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Analysis of genetic diversity of *Borrelia burgdorferi* sensu lato and *Borrelia miyamotoi* across Central and Eastern Europe using multilocus sequence typing (MLST) in range of Slovakia and data from the MLST database with focus on Central and Eastern Europe

Master thesis

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Annotation

The study was based on tick collection, DNA extraction, PCR amplification, sequencing and computational analyses of *Borrelia burgdorferi* sensu lato and *B. miyamotoi*. The study included evaluation on the prevalence of *Borrelia burgdorferi* sensu lato and *B. miyamotoi* in *Ixodes ricinus* ticks across the Slovak Republic based on the collection of ticks from 2017. The spirochetes were further classified into genospecies as well as sequence types (ST) according to MLST method by amplifying and sequencing eight housekeeping genes. Subsequently, population-genetic relationships of *Borrelia* tick-borne pathogens across Central and Eastern Europe were analysed.

Declaration

I declare that I am the author of this qualification thesis and that in writing it I have used the sources and literature displayed in the list of used sources only.

České Budějovice, 13.04. 2022.

Signature.....

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ABBREVIATIONS

ACA	acrodermatitis chronica atrophicans
ANI	average nucleotide identity
BIC	Bayesian Information Criterion
CC	clonal complex/cluster
CCs	clonal complexes/clusters
DLVs	double locus variants
EDTA	ethylenediaminetetraacetic acid
EM	erythema migrans
FlaA/B	flagellin A/B
goeBURST	globally optimized implementation of the eBURST algorithm
HTBRF	hard-tick-borne relapsing fever
IGS	intergenic spacer
LB	Lyme borreliosis
LBRF	louse-borne relapsing fever
LD	Lyme disease
LG	Lyme group
LNB	Lyme neuroborreliosis
LPS	lipopolysaccharide
MLEE	multi-locus enzyme electrophoresis
MLSA	multilocus sequence analysis
MLST	multilocus sequence typing
MST	minimum-spanning tree
NT29	ribotype
NWF	nuclease free water
OsP	outer surface/membrane protein
OspA/C	outer surface protein A or C
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
POCP	percentage of conserved proteins
QPweb	Parasitology on the Web

REP/G	Echidna-reptile group
RF	relapsing fever
RFG	Relapsing fever group
RFLP	restriction fragment length polymorphism
SG	singleton
s.l.	sensu lato
SLVs	single locus variants
SNP	single nucleotide polymorphism
s.s.	sensu stricto
SSCP	single-stranded conformation polymorphism
ST/s	sequence type/s
STBRF	soft-tick-borne relapsing fever
TBEV	tick-borne encephalitis virus
TBRF	tick-borne relapsing fever
TLVs	triple locus variants
VNTRs	variable-number tandem repeats
WGS	whole genome sequencing

CONTENTS

1. INTRODUCTION

Borreliae organisms are divided into three distinct groups (Appendix 1). First group is associated with Lyme disease (LD) or Lyme borreliosis (LB), i.e., the Lyme group (LG). The second group of *Borrelia* organisms includes aetiological agents of relapsing fever disease (RF or RFG). A novel group has recently been described as the Echidna-Reptile group (REP or REPG) (Binetruy et al. 2020; Trevisan et al. 2021).

Several members of the *Borrelia burgdorferi sensu lato* (s.l.) complex are causative agents of LB, which is considered the most prevalent tick-borne disease in the Northern Hemisphere (Rosenberg et al. 2018; Sykes & Makiello 2017; van den Wijngaard et al. 2017). The complex currently includes more than 20 borrelial genospecies (Margos et al. 2020). These zoonotic agents are maintained in natural foci by the transmission between an ixodid vector (Acari: Ixodidae) and a vertebrate reservoir host, whereas over 40 mammal species are considered reservoir hosts for the *B. burgdorferi* s.l. complex. (Gassner et al. 2013; Gorelova et al. 1995; Hengge et al. 2003; Piesman et al. 2004). In Europe, the predominant pathogenic genospecies are: *B. garinii*, *B. afzelii*, and *B. burgdorferi* sensu stricto (s.s.) (Margos et al. 2009). The clinical features vary among individual genospecies. Lyme neuroborreliosis (LNB) is mostly associated with *B. garinii*, whereas *B. afzelii* can cause the chronic dermatological disease, namely, acrodermatitis chronica atrophicans (ACA) (Bazovská et al. 2011; Brisson et al. 2011; Fingerle et al. 2008; Margos et al. 2013; Ornstein et al. 2002; Ogrinc et al. 2013). Clinical cases of LNB occur more frequently in the Old World in comparison to the New World (Hengge et al. 2003). The most common manifestation of *B. burgdorferi* s.s. is arthritis (Rudenko et al. 2011; Wang et al. 1999). Pathogenic genospecies of *B. burgdorferi* s.l. complex are frequently linked with skin lesions in its acute phase of infection, also called, erythema migrans (EM). Reports indicate briefer duration of EM when infected by *B. burgdorferi* s.s. compared to infection by *B. afzelii* which also maintain the EM skin rash (Strle et al. 1999).

Moreover, it has been observed that not all strains within a specific genospecies cause disseminated form of the disease (Baranton et al. 2001; Wormser et al. 2008). The genetic differences between the genospecies may be connected with pathogenicity, ecology, geographic distribution, vector competence and species-specific relationships to the host reservoir, and host range (Barbour 2014; Krause et al. 2015). Therefore, it is of importance to identify those factors in correlation with specific genotypes and their clinical relevance.

Another major clade of genospecies in the genus *Borrelia* is a relapsing fever group, (RF) which is mostly transmitted by argasid ticks and lice (Talagrand-Reboul et al. 2018; Trevisan et al. 2021). In 1995, *B. miyamotoi* was described in ixodid tick (*Ixodes persulcatus*) in Japan (Fukunaga et al. 1995). Hence it is the only member of the relapsing fever group for which an ixodid tick (i.e, *I. ricinus*) serve as the main vector in Europe (Cutler et al. 2019; Kubiak et al 2021; Layzell et al. 2018; Sawczyn-Domańska 2021; Siński et al. 2016). Subsequently, the genospecies has been detected in other tick genospecies associated with Lyme disease among the Holarctic region, namely *I. scapularis*, *I. pacificus*, *I. ovatus*, and *I. pavlovskyi* (Crowder et al. 2014; Dibernardo et al. 2014; Fedorova et al. 2014; Fukunaga et al. 1995; Geller et al. 2012; Keesing et al. 2021; Korenberg et al. 2010; McVicar et al. 2022; Mun et al. 2006; Salkeld et al. 2014; Scoles et al. 2001; Takano et al. 2010; Tokarz et al. 2010; Wagemakers et al. 2017; Xu et al. 2021).

Human *B. miyamotoi* infections may cause severe disease, including meningoencephalitis (Gugliotta et al. 2013; Henningson et al. 2019; Hovius et al. 2013; Krause et al. 2013; Krause et al. 2015; Platonov et al. 2011). Nevertheless, the clinical manifestations are non-specific (Kubiak et al. 2021). Therefore, detection of the pathogen by specific diagnostic methods, such as antibody assay, PCR, or *in vitro* cultivation is of high importance (Franck et al. 2020; Harris et al. 2019; Karan et al. 2018; Koetsveld et al. 2017; Koetsveld et al. 2018; Tokarz et al. 2020).

As interspecies and intraspecies diversity of the genus *Borrelia* in association with its pathogenic character increases, an emphasis is put on accurate strain typing within the genospecies. Therefore, a method called Multilocus sequence typing (MLST), with a high discriminatory power is used. The MLST scheme describes a sequence information of all eight housekeeping loci of *Borrelia*, to analyse differences between and within genospecies. (Margos et al. 2008). It is considered that not all genotypes within a genospecies of *B. burgdorferi* s.l. cause clinical symptoms in patients. Therefore, it is important to identify the geographical distribution of genospecies causing LB as well as the spatial distribution of the specific genotypes. Comparison of different sequence types (STs) of *Borrelia* obtained from questing ticks, reservoir hosts and patients (with specific symptoms of Lyme disease) can help identify risk regions of infection) (Gallais et al. 2018; Hanincová et al. 2013)

Considering the fact, that the most prevalent genospecies are: *B. afzelii*, *B. garinii*, and *B. burgdorferi* s.s. (Appendix 1) (Margos et al. 2009), a higher genetic diversity and thus

division into different clusters is assumed in *B.* afzelii, in relation to different regions of Europe (and Asia) due to the reservoir host (rodent) and its limited ability to migrate (Humair & Gern 2000; Kurtenbach et al. 2002; Gallais et al. 2018). This may also be related to the potential difference in severity of symptoms in patients infected with different STs. In the case of *B. garinii*, spatial mixing of ST within different regions of Europe is expected due to the migration routes of the same reservoir hosts, i.e., birds (Humair & Gern 2000; Kurtenbach et al. 2002; Miyamoto et al. 2002). Division of clusters of European and Asian STs are expected. (Vollmer et al., 2011). A free distribution of STs of *B. burgdorferi* s.s. is assumed among Europe (Humair & Gern 2000; Kurtenbach et al. 2002).

The genetic variability of *B. miyamotoi* in terms of MLST is not yet well explored, so it is desirable to contribute new data to help determine further relationships on the risk of potential infection with clinical symptoms in the middle (and Eastern) Europe.

2. THEORETICAL FRAMEWORK

2.1 Classification and geographical distribution of *Borrelia burgdorferi* s.l. and *B. miyamotoi*

B*orrelia burgdorferi* s.l. complex belongs to the genus *Borrelia* (Spirochaetales: Spirochaetacea). Currently, *Borrelia burgdorferi* s.l. includes 22 genospecies, out of which 11 are recognized in the Old World (*B. afzelii, B. bavariensis, B. garinii, B. japonica, B. lusitaniae, B. sinica, B. spielmanii, B. tanukii, B. turdi, B. valaisiana* and *B. yangtzenzis*). Other four genospecies from the *B. burgdorferi* s.l. complex are strictly associated with the New World only (*B. americana, B. andersonii, B. californiensis,* and *B. kurtenbachii*). *B. burgdorferi* s.s., *B. bissettiae* and *B. carolinensis* are identified from both Eurasia and the USA (Rudenko et al. 2011; Trevisan et al. 2021; Waindok et al. 2017).

Spirochetes of the genus *Borrelia* are divided into three distinct groups, namely, LG, RF, and REP. The first group consists of members responsible for LD with occurrence in the temperate zone of the Holarctic region (Margos et al. 2011). The life cycle of LB group of spirochetes involves hard ticks (vectors) in transmission to vertebrates (reservoir hosts) (Hengge et al. 2003; Piesman & Gern 2004).

The second, the causative agent of RF in humans, was generally known to occur from subtropical to tropical regions (Ogden et al. 2014). The vast majority of RFG of *Borrelia* includes argasid (soft) ticks as vectors. However, a RF pathogen belonging to *Borrelia* group, *B. miyamotoi*, is known to be transmitted by the same Ixodidae invertebrate that serve as vector for LG of *Borrelia* spirochetes (Kubiak et al. 2021; McVicar et al. 2022; Sawczyn-Domańska 2021; Wagemakers et al. 2017; Xu et al. 2021). *B. miyamotoi,* as a RF genospecies, occurs sympatrically with the LG of *Borrelia* in The Old and the New World.

Borrelia miyamotoi spirochetes belong to the RFG along with 33 recent valid genospecies (Jakab et al. 2022). As mentioned before, *B. miyamotoi* is found primarily in the temperate zone of both the Old and the New World (Krause et al. 2015; Ogden et al. 2014). Additional 10 genospecies occur in the Old World, i.e., *B. crocidurae*, *B. duttoni*, *B. graingeri*, *B. harveyi*, *B. tillae*, *B. baltazardii*, *B. caucasica*, *B. hispanica*, *B. latyschewii*, and *B. persica*. In the New World, eight other taxa are included, namely: *B. brasiliensis*, *B. coriaceae*, *B. dugesii*, *B. hermsii*, *B. parkeri*, *B. turicatae*, *B. mazzotti*, and *B. venezuelensis*. Genospecies *B. recurrentis* and *B. anserina* are distributed worldwide. *B.* theileri is

associated with northern and southern America, Africa and Australia (Talagrand-Reboul et al. 2018).

Study in 2014 has suggested to re-classify the LG and RFG into two genera, i. e. *Borrelia* and *Borreliella* (Adelou & Gupta 2014), within a newly formed family of *Borreliaceae* (order: Spirochetales) (Barbour et al. 2018). The separation of these groups of spirochetes is based on analysis of phylogenetic and phenotypic aspects using method of average nucleotide identity (ANI). In recent research, another approach was used to study the *Borrelia* genus/genera based on the pairwise analysis of percentage of conserved proteins (POCP) (Margos et al. 2018a). Based on the study, it was proposed to not separate the individual groups into genera and rather re-unite them into the genus *Borrelia*.

Geographical distribution, diversity of vectors and host specificity of *Borrelia burgdorferi* s.l. and relapsing fever-related spirochetes, *B. miyamotoi* (Fukunaga et al. 1995; Gugliotta et al. 2013; Platonov et al. 2011; Wagemakers et al. 2017), is shown in Appendix 1.

2.1.1 General characteristics

Borrelia is a spiral-shaped bacterium considered to have a diderm (dual-membrane) cell envelope with a peptidoglycan layer situated between the outer and inner membrane (Barbour & Hayes 1986). The doubling time of borreliae spirochete takes from 24 to 48 hours (Zückert 2007).

Borrelia spirochetes are not classified as either Gram−positive or Gram−negative. The bacterium does not possess a rigid cellular wall and a lipopolysaccharide (LPS) substance, the major component of the outer membrane of Gram−negative bacteria. The LPS is mainly responsible for structural integrity and protection from the environment (Zhang et al. 2013). Instead of LPS, *Borrelia* spirochetes contain immunoreactive glycolipids at the outer surface, essential for a host adaptation (Ben-Menachem et al. 2003; Radolf et al. 2012; Caimano et al. 2016).

The *Borrelia* cell may reach up to 10–40 μm in length, and 0.2–0.5 μm in diameter. The movement of bacterium is caused by 7–14 periplasmic flagella, longitudinally coiled around the helical protoplasmic cylinder between the peptidoglycan layer and the outer membrane sheath. The bundled flagella provide a screw-like motion and enable the cell to move in

media with various viscosity level (Motaleb 2000). Hence there is a link between the features of flagella and a high virulence factor of the *Borrelia* (Botkin et al. 2006; Harman et al. 2012; Sellek et al. 2002) as it has been implicated in several other spirochaetal genospecies such as *Treponema pallidum* (Lux et al. 2001; Botkin et al, 2006), *Leptospira interrogans* (Lambert et al. 2012), *Brachyspira pilosicoli* (Nakamura et al. 2006). The flagellar appendages consist predominantly of two types of outer layer flagellin proteins, major flagellin B (FlaB (41 kDa) and minor flagellin A (FlaA) (38 kDa) (Ge et al. 1998) forming up to 14 % and less than 0.5 % of the total protein of the cell, respectively (Ge et al. 1998; Motaleb et al. 2004). In most spirochaetal bacteria, FlaA forms a cover that surrounds the core of FlaB (Li et al. 2008). As suggested by Gilmore et al (1999), FlaA could potentially perform as useful antigen in detection of antibodies in early stage of LD. Nonetheless, other studies revealed the FlaA have a limited role for IgM serodiagnosis. In IgG serology, it has been shown that FlaA protein might be a sensitive antigen in diagnosis of disseminated LD (Ge et al, 1997; Panelius et al. 2001).

A study from 2013 indicates that FlaB filaments of borrelial flagella are important for virulence. The ability to establish infection of a motile wild-type FlaB with a flat-wave morphology was compared to independently isolated nonmotile rod-shaped FlaB mutants. The analysis revealed a decrease of viability of the spirochetes with a FlaB-inactived filament in fed ticks (*Ixodes scapularis*) and inability to establish infection in a rodent host (*Mus musculus*) after inoculation of the pathogen. In contrast, the wild-type cells were reisolated from both inoculated mice and fed ticks. The research indicates the importance of the shape of the periplasmic flagella and the possibility of movement related to survival of spirochetes in the arthropod vector and infection of the vertebrate host (Sultan et al. 2013).

The metabolic activity of *Borrelia* cell is limited. The *Borrelia* spirochetes are not capable of synthesis of amino acids, fatty acids, enzyme cofactors, and nucleotides. It is considered that the genes encoding the enzymes for these mechanisms disappeared during the coevolution with the *Borrelia* invertebrate vector and the vertebrate host (Fraser et al. 1997; Skare et al. 2010). As a substitute, the *borrelial* spirochetes possess at least 52 known genes encoding transporters and eventually binding proteins of amino acids, carbohydrates, and peptides (Saier & Paulsen 2000). Since the *Borrelia* cells lack the genes essential for encoding the components for the citric acid cycle and oxidative phosphorylation, the energy

is produced by glycolysis and the fermentation of sugars to lactic acid (Fraser et al. 1997; Gherardini et al. 2010).

The bacterium is microaerophilic and capable to survive without iron in compare to many other pathogenic bacteria acquiring iron to persist. Instead, *Borrelia* spirochetes are using manganese to substitute iron-sulfur cluster enzymes (Troxell et al. 2012).

2.1.2 Genome characteristics

The genome of *Borrelia* cell consists of approximately 950 kb linear chromosome and numerous circular and linear plasmids with sizes ranging from 9 kb to 62 kb (Barbour & Garon 1987; Casjens et al. 2012). Majority of housekeeping genes are found on the chromosome. Lipoproteins-encoding genes expressed on the outer membrane of the bacteria are on the plasmids. The importance of lipoproteins is significant, despite the low abundance with only 7.8 % of all open reading frames. Lipoproteins of *Borrelia* are considered to mediate transition through the sylvatic (enzootic) cycle during which the proteins are expressed differently (Radolf et al. 2012; Samuels 2011; Singh & Girschick 2004). Linear plasmids differ from each other distinctively. Nevertheless, all linear plasmids include multiple copies of paralogs (paralogous genes) (Casjens et al. 2012; Fraser et al. 1997). Several linear and circular plasmids are not fundamental for *in vitro* propagation, including plasmids lp28-1, lp25 and various plasmids of the cp32 family (Brisson et al. 2012; Labandeira-Rey & Skare 2001; Purser et al. 2003). Nonetheless, the above-mentioned plasmids are crucial for the enzootic cycle. In other words, *in vitro* manipulation of *Borrelia* spirochetes may lead to loss of essential plasmids. Another circular plasmid, cp26 is known to be required for *in vitro* growth. In addition, the plasmid carries a membrane receptor, the outer surface protein C (OspC) (Sadziene et al. 1993), which maintains transmission of the spirochete from its specific vector to vertebrate host and early infection establishment (Grimm et al. 2004; Stewart et al. 2006; Tilly et al. 2007). The OspC is induced in the spirochete during nymphal tick feeding and during early phase of infection in mammals (Carrasco et al. 2015; Pal et al. 2004).

2.1.3 Eco−epidemiology of *B. burgdorferi* s.l. and *B. miyamotoi*

Eco−epidemiology of *Borrelia* is characterized by dynamic interaction between the pathogen, vector and reservoir host (Fig. 1.) (Centers for Disease Control and Prevention 2011; Estrada-Peña & Fernández-Ruiz 2022; Obiegala et al. 2017; Rizzoli et al. 2014). The sylvatic cycle of the bacterium is closely linked to landscape and climatic factors of its invertebrate and vertebrate hosts, which broadly determine the spirochete ecological niches (Kurtenbach et al. 2006).

Borrelia burgdorferi s.l. is transmitted by *Ixodes* spp. ticks. The main vector is considered to be *I. ricinus* (Cotté et al. 2010). Ixodid natural cycle includes four stages − egg, larvae, nymph, and adult. After hatching from eggs, ixodid ticks take one blood meal per stage from larval to adult phase (male/female). The life cycle can be completed up to three years (1 life stage/year) (Fourie & Horak 1994; Piesman 2002; Grigoryeva & Stanyukovich 2016).

Adults are sexually dimorphic in both morphology and behaviour. Males occasionally attach and feed for short times (Eisen et al. 2012; Apanaskevich & Oliver 2014). Females take one large blood meal in two phases, which is divided into slow (4−5 days) and rapid (up to 24 hours) food intake. During the feeding process of female, the volume of the body increases about 100-fold as compared to fasting. As males mostly do not take any blood meal, their body proportions remain without significant changes (Apanaskevich & Oliver 2014). Adults are able to mate on or off host as the bloodmeal is not required to achieve the gonotrophic cycle (Kiszewski et al. 2001). After mating, females complete the life cycle by laying eggs on the ground and die (Apanaskevich & Oliver 2014).

Larval ticks and nymphs feed predominantly on small mammals and birds. Adult ticks feed mostly on large mammals (Kurtenbach et al. 2006; Piesman, & Schwan 2010). The infection of *Borrelia* to tick is transmitted through the bloodmeal from an infected animal. Maintenance of spirochetes is retained trans-stadially after each feeding and moult (Brisson et al. 2012).

In case of relapsing-fever group genospecies, e.g., *B. miyamotoi*, the spirochetes are capable of transovarial transmission (Breuner et al. 2018).

Another possibility of transmission includes co-feeding between ticks in close location on a susceptible or non-susceptible vertebrate (Kurtenbach et al. 2002b; Ogden et al. 1997).

For *B. burgdorferi* s.l., the preference of larval and nymphal stages of ticks for small to medium sized reservoir hosts (mammals, birds or lizards), respectively, is crucial for maintaining persistent infection of the bacteria in their natural transmission cycles (Mannelli et al. 2012). Small mammals are considered the primary reservoirs of the pathogen (Gern et al. 1998; Hanincová et al. 2003; Humair & Gern 1998; Hofmeester et al. 2016; Ostfeld et al. 2014). Nevertheless, birds as reservoir hosts play a significant role in the spread of the pathogen within distant habitats, based on their migratory pathways (Hanincová et al. 2003; Kurtenbach et al. 2006; Piesman, & Schwan 2010; Tarageľová et al. 2008). Other common reservoir hosts for *B. burgdorferi* s.l. are hedgehogs, squirrels, and lizards (Gern et al. 1997; Majláthová et al. 2006; Pisanu et al. 2014). The adult stage of tick usually feed on larger mammals which are incompetent to support the survival of the spirochetes. Nonetheless, large mammals such as deer are essential for maintenance of tick population due to the fact that adult ticks mate on the vertebrates. (Brisson et al. 2012; Mannelli et al. 2012; Piesman, & Schwan 2010, Telford et al. 1988).

B. burgdorferi s.l is mostly transmitted to the mammalian host through a nymphal stage of an *Ixodes* tick. Depending on the genospecies of *Borrelia*, transmission of the pathogen to humans is possible, while it is considered a dead-end host, unlikely to continue the transmission of the bacterium to other hosts (Brisson et al. 2012; Radolf et al. 2012). A nymphal stage is considered to be responsible for majority of infection to humans, owing to the fact that the size of nymphs causes difficult and almost impossible detection on the skin of the human host (Stańczak et al. 1999; Vassallo & Pérez-Eid 2002; Wilhelmsson et al. 2013). There is no evidence whether infected humans may establish transmission of *Borrelia* to feeding tick.

If the tick attached to the skin is removed within 24 hours, the maintenance of the pathogen in the human host is considered to be low (Cook 2014; des Vignes et al. 2001; Eisen 2018; Hojgaard et al. 2008; Hofhuis et al. 2017). There are several views on the transmission of the spirochetes from an ixodid tick to the host. One hypothesis is considering the possibility of the pathogen to be transmitted into the skin of the host through the tick

saliva as it replicates enough due to reactivation caused by the new blood energy source from the host as the thick feeds (Coburn et al. 2021; Ribeiro et al. 1987). A recent study (Pospisilova et al. 2019) of transmission cycle of *B. afzelii* supports another hypothesis (Benach et al. 1987), suggesting a transmission of spirochetes from the gut via mouth parts of the ixodid tick by a regurgitation process, whereas salivary glands are not found to be important in the infectivity of the pathogen. However, the risk of infection increases significantly within 72 hours of tick attachment to the human skin (Cook 2014; Eisen 2018; Hojgaard et al. 2008; Tilly et al. 2008; Zhou et al. 2021).

