e00153-10.

Baril, C., Richaud, C., Baranton, G. and Saint, G.I.S. (1989) Linear chromosome of *Borrelia burgdorferi*. Res Microbiol, 140: 507-16.

Bates, D., Mächler, M., Bolker, M.B. and Walker, S.C. (2015) Fitting linear mixed-effects models using lme4. J Stat Softw, 67: 48.

Battisti, J.M., Bono, J.L., Rosa, P.A., Schrumpf, M.E., Schwan, T.G. and Policastro, P.F. (2008) Outer surface protein A protects Lyme disease spirochetes from acquired host immunity in the tick vector. Infect Immun, 76: 5228-37.

Baumgarten, B.U., Rollinghoff, M. and Bogdan, C. (1999) Prevalence of *Borrelia burgdorferi* and granulocytic and monocytic ehrlichiae in *Ixodes ricinus* ticks from southern Germany. J Clin Microbiol, 37: 3448-51.

Becker, N.S., Margos, G., Blum, H., Krebs, S., Graf, A., Lane, R.S., et al. (2016) Recurrent evolution of host and vector association in bacteria of the *Borrelia burgdorferi* sensu lato species complex. BMC Genomics, 17: 734.

Bellet-Edimo, R., Betschart, B. and Gern, L. (2005) Frequency and efficiency of transovarial and subsequent transstadial transmissions of *Borrelia burgdorferi* sensu lato in *Ixodes ricinus* ticks.

Ben Said, M., Belkahia, H., Alberti, A., Abdi, K., Zhioua, M., Daaloul-Jedidi, M. and Messadi, L. (2016) First molecular evidence of *Borrelia burgdorferi* sensu lato in goats, sheep, cattle and camels in Tunisia. Ann Agric Environ Med, 23: 442-47.

Blazejak, K., Raulf, M.K., Janecek, E., Jordan, D., Fingerle, V. and Strube, C. (2018) Shifts in *Borrelia burgdorferi* (s.l.) geno-species infections in *Ixodes ricinus* over a 10-year surveillance period in the city of Hanover (Germany) and *Borrelia miyamotoi*-specific reverse line blot detection. Parasit Vectors, 11: 304.

Boden, K., Lobenstein, S., Hermann, B., Margos, G. and Fingerle, V. (2016) *Borrelia miyamotoi*-associated neuroborreliosis in immunocompromised person. Emerg Infect Dis, 22: 1617-20.

Bormane, A., Lucenko, I., Duks, A., Mavtchoutko, V., Ranka, R., Salmina, K. and Baumanis, V. (2004) Vectors of tick-borne diseases and epidemiological situation in Latvia in 1993-2002. Int J Med Microbiol, 293 Suppl 37: 36-47.

Brisson, D. and Dykhuizen, D.E. (2004) OspC diversity in *Borrelia burgdorferi*: different hosts are different niches. Genetics, 168: 713-22.

Bruyndonckx, N., Henry, I., Christe, P. and Kerth, G. (2009) Spatio-temporal population genetic structure of the parasitic mite *Spinturnix bechsteini* is shaped by its own demography and the social system of its bat host. Mol Ecol, 18: 3581-92.

Buczek, A., Ciura, D., Bartosik, K., Zając, Z. and Kulisz, J. (2014) Threat of attacks of *Ixodes ricinus* ticks (Ixodida: Ixodidae) and Lyme borreliosis within urban heat islands in south-western Poland. Parasites & vectors, 7: 562-62.

Bunikis, J., Garpmo, U., Tsao, J., Berglund, J., Fish, D. and Barbour, A.G. (2004) Sequence typing reveals extensive strain diversity of the Lyme borreliosis agents *Borrelia burgdorferi* in North America and *Borrelia afzelii* in Europe. Microbiology, 150: 1741-55.

Burgdorfer, W., Barbour, A.G., Hayes, S.F., Benach, J.L., Grunwaldt, E. and Davis, J.P. (1982) Lyme disease-a tick-borne spirochetosis? Science, 216: 1317-9.

Burgess, E.C. (1988) *Borrelia burgdorferi* infection in Wisconsin horses and cows. Ann N Y Acad Sci, 539: 235-43.

Burgess, E.C., Gendron-Fitzpatrick, A. and Wright, W.O. (1987) Arthritis and systemic disease caused by *Borrelia burgdorferi* infection in a cow. J Am Vet Med Assoc, 191: 1468-70.

Busch, U., Hizo-Teufel, C., Boehmer, R., Fingerle, V., Nitschko, H., Wilske, B. and Preac-Mursic, V. (1996) Three species of *Borrelia burgdorferi* sensu lato (*B. burgdorferi* sensu stricto, *B afzelii*, and *B. garinii*) identified from cerebrospinal fluid isolates by pulsed-field gel electrophoresis and PCR. J Clin Microbiol, 34: 1072-8.

Canica, M.M., Nato, F., du Merle, L., Mazie, J.C., Baranton, G. and Postic, D. (1993) Monoclonal antibodies for identification of *Borrelia afzelii* sp. nov. associated with late cutaneous manifestations of Lyme borreliosis. Scand J Infect Dis, 25: 441-8.

Casjens, S. (2000) *Borrelia* genomes in the year 2000. J Mol Microbiol Biotechnol, 2: 401-10.

Casjens, S. and Huang, W.M. (1993) Linear chromosomal physical and genetic map of *Borrelia burgdorferi*, the Lyme disease agent. Mol Microbiol, 8: 967-80.

Casjens, S.R., Fraser-Liggett, C.M., Mongodin, E.F., Qiu, W.G., Dunn, J.J., Luft, B.J. and Schutzer, S.E. (2011) Whole genome sequence of an unusual *Borrelia burgdorferi* sensu lato isolate. J Bacteriol, 193: 1489-90.

Casjens, S.R., Gilcrease, E.B., Vujadinovic, M., Mongodin, E.F., Luft, B.J., Schutzer, S.E., et al. (2017) Plasmid diversity and phylogenetic consistency in the Lyme disease agent *Borrelia burgdorferi*. BMC Genomics, 18: 165.

Casjens, S.R., Mongodin, E.F., Qiu, W.G., Luft, B.J., Schutzer, S.E., Gilcrease, E.B., et al. (2012) Genome stability of Lyme disease spirochetes: Comparative genomics of *Borrelia burgdorferi* plasmids. PLoS One, 7: e33280.

Cayol, C., Koskela, E., Mappes, T., Siukkola, A. and Kallio, E.R. (2017) Temporal dynamics of the tick *Ixodes ricinus* in northern Europe: Epidemiological implications. Parasit Vectors, 10: 166.

Chvostáč, M., Špitalská, E., Václav, R., Vaculová, T., Minichová, L. and Derdáková, M. (2018) Seasonal Patterns in the Prevalence and Diversity of Tick-Borne *Borrelia burgdorferi* Sensu Lato, *Anaplasma phagocytophilum* and *Rickettsia spp.* in an Urban Temperate Forest in South Western Slovakia. Intern J Environ Res Public Health, 15: 994.

Cohen, N.D., Heck, F.C., Heim, B., Flad, D.M., Bosler, E.M. and Cohen, D. (1992) Seroprevalence of antibodies to *Borrelia burgdorferi* in a population of horses in central Texas. J Am Vet Med Assoc, 201: 1030-4.

Coipan, C.E., van Duijvendijk, G.L.A., Hofmeester, T.R., Takumi, K. and Sprong, H. (2018) The genetic diversity of *Borrelia afzelii* is not maintained by the diversity of the rodent hosts. Parasit Vectors, 11: 454.

Coipan, E.C., Jahfari, S., Fonville, M., Maassen, C.B., J., v.d.G., Takken, W., et al. (2013) Spatiotemporal dynamics of emerging pathogens in questing *Ixodes ricinus*. Front Cell Infec Mi, 3: 36-36.

Coipan, E.C., Jahfari, S., Fonville, M., Oei, G.A., Spanjaard, L., Takumi, K., et al. (2016) Imbalanced presence of *Borrelia burgdorferi* s.l. multilocus sequence types in clinical manifestations of Lyme borreliosis. Infect Genet Evol, 42: 66-76.

Comstedt, P., Asokliene, L., Eliasson, I., Olsen, B., Wallensten, A., Bunikis, J. and Bergstrom, S. (2009) Complex population structure of Lyme borreliosis group spirochete *Borrelia garinii* in subarctic Eurasia. PLoS One, 4: e5841.

Comstedt, P., Bergstrom, S., Olsen, B., Garpmo, U., Marjavaara, L., Mejlon, H., et al. (2006) Migratory passerine birds as reservoirs of Lyme borreliosis in Europe. Emerg Infect Dis, 12: 1087-95.

Comstedt, P., Jakobsson, T. and Bergström, S. (2011) Global ecology and epidemiology of *Borrelia garinii* spirochetes. Infect Ecol Epidemiology, 1: 10.3402/iee.v1i0.9545.

Cook, M.J. (2015) Lyme borreliosis: A review of data on transmission time after tick attachment. Int J Gen Med, 8: 1-8.

Couper, D., Margos, G., Kurtenbach, K. and Turton, S. (2010) Prevalence of *Borrelia* infection in ticks from wildlife in south-west England. Veterinary Record, 167: 1012.

Cutler, S.J., Ruzic-Sabljic, E. and Potkonjak, A. (2017) Emerging *Borreliae* - Expanding beyond Lyme borreliosis. Mol Cell Probes, 31: 22-27.

Demaerschalck, I., Ben Messaoud, A., De Kesel, M., Hoyois, B., Lobet, Y., Hoet, P., et al. (1995) Simultaneous presence of different *Borrelia burgdorferi* genospecies in biological fluids of Lyme disease patients. J Clin Microbiol, 33: 602-8.

Derdakova, M. and Lencakova, D. (2005) Association of genetic variability within the *Borrelia burgdorferi* sensu lato with the ecology, epidemiology of Lyme borreliosis in Europe. Ann Agric Environ Med, 12: 165-72.

Dessau, R.B., van Dam, A.P., Fingerle, V., Gray, J., Hovius, J.W., Hunfeld, K.P., et al. (2018) To test or not to test? Laboratory support for the diagnosis of Lyme borreliosis: A position paper of ESGBOR, the ESCMID study group for Lyme borreliosis. Clin Microbiol Infect, 24: 118-24.

Dinnis, R.E., Seelig, F., Bormane, A., Donaghy, M., Vollmer, S.A., Feil, E.J., et al. (2014) Multilocus sequence typing using mitochondrial genes (mtMLST) reveals geographic population structure of *Ixodes ricinus* ticks. Ticks Tick Borne Dis, 5: 152-60.

Durand, J., Herrmann, C., Genne, D., Sarr, A., Gern, L. and Voordouw, M.J. (2017) Multistrain infections with Lyme borreliosis pathogens in the tick vector. Appl Environ Microbiol, 83.

Durand, J., Jacquet, M., Paillard, L., Rais, O., Gern, L. and Voordouw, M.J. (2015) Cross-immunity and community structure of a multiple-strain pathogen in the tick vector. Appl Environ Microbiol, 81: 7740-52.

Dykhuizen, D.E. and Baranton, G. (2001) The implications of a low rate of horizontal transfer in *Borrelia*. Trends Microbiol, 9: 344-50.

Ehrmann, S., Ruyts, S.C., Scherer-Lorenzen, M., Bauhus, J., Brunet, J., Cousins, S.A.O., et al. (2018) Habitat properties are key drivers of *Borrelia burgdorferi* (s.l.) prevalence in *Ixodes ricinus* populations of deciduous forest fragments. Parasites & Vectors, 11: 23-23.

Eisen, L. and Lane, R.S. (2002) Vectors of *Borrelia burgdorferi* sensu lato. In Lyme Borreliosis: Biology, Epidemiology, and Control.

Enkelmann, J., Böhmer, M., Fingerle, V., Siffczyk, C., Werber, D., Littmann, M., et al. (2018) Incidence of notified Lyme borreliosis in Germany, 2013–2017. Scientific Reports, 8: 1--8.

Enright, M.C. and Spratt, B.G. (1998) A multilocus sequence typing scheme for *Streptococcus pneumoniae*: identification of clones associated with serious invasive disease. Microbiology, 144 (Pt 11): 3049-60.

Enright, M.C. and Spratt, B.G. (1999) Multilocus sequence typing. Trends Microbiol, 7: 482-7.

Estrada-Peña, A., Ayllón, N. and de la Fuente, J. (2012) Impact of climate trends on tick-borne pathogen transmission. Frontiers Physiol, 3: 64-64.

Estrada-Pena, A., Cutler, S., Potkonjak, A., Vassier-Tussaut, M., Van Bortel, W., Zeller, H., et al. (2018) An updated meta-analysis of the distribution and prevalence of *Borrelia burgdorferi* s.l. in ticks in Europe. Int J Health Geogr, 17: 41.

Estrada-Pena, A., Martinez, J.M., C., S.A., Quilez, J. and Del Cacho, E. (2004) Phenology of the tick, *Ixodes ricinus*, in its southern distribution range (central Spain). Med Vet Entomol, 18: 387-97.

Estrada-Pena, A., Ortega, C., Sanchez, N., Desimone, L., Sudre, B., Suk, J.E. and Semenza, J.C. (2011) Correlation of *Borrelia burgdorferi* sensu lato prevalence in questing *Ixodes ricinus* ticks with specific abiotic traits in the western palearctic. Appl Environ Microbiol, 77: 3838-45.

Estrada-Peña, A. and Venzal, J.M. (2006) Changes in habitat suitability for the tick *Ixodes ricinus* (Acari: Ixodidae) in Europe (1900–1999). EcoHealth, 3: 154-62.

Etti, S., Hails, R., Schafer, S.M., De Michelis, S., Sewell, H.S., Bormane, A., et al. (2003) Habitat-specific diversity of *Borrelia burgdorferi* sensu lato in Europe, exemplified by data from Latvia. Appl Environ Microbiol, 69: 3008-10.

Falco, R.C. and Fish, D. (1991) Horizontal movement of adult *Ixodes dammini* (Acari: Ixodidae) attracted to CO2-baited traps. J Med Entomol, 28: 726-9.

Feil, E.J., Li, B.C., Aanensen, D.M., Hanage, W.P. and Spratt, B.G. (2004) eBURST: Inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J Bacteriol, 186: 1518-30.

Feil, E.J. and Spratt, B.G. (2001) Recombination and the population structures of bacterial pathogens. Annu Rev Microbiol, 55: 561-90.

Fingerle, V., Eiffert, H., Gessner, A., Göbel, U.B., Hofmann, H., Hunfeld, K.P., et al. (2017) MiQ12 Lyme-borreliose. In Therapie und Verlauf neurologischer Erkrankungen. Podbielski, A., Herrmann, M., Kniehl, E., Mauch, H. and Rüssmann, H.H. (eds). München, Jena: Urban & Fischer Verlag. 1-68.

Fingerle, V., Hauser, U., Liegl, G., Petko, B., Preac-Mursic, V. and Wilske, B. (1995) Expression of outer surface proteins A and C of *Borrelia burgdorferi* in *Ixodes ricinus*. J. Clin. Microbiol., 33: 1867-69.

Fingerle, V., Laux, H., Munderloh, U., Schulte-Spechtel, U. and Wilske, B. (2000) Differential expression of outer surface proteins A and C by individual *Borrelia burgdorferi* in different genospecies. Med Microbiol Immunol 189: 59-66

Fingerle, V., Liegl, G., Munderloh, U. and Wilske, B. (1998) Expression of outer surface proteins A and C of *Borrelia burgdorferi* in *Ixodes ricinus* ticks removed from humans. Med Microbiol Immunol, 187: 121-6.

Fingerle, V., Rauser, S., Hammer, B., Kahl, O., Heimerl, C., Schulte-Spechtel, U., et al. (2002) Dynamics of dissemination and outer surface protein expression of different European *Borrelia burgdorferi* sensu lato strains in artificially infected *Ixodes ricinus* nymphs. J Clin Microbiol, 40: 1456-63.

Fingerle, V., Schulte-Spechtel, U.C., Ruzic-Sabljic, E., Leonhard, S., Hofmann, H., Weber, K., et al. (2008) Epidemiological aspects and molecular characterization of *Borrelia burgdorferi* s.l. from southern Germany with special respect to the new species *Borrelia spielmanii* sp. nov. Int J Med Microbiol, 298: 279-90.

Fraser, C.M., Casjens, S., Huang, W.M., Sutton, G.G., Clayton, R., Lathigra, R., et al. (1997) Genomic sequence of a Lyme disease spirochaete, *Borrelia burgdorferi*. Nature, 390: 580-6.

Fritz, L.C. and Kjemtrup, A.M. (2003) Lyme borreliosis. Vet Med Today, 223: 1261-70.

Fukunaga, M., Hamase, A., Okada, K. and Nakao, M. (1996) *Borrelia tanukii* sp. nov. and *Borrelia turdae* sp. nov. found from ixodid ticks in Japan: rapid species identification by 16S rRNA gene-targeted PCR analysis. Microbiol Immunol, 40: 877-81.

Fukunaga, M., Takahashi, Y., Tsuruta, Y., Matsushita, O., Ralph, D., McClelland, M. and Nakao, M. (1995) Genetic and phenotypic analysis of *Borrelia miyamotoi* sp. nov., isolated from the ixodid tick *Ixodes persulcatus*, the vector for Lyme disease in Japan. Int J Syst Bacteriol, 45: 804-10.

Galfsky, D., Król, N., Pfeffer, M. and Obiegala, A. (2019) Long-term trends of tick-borne pathogens in regard to small mammal and tick populations from Saxony, Germany. Parasites & Vectors, 12: 131-31.