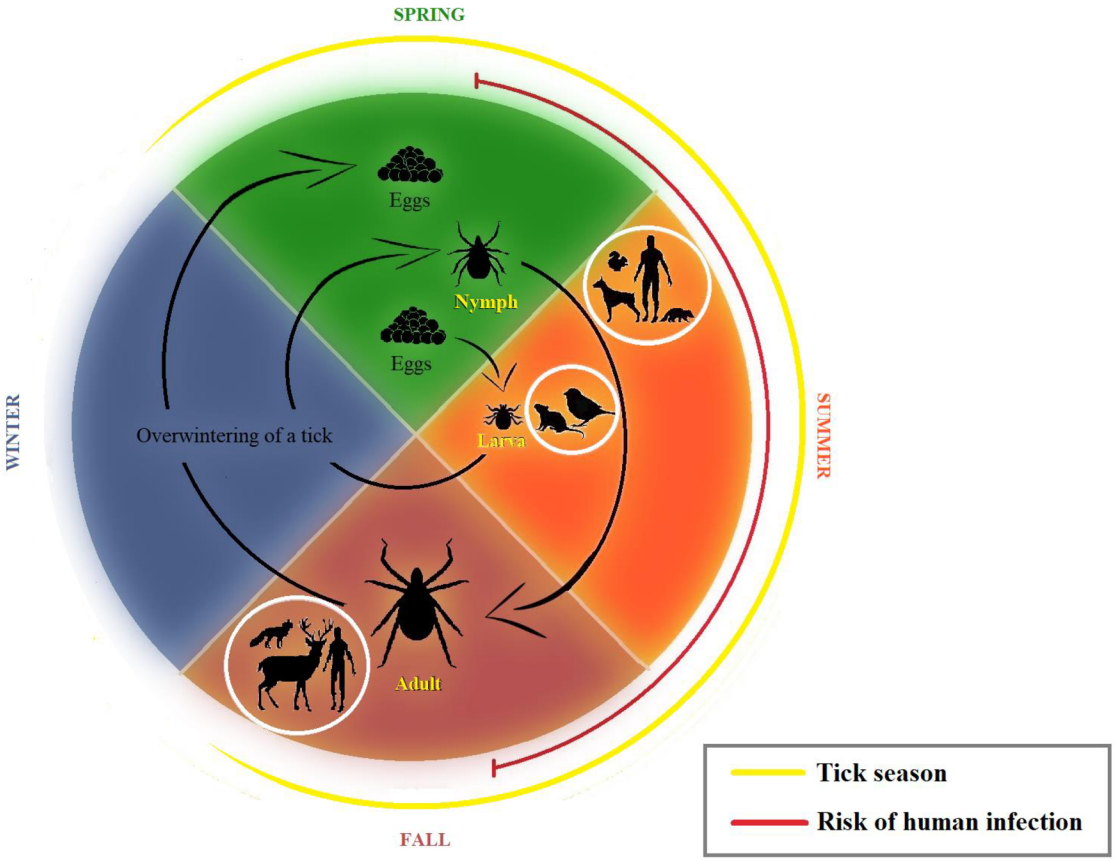


Fig. 1. Brief scheme of life cycle of *Ixodes* ticks in association with their hosts and appearance during individual seasons of the year (yellow line). White circles display vertebrate hosts of individual stages of the ticks. Red line represents the greatest risk of human infection (created in GIMP version 2.10.30).

The ecology of *B. miyamotoi*, belonging to the relapsing fever group of spirochetes, is poorly known, thus it is subject of observation. Various species of rodents and birds have been detected as reservoir hosts (Barbour et al. 2009; Hamer et al. 2012; Scoles et al. 2001).

The study from Barbour et al. (2009) analysed tissues and blood of captured mice (*Peromyscus leucopus*) in eastern Connecticut. The prevalence and abundance of *B. burgdorferi* s.l. and *B. miyamotoi* of infected reservoir hosts was evaluated. The study resulted in higher prevalence of *B. burgdorferi* in the blood (12 %) and skin tissues (76 %) of mice in compare to infection of *B. miyamotoi* with the prevalence of 7 % and 2 %, respectively. However, the analysis of blood samples of mice revealed that the densities of *B. miyamotoi* were higher than the densities of *B. burgdorferi* spirochetes. Taking into account that *B. miyamotoi* may be transmitted transovarially, there is a possibility to occur in unfed larvae (Krause et al. 2015; Rollend et al. 2012). The occurrence of *B. burgdorferi* s.l. in blood of host is considered to be accidental (Tilly et al. 2008) in contrast to RFG of *Borrelia* (Brouqui et al. 2005; Wyplosz et al. 2005). The peak of a seasonal curve of transmission may potentially extend in case of possibility of Ixodid larvae to transmit the infection to vertebrate hosts, as it is implied in a report from Japan (Takano et al. 2014).

The ixodid vector is also known to transmit other pathogens, causing infections, such as anaplasmosis, babesiosis, rickettsiosis, and tick-borne encephalitis virus (TBEV) (Ecker et al. 1999; Lindquist 2014; Schorn et al. 2011; Wójcik-Fatla et al. 2016).

2.1.4 Prevalence and distribution of *B. burgdorferi* s.l. and *B. miyamotoi* in Europe

As mentioned before, the complex of *Borrelia burgdorferi* sensu lato includes genospecies known to be the causative agents of LD (Appendix 1) the most prevalent tick-borne disease in the Holarctic region (Mead 2015). As the disease is caused predominantly by *B. afzelii*, *B. garinii*, and B. *burgdorferi sensu stricto* (s.s.) in Europe, this chapter will be focused mostly on these genospecies of the Old World (Margos et al. 2009).

It is estimated that there are over 65 000 new cases of LD yearly in Europe, whereas the highest incidence is reported in Germany, Austria, Slovenia, and Sweden (Schotthoefer & Frost 2015). Many factors contributing to the increase of incidence of tick-borne diseases have been observed. In general, the drivers of spread and changes in distribution of *I. ricinus*

are divided into three categories. Those are factors related to: climatic changes, distribution of tick hosts (mainly cervids), anthropogenic−induced changes, and other ecological changes (Medlock et al. 2013). It is considered that an adaption of ticks to spread from their natural ecosystem to urban and suburban areas may be involved (Akimov & Nebogatkin 2016).

A comprehensive analysis of scientific reports in range from 1991 to 2017 evaluated the prevalence of tick-borne pathogens in field-collected ticks *I. ricinus* from European urban and suburban areas depending on climatic zones (Grochowska et al. 2020). The highest median incidence rate of *B. afzelii*, considered the most prevalent pathogen among the complex of *B. burgdorferi* s.l. in Europe (Grygorczuk et al. 2013; Stanek et al. 2012), was in the cold temperate climate zone (Grochowska et al. 2020). Other frequent pathogens of *B. burgdorferi* s.l., e.g, *B. garinii* and *B. burgdorferi* s.s. were mostly reported from Central and Western Europe.

The meta-analysis of *B. burgdorferi* s.l. prevalence in questing tick *I. ricinus* in Europe from 2010 to 2016 revealed that the mean prevalence of the *B. burgdorferi* complex in *I. ricinus* reached 12,3 % (Strnad et al. 2017). The study supports previous results showing the prevalence in adult ticks was higher than that in nymphs with 14.9 % and 11.8 %, respectively (Hubálek & Halouzka 1998; Rauter & Hartung 2005). An association was found between the prevalence of *B. burgdorferi* s.l. in ticks and the specific European areas. The results showed increasing prevalence of infected adults and nymphal ticks from west to the east of Europe (Rauter & Hartung 2005; Strnad et al. 2017). The geographical trend is considered to be associated with the high prevalence of ixodid ticks in Central Europe. Moreover, the results of prevalence from northern-limited distribution area of ixodid ticks indicate significantly higher values in compare to the rest of European locations (Strnad et al. 2017). According to the data, the most frequently transmitted pathogen is *B. afzelii*, followed by *B. garinii*, *B. valaisiana*, and B. *burgdorferi* s.s. (Strnad et al. 2017).

Several studies including the prevalence data of *B. miyamotoi* in ixodid ticks in Europe and the U.S., indicate three distinct genotypes based upon their geographic distribution and vector-specifity (Barbour et al. 2009; Geller et al. 2012; Mun et al. 2006; Platonov et al. 2011; Scott et al. 2010). These genetic groups differ in variations within the genographic groups. There was observed no genotypic difference between the B. *miyamotoi* found in *I. scapularis* and *I. pacificus* from distant location of range from eastern to midwest US and

the state of California, respectively (Crowder et al. 2014; Mun et al. 2006). Nonetheless, an analysis of sequenced gene of the 16S rRNA of *B. miyamotoi* strains collected in Europe and the United States showed affinity to occur in different clusters.

Moreover, strains of *B. miyamotoi* within the Old World differed in associaton with geographically distinct areas. For instance, the Japanese strain FR64b differed from isolates found in *I. ricinus* in Europe, whereas *B. miyamotoi* isolates of ticks from Russia grouped with European isolates (Platonov et al. 2011).

Studies involving data related to *B. miyamotoi* indicate low prevalence of the pathogen in host-seeking ticks. In Europe, the prevalence of *B. miyamotoi* is approximately 2 % in questing ticks (Crowder et al. 2014; Geller et al. 2012).

According to a comprehensive study of Grochowska et al. (2020) mentioned above *Borrelia miyamotoi* did not reveal significant differences of prevalence in individual climate zones of Europe.

2.1.5 Pathogenity and therapy of *B. burgdorferi* s.l. − focused on Lyme borreliosis

Lyme disease (LD), or Lyme borreliosis (LB) is a multisystemic disorder caused by several genospecies from the *Borrelia burgdorferi* s.l. complex with approximately 85 000 and 300 000 new cases per year in the Old World (Europa and Asia) and northern America, respectively (Brisson 2012; Hinckley et al. 2014; Seifert et al. 2015; Sykes & Makiello 2017; van den Wijngaard et al. 2017; Wilske 2005). A typical sign (60−80% of cases) of infection is an expanding skin rash reaching up to 30 cm, also known as erythema migrans (EM) (Steere et al. 2003a; Zane et al. 2006). The redness of skin usually occurs approximately a week after the tick bite. It is mostly localised at the site of the tick bite ((Aucott 2015; Schwartz et al. 2017)). Another of the early symptoms may be of neurological origin and manifests as neck pain (Bigi et al. 2020; Hieber et al. 2020). Other early signs (up to 30 days) include nonspecific symptoms such as fatigue, fever and headache. Symptoms mentioned above are characteristic for an early Lyme disease. If left untreated, late Lyme disease may occur, including neck stiffness, additional EMs, facial palsy at one or both sides of face and swelling of joints (mostly the knees). Other severe symptoms include heart palpitations, intermittent and shooting paints, nerve pain and inflammation of the brain and spinal cord. Despite the treatment, up to 20 % of patients

develop joint pain, problems with short-term memory and tiredness. Repeated periods of joint pain and swelling of large joints may appear even years after the first signs of LB (Aucott 2015; Schwartz et al. 2017). Asymptomatic infection may also occur, although the prevalence is low. For instance, the prevalence of asymptomatic cases is less than 7 % in the USA (Biesiada et al. 2012; Steere et al. 2003b).

The clinical features of LB in both continents are very similar, although differing in various aspects due to the greater variety of genospecies in Europe. From five genospecies of *Borrelia burgdorferi* s.l. known to cause LB in Europe (*B. afzelii*, *B. garinii*, *B. spielmanii*, and *B. burgdorferi* sensu stricto (s.s)), *B. spielmanii* is associated only with EM, while *B. afzelii* is the only known pathogen causing the chronic dermatological condition leading to atrophy of the skin, called acrodermatitis chronica atrophicans. Studies suggest that the *B. burgdorferi* sensu stricto can apparently give rise to all of the clinical manifestations of borreliosis, while *B. garinii* and *B. bavariensis* are more often associated with Lyme neuroborreliosis (LNB) (Bazovská et al. 2011; Brisson et al. 2011; Fingerle et al. 2008; Margos et al. 2013; Ogrinc et al. 2013; Ornstein et al. 2002).

Studies indicate that the geographical distribution of individual *Borrelia burgdorferi* s.l. genospecies correlate with the neuroborreliosis and its specific symptoms. Manifestation of LNB occurs more frequently in Europe in comparison to the northern America (Hengge et al. 2003). Among others, another genospecies causing neuroborreliosis is *B. afzelii*. Nonetheless, neurological manifestations of *B. afzelii* result in different and often less specific symptoms in comparison to clinical symptoms of *B. garinii* (Carlsson et al. 2003; Logar et al. 2004). Patients infected with *B. garinii* show a distinct clinical manifestation of painful meningoradiculitis (Bannwarth syndrome) and facial palsy (Ogrinc et al. 2016), while neurological diagnoses of *B. afzelii* infections are generally nonspecific and more difficult to diagnose (Strle et al. 2006).

Two European genospecies of *B. burgdorferi* s.l., i.e., *B. lusitaniae* (Collares-Pereira et al. 2004; de Carvalho et al. 2008) and *B. valaisiana* (Diza et al. 2004), were suspected human pathogens. The role of potential pathogenicity of *B. lusitaniae* is unclear, as the clinical symptoms do not match those of LD (Collares-Pereira et al. 2004). The concept of *B. valaisiana* as human pathogenic is not highly supported. There are only 12 reported cases in 20 years of *B. valaisiana* in human patients suffering from LB clinical manifestations, provided only with DNA evidence of the genospecies (Margos et al. 2017b). *B. valaisiana* is

considered to be unlikely human pathogenic, as the prevalence of the genospecies is highly frequent in *I. ricinus* ticks, known to bite humans (Estrada-Peña et al. 2018). Thus, there should be more evidence of *B. valaisiana* infecting humans.

The genospecies of *B. burgdorferi* s. l. complex found in the Old and the New World, *B. bissettiae*, has been detected in both human patients and questing ticks. However, the pathogenicity of the genospecies remains unclear (Girard et al. 2011; Hulínská et al. 2007; Picken et al. 1996; Rudenko et al. 2008; Strle et al. 1997; Tappe et al. 2014).

Clinical features of U.S patients with *Borrelia burgdorferi* s.s. have shown briefer duration of EM in compare to *B. afzelii* in European patients, although systemic manifestations were more likely to occur in the U.S. patients (Strle et al. 1999).

The genospecies *B. burgdorferi* s.s. present in the U.S. is also associated with major cases of both Lyme arthritis and LNB (Tijsse-Klasen, et al. 2013). Several studies imply that specific genotypes of *B. burgdorferi* s.s in the U.S. vary in their ability to cause LB or dissemination to blood or cerebrospinal fluid (CSF) (Brisson et al. 2011; Wormser & Halperin 2013). For instance, analyses of patients suffering from disseminate disease in endemic areas of the U.S revealed higher prevalence of infection by the RST1 strain in compare to RST2, and RST3 strain (Jones et al. 2006). In addition, blood or CSF dissemination was mostly associated with specific *ospC* genotypes, i.e, A, B, I, and K. These finding are related to ecological and epidemiological aspects of individual subtypes. (Alghaferi et al. 2005; Earnhart et al. 2005; Seinost et al. 1999; Wormser et al. 2008). Nevertheless, *B. burgdorferi* s.s. symptoms in Europe are matching to clinical manifestations caused by *B. afzelii* or *B. garinii* (Cerar et al. 2016).

Infection usually occurs from late spring to early autumn due to the highest activity of the vector of LB (ticks) during these seasons. (Cruz et al. 2013; Darcy et al. 2013).

As mentioned above, antibiotic drugs are the primary choice of LB treatment. When used properly, recurrences are considered to be rare. Nevertheless, reinfections are possible after other tick bites due to no lasting immunity after previous infection (Rauer et al. 2012; Wright et al. 2012). Standard treatment includes oral antibiotics, i.e., doxycycline, amoxicillin or cephalosporins. Patients with specific cardiac or neurological complications may use ceftriaxone or penicillin intravenously. Doxycycline is recommended to be applied in treatment for less than 21 days. In case of intolerance of generally used drugs, they are replaced by one of the following: clarithromycin, erythromycin or macrolides azithromycin.

Nevertheless, alternative antibiotics mentioned above are less reliable in treatment of LB (Hu 2016; Kowalski et al. 2010; Sanchez et al. 2016; Stupica et al. 2012; Wormser et al. 2006).

2.1.6 Pathogenity and therapy of *B. miyamotoi*

The relapsing fever-related organism, *Borrelia miyamotoi*, has been recently shown to be a human pathogen (Hovius et al. 2013; Chowdri et al. 2013; Krause et al. 2013; Platonov et al. 2011). Reports of human cases infected with *B. miyamotoi* have been reported in temperate zones of the Old World, e.g., Asia, Europe, and the New World, eastern North America, specifically (Hovius et al. 2013; Chowdri et al. 2013; Molloy et al. 2015; Platonov et al. 2011; Sato et al. 2014).

The report from Russia indicates that *B. miyamotoi* may cause symptoms similar to the relapsing fever (Platonov et al. 2011). Simultaneously, skin lesions such as erythema migrans associated with infection caused by *B. burgdorferi* s.l. may occur. Reports of clinical cases of *B. miyamotoi* occurred in both healthy and immunocompromised patients in both the Old and the New World (Gugliotta et al. 2013; Hovius et al. 2013; Chowdri et al. 2013; Krause et al. 2013; Platonov et al. 2011). Serological tests used for detection of standard LD are not reliable to identify antibodies against *B. miyamotoi*. Therefore, data extension of the infection rate in ticks of specific regions may help to take into account a possibility of *B. miyamotoi* infection and its related symptoms (Scoles et al. 2001).

The pathogenity of *B. miyamotoi* is associated with meningoencephalitis, which causes an elevation of proteins as a result of cerebrospinal fluid pleocystosis. These symptoms appear in both acute and chronic manifestations of the disease (Boden et al. 2016; Gugliotta et al. 2013; Hovius et al. 2013;).

The most reported clinical symptoms of *B. miyamotoi* occur in acute form. The symptoms of the acute infection with *B. miyamotoi* may include fever and influenza-like disease which can evolve to relapses. Another symptom is the skin rash similar to Erythema migrans of LD. Acute symptoms also include arthralgia, malaise, fatigue, headache, chills, and myalgia (Platonov et al. 2011).

Treatment of clinical manifestations of *B. miyamotoi* infection depends on the specific symptoms. In general, doxycycline is applied in adults. When more severe symptoms occur, such as meningoencephalitis, ceftriaxone or penicillin is used instead (Platonov et al. 2011;

Gugliotta et al. 2013; Krause et al. 2013; Krause et al. 2014; Hovius et al. 2013; Chowdri et al. 2013; Sato et al. 2014).

2.2 Typing methods

Typing methods are vital tools in diagnostics and evaluation of clinical and epidemiological studies, pathogen evolution and population genetics.

2.2.1 Phenotypic typing methods

Traditional typing techniques are based on phenotype. Phenotypic typing methods applied for *Borrelia* speciation include serotyping, which uses the principle of heterogeneity of outer membrane proteins (OsP). Outer surface protein A (OspA) is one of the major membrane lipoproteins of *Borrelia burgdorferi*. Individual serotypes of the protein are linked to various manifestations of Lyme borreliosis (Hilton et al., 1996; Wilske et al., 1993; Wang et al., 1997). Another marker commonly used in molecular diagnostics is (OspC), a lipoprotein that is the predominant antigen in the early stages of borreliosis and has higher heterogeneity than the OspA protein (Aguero-Rosenfeld et al., 1996; Pavia et al., 1996). See more information about OsP proteins in Appendix 2.

Another phenotypic technique is multi-locus enzyme electrophoresis (MLEE), characterized by the use of different electrophoretic mobilities of intracellular enzymes. This method leads to differentiating of organisms by generating an electrophoretic type (Mallik, 2014; Stanley & Wilson, 2003). Phenotypic approaches, such as OspC typing is mostly used to analyse strains within the same genospecies and for disease associated researches (Seinost et al., 1999; Wormser et al., 2008). Phenotypic methods have been used for several decades. However, they do not always provide optimal discriminatory power (purpose of a study).

2.2.2 Molecular typing methods

Currently, genotypic typing study differences in organisms at the molecular level, specifically in chromosomal and extrachromosomal site of a nucleic acid (Sabat et al., 2013). This diagnostic method has a high discriminative ability and efficiency on a wider range of organisms in comparison to phenotypic approach (Patel & Graham, 2007). Genotypic molecular techniques include: DNA-DNA hybridization, single-stranded conformation polymorphism (SSCP), rRNA restriction (ribotyping), sequencing of rRNA / DNA conserved genes, species-specific polymerase chain reaction (PCR), restriction fragment

length polymorphism (RFLP), pulsed-field gel electrophoresis (PFGE), plasmid fingerprinting, quantitative real-time PCR and melting point analysis, MLST and multilocus sequence analysis (MLSA), and whole genome sequencing (WGS) (Wang et al., 2014). The selection of an appropriate method depends on various factors: aims and objectives of the study, the level of discriminatory power required, origin of the samples (tissue, blood, tick), and laboratory conditions (Dolange et al. 2021).

For instance, diagnostic sensitivity of PCR method may differ according to stage of the disease in human patients and the anatomical location of the manifestation of the disease. PCR diagnostic method of *Borrelia* is regarded to be useful for LA from synovial fluid or tissue, and for ACA from skin biopsy (11-16). Molecular detection of *Borrelia* from blood of patients may be diagnosed by collecting (whole-EDTA) blood samples, extraction of the nucleic acid, and real-time PCR based on the targeted genes (for instance, ospA or flab for *B. burgdorferi* s.l., and flagellin for *B. miyamotoi*) (Geebelen et al. 2022; Hovius et al. 2013). Nevertheless, PCR detection of the pathogen DNA from blood of patients has not been considered significantly successful due to low level of the bacteria in blood circulation (Schutzer et al. 2018; van Dam 2011; Wilske et al. 2007). Identifcation of *Borrelia* from ticks by PCR method is considered to be reliable (Lager et al. 2017; Rodríguez et al. 2015; Ušanović et al. 2021). The approach approximates the probability of infection which, if positive, may lead to preventive treatment of the potential disease (Faulde et al. 2014; Piesman & Hojgaard et al. 2012).

The whole genome-based techniques such as WGS are most frequently used for differentiation of *B. burgdorferi* strains between and within genospecies in relation to borrelial origin and geographical zone (Vitorino et al., 2008; Hoen et al., 2009; Margos et al., 2011; Ogden et al., 2013; Vollmer et al., 2011, 2013).

Our study includes techniques, which are widely used for molecular epidemiology and evolution of *Borrelia* and clinical presentation of LB and its genotypes in relation to borrelial strains. Therefore, the following chapters are primarily focused on these methods, i.g., PCR, RFLP and MLST/ MLSA.

2.2.2.1 PCR-based approach

Within molecular techniques, PCR-based methods are the most widely used to confirm the presence of *B. burgdorferi* (RuŽić-Sabljić & Cerar, 2017). The PCR sensitivity may vary depending on the type of obtained material (blood, tissue, cerebrospinal / synovial fluid), the DNA extraction protocol, the PCR target and PCR approach. PCR techniques used for detection of *Borrelia* infection include: PCR, nested PCR, real time PCR, PCR-RFLP, digital PCR, etc.) (Bonin, 2016; RuŽić-Sabljić & Cerar, 2017; van Dam, 2011). This research used several approaches to identify borrelial pathogen in ticks, therefore the following chapters are dedicated to these methods.

2.2.2.1.1 Touchdown PCR

Touchdown PCR is a method where the amplifying of non-specific sequences may be highly reduced by process of lowering the annealing temperature every additional set of cycles. The specifity of the PCR reaction is increased at higher temperatures around the melting point of the specific primers. By decreasing the temperature of primer annealing, the efficiency of the PCR method ins increased. It is a rapid method to optimize PCR with aim to improve the results by increasing of sensitivity and specifity with no need to redesign the primers (Green & Sambrook 2018; Korbie & Mattick 2008). For instance, the touchdown PCR method may be used for amplification of the 5S–23S rRNA intergenic spacer of *B. burgdorferi* s.l. (Derdáková et al. 2003; Schwarz et al. 2012)

2.2.2.1.2 PCR – RFLP

Detection of the presence of representatives of the *B. burgdorferi* complex by PCR-RFLP is possible by amplification of two intergenic spacer (IGS) rRNA sequences (Fukunaga & Sohnaka, 1992) or the flagellin genes (Wodecka, 2011). There are two sets of rRNA genes in the genome of *B. burgdorferi*. (Fukunaga & Sohnaka, 1992), the 5S-23S (*rrfA-rrlB*) rRNA of the intergenic spacer (Derdáková et al., 2003; Lee et al., 2000) and the 16S-23S rRNA (*rrs-rrlA*) (Liveris et al., 1999; Ranka et al., 2004; Wang et al., 2014).

In the first step of PCR-RFLP, the *rrs-rrlA* or *rrfA-rrlB* intergenic spacer regions of a given RNA segment are amplified by PCR. First, the strands of the RNA template are denatured. Subsequently, target oligonucleotides (primers) are binding to a particular region

of the RNA sequence. The polymerase synthetizes the complementary strand at the point of annealing primers in the 5′ - 3′ direction. The process of each PCR reaction relies on different temperature conditions. To obtain a sufficient amount of amplified DNA, the steps are cyclically repeated in the same order. The second step of PCR-RFLP is to digest the DNA fragments of positive PCR samples by restriction enzymes. In particular, Tru1I or MseI endonucleases are currently used to cleave specific nucleotide sequence sites. The restriction enzymes separate the sequences to form fragments in the same region: 5′....T ↓ TAA .... 3′ and 3′....AAT ↑ T....5′. The length of the fragments is specific to individual genospecies or strains of *Borrelia*. In their phylogenetic development, nucleotide substitutions, deletions and insertions gradually began to appear in the given regions, which resulted in the formation or elimination of cleavage sites of the endonucleases. The separation of the fragments is visualized by agarose or polyacrylamide gel electrophoresis (Postic et al., 1994; Wang et al., 2014).

2.2.2.1.3 Nested-PCR

The nested-PCR molecular method is a modification of simple PCR in order to minimize amplification process of unexpected primer binding sites, leading to non-specific binding in products. The approach may also provide an increase of the product amount. Instead of necessity of culture, the procedure allows direct use of DNA extracted from environmental (vectors, reservoir hosts) and patient samples (Wang et al., 2014).

Unlike a simple PCR consisting of one amplification round with one primer set, nested PCR involves two successive runs of two sets of primers. The target DNA undergoes the first run with the first set of primers. The first-round amplification product (amplicons) is used as a template for a second amplification round with the second set of primers. The primers of the second amplification step bind to the target sequence within the amplified sequence of the first reaction. The products of the first round mostly contain the target sequence and its surrounding sequences, leading to reduction of the amount of nonspecific binding in final products. The second amplification step may significantly enhance sensitivity and specificity of the PCR method. Nevertheless, the contamination of the target product may be increased due to additional manipulation of amplicons. Therefore, it is highly recommended to undergo individual parts of protocol in entirely separate rooms (Carr et al., 2010; van Pelt-Verkuil et al., 2008; Wanger et al., 2017).

2.1.2 MLST and MLSA methods

MLST and subsequent MLSA are reliable and highly discriminating typing approaches, based on amplification and sequence analyses of internal fragments of several housekeeping genes (~ 500 bp) (Urwin & Maiden, 2003). Sequence analysis of multiple genes significantly increases discriminatory power, compared to methods using a single locus sequence analysis. The approach was originally developed by Spratt and Maiden (1998) to improve molecular techniques for epidemiological purposes and to overcome a poor portability of obtained data among laboratories (Enright & Spratt, 1999; Maiden et al., 1998; Urwin & Maiden, 2003). Since then, MLSA has been used for epidemiological analyses, phylogenetic relationships, population and landscape genetic studies of many bacterial genospecies and other haploid organisms (Arvand et al., 2007; Jolley et al., 2000; Kaczmarek et al., 2017; Patiño et al., 2018; Wareing et al., 2003). Data obtained by MLST are placed to a global database for molecular typing and microbial genome diversity PubMLST (PubMLST, n.d.), allowing to share and analyse the data in target studies. The MLST is commonly used to study both interspecies and intraspecies relationships of organisms, thus allowing to study the organisms at the genus level (Bishop et al., 2009).

The MLST analysis of *Borrelia* may include maximum of eight housekeeping loci (*clpA*, *clpX*, *nifS*, *pepX*, *pyrG*, *recG*, *rplB*, and *uvrA*) (Tab. I) localized at the main linear chromosome, which undergo slow evolution and provide a high intraspecies discriminatory power (Gallais et al. 2018; Margos et al. 2008; Urwin & Maiden, 2003).

Tab. I. Housekeeping genes of *Borrrelia* bacteria used for MLST and MLSA.

Locus	Product
clpA	Clp-A protease subunit
clpX	Clp-X protease subunit
nifS	Cysteine desulfurase
pepX	Dipeptidylaminopeptidase
pyrG	CTP synthase
recG	DNA recombinase
rplB	50 S ribosomal protein
uvrA	A- exonuclease ABC subunit

(according to Margos et al. 2008)

The MLST method involves collection of samples, DNA extraction, amplification of targeted genes and sequencing (Fig. 2). The approach requires an analysis of single point mutations of the sequences, since the single base difference represents a new allele. Therefore, the housekeeping loci need to be sequenced in both forward and reverse directions. Based on the quality of forward and reverse sequences, amplicons may be classified as single strains or strains containing mixed infections (Margos et al. 2018b).

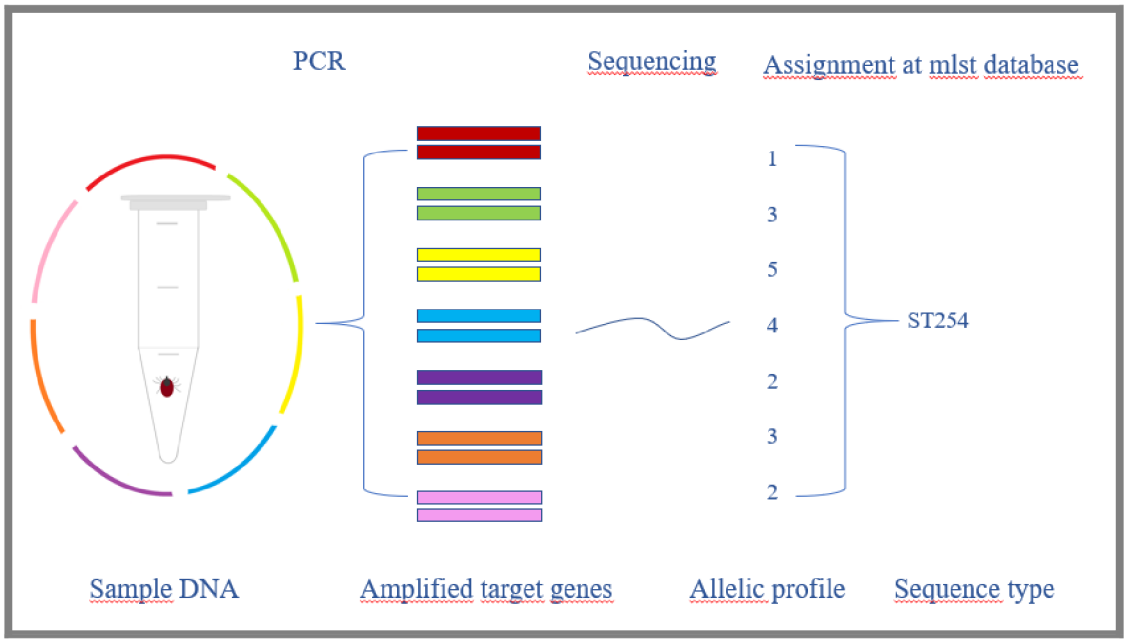
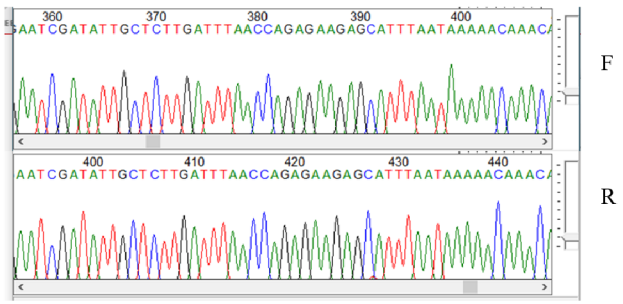


Fig. 2: A scheme showing the procedure of the MLST (created in GIMP version 2.10.30, according to protol of Margos et al. 2018b).

Sequences meeting the required quality (Fig. 3-A) are compared to the data available in the MLST database. Subsequently, these sequences obtain specific allele numbers.

A)



B)

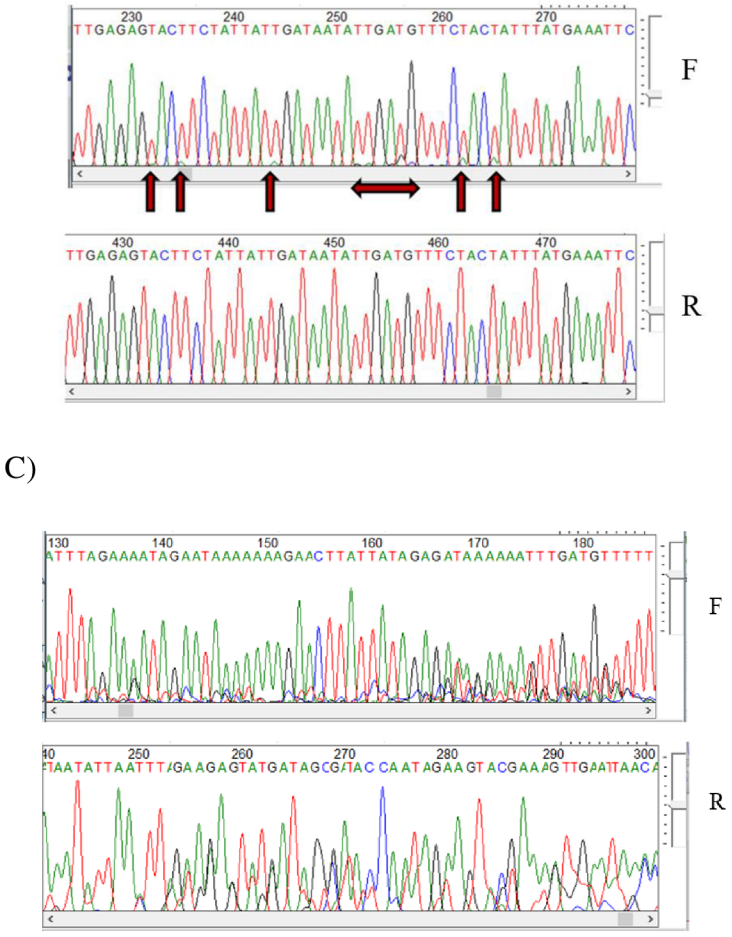


Fig. 3: Examples of sequences of different quality using MEGA-X software (version 10.0.5): (A) The forward and reverse sequence are of sufficient quality. (B) In case of backround in one sequence (arrows), the data may be used as long as the second sequence is good. (C) Sequences reads of bad quality. Such sequences cannot be included in the analysis.
sequence directions: F-forward, R-reverse

In case of successful completion of allelic numbers for all 8 genes of a given isolate, the isolate is characterized by the allelic profile. Subsequently, the allelic profile is attributed to a ST. Isolates consisting of not yet described alleles obtain specific allele numbers, thus they are registered as novel STs to the MLST database. The new sequence types are also assigned to allelic profiles that contain already registered alleles, but in yet unknown combination of allele types. (Margos et al., 2018). The STs substitute a small proportion of the 'conserved' parts of the observed genome. Nonetheless, a big amount of STs of bacteria determine the importance of an expandable means of summarizing and comparing data (Maiden, 2006; Pérez-Losada et al., 2013). Concatenated sequences of the housekeeping genes can be used

in phylogenetic analyses, population genetics, and other fields of study, aiming to observe intraspecies and interspecies relationships. Sequences ought to be investigated using appropriate software (e.g., MEGA-X software, Smartgene Databases, SeqMan Pro) (Margos et al., 2011).

The housekeeping genes chosen for MLST are subject to various criteria. The genes are selected throughout the genome to reduce possible local bias that may appear in the genome. As the adjacent genes may be influenced by strong selection pressure, the surrounding genes should have similar functions as the housekeeping genes. Moreover, genetic diversity of selected genes should be in balance to provide equivalent contribution to target analyses. In other words, there should not appear any gene dominating a phylogenetic tree, thus gives a false result (Margos et al., 2011; Urwin & Maiden, 2003).

In addition, MLST analysis may be developed only among closely related bacteria due to the variability of house-keeping genes among different bacterial organisms. This method alone is not suitable for all typing purposes, such as characterization of single-clone pathogens, reproducing by binary fission, such as *Bacillus anthracis* (Jolley, 2012; Priest et al. 2003). In such case, MLST can provide sufficient results with additional typing methods that determine more variable loci, such as antigen genes (Dingle et al., 2008; Jolley & Maiden, 2007,) or variable-number tandem repeats (VNTRs) (Adair et al., 2000).

The exact geographic origins of clinical isolates of *B. burgdorferi* are often not possible to resolve. Nonetheless, isolates from ticks can be supplemented by geographical information, thus serving as a useful resource for population studies of *Borrelia*. As the cultured isolates may provide an unclear picture toward various strains (Liveris et al., 1999; Norris et al., 1997), direct genotyping of ticks or hosts samples is desirable. Given that the MLST approach includes the amplification of borreliae housekeeping genes, which can be obtained by DNA extraction without prior cultivation of the bacteria, this method is suitable and increasingly popular for complex analyses (Margos et al., 2008).

The study from 2008 revealed that the housekeeping genes of *Borrelia* evolve differently in comparison to widely used IGS (*rrs-rrlA*) and OspC (Margos et al., 2008). Furthermore, the analysis demonstrates the USA and European populations of *B. burgdorferi* correspond to distinct lineages. Previous studies were based on varieties

of OspC (Marti et al., 1997), PCR and pulsed-field gel electrophoresis (PFGE) (Foretz et al., 1997). According to analysed data by MLSA, it is suggested that the common ancestor of *B. burgdorferi* evolved in Europe rather than in North America as previously proposed. As the housekeeping genes are considered to evolve slowly, the individual populations of *B. burgdorferi* have been probably separated for a long time (i.e., probably millions of years) (Margos et al., 2008).

The MLST method contributed to revealing of significant inter-specific and intra-specific genetic differences. The approach led to characterization of new genospecies *B. burgdorferi* sensu lato, such as *B. bavariensis* (Margos et al., 2009). This genospecies has previously been identified as serotype OspA 4, belonging to the genospecies *B. garinii* (a bird-associated ecotype). As the new genospecies differs from *B. garinii* by its affinity for rodents as reservoir hosts, it falls into a different ecotype. Furthermore, in a phylogenetic tree of MLSA, the OspA 4 emerged at the base of the *B. garinii* clade. Therefore, it is considered that the specialization to rodents is a more ancient trait than affinity to birds as reservoir hosts (Margos et al., 2009).

The genospecies *B. kurtenbachii*, formerly known as strain type 25015T was part of the genospecies *B. bissettiae*. (Anderson et al., 1990; Postic et al., 1998). The genetic diversity and ecology of the strain, originally found in Illinois and New York, the USA, was so high that this strain was re-qualified as a separate genospecies (Margos et al., 2010). Vectors of *B. bissettiae* include *I. pacificus*, *I. spinipalpis* and *I. affinis* in comparison to *B. kurtenbachii*, so far detected only in *I. scapularis* vector. This fact is important from an epidemiological point of view, as there are more frequent cases of *I. scapularis* tick bites in humans than of vectors of *B. bissettiae*. Even though the genetic similarity to *B. kurtenbachii* has been identified in clinical samples in past (Picken et al., 1996), there is no evidence of the genospecies in recent clinical studies, thus the pathogenicity of the genospecies remains unknown (Margos et al., 2014).

The above-mentioned studies were based on amplification and subsequent analysis of eight "house-keeping" genes. Identification of a new genospecies *B. spielmanii*, formerly known as *'Borrielia spielmani'* (Richter et al., 2004) has been confirmed by analysis of seven loci: *rrs, hbb, groEL, recA, fla, ospA* and *rrf-rrl* intergenic spacer (Richter et al., 2006).

Another genospecies, *B. californiensis* (Postic et al., 2007) has also been classified by using this method.

The study of Park et al. (2018) is the first report of *Borrelia yangtzensis* by MLST in South Korea in spite of the fact that the isolate was firstly classified as *B. valaisana* by analyses of OspA and 5S-23S IGS. The genospecies has previously been reported in China and Japan (Bacon et al., 2008; Chu et al., 2008; Kawabata et al., 2013; Masuzawa et al., 2001). It is considered that *B. yangtzensis* observed in this study will be more closely related to Chinese strains than Japanese strains.

Another MLST study revealed in 2017 the first documentation of *B. bavariensis* outside Eurasia. Moreover, it is the first evidence of the genospecies in *I. uriae* and its associated seabird hosts. Samples of ticks were collected from seabird colonies of Newfoundland and Labrador, Canada, during three calendar years. Previously known as a rodent-specific *Borrelia* ecotype, *B. bavariensis* has been associated only with *I. persulcatus* and *I. ricinus* ticks (Munro et al., 2017).

In general, most MLST studies among Europe have focused on pathogenicity at the inter-species level or have compared the isolates with the American *B. burgdorferi* strains. The comparative analysis of *B. afzelii* (the predominant genospecies in Europe) and *B. burgdorferi* sensu stricto (s.s.) (located in Europe and the USA) revealed an overlap of clinical samples of the genospecies among Europe and the USA. Nonetheless, there was no overlap of the populations isolated from ticks. *Borrelia* STs identified in Europe and the USA may vary in clinical symptomatics. The European strains of *B. burgdorferi* s.s. are more frequently associated with neuroborreliosis than the USA related strains. Further results showed that *B. afzelii* caused disseminated infection to a significantly lesser extent than *B. burgdorferi* s.s. from both Europe and the USA. (Jungnick et al., 2015). The fact that there is a diversity in clinical symptomatics of various genotypes of *Borrelia* from Europe and the USA associated with inflammatory levels was supported by research of Cerar et al. (2016).

A correlation was found among phylogeny and pathogenicity of *B. burgdorferi* s.s. strains isolated from human patients of New York and Wisconsin. The study characterized 146 strains of *B. burgdorferi* s.s divided into various clonal complexes with different

dissemination properties. Several complexes were related to hematogenous dissemination, while others were associated with localized skin infection. The data suggest that MLST is better method to predict the implications of the types of infection than OspC typing techniques (Hanincová et al., 2013).

The results are supported by other recent study, indicating the pathogenic properties of *B. burgdorferi* s.l. in correlation with the phylogenetic signal (Copain et al., 2016). Pathogenicity of samples of LD patients across the Europe and *I. ricinus* samples from the Netherlands have been investigated at the intra-species level. Six STs (associated with *B. afzelii*, *B. bavariensis*) and five IGS haplotypes were associated with the LD in humans.

Using a sequence-based approach, the research on genetic diversity and phylogeny of *B. afzelii* strains of Europe also indicates different dissemination properties of LB in humans among various *Borrelia* genospecies. Despite the fact that the MLST profiles comparison of the study showed low genetic differentiation among clinical *B. afzelii* strains in Europe, two STs were showing significant correlation with clinical symptoms, such as erythema migrans, whereas another ST was more frequently identified in strains related to disseminated manifestations, mainly neuroborreliosis (Gallais et al., 2018).

In extensive study of Mukhacheva & Kovalev (2013) had been analyzed tick isolates of almost all regions of Russia and 16 new STs were identified by MLST approach. The study revealed that *B. afzelii* isolates of this research are of Asian origin. Moreover, the *B. afzelii* STs indicated low territorial mixing. STs of *B. bavariensis* and *B. garinii* had a random distribution among different areas. According to MLST, the previously identified genomic groups of *B. garinii* have been re-classified to exact genospecies, including a genomic group NT29 (ribotype, formerly known as Asia-type of *B. garinii* (Postic et al. 1997)) corresponding to *B. bavariensis*.

Phylogeographic analysis of *B. lusitaniae* in Portugal showed forming of genetically different populations of two regions at a distance of 130 km. On the contrary, no significant phylogeographic variants were found based on the ospA and ospC analyses (Vitorino et al. 2008).

A new genospecies, *B. mayonii*, has been detected in 2016, when genetic-distance analysis of formerly known type strains MN14-1420 and MN14-1539 was performed using MLST data of the isolates gathered from human patients (Pritt et al. 2016). The genetic-distance analysis showed the genospecies belongs to the *B. burgdorferi* s.l. complex with a 94.7-94.9 % similarity to *B. burgdorferi* s.s. (Pritt et al. 2016).

In case of *B. valaisiana*, two new genospecies were delineated by MLSA, namely, B. *californiensis*, and B. *yangtze* (Margos et al. 2015b; Postic et al .2007).

2.1.3 Population-genetic analysis using PHYLOViZ 2

The PHYLOViZ 2 software is widely used for population and epidemiological studies as it allows processing various data sources and visualize the potential evolutionary relationships among isolates (Francisco et al. 2012). The software provides the ability to analyse data obtained by different molecular methods and allelic profile data, such as MLST (Maiden et al. 1998), multilocus variable number of tandem repeats analysis (MLVA) (Lindstedt 2005), and single nucleotide polymorphism (SNP) (Ribeiro-Gonçalves et al. 2016; Wang et al. 1998). Specific sequence (typing) or auxiliary (isolate) data including geographic, demographic or clinical details of patients can be integrated. Data sets may be directly retrieved from online databases within PHYLOViZ (in Excel or FASTA format), e.g., PubMLST (PubMLST, n.d.) or Pasteur MLST Databases (Institut Pasteur MLST databases and software, n.d.).

In case of MLST approach, a specific number is assigned to each unique sequence (allele) and to individual combinations of integers (the sequence type (ST)). Weakly related or unrelated STs include few to none identical alleles. On the other hand, STs comprising of identical alleles, excluding one or two, are considered to be closely related (Cooper & Feil 2004).

Once both isolate and typing data are loaded, the content may be displayed in a table or a tree view. Then the goeBURST algorithm (globally optimized implementation of the eBURST algorithm (Feil et al. 2004) and an expansion of goeBURST algorithm, the Minimum Spanning Tree (MST) approach may be applied to infer the possible evolutionary relationships between individual isolates (allelic profiles) (Francisco et al. 2009). The algorithm is a graphic matroid approach used for multilocus typing techniques generating

data which can be interpreted by sequence of numbers or characters. An analysis of input data by goeBURST algorithm generates an unrooted tree, based on differences of the allelic profiles. The goeBURST rules are extended to any number of loci to create a single tree. The algorithm is processing all the possible MST-like trees resulting in identification of one suitable tree which meets the evolutionary model supporting the rules of the goeBURST approach (Francisco et al. 2012).

Individual isolates are categorised into clonal complexes (CCs), based on sharing at least one allele or singletons (not related STs to any other (SG)) (Turner & Feil 2007). Subsequently, existing links can be explored. CCs are characterised as follows: individual genotypes may increase their number in the population for various reasons such as random genetic drift or a fitness advantage. Such genotypes become founder clones in the population, followed by genetic changes, as a consequence of mutation and recombination. The diversification of the genotypes is resulting in formation of clusters comprising of phylogenetically related strains. STs consisting of identical housekeeping gene set as the founding genotype, except one housekeeping gene sequence, are classified as single locus Francisco et al. 2009). The goeBURST algorithmic implementation is providing optimal resolution for the layout of links between STs. All potential links are enumerated using the Kruskal algorithm (Francisco et al. 2012; Kruskal et al. 1956).

In case of MLST analysis, the connections are categorised based on different number of locus variants. The software provides any possible distance to compare those links. Thus, there is a function to compare each connection by implementation of any distance option and calculation of a level for each link (Francisco et al. 2012).

The MST is using different distance metrics resulting in visualization of the individual STs formed into various groups at any linkage level. The tree is using the Prim algorithm (Prim et al. 1957) to avoid computation of all links, growing quadratically with the number of nodes. The requirement of the Prim algorithm is to generate as many connections as the number of nodes. Another implementation is an incremental addition of nodes to the force directed display according to a Breadth First Search approach. The usage of these extensions and the optimization of the MST visualization makes the approach more scalable for large data sets (including thousands of nodes). The analysis also allows to display isolates formed at SLV level which is the standard result of goeBURST approach (Francisco et al. 2012).

The PHYLOViZ software is able to process large number of loci and establish connection of isolates at any linkage level. Therefore, it is especially useful not only for MLST isolates, but also for data of MLVA or SNP method. In addition, using the same approach (or algorithms) to various typing techniques mentioned above prevents anomalies or dissimilarities as a result of applying the same primary evolutionary models and premises. Therefore, the algorithms used in PHYLOViZ software may provide more transparent comparison of results of various molecular typing methods (Francisco et al. 2012).

3. AIMS OF STUDY

1. Detection and evaluation of the prevalence of *B. burgdorferi* s.l. and *B. miyamotoi* in *Ixodes ricinus* ticks collected in 2017 from four main localities (additional locality in case of *B. miyamotoi* is Drienovec) across the Slovak Republic.

2. Analysis of the samples collected in 2017 from Slovak Republic by multilocus sequence typing (MLST) method and submitting the obtained data to the worldwide MLST database (PubMLST).