Gasmi, S., Ogden, N.H., Ripoche, M., Leighton, P.A., Lindsay, R.L., Nelder, M.P., et al. (2019) Detection of municipalities at-risk of Lyme disease using passive surveillance of *Ixodes scapularis* as an early signal: A province-specific indicator in Canada. PLoS One, 14: e0212637.

Geller, J., Nazarova, L., Katargina, O. and Golovljova, I. (2013) *Borrelia burgdorferi* sensu lato prevalence in tick populations in Estonia. Parasit Vectors, 6: 202.

Gelman, A. and Su, Y.-S. (2016) Data analysis using regression and multilevel/hierarchical models [Internet].

Gern, L., Estrada-Pena, A., Frandsen, F., Gray, J.S., Jaenson, T.G., Jongejan, F., et al. (1998) European reservoir hosts of *Borrelia burgdorferi* sensu lato. Zentralbl Bakteriol, 287: 196-204.

Gern, L. and Humair, P.F. (2002) "Ecology of *Borrelia burgdorferi* sensu lato in Europe." In *Lyme Borreliosis: Biology, Epidemiology and Control,* edited by O. K. J.S Gray, R.S.l.a.G.S.]: CABI Publishing.

Gern, L. and Rais, O. (1996) Efficient transmission of *Borrelia burgdorferi* between cofeeding *Ixodes ricinus* ticks (Acari: Ixodidae). J Med Entomol, 33: 189-92.

Gern, L. and Sell, K. (2009) Isolation of *Borrelia burgdorferi* sensu lato from the skin of the European badger (Meles meles) in Switzerland. Vector Borne Zoonotic Dis, 9: 207-8.

Gevers, D., Cohan, F.M., Lawrence, J.G., Spratt, B.G., Coenye, T., Feil, E.J., et al. (2005) Opinion: Re-evaluating prokaryotic species. Nat Rev Microbiol, 3: 733-9.

Gibbs, C.P., Livey, I. and Dorner, F. (1996) The role of recombination in ospC variation in Lyme disease *Borrelia*. acta dermatovenerologica A.P.A., 5: 179-83.

Ginsberg, H.S., Buckley, P.A., Balmforth, M.G., Zhioua, E., Mitra, S. and Buckley, F.G. (2005) Reservoir competence of native North American birds for the Lyme disease spirochete, *Borrelia burgdorfieri*. J Med Entomol, 42: 445-9.

Gofton, A.W., Margos, G., Fingerle, V., Hepner, S., Loh, S.M., Ryan, U., et al. (2018) Genome-wide analysis of *Borrelia turcica* and '*Candidatus* Borrelia tachyglossi' shows relapsing fever-like genomes with unique genomic links to Lyme disease *Borrelia*. Infect Genet Evol, 66: 72-81.

Goldstein, S.F., Charon, N.W. and Kreiling, J.A. (1994) *Borrelia burgdorferi* swims with a planar waveform similar to that of eukaryotic flagella. Proc Natl Acad Sci U S A, 91: 3433-7.

Gray, J.S., Dautel, H., Estrada-Pena, A., Kahl, O. and Lindgren, E. (2009) Effects of climate change on ticks and tick-borne diseases in europe. Interdiscip Perspect Infect Dis, 2009: 593232.

Grimm, D., Tilly, K., Byram, R., Stewart, P.E., Krum, J.G., Bueschel, D.M., et al. (2004) Outer-surface protein C of the Lyme disease spirochete: a protein induced in ticks for infection of mammals. Proc Natl Acad Sci U S A, 101: 3142-7.

Guglielmone, A.A., Robbins, R.G., Apanaskevich, D.A., Petney, T.N., Estrada-Pena, A., Horak, I.G., et al. (2010) The Argasidae, Ixodidae and Nuttalliellidae (Acari: Ixodida) of the world: A list of valid species names. Zootaxa, 2528: 1-28.

Güner, E.S., Hashimoto, N., Kadosaka, T., Imai, Y. and Masuzawa, T. (2003) A novel, fast-growing *Borrelia* sp. isolated from the hard tick *Hyalomma aegyptium* in Turkey. Microbiology, 149: 2539-44.

Gupta, R.S., Mahmood, S. and Adeolu, M. (2013) Erratum: A phylogenomic and molecular signature based approach for characterization of the phylum spirochaetes and its major clades: proposal for a taxonomic revision of the phylum. Front Microbiol, 4: 322.

Guy, E.C. and Stanek, G. (1991) Detection of *Borrelia burgdorferi* in patients with Lyme disease by the polymerase chain reaction. J Clin Pathol, 44: 610-1.

Gylfe, A., Olsen, B., Strasevicius, D., Ras, N.M., Weihe, P., Noppa, L., et al. (1999) Isolation of Lyme disease *Borrelia* from puffins (*Fratercula arctica*) and seabird ticks (*Ixodes uriae*) on the Faeroe Islands. J Clin Microbiol, 37: 890-6.

Halos, L., Jamal, T., Vial, L., Maillard, R., Suau, A., Le Menach, A., et al. (2004) Determination of an efficient and reliable method for DNA extraction from ticks. Vet Res, 35: 709-13.

Hanincova, K., Kurtenbach, K., Diuk-Wasser, M., Brei, B. and Fish, D. (2006) Epidemic spread of Lyme borreliosis, northeastern United States. Emerg Infect Dis, 12: 604-11.

Hanincova, K., Mukherjee, P., Ogden, N.H., Margos, G., Wormser, G.P., Reed, K.D., et al. (2013) Multilocus sequence typing of *Borrelia burgdorferi* suggests existence

of lineages with differential pathogenic properties in humans. PLoS One, 8: e73066.

Hansford, K.M., Fonville, M., Gillingham, E.L., Coipan, E.C., Pietzsch, M.E., Krawczyk, A.I., et al. (2017) Ticks and *Borrelia* in urban and peri-urban green space habitats in a city in southern England. Ticks Tick Borne Dis, 8: 353-61.

Haven, J., Vargas, L.C., Mongodin, E.F., Xue, V., Hernandez, Y., Pagan, P., et al. (2011) Pervasive recombination and sympatric genome diversification driven by frequency-dependent selection in *Borrelia burgdorferi*, the Lyme disease bacterium. Genetics, 189: 951-66.

Head, S.R., Komori, H.K., LaMere, S.A., Whisenant, T., Van Nieuwerburgh, F., Salomon, D.R. and Ordoukhanian, P. (2014) Library construction for next-generation sequencing: Overviews and challenges. Biotechniques, 56: 61-4, 66, 68, passim.

Herrmann, C. and Gern, L. (2015) Search for blood or water is influenced by *Borrelia burgdorferi* in *Ixodes ricinus*. Parasites & Vectors, 8: 6.

Herrmann, C., Gern, L. and Voordouw, M.J. (2013) Species co-occurrence patterns among Lyme borreliosis pathogens in the tick vector *Ixodes ricinus*. Appl Environ Microbiol, 79: 7273-80.

Hillyard, P.D. (1996) Ticks of North-West Europe: keys and notes for identification of the species: Published for the Linnean Society of London and the Estuarine and Coastal Sciences Association by Field Studies Council. Med Vet Entomol ,11: 102-103

Hoen, A.G., Margos, G., Bent, S.J., Diuk-Wasser, M.A., Barbour, A., Kurtenbach, K. and Fish, D. (2009) Phylogeography of *Borrelia burgdorferi* in the eastern United States reflects multiple independent Lyme disease emergence events. Proc Natl Acad Sci U S A, 106: 15013-8.

Hoen, A.G., Rollend, L.G., Papero, M.A., Carroll, J.F., Daniels, T.J., Mather, T.N., et al. (2009) Effects of tick control by acaricide self-treatment of white-tailed deer on host-seeking tick infection prevalence and entomologic risk for *Ixodes scapularis*-borne pathogens. Vector Borne Zoonotic Dis, 9: 431-8.

Hofmann, H., Rauer, S., V., F., Huppertz, H.I., Hunfeld, K.P., Krause, A. and Ruf, B. (2016) AWMF-Leitlinie "kutane lyme borreliose". AWMF online, Das Portal der wissenschaftlichen Medizin:
1-65.

Hoornstra, D., Koetsveld, J., Sprong, H., Platonov, A.E. and Hovius, J.W. (2018) *Borrelia miyamotoi* disease in an immunocompetent patient, western Europe. Emerg Infect Dis, 24: 1770-72.

Hubalek, Z. (2009) Epidemiology of Lyme borreliosis. Curr Probl Dermatol, 37: 31-50.

Hubalek, Z. and Halouzka, J. (1998) Prevalence rates of *Borrelia burgdorferi* sensu lato in host-seeking *Ixodes ricinus* ticks in Europe. Parasitol Res, 84: 167-72.

Humair, P.F., Postic, D., Wallich, R. and Gern, L. (1998) An avian reservoir (*Turdus merula*) of the Lyme borreliosis spirochetes. Zentralbl Bakteriol, 287: 521-38.

Illumina. (2017) Nextera™ DNA flex library preparation kit.

Illumina. (2019) Key differences between next-generation sequencing and Sanger sequencing.

Ivanova, L.B., Tomova, A., Gonzalez-Acuna, D., Murua, R., Moreno, C.X., Hernandez, C., et al. (2014) *Borrelia chilensis*, a new member of the *Borrelia burgdorferi* sensu lato complex that extends the range of this genospecies in the southern hemisphere. Environ Microbiol, 16: 1069-80.

Jacquot, M., Gonnet, M., Ferquel, E., Abrial, D., Claude, A., Gasqui, P., et al. (2014) Comparative population genomics of the *Borrelia burgdorferi* species complex reveals high degree of genetic isolation among species and underscores benefits and constraints to studying intra-specific epidemiological processes. PloS one, 9: e94384-e84.

James, M.C., Gilbert, L., Bowman, A.S. and Forbes, K.J. (2014) The heterogeneity, distribution, and environmental associations of *Borrelia burgdorferi* sensu lato, the agent of Lyme borreliosis, in Scotland. Front Public Health, 2: 129.

Jauris-Heipke, S., Liegl, G., Preac-Mursic, V., Rössler, D., Schwab, E., Soutschek, E., et al. (1995) Molecular analysis of genes encoding outer surface protein C (OspC) of *Borrelia burgdorferi* sensu lato: Relationship to ospA genotype and evidence of lateral gene exchange of ospC. J clin Microbiol, 33: 1860-66.

Johnson, R.C., Schmid, G.P., Hyde, F.W., Steigerwalt, A.G. and Brenner, D.J. (1984) *Borrelia burgdorferi* sp. nov.: Etiologic agent of Lyme disease Systematic Bacteriology, 34.

Jones, L.D., Davies, C.R., Steele, G.M. and Nuttall, P.A. (1987) A novel mode of arbovirus transmission involving a nonviremic host. Science, 237: 775-7.

Jouda, F., Perret, J.L. and Gern, L. (2004) Density of questing *Ixodes ricinus* nymphs and adults infected by *Borrelia burgdorferi* sensu lato in Switzerland: Spatio-temporal pattern at a regional scale. Vector Borne Zoonotic Dis, 4: 23-32.

Jouda, F., Perret, J.L. and Gern, L. (2004) *Ixodes ricinus* density, and distribution and prevalence of *Borrelia burgdorferi* sensu lato infection along an altitudinal gradient. J Med Entomol, 41:
162-9.

Jungnick, S., Margos, G., Rieger, M., Dzaferovic, E., Bent, S.J., Overzier, E., et al. (2015) *Borrelia burgdorferi* sensu stricto and *Borrelia afzelii*: Population structure and differential pathogenicity. Int J Med Microbiol, 305: 673-81.

Kampen, H., Rotzel, D.C., Kurtenbach, K., Maier, W.A. and Seitz, H.M. (2004) Substantial rise in the prevalence of Lyme borreliosis spirochetes in a region of western Germany over a 10-year period. Appl Environ Microbiol, 70: 1576-82.

Karan, L., Makenov, M., Kolyasnikova, N., Stukolova, O., Toporkova, M. and Olenkova, O. (2018) Dynamics of spirochetemia and early PCR detection of *Borrelia miyamotoi*. Emerg Infect Dis, 24: 860-67.

Kawabata, H., Masuzawa, T. and Yanagihara, Y. (1993) Genomic analysis of *Borrelia japonica* sp. nov. isolated from *Ixodes ovatus* in Japan. Microbiol Immunol, 37: 843-8.

Keirans, J.E., Hutcheson, H.J., Durden, L.A. and Klompen, J.S. (1996) *Ixodes* (*Ixodes*) *scapularis* (Acari:Ixodidae): redescription of all active stages, distribution, hosts, geographical variation, and medical and veterinary importance. J Med Entomol, 33: 297-318.

Kenedy, M.R., Lenhart, T.R. and Akins, D.R. (2012) The role of *Borrelia burgdorferi* outer surface proteins. FEMS Immunol Med Microbiol, 66: 1-19.

Kimura, M. (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol, 16: 111-20.

Kingry, L.C., Anacker, M., Pritt, B., Bjork, J., Respicio-Kingry, L., Liu, G., et al. (2018) Surveillance for and discovery of *Borrelias* species in US patients suspected of tickborne illness. Arch Clin Infect Dis, 66: 1864-71.

Kirstein, F., Rijpkema, S., Molkenboer, M. and Gray, J.S. (1997) Local variations in the distribution and prevalence of *Borrelia burgdorferi* sensu lato genomospecies in *Ixodes ricinus* ticks. Appl Environ Microbiol, 63: 1102-06.

Kocan, K.M., de la Fuente, J. and Coburn, L.A. (2015) Insights into the development of *Ixodes scapularis:* a resource for research on a medically important tick species. Parasites & vectors, 8: 592-92.

Krause, P.J., Fish, D., Narasimhan, S. and Barbour, A.G. (2015) *Borrelia miyamotoi* infection in nature and in humans. Clin Microbiol Infect, 21: 631-9.

Krupka, I. and Straubinger, R.K. (2010) Lyme borreliosis in dogs and cats: background, diagnosis, treatment and prevention of infections with *Borrelia burgdorferi* sensu stricto. Vet Clin North Am Small Anim Pract, 40: 1103-19.

Kubiak, K. and Dziekonska-Rynko, J. (2006) Seasonal activity of the common European tick, *Ixodes ricinus* (Linnaeus, 1758), in the forested areas of the city of Olsztyn and its surroundings. Wiad Parazytol, 52: 59-64.

Kumaran, D., Eswaramoorthy, S., Luft, B.J., Koide, S., Dunn, J.J., Lawson, C.L. and Swaminathan, S. (2001) Crystal structure of outer surface protein C (OspC) from the Lyme disease spirochete, *Borrelia burgdorferi*. Embo j, 20: 971-8.

Kurtenbach, K., De Michelis, S., Etti, S., Schafer, S.M., Sewell, H.S., Brade, V. and Kraiczy, P. (2002) Host association of *Borrelia burgdorferi* sensu lato-the key role of host complement. Trends Microbiol, 10: 74-9.

Kurtenbach, K., De Michelis, S., Sewell, H.S., Etti, S., Schafer, S.M., Hails, R., et al. (2001) Distinct combinations of *Borrelia burgdorferi* sensu lato genospecies

found in individual questing ticks from Europe. Appl Environ Microbiol, 67: 4926-9.

Kurtenbach, K., De Michelis, S., Sewell, H.S., Etti, S., Schafer, S.M., Holmes, E., et al. (2002) The key roles of selection and migration in the ecology of Lyme borreliosis. Int J Med Microbiol, 291 Suppl 33: 152-4.

Kurtenbach, K., Hanincova, K., Tsao, J.I., Margos, G., Fish, D. and Ogden, N.H. (2006) Fundamental processes in the evolutionary ecology of Lyme borreliosis. Nat Rev Microbiol, 4: 660-9.

Kurtenbach, K., Peacey, M., Rijpkema, S.G., Hoodless, A.N., Nuttall, P.A. and Randolph, S.E. (1998) Differential transmission of the genospecies of *Borrelia burgdorferi* sensu lato by game birds and small rodents in England. Appl Environ Microbiol, 64: 1169-74.

Lane, R.S. and Lavoie, P.E. (1988) Lyme borreliosis in California. Acarological, clinical, and epidemiological studies. Ann N Y Acad Sci, 539: 192-203.

Lappin, M.R., Chandrashekar, R., Stillman, B., Liu, J. and Mather, T.N. (2015) Evidence of *Anaplasma phagocytophilum* and *Borrelia burgdorferi* infection in cats after exposure to wild-caught adult *Ixodes scapularis*. J Vet Diagn Invest, 27: 522-5.

Larsen, M.V., Cosentino, S., Rasmussen, S., Friis, C., Hasman, H., Marvig, R.L., et al. (2012) Multilocus sequence typing of total-genome-sequenced bacteria. J Clin Microbiol, 50: 1355-61.

Le Fleche, A., Postic, D., Girardet, K., Peter, O. and Baranton, G. (1997) Characterization of *Borrelia lusitaniae* sp. nov. by 16S ribosomal DNA sequence analysis. Int J Syst Bacteriol, 47: 921-5.

LeFebvre, R.B., Perng, G.C. and Johnson, R.C. (1989) Characterization of *Borrelia burgdorferi* isolates by restriction endonuclease analysis and DNA hybridization. J Clin Microbiol, 27: 636-9.

Lejal, E., Marsot, M., Chalvet-Monfray, K., Cosson, J.F., Moutailler, S., Vayssier-Taussat, M. and Pollet, T. (2019) A three-years assessment of *Ixodes ricinus*-borne pathogens in a French peri-urban forest. bioRxiv: 597013.