3. Population-genetic analysis of obtained data of *B. burgdorferi* s.l. and *B. miyamotoi* of this study in comparison to samples obtained from patients and questing ticks already deposited in the MLST database, mainly in range of Central and Eastern Europe. Observation of STs of individual genospecies and their possible associations with the symptoms of Lyme disease will be observed.

4 MATERIAL AND METHODS

4.1 Collection of material

Ixodes ricinus ticks were collected by the drag sampling method according to Falco & Fish (1992). The material was obtained using a white corduroy flag of 1 x 1 meter. Sampling of ticks took at least 1 hour at each locality. The ticks were stored in 70 % ethanol until further analyses.

Ticks were collected at turn of April/ May, May/June, and October 2017 at following regions of Slovakia (Fig.4): Košice (an urban area, 48° 44' 47.140" N, 21° 16' 50.31" E), Malacky (an urban park "Zámocký park" 48° 26' 18.37" N, 17° 01' 50.83" E), Vrbovce (a rural area, 48° 48' 52.59" N, 17° 27' 56.36" E) and Bratislava (a suburban forest park "Železná studnička", 48° 10' 40.27" N, 17° 4' 24.35" E).

Additional material of questing ticks and ticks collected from birds was provided by R. Václav as the tick samples contained DNA of *B. miyamotoi* which prevalence is generally considered to be low. The material was obtained at Drienovec study site in April and June 2017 (woody wetland and forest-meadow ecotones, 48° 36' 56.02" N, 20° 54' 54.69" E). The majority of a tick collection of Drienovec (excluding *B. miyamotoi*) was analysed in different study (Mtierová et al. 2020). Therefore, data of the study will predominantly be separated from the data we obtained.

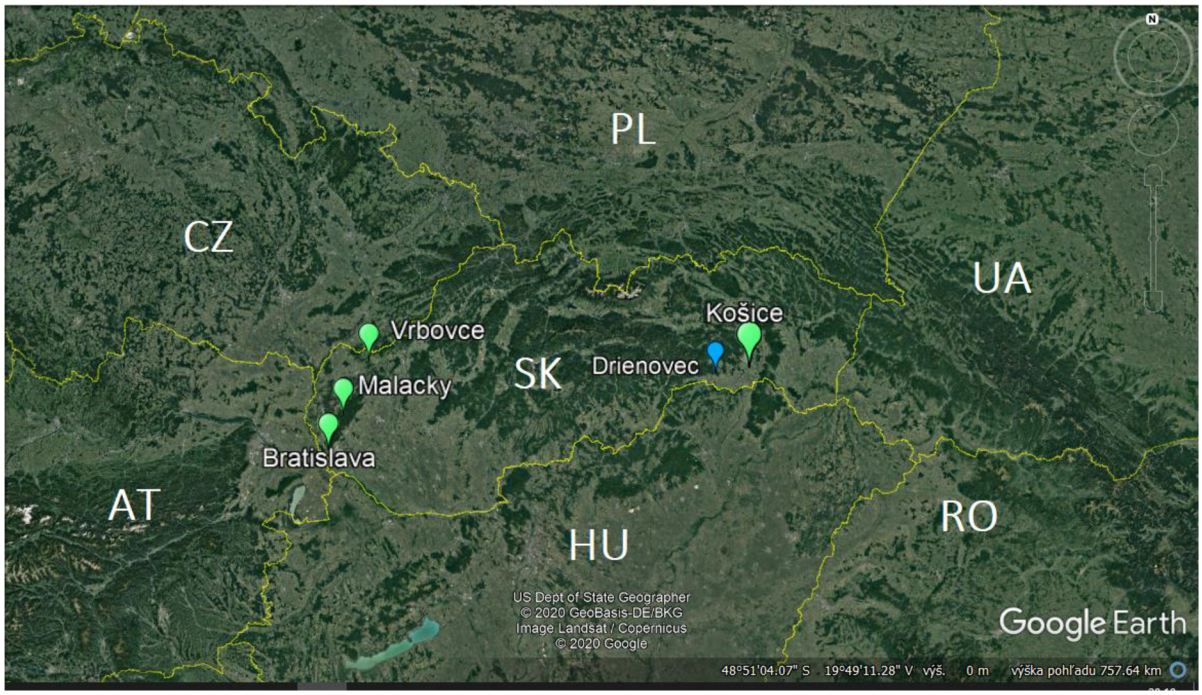


Fig. 4: Study sites of Slovakia 2017: Bratislava, Malacky, Vrbovce, Košice and Drienovec (*B. miyamotoi* tick samples). AT – Austria, CZ – Czech Republic, HU – Hungary, PL – Poland, SK – Slovakia, RO – Romania, UA – Ukraine (edited in GoogleEarth version 7.3.3.7673)

4.2 DNA extraction

Isolation of the DNA from the collected ticks was performed by alkaline hydrolysis in a laminar box (HELAGO, Slovakia) according to Guy & Stanek (1991). A 1.25 % ammonium hydroxide solution was prepared by diluting ammonia (Slavus, Slovakia) with MilliQ water. Collected ticks were dried on filter paper prior to DNA extraction. Individual ticks were identified and divided into groups based on their developmental stage. Subsequently, ticks were individually placed into 1.5 ml Safelock tubes (Eppendorf, Germany) containing a specific volume of 1.25 % ammonium hydroxide. A volume of 500 µl of ammonium hydroxide was used for imagoes and 250 µl for the nymphs. Individual ticks were cut into small pieces with a disposable sterile scalpel (Sarstedt, Germany) and homogenized in the solution of ammonium hydroxide. Locked homogenizates of ticks were incubated for 30 minutes at 100 ° C in thermostat (Stuart, UK). Samples were incubated for

additional 15−20 minutes at the same temperature with the lids open until half of the initial volume had evaporated. The genomic DNA was stored at 4 °C.

4.3 Detection of *I. ricinus* DNA

To confirm successful DNA extraction, a fragment of cytochrome *b* mitochondrial gene (see Tab. II) with specificity for *I. ricinus* DNA was amplified by a PCR method (Black & Roehrdanz, 1998). The amplification reaction mixture was prepared in a sterile PCR cabinet (Bioair Instruments Aura PCR, Italy) using the Bioron Superhot Master Mix Kit (Bioron, Germany). Reaction mix contained 12.5 µl MasterMix (1X), 1 µl $MgCl_2$ (1mM), 0.5 µl forward primer (200 nM), 0.5 µl reverse primer (200 nM), 5.5 µl nuclease free water (NFW). 5 µl of the DNA template was added to 20 µl of the reaction mixture. As a positive control, a DNA template positive for the presence of *I. ricinus* DNA was used.

Two negative controls were used within the PCR reaction. 5 µl of NFW was added to the non-template mixture instead of the DNA template. The first negative control was prepared in a PCR cabinet where the reaction mixture was prepared. A second negative control was prepared outside the sterile box in a separate room used for the addition of the DNA template.

DNA amplification was performed in MJ mini personal thermal cycler (Bio-Rad, California, USA) with program settings according to the Tab. III.

Tab. II. Oligonucleotide primers sequences used for detection of the *cytochrome b* gene.

Type of oligonucleotide	**Direction**	**5′− 3′ primer sequence**	**Length [bp]**
Cytb1	Forward	TTTTAGCAATAAACTTTTCAAG	620
Cytb2	Reverse	AATAAAAAATATCATTCTGG	

Tab III. PCR settings for the cytochrome b amplification.

	Temperature [°C]	Time [min]	Number of cycles
Initialisation	95	5:00	
Denaturation	94	0:15	36x
Annealing	57	0:40	
Extention	72	0:30	
Final extention	72	5:00	
Hold	4	∞	

4.4 Detection of *B. burgdorferi* s.l. DNA

4.4.1 Touchdown PCR

Touchdown PCR amplification of the *5S–23S* rRNA intergenic spacer (length: 250-300 bp) of *B. burgdorferi* s.l. according to protocol of Derdáková et al. (2003) was applied for positive samples of *I. ricinus* DNA from previous analysis. The reaction mixture contained following ingredients, using PCR kit HotStarTaq Plus DNA polymerase (Qiagen, Germany): 2.5 µl CoralLoad PCR Buffer (1X), 1µl $MgCl_2$ (1 mM), 0.5 µl dNTP (200 µM), 0.125 µl Taq polymerase (5U/ µl), 0.5 µl of each primer (200 nM) (see Tab. IV) and 14.875 µl MiliQ water. DNA template of 5 µl was added to the 20 µl PCR mix. Nuclease-free water (NFW) (Qiagen, Germany) was used for negative control. DNA from *Borrelia* was used for positive control.

Tab. IV. Primer sequences used for detection of *B. burgdorferi* s.l. DNA.

Name of oligonucleotid	Direction	5´– 3´ primer sequence
IGSa	Forward	CGACCTTCTTCGCCTTAAAGC
IGSb	Reverse	AGCTCTTATTCGCTGATGGTA

The PCR reaction was performed in MJ mini personal thermal cycler (Bio-Rad, California, USA) with the settings shown in Tab V.

Tab. V. Touchdown PCR settings for *B. burgdorferi* s.l. gene amplification.

	Temperature [°C]	Time [min]	Number of cycles
Initialisation	95	05:00	
Denaturation	94	00:15	6 x
Annealing	61 (-0.2 °C/cycle)	00:25	
Extention	72	00:30	
Denaturation	94	00:15	6 x
Annealing	60 (-0.4 °C/cycle)	00:25	
Extention	72	00:30	
Denaturation	94	00:15	30 x
Annealing	58	00:25	
Extention	72	00:30	
Final extension	72	05:00	
Hold	4	∞	

4.4.2 Agarose gel electrophoresis

Results of PCR reactions were evaluated by 1.5 % agarose gel electrophoresis, using GoodView (SBS genetech, China) for separation and size determination of the PCR products. 5 µl of each product was loaded on the gel and run at 120 V for 35 in 1X TAE solution minutes using Bio-Rad PowerPac^{TM} Basic (USA, California). Size of the amplified product was determined according to the 100 bp DNA ladder (ThermoFisher Scientific, Massachusetts, USA). Results were visualized by UV trans-illuminator (Vilber Lourmant, Collégien, France), at a wavelength of 312 nm.

4.4.3 Restriction fragment length polymorphism (RFLP) analysis

Positive samples were classified to genospecies of *B. burgdorferi* s.l. and relapsing fever group of *Borrelia* based on protocol of Derdáková et al. (2003) and Ondrisková (2012). Restriction enzyme *TruI* (*MseI* isoschizomer, 5U/µl) was applied to cleave amplified PCR products of 5S – 23S rRNA intergenic spacer. Restriction enzyme cleavage sites were 5'...TᵛT AA...3' and 3'...AA T^T...5'. Two reagents were added to the remaining PCR product volume (13 µl): 1.5 µl FastDigest buffer (10X, Thermo Fisher Scientific, USA,

Massachusetts) and 0.5 µl fast digestive enzyme *TruI/MseI* (5U/µl, *Tru1*, Fast Digest, ThermoFisher Scientific, Massachusetts, USA). Samples were centrifuged for 1 min. at 12000 rpm (HETTICH, Nemecko). Samples were incubated for 5 min. at 65 °C (Bio TDB-100, Biosan, Latvia) when *TruI* was applied. The *MseI* treated samples were heated for 5 min. at 37 °C, followed by incubation for 5 min. at 65 °C. Products were centrifuged for 30 sec. at 12000 rpm.

4.4.4 Polyacrylamide gel electrophoresis (PAGE)

Products of RFLP analysis were visualized by electrophoretic separation (Ondrisková 2012). Ultra Low Range DNA ladder (ThermoFisher Scientific, USA) was used to read the length of products fragments. For the separation of rRNA fragments was used Spreadex® Ready-to-Use Gel Wide Mini (Spreadex® EL 300, Elchrom Scientific, Switzerland) in 1X TAE. Electrophoresis was run at 90 V for 150 min. (Origins, Elchrom Scientific, Switzerland). Subsequently, the gel was transferred to 225 ml TAE (1X) stained with 45 µl DNA SYBRGreen I Nucleic Acid Gel Stain (ThermoFisher Scientific, Massachusetts, USA). The gel was stained on a shaker (OS-10, Biosan, Latvia) in the dark for 45 min at 70 rpm. Results were visualized with UV-transilluminator (Vilber Lourmant, France) at a wavelength 312 nm.

4.5 Detection of *B. miyamotoi* DNA

4.5.1 Real-Time PCR

Samples of *I. ricinus* DNA (Section 4.3) were analysed by real-time PCR amplification of the *16S* rRNA gene (length: 1256 bp) for presence of *B. miyamotoi* according to protocol of Platonov et al. (2011). The ingredients of the reaction mixture were following: 12.5 µl Super Hot Master mix (Bioron, Germany), 0.625 µl MgCl2, 1.8 µl of each primer (see Tab. VI), 0.5 µl dye-labeled probe (see Tab. VI), and 2.775 µl of MiliQ water. 5 µl of DNA template was added to the 20 µl PCR mix. Each real-time PCR run included negative (NFW) (Qiagen, Germany) and positive (DNA from *Borrelia*) control.

Tab. VI. Primer sequences used for detection of *B. miyamotoi* DNA and the correnspondingdye-labeled probe.

Name of primer	Direction	5´– 3´ primer sequence
Bmp41	Forward	TTGCTTGTGCAATCATAGCC
Bmp41R	Reverse	GCAAATCTTGGTGCTTTTCAA

Dye-labeled probe	Cy5-AGATGCCACAATTTCATCTGTCATTA-BBQ-650-3

The PCR reaction was performed in CFX96™ Real-Time System (Bio-Rad, California, USA) with the settings shown in Tab VII.

Tab. VII. Settings of real-time PCR of *B. miyamotoi 16S* rRNA gene amplification.

	Temperature [°C]	Time [min]	Number of cycles
Initialisation	95	03:00	
Denaturation	95	00:20	10 x
Annealing	67	00:50	
Extention	72	00:20	
Denaturation	95	00:20	40 x
Annealing	60	00:50	
Extention	72	00:20	

4.6 Statistical analysis

For samples for which *B. burgdorferi* s.l. genospecies were not accurately identified by RFLP, identification was further specified by MLST. Subsequently, prevalences were evaluated. The associations between prevalences and distribution of individual genospecies at each sampling area were tested by a chi-square analysis. Data on the prevalence of individual species from the four collection sites were used to evaluate the above-mentioned relationship. Analytical software, Quantitative Parasitology on the Web (QPweb), was used to analyse the data (Reiczigel et al. 2019). $P < 0.05$ was considered a statistically significant difference.

4.7 MLST

Samples positive for *B. burgdorferi* s.l. (based on RFLP) and *B. miyamotoi* (based on real Time PCR) were subjected to MLST and MLSA, according to the modified protocol of Margos et al. (2010).

4.7.1 PCR

The number of studied samples of *B. burgdorferi* s.l. and *B. miyamotoi* was gradually reduced, based on unsuccessful amplification of individual genes or non-standard results of sequences.

4.7.1.1 Nested–PCR of eight housekeeping genes of *B. burgdorferi* s.l.

The specifity and sensitivity of the gene amplification was increased by using nested and semi–nested PCR method (Wang et al. 2014). The amplified loci included: *clpA, clpX, nifS, pepX, pyrG, recG, rplB* and *uvrA*. The PCR kit HotStarTaq Plus DNA polymerase (Qiagen, Germany) was used for the amplification of targets. The first round of PCR included following reagents (per sample): 2.5 µl CoralLoad Buffer (10X), 1µl $MgCl_2$ (25 mM), 1 µl dNTP (10 µM), 0.15 µl Taq polymerase (5U/ µl), 1 µl outer forward primer (10 pmol/ µl), 1 µl outer reverse primer (10 pmol/ µl), 15.85 µl NFW. 2.5 µl template DNA (~25ng/ µl) was added to 22.5 µl reaction mixture in a separate room (n°2). In the second round of PCR, the reaction volume was adjusted to 30 µl and the reagent ratio was as follows: 3 µl CoralLoad Buffer (10X), 1.5 µl $MgCl_2$ (25 mM), 1.5 µl dNTP (10 µM), 0.27 µl Taq polymerase (5U/ µl), 1.5 µl each inner primer (10 pmol/ µl), 17.73 µl NFW and 3 µl of the PCR product from the first round. Master mixes for both PCR rounds were prepared in the same room (n°1) in the sterile PCR cabinet (Bioair Instruments Aura PCR, Italy). The first round PCR product was added to the second round PCR mix in a separate room (n°3). The process was applied to reduce the risk of contamination of individual PCR reactions. The method involved three negative controls. Each negative control was set up in individual rooms (n°1–n°3) designed for samples handling.

The primer sequences are shown below (Tab. VIII).

Tab. VIII. Sequences used for amplification of eight house-keeping genes of *B. burgdorferi* s.l. genospecies.

Gene	**5´– 3´ primer sequence (OF/OR/IF/IR)[a]**		**Product length [bp]**
clpA	OF	AAAGATAGATTTCTTCCAGAC	579
	OR	GAATTTCATCTATTAAAAGCTTTC	
	IF	GACAAAGCTTTTGATATTTTAG	
	IR	CAAAAAAAACATCAAATTTTCTATCTC	
clpX	OF	GCTGCAGAGATGAATGTGCC	624
	OR	GATTGATTTCATATAACTCTTTTG	
	IF	AATGTGCCATTTGCAATAGC	
	IR	TTAAGAAGACCCTCTAAAATAG	
nifS	OF	ATGGATTTCAAACAAATAAAAAG	564
	OR	GATATTATTGAATTTCTTTTAAG	
	IF	Same as 'OF'	
	IR	GTTGGAGCAAGCATTTTATG	
pepX	OF	ACAGAGACTTAAGCTTAGCAG	570
	OR	GTTCCAATGTCAATAGTTTC	
	IF	TTATTCCAAACCTTGCAATCC	
	IR	TGTGCCTGAAGGAACATTTG	
pyrG	OF	GATTGCAAGTTCTGAGAATA	603
	OR	CAAACATTACGAGCAAATTC	
	IF	GATATGGAAAATATTTTATTTATTG	
	IR	AAACCAAGACAAATTCCAAG	
recG	OF	CCCTTGTTGCCTTGCTTTC	651
	OR	GAAAGTCCAAAACGCTCAG	
	IF	CTTTAATTGAAGCTGGATATC	
	IR	CAAGTTGCATTTGGACAATC	

rplB	OF	TGGGTATTAAGACTTATAAGC	624
	OR	GCTGTCCCCAAGGAGACA	
	IF	CGCTATAAGACGACTTTATC	
	IR	Same as 'OR'	
uvrA	OF	GAAATTTTAAAGGAAATTAAAAGTAGGCTTAA	570
	OR	CAAGGAACAAAAACATCTGG	
	IF	GCTTAAATTTTTAATTGATGTTGG	
	IR	CCTATTGGTTTTTGATTTATTTGAATAA	

[a]OF- outer forward/ OR- outer reverse/ IF- inner forward/ IR- inner reverse

The PCR reaction was performed in a Bio-Rad T100^{TM} Thermal Cycler (California, USA). Program settings for amplification of *clpA, clpX, nifS, pepX, pyrG, rp1B,* and *uvrA* genes were different for the first (Tab. IX) and the second round (Tab. X). The thermocycler conditions are shown in the tables below.

Tab IX. First round PCR settings for genes *clpA, clpX, nifS, pepX, pyrG, rplB* and *uvrA*.

	Temperature [°C]	Time [min]	Number of cycles
Initialisation	95	05:00	
Denaturation	94	00:30	9 x
Annealing	55 (-1 °C/cycle)	00:30	
Extention	72	01:00	
Denaturation	94	00:30	37 x
Annealing	48	00:30	
Extention	72	00:30	
Final extention	72	05:00	
Hold	4	∞	

Tab. X. Second round PCR settings for genes *clpA, clpX, nifS, pepX, pyrG, rplB* and *uvrA*.

	Temperature [°C]	Time [min]	Number of cycles
Initialisation	95	05:00	
Denaturation	94	00:30	10 x
Annealing	60 (-1 °C/cycle)	00:30	

Extention	72	01:00	
Denaturation	94	00:30	37 x
Annealing	52	00:30	
Extention	72	00:30	
Final extention	72	05:00	
Hold	4	∞	

For recG, a nested-PCR was used with different settings of the PCR protocol in compare to settings used for genes mentioned above (Tab. XI). The same protocol was applied for both 1st and the second round of amplification of the *recG* house-keeping gene.

Tab. XI. PCR settings for *recG* gene.

	Temperature [°C]	Time [min]	Number of cycles
Initialisation	95	05:00	
Denaturation	94	00:15	40 x
Annealing	55	00:30	
Extension	72	01:00	
Final extension	72	05:00	
Hold	4	∞	

4.7.1.2 PCR of eight housekeeping genes of *B. miyamotoi*

The *B. miyamotoi*-positive ticks were subjected to amplification and sequencing of all eight housekeeping genes using the protocol described by Margos et al. (2015) with modifications.

The amplification of 2 gene targets *clpA* and *nifs*, was performed using PCR kit HotStarTaq Plus DNA polymerase (Qiagen, Germany). The reaction volume was adjusted to 30 µl: 3 µl CoralLoad Buffer (10X), 1.5 µl $MgCl_2$ (2.5 mM), 1.5 µl dNTP (1 µM), 0.27 µl Taq polymerase (5U/ µl), 1.5 µl each inner primer (500 nM) (Tab. XIV), 17.73 µl NFW and 3 µl template DNA (~10 ng/ µl).

The PCR amplification of six remaining gene fragments (*clpX, pepX, pyrG, recG, rplB* and *uvrA*) was performed using PCR kit HotStarTaq Plus DNA polymerase (Qiagen, Germany): 2.5 µl CoralLoad PCR Buffer (10X), 1µl $MgCl_2$ (2.5 mM), 1 µl dNTP (1 µM), 0.15 µl Taq polymerase (5U/ µl), 1 µl each outer primer (500 nM), 15.85 µl NFW and 2.5 µl

template DNA (~10 ng/ µl). Primers used for the first PCR reaction are given below (Tab. XII).

Tab. XII. Outer primers used for amplification of *B. miyamotoi* house-keeping genes.

Gene	**5´– 3´ primer sequence (F/R)[a]**		**Size of fragment [bp]**
clpA	F	TTGATCTCTTAGATGATCTTGG	783
	R	CAAACATAAACCTTTTCAGCCTTTAATA	
clpX	F	CTGTTGCYATTTGTTTTGAATGC(Y)TC	1173
	R	TAAAGTTCTTTTGCCCAAGG	
nifS	F	GAAAAAGTAAACTCCCTCAGAAAGG	861
	R	CAATGATGCCTGCAATATTTGGTG	
pepX	F	AGAGAYTTAAGYTTAKCAGG	841
	R	GTTTCTCTTAAAGAYTGCATTCC	
pyrG	F	CTTYTAGTWATTGARATTGGTGGT	846
	R	CAGCATCAAYTATRCCACAAAC	
recG	F	CTAGYATTCCTYTAATTGAGGC	871
	R	TTCRGTTAAAGGTTCCTTATAAAG	
rplB	F	ATTAAGACTTATARGCCAAAAAC	743
	R	GGCTGNCCCCAAGGWGAT	
uvrA	F	GCTKAAATTTTTRATTGATGTTGGA	871
	R	CARGGAACAAAAACATCRGGC	

*F-forward, R-reverse

For *clpA* and *nifS* targets, a touchdown PCR was performed (Tab XIII). For the remaining six genes (*clpX*, *pepX*, *pyrG*, *recG*, *rplB* and *uvrA*), a nested PCR was carried out.

Tab. XIII. PCR conditions for housekeeping genes *clpA* and *nifS*.

	Temperature	**Time [min]**	**Number of**
Initialisation	95	5:00	
Denaturation	94	00:30	9 x
Annealing	58 (-1 °C/cycle)	00:30	
Extension	72	01:00	
Denaturation	95	00:30	39 x
Annealing	53	00:30	
Extension	72	01:00	
Final extension	72	05:00	
Hold	15	∞	

The reaction volume of the remaining genes in the second round of PCR was adjusted to 30 µl. The reagent ratio was as follows: 3 µl CoralLoad Buffer (10X), 1.5 µl $MgCl_2$ (2.5 mM), 1.5 µl dNTP (1 µM), 0.27 µl Taq polymerase (5U/ µl), 1.5 µl each inner primer (500 nM) (Tab. XIV) and 17.73 µl NFW. The PCR product of 3 µl from the first round was included in a nested PCR reaction. Master mixes for both PCR rounds were prepared in the same room (n°1) in the sterile PCR cabinet (Bioair Instruments Aura PCR, Italy). The individual steps of preparation of the PCR reactions have been done separately as in the chapter 4.6.1.1.

Tab. XIV. Sequencing and inner primers for *B. miyamotoi*.