Lemoine, M., Cornetti, L. and Tschirren, B. (2018) Does *Borrelia burgdorferi* sensu lato facilitate the colonisation of marginal habitats by *Ixodes ricinus*? A correlative study in the Swiss Alps. bioRxiv: 273490.

Liebisch, G., Sohns, B. and Bautsch, W. (1998) Detection and typing of *Borrelia burgdorferi* sensu lato in *Ixodes ricinus* ticks attached to human skin by PCR. J clin Microbiol, 36: 3355-58.

Lin, T., Oliver, J.H.J. and Gao, L. (2002) Genetic diversity of the outer surface protein C gene of southern *Borrelia* isolates and its possible epidemiological, clinical, and pathogenetic implications. J clin Microbiol, 40: 2572-83.

Lindgren, E. and Jaenson, T.G.T. (2006) Lyme borreliosis in Europe : Influences of climate and climate change, epidemiology, ecology and adaptation measures.

Livey, I., Gibbs, C.P., Schuster, R. and Dorner, F. (1995) Evidence for lateral transfer and recombination in OspC variation in Lyme disease *Borrelia*. Mol Microbiol, 18: 257-69.

Loh, S.M., Gofton, A.W., Lo, N., Gillett, A., Ryan, U.M., Irwin, P.J. and Oskam, C.L. (2016) Novel *Borrelia* species detected in echidna ticks, *Bothriocroton concolor,* in Australia. Parasit Vectors, 9: 339.

Lohr, B., Fingerle, V., Norris, D.E. and Hunfeld, K.P. (2018) Laboratory diagnosis of Lyme borreliosis: Current state of the art and future perspectives. Crit Rev Clin Lab Sci, 55: 219-45.

Maiden, M.C. (2006) Multilocus sequence typing of bacteria. Annu Rev Microbiol, 60: 561-88.

Maiden, M.C., Bygraves, J.A., Feil, E., Morelli, G., Russell, J.E., Urwin, R., et al. (1998) Multilocus sequence typing: A portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci U S A, 95: 3140-5.

Mannelli, A., Bertolotti, L., Gern, L. and Gray, J. (2012) Ecology of *Borrelia burgdorferi* sensu lato in Europe: Transmission dynamics in multi-host systems, influence of molecular processes and effects of climate change. FEMS Microbiol Rev, 36: 837-61.

Marchant, A., Le Coupanec, A., Joly, C., Perthame, E., Sertour, N., Garnier, M., et al. (2017) Infection of *Ixodes ricinus* by *Borrelia burgdorferi* sensu lato in peri-urban forests of France. PloS one, 12: 1-28.

Marconi, R.T., Hohenberger, S., Jauris-Heipke, S., Schulte-Spechtel, U., LaVoie, C.P., Rössler, D. and Wilske, B. (1999) Genetic analysis of *Borrelia garinii* ospA serotype 4 strains associated with neuroborreliosis: Evidence for extensive genetic homogeneity. J clin Microbiol, 37: 3965-70.

Marconi, R.T., Liveris, D. and Schwartz, I. (1995) Identification of novel insertion elements, restriction fragment length polymorphism patterns, and discontinuous 23S rRNA in Lyme disease spirochetes: Phylogenetic analyses of rRNA genes and their intergenic spacers in *Borrelia japonica* sp. nov. and genomic group 21038 (*Borrelia andersonii* sp. nov.) isolates. J Clin Microbiol, 33: 2427-34.

Margos, G., Chu, C.Y., Takano, A., Jiang, B.G., Liu, W., Kurtenbach, K., et al. (2015) *Borrelia yangtzensis* sp. nov., a rodent-associated species in Asia, is related to *Borrelia valaisiana*. Int J Syst Evol Microbiol, 65: 3836-40.

Margos, G., Fedorova, N., Kleinjan, J.E., Hartberger, C., Schwan, T.G., Sing, A. and Fingerle, V. (2017) *Borrelia lanei* sp. nov. extends the diversity of *Borrelia* species in California. Int J Syst Evol Microbiol, 67: 3872-76.

Margos, G., Gatewood, A.G., Aanensen, D.M., Hanincova, K., Terekhova, D., Vollmer, S.A., et al. (2008) MLST of housekeeping genes captures geographic

population structure and suggests a European origin of *Borrelia burgdorferi*. Proc Natl Acad Sci U S A, 105: 8730-5.

Margos, G., Gofton, A.W., Wibberg, D., Dangel, A., Marosevic, D., Loh, S.M., et al. (2018) The genus *Borrelia* reloaded. PloS one, 13: e0208432-e32.

Margos, G., Hepner, S., Mang, C., Marosevic, D., Reynolds, S.E., Krebs, S., et al. (2017) Lost in plasmids: Next generation sequencing and the complex genome of the tick-borne pathogen *Borrelia burgdorferi*. BMC Genomics, 18: 422.

Margos, G., Hojgaard, A., Lane, R.S., Cornet, M., Fingerle, V., Rudenko, N., et al. (2010) Multilocus sequence analysis of *Borrelia bissettii* strains from North America reveals a new *Borrelia* species, *Borrelia kurtenbachii*. Ticks Tick Borne Dis, 1: 151-8.

Margos, G., Lane, R.S., Fedorova, N., Koloczek, J., Piesman, J., Hojgaard, A., et al. (2016) *Borrelia bissettiae* sp. nov. and *Borrelia californiensis* sp. nov. prevail in diverse enzootic transmission cycles. Int J Syst Evol Microbiol, 66: 1447-52.

Margos, G., Sing, A. and Fingerle, V. (2017) Published data do not support the notion that *Borrelia valaisiana* is human pathogenic. Infection, 45: 567-69.

Margos, G., Tsao, J.I., Castillo-Ramirez, S., Girard, Y.A., Hamer, S.A., Hoen, A.G., et al. (2012) Two boundaries separate *Borrelia burgdorferi* populations in North America. Appl Environ Microbiol, 78: 6059-67.

Margos, G., Vollmer, S.A., Cornet, M., Garnier, M., Fingerle, V., Wilske, B., et al. (2009) A new *Borrelia* species defined by multilocus sequence analysis of housekeeping genes. Appl Environ Microbiol, 75: 5410-6.

Margos, G., Vollmer, S.A., Ogden, N.H. and Fish, D. (2011) Population genetics, taxonomy, phylogeny and evolution of *Borrelia burgdorferi* sensu lato. Infect Genet Evol, 11: 1545-63.

Margos, G., Wilske, B., Sing, A., Hizo-Teufel, C., Cao, W.C., Chu, C., et al. (2013) *Borrelia bavariensis* sp. nov. is widely distributed in Europe and Asia. Int J Syst Evol Microbiol, 63: 4284-8.

Masuzawa, T., Takada, N., Kudeken, M., Fukui, T., Yano, Y., Ishiguro, F., et al. (2001) *Borrelia sinica* sp. nov., a Lyme disease-related *Borrelia* species isolated in China. Int J Syst Evol Microbiol, 51: 1817-24.

Matuschka, F.R., Eiffert, H., Ohlenbusch, A. and Spielman, A. (1994) Amplifying role of edible dormice in Lyme disease transmission in central Europe. J Infec Dis, 170: 122-27.

Matuschka, F.R., Fischer, P., Heiler, M., Blumcke, S. and Spielman, A. (1992) Stage-associated risk of transmission of the Lyme disease spirochete by European Ixodes ticks. Parasitol Res,
78: 695-8.

McCoy, K.D., Boulinier, T., Tirard, C. and Michalakis, Y. (2001) Host specificity of a generalist parasite: Genetic evidence of sympatric host races in the seabird tick *Ixodes uriae*. J Evol Biol,
14: 395-405.

Mechai, S., Margos, G., Feil, E.J., Barairo, N., Lindsay, L.R., Michel, P. and Ogden, N.H. (2016) Evidence for host-genotype associations of *Borrelia burgdorferi* sensu stricto. PLoS One, 11: e0149345.

Mechai, S., Margos, G., Feil, E.J., Lindsay, L.R., Michel, P., Kotchi, S.O. and Ogden, N.H. (2018) Evidence for an effect of landscape connectivity on *Borrelia burgdorferi* sensu stricto dispersion in a zone of range expansion. Ticks and Tick-borne Diseases, 9: 1407-15.

Mechai, S., Margos, G., Feil, E.J., Lindsay, L.R. and Ogden, N.H. (2015) Complex population structure of *Borrelia burgdorferi* in southeastern and south central Canada as revealed by phylogeographic analysis. Appl Environ Microbiol, 81: 1309.

Mejlon, H.A. and Jaenson, T.G. (1993) Seasonal prevalence of *Borrelia burgdorferi* in *Ixodes ricinus* in different vegetation types in Sweden. Scand J Infect Dis, 25: 449-56.

Merilainen, L., Herranen, A., Schwarzbach, A. and Gilbert, L. (2015) Morphological and biochemical features of *Borrelia burgdorferi* pleomorphic forms. Microbiology, 161: 516-27.

Milutinovic, M., Radulovic, Z., Tomanovic, C. and Tomanovic, Z. (2006) Seasonal distribution of *Borrelia* in *Ixodes ricinus* ticks in the Belgrade region, Serbia. Arch Biol Sci Belgrade, 58: 183-86.

Mitani, H., Talbert, A. and Fukunaga, M. (2004) New world relapsing fever *Borrelia* found in *Ornithodoros porcinus* ticks in central Tanzania. Microbiol Immunol, 48: 501-5.

Morellet, N., Moorter, B., Cargnelutti, B., Angibault, J.M., Lourtet, B., Merlet, J., et al. (2011) Landscape composition influences roe deer habitat selection at both home range and landscape scale. Landscape Ecology, 26: 999-1010.

Motaleb, M.A., Corum, L., Bono, J.L., Elias, A.F., Rosa, P., Samuels, D.S. and Charon, N.W. (2000) *Borrelia burgdorferi* periplasmic flagella have both skeletal and motility functions. Proc Natl Acad Sci U S A, 97: 10899-904.

Moutailler, S., Valiente Moro, C., Vaumourin, E., Michelet, L., Tran, F.H., Devillers, E., et al. (2016) Co-infection of Ticks: The Rule Rather Than the Exception. PLoS Negl Trop Dis, 10: e0004539.

Mysterud, A., Stigum, V.M., Jaarsma, R.I. and Sprong, H. (2019) Genospecies of *Borrelia burgdorferi* sensu lato detected in 16 mammal species and questing ticks from northern Europe. Scientific Reports, 9: 5088.

Nahimana, I., Gern, L., Blanc, D., Praz, G., Francioli, P. and Péter, O. (2004) Risk of *Borrelia burgdorferi* infection in western Switzerland following a tick bite. European journal of clinical microbiology & infectious diseases, 23: 603-8.

Nakagawa, S. and Schielzeth, H. (2010) Repeatability for gaussian and non-gaussian data: A practical guide for biologists. Biol Rev Camb Philos Soc, 85: 935-56.

Ned, K. (2015) One-tailed or two-tailed P values in PLS-SEM? International Journal of e-Collaboration (IJeC), 11: 1-7.

Neelakanta, G., Li, X., Pal, U., Liu, X., Beck, D.S., DePonte, K., et al. (2007) Outer surface protein B is critical for *Borrelia burgdorferi* adherence and survival within *Ixodes* ticks. PLoS Pathog, 3: 33.

Nefedova, V.V., Korenberg, E.I., Gorelova, N.B. and Kovalevskii, Y.V. (2004) Studies on the transovarial transmission of *Borrelia burgdorferi* sensu lato in the taiga tick *Ixodes persulcatus*. Folia Parasitol (Praha), 51: 67-71.

Ni, X.B., Jia, N., Jiang, B.G., Sun, T., Zheng, Y.C., Huo, Q.B., et al. (2014) Lyme borreliosis caused by diverse genospecies of *Borrelia burgdorferi* sensu lato in northeastern China. Clin Microbiol Infect, 20: 808-14.

Ogden, N.H., Lindsay, L.R., Hanincova, K., Barker, I.K., Bigras-Poulin, M., Charron, D.F., et al. (2008) Role of migratory birds in introduction and range expansion of *Ixodes scapularis* ticks and of *Borrelia burgdorferi* and *Anaplasma phagocytophilum* in Canada. Appl Environ Microbiol, 74: 1780-90.

Ogden, N.H., Margos, G., Aanensen, D.M., Drebot, M.A., Feil, E.J., Hanincová, K., et al. (2011) Investigation of genotypes of *Borrelia burgdorferi* in *Ixodes scapularis* ticks collected during surveillance in Canada. Appl Environ Microbiol, 77: 3244-54.

Okeyo, M., Hartberger, C., Margos, G., Straubinger, R.K., Sing, A. and Fingerle, V. (2019) Comparison of methods for economic and efficient tick and *Borrelia* DNA purification. Ticks and Tick-borne Diseases, 10: 1041-45.

Olsen, B., Duffy, D.C., Jaenson, T.G., Gylfe, A., Bonnedahl, J. and Bergstrom, S. (1995) Transhemispheric exchange of Lyme disease spirochetes by seabirds. J Clin Microbiol, 33: 3270-4.

Olsen, B., Jaenson, T.G. and Bergstrom, S. (1995) Prevalence of *Borrelia burgdorferi* sensu lato-infected ticks on migrating birds. Appl Environ Microbiol, 61: 3082-7.

Ostfeld, R.S., Canham, C.D., Oggenfuss, K., Winchcombe, R.J. and Keesing, F. (2006) Climate, deer, rodents, and acorns as determinants of variation in Lyme-disease risk. PLoS biology, 4: e145-e45.

Pal, U., Li, X., Wang, T., Montgomery, R.R., Ramamoorthi, N., Desilva, A.M., et al. (2004) TROSPA, an *Ixodes scapularis* receptor for *Borrelia burgdorferi*. Cell, 119: 457-68.

Parola, P. and Raoult, D. (2001) Ticks and tickborne bacterial diseases in humans: an emerging infectious threat. Clin Infect Dis, 32: 897-928.

Patrican, L.A. (1997) Absence of Lyme disease spirochetes in larval progeny of naturally infected *Ixodes scapularis* (Acari:Ixodidae) fed on dogs. J Med Entomol, 34: 52-5.

Pawelczyk, A. and Sinski, E. (2004) Prevalence of *Ixodes ricinus* infection with *Borrelia burgdorferi* s.l. seasonal and annual variations. Wiad Parazytol, 50: 253-8.

Pearson, T., Giffard, P., Beckstrom-Sternberg, S., Auerbach, R., Hornstra, H., Tuanyok, A., et al. (2009) Phylogeographic reconstruction of a bacterial species with high levels of lateral gene transfer. BMC Biol, 7: 78.

Pérez, D., Kneubühler, Y., Rais, O., Jouda, F. and Gern, L. (2011) *Borrelia afzelii* ospC genotype diversity in *Ixodes ricinus* questing ticks and ticks from rodents in two Lyme borreliosis endemic areas: Contribution of co-feeding ticks. Ticks and Tick-borne Diseases, 2: 137-42.

Pfaffle, M., Littwin, N., Muders, S.V. and Petney, T.N. (2013) The ecology of tick-borne diseases. Int J Parasitol, 43: 1059-77.

Pfaffle, M., Petney, T., Skuballa, J. and Taraschewski, H. (2011) Comparative population dynamics of a generalist (*Ixodes ricinus*) and specialist tick (*I. hexagonus*) species from European hedgehogs. Exp Appl Acarol, 54: 151-64.

Piesman, J. and Gern, L. (2004) Lyme borreliosis in Europe and North America. Parasitology,
129 S191-220.

Pillemer, D.B. (1991) One- versus two-tailed hypothesis tests in contemporary educational research. Educational Researcher, 20: 13-17.

Platonov, A.E., Karan, L.S., Kolyasnikova, N.M., Makhneva, N.A., Toporkova, M.G., Maleev, V.V., et al. (2011) Humans infected with relapsing fever spirochete *Borrelia miyamotoi*, Russia. Emerg Infect Dis, 17: 1816-23.

Postic, D., Edlinger, C., Richaud, C., Grimont, F., Dufresne, Y., Perolat, P., et al. (1990) Two genomic species in *Borrelia burgdorferi*. Res Microbiol, 141: 465-75.

Postic, D., Garnier, M. and Baranton, G. (2007) Multilocus sequence analysis of atypical *Borrelia burgdorferi* sensu lato isolates-description of *Borrelia californiensis* sp. nov., and genomospecies 1 and 2. Int J Med Microbiol, 297: 263-71.

Postic, D., Ras, N.M., Lane, R.S., Hendson, M. and Baranton, G. (1998) Expanded diversity among Californian *Borrelia* isolates and description of *Borrelia bissettii* sp. nov. (formerly *Borrelia* group DN127). J Clin Microbiol, 36: 3497-504.

Pritt, B.S., Mead, P.S., Johnson, D.K.H., Neitzel, D.F., Respicio-Kingry, L.B., Davis, J.P., et al. (2016) Identification of a novel pathogenic *Borrelia* species causing Lyme borreliosis with unusually high spirochaetaemia: A descriptive study. Lancet Infect Dis, 16: 556-64.

Qiu, W.G., Bruno, J.F., McCaig, W.D., Xu, Y., Livey, I., Schriefer, M.E. and Luft, B.J. (2008) Wide distribution of a high-virulence *Borrelia burgdorferi* clone in Europe and North America. Emerg Infect Dis, 14: 1097-104.

Radolf, J.D., Caimano, M.J., Stevenson, B. and Hu, L.T. (2012) Of ticks, mice and men: understanding the dual-host lifestyle of Lyme disease spirochaetes. Nat Rev Microbiol, 10: 87-99.

Randolph, S. (1994) The relative contributions of transovarial and transstadial transmission to the maintenance of tick-borne diseases. Springer link, 260: 131-34.

Randolph, S.E. (2004) Tick ecology: Processes and patterns behind the epidemiological risk posed by ixodid ticks as vectors. Parasitology, 129 Suppl: S37-65.

Randolph, S.E. (2008) The impact of tick ecology on pathogen transmission dynamics. In Ticks Biology, Disease and Control. Bowman, A.S. and Nuttall, P.A. (eds). UK: Cambridge University Press, 40-72.

Randolph, S.E., Green, R.M., Hoodless, A.N. and Peacey, M.F. (2002) An empirical quantitative framework for the seasonal population dynamics of the tick *Ixodes ricinus*. Int J Parasitol, 32: 979-89.

Ras, N.M., Postic, D., Foretz, M. and Baranton, G. (1997) *Borrelia burgdorferi* sensu stricto, a bacterial species "made in the U.S.A."? Int J Syst Bacteriol, 47: 1112-7.

Rauer, S., Kastenbauer, S., Fingerle, V., Hunfeld, K.P., Huppertz, H.I. and Dersch, R. (2018) Neuroborreliose. Dtsch Arztebl International, 115: 751-56.

Rauter, C. and Hartung, T. (2005) Prevalence of *Borrelia burgdorferi* sensu lato genospecies in *Ixodes ricinus* ticks in Europe: a metaanalysis. Appl Environ Microbiol, 71: 7203-16.

Rauter, C., Oehme, R., Diterich, I., Engele, M. and Hartung, T. (2002) Distribution of clinically relevant *Borrelia genospecies* in ticks assessed by a novel, single-run, real-time PCR. J Clin Microbiol, 40: 36-43.

Remesar, S., Fernandez, P.D., Venzal, J.M., Perez-Creo, A., Prieto, A., Estrada-Pena, A., et al. (2019) Tick species diversity and population dynamics of *Ixodes ricinus* in Galicia (north-western Spain). Ticks Tick Borne Dis, 10: 132-37.

Reye, A.L., Hübschen, J.M., Sausy, A. and Muller, C.P. (2010) Prevalence and seasonality of tick-borne pathogens in questing *Ixodes ricinus* ticks from Luxembourg. Appl Environ Microbiol, 76: 2923.

Reye, A.L., Stegniy, V., Mishaeva, N.P., Velhin, S., Hubschen, J.M., Ignatyev, G. and Muller, C.P. (2013) Prevalence of tick-borne pathogens in *Ixodes ricinus* and *Dermacentor reticulatus* ticks from different geographical locations in Belarus. PLoS One, 8: 54476-85.

Richter, D., Allgower, R. and Matuschka, F.R. (2002) Co-feeding transmission and its contribution to the perpetuation of the Lyme disease spirochete *Borrelia afzelii*. Emerg Infect Dis, 8: 1421-5.

Richter, D., Debski, A., Hubalek, Z. and Matuschka, F.R. (2012) Absence of Lyme disease spirochetes in larval *Ixodes ricinus* ticks. Vector Borne Zoonotic Dis, 12: 21-7.

Richter, D., Postic, D., Sertour, N., Livey, I., Matuschka, F.R. and Baranton, G. (2006) Delineation of *Borrelia burgdorferi* sensu lato species by multilocus sequence analysis and confirmation of the delineation of *Borrelia spielmanii* sp. nov. Int J Syst Evol Microbiol, 56: 873-81.

Richter, D., Schlee, D.B. and Matuschka, F.R. (2011) Reservoir competence of various rodents for the Lyme disease spirochete *Borrelia spielmanii*. Appl Environ Microbiol, 77: 3565-70.

Rijpkema, S. and Bruinink, H. (1996) Detection of *Borrelia burgdorferi* sensu lato by PCR in questing *Ixodes ricinus* larvae from the Dutch North Sea island of Ameland. Exp Appl Acarol, 20: 381-5.

Rijpkema, S., Nieuwenhuijs, J., Franssen, F.F. and Jongejan, F. (1994) Infection rates of *Borrelia burgdorferi* in different instars of *Ixodes ricinus* ticks from the Dutch North Sea Island of Ameland. Exp Appl Acarol, 18: 531-42.

Rijpkema, S.G., Tazelaar, D.J., Molkenboer, M.J., Noordhoek, G.T., Plantinga, G., Schouls, L.M. and Schellekens, J.F. (1997) Detection of *Borrelia afzelii*, *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* and group VS116 by PCR in skin biopsies of patients with erythema migrans and acrodermatitis chronica atrophicans. Clin Microbiol Infect, 3: 109-16.

Rodríguez, I., Fraga, J., Noda, A.A., Mayet, M., Duarte, Y., Echevarria, E. and Fernández, C. (2014) An Alternative and Rapid Method for the Extraction of Nucleic Acids from Ixodid Ticks by Potassium Acetate Procedure. Braz. arch. biol. technol, 57: 542-47.

Rosselló-Mora, R. (2006) DNA-DNA reassociation methods applied to microbial taxonomy and their critical evaluation. In Molecular Identification, Systematics, and Population Structure of Prokaryotes. Stackebrandt, E. (ed.). Berlin, Heidelberg: Springer Berlin Heidelberg. 23-50.

Rudenko, N., Golovchenko, M., Grubhoffer, L. and Oliver, J.H.J. (2009) *Borrelia carolinensis* sp. nov., a new (14th) member of the *Borrelia burgdorferi* Sensu Lato complex from the southeastern region of the United States. J Clin Microbiol, 47: 134-41.

Rudenko, N., Golovchenko, M., Lin, T., Gao, L., Grubhoffer, L. and Oliver, J.H.J. (2009) Delineation of a new species of the *Borrelia burgdorferi* sensu lato complex, *Borrelia americana* sp. nov. J Clin Microbiol, 47: 3875-80.

Saint, G.I., Old, I.G. and Davidson, B.E. (1994) Molecular biology of the *Borrelia*, bacteria with linear replicons. Microbiology, 140 (Pt 8): 1803-16.

Samuels, D. and Radolf, J. (2010) *Borrelia*: Molecular Biology, Host Interaction and Pathogenesis, Norfok, UK: Caister Academic Press

Sanger, F., Nicklen, S. and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci U S A, 74: 5463-7.

Schildkraut, C.L., Marmur, J. and Doty, P. (1961) The formation of hybrid DNA molecules and their use in studies of DNA homologies. J Mol Biol, 3: 595-617.

Schillberg, E., Lunny, D., Lindsay, L.R., Nelder, M.P., Russell, C., Mackie, M., et al. (2018) Distribution of *Ixodes scapularis* in northwestern Ontario: Results from active and passive surveillance activities in the northwestern health unit catchment area. Intern J Environ Res Public Health, 15: 2225.

Schmid, G.P., Steigerwalt, A.G., Johnson, S., Barbour, A.G., Steere, A.C., Robinson, I.M. and Brenner, D.J. (1984) DNA characterization of Lyme disease spirochetes. Yale J Biol Med, 57: 539-42.

Schrauber, E.M. and Ostfeld, R.S. (2002) Modeling the effects of reservoir competence decay and demographic turnover in Lyme Disease Ecology, 12: 1142-62.

Schutzer, S.E., Fraser-Liggett, C.M., Casjens, S.R., Qiu, W.G., Dunn, J.J., Mongodin, E.F. and Luft, B.J. (2011) Whole-genome sequences of thirteen isolates of *Borrelia burgdorferi*. J Bacteriol, 193: 1018-20.

Schwaiger, M., Peter, O. and Cassinotti, P. (2001) Routine diagnosis of *Borrelia burgdorferi* (sensu lato) infections using a real-time PCR assay. Clin Microbiol Infect, 7: 461-9.

Schwan, T.G. and Piesman, J. (2000) Temporal changes in outer surface proteins A and C of the Lyme disease-associated spirochete, *Borrelia burgdorferi*, during the chain of infection in ticks and mice. J Clin Microbiol, 38: 382-8.

Schwan, T.G., Piesman, J., Golde, W.T., Dolan, M.C. and Rosa, P.A. (1995) Induction of an outer surface protein on *Borrelia burgdorferi* during tick feeding. PNAS USA, 92: 2909-13.

Schwartz, A.M., Hinckley, A.F., Mead, P.S., Hook, S.A. and Kugeler, K.J. (2017) Surveillance for Lyme disease - United States, 2008-2015. MMWR Surveill Summ, 66: 1-12.

Schwartz, I., Varde, S., Nadelman, R.B., Wormser, G.P. and Fish, D. (1997) Inhibition of efficient polymerase chain reaction amplification of *Borrelia burgdorferi* DNA in blood-fed ticks. Am J Trop Med Hyg, 56: 339-42.

Schwarz, A., Hönig, V., Vavrušková, Z., Grubhoffer, L., Balczun, C., Albring, A. and Schaub, G.A. (2012) Abundance of *Ixodes ricinus* and prevalence of *Borrelia burgdorferi* s.l. in the nature reserve Siebengebirge, Germany, in comparison to three former studies from 1978 onwards. Parasites & vectors, 5: 268-68.

Seinost, G., Dykhuizen, D.E., Dattwyler, R.J., Golde, W.T., Dunn, J.J., Wang, I.N., et al. (1999) Four clones of *Borrelia burgdorferi* sensu stricto cause invasive infection in humans. Infec Immun, 67: 3518-24.

Seinost, G., Golde, W.T., Berger, B.W., Dunn, J.J., Qiu, D., Dunkin, D.S., et al. (1999) Infection with multiple strains of *Borrelia burgdorferi* sensu stricto in patients with Lyme disease. Arch Dermatol, 135: 1329-33.

Sormunen, J.J., Klemola, T., Hänninen, J., Mäkelä, S., Vuorinen, I., Penttinen, R., et al. (2018) The importance of study duration and spatial scale in pathogen detection-evidence from a tick-infested island. Emerg Microb Infect, 7: 189-89.

Stackebrandt, E. (2003) The richness of prokaryotic diversity: There must be a species somewhere. Food Technol Biotechnol, 1: 17-22.

Stackebrandt, E., Frederiksen, W., Garrity, G.M., Grimont, P.A., Kampfer, P., Maiden, M.C., et al. (2002) Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. Int J Syst Evol Microbiol, 52: 1043-7.

Stanek, G., Fingerle, V., Hunfeld, K.P., Jaulhac, B., Kaiser, R., Krause, A., et al. (2011) Lyme borreliosis: Clinical case definitions for diagnosis and management in Europe. Clin Microbiol Infect,
17: 69-79.

Stanek, G. and Strle, F. (2009) Lyme borreliosis: A European perspective on diagnosis and clinical management. Curr Opin Infect Dis, 22: 450-4.

Steere, A.C. (2001) Lyme disease. N Engl J Med, 345: 115-25.

Steere, A.C., Malawista, S.E., Hardin, J.A., Ruddy, S., Askenase, W. and Andiman, W.A. (1977) Erythema chronicum migrans and Lyme arthritis. The enlarging clinical spectrum. Ann Intern Med, 86: 685-98.

Stevenson, B., Fingerle, V., Wormser, G.P. and Margos, G. (2019) Public health and patient safety concerns merit retention of Lyme borreliosis-associated spirochetes within the genus *Borrelia*, and rejection of the genus novum *Borreliella*. Ticks Tick Borne Dis, 10: 1-4.

Strle, F., Cheng, Y., Nelson, J.A., Picken, M.M., Bouseman, J.K. and Picken, R.N. (1995) Infection rate of *Ixodes ricinus* ticks with *Borrelia afzelii, Borrelia garinii*, and *Borrelia burgdorferi* sensu stricto in Slovenia. Eur J Clin Microbiol Infect Dis, 14: 994-1001.

Strnad, M., Honig, V., Ruzek, D., Grubhoffer, L. and Rego, R.O.M. (2017) Europe-wide meta-analysis of *Borrelia burgdorferi* sensu lato prevalence in questing *Ixodes ricinus* ticks. Appl Environ Microbiol, 83.(15):e00609-17

Szekeres, S., Lugner, J., Fingerle, V., Margos, G. and Foldvari, G. (2017) Prevalence of *Borrelia miyamotoi* and *Borrelia burgdorferi* sensu lato in questing ticks from a recreational coniferous forest of East Saxony, Germany. Ticks Tick Borne Dis, 8: 922-27.

Takano, A., Fujita, H., Kadosaka, T., Konnai, S., Tajima, T., Watanabe, H., et al. (2011) Characterization of reptile-associated *Borrelia* sp. in the vector tick, *Amblyomma geoemydae,* and its association with Lyme disease and relapsing fever *Borrelia spp*. Environ Microbiol Rep, 3: 632-7.

Takano, A., Goka, K., Une, Y., Shimada, Y., Fujita, H., Shiino, T., et al. (2010) Isolation and characterization of a novel *Borrelia* group of tick-borne *Borreliae* from imported reptiles and their associated ticks. Environ Microbiol, 12: 134-46.

Takken, W., van Vliet, A.J., Verhulst, N.O., Jacobs, F.H., Gassner, F., Hartemink, N., et al. (2017) Acarological Risk of *Borrelia burgdorferi* sensu lato infections across space and time in the Netherlands. Vector Borne Zoonotic Dis, 17: 99-107.

TäLleklint, L. and Jaenson, T.G.T. (1996) Seasonal variations in density of questing *Ixodes ricinus* (Acari: Ixodidae) nymphs and prevalence of infection with *B. burgdorferi* s.l. in south central Sweden. J. Med. Entomol., 33: 592-97.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. (2013) MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol Biol Evol, 30: 2725-9.

Team, R.C. (2019) 'R: A language and environment for statistical computing. R foundation for statistical computing, Vienna, Austria'. https://www.gbif.org/tool/81287/r-a-language-and-environment-for-statistical-computing.

Urwin, R. and Maiden, M.C. (2003) Multi-locus sequence typing: a tool for global epidemiology. Trends Microbiol, 11: 479-87.

van Dam, A.P., Oei, A., Jaspars, R., Fijen, C., Wilske, B., Spanjaard, L. and Dankert, J. (1997) Complement-mediated serum sensitivity among spirochetes that cause Lyme disease. Infect Immun, 65: 1228-36.

van den Wijngaard, C.C., Hofhuis, A., Simões, M., Rood, E., van Pelt, W., Zeller, H. and Van Bortel, W. (2017) Surveillance perspective on Lyme borreliosis across the European Union and European economic area. Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin, 22: 30569.

van Duijvendijk, G., Coipan, C., Wagemakers, A., Fonville, M., Ersöz, J., Oei, A., et al. (2016) Larvae of *Ixodes ricinus* transmit *Borrelia afzelii* and *B. miyamotoi* to vertebrate hosts. Parasites & Vectors, 9: 97.

Vandamme, P., Pot, B., Gillis, M., de Vos, P., Kersters, K. and Swings, J. (1996) Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiol Rev, 60: 407-38.

Venczel, R., Knoke, L., Pavlovic, M., Dzaferovic, E., Vaculova, T., Silaghi, C., et al. (2016) A novel duplex real-time PCR permits simultaneous detection and differentiation of *Borrelia miyamotoi* and *Borrelia burgdorferi* sensu lato. Infection, 44: 47-55.

Venter, J.C., Adams, M.D., Myers, E.W., Li, P.W., Mural, R.J., Sutton, G.G., et al. (2001) The sequence of the human genome. Science, 291: 1304-51.

Vollmer, S.A. (2010) 'Towards the origin of Lyme borreliosis', Doctor of Philosophy, University of Bath.

Vollmer, S.A., Bormane, A., Dinnis, R.E., Seelig, F., Dobson, A.D.M., Aanensen, D.M., et al. (2011) Host migration impacts on the phylogeography of Lyme borreliosis spirochaete species in Europe. Environ Microbiol, 13: 184-92.

Vollmer, S.A., Feil, E.J., Chu, C.Y., Raper, S.L., Cao, W.C., Kurtenbach, K. and Margos, G. (2013) Spatial spread and demographic expansion of Lyme borreliosis spirochaetes in Eurasia. Infect Genet Evol, 14: 147-55.

Voordouw, M.J. (2015) Co-feeding transmission in Lyme disease pathogens. Parasitology, 1
42: 290-302.

Vuong, H.B., Canham, C.D., Fonseca, D.M., Brisson, D., Morin, P.J., Smouse, P.E. and Ostfeld, R.S. (2014) Occurrence and transmission efficiencies of *Borrelia burgdorferi* OspC types in avian and mammalian wildlife. Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases, 27: 594-600.

Walter, K., Carpi, G., Caccone, A. and Diuk-Wasser, M.A. (2017) Genomic insights into the ancient spread of Lyme disease across North America.

Wang, G., Dankert, J. and van Dam, A.P. (1999) Evidence for frequent OspC gene transfer between *Borrelia valaisiana* sp. nov. and other Lyme disease spirochetes. FEMS Microbiol Lett, 177: 289-96.

Wang, G., Liveris, D., Mukherjee, P., Jungnick, S., Margos, G. and Schwartz, I. (2014) Molecular typing of *Borrelia burgdorferi*. Current protocols in microbiology, 34: 12C.5.1-12C.5.31.

Wang, G., van Dam, A.P., Le Fleche, A., Postic, D., Peter, O., Baranton, G., et al. (1997) Genetic and phenotypic analysis of *Borrelia valaisiana* sp. nov. (*Borrelia* genomic groups VS116 and M19). Int J Syst Bacteriol, 47: 926-32.

Wang, G., van Dam, A.P., Schwartz, I. and Dankert, J. (1999) Molecular typing of *Borrelia burgdorferi* sensu lato: taxonomic, epidemiological, and clinical implications. Clin Microbiol Rev,
12: 633-53.