Gene	5´– 3´ primer sequence (F/R)[a]	
clpA	F	TTGATCTCTTAGATGATCTTGG
	R	CAAACATAAACCTTTTCAGCCTTTAATA
clpX	F	TTATCTGTTGCTGTTTATAATC
	R	TTCAAACATAACATCTTTAAGTAATTCTTC
nifS	F	GAAAAAGTAAACTCCCTCAGAAAGG
	R	CAATGATGCCTGCAATATTTGGTG
pepX	F	AGAGACTTAAATTTAGCAGGAGTTG
	R	TGCATTCCCCACATTGGAGTTC
pyrG	F	TTTAGTAATTGAGATTGGTGGTAC
	R	TATTCCACAAACATTACGAGC
recG	F	TAGCATTCCTTTAGTTGAGGC
	R	CTCAGCATGCTCAACTACC
rplB	F	GACTTATAGGCCAAAAACTTC
	R	GATACAGGATGACGACCACC
uvrA	F	TTAAATTTTTAATTGATGTTGGACT
	R	TCTGTAAAAAACCCAACATAAGTTGC

[a]F-forward, R-reverse

The first round of PCR for the six remaining genes included two sets of cycles (Tab. XV). The first set of cycles included a touchdown PCR with an annealing temperature at 60°C decreasing with 1°C each cycle (9x). Additional set of cycles included different annealing temperatures for individual genes. Other settings of the PCR reaction were the same for all six genes.

Tab XV: First round PCR conditions for remaining genes of *B. miyamotoi*: *clpX, pepX, pyrG, recG, rplB* and *uvrA*.

	Temperature [°C]	Time [min]	Number of cycles
Initialisation	95	5:00	
Denaturation	94	00:30	9 x
Annealing	60 (-1°C/cycle)	00:30	
Extension	72	01:00	
Denaturation	95	00:30	39 x
Annealing	*	00:30	
Extension	72	01:00	
Final extension	72	05:00	
Hold	15	∞	

*55°C = *clpX*, 52°C = *pepX*, *recG*, 58°C = *rplB*, 54°C = *uvrA*

Settings of the second round of PCR of the remaining genes are shown below (Tab XVI).

Tab XVI: PCR settings of the second round of amplification of *B. miyamotoi* genes: *clpA, clpX, nifS, pepX, pyrG, rplB* and *uvrA*.

	Temperature [°C]	Time [min]	Number of cycles
Initialisation	95	5:00	
Denaturation	94	00:30	34 x
Annealing	*	00:30	
Extension	72	01:00	
Final extension	72	05:00	
Hold	15	∞	

*55°C = *clpX*, 52°C = *pepX*, *recG*, 58°C = *rplB*, 54°C = *uvrA*

4.7.2 Evaluation of PCR products by agarose gel electrophoresis

The amplified DNA was evaluated by Serva DNA Stain G (SERVA Electrophoresis GmbH, Germany)-stained 1.5 % agarose gel (in 1X TAE). Further steps were followed by the same procedure as in section 4.5.

4.7.3 Purification

Products of successful amplification of individual genes were purified after visualization of the results according to the adapted protocol of purification kit NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, Germany). DNA was diluted with 25 µl NFW and bound to 100 µl Buffer NTI. Subsequently, the entire volume of the product was transferred to a NucleoSpin® Gel and PCR Clean-up Column, placed in a collection tube (2ml). The content was centrifuged for 1 min. at 11000 rpm (Hettich Mikro 120, Andreas Hettich GmbH & Co. KG, Germany) and the flow-through was discarded. DNA was 2 x washed by adding 600 µl (each time) buffer NT3 to the column and centrifuged under the same conditions as before. The flow-through was discarded each time. Centrifugation was repeated two more times to remove the buffer completely. The column was transferred into a new 1.5 ml microcentrifuge tube (Eppendorf, Germany). The content was dried for 2 min. at 60 °C (Stuart® block heater SBH130D/3, Cole-Palmer Ltd., UK) with lids opened. The evaporation was performed to remove residual ethanol (NT3 Buffer) which might inhibit enzymatic reactions. DNA was eluted by adding 30 µl NFW at the silica membrane and incubated for 2 min. at room temperature. The content was centrifuged for 3 min. at 14 000 rpm.

Presence of DNA was confirmed by agarose gel electrophoresis. Since the purified products did not contain any binding dye necessary for their visualisation, samples were prepared for gel electrophoresis under following conditions: 5 µl DNA was mixed with 2 µl 6X Loading Dye ThermoFisher Scientific. Massachusetts, USA) and transferred to the 1.5 % agarose gel. The electrophoresis was run at 120 V for 35 min. Results were visualized using UV-transilluminator (Vilber Lourmant, France) at a wavelength of 312 nm.

Purified products were stored at -20°C until further analysis.

4.7.4 Sequencing

Products of purification were prepared for Sanger sequencing by commercial company (GATC Biotech by Eurofins Genomics) in forward and reverse direction as follows: amount of 5 µl of purified product was mixed with 5 µl of specific inner forward primer (5 pmol/ µl) in 1.5 ml Safelock microcentrifuge tube (Eppendorf, Germany). Another 5 µl of purified product was mixed with 5 µl of inner reverse primer (5 pmol/ µl) in separate safelock tube

(1.5 ml). Samples were centrifuged 30 sec. at 10 000 rpm. Primers of same sequences as for previously perfomed PCR reactions were used. Such prepared samples were sent to the sequencing company.

4.8 MLSA

The results of sequences were available and downloaded in AB1 format from www.eurofinsgenomics.eu. Forward and reverse sequences for each sample were opened in MEGA-X software (version 10.0.5). Sequence fragments were checked for undesirable backrounds or mixed strain infections. Such samples were not included in further analyses. Sequences of sufficient quality were compared to sequences defined in the MLST database (www.publmlst.org). When sequences of samples from our study matched alleles already present in the database, the allele number was given. Allele numbers of all eight house-keeping genes determined the ST. Examined samples generating novel alleles or STs have been registered to the MLST database in order to obtain specific allele or ST numbers.

4.8.1 Population-genetic analysis based on the MLST data

The *Borrelia* genospecies detected in this study, to which STs were assigned (more information is available in Appendix 3), were individually analysed using the PHYLOViZ 2 program (Nascimento et al. 2017).

Genetic variability of specific *Borrelia* genospecies was analysed predominantly in range of Central and Eastern Europe with extended data of samples obtained in this study from Slovakia.

There are different views and distribution of countries of Central and Eastern Europe, as well, as for other parts of the continent. The countries are grouped according to historical perspective, geographical and cultural reasons, or economical standards.

In this research, countries of Central and Eastern Europe are identified according to a globally used and recognized scheme in the scientific field, The World of Factbook. According to the previously mentioned standard, the Central Europe includes following countries: Austria, Czech Republic, Germany, Hungary, Liechtenstein, Poland, Slovakia,

Slovenia, and Switzerland. Eastern Europe include Belarus, Estonia, Latvia, Lithuania, Moldova, Russia, and Ukraine.

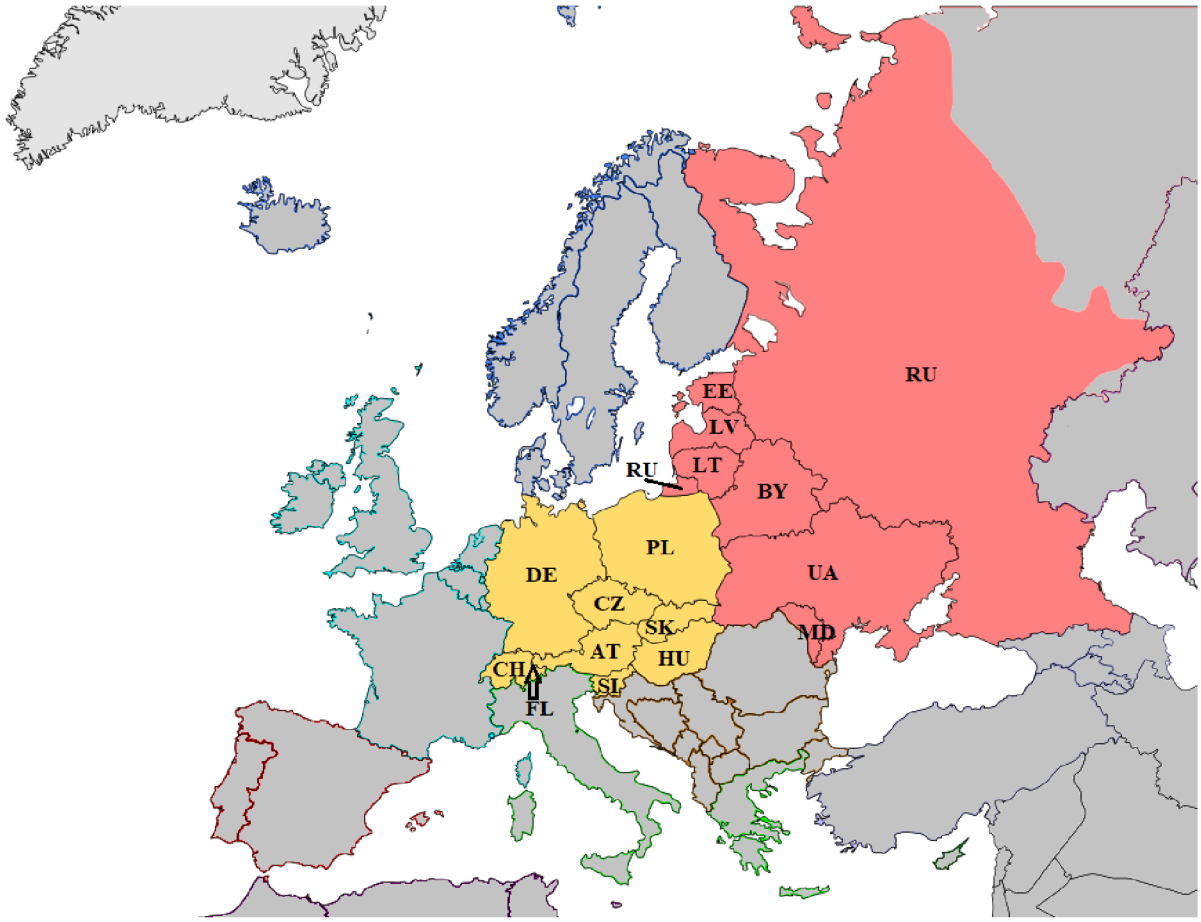


Fig. 5: Structure of Central and Eastern Europe countries used in this study, according to The World Factbook (Central Intelligence Agency, n.d. and edited in CC BY 3.0 program). Yellow backround = Central Europe: AT-Austria, CZ-Czech Republic, DE-Germany, FL-Liechtenstein, HU- Hungary, CH-Switzerland, PL-Poland, SI- Slovenia, SK-Slovakia
Pink backround = Eastern Europe: BY-Belarus, EE-Estonia, LT-Lithuania, LV-Latvia, MD-Moldova, RU-Russia, UA-Ukraine

An identification of clonal clustering of STs of the individual genospecies of this study with the STs (of exact genospecies) from the PubMLST database was performed using the goeBURST Distance analysis. Subsequently created trees served as visualizations of the relationships among STs at a global level. For individual analyses, all records of exact genospecies with a complete MLST profile were extracted from the PubMLST database as of December 2020. The clonal clusters (CCs) were set up at the level of single-locus variants (SLVs). In order to generate the minimum-spanning tree (MST), the goeBURST Full MST

algorithm was applied. The tree was built up at the maximum level of locus variants (level 8). Further relationships between individual samples were visualised by additional data on the source of isolate (tick/ human) and the country of origin.

Data used for population-genetic analysis of *B. afzelii* included isolates collected in one country category which was defined as Unknown. Detailed search of this category revealed the samples of this group were from Germany. Therefore, the data of Unknown category were assigned to Germany in this study.

4.8.2 Phylogenetic analysis based on the MLST data

The analysis included sequenced samples of *B. burgdorferi* s.l. and *B. miyamotoi* of this study (from Slovakia, 2017) in addition to isolates from the MLST database (PubMLST, n.d.) to December 2020. Only samples comprising of all 8 house-keeping genes were included. ClustalW algorithm was applied in BioEdit software (Hall 1999) to generate a multiple sequence alignment. The default parameters of the algorithm were used. The analysis was performed in MEGA-X software (version 10.0.5) (Kumar et al. 2018).

- Gap opening penalty: 15
- Gap extension penalty: 6.66
- Transition weight: 0.5

Individual samples of specific *Borrelia* genospecies for the phylogenetic analysis of *B. burgdorferi* s.l. can be found in Appendix 3. The data set included the sequences of *B. burgdorferi* s.l. that were identified in this study, in Slovakia. Other samples of the analysis comprised additional frequented and potentially pathogenic genospecies of *B. burgdorferi* s.l. in Europe. As some genospecies occur throughout the Holarctic region, samples from countries far from Europe have also been added to the analysis. The database for this analysis includes isolated from human patients as well as of vectors. An isolate of a non-pathogenic genospecies from South America, *B. chilensis*, was used as an outgroup.

95 STs of the *B. burgorferi* s.l. complex were retrieved from the database, including STs of our study. One representative was selected for each ST, which was repeated in this study. The phylogenetic analysis was constructed using the Maximum Likelihood method

according to Best DNA Model with respect to Bayesian Information Criterion (BIC) (Tamura & Nei 1993). The analysis was inferred by using the GTR+G+I model, in other words, General Time Reversible substitution model with rates among sites set at Gamma Distributed With Invariant Sites (discrete Gamma categories n = 5, parameter = 0.5612) (Tamura & Nei 1993). The phylogenetic tree was obtained by applying Neighbor-Join and BioNJ approach to a matrix of pairwise distances. The total number of positions used in the final dataset was 4788. Pairwise distances were estimated using the Maximum Composite Likelihood method. The Bootstrap value was set to 1000 replicates.

Samples used for *B. miyamotoi* phylogenetic analysis included isolates of our study along with isolates of RFG genospecies. An isolate of a genospecies *B. burgdorferi* s.l. complex, i.e., *B. burgdorferi* s.s. was chosen as outgroup. The required data were retrieved from the MLST database and processed in the MEGA X. program. 19 nucleotide sequences (16 STs) of the RF *Borrelia* complex were selected for the analysis, including samples from our study (Appendix 4). One ST of *B. burgdorferi* s.s was chosen for outgroup. Evolutionary analysis was inferred by using the Maximum Likelihood statistical method according to Best DNA Model with respect to BIC. GTR+G model was chosen with a discrete Gamma distribution comprising 5 rate categories (parameter = 0.3795). The phylogenetic tree was obtained by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances (Fig. 9). The distances were estimated based on the MCL approach. The total number of positions used in the final dataset was 4788 (Kumar et al. 2018). The Bootstrap method was set to 1000 replicates.

5. RESULTS

5.1 Collection of material

Excluding Drienovec, 653 *I. ricinus* ticks were collected from April to October 2017 in four sites of Slovakia (Tab. XVII). The dominant tick life stage was represented by nymphs with overall collection of 358 individuals (54.8 %), followed by females (n = 160; 24.5 %) and males (n = 128; 19.6 %). Ticks for this research were collected in nymphal and adult (male/female) stage only.

The highest number of ticks was collected in Malacky (n = 216) and Košice (n = 216), representing together 66.2 % of total ticks, followed by Bratislava (n = 119; 18.2 %), and Vrbovce (n = 102; 15.6 %). Dates of collection of ticks at specific locations are shown in appendices (Appendix 5).

Tab. XVII. Number of collected *I. ricinus* ticks from each study area of Slovakia.

	Locality	Life stage of a tick			Total
		Nymph	Adult		
			Male	Female	
Questing ticks	Bratislava	33	32	54	119
	Košice	51	78	87	216
	Malacky	195	9	12	216
	Vrbovce	86	9	7	102

Predominantly, *I. ricinus* genospecies have been identified from all study sites. In Malacký park and Vrbovce, *Dermacentor reticulatus* was identified within six and 13 individuals, respectively. The genospecies *D. reticulatus* was not further analysed for this study.

5.2 Prevalence of *B. burgdorferi* s.l. in questing ticks of Bratislava, Košice, Malacky, and Vrbovce.

Based on touchdown PCR amplification of the *5S–23S* rRNA intergenic spacer of *I. ricinus* samples and MLST, *B. burgdorferi* s.l. infections in ticks were identified.

The analyses of questing ticks from four study sites revealed 28.4 % (n = 186/653) positive cases of *B. burgdorferi* s.l. (Tab. XVIII). Out of total number of questing ticks, the highest prevalence was detected in nymphs 13.9 %, followed by females 8.7 % and males 5.8 %. Within a particular stage of the tick, the prevalences were as follows: the highest prevalence of the pathogen was found in females 35.6 % (n = 57/160), followed by males 29.6 % (n = 38/128), and nymphs 24.9 % (n = 91/365).

For more data focused on prevalence of individual genospecies at exact study sites see appendices 6−9.

Tab. XVIII. Prevalence of *B. burgdorferi* s.l. in questing ticks at four study sites (Bratislava, Košice, Malacky, and Vrbovce) of Slovakia.

Tick life stage	N	Positive cases of specific areas				Prevalence in relation to specific tick stage (%)	Prevalence in relation to total number of ticks (%)
		Bratislava	Košice	Malacky	Vrbovce		
Nymph	365	11	15	41	24	24.9	13.9
Male	128	7	31	-	-	29.6	5.8
Female	160	16	36	3	2	35.6	8.7
Total	653						28.4

N- total number of analysed samples, (%)-prevalence of positive samples

The prevalences of positive cases of *Borrelia* spp. in questing ticks at specific study sites were following: 37.9 % (Košice), 28.5 % (Bratislava), 25.5 % (Vrbovce), 20.4 % (Malacky).

5.3 Positivity of *B. burgdorferi* s.l. genospecies in Bratislava, Košice, Malacky, and Vrbovce.

According to the RFLP analysis (RFLP profiles are shown in Fig. 5), six genospecies of *B. burgdorferi* s.l. pathogen have been detected, including: *B. afzelii*, *B. garinii*, *B. burgdorferi* s.s., *B. valaisiana*, *B. bavariensis*, and *B. spielmanii*.

The following graph (Graph 1) shows prevalence of exact genospecies of *B. burgdorferi* s.l. in individual study sites of questing ticks.

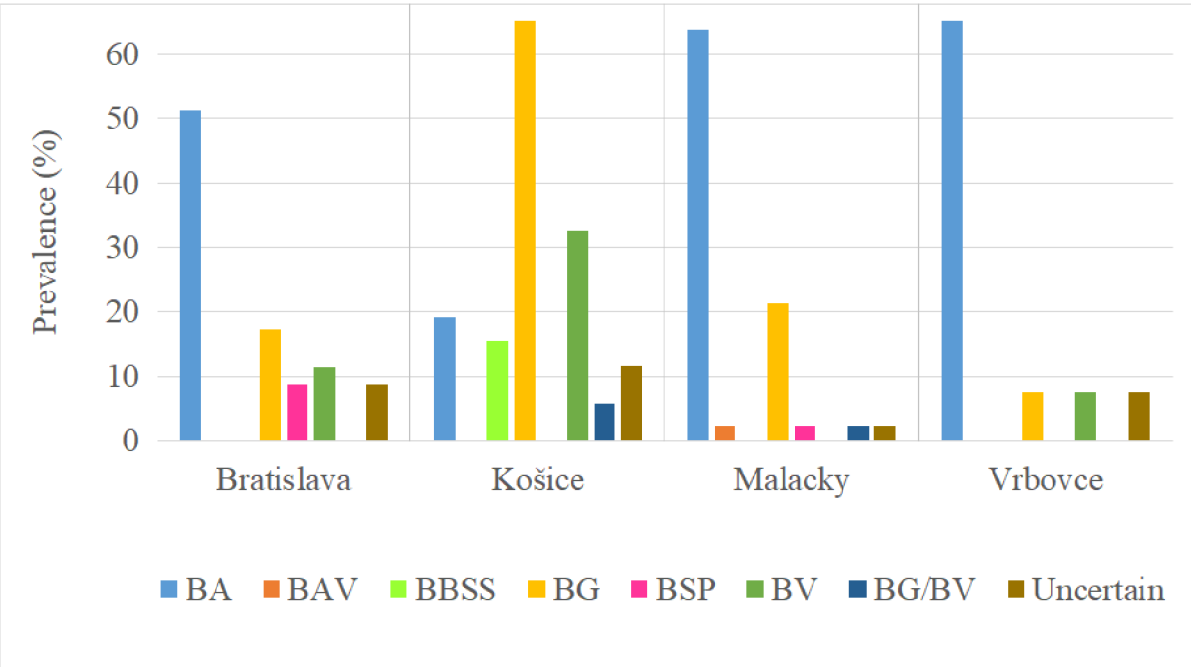
In Bratislava, 34 (28.5 %) samples of questing ticks were positive. The collection of borrelial genospecies included in descending order of positive cases: *B. afzelii* (n = 18), *B. garinii* (n = 6), *B. valaisiana* (n = 4), and *B. spielmanii* (3). Another 3 positive cases remained unclear as they could not be identified by species.

Out of 82 positive samples (37.9 %) in Košice, the genospecies collection of *B. burgdorferi* s.l. was following: *B. afzelii* (n = 10), *B. burgdorferi* s.s. (n = 8), *B. garinii* (n = 38), and *B. valaisiana* (n = 17). Three ticks were coinfected by two genospecies, specifically, *B. garinii* and *B. valaisiana*. Six tick samples carried uncertain borrelial infection.

Sample collection of Malacky with 44 (20.4 %) positive cases revealed the highest diversity of borrelial genospecies among all four study sites, comprising of 30 cases of infection by *B. afzelii*, 10 cases of *B. garinii*, one case of *B. bavariensis* and one case of *B. spielmanii*. One tick was infected by two genospecies, specifically by *B. garinii* along with *B. valaisiana*. Another tick sample was identified positive with uncertain identification of exact genospecies.

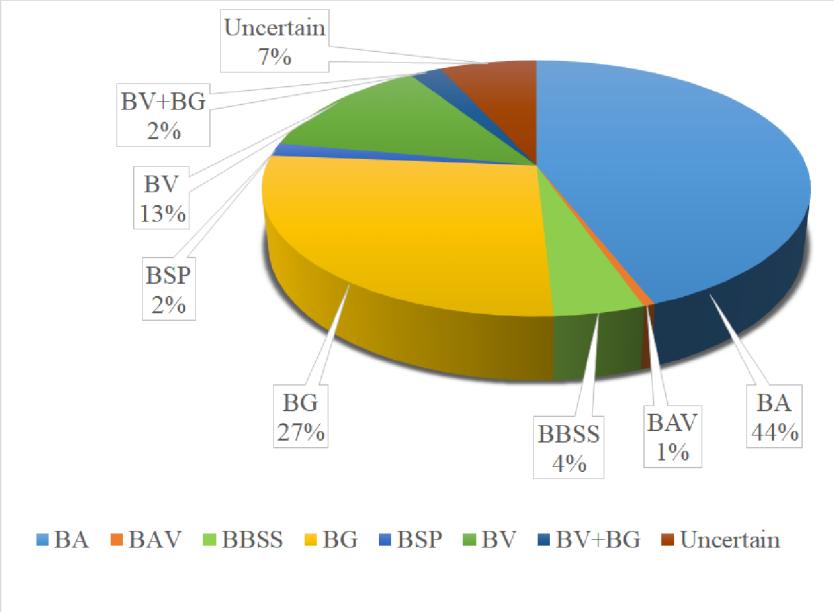
From 102 collected ticks in Vrbovce, 25.5 % ticks possessed borrelial DNA. The analysis determined 26 positive samples of infection by four exact *Borrelia* genospecies, which is the lowest diversity of genospecies among the four study areas. The genospecies were: *B. afzelii* (n = 20), *B. garinii* (n = 2), *B. valaisiana* (n = 2). In two positive cases was not possible to identify exact genospecies of *Borrelia*.

More data related to the prevalences of exact genospecies of individual study sites are shown in Appendices (4−7).



Graph 1. Prevalence of genospecies of *B. burgdorferi* s.l. in *I. ricinus* at individual study sites of this research: Bratislava, Košice, Malacky, and Vrbovce. BA- *B. afzelii*, BAV- *B. bavariensis*, BBSS- *B. burgdorferi* s.s., BG- *B. garinii*, BSP- *B. spielmanii*, BV- *B. valaisiana*, BG/BV- *B. garinii/B. valaisiana*, Uncertain-uncertain identification of genospecies of *B. burgdorferi* s.l. complex.