Wang, G., van Dam, A.P., Spanjaard, L. and Dankert, J. (1998) Molecular typing of *Borrelia burgdorferi* sensu lato by randomly amplified polymorphic DNA fingerprinting analysis. J Clin Microbiol, 36: 768-76.

Wang, I.N., Dykhuizen, D.E., Qiu, W., Dunn, J.J., Bosler, E.M. and Luft, B.J. (1999) Genetic diversity of ospC in a local population of *Borrelia burgdorferi* sensu stricto. Genetics, 151: 15-30.

Wayne, L.G. (1988) International committee on systematic bacteriology: Announcement of the report of the ad hoc committee on reconciliation of approaches to bacterial systematics. Zentralbl Bakteriol Mikrobiol Hyg A, 268: 433-4.

Westbury, M. (2018) 'Unraveling evolution through next generation sequencing', doctor rerum naturalium, Potsdam University.

Wielinga, P.R., Gaasenbeek, C., Fonville, M., de Boer, A., de Vries, A., Dimmers, W., et al. (2006) Longitudinal analysis of tick densities and *Borrelia*, *Anaplasma*, and *Ehrlichia* infections of *Ixodes ricinus* ticks in different habitat areas in the Netherlands. Appl Environ Microbiol, 72: 7594-601.

Wilk, M.B. and Shapiro, S.S. (1965) An analysis of variance test for normality (complete samples). Biometrika, 52: 591-611.

Will, G., Jauris-Heipke, S., Schwab, E., Busch, U., Rossler, D., Soutschek, E., et al. (1995) Sequence analysis of ospA genes shows homogeneity within *Borrelia burgdorferi* sensu stricto and *Borrelia afzelii* strains but reveals major subgroups within the *Borrelia garinii* species. Med Microbiol Immunol, 184: 73-80.

Wilske, B., Busch, U., Fingerle, V., Jauris-Heipke, S., Preac Mursic, V., Rossler, D. and Will, G. (1996) Immunological and molecular variability of ospA and ospC. Implications for *Borrelia* vaccine development. Infection, 24: 208-12.

Wilske, B., Preac-Mursic, V., Gobel, U.B., Graf, B., Jauris, S., Soutschek, E., et al. (1993) An OspA serotyping system for *Borrelia burgdorferi* based on reactivity with monoclonal antibodies and OspA sequence analysis. J Clin Microbiol, 31: 340-50.

Wilske, B., Preac-Mursic, V., Jauris, S., Hofmann, A., Pradel, I., Soutschek, E., et al. (1993) Immunological and molecular polymorphisms of ospC, an immunodominant major outer surface protein of *Borrelia burgdorferi*. Infect Immun, 61: 2182-91.

Woodman, M.E., Cooley, A.E. and Stevenson, B. (2008) Production of outer surface protein A by *Borrelia burgdorferi* during transmission from infected mammals to feeding ticks is insufficient to trigger OspA seroconversion. FEMS Immunol Med Microbiol, 54: 277-82.

Wormser, G.P., Dattwyler, R.J., Shapiro, E.D., Halperin, J.J., Steere, A.C., Klempner, M.S., et al. (2006) The clinical assessment, treatment, and prevention of Lyme disease, human granulocytic anaplasmosis, and babesiosis: Clinical practice guidelines by the infectious Diseases Society of America. Arch Clin Infect Dis, 43: 1089-134.

Wormser, G.P., McKenna, D., Carlin, J., Nadelman, R.B., Cavaliere, L.F., Holmgren, D., et al. (2005) Brief communication: hematogenous dissemination in early Lyme disease. Ann Intern Med,
142: 751-5.

Yang, X.F., Pal, U., Alani, S.M., Fikrig, E. and Norgard, M.V. (2004) Essential role for ospA/B in the life cycle of the Lyme disease spirochete. J Exp Med, 199: 641-8.

Appendix

Appendix 1: List of primers used in this study

Tick Cytochrome C oxidase subunit I primers

Primer Foward	5`-ATTTTACCGCGATGAYTWTWCTC-3`
Primer Revers	5`-ATTTTACCGCGATGAYTWTWCTC-3`

OspC PCR

OspCF306	59- ATG AAA AAG AAT ACA TTA AGT GC-39 (Positions 306-328 of U01894)
OspCR963	59- ATT AAT CTT ATA ATA TTG ATT TTA ATT AAG G-39 (963-933)
OspCR948	59- TTG ATT TTA ATT AGG GTT TTT TTG G -39 (948-924)

Primers and probes for duplex PCR (Schwaiger et al., 2001, (Venczel et al., 2016))

Forward primer FlaF1A	5´- AGC AAA TTT AGG TGC TTT CCA A-3`
Reverse primer FlaR1	5´- GCA ATC ATT GCC ATT GCA GA-3`
Probe FlaProbe1	FAM-5´- TGC TAC AAC CTC ATC TGT CAT TGT AGC ATC TTT TAT TTG–BBQ-xxx

Primer for MLST PCR (Wang et al. 2014, see also http://pubmlst.org/borrelia)

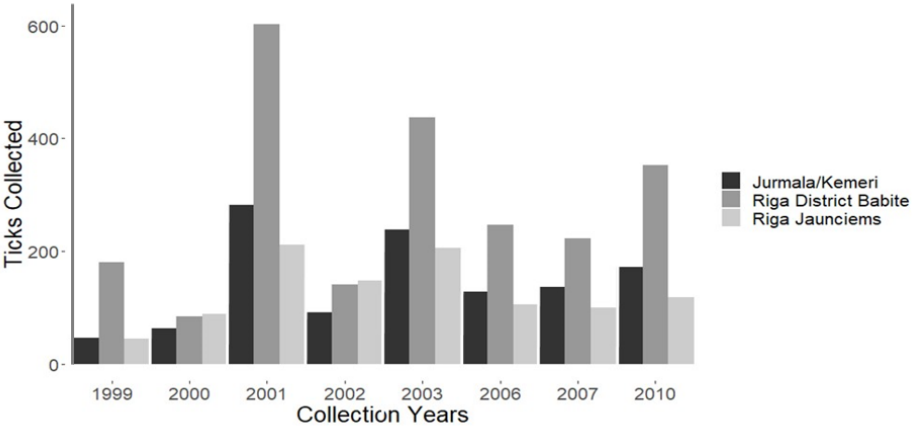
Forward primer BM F	5´-TTG CTT GTG CAA TCA TAG CC-3´
Reverse primer BM R	5´-GCA AAT CTT GGT GCT TTT CAA-3´
Probe FlaProbe1	5´-Cy5-AGA TGC CAC AAT TTC ATC TGT CAT TA-BBQ-650-3´

Gene	5‘ – 3‘ primer sequence
clpA	
IF*	5‘-GACAAAGCTTTTGATATTTTAG-3‘
IR*	5‘-CAAAAAAAACATCAAATTTTCTATCTC-3‘
OF*	5‘-AAAGATAGATTTCTTCCAGAC-3‘
OR*	5‘-GAATTTCATCTATTAAAAGCTTTC-3‘
clpX	
IF	5‘-AATGTGCCATTTGCAATAGC-3‘
IR	5‘-TTAAGAAGACCCTCTAAAATAG-3‘
OF	5‘-GCTGCAGAGATGAATGTGCC-3‘
OR	5‘-GATTGATTTCATATAACTCTTTTG-3‘
nifS	
IF	Same as OF
IR	5‘-GTTGGAGCAAGCATTTTATG-3‘
OF	5‘-ATGGATTTCAAACAAATAAAAAG-3‘
OR	5‘-GATATTATTGAATTTCTTTTAAG-3‘
pepX	
IF	5‘-TTATTCCAAACCTTGCAATCC-3‘
IR	5‘-TGTGCCTGAAGGAACATTTG-3‘
OF	Same as IF
OR	5‘-GTTCCAATGTCAATAGTTTC-3‘

Gene	5‘ – 3‘ primer sequence
pyrG	
IF	5‘-GATATGGAAAATATTTTATTTATTG-3‘
IR	5‘-AAACCAAGACAAATTCCAAG-3‘
OF	5‘-GATTGCAAGTTCTGAGAATA-3‘
OR	5‘-CAAACATTACGAGCAAATTC-3‘
recG	
IF	5‘-CTTTAATTGAAGCTGGATATC-3‘
IR	5‘-CAAGTTGCATTTGGACAATC-3‘
OF	5‘-CCCTTGTTGCCTTGCTTTC-3‘
OR	5‘-GAAAGTCCAAAACGCTCAG-3‘
rplB	
IF	5‘-CGCTATAAGACGACTTTATC-3‘
IR	Same as OR
OF	5‘-TGGGTATTAAGACTTATAAGC-3‘
OR	5‘-GCTGTCCCCAAGGAGACA-3‘
uvrA	
IF	5‘-GCTTAAATTTTTAATTGATGTTGG-3‘
IR	5‘-CCTATTGGTTTTTGATTTATTTG-3‘
OF	5‘-GAAATTTTAAAGGAAATTAAAAGTAG-3‘
OR	5‘-CAAGGAACAAAAACATCTGG-3‘

*IF = inner forward; IR = inner reverse; OF = outer forward; OR = outer reverse

Appendix 2: Total number of ticks analyzed in the whole study



Appendix 3: Samples used in this study with at least one MLST gene positive

Samples used in this study with at least one MLST gene positive												
	Strain	**Species**	**Collection Site**	**Year**	**ClpA**	**ClpX**	**nifS**	**pepX**	**pyrG**	**recG**	**rplB**	**uvrA**
1	9-26-55	*B. burgdorferi s.s.*	Babite	1999	14	1	11	1	168	1	8	10
2	9-26-08	*B. garinii*	Babite	1999	43	28	30	90	87	42	28	229
3	9-22-37	*B. afzelii*	Kemeri	1999	36	212	23	30	92	27	23	29
4	9-22-36	*B. afzelii*	Kemeri	1999	43	28	34	90	87	36	28	34
5	9-22-27	*B. garinii*	Kemeri	1999	40	213	26	36	28	34	28	31
6	9-22-21	*B. afzelii*	Kemeri	1999	183	24	23	31	27	27	23	29
7	9-12-26	*B. afzelii*	Babite	1999	109	24	25	31	92	52	23	28
8	9-12-11	*B. burgdorferi s.s.*	Babite	1999	15	1	1	1	168	1	1	1
9	9-26-20	*B. garinii*	Babite	1999	42	27	29	92	29	36	27	33
10	9-25-62	*B. burgdorferi s.s.*	Babite	1999	1	1	1	1	1	1	1	1
11	9-22-34	*B. garinii*	Kemeri	1999	42	27	29	38	29	80	27	33
12	9-22-17	*B. burgdorferi s.s.*	Kemeri	1999	14	1	11	1	1	1	1	10
13	9-22-16	*B. valaisiana*	Kemeri	1999	49	36	36	45	38	44	35	40

Samples used in this study with at least one MLST gene positive												
	Strain	**Species**	**Collection Site**	**Year**	**ClpA**	**ClpX**	**nifS**	**pepX**	**pyrG**	**recG**	**rplB**	**uvrA**
14	9-22-09	*B. burgdorferi s.s.*	Kemeri	1999	14	1	11	1	1	1	1	10
15	9-22-04	*B. garinii*	Kemeri	1999	43	28	30	90	87	36	28	34
16	9-20-24	*B. bavariensis*	Jaunciems	1999	41	26	27	37	28	35	26	32
17	0-14-06	*B. afzelii*	Babite	2000	39	24	23	87	92	27	23	78
18	0-14-10b	*B. garinii*	Babite	2000	42	27	29	38	29	39	80	33
19	0-14-26	*B. afzelii*	Babite	2000	51	24	23	86	85	92	23	29
20	0-14-36	*B. garinii*	Babite	2000	45	33	34	36	36	38	30	38
21	0-14-37	*B. afzelii*	Babite	2000	35	24	24	32	21	27	208	28
22	0-14-39	*B. afzelii*	Babite	2000	39	24	24	31	22	92	23	28
23	0-8-03	*B. burgdorferi s.s.*	Kemeri	2000	14	1	11	1	1	10	1	10
24	0-4	*B. afzelii*	Jaunciems	2000	109	24	24	85	90	91	24	29
25	0-13-41	*B. burgdorferi s.s.*	Babite	2000	1	1	1	1	1	1	1	1
26	1-8-29	*B. garinii*	Kemeri	2001	46	214	29	43	98	40	31	37
27	1-8-50	*B. valaisiana*	Kemeri	2001	50	36	37	45	234	44	36	40
28	1-8-12b	*B. afzelii*	Kemeri	2001	172	24	23	85	98	27	24	28
29	1-8-28	*B. afzelii*	Kemeri	2001	109	24	24	31	92	246	23	28
30	1-29-12	*B. afzelii*	Jaunciems	2001	37	215	24	31	22	91	23	29

Samples used in this study with at least one MLST gene positive												
	Strain	**Species**	**Collection Site**	**Year**	**ClpA**	**ClpX**	**nifS**	**pepX**	**pyrG**	**recG**	**rplB**	**uvrA**
31	1-29-17	*B. valaisiana*	Jaunciems	2001	257	37	37	86	39	93	36	40
32	1-29-35b	*B. afzelii*	Jaunciems	2001	109	24	24	85	90	91	209	29
33	1-29-60	*B. afzelii*	Jaunciems	2001	36	24	23	89	95	27	23	28
34	1-29-22	*B. afzelii*	Jaunciems	2001	36	24	23	89	118	27	23	28
35	1-29-30a	*B. afzelii*	Jaunciems	2001	38	24	25	32	90	247	24	28
36	1-28	*B. afzelii*	Jaunciems	2001	36	24	24	32	21	31	23	30
37	1-28-18	*B. afzelii*	Jaunciems	2001	37	24	24	31	235	248	23	28
38	1-28-27	*B. afzelii*	Jaunciems	2001	37	24	25	31	236	92	23	28
39	1-41-07	*B. afzelii*	Babite	2001	51	24	23	86	85	27	23	230
40	1-41-18	*B. afzelii*	Babite	2001	36	24	23	86	237	27	23	28
41	1-38-13	*B. afzelii*	Babite	2001	258	24	23	203	26	96	23	156
42	1-37-13	*B. afzelii*	Babite	2001	37	24	24	88	22	92	23	28
43	1-32-30	*B. burgdorferi s.s.*	Kemeri	2001	14	1	11	1	168	10	1	10
44	1-32-49	*B. afzelii*	Kemeri	2001	109	24	24	31	22	246	24	28
45	1-32-53	*B. garinii*	Kemeri	2001	47	73	33	90	91	76	32	33
46	1-31-43	*B. afzelii*	Kemeri	2001	259	24	24	31	22	92	23	28

Samples used in this study with at least one MLST gene positive												
	Strain	**Species**	**Collection Site**	**Year**	**ClpA**	**ClpX**	**nifS**	**pepX**	**pyrG**	**recG**	**rplB**	**uvrA**
47	1-31LT	*B. garinii*	Kemeri	2001	40	216	26	36	27	250	25	31
48	1-5-26	*B. valaisiana*	Jaunciems	2001	50	35	37	45	38	44	35	40
49	1-5-15	*B. valaisiana*	Jaunciems	2001	50	37	37	45	39	93	36	40
50	1-5-29	*B. afzelii*	Jaunciems	2001	131	82	24	88	92	27	23	28
51	1-29-03	*B. valaisiana*	Jaunciems	2001	49	35	35	45	38	43	35	40
52	1-29-08	*B. garinii*	Jaunciems	2001	44	29	31	40	31	37	80	35
53	1-29-29	*B. afzelii*	Jaunciems	2001	37	24	24	31	98	92	23	28
54	1-29-43a	*B. afzelii*	Jaunciems	2001	48	34	34	44	37	42	33	39
55	1-4-09	*B. afzelii*	Jaunciems	2001	37	24	24	31	22	92	23	28
56	1-14	*B. valaisiana*	Babite	2001	50	38	36	45	38	44	35	40
57	1-19-17	*B. afzelii*	Babite	2001	39	24	24	31	22	92	23	28
58	1-38-03	*B. garinii*	Babite	2001	42	27	29	38	29	39	27	33
59	1-37-19	*B. valaisiana*	Babite	2001	49	37	37	45	39	45	36	40
60	1-31-25	*B. afzelii*	Kemeri	2001	36	24	23	31	92	27	23	29
61	1-31-42	*B. afzelii*	Kemeri	2001	37	24	24	31	22	92	23	28
62	2-47-03LT	*B. afzelii*	Jaunciems	2002	35	24	23	86	22	27	23	28
63	2-02-11L	*B. afzelii*	Babite	2002	36	24	23	87	92	27	23	78

Samples used in this study with at least one MLST gene positive												
	Strain	**Species**	**Collection Site**	**Year**	**ClpA**	**ClpX**	**nifS**	**pepX**	**pyrG**	**recG**	**rplB**	**uvrA**
64	2-16-21L	*B. afzelii*	Babite	2002	36	24	23	87	92	27	23	78
65	2-08-09LT	*B. afzelii*	Babite	2002	36	82	24	88	92	27	23	28
66	2-53LT	*B. afzelii*	Jaunciems	2002	37	24	23	31	22	92	23	30
67	2-58-15L	*B. afzelii*	Jaunciems	2002	37	24	25	31	238	92	23	30
68	2-54-15L	*B. afzelii*	Jaunciems	2002	38	24	25	32	90	29	24	28
69	2-27LT	*B. garinii*	Babite	2002	44	29	76	40	31	37	80	77
70	2-16-17L	*B. lusitaniae*	Kemeri	2002	261	22	94	123	18	118	74	102
71	2-45-9LT	*B. afzelii*	Babite	2002	36	24	24	31	22	92	23	28
72	2-32-12L	*B. afzelii*	Babite	2002	109	24	23	31	23	30	23	30
73	2-18-21L	*B. valaisiana*	Babite	2002	50	35	37	45	38	44	35	40
74	3-5-17	*B. burgdorferi s.s.*	Kemeri	2003	14	1	11	1	1	10	1	10
75	3-8-06	*B. afzelii*	Kemeri	2003	36	24	23	37	92	27	23	231
76	3-8-07	*B. afzelii*	Kemeri	2003	35	24	22	32	98	27	23	28
77	3-17-10	*B. garinii*	Kemeri	2003	48	34	90	44	37	111	33	39
78	3-14-23	*B. afzelii*	Jaunciems	2003	131	217	24	88	92	27	23	28
79	3-8-05	*B. burgdorferi s.s.*	Kemeri	2003	14	1	11	1	1	10	1	10