Among *B. burgdorferi* s.l. positive samples from questing ticks, *B. afzelii* (n = 78) was the predominant genospecies, followed by *B. garinii* (n = 48) *B. valaisiana* (n = 23), *B. burgdorferi* s.s. (n = 8), *B. spielmanii* (n = 3), and *B. bavariensis* (n = 1) (Graph 2). Coinfection of *B. garinii* and *B. valaisiana* appeared in 4 cases. No exact genospecies were identified for 12 positive tick samples.



Graph 2. Prevalence of individual genospecies of *B. burgdorferi* s.l. in *I. ricinus* of this research: BA- *B. afzelii*, BAV- *B. bavariensis*, BBSS- *B. burgdorferi* s.s., BG- *B. garinii*, BSP- *B. spielmanii*, BV- *B. valaisiana*, BV+BG- *B. valaisiana* and *B. garinii*, Uncertain- uncertain identification of genospecies of *B. burgdorferi* s.l. complex.

An association between *Borrelia* genospecies and its locality of occurrence was observed according to chi-squared test, $\chi 2 = 84.26$, df = 21 (expected value of chi-squared distribution = 32.665).

5.4 Prevalence and positivity of *B. miyamotoi* in Bratislava, Košice, Malacky, and Vrbovce.

B. miyamotoi from RFG of *Borrelia* was identified in the above–mentioned *I. ricinus* samples by real-time PCR amplification of the *16S* rRNA gene. The analysis of questing ticks from four study sites revealed 1.2 % (8/653) positive cases of *B. miyamotoi* (Tab. XVIX). The highest prevalence of *B. miyamotoi* was in nymphs 0.7 %, followed by females 0.4 %. Within a particular stage of the tick, the prevalences were as follows: in females 1.8 %, and nymphs 1.3 %. There was no positive case of *B. miyamotoi* in male ticks.
One case of *B. miyamotoi* in nymph tick was in co-infection with *B. afzelii*.

Tab. XVIX. Prevalence of *B. burgdorferi* s.l. in questing ticks at four study sites (Bratislava, Košice, Malacky, and Vrbovce) of Slovakia.

Tick life stage	N	Positive cases of specific areas				Prevalence in relation to specific tick stage (%)	Prevalence in relation to total number of ticks (%)
		Bratislava	Košice	Malacky	Vrbovce		
Nymph	365	-	-	4	1	1.3	0.7
Male	128	-	-	-	-	0	0
Female	160	1	2	-	-	1.8	0.4
Total	653						

5.5 Prevalence and positivity of *B. miyamotoi* in Drienovec

From Drienovec, *B. miyamotoi* samples were provided from five questing ticks for this study. Three samples were collected during April. One *I. ricinus* tick sample was collected in June, and one sample was collected in July. One tick isolate (from April) was coinfected with *B. afzelii*.

Additional two samples were provided from ticks fed on blackbirds (*Turdus merula*). One sample from tick fed on *T. merula* was collected in April, another in June. Both ticks were infected with genospecies of *B. burgdorferi* s.l. complex. The sample collected in April was coinfected with *B. garinii*. The sample from June was coinfected with *B. valaisiana*.

A total of 624 *I. ricinus* ticks were collected in Drienovec. 369 ticks were collected from birds, and 255 ticks were collected from vegetation. Bird species infested with ticks were following: *Turdus merula*, *Erithacus rubecula*, *Coccothraustes coccothraustes*, *Parus major*, *Turdus philomelos*, *Garrulus glandarius*, *Luscinia megarhynchos*, *Chloris chloris*, *Fringilla coelebs*, *Phylloscopus collybita*, *Phylloscopus trochilus*, *Sylvia atricapilla*, *Sylvia communis*, *Troglotydes troglodytes*, and *Prunella modularis* (Mtierová et al. 2020).

Prevalences of *B. miyamotoi* reached 0.5 % (co-infections only) in ticks samples collected from birds and 1.9 % from questing ticks (including 0.3 % of co-infections with *B. burgdorferi* s.l.).

B. burgdorferi s.l., collected from ticks fed on birds, was 43.3 %. Representation of *B. burgdorferi* s.l. genospecies in descending order of prevalence was as follows: *B. garinii* (22.4 %), *B. valaisiana* (10.8 %), *B. afzelii* (2.4 %). *B. lusitaniae* and *B. spielmanii* were represented in the same prevalence of 0.27 % for each. The following co-infections were observed: *B. garinii* with *B. valaisiana* (4.8 %), *B. garinii* with *B. afzelii* (0.8 %), *B. garinii* and *B. lusitaniae* (0.2 %), and *B. garinii* with *B. bavariensis* (0.2 %). Another co-infection was identified of *B. valaisiana* with uncertain genospecies (0.8 %).

Prevalence of *B. burgdorferi* s.l., from questing *I. ricinus* ticks was 26.2 %. Prevalence of *B. burgdorferi* s.l. exact genospecies in descending order was following: *B. afzelii* (16.0 %), *B. garinii* (4.3 %), and *B. valaisiana* (3. 9 %). Co-infection of *B. garinii* and *B. valaisiana* occured in 4.3 % cases. Co-infection of *B. afzelii* and *B. valaisiana* was detected in 0.3 % cases.

5.6 MLST

Out of 186 (28.4 %) positive samples of *Borrelia* in questing ticks, a representative group of 104 samples was analysed by MLST method. Amplification of individual genes and their subsequent sequencing reduced the number of analysed samples, based on several criteria. The first parameter was the presence of individual genes and thus their possibility of amplification (Fig.5). Subsequent sequencing and evaluation of results in MEGA-X software (Version 10.0.5) showed if the gene met the MLST parameters. Samples that could not be sequenced for all (8) housekeeping genes or revealed a mixed infection were not registered to the MLST database and were not used in further analyses.

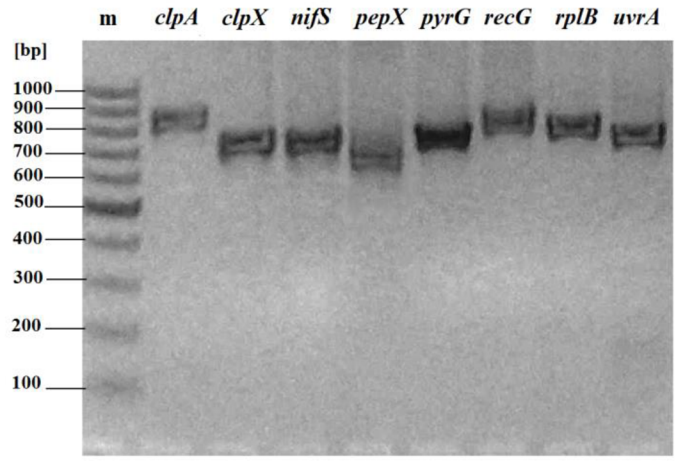


Fig. 5. MLST profile of eight housekeeping genes (*clpA, clpX, nifS, pepX, pyrG, recG, rplB, uvrA*) of *B. burgdorferi* s.l. complex. m-100 bp ladder

Based on the MLST method, 24 samples were sequenced for all housekeeping genes and specific STs were given (Appendix 10). These STs are available at the worldwide database for molecular typing and microbial genome diversity (PubMLST, n.d.). Sequenced samples included following genospecies: *B. afzel*ii (8), *B. burgdorferi* s.s. (3), *B. garinii* (10). Three samples of *B. miyamotoi* were given the STs. One new allele (in yellow) has been identified within samples of *B. miyamotoi*. No new alleles have been discovered in *B. burgdorferi* s.l. complex.

For *B. afzelii* representatives, two new STs were assigned. Genotyped samples included all four studied areas (Bratislava, Košice, Vrbovce, and Malacky). Sequenced samples represented different stages of the tick's life. Samples of Malacky and Vrbovce came from nymphs. The sample of Košice was isolated from a male tick. Samples from Bratislava came from both nymphs and adults (1x male, 2x female). Four samples from Bratislava possessed identical allelic profiles. Therefore, identical STs were registered for these samples. Two new STs (ST944 and ST945) of *B. afzelii* of this study are considered to be firstly recorded in the database.

In the case of *B. burgdorferi* s.s., three samples from Košice were sequenced within all eight housekeeping genes. The samples came from a nymph, male, and female. Isolate from a nymph was assigned ST24. Samples from adult ticks were characterized by one allelic profile (ST20). In addition to new records in Slovakia, the ST20 is also detected in France,

Latvia, Germany, Ukraine, and Switzerland. Predominantly, ST20 was identified from tick vectors. In Germany, this ST was identified also in LB patients. Specifically, borrelial DNA was isolated from skin (two cases), CSF (seven cases) and joints.

Amplification and sequencing of all eight housekeeping genes of *B. garinii* were completed on samples from three areas: Malacky (4), Košice (5) and Bratislava (1). Sources of all samples of Malacky were nymphal ixodid ticks. Ticks from Košice were in adult stage: females (4) and males (1). Borrelial DNA isolated from Bratislava was identified in a female tick. In total, eight STs were assigned to the samples. Two samples from Košice had identical allelic profiles (ST251). Other two samples with identical ST (86) were detected in two study sites, specifically, Košice and Malacky. A new ST (953) was assigned to an isolate from female tick from Košice site. The ST was reported for the first time in the PubMLST database.

In case of *B. miyamotoi* genotyping, one new allele (296) was discovered. The new allele was identified in questing tick (GenBank accession number: MW815877) and a tick from a bird (GenBank accession number: MW815878) at the same location (ST956) of Drienovec (Tab XVX). The isolate from a bird-fed tick was coinfected by *B. garinii*. Another new ST of *B. miyamotoi* was discovered in Malacky from a questing tick (ST635). All three samples were isolated from nymphs. The deposited ST956 of *B. miyamotoi* of this study was reported for the first time in the PubMLST database.

Tab XVX. New alleles identified in *B. miyamotoi* samples of this study with given GenBank accession numbers.

isolate	**region**	**tick source/host**	**allele**	**GenBank accession number**
DNQ107	Drienovec	nymph	296	MW815877
1DIVN4	Drienovec	nymph/*Turdus merula* ♂	296	MW815878

5.7 Population-genetic analysis

As mentioned before (chapter 4.8.1.), genospecies with complete MLST profiles and STs of this study were used for population-genetic analyses. In addition, samples of exact genospecies with complete MLST profiles from the PubMLST database were included. Specifically, 568, 1344, 469, and 38 samples (including samples obtained in this study) were used for analyses of *B. afzelii*, *B. burgdorferi* s.s., *B. garinii*, and *B. miyamotoi*, respectively. 10 schemes related to population genetic analyses of individual species can be found in the appendices and also in the zip file with higher resolution if necessary (Appendices 12−15, 17−19, and 21).

5.7.1 *B. afzelii*

GoeBURST analysis was performed for all STs of *B. afzelii* deposited in the PubMLST database. The analysis was constructed at SLV level.

Out of 10 STs in Slovakia in total, seven STs were assigned to six CCs. Another three STs were desribed as SGs (Appendices 11−12).

Four STs of our study were localised in four CCs (ST170, ST540, ST945, ST463). One ST of our study (ST 944) was characterised as a SG. All four CCs included STs from countries of Western, Central, and Eastern Europe. STs of the first CC (CC 0) were distributed in range of Austria, France, Germany, Italy, Latvia, Slovakia, Slovenia, Switzerland, the Netherlands, the United Kingdom, and Finland. The second clonal group (CC 5) included STs of following countries: France, Germany, Italy, Latvia, Slovakia, Slovenia, Switzerland, and the United Kingdom. CC 6 comprised of six countries: France, Germany, Latvia, Slovakia, Serbia, and the United Kingdom. Countries of CC 8 were following: Austria, France, Germany, Latvia, Slovakia, Slovenia, and the Netherlands.

Genotypes obtained in our study were identified from both ticks and human patients of LB (Appendix 14). ST540 was identified in Slovakia (source: tick), Germany (source: human), and Slovenia (source: human). One genotype (ST479) from previous study of Slovakia was obtained in both tick (Slovakia) and human patient (Slovenia).

The gene distribution of *B. afzelii* using our isolates and isolates from the database was as follows. Genotypes of *B. afzelii* from MLST database were distributed in 159 units at

SLV, whereas 34 groups were classified as CCs, and 125 were SGs. STs of Central and Eastern Europe occured in 27 CCs and 79 SGs (Appendix 13). As of number of *B. afzelii* samples of the database, 54.92 % samples were assigned to the database from Central and Eastern Europe (33.27 % and 21.65 %, respectively). Following countries of Central Europe are included in the database: Germany (16.55 % + Unknown 0.53 %), Slovenia (7.22 %), Switzerland (3.17 %), Austria (2.99 %), Slovakia (2.46 %), Czech Republic (0.18 %), Hungary (0.18 %), and Poland (0.18 %). Three countries of Eastern Europe are included in this study: Latvia (11.44 %), Russia (9.33 %) and Ukraine (0.7%).

Out of 34 clonal groups generated by using SLV in goeBURST algorithm, three CCs included only STs occurring in Eastern Europe, specifically, Russia (CC 12, 13, and 26). Other three CCs comprised countries only of Central Europe: Germany (CC 22 and 25) and one ST of Slovenia along with another ST of Hungary (CC 28). 20 other clonal groups included STs of wide range of European countries. Excluding Central and Eastern Europe, two CCs included STs only of France (CC 23 and 27). One CC comprised STs only of Norway (CC 23). One clonal group (CC 20) was characterised with STs only from the United Kingdom. Three CCs included genotypes only of Asia (CC 19, 21, and 33), including China, Japan, and Kazakhstan.

In total, 325 STs of *B. afzelii* were described worldwide, including 89 STs in Central Europe, 81 STs in Eastern Europe, and 111 STs in other countries of Europe only. 26 STs were distributed among Europe, of which five STs occurred in Central and Eastern Europe only (Appendix 14).

Countries with complete MLST profile of *B. afzelii* excluding areas of Central and Eastern Europe were following: the United Kingdom, Italy, former Yugoslavia, France, the Netherlands, Finland, Sweden, Norway, and Serbia. Asian countries identifying STs of *B. afzelii* were Kazakhstan, Mongolia, Japan, China, and South Korea. No evidence of countries of other continents is available as *B. afzelii* is considered to be a genospecies occurring only in Eurasia within the Holarctic region. Central Europe and other parts of Eurasia (excluding Eastern Europe) matched 16 equal STs, including 9 STs (locality: Austria, France, Germany, Italy, Latvia, Slovenia, and Switzerland), found in ticks and human patients of LB. Other six genotypes were identifiend only from human patients (locality: France, Germany, Slovenia). ST215 from tick source was found in Latvia, Italy,

and Czech Republic. Another ST (463) identified in tick from Latvia, was also identified in Germany in human patients of LB. ST570 was identified in both Latvia and Slovenia, in tick and human source, respectively.

Within Central Europe, *B. afzelii* samples extracted from human tissues (n = 123) have been detected in Germany, Austria, Poland, Slovenia, and Switzerland. Majority of samples from human sources were obtained in Germany (73 + 3 (Unknown) /123). Genotypes of *B. afzelii* from human patients occured in 18 CCs of Central Europe, specifically, CC 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 11, 14, 17, 22, 25, 28, 29, and 30. Other associated countries within the CCs were Czech Republic and Slovakia. There is no evidence of human source of *B. afzelii* samples in the PubMLST database in range of Eastern Europe. Nevertheless, several clonal complexes, including samples of countries of Eastern Europe (namely Latvia and Russia), consist of data of both human and tick source of samples. The clonal groups including data of Latvia were following: 0, 1, 2, 3, 4, 5, 6, 8, 11, and 15. Samples from Russia within clonal groups including data of human sources were CC 3 and CC 9. Other countries assocciated with clonal groups including samples from human patients of LB were following: Finland, Italy, Norway, Serbia, Sweden, the Netherlands, and the United Kingdom. To date, data of remaining countries were not detected in human patients of LB and did not occur in clonal groups associated with the disease: Croatia, Japan, Mongolia, South Korea, and Ukraine.

The minimum-spanning tree of *B. afzelii* in association with human source of the specific genotypes in Central and Eastern Europe is shown in Appendix 14. Out of the total number of 325 STs of *B. afzelii* worldwide, 35.37% of STs were also diagnosed from human patients. Of the total number of STs, 8.54% of STs were identified from more than one country, including samples obtained from patients. Specifically, *B. afzelii* samples were processed by MLST in 26 countries, 30.77% of which also contained samples from patients: Austria, China, France, Former Yugoslavia, Germany, Poland, Slovenia, and Switzerland. Another 19.23% of the countries contained STs of the above-mentioned countries, while they have been obtained from questing ticks or from reservoir hosts: Italy, Latvia, Serbia, Slovakia, Sweden, and the United Kingdom.

5.7.2 *B. burgdorferi* s.s.

The goeBURST analysis of *B. burgdorferi* s.s. profiles at SLV defined 29 clonal groups (CCs) and 53 SGs.

There were four records of *B. burgdorferi* s.s. from Slovakia in the MLST database. Three records of this study (Košice site) were assigned to two CCs. ST20 (two isolates) was assigned to CC 0 (Appendix 15). ST24 (one sample) was assigned to CC 5. ST20 of our study was in one clonal group with Austria, Germany, Slovenia, Switzerland, Latvia, Ukraine, Italy, France, the United Kingdom, and Canada. ST24 was also detected in Germany and France and it is in one clonal group with other STs from Germany, Latvia, and the United Kingdom. Both STs found in Slovakia have also been detected in isolates from patients from Germany (Appendix 17 – 18).

Isolate of *B. burgdorferi* s.s. of Europe deposited in the database were also detected in Italy, Finland, and Norway. The vast majority of the samples came from the USA (47.77 %) and Canada (41.39 %) (Appendix 17).

At level six, three groups were formed in the dataset, including a large group of STs of all above mentioned countries, one group of three STs of the USA, and one set with geographic affinity to Europe (Finland, France, Germany, Italy, Norway and Switzerland). At level five, a new group of samples of Canada and the USA appeared.

Additional four groups and five SGs show up at level four, including another complex with geographic affinity to Europe (France, Germany, Latvia, Slovakia (ST24) and the United Kingdom). All other groups and vast majority of SGs comprised samples of Canada and the USA. One ST of Germany detached from the "European" group, previously formed at level 6.

The complex of MLST profiles was divided into 13 groups and 14 SGs at level three. 10 groups consisted of North American countries. Two groups consisted of European countries. One group, consisting of the largest number of STs (94 STs), contained STs that were isolated from both European and North American countries.

24 groups and 22 SG have shown difference in three loci of their profiles (level 2). At this level, a group of mostly European countries was formed. The complex consisted of 11 countries: Austria, Canada, Germany, Italy, France, Latvia, Slovakia, Slovenia, Switzerland, the United Kindgom, and Ukraine. A sub-group founder of common nodes was ST20 (38 samples, including 2 samples of this study), deposited from France, Germany, Latvia, Slovakia, Switzerland, and Ukraine. Another complex was predominantly formed by samples of Northern America (USA and Canada), including ST1 identified also in Germany and Latvia. At SLV level, several other groups and SGs appeared.

Almost 16 % (n = 217) of *B. burgdorferi* s.s. samples were collected from human patients of LB (Appendix 17). Vast majority of the samples were obtained in the United States (82 %). Other countries comprising of samples from human tissues were Germany, Italy, and Slovenia. Nearly 37 % of all 176 STs of *B. burgdorferi* s.s. genospecies occured in samples from human source. 21 STs were collected from human patients only in USA. Other 33 STs of the human source were found in both USA and Canada. ST1 was collected from human patients in USA and Germany, whereas other two countries detected this ST from ticks, specifically, Latvia and Canada. Two STs (ST20 and ST21) were characterised in wide range of European countries, including collection of samples obtained from human patients in Germany. ST20 was also identified in France, Latvia, Slovakia, Switzerland, and the United Kingdom. ST21 was detected along with Germany in Austria, France, Latvia, Italy, and Slovakia. Samples of *B. burgdorferi* s.s. from Germany revealed two other STs (ST24 and ST284) also detected in men, along with France and the United Kingdom, respectively. Two and only STs of *B. burgdorferi* s.s. in Slovenia were of human patients of LB. One out of four STs (ST332) of Italy came from samples of human patients.

Out of 176 STs of *B. burgorderi* s. s., 36.93 % STs were isolated from human patients. 13 countries contained samples of *B. burgdorferi* s.s. within the MLST database, while four countries (38.46%) included STs identified also from patients: Canada, Germany, Italy, and Slovenia. These STs were also identified in six other countries (46.15%) only from questing ticks: France, Latvia, Slovakia, Switzerland, Ukraine, and the United Kingdom.

5.7.3 *B. garinii*

The goeBURST analysis of *B. garinii*, constructed at SLV level, assigned eight completely sequenced samples of the genospecies of this study into eight individual CCs (Appendix 19−20). Clonal group including isolates of this study (CC 0) from both Malacky and Vrbovce (ST86) comprised of following countries: Austria, Finland, Former Yugoslavia, France, Germany, Italy, Latvia, Russia, Serbia, Slovakia, Switzerland, Sweden, Ukraine, and the United Kingdom. CC 2 included STs deposited from Czech Republic, France, Latvia, Slovakia, the Netherlands, and the United Kingdom. Distribution of STs in the clonal group 5 was as follows: Finland, France, Latvia, Serbia, Slovakia, and Switzerland. CC 7 included STs from Finland, Germany, Latvia, Slovenia, Slovakia, Switzerland, and the United Kingdom. CC8 comprised of STs from Germany, Hungary, Latvia, Slovakia, and the United Kingdom.STs of CC 9 were in range of Finland, Germany, Slovakia, Switzerland, and the United Kingdom. CC 16 included isolates from Germany, Latvia, Slovakia, Switzerland, the Netherlands, the United Kingdom, and Ukraine. The last clonal group (C 22), of STs including isolates from our study comprised of three countries: Latvia, Slovakia, and Ukraine. One ST of the study was characterised as a SG (Košice study site).

Four STs of our study have also been previously detected in isolates from human patiens of LB in various countries. ST245, ST246, and ST251 have been described in patients from Germany. ST180 has been detected in patient from Slovenia.

All STs of *B. garinii* from MLST database were distributed into 90 units at SLV, including 24 CCs and 65 SGs. STs of Central and Eastern Europe occured in 33 CCs and 50 SGs (Appendix 19−20). As of number of *B. garinii* samples of the database, 58.75 % samples came from Central and Eastern Europe, in descending order: Germany (14.74 %), Russia (13.46 %), Latvia (10.26 %), Switzerland (9.4 %), Slovakia (7.48 %), Slovenia (1.5 %). Czech Republic (0.85 %), Ukraine (0.43 %), Austria (0.21 %), Estonia (0.21 %), and Hungary (0.21 %).

Out of 24 CCs, 17 clonal groups comprised STs occurring only in Europe. One clonal group comprised samples occurring in Eastern Europe, specifically, Latvia and Estonia (CC 22). Three CCs included genotypes present only in Finland (CCs 10, 18, 19). Two groups included genotypes discovered in both Europe and North America (Canada) (CC 1 and CC

11). Two CCs were present only in Canada (20, 21). Three clonal groups consisted of samples found only in Asia (CC 5, 14, 17, 24), including China, Japan, and Russia.

In total, 158 STs of *B. garinii* were identified worldwide, including 50 STs in Central Europe, 62 STs in Eastern Europe, and 96 STs in other countries (Appendix 21). In Central and Eastern Europe have been found 14 identical STs, including seven STs extracted from both ticks and human patients.

Other countries revealing *B. garinii* with complete MLST profile from Europe were following: the United Kingdom, Italy, former Yugoslavia, France, Netherlands, Finland, Sweden, Norway, Serbia, and Denmark. Asian countries identifying STs of *B. garinii* were Mongolia, Japan, China, South Korea. Another country belonging to this database was Canada. Central Europe and other parts of the Holarctic region (excluding Eastern Europe) matched 20 equal STs, including 11 STs found also in human patients. 22 STs were idenified in both Eastern Europe and other countries of the Holarctic region (excluding Central Europe), consisting of one STs found in both ticks and human patients.

Within Central Europe, *B. garinii* isolates extracted from human tissues have been detected in Germany and Slovenia. Genotypes of *B. garinii* from human patients occured in eight CCs, specifically, CC 0, 1, 3, 6, 8, 9, 11, 16. The vast majority of these samples were identified in Germany (54/58). No data of human source of *B. garinii* tick isolate in specific country of Eastern Europe appeared in the MLST database. In general, the CCs mentioned above consist of isolates of following countries: Former Yugoslavia, Germany, Austria, Canada, Czech Republic, Finland, France, Hungary, Italy, Latvia, Norway, Russia, Serbia, Slovakia, Slovenia, Sweden, Switzerland, the Netherlands and the United Kingdom. The minimum-spanning tree of *B. garinii* in association with human source of the specific genotypes in Central and Eastern Europe is shown in Appendix 21.