Samples used in this study with at least one MLST gene positive												
	Strain	**Species**	**Collection Site**	**Year**	**ClpA**	**ClpX**	**nifS**	**pepX**	**pyrG**	**recG**	**rplB**	**uvrA**
80	6-44-09L	*B. afzelii*	Babite	2006	36	24	23	86	22	27	23	28
81	6-42-24L	*B. afzelii*	Jaunciems	2006	36	24	23	86	22	27	23	28
82	6-12-18L	*B. afzelii*	Kemeri	2006	36	24	23	30	92	27	23	29
83	6-06-18L	*B. afzelii*	Kemeri	2006	36	24	23	30	92	27	23	29
84	6-07-24L	*B. afzelii*	Jaunciems	2006	36	24	23	31	92	89	23	29
85	6-33-21L	*B. afzelii*	Jaunciems	2006	37	24	24	31	22	92	23	28
86	6-37-21L	*B. afzelii*	Jaunciems	2006	37	24	24	31	22	92	23	28
87	6-35-21L	*B. afzelii*	Jaunciems	2006	51	24	23	86	85	27	23	29
88	6-19-18L	*B. afzelii*	Kemeri	2006	51	24	23	86	85	27	23	29
89	6-22-18L	*B. afzelii*	Kemeri	2006	51	24	23	86	85	27	23	29
90	6-34-24L	*B. afzelii*	Jaunciems	2006	102	24	24	31	22	92	23	28
91	6-10-06L	*B. garinii*	Babite	2006	42	27	29	38	29	36	27	33
92	6-50-06L	*B. garinii*	Babite	2006	42	27	29	38	29	36	27	33
93	6-14-06L	*B. garinii*	Babite	2006	31	80	78	99	81	39	79	87
94	6-10-09L	*B. garinii*	Babite	2006	42	27	29	92	29	36	27	33
95	6-23-06L	*B. garinii*	Babite	2006	43	28	30	90	87	36	28	34

Samples used in this study with at least one MLST gene positive												
	Strain	**Species**	**Collection Site**	**Year**	**ClpA**	**ClpX**	**nifS**	**pepX**	**pyrG**	**recG**	**rplB**	**uvrA**
96	6-25-06L	*B. garinii*	Babite	2006	43	28	30	90	87	36	28	34
97	6-21-18L	*B. garinii*	Kemeri	2006	43	28	30	90	87	36	28	34
98	6-38-09L	*B. garinii*	Babite	2006	44	29	31	40	31	87	80	77
99	6-10-112L	*B. garinii*	Babite	2006	46	76	73	43	34	40	31	37
100	6-23-03L	*B. garinii*	Babite	2006	47	32	33	42	91	86	32	36
101	6-23-09L	*B. garinii*	Babite	2006	47	32	33	42	91	86	32	36
102	6-12-09L	*B. garinii*	Babite	2006	47	32	33	42	35	88	32	36
103	6-29-09L	*B. garinii*	Babite	2006	48	34	34	44	27	42	33	39
104	6-92-12L	*B. garinii*	Babite	2006	48	76	29	43	34	42	31	37
105	6-66-12L	*B. garinii*	Babite	2006	99	77	36	91	88	84	75	33
106	6-13-06L	*B. valaisiana*	Babite	2006	49	35	35	45	38	43	35	40
107	6-18-09L	*B. valaisiana*	Babite	2006	50	36	36	45	38	44	35	40
108	6-35-03L	*B. valaisiana*	Babite	2006	50	36	36	45	38	44	35	40
109	6-36-03L	*B. valaisiana*	Babite	2006	50	39	36	45	38	44	35	40
110	6-21-03L	*B. valaisiana*	Babite	2006	50	37	37	45	39	45	36	40
111	6-16-18L	*B. valaisiana*	Kemeri	2006	50	39	74	45	38	44	35	40
112	6-10-412L	*B. valaisiana*	Babite	2006	96	75	36	98	84	44	78	86

Samples used in this study with at least one MLST gene positive												
	Strain	**Species**	**Collection Site**	**Year**	**ClpA**	**ClpX**	**nifS**	**pepX**	**pyrG**	**recG**	**rplB**	**uvrA**
113	6-39-09L	*B. valaisiana*	Babite	2006	96	75	36	98	84	44	78	86
114	6-84-12L	*B. valaisiana*	Babite	2006	96	75	36	98	84	44	78	86
115	7-50-21L	*B. afzelii*	Jaunciems	2007	36	24	23	86	22	27	23	28
116	7-14-24L	*B. afzelii*	Jaunciems	2007	36	24	23	31	92	27	23	29
117	7-05-15L	*B. afzelii*	Kemeri	2007	36	24	23	31	85	27	23	29
118	7-59-18L	*B. afzelii*	Kemeri	2007	37	24	24	31	22	92	23	28
119	7-46-18L	*B. afzelii*	Kemeri	2007	39	24	24	31	22	92	23	28
120	7-16-18L	*B. afzelii*	Kemeri	2007	109	24	23	89	22	27	23	28
121	7-2-721L	*B. afzelii*	Jaunciems	2007	51	24	23	28	85	27	23	29
122	7-29-15L	*B. afzelii*	Kemeri	2007	51	24	23	86	85	91	23	29
123	7-19-24L	*B. afzelii*	Jaunciems	2007	109	24	24	85	90	91	24	29
124	7-15-09L	*B. burgdorferi s.s.*	Babite	2007	15	9	12	8	1	17	8	16
125	7-43-06L	*B. burgdorferi s.s.*	Babite	2007	15	9	12	8	1	17	8	16
126	7-25-06L	*B. burgdorferi s.s.*	Babite	2007	15	9	12	8	1	83	8	16
127	7-09-03L	*B. garinii*	Babite	2007	42	27	29	38	29	36	27	33
128	7-42-15L	*B. garinii*	Kemeri	2007	42	27	29	38	29	36	27	33

Samples used in this study with at least one MLST gene positive												
	Strain	**Species**	**Collection Site**	**Year**	**ClpA**	**ClpX**	**nifS**	**pepX**	**pyrG**	**recG**	**rplB**	**uvrA**
129	7-36-06L	*B. garinii*	Babite	2007	44	29	31	40	31	37	29	35
130	7-53-09L	*B. garinii*	Babite	2007	44	29	31	40	31	37	29	35
131	7-16-09L	*B. garinii*	Babite	2007	45	30	32	41	32	38	30	36
132	7-08-09L	*B. garinii*	Babite	2007	31	80	78	99	81	39	79	87
133	7-64-18L	*B. garinii*	Kemeri	2007	42	27	29	38	81	36	27	33
134	7-45-03L	*B. garinii*	Babite	2007	43	28	30	39	30	78	28	34
135	7-41-09L	*B. garinii*	Babite	2007	43	28	30	39	88	87	28	34
136	7-04-06L	*B. garinii*	Babite	2007	44	29	31	40	31	37	80	35
137	7-39-12L	*B. garinii*	Babite	2007	47	73	33	42	91	76	32	36
138	7-24-09L	*B. garinii*	Babite	2007	48	34	34	44	27	42	33	39
139	7-44-15L	*B. garinii*	Kemeri	2007	95	74	34	96	83	78	77	85
140	7-58-03L	*B. garinii*	Babite	2007	95	29	34	91	89	78	77	85
141	7-44-03L	*B. garinii*	Babite	2007	95	74	34	96	89	78	77	85
142	7-14-12L	*B. lusitaniae*	Babite	2007	101	21	20	27	86	85	74	81
143	7-20-09L	*B. valaisiana*	Babite	2007	49	35	35	45	38	43	35	40
144	7-30-09L	*B. valaisiana*	Babite	2007	50	36	36	45	38	44	35	40
145	7-40-03L	*B. valaisiana*	Babite	2007	50	36	36	45	38	44	35	40

Samples used in this study with at least one MLST gene positive												
	Strain	**Species**	**Collection Site**	**Year**	**ClpA**	**ClpX**	**nifS**	**pepX**	**pyrG**	**recG**	**rplB**	**uvrA**
146	7-25-09L	*B. valaisiana*	Babite	2007	49	35	72	45	38	43	35	40
147	7-2-609L	*B. valaisiana*	Babite	2007	50	35	37	45	38	44	35	40
148	7-38-15L	*B. valaisiana*	Kemeri	2007	50	35	37	45	38	44	35	40
149	7-39-15L	*B. valaisiana*	Kemeri	2007	50	35	37	45	38	44	35	40
150	7-15-12L	*B. valaisiana*	Babite	2007	50	36	37	45	38	44	35	40
151	7-56-18L	*B. valaisiana*	Kemeri	2007	50	36	37	45	38	44	35	40
152	7-22-09L	*B. valaisiana*	Babite	2007	110	39	36	45	38	81	35	40
153	7-24-03L	*B. valaisiana*	Babite	2007	96	37	37	45	39	79	36	86
154	7-15-15L	*B. valaisiana*	Kemeri	2007	96	37	37	45	39	79	36	86
155	7-34-09L	*B. valaisiana*	Babite	2007	96	75	36	98	38	82	78	86
156	10-26-29	*B. garinii*	Babite	2010	260	218	81	91	88	249	82	33
157	10-22-22	*B. garinii*	Babite	2010	43	28	30	39	30	36	28	34
158	10-22-04	*B. garinii*	Babite	2010	42	27	29	92	29	36	27	33
159	10-24-07	*B. valaisiana*	Babite	2010	50	38	36	45	38	44	35	40
160	9-26-41	*B. garinii*	Babite	1999	24		29	91				33
161	9-26-37	*B. garinii*	Babite	1999	43	28	30	90	32			

Samples used in this study with at least one MLST gene positive												
	Strain	**Species**	**Collection Site**	**Year**	**ClpA**	**ClpX**	**nifS**	**pepX**	**pyrG**	**recG**	**rplB**	**uvrA**
162	9-26-29	*B. valaisiana*	Babite	1999		35			38	43		40
163	9-26-28	*B. afzelii*	Babite	1999	35	135	22	32	20	23		
164	9-26-26	*B. afzelii*	Babite	1999			25		90	29	24	
165	9-26-02	*B. afzelii*	Babite	1999		24			26			
166	9-25-54	*B. garinii*	Babite	1999	99	77	29			39		33
167	9-25-39	*B. garinii*	Babite	1999	47	177						
168	9-25-36	*B. burgdorferi s.s.*	Babite	1999		1	1	1	168		1	1
169	9-25-33	*B. afzelii*	Babite	1999	36	23	24			27	23	
170	9-22-20	*B. garinii*	Kemeri	1999	112	80	78	31	23			87
171	9-20-28	*B. afzelii*	Jaunciems	1999	37	40	24		98			131
172	9-20-22	*B. afzelii*	Jaunciems	1999	36	24	23		98	27	23	28
173	9-12-33	*B. afzelii*	Babite	1999					163	52		28
174	9-25-35	*B. afzelii*	Babite	1999						27		
175	9-12-22	*B. lusitaniae*	Babite	1999								
176	9-25-20	*B. afzelii*	Babite	1999			24				23	
177	0-4-12	*B. afzelii*	Jaunciems	2000	109		24		90		24	29
178	0-4-18	*B. afzelii*	Jaunciems	2000	36			88		27		28

Samples used in this study with at least one MLST gene positive												
	Strain	**Species**	**Collection Site**	**Year**	**ClpA**	**ClpX**	**nifS**	**pepX**	**pyrG**	**recG**	**rplB**	**uvrA**
179	0-8-09	*B. afzelii*	Kemeri	2000	109	24	24	31	97	27		30
180	0-8-19	*B. valaisiana*	Kemeri	2000	49			46	38	43		
181	0-8-30	*B. afzelii*	Kemeri	2000	36	23	22	203	26	96	23	
182	0-8-36	*B. garinii*	Kemeri	2000	42		29	43	29	36	77	37
183	0-14-03	*B. afzelii*	Babite	2000		24	24		22		23	28
184	0-14-10	*B. garinii*	Babite	2000		33	34	36	36	38		38
185	0-14-11	*B. valaisiana*	Babite	2000			37	45	39	79	35	86
186	0-14-22	*B. garinii*	Babite	2000	43	28			87	36		34
187	0-14-38	*B. afzelii*	Babite	2000	95			96	89	78		
188	0-4-37	*B. valaisiana*	Jaunciems	2000				45	38			
189	0-5-10	*B. afzelii*	Jaunciems	2000			24			92		
190	0-5-19	*B. afzelii*	Jaunciems	2000	38							
191	0-5-23	*B. afzelii*	Jaunciems	2000	109				23			78
192	0-7-02	*B. afzelii*	Kemeri	2000				86		27		
193	1-5-10	*B. afzelii*	Jaunciems	2001	109	82		88		52		28
194	1-5-28	*B. afzelii*	Jaunciems	2001	37		37	31				

Samples used in this study with at least one MLST gene positive												
	Strain	**Species**	**Collection Site**	**Year**	**ClpA**	**ClpX**	**nifS**	**pepX**	**pyrG**	**recG**	**rplB**	**uvrA**
195	1-5-34	*B. afzelii*	Jaunciems	2001	109	24	24	31	92			
196	1-5-28	*B. afzelii*	Jaunciems	2001	37			31				
197	1-5-32	*B. afzelii*	Jaunciems	2001	109			88	23	30		
198	1-5-2	*B. afzelii*	Jaunciems	2001	131							
199	1-8-45	*B. garinii*	Kemeri	2001	44	156	31		38	40		35
200	1-8-27	*B. afzelii*	Kemeri	2001	36		23		38	27	24	
201	1-8-39	*B. valaisiana*	Kemeri	2001	50			45		44	35	
202	1-8-47	*B. valaisiana*	Kemeri	2001			37			79	35	
203	1-8-18	*B. garinii*	Kemeri	2001			31		34	40		
204	1-8-28b	*B. afzelii*	Kemeri	2001	37		81	31	98	91		
205	1-8-46	*B. garinii*	Kemeri	2001	116			39	31	36		
206	1-8-54	*B. afzelii*	Kemeri	2001	109		25					
207	1-8-39b	*B. burgdorferi s.s.*	Kemeri	2001	15		12	8	1	17	8	16
208	1-29-28	*B. garinii*	Jaunciems	2001	40	25	26	36	27	34	25	31
209	1-29-27	*B. afzelii*	Jaunciems	2001	36		23		95	27	23	
210	1-29-43b	*B. garinii*	Jaunciems	2001	47	73	33		91	76	32	
211	1-29-17b	*B. valaisiana*	Jaunciems	2001	96	37				44		

Samples used in this study with at least one MLST gene positive												
	Strain	**Species**	**Collection Site**	**Year**	**ClpA**	**ClpX**	**nifS**	**pepX**	**pyrG**	**recG**	**rplB**	**uvrA**
212	1-29-30	*B. garinii*	Jaunciems	2001	112			99		39		
213	1-28-17	*B. afzelii*	Jaunciems	2001	51				85	27		29
214	1-28-25	*B. afzelii*	Jaunciems	2001	109	24	24			91	24	29
215	1-28-12b	*B. garinii*	Jaunciems	2001	47		33		91		32	
216	1-28-05	*B. afzelii*	Jaunciems	2001	37	24		31	96	92		
217	1-4-07	*B. afzelii*	Jaunciems	2001	37		24		98	27		
218	1-13-20	*B. burgdorferi s.s.*	Babite	2001	14							10
219	1-13	*B. afzelii*	Babite	2001	35							221
220	1-13-42	*B. garinii*	Babite	2001	42	27				39		33
221	1-13-16	*B. garinii*	Babite	2001	42							
222	1-14-44	*B. garinii*	Babite	2001	99				108	202		33
223	1-14-45	*B. garinii*	Babite	2001	95		34		89	78		154
224	1-14-48	*B. afzelii*	Babite	2001	51			86		27		29
225	1-14-47	*B. afzelii*	Babite	2001	36					27	23	28
226	1-14-39	*B. burgdorferi s.s.*	Babite	2001		9	12	8				
227	1-14-42	*B. garinii*	Babite	2001	46		29		34	39		33