Of all STs of *B. garinii* identified in the MLST database, 11.98 % of STs have been identified from patients. Isolates of *B. garinii* are registered from 26 countries in the MLST database, including four countries (15.38 %) with evidence of human patient samples of *B. garinii*: Former Yugoslavia, Germany, Japan, and Slovenia. STs detected in samples of patients from above-mentioned countries have also been found in another 19 countries (73.08%): Austria, Canada, China, Czech Republic, Denmark, Finland, France, Hungary,

Italy, Latvia, Norway, Russia, Serbia, Slovakia, Sweden, Switzerland, The Netherlands, The United Kingdom, and Ukraine.

5.7.4 *B. miyamotoi*

The genospecies *B. miyamotoi* was completely sequenced and deposited into MLST database from six countries so far, i.e., Mongolia, USA, Japan, Slovakia, Germany, and Russia. GoeBURST analysis constructed at SLV level resulted in one CC and six SGs (Fig. 6) Due to low number of *B. miyamotoi* isolates, SGs were included in the schemes as well. Both STs obtained in this study (ST635, ST956) belong to the CC 0 along with isolates from another country of Central Europe, specifically, Germany (ST635).

Another ST identified in more than one country was identified as ST633, in Mongolia, Japan, and Russia. Mongolia, Russia and Germany assigned one ST of *B. miyamotoi* into the database of MLST. The USA and Slovakia deposited two STs into the database. Japan assigned the majority STs, specifically, 4 STs.

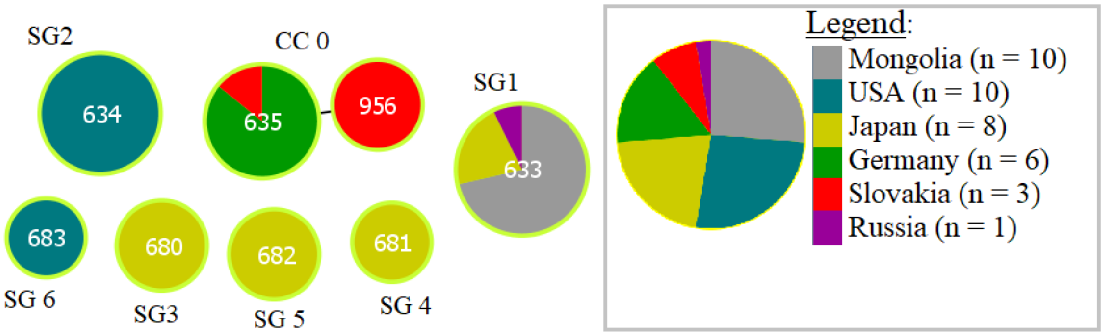


Fig. 6. GoeBURST analysis of *B. miyamotoi* at SLV level. Node size reflect the number of isolates characterised by specific ST (number in a node). Light green edge of node-group founder, black link-without recourse to tiebreak rules. CC- clonal complex, SG- singleton, (n)-number of isolates deposited in the MLST database (created in GIMP version 2.10.30 with data from Phyloviz 2 software).

The further linking of allelic profiles occurred at the TLV level for two STs from Japan (ST681 and ST682). Another linkage occurred for ST from Japan (ST680) and ST633 at the locus variant level 5. The next and overall connection of all STs of *B. miyamotoi* occurred at level 8.

The only genotype isolated from human patient of LB was the ST634, from the USA (Fig. 7).

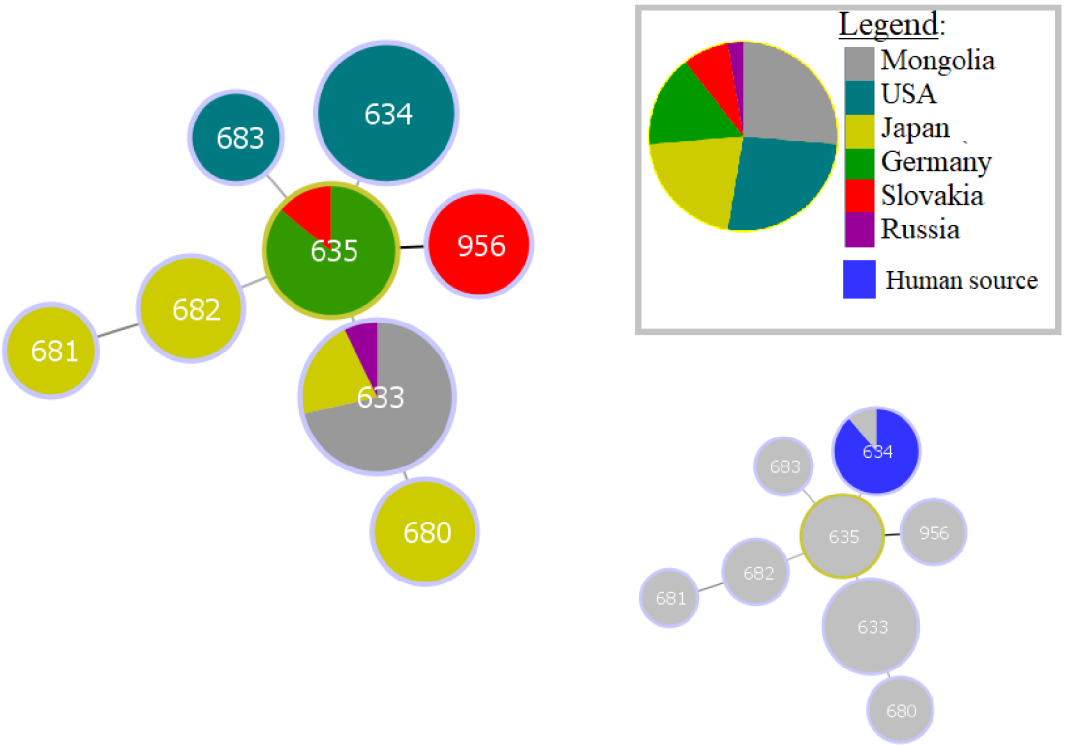


Fig. 7. Minimum-spanning tree of *B. miyamotoi* STs using goeBURST Full MST algorithm, build up at the maximum level of locus variants, i.e., 8 levels. The tree miniature at the bottom highlights ST identified in isolates from LD human patients (blue colour). Blue-coloured are within the node reflects the number of isolates (n = 8) for the given ST. Individual range of coloured area of the main tree also reflects the number of isolates for the given ST. Node size reflect the number of isolates characterised by specific ST (number in a node). The links between nodes are of black, dark grey or light grey colour. Black links reflect less differences between nodes in comparison to lighter grey links. Borders of nodes are coloured in dark green (sub-group founder) and light blue (common node) (created in GIMP version 2.10.30 with data from Phyloviz 2 software).

5.8 Phylogenetic analysis of *B. burgdorferi* s.l. group based on the MLST data

The phylogenetic relationships of *B. afzelii, B. garinii* and *B. burgdorferi* s.s. isolates obtained in this study were further analysed along with other STs of the *B. burgorferi* s.l. complex (Fig. 8). The rate variation approach (+I) of GTR+G+I analysis recognised 37.30 % sites to be evolutionary invariable. The highest log likelihood was -22821.50.

Branching of genospecies with a common ancestor with bootstrap value at 100 % was observed among *B. bissetiae* − *B. kurtenbachii* and *B. garinii* – *B. valaisiana*. The branches delimiting the genospecies were formed at bootstrap values of 100 %. *B. garinii* isolates from our study clustered with other *B. garinii* samples at various levels. A separate line at a bootstrap value of 79 % was observed for an isolate from Slovakia, Košice study site (ST243) and the Netherlands (ST746). Another isolate from Košice (ST251) clustered with an isolate from Germany (ST482). The third isolate retrieved from Košice region clustered with isolates from Finland (ST89), Estonia (ST750), and another isolate from Slovakia (ST185) at 99 % boostrap value. An isolate from Malacky region (ST180) clustered with an isolate from Finland (ST749) at 100 % bootstrap value. Another isolate from Malacky (ST245) clustered with an isolate from the United Kingdom (ST325) at 100 % bootstrap value. The third isolate from Malacky region (ST86) clustered with isolates from Germany (ST574), Czech Republic (ST177), Ukraine (ST175), and Former Yugoslavia (ST87).

B. burgdorferi s.s. isolates of our study were divided into two separate clusters. The first isolate from Košice study site emerged in a group with an isolate from Latvia (ST161). The second isolate (ST20) from Košice clustered with isolates from Canada (ST314), Slovenia (546), Italy (ST332), and Ukraine (ST904) at 81 % bootstrap value.

B. afzelii isolate of Bratislava, Slovakia (ST463) clustered with various STs detected in Serbia, Ukraine, and France at 95 % bootstrap value. Isolate from Košice, Slovakia (ST540) clustered with ST72 from Switzerland at 96 % bootstrap value. New ST (ST945) clustered with Asian and European STs at 31 % bootstrap value. Isolate from Malacky (ST170) paired with an isolate from Slovenia (ST570) at 70 % boostrap value.

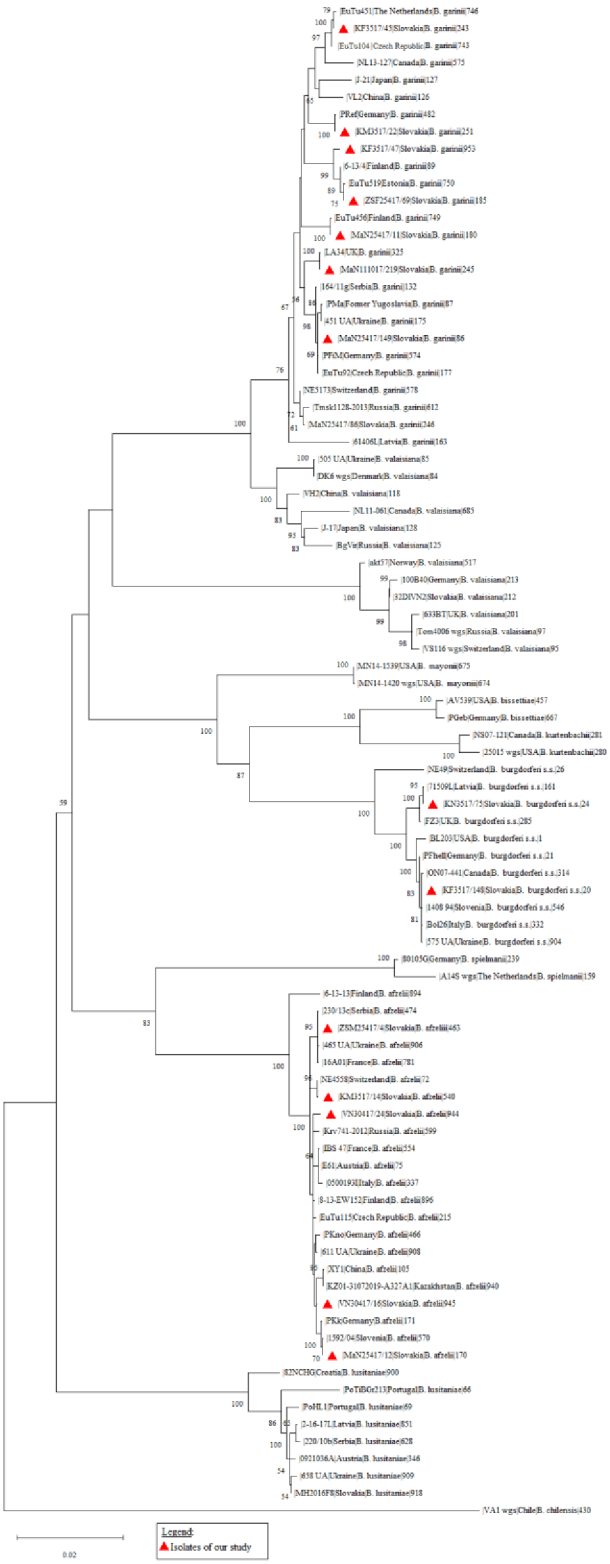


Fig. 8. Phylogenetic tree of *B. burgorferi* s.l. including concatenated sequences of eight housekeeping genes of isolates from our study along with isolates retrieved from the MLST database.

5.9 Phylogenetic analysis of RF *Borrelia* group including *B. miyamotoi* based on the MLST data

The phylogenetic relationships of *B. miyamotoi* isolates obtained in this study were further analysed along with other genospecies of the RFG of *Borrelia*. By using the Maximum Likelihood method, the highest log likelihood was evaluated as -20695.06. The *B. miyamotoi* cluster was divided into several groups based on the geographical distribution of the STs. The clusters were divided into two asian, one american and one European group. The European group was formed with a common ancestor at 100 % bootstrap value. The group included two STs, whereas ST956 was identified in Slovakia only. ST635 was identified from both Germany and Slovakia.

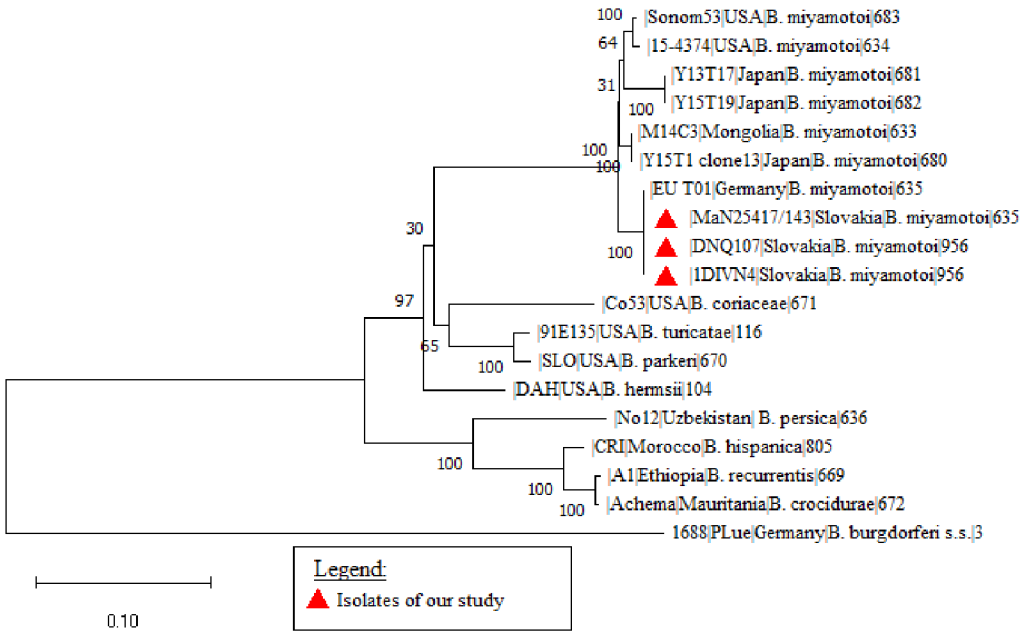


Fig. 9. Phylogenetic tree of RF *Borrelia* complex comprising concatenated sequences of eight housekeeping genes of isolates from our study along with isolates retrieved from the MLST database. *B. burgdorferi* s.s. from *B. burgdorferi* s.l. complex was used for outgroup.

6. DISCUSSION

This study was focused on the genetic diversity of *B. burgdorferi* s.l. and *B. miyamotoi* within Central and Eastern Europe. Part of the experimental work was the data extension of Central Europe by tick collection during the tick season (april-october) of 2017 in four areas of Slovakia.

Significant differences in prevalences of the six genospecies analysed were observed. *B. afzelii* (44 %) and *B. garinii* (27 %) were the most frequently detected genospecies, followed by *B. valaisiana* (13 %), *B. burgdorferi* s.s. (4 %), *B. spielmanii* (2 %), and *B. bavariensis* (1.0%). The obtained results are in correlation with the meta-analysis of Strnad et al. (2017) with the following values: *B. afzelii* (46.6 %), *B. garinii* (23.8 %), *B. valaisiana* (11.4 %), *B. burgdorferi* s.s. (10.2 %), *B. spielmanii* (1.7 %), and *B. bavariensis* (2.0%). Prevalence of *B. burgdorferi* s.s. in Slovakia was more than two and a half times lower when compared to the result of prevalence of the whole Europe (Strnad et al. 2017).

The chi-squared statistic (84.26) computed from the observation was significantly higher than the expected value of chi-squared distribution with 21 degrees of freedom (32.665). Therefore, we can safely reject the null hypothesis and conclude that there exists an association between *Borrelia* genospiecies and its locations.

B. miyamotoi with prevalence of 1.2 % in *I. ricinus* questing ticks along with ticks fed on *T. merula* birds in Slovakia (including Drienovec) was rarely detected. The highest prevalence of *B. miyamotoi* was in nymphs 0.7 %, followed by adults 0.4 %. The pathogen has been detected in approx. 1.87 % nymphs and 3.65 % adults in Europe (Kubiak et al. 2021). The results of overall prevalence are consistent with detection rates of *B. miyamotoi* in Europe (≈ 2 %), according to recent and earlier studies (Crowder et al. 2014; Geller et al. 2012; Hulínská et al. 2007; Kubiak et al. 2021). In study of Crowder et al. (2014), infection rates varied by countries and regions. For instance, prevalences in Czech Republic ranged from 0 % to 3.2 %. In this study, the values of infection rates were not that significant, with prevalences ranging from 0.1 % to 0.6 %. Infection rates of *B. miyamotoi* in recent studies of Slovakia ranged from 0. 75 % to 1 % (Hamšíková et al. 2017; Vaculová et al. 2019).

Pathogenicity and distribution of specific genospecies of *B. burgdorferi* s.l. has long been relatively well known (Dantas-Torres et al. 2012; Diterich & Hartung 2001; Mysterud et al. 2017; Rizzoli et al. 2014). *B. miyamotoi* has only been associated with pathogenicity since 2011, in a worldwide scale (Krause et al. 2013; Molloy et al. 2015; Platonov et al.

2011; Sato et al. 2014;), also including Europe (Hansford et al. 2015; Hovius et al., 2013; Jahfari et al., 2016). Since *B. miyamotoi* was considered to be the cause of one of the emerging tick-borne diseases (Cutler et al. 2019; Telford et al. 2015), this work has been also focused on the detection of this species in the collection of *I. ricinus* ticks in Slovakia. *B. miyamotoi* samples from *I. ricinus* tick collections were used in the study, including the 4 areas mentioned above, as well as from the Drienovec area, originating from the collection of another study (Mtierová et al. 2020). *B. burgdroferi* s.l. was also detected during the sampling for study of Mtierová (2020). Since the results of *B. burgdorferi* s.l. were part of previously-mentioned study, the prevalences were only briefly described and were not further included in the results of this work.

The overall mean prevalence of *B. burgdorferi* s.l. in Slovakia was 28.4 %. According to a meta-analysis of prevalence of *B. burgdorferi* s.l. in ticks of Europe including reports from 2010 to 2016, the prevalence was more than twice as high as the overall mean prevalence of *B. burgdorferi* s.l. (12.3 %) in Europe (Strnad et al. 2017). In the meta-analysis, the highest prevalence was evaluted for Central Europe (19.3 %), which is more in correlation with our results. The prevalence of adult ticks (14.5 %) was slightly higher thant that in nymphal ticks (14.0 %), a finding in accordance with other studies (Hubálek & Halouzka 1998; Rauter & Hartung et al. 2005; Strnad et al. 2017).

Out of the overall prevalence of *B. burgdorferi* s.l. genospecies, 12 tick samples showed unclear positivity of *B. burgdorferi* s.l. in the PCR analysis. Neither RFLP nor MLST analysis could reveal specific *Borrelia* genospecies of these samples. Therefore, it should be considered that the resulting diversity and prevalences of *Borrelia* genospecies may differentiate among the studied regions. Four genospecies were distributed in all studied areas of Slovakia, i.e., *B. afzelii*, *B. garinii*, *B. valaisiana* and *B. miyamotoi*. The extensive distribution of these genospecies is consistent with their reservoir hosts and their ability to migrate to such distant areas and thus spread the pathogen to other populations (Humair & Gern 2000; Kurtenbach et al. 2002; Gallais et al. 2018). *B. spielmanii* was confirmed in Bratislava and Malacky only. *B. burgdorferi* s.s. was confirmed only in Košice.

23 % of samples (total n = 104) were sequenced by MLST method for all eight housekeeping genes and specific STs were given. In some cases, positive samples were sequenced repeatedly if the first sequencing was unsuccessful for one of the forward or reverse directions.

STs were successfully given to following genospecies of *B. burgdorferi* s.l.: *B. afzelii* (8), *B. burgdorferi* s.s. (3), and *B. garinii* (10).

Three samples of *B. miyamotoi* were sequenced for all eight housekeeping genes. One new allele was discovered in *B. miyamotoi* (Appendix. 9). This paper reports the first STs of *B. miyamotoi* in Slovakia, while the new ST is the only evidence in the database so far.

Eight out 14 records (10 STs) of *B. afzelii* in total are added from this study to the pubmlst database, including two new STs. Each ST of *B. afzelii* in Slovakia is reported from specific areas only (STs are not located in multiple locations of Slovakia).

There were 5 records (3 STs) of *B. burgdorferi* s.s., including three samples of our study. All samples were from study sites of Košice.

Most samples were of *B. garinii* (38; 17 STs), including one new ST of our study. The new ST, ST953, was observed in two study sites, specifically, Bratislava, and Košice. There are two other cases of distribution of individual STs at multiple locations: ST902 of our study was detected in Košice and Malacky. Another ST, ST86, was observed in Drienovec and Martinské hole.

As the identification of *B. miyamotoi* in Europe has only recently taken place (Margos et al. 2015a; Răileanu et al. 2020; Iwabu-Itoh et al. 2017) and the sequencing method is not yet very widespread for this genospecies, it was necessary to design a protocol that would allow us to amplify and subsequently sequence a set of 8 house-keeping genes. The parameters of the amplification of specific genes varied.

In the case *of B. miyamotoi*, 3 isolates were sequenced, including two samples of the same allelic profiles. This genetic set was isolated from a tick fed on bird, *Turdus merula*, and from a questing tick in the same area, Drienovec. *Turdus merula* is able to migrate to a distance up to 2000 km, so there is a presumption that this genetic set has the potential to be distributed to distant areas (Schwabl 1983). A third isolate from Drienovec was assigned the same ST as several isolates from ticks in Germany. Since *B. miyamotoi* is transmitted not only by rodents but also by birds, the migratory routes of birds suggest an explanation for the distribution of this ST. Widespread distribution of individual genetic sets was observed within the MLST database also in other STs.

Isolates that revealed mixed infection were not further sequenced in remaining genes, while such samples are not included in the database. Nevertheless, several isolates contained

infection of more than one genospecies of *B. burgdorferi* s.l. complex, which appeared to be infected with one genospecies only by the RFLP method.

This study confirms that the MLST method is more time-consuming and costly than RFLP, but more accurate and reliable (Hathaway et al. 2007, Li et al. 2009).

Analysis of STs by the goeBURST approach of individual genospecies of *B. burgdorferi* s.l. and *B. miyamotoi* characterised the potential relatedness of individual isolates from different countries based on the similarity of allelic profiles.

Four out of five STs of *B. afzelii* detected in our study were assigned to six CCs, which included STs from countries of Western, Central, and Eastern Europe. One ST was characterised as a SG. In the minimum-spanning tree was observed one STs (ST540) of Slovakia from this study in association with samples from other countries (Germany, Slovenia) retrieved from human patients suffering from LB. The recorded data indicate a relatively high genetic diversity of *B. afzelii* in Central and Eastern Europe, given that of the 325 ST worldwide, 27.3% are from Central Europe and 24.9% are from Eastern Europe. Of the 26 STs distributed across Europe, five STs occurred in the Central and Eastern EU, suggesting that *B. afzelii* genotypes may "migrate" over long distances. One of these STs was registered in Serbia, Latvia, and Germany. Isolates of Germany came from a human patient suffering from LB. Therefore, the ST may be related to pathogenicity of *B. afzelii* as the connection of specific STs with pathogenic manifestations of Lyme disease is suggested (Jungnick et al. 2015). Another ST isolated from both ticks and patients occurred in Latvia, Germany, and France. Another ST was diagnosed from a human patient in Slovenia and was also isolated from a tick in Slovakia. Within Central Europe, *B. afzelii* samples extracted from human tissues (n = 123) have been detected in Germany, Austria, Poland, Slovenia, and Switzerland. Majority of samples from human sources were obtained in Germany). Genotypes of *B. afzelii* from human patients occured in 18 out of 27 CCs of Central Europe. Other associated countries within the CCs were Czech Republic and Slovakia. Although there is no evidence of human source of *B. afzelii* samples in the PubMLST database in range of Eastern Europe, several clonal complexes consist of data from Eastern Europe and STs found in human patients from other parts of Europe.