Samples used in this study with at least one MLST gene positive												
	Strain	**Species**	**Collection Site**	**Year**	**ClpA**	**ClpX**	**nifS**	**pepX**	**pyrG**	**recG**	**rplB**	**uvrA**
228	1-14-37	*B. afzelii*	Babite	2001			36	49		91		
229	1-14-43	*B. garinii*	Babite	2001				90	29	39		33
230	1-15-48	*B. garinii*	Babite	2001	43	28		90	87			34
231	1-15-32	*B. valaisiana*	Babite	2001	96			45				
232	1-19-11	*B. afzelii*	Babite	2001		82	23	31	92	27	23	28
233	1-19-18	*B. garinii*	Babite	2001	42	28		39	30	36	28	34
234	1-19-41	*B. burgdorferi s.s.*	Babite	2001	14	1	11	1	1	1	1	
235	1-19-34	*B. afzelii*	Babite	2001	39				85	92		
236	1-19-43	*B. afzelii*	Babite	2001	36	24				27		
237	1-18-20	*B. garinii*	Babite	2001				112	108	54		33
238	1-18-21	*B. garinii*	Babite	2001	116					125		33
239	1-18-30	*B. valaisiana*	Babite	2001				45	38			40
240	1-18-15	*B. garinii*	Babite	2001	42							33
241	1-18-16	*B. afzelii*	Babite	2001		24	23	31			24	
242	1-18-39	*B. afzelii*	Babite	2001	36		24	88			23	
243	1-41-09	*B. afzelii*	Babite	2001	131	24	23	85	22	27		28
244	1-41-11	*B. garinii*	Babite	2001		24		42				136

Samples used in this study with at least one MLST gene positive												
	Strain	**Species**	**Collection Site**	**Year**	**ClpA**	**ClpX**	**nifS**	**pepX**	**pyrG**	**recG**	**rplB**	**uvrA**
245	1-41-22	*B. valaisiana*	Babite	2001		39		45		44	39	86
246	1-41-19	*B. valaisiana*	Babite	2001		75				44		86
247	1-40-20	*B. afzelii*	Babite	2001	39	24	24		22	92	23	
248	1-40-31	*B. afzelii*	Babite	2001	36		23	49	26	91	23	
249	1-40-26	*B. garinii*	Babite	2001	112	80				38		50
250	1-40-18	*B. garinii*	Babite	2001	44							
251	1-42-05	*B. afzelii*	Babite	2001	109	24		85	22	91		29
252	1-42-14	*B. afzelii*	Babite	2001		82						28
253	1-42-07	*B. afzelii*	Babite	2001		24		88				
254	1-39-03	*B. afzelii*	Babite	2001	131	24	23	31	22	27		
255	1-39-12	*B. afzelii*	Babite	2001	36		24	86		91		
256	1-38	*B. garinii*	Babite	2001	47			38				
257	1-38-04	*B. garinii*	Babite	2001	42	27				39		33
258	1-38-19	*B. afzelii*	Babite	2001	36	24		31		27		
259	1-38-21	*B. garinii*	Babite	2001	99					54		
260	1-38-28	*B. garinii*	Babite	2001	116	34				42		

Samples used in this study with at least one MLST gene positive												
	Strain	**Species**	**Collection Site**	**Year**	**ClpA**	**ClpX**	**nifS**	**pepX**	**pyrG**	**recG**	**rplB**	**uvrA**
261	1-37-11	*B. garinii*	Babite	2001	44	156	31		31	37	35	35
262	1-37-10	*B. valaisiana*	Babite	2001	50	36	36		38	44		40
263	1-37-05	*B. garinii*	Babite	2001	99	77	81		88	84	82	33
264	1-32-08	*B. burgdorferi s.s.*	Kemeri	2001	14		11	8	1	1	1	10
265	1-32-25	*B. afzelii*	Kemeri	2001	51	24	23		85	27		29
266	1-32-31	*B. burgdorferi s.s.*	Kemeri	2001	14	1		1	168	1	1	10
267	1-32-19	*B. valaisiana*	Kemeri	2001		35	37	45				
268	1-31-06	*B. garinii*	Kemeri	2001		27	29	38	29	39	27	39
269	1-31-59	*B. garinii*	Kemeri	2001	43	28	30		87	38		34
270	1-31-49	*B. garinii*	Kemeri	2001	44	156	31	40	22	37		
271	1-31-46	*B. garinii*	Kemeri	2001	44							
272	1-31-03	*B. valaisiana*	Kemeri	2001							35	
273	1-31-07	*B. garinii*	Kemeri	2001		29	31		208			
274	1-30-12	*B. afzelii*	Jaunciems	2001	37	24		31	96	92	23	30
275	1-30-22	*B. garinii*	Jaunciems	2001	42		29				27	
276	1-9-18	*B. afzelii*	Kemeri	2001	131		23			92	23	29
277	1-13	*B. afzelii*	Babite	2001	35							41

Samples used in this study with at least one MLST gene positive												
	Strain	**Species**	**Collection Site**	**Year**	**ClpA**	**ClpX**	**nifS**	**pepX**	**pyrG**	**recG**	**rplB**	**uvrA**
278	1-13-42	*B. garinii*	Babite	2001	42	27				39		
279	1-13-20	*B. burgdorferi s.s.*	Babite	2001	14							10
280	1-13-45	*B. garinii*	Babite	2001					29			
281	1-7-46	*B. garinii*	Kemeri	2001	42							
282	1-13-23	*B. garinii*	Babite	2001					29			
283	1-13-38	*B. garinii*	Babite	2001					87			
284	1-4-05	*B. garinii*	Jaunciems	2001					37			
285	1-13-08	*B. valaisiana*	Babite	2001				45				
286	1-14-43	*B. garinii*	Babite	2001					29	39		33
287	1-15-39	*B. afzelii*	Babite	2001			23		22			
288	1-15	*B. afzelii*	Babite	2001				86				28
289	1-15-11	*B. valaisiana*	Babite	2001								86
290	1-17-26	*B. garinii*	Babite	2001	42							
291	1-17-30	*B. garinii*	Babite	2001		156			31			
292	1-18	*B. garinii*	Babite	2001	43					38		34
293	1-18-11	*B. burgdorferi s.s.*	Babite	2001						17		

Samples used in this study with at least one MLST gene positive												
	Strain	**Species**	**Collection Site**	**Year**	**ClpA**	**ClpX**	**nifS**	**pepX**	**pyrG**	**recG**	**rplB**	**uvrA**
294	1-31-03	*B. valaisiana*	Kemeri	2001				45	43		35	
295	1-39-10	*B. garinii*	Babite	2001					208			
296	1-14-16	*B. garinii*	Babite	2001	42							
297	3-5-24	*B. garinii*	Kemeri	2003	45	33	49		36	54		33
298	3-17	*B. afzelii*	Kemeri	2003	109	24		87	118	27	23	29
299	3-9-19	*B. afzelii*	Babite	2003	109	24	23		92		23	78
300	3-17-14	*B. garinii*	Kemeri	2003	46		29	43		42	31	37
301	3-9-02	*B. garinii*	Babite	2003	43	23					77	
302	3-8-24	*B. valaisiana*	Kemeri	2003		35	37	45	38			
303	3-9-11	*B. burgdorferi s.s.*	Babite	2003	14	1	11		168	1		
304	3-3-41	*B. afzelii*	Jaunciems	2003		24	25	87	96			
305	3-02-03	*B. afzelii*	Jaunciems	2003				88				
306	3-6-75	*B. afzelii*	Kemeri	2003		24			98			
307	3-6-77	*B. afzelii*	Kemeri	2003				88	92		23	
308	3-9-17b	*B. afzelii*	Babite	2003	109							
309	3-8-17	*B. garinii*	Kemeri	2003							28	
310	3-5-11	*B. garinii*	Kemeri	2003						38		

Samples used in this study with at least one MLST gene positive												
	Strain	**Species**	**Collection Site**	**Year**	**ClpA**	**ClpX**	**nifS**	**pepX**	**pyrG**	**recG**	**rplB**	**uvrA**
311	3-12-24	*B. bavariensis*	Babite	2003						35		
312	3-9-13	*B. garinii*	Babite	2003						39		
313	3-5-13	*B. garinii*	Kemeri	2003	48							
314	10-17-17	*B. garinii*	Babite	2010	44	156	31	40	31	37	80	
315	10-15-17	*B. afzelii*	Babite	2010	109	24	24	86		91	23	28

Appendix 4: Total number of samples used for spatio-temporal distribution analysis

Species	Babite	Jaunciems	Kemeri
B. afzelii	51	47	33
B. bavariensis	1	1	0
B. garinii	76	4	27
B. lusitaniae	2	0	1
B. valaisiana	30	5	13
B. burgdorferi s.s.	15	0	9
Total	**175**	**57**	**83**

Appendix 5: Temporal distribution of *B. burgdorferi* species in Babite

Babite	1999	2000	2001	2002	2003	2006	2007	2010	Total
B. afzelii	9	6	26	5	2	1		2	51
B. bavariensis					1				1
B. garinii	7	3	33	1	2	14	12	4	76
B. lusitaniae	1						1		2
B. valaisiana	1		10	1		8	9	1	30
*Bb*ss	4	1	6		1		3		15
Total	**22**	**10**	**75**	**7**	**6**	**23**	**25**	**7**	**175**

Appendix 6: Temporal distribution of *B. burgdorferi* species in Jaunciems

Jaunciems	1999	2000	2001	2002	2003	2006	2007	2010	Total
B. afzelii	2	6	21	4	4	6	4		47
B. bavariensis	1								1
B. garinii			4						4
B. lusitaniae									0
B. valaisiana		1	4						5
Bbss									0
Total	**3**	**7**	**29**	**4**	**4**	**6**	**4**	**0**	**57**

Appendix 7: Temporal distribution of *B. burgdorferi* species in Kemeri

Kemeri	1999	2000	2001	2002	2003	2006	2007	2010	Total
B. afzelii	3	3	13		5	4	5		33
B. bavariensis									0
B. garinii	4	1	12		6	1	3		27
B. lusitaniae				1					1
B. valaisiana	1	1	5		1	1	4		13
Bbss	2	1	4		2				9
Total	**10**	**6**	**34**	**1**	**14**	**6**	**12**	**0**	**83**

Appendix 8: OspC major groups of *B. afzelii*

Isolate No.		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
		0-5-10-*B. afzelii*	0-8-30-*B. afzelii*	1-8-20-*B. afzelii*	0-14-03-*B. afzelii*	0-14-37-*B. afzelii*	0-14-39-*B. afzelii*	1-28-25-*B. afzelii*	1-28-*B. afzelii*	1-29-35b-*B. afzelii*	1-42-05-*B. afzelii*	1-29-29-*B. afzelii*	1-31-25-*B. afzelii*	1-29-22-*B. afzelii*	1-37-13-*B. afzelii*	3-8-11-*B. afzelii*	3-8-06-*B. afzelii*	1-31-42-*B. afzelii*	3-8-07-*B. afzelii*	10-15-17-*B. afzelii*	1-40-20-*B. afzelii*	1-28-05-*B. afzelii*	1-29-27-*B. afzelii*	9-20-22-*B. afzelii*	1-41-09-*B. afzelii*	1-32-25-*B. afzelii*
1	0-5-10-*B. afzelii*																										
2	0-8-30-*B. afzelii*	0,150														0,02											
3	1-8-20-*B. afzelii*	0,124	0,140													0,08											
4	0-14-03-*B. afzelii*	0,000	0,150	0,124																							

Isolate No.		1	2	3	4	5	6	7	8	9	10
		0-5-10-*B. afzelii*	0-8-30-*B. afzelii*	1-8-20-*B. afzelii*	0-14-03-*B. afzelii*	0-14-37-*B. afzelii*	0-14-39-*B. afzelii*	1-28-25-*B. afzelii*	1-28-*B. afzelii*	1-29-35b-*B. afzelii*	1-42-05-*B. afzelii*
5	0-14-37-*B. afzelii*	0,106	0,160	0,134	0,106						
6	0-14-39-*B. afzelii*	0,000	0,150	0,124	0,000	0,106					
7	1-28-25-*B. afzelii*	0,120	0,136	0,003	0,120	0,130	0,120				
8	1-28-*B. afzelii*	0,106	0,160	0,134	0,106	0,000	0,106	0,130			
9	1-29-35b-*B. afzelii*	0,124	0,140	0,000	0,124	0,134	0,124	0,003	0,134		
10	1-42-05-*B. afzelii*	0,124	0,140	0,000	0,124	0,134	0,124	0,003	0,134	0,000	
26	1-32-25-*B. afzelii*										
25	1-41-09-*B. afzelii*										
24	9-20-22-*B. afzelii*										
23	1-29-27-*B. afzelii*										
22	1-28-05-*B. afzelii*										
21	1-40-20-*B. afzelii*										
20	10-15-17-*B. afzelii*										
19	3-8-07-*B. afzelii*										
18	1-31-42-*B. afzelii*										
17	3-8-06-*B. afzelii*										
16	3-8-11-*B. afzelii*										
15	1-37-13-*B. afzelii*										
14	1-29-22-*B. afzelii*										
13	1-31-25-*B. afzelii*										
12	1-29-29-*B. afzelii*										
10	1-42-05-*B. afzelii*										
9	1-29-35b-*B. afzelii*										
8	1-28-*B. afzelii*										
7	1-28-25-*B. afzelii*										
6	0-14-39-*B. afzelii*										
5	0-14-37-*B. afzelii*										
4	0-14-03-*B. afzelii*										
3	1-8-20-*B. afzelii*										
2	0-8-30-*B. afzelii*										
1	0-5-10-*B. afzelii*										

12	1-29-29-*B. afzelii*	0,000	0,150	0,124	0,000	0,106	0,000	0,120	0,106	0,124	0,124																
13	1-31-25-*B. afzelii*	0,118	0,146	0,126	0,118	0,156	0,118	0,126	0,156	0,126	0,126	0,118															
14	1-29-22-*B. afzelii*	0,124	0,140	0,000	0,124	0,134	0,124	0,003	0,134	0,000	0,000	0,124	0,126														
15	1-37-13-*B. afzelii*	0,000	0,150	0,124	0,000	0,106	0,000	0,120	0,106	0,124	0,124	0,000	0,118	0,124													
16	3-8-11-*B. afzelii*	0,164	0,158	0,085	0,164	0,138	0,164	0,082	0,138	0,085	0,085	0,164	0,165	0,085	0,164												
17	3-8-06-*B. afzelii*	0,118	0,146	0,126	0,118	0,156	0,118	0,126	0,156	0,126	0,126	0,118	0,000	0,126	0,118	0,165											
18	1-31-42-*B. afzelii*	0,000	0,150	0,124	0,000	0,106	0,000	0,120	0,106	0,124	0,124	0,000	0,118	0,124	0,000	0,164	0,118										

Isolate No.																									
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
19	3-8-07-*B. afzelii*	0,162	0,156	0,083	0,162	0,136	0,162	0,080	0,136	0,083	0,083	0,163	0,162	0,163	0,083	0,162	0,002	0,163	0,162						
20	10-15-17-*B. afzelii*	0,162	0,156	0,083	0,162	0,136	0,162	0,080	0,136	0,083	0,083	0,163	0,162	0,163	0,083	0,162	0,002	0,163	0,162	0,000					
21	1-40-20-*B. afzelii*	0,000	0,150	0,124	0,000	0,106	0,000	0,120	0,106	0,124	0,124	0,118	0,000	0,118	0,124	0,000	0,164	0,118	0,000	0,162	0,162				
22	1-28-05-*B. afzelii*	0,124	0,156	0,134	0,124	0,140	0,124	0,130	0,140	0,134	0,134	0,148	0,124	0,148	0,134	0,124	0,159	0,148	0,124	0,157	0,157	0,124			
23	1-29-27-*B. afzelii*	0,124	0,156	0,134	0,124	0,140	0,124	0,130	0,140	0,134	0,134	0,148	0,124	0,148	0,134	0,124	0,159	0,148	0,124	0,157	0,157	0,124	0,000		
24	9-20-22-*B. afzelii*	0,120	0,083	0,116	0,120	0,124	0,120	0,116	0,124	0,116	0,116	0,130	0,120	0,130	0,116	0,120	0,146	0,130	0,120	0,138	0,144	0,120	0,138	0,138	
25	1-41-09-*B. afzelii*																								
26	1-32-25-*B. afzelii*																								

25	1-41-09-*B. afzelii*	0,120	0,083	0,116	0,120	0,124	0,120	0,116	0,124	0,116	0,116	0,120	0,130	0,116	0,120	0,146	0,130	0,120	0,144	0,144	0,120	0,138	0,138	0,000		
26	1-32-25-*B. afzelii*	0,177	0,161	0,134	0,177	0,178	0,177	0,134	0,178	0,134	0,134	0,177	0,157	0,134	0,177	0,142	0,157	0,177	0,140	0,140	0,177	0,193	0,193	0,144	0,144	

Group	Isolate No.							Type
Group 1	1	4	6	12	15	18	21	afzeLA1
Group 2	3	7	9	10		14		afzeLA2
Group 3	5	8						afzeLA3
Group 4	16	19	20					afzeLA4
Group 5	22	23						afzeLA5
Group 6	24	25						afzeLA6
Group 7	2							afzeLA7
Group 8	13							afzeLA8
Group 9	17							afzeLA9
Group 10	26							afzeLA10

Appendix 9: OspC major groups of *B. burgdorferi* s.s.

Isolate No.		1	2	3	4
		0-13-41-*B. burgdorferi* s.s.	9-25-70-*B. burgdorferi* s.s.	1-32-30-*B. burdgorferi* s.s.	3-5-17-*B. burgdorferi* s.s.
1	0-13-41-*B. burgdorferi* s.s.				
2	9-25-70-*B. burgdorferi* s.s.	0,231			
3	1-32-30-*B. burdgorferi* s.s.	0,181	0,243		
4	3-5-17-*B. burgdorferi* s.s.	0,181	0,243	0,000	

Group	Isolate No.		Type
Group 1	3	4	OspC MT Q
Group 2	1		OspC MT A
Group 3	2		xxx

Appendix 10: OspC major groups of *B. garinii*

Isolate No.		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
		1-25-53-*B. garinii*	1-28-12b-*B. garinii*	0-14-42-*B. garinii*	10-22-22-*B. garinii*	1-41-28-*B. garinii*	1-29-08-*B. garinii*	1-8-45-*B. garinii*	9-26-26-*B. garinii*	1-37-11-*B. garinii*	1-15-48-*B. garinii*	1-29-28-*B. garinii*	1-32-53-*B. garinii*	10-22-03-*B. garinii*	9-22-27-*B. garinii*	0-8-36-*B. garinii*	9-26-41-*B. garinii*	9-22-36-*B. garinii*	1-29-30-*B. garinii*
1	1-25-53-*B. garinii*																		
2	1-28-12b-*B. garinii*	0,000															0,02		
3	0-14-42-*B. garinii*	0,000	0,000														0,08		
4	10-22-22-*B. garinii*	0,132	0,132	0,132															
5	1-41-28-*B. garinii*	0,132	0,132	0,132	0,000														

Isolate No.		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
		1-25-53-*B. garinii*	1-28-12b-*B. garinii*	0-14-42-*B. garinii*	10-22-22-*B. garinii*	1-41-28-*B. garinii*	1-29-08-*B. garinii*	1-8-45-*B. garinii*	9-26-26-*B. garinii*	1-37-11-*B. garinii*	1-15-48-*B. garinii*	1-29-28-*B. garinii*	1-32-53-*B. garinii*	10-22-03-*B. garinii*	9-22-27-*B. garinii*	0-8-36-*B. garinii*	9-26-41-*B. garinii*	9-22-36-*B. garinii*	1-29-30-*B. garinii*
6	1-29-08-*B. garinii*	0,070	0,070	0,070	0,136	0,136													
7	1-8-45-*B. garinii*	0,070	0,070	0,070	0,136	0,136	0,000												
8	9-26-26-*B. garinii*	0,070	0,070	0,070	0,136	0,136	0,000	0,000											
9	1-37-11-*B. garinii*	0,070	0,070	0,070	0,136	0,136	0,000	0,000	0,000										
10	1-15-48-*B. garinii*	0,164	0,164	0,164	0,118	0,118	0,149	0,149	0,149	0,149									
11	1-29-28-*B. garinii*	0,114	0,114	0,114	0,098	0,098	0,116	0,116	0,116	0,116	0,099								

12	1-32-53-*B. garinii*	0,000	0,000	0,000	0,132	0,132	0,070	0,070	0,070	0,070	0,164	0,114						
13	10-22-03-*B. garinii*	0,164	0,164	0,164	0,118	0,118	0,149	0,149	0,149	0,149	0,000	0,099	0,164					
14	9-22-27-*B. garinii*	0,114	0,114	0,114	0,098	0,098	0,116	0,116	0,116	0,116	0,099	0,000	0,114	0,099				
15	0-8-36-*B. garinii*	0,149	0,149	0,149	0,141	0,141	0,134	0,134	0,134	0,134	0,149	0,145	0,149	0,149	0,145			
16	9-26-41-*B. garinii*	0,166	0,166	0,166	0,120	0,120	0,151	0,151	0,151	0,151	0,002	0,101	0,166	0,002	0,101	0,147		
17	9-22-36-*B. garinii*	0,164	0,164	0,164	0,118	0,118	0,149	0,149	0,149	0,149	0,000	0,099	0,164	0,000	0,099	0,149	0,002	
18	1-29-30-*B. garinii*	1,162	1,162	1,162	1,167	1,167	1,205	1,205	1,205	1,205	1,194	1,151	1,162	1,194	1,151	1,148	1,203	1,194

OspC major groups of *B. garinii*

Group	Isolate No.				Type
Group 1	1	2	3	12	garLG1
Group 2	4	5			garLG2
Group 3	6	7	8	9	garLG3
Group 4	10	13	16	17	garLG4
Group 5	11	14			garLG5
Group 6	15				garLG6
Group 7	18				garLG7

Appendix 11: OspC major groups of *B. valaisiana*

Isolate No.		1	2	3	4
		10-24-07-*B. valaisiana*	1-29-03-*B. valaisiana*	1-14-39-*B. valaisiana*	1-37-10-*B. valaisiana*
1	10-24-07-*B. valaisiana*				
2	1-29-03-*B. valaisiana*	0,114			
3	1-14-39-*B. valaisiana*	0,190	0,221		
4	1-37-10-*B. valaisiana*	0,159	0,226	0,249	

Group	Isolate No.	Type
Group 1	1	valLV1
Group 2	2	valLV2
Group 3	3	valLV3
Group 4	4	valLV4

Appendix 12: Gadgetry used

Gadget	Name	Manufacturer	Office	Additional specification
Centrifuge	Centrifuge 5424	Eppendorf	Hamburg	
Centrifuge	PCV-2400	Grant Instruments	Cambridge, UK	
Centrifuge	Ministar Silverline	VWR	Darmstadt	
Centrifuge	Centrifuge 5210R	Eppendorf	Hamburg	
Centrifuge	PerfectSpin P	PeqLab Biotechnologie	Erlangen	
DNA-Extraction maschine	BioSprint 96	QIAGEN®	Venlo, Netherlands	
DNA-Extraction maschine	Maxwell® 16	Promega	Fitchburg, WI, USA	
Fluorometer	Qubit® 3.0 Fluorometer	Thermo Fisher Scientific	Waltham, MA, USA	
Fluorometer	Qubit30	Thermo Fisher Scientific	Waltham, MA, USA	
Freezer	Ultra-Low Temperature Freezer U725 innova®	Eppendorf	Hamburg	- 80°C
Fridge	Mediline	Liebherr	Biberach an der Riß	
Fridge	Premium	Liebherr	Biberach an der Riß	

Gadget	Name	Manufacturer	Office	Additional specification
Gel carrier	L4	PeqLab Biotechnologie	Erlangen	horizontal
Gel chamber	Standard chamber	PeqLab Biotechnologie	Erlangen	20 Zähne, 1,5 mm, 22 µL 24 Zähne, 1,5 mm, 17 µL
Gel chamber	Mikrotiter chamber	PeqLab Biotechnologie	Erlangen	42 Zähne, 1,5 mm, 16 µL
Gel chamber	PerfectBlue™ Gelsystem Mini L	PeqLab Biotechnologie	Erlangen	horizontal
Gel chamber	Sub-Cell® Model 192	Bio-Rad	München	big
Gel rack	MultiCast Cast rack	PeqLab Biotechnologie	Erlangen	
Magnetic base	MM-Separator M96 P	Magtivio	Niederlande	für 96-Well Platte
Magnetic base	DynaMagTM-Spin	Thermo Fisher Scientific	Waltham, MA, USA	für Tubes
Microwave	HF 22024	Siemens	München	
Nucleic Acid System	2200 TapeStation	Agilent Technologies	Oberhaching	High Sensitivity Tape
Nucleic Acid System	Fragment Analyzer	Advanced Analytical	Oberhaching	
PCR Plate Sealer	PX1™	Bio-Rad	München	
PCR Workstation	UV2 PCR Workstation	VWR	Radnor, PA, USA	
PCR-Cycler	Mastercycler® nexus gradient	Eppendorf	Hamburg	conventional PCR
PCR-Cycler	2720 Thermal Cycler	Applied Biosystems	Foster City, CA, USA	conventional PCR

Gadget	Name	Manufacturer	Office	Additional specification
PCR-Cycler	Mx3005P	Agilent Technologies	Oberhaching	real-time PCR
PCR-Cycler	Mx3000P	Agilent Technologies	Oberhaching	real-time PCR
Pipette	Multipette® plus	Eppendorf	Hamburg	
Pipette	Loading Tip Transer Tool	Agilent Technologies	Oberhaching	
Pipette	Research plus	Eppendorf	Hamburg	2-20 µL
Pipette	Xplorer	Eppendorf	Hamburg	Multi-Channel 1-10 µL 15-300 µL
Pipette	Reference	Eppendorf	Hamburg	0,5-10 µL 10-100 µL 100-1000 µL
Scale	EW 1500-2M	Kern und Sohn GmbH	Balingen	
Shaker	Thermomixer comfort	Eppendorf	Hamburg	DNA-Extraktion über Nacht
Sequencing gadget	MiSeq	Illumina	San Diego, CA, USA	
Spectro-photometer	NanoDrop 1000	PeqLab Biotechnologie	Erlangen	
UV-Gel-Document-ation system	Vilber lourmat	LTF Labor-technik GmbH & Co KG	Wasserburg	
Vortex	MS3	IKA	Staufen im Breisgau	
Vortex	Vortex Mixer	VWR	Darmstadt	
Vortex	Lab Dancer S40	VWR	Darmstadt	

Appendix 13: Consumables

Material	Manufacturer	Kat. No.	Additional specification
Al-PCR-Folie	Bio-Rad	1814040	PCR Plate Sealer
Assay tubes	Thermo Fisher Scientific	Q32586	for measurement with Qubit30
DNA LoBind Tubes	Eppendorf	0030108051	1,5 mL
Microplate 96-Well	Qiagen	1031656	
PCR Single Cap 8-er Soft Strips	Biozym	710971	0,2 mL
PCR-Folie	Ratiolab	6018610	
PCR-Folie	PeqLab Biotechn.	PEQL82-1170-A	
PCR-Folie	MicroAmp™	4311971	real-time
PCR-Platte	4titude®	4-ti-0760	for Fragment Analyzer
PCR-Platte	Eppendorf	0030129334	conventional
PCR-Platte	Appl. Biosystems®	4306737	real-time
Pipette tips	Eppendorf	022491202	10 µL M PCR clean/sterile
Pipette tips	Biozym	VT0210 VT0230 VT0260	10 µL, steril 100 µL, steril 1000 µL, steril
Pipette tips (Combitips advanced®)	Eppendorf	0030089405 0030089413 0030089634 0030089448	0,1 mL; 0,2 mL; 0,5 mL; 2,5 mL; Used for Multipette® plus
Pipette tips (Loading Tips)	Agilent Technologies	5067-5153	Used for 2200 TapeStation
Safe-Lock Tube	Eppendorf	0030121.589 0030120.094	1,5 mL 2,0 mL

Appendix 14: Kits

Material	Manufacturer	Kat. No.	Additional specification
BioSprint 96 One-For-All Vet Kit	QIAGEN®	947057	Device: BioSprint 96
High Pure PCR Template Preparation Kit of Roche	Roche	11796828001	
High Sensitivity NGS Fragment Analysis Kit	Advanced Analytical	DNF-474	Device: Fragment Analyzer
HotStar Taq® Master Mix Kit	QIAGEN®	203445	HotStar Taq® Master Mix, RNase-free Water
Index Adapter Replacement Caps	Illumina	15026762	
Index Kit	Illumina	15055293 15055294	24 samples 96 samples
Maxwell® 16 LEV Blood DNA Kit	Promega	AS1290	Device: Maxwell® 16
MiSeq® Reagent Micro Kit v2	Illumina	15036715	
MiSeq® Reagent Nano Kit v2	Illumina	15036714	
MiSeq® v2 Reagent Kit	Illumina	15033624	300 Cycles, 500 Cycles
Nextera® XT Library Prep Kit 96 sample	Illumina	15032354 15032355	Box 1 von 2 Box 2 von 2
Qubit® ds DNA BR Assay Kit	Thermo Fisher Scientific	Q32853	
Qubit® ds DNA HS Assay Kit	Thermo Fisher Scientific	Q32854	

Appendix 15: Chemicals

Chemical	Manufacturer	Kat. No.
10 x PBS	Roth	1058.1
10 x TBE	Roth	3061.1
Agarose	Biozym	840004
Agencourt® AMPure® XP	Beckman Coulter	A63880
Ethanol	Fisher Scientific	E/0650DF/17
GelRed™ Nucleic Acid	Biotium	41003
Gene Ruler 50 bp	Thermo Fisher Scientific	SM0371
High Sensitivity D1000 Ladder	Agilent Technologies	5067-5587
High Sensitivity D1000 Sample Buffer	Agilent Technologies	5190-6504
High Sensitivity D1000 Screen Tape	Agilent Technologies	5067-5584
Lysis Solution TLS	AJ Innuscreen GmbH - Analytik Jena AG	innuPREP DNA micro Kit: 845-KS-1010010
$MgCl_2$ (25mM)	Thermo Fisher Scientific	R0971
Nuclase free water	Promega	P1193
Orange DNA Loading Dye (6x)	Thermo Fisher Scientific	R0631
Polyethylenglykol	Roth	0263.1

Appendix 16: STs Analysed in this study.

Illustrataes the frequency of STs, their origin and years of collection used in the PhyloViz analysis for the construction of minimum spanning tree

Species	ST	Frequency	Year	Region
B. burgdorferi s.s.	1	2	1999, 2000	Babite
B. burgdorferi s.s.	20	2	1999	Kemeri
B. burgdorferi s.s.	21	3	2003 (2), 2000	Kemeri
B. bavariensis	84	1	1999	Jaunciems
B. garinii	86	4	2006 (2), 2007 (2)	Babite 3, Kemeri 1
B. garinii	87	1	2001	Babite
B. garinii	88	1	2010	Babite
B. garinii	89	2	2007	Babite
B. garinii	90	1	2007	Babite
B. garinii	93	1	2001	Babite
B. valaisiana	96	3	2001, 2006, 2007	Jaunciems 1, Babite 2
	97	4	2006 (2), 2007 (2)	Babite
	99	1	2001	Babite
	100	2	2001, 2010	Babite
	102	1	2006	Babite
	103	1	1999	Kemeri
B. burgdorferi s.s.	161	2	2007	Babite
	162	1	2007	Babite
B. garinii	163	2	2006, 2007	Babite
B. afzelii	165	3	2006 (2), 2007	Babite 1, Jaunciems 2
	166	3	2001, 2006, 2007	Kemeri 2, Jaunciems
	167	1	2006	Kemeri
	168	1	2007	Kemeri
	169	1	2006	Jaunciems
	170	5	2006 (2), 2007, 2001 (2)	Jaunciems 3, Kemeri 2

Species	ST	Frequency	Year	Region
	171	3	2007, 2001 (2)	Kemeri, Babite 2
B. garinii	175	1	1999	Kemeri
	177	3	1999, 2006, 2010	Babite
	178	1	2007	Kemeri
	180	4	1999, 2006 (3)	Kemeri 2, Babite 2,
	181	1	2007	Babite
	182	1	2007	Babite
	184	2	2001, 2007	Jaunciems, Babite
	185	1	2006	Babite
	186	1	2006	Babite
	187	1	2007	Babite
	188	2	2006	Babite
	189	1	2006	babite
	190	2	2006, 2007	Babite
	193	1	2006	Babite
B. valaisiana	197	1	2007	Babite
	199	5	2001 (1), 2002 (1), 2007 (3)	Babite 2, Kemeri 2, Jaunciems
	201	2	2001, 2006	Jaunciems 2, Babite
	202	1	2006	Kemeri
B. valaisiana	203	2	2007	Kemeri, Babite
B. afzelii	204	1	2007	Kemeri
B. valaisiana	206	1	2007	Babite
B. garinii	207	1	2007	Kemeri
B. garinii	208	1	2007	Babite
B. garinii	209	1	2007	Babite
B. valaisiana	211	2	2007	Babite, Kemeri
	212	3	2006	Babite
	213	1	2007	Babite
B. garinii	214	1	2006	Babite
B. afzelii	215	3	2006	Jaunciems, Kemeri 2
	216	1	2007	Jaunciems
	217	1	2007	Kemeri
B. afzelii	219	1	2006	Jaunciems
	220	2	2007, 2000	Jaunciems
	244	1	2001	Jaunciems
	463	1	2002	Jaunciems
	554	1	2002	Babite

Species	ST	Frequency	Year	Region
	570	1	2001	Jaunciems
	571	1	2001	Jaunciems
	707	1	2002	Babite
B. burgdorferi s.s.	807	1	1999	Babite
B. garinii	808	1	1999	Babite
B. afzelii	809	1	1999	Kemeri
	810	1	1999	Kemeri
	811	1	1999	Kemeri
	812	1	1999	Kemeri
	813	1	1999	Babite
B. burgdorferi s.s.	814	1	1999	Babite
B. afzelii	815	1	2001	Babite
B. garinii	816	1	2000	Babite
B. afzelii	817	1	2001	Babite
	818	1	2001	Babite
B. garinii	819	1	2001	Kemeri
B. valaisiana	820	1	2001	Kemeri
B. afzelii	821	1	2001	Kemeri
	822	1	2001	Kemeri
	823	1	2001	Jaunciems
B. valaisiana	824	1	2001	Jaunciems
B. afzelii	825	1	2001	Jaunciems
	826	1	2001	Jaunciems
	827	1	2001	Jaunciems
	828	1	2001	Jaunciems
	829	1	2001	Jaunciems
B. afzelii	830	1	2001	Jaunciems
	831	1	2001	Jaunciems
	832	1	2001	Babite
	833	1	2001	Babite
	834	1	2001	Babite
	835	1	2001	Babite
B. burgdorferi s.s.	836	1	2001	Kemeri
B. afzelii	837	1	2001	Kemeri
B. garinii	838	1	2001	Kemeri
B. afzelii	839	1	2001	Kemeri
B. garinii	852	1	2001	Kemeri
B. afzelii	840	1	2003	Kemeri
	841	1	2003	Kemeri

Appendix

Species	ST	Frequency	Year	Region
B. garinii	842	1	2003	Kemeri
B. afzelii	843	1	2003	Jaunciems
B. garinii	844	1	2010	Babite
B. afzelii	845	1	2002	Jaunciems
	846	2	2002	Babite
	847	1	2002	Babite
	848	1	2002	Jaunciems
	849	1	2002	Jaunciems
B. garinii	850	1	2002	Babite

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