Full MST analysis suggested a higher coherence of STs of Russia and Mongolia based on the relatedness of allelic profiles. On the other hand, random distribution of STs was observed among Europe. The results are supported by previous studies, which assume that

the factors influencing the distribution are: transport, geographical barrier, and reservoir hosts (James et al. 2014; Pukhovskaya et al. 2019). Free distribution of STs was observed in terms of genetic variability among Europe, which is in correlation with study of Gallais et al. (2018).

Two STs of *B. burgdorferi* s.s. of our study were assigned to two individual CCs. ST20 of our study was detected also in countries among Europe and Canada. ST24 of the study was observed in Europe only. Both STs were detected also from samples from human patients (Germany) suffering from LB.

Vast majority of the samples (89.1 %) came from the northern America. At SLV level, two out of 13 groups consisted of European countries only. A group, consisting of the largest number of STs (94 STs), contained STs from both European and North American countries (Hanincová et al. 2006). The significantly higher number of STs, thus the genetic diversity, of *B. burgdorferi* s.s. in North America compared to Europe correlates with the fact that in Europe is a lower prevalence of the genospecies than in the USA and Canada (Marques et al. 2021).

Seven out of eight STs *of B. garinii* of this study were assigned to individual CCs. One ST was a SG. In the minimum-spanning tree were observed five STs (ST180, ST86, ST246, ST245, and ST251) of Slovakia from this study in association with samples from other countries of Europe retrieved from human patients suffering from LB. The recorded data indicate a relatively high genetic diversity of *B. garinii* in Central and Eastern Europe, given that of the 170 ST in Europe, 31.6 % are from Central Europe and 39.2 % are from Eastern Europe. Within Central Europe, *B. garinii* samples extracted from human tissues have been detected in Germany and Slovenia. Genotypes of *B. garinii* from human patients occured in eight out of 24 CCs of Europe.

In case of *B. garinii*, spatial mixing of STs of Europe was observed and Asian STs were in more distant clusters, as expected (Humair & Gern 2000; Kurtenbach et al. 2002; Munro et al. 2018; Miyamoto et al. 2002; Vollmer et al., 2011).

According to analysis of *B. miyamotoi*, there were six SGs in total and one clonal complex. The clonal complex consisted only of two STs of this study. One of the STs (ST635) was also detected in Germany. There were two SGs of STs of the USA, 3 SGs of STs of Japan, and one SG consisting of samples from Mongolia, Japan, and Russia only. As there were only 38 samples of *B. miyamotoi* in the pubmlst worldwide, it is not possible to

evaluate significant intraspecies relationships on genetic variability of STs of *B. miyamotoi* so far. Only one sample from the USA was detected in human patient.

Phylogenetic relationships of *B. burgdorferi* s.l. were analysed including isolates of *B. afzelii, B. garinii* and *B. burgdorferi* s.s. Isolates of *B. garinii* in this study clustered with other *B. garinii* samples at various levels, including STs distributed among all Europe. One isolate of our study of *B. burgdorferi* s.s. clustered with isolate from Canada and European countries. The second isolate clustered with ST from Latvia. STs of *B. afzelii* of this study clustered with STs among Europe, excluding one new ST of this study. The new ST (ST945) clustered with Asian and European STs. Nevertheless, the bootstrap value was only 31 %. The phylogenetic relationships are in correlation with other studies (Margos et al. 2018b; Sabitova et al. 2018).

As the number of RFG samples is still relatively low in the database, all available RFG genospecies from all over the world were included in the analysis of *B. miyamotoi*. The analysis based on sequences of eight housekeeping genes of *B. miyamotoi* divided the isolates into dinstinctive groups of Asia, North America, and Europe, resulting in monophyletic clade among RF group of *Borrelia*. The results correllates with results of Kuleshov et al. (2020). Nevertheless, it is assumed that genotypes of *B. miyamotoi* are probably not associated with geographic origin, but with host range, vector competence, and pathogenicity (Barbour 2014; Krause et al. 2015).

7. CONCLUSIONS

The MLST approach has been shown to be suitable for the detection of *B. burgdorferi* s.l. complex. Moreover, it is possible to share data and subject them to the required analyses thanks to a globally accessible database. In our study, STs (n = 24) of three genospecies of *B. burgdorferi* s.l. (*B. afzelii*, *B. garinii*, *B. burgdorferi* s.s.) and *B. miyamotoi* belonging to the RFG of the genus *Borrelia* were identified in Slovakia (Appendix. 9). Individual genospecies were analysed by population genetic analysis using goeBURST and Full MST approach, along with data obtained from the database.

Full MST analysis of *B. afzelii* revealed free distribution of STs of this study (detected in Slovakia) among Europe. In general, a free distribution of *B. afzelii* genotypes was observed across Europe with an indication of clustering of previously detected genotypes in Russia. No visible clustering of Central and Eastern European samples of *B. burgdorferi* s.s. of our study (and in general) was observed, which correlates with previous studies. MST analysis of *B. burgdorferi* s.s. formed two distinct clusters of European STs, while the majority of STs of the analysis were detected in northern America. The prevalence of samples from northern America was significantly higher than European ones. Spatial mixing of STs *B. garinii* of our study (and in general) within different regions of Central and Eastern Europe was observed as expected. Asian STs were formed into clusters, divided from European clusters. Several genotypes of *B. afzelii* (ST540), *B. burgdorferi* s.s. (ST20, ST24), and *B. garinii* (ST180, ST245, ST246, and ST251), of this study may be associated with LB, as they have been previously detected in samples from patients of LB in various countries.

It is not yet significantly possible to determine the intraspecies relationships of *B. miyamotoi* genetic variability using MLST due to the low number of samples in the database so far. However, this study has so far contributed the first isolates from Slovakia into the database, thus it is the third country of Eurasia in the database with completely sequenced records of *B. miyamotoi* for all 8 housekeeping genes. There is no evidence of specific genotype of *B. miyamotoi* detected in Slovakia (or Europe, in general) to be obtained from human patient.

Phylogenetic analysis of *B. burgdorferi* s.l. supports the results of previous population-genetic analyses of this study in terms of genetic variability and its distribution across

Europe, in the case of *B. burgdorferi* s.s. with regard to worldwide distribution. In the case of *B. miyamotoi*, the relationships correlate with the previous studies.

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9. APPENDICES

Appendix 1. List of the currently valid genospecies of *Borreliae* divided into three groups: LG, REPG, and tick-borne RFG with the specific vectors and hosts included in their life cycle, and geographical distribution. In addition, RFG category is divided into subgroups according to tick-vector transmission of individual genospecies, namely, STBRF, HTBRF, LBRF, and the relapsing fever group associated with avian hosts.

Borrelia species	Vector (Ixodes tick)	Host (Reservoir)	Geographic distribution	Reference
Lyme group				
B. afzelii	*Ixodes persulcatus, I. ricinus*	rodents	Europe, Asia	Canica et al. 1993
B. americana	*I. minor, I. pacificus*	birds	United States	Rudenko et al. 2011
B. andersonii	*I. dentatus*	cottontail rabbit	United States	Marconi et al. 1995
B. bavariensis	*I. ricinus*	rodents	Europe, North America	Margos et al. 2009; Munro et al. 2017
B. bissettiae	*I. pacificus, I. spinipalpis, I. affinis, I. auritulus*	rodents	Europe, United States	Margos et al. 2016
B. burgdorferi s.s.	*I. pacificus, I. ricinus, I. scapularis*	birds, lizards, big mammals, rodents	Europe, United States	Baranton et al. 1992
B. californiensis	*I. pacificus, I. spinipalpis, I. jellisoni*	kangaroo rat, mule deer	United States	Postic et al. 2007
B. carolinensis	*I. minor*	birds, rodents	United States	Rudenko et al. 2009a
B. chilensis	*I. stilesi*	long-tailed rice rats	Chile, South America	Ivanova et al. 2014

B. finlandensis	*I. ricinus*	unknown	Europe	Casjens et al. 2011
B. garinii	*I. ricinus, I. persulcatus*	birds, lizards, rodents	Europe, Asia, Arctic-Antarctic circles	Baranton et al. 1992
B. ibitipoquensis	*I. paranaensis*	birds	Brazil	Muñoz-Leal et al. 2020
B. japonica	*I. ovatus*	rodents	Japan	Kawabata et al. 1993
B. kurtenbachii	*I. scapularis*	rodents	Europe, United States	Margos et al. 2010
B. lanei	*I. pacificus, I. spinipalpis*	rabbits	United States	Margos et al. 2017a
B. lusitaniae	*I. ricinus*	lizards, rodents	Europe, North Africa	Le Fleche et al. 1997
B. mayonii	*I. scapularis*	unknown	midwestern United States	Pritt et al. 2016
B. sinica	*I. ovatus*	rodents	China	Masuzawa et al. 2001
B. spielmanii	*I. ricinus*	rodents	Europe	Richter et al. 2006
B. tanukii	*I. tanuki*	unknown-possibly dogs, cats	Japan	Fukunaga et al. 1996
B. turdi	*I. turdus*	birds	Japan	Fukunaga et al. 1996
B. valaisiana	*I. granulatus, I. ricinus*	birds, lizards	Europe, Asia	Wang et al. 1997
B. yangtzenzis	*I.granulatus, Haemaphysalis longicornis*	rodents	China	Chu et al. 2008; Margos et al. 2015b

Relapsing fever group -STBRF				
B. armenica	*Ornithodoros verrucosus*	rodents	Eastern Europe	Filatov et al. 2020
B. babylonensis	*O. verrucosus*	rodents	Eastern Europe, Russia	Goubau et al. 1984
B. baltazardii	*O. tholozani*	unknown	Iran	Karimi et al. 1979; Moradi-Asl et al. 2020
B. brasiliensis	*O. brasiliensis*	dogs, armadillos	Southern Brazil	Reck et al. 2013
B. caucasica	*O. verrucosus*	unknown	Southeast Europe, western Asia	Filatov et al. 2020; Kandelaki 1935
B. coriacea	*O. coriaceus*	deer, dogs	Western North America	Russell et al. 1987
B. crocidurae	*O. marocanus*, *O. erraticus*, *O. sonrai*	rodents, shrews	Western, Northern Africa	Jakab et al. 2022; Souidi et al. 2014
B. dugesii	*O. dugesii*	rodents	Mexico	Guzmán-Cornejo et al. 2019
B. duttonii	*O. moubata*	chicken, pigs	Central, Eastern, and Southern Africa	Diatta et al. 2012; Holten et al. 1997; Obolo-Mvoulouga et al. 2018

B. graingeri	*O. graingeri*	unknown	Kenya	Heisch 1953
B. hermsii	*O. hermsi*	rodents, squirrels	USA, Canada	Ogden et al. 2014
B. hispanica	*O. erraticus, O. marocanus, O. occidentalis*	mammals (cats, dogs, cattle, hedgehogs, pigs, rodents, sheep, warblers	Iberian Peninsula and Northern Africa	Heida et al. 2019, Jakab et al. 2022; Nordstrand et al. 2007
B. latyschewii	*O. tartakovsky*	birds	Iran, Middle East	Baltazard et al. 1955; Jakab et al. 2022
B. mazzottii	*O. talaje*	rodents	Mexico, Central America, and western USA	Davis 1956; Jakab et al. 2022
B. merionesi	*O. costalis*, *O. merionesi*	rodent, monkeys	Morocco and Atlantic coastal areas of the Sahara desert	Diatta et al. 2012; Trappe et al. 2013

B. microtti	*O. erraticus*	Hedgehogs, rodents, toads	Iran, Afghanistan, Eastern Africa	Assmar et al. 2002; Jakab et al. 2022
B. osphepa	*O. ophenicus*	unknown	South America	Jakab et al. 2022
B. parkeri	*O. parkeri*	horses	Western US	Davis 1962; Gage et al. 2001; Jakab et al. 2022
B. persica	*O. tholozani*	Dogs, cats	Central Asia, Middle East, Egypt, and India	Colin de Verdiere et al. 2011; Baneth et al. 2016
B. queenslandica	*O. gurneyi*	rodents, kanga roos	Australia	Hussain-Yusuf et al. 2020
B. sogdiana	*O. papillipes*	rodents	Uzbekistan, Kyrgyzstan	Jakab et al. 2022
B. tillae	*O. zumpti*	rodents	Southern Africa	Geigy & Aeschlimann 1965
B. turicatae	*O. turicata*	birds, mammals, reptiles	British Columbia (Canada), southwestern and south-central US and	Donaldson et al. 2016; Jakab et al. 2022; Krishnavajhala et al. 2018

			Mexico	
B. turcica	*Amblyomma aureolatum*, *A. longirostre*, *Hy. aegyptium*	birds, camels, cattle, tortoises	Greece, Turkey	Jakab et al. 2022
B. venezualensis	*O. rudis*	unknown	Central America and northern South America, Venezuela, Brazil, Colombia, Panama	Muñoz-Leal et al. 2018
Candidatus B. johnsonii	*Carios kelleyi*	bats	Canada, US, China, Mexico, Cuba, Costa Rica, Chile	Kingry et al. 2018; Li et al. 2021; Nadolny et al. 2021
C. B. kalaharica	*O. savignyi*	unknown	Africa (Kalahari desert)	Cutler et al. 2018

Relapsing fever group -HTBRF

B. lonestari	*Am. americanum*	deer, migratory birds, wild	USA (Missouri, California)	Allan et al. 2010; Moyer et al. 2006;

		turkeys		Krishnavajhala et al. 2017
B. miyamotoi	*I. persulcatus*, *I. ricinus*, *I. scapularis*, *I. pacificus*	rodents, birds	Asia, Europe, USA	Fukunaga et al. 1995; Gugliotta et al. 2013
B. theileri	*Rhipicephalus* sp., *Marg aropus australis*	cattle, sheep, goats, horses	Africa, Australia, Brazil, northern South America	Callow 1967; Guglielmone et al. 2014; Laveran 1903; Theiler 1905
Avian associated relapsing fever group				
B. anserina	*Argas* sp.	Birds	Worldwide	Sakharoff 1891; Thomas et al. 2002

Echidna-reptile group				
B. turcica	*Hy. aegyptium*	Reptiles-tortoises	Southeastern Europe, Japan	Guner et al. 2003; Kalmar et al. 2015
B. tachyglossi	*Bothriocroton concolor*	Mammals-echidnas	Australia	Loh et al. 2016

Infectious potential for humans

Uncertain infectious potential for humans

Non-infectious genospecies for humans

Appendix 2. Diagnostically significant OsP proteins of *Borrelia* (retrieved and updated according to Zajkowska et al. 2014).

protein	Size [kDa]
OspA	34
OspB	31
OspC	21-24
OspE	19,2
OspF	26,1
BmpA	39
P83/100	83 -100
BBA 36	24
BBO 323	42
Crasp 3	21
VIsE	35
pG	22
HSP60/75	60 -75
P41	41
P41 int	14
P66	-

BBK32/p35	-
FlaA	37 -38
BBK50/p37	-
DbpA/Osp17	17 -18
DbpB	-
OspE	19,2
P21	-
P22	-
OspF	26,1
P30	-
OspA	34
OspB	31
P14/18/28/58/93	-

Appendix 3. List of the specific samples used in the phylogenetic analysis of *B. burgdorferi* s.l. including information about the country of origin, exact species, source, and ST of the samples.

ID	**Country**	**Species**	**Source**	**ST**
3165	Slovakia	*B. afzelii*	tick	170
3166	Slovakia	*B. afzelii*	tick	540
3167	Slovakia	*B. afzelii*	tick	944
3168	Slovakia	*B. afzelii*	tick	945
3169	Slovakia	*B. afzelii*	tick	463
1618	Germany	*B. afzelii*	human	171
1579	Slovenia	*B. afzelii*	human	570

2493	Finland	*B. afzelii*	tick	894
1888	Austria	*B. afzelii*	human	75
2098	France	*B. afzelii*	human	554
2590	Ukraine	*B. afzelii*	tick	908
1167	Italy	*B. afzelii*	human	337
248	Serbia	*B. afzelii*	human	474
2132	France	*B. afzelii*	human	781
1751	Russia	*B. afzelii*	tick	599
2674	Switzerland	*B. afzelii*	tick	72
1653	Germany	*B. afzelii*	human	466
3164	Kazakhstan	*B. afzelii*	tick	940
220	China	*B. afzelii*	tick	105
2480	Czech Republic	*B. afzelii*	tick	215
2495	Finland	*B. afzelii*	tick	896
2588	Ukraine	*B. afzelii*	tick	906
3176	Slovakia	*B. garinii*	tick	180
3177	Slovakia	*B. garinii*	tick	246
3178	Slovakia	*B. garinii*	tick	86
3179	Slovakia	*B. garinii*	tick	245
3181	Slovakia	*B. garinii*	tick	243
3182	Slovakia	*B. garinii*	tick	953
3183	Slovakia	*B. garinii*	tick	251
3184	Slovakia	*B. garinii*	tick	185
58	Germany	*B. garinii*	human	574
1123	Japan	*B. garinii*	human	127
1977	Former Yugoslavia	*B. garinii*	human	87
62	Serbia	*B. garinii*	tick	132
358	Latvia	*B. garinii*	tick	163
211	China	*B. garinii*	tick	126
1055	UK	*B. garinii*	tick	325
2451	Czech Republic	*B. garinii*	tick	177
1791	Russia	*B. garinii*	tick	612
1916	Canada	*B. garinii*	tick	575

2583	Ukraine	*B. garinii*	tick	175
2513	Czech Republic	*B. garinii*	tick	743
2463	The Netherlands	*B. garinii*	tick	746
2473	Finland	*B. garinii*	tick	749
2475	Estonia	*B. garinii*	tick	750
1985	Germany	*B. garinii*	human	482
2491	Finland	*B. garinii*	tick	89
2683	Switzerland	*B. garinii*	human	578
3175	Slovakia	*B. burgdorferi* s.s.	tick	20
3173	Slovakia	*B. burgdorferi* s.s.	tick	24
83	USA	*B. burgdorferi* s.s.	human	1
1553	Slovenia	*B. burgdorferi* s.s.	human	546
1702	Germany	*B. burgdorferi* s.s.	human	21
1066	Italy	*B. burgdorferi* s.s.	human	332
961	Canada	*B. burgdorferi* s.s.	tick	314
1154	Austria	*B. burgdorferi* s.s.	tick	21
45	France	*B. burgdorferi* s.s.	tick	24
48	Switzerland	*B. burgdorferi* s.s.	tick	26
1152	Italy	*B. burgdorferi* s.s.	human	27
341	Latvia	*B. burgdorferi* s.s.	tick	161
1030	UK	*B. burgdorferi* s.s.	tick	285
2585	Ukraine	*B. burgdorferi* s.s.	tick	904
1818	Serbia	*B. valaisiana*	tick	203
2671	Slovakia	*B. valaisiana*	tick	212
2802	Russia	*B. valaisiana*	tick	97
2803	Switzerland	*B. valaisiana*	tick	95
302	UK	*B. valaisiana*	tick	201
1523	Norway	*B. valaisiana*	tick	517
2085	Germany	*B. valaisiana*	tick	213
2801	The Netherlands	*B. spielmanii*	human	159
475	Germany	*B. spielmanii*	tick	239
136	Portugal	*B. lusitaniae*	human	69
1182	Austria	*B. lusitaniae*	tick	346

2500	Croatia	*B. lusitaniae*	tick	900
3084	Latvia	*B. lusitaniae*	tick	851
1815	Serbia	*B. lusitaniae*	tick	628
2653	Slovakia	*B. lusitaniae*	tick	919
1120	Japan	*B. bavariensis*	human	128
2702	Denmark	*B. bavariensis*	human	84
2593	Ukraine	*B. bavariensis*	tick	85
1902	Canada	*B. bavariensis*	tick	685
204	China	*B. bavariensis*	tick	118
1840	Russia	*B. bavariensis*	tick	125
1064	Mongolia	*B. bavariensis*	tick	128
1287	USA	*B. bissettiae*	tick	457
1874	Germany	*B. bissettiae*	human	667
2799	USA	*B. mayonii*	human	674
1886	USA	*B. mayonii*	human	675
2763	USA	*B. kurtenbachii*	tick	280
898	Canada	*B. kurtenbachii*	tick	281
2804	Chile	*B. chilensis*	tick	430

 ST detected in Slovakia in this study

Appendix 4. List of the specific borrelial samples used in the phylogenetic analysis of *Borrelia miyamotoi* within RFG of *Borrelia*, including information about the country of origin, exact species, source, and ST of the samples. *B. burgdorferi* s.s. belonging to the *B. burgdorferi* s.l. complex was chosen for outgroup.

ID	Country	Species	Source	ST
2020	Japan	*B. miyamotoi*	tick	680
2021	Japan	*B. miyamotoi*	tick	681
2023	Japan	*B. miyamotoi*	tick	682
1898	USA	*B. miyamotoi*	tick	683
2066	USA	*B. miyamotoi*	human	634
3188	Slovakia	*B. miyamotoi*	tick	956
3186	Slovakia	*B. miyamotoi*	tick	635
1279	Germany	*B. miyamotoi*	tick	635
2011	Mongolia	*B. miyamotoi*	tick	633
1878	USA	*B. coriaceae*	tick	671
56	USA	*B. hermsii*	human	104
1877	USA	*B. parkeri*	animal	670
57	USA	*B. turicatae*	tick	116
1876	Ethiopia	*B. recurrentis*	human	669
1280	Uzbekistan	*B. persica*	tick	636
2485	Morocco	*B. hispanica*	tick	805
1879	Mauritania	*B. crocidurae*	tick	672
1688	Germany	*B. burgdorferi* s.s.	human	3

ST detected in Slovakia in this study

Appendix 5. Dates of collections of ticks at specific study sites.

Season	Area			
	Bratislava	**Košice**	**Malacky**	**Vrbovce**
Spring	25.4.	3.5.	25.4.	30.4.
Summer	-		1.6.	20.5
Autumn	2.10.	october	11.10.	4.10.
				26.10.

Appendix 6. Number of positive cases of exact *B. burgdorferi* s.l. genospecies found in *I. ricinus* ticks in individual life stages in Bratislava.

Borrelia genospecies		**Life stage of a tick**			**Total N°**
		Nymph[a]	**Adult**		
			Male[b]	**Female[c]**	
Exact genospecies	*B. afzelii*	8	1	9	18
	B. garinii	2	2	2	6
	B. valaisiana	-	3	1	4
	B. spielmanii	-	1	2	3
Uncertain	1	-	2	3	

N°- total number of positive cases of exact genospecies, a–33 nymphs in total (positive + negative cases), b-32 males in total (positive + negative cases), c-54 females in total (positive + negative cases)

Appendix 7. Number of positive cases of exact *B. burgdorferi* s.l. genospecies found in *I. ricinus* ticks in individual life stages in Košice.

Borrelia genospecies		**Life stage of a tick**			**Total N°**
		Nymph[a]	**Adult**		
			Male[b]	**Female[c]**	
Exact genospecies	*B. afzelii*	2	3	5	10
	B. burgdorferi s.s.	1	3	4	8
	B. garinii	7	14	17	38
	B. valaisiana	3	7	7	17
Co-infection	*B. garinii/ B. valaisiana*	-	1	2	3
Uncertain genospecies		2	3	1	6

N°- total number of positive cases of exact genospecies, a-51 nymphs in total (positive + negative cases), b-78 males in total (positive + negative cases), c-87 females in total (positive + negative cases)

Appendix 8. Number of positive cases of exact *B. burgdorferi* s.l. genospecies found in *I. ricinus* ticks in individual life stages in Malacky.

Borrelia genospecies		**Life stage of a tick**			**Total N°**
		Nymph[a]	**Adult**		
			Male[b]	**Female[c]**	
Exact genospecies	*B. afzelii*	29	-	1	30
	B. bavariensis	1	-	-	1
	B. garinii	9	-	1	10
	B. spielmanii	-	-	1	1
Co-infection	*B. garinii/ B. valaisiana*	1	-	-	1
Uncertain genospecies		1	-	-	1

a-195 nymphs in total (positive + negative cases), b–9 males in total (positive + negative cases), c–12 females in total (positive + negative cases)

Appendix 9. Number of positive cases of exact *B. burgdorferi* s.l. genospecies found in *I. ricinus* ticks in individual life stages in Vrbovce.

Borrelia genospecies		**Life stage of a tick**			**Total N°**
		Nymph[a]	**Adult**		
			Male[b]	**Female[c]**	
Exact genospecies	*B. afzelii*	19	-	1	20
	B. garinii	2	-	-	2
	B. valaisiana	2	-	-	2
Uncertain genospecies		2	-	-	2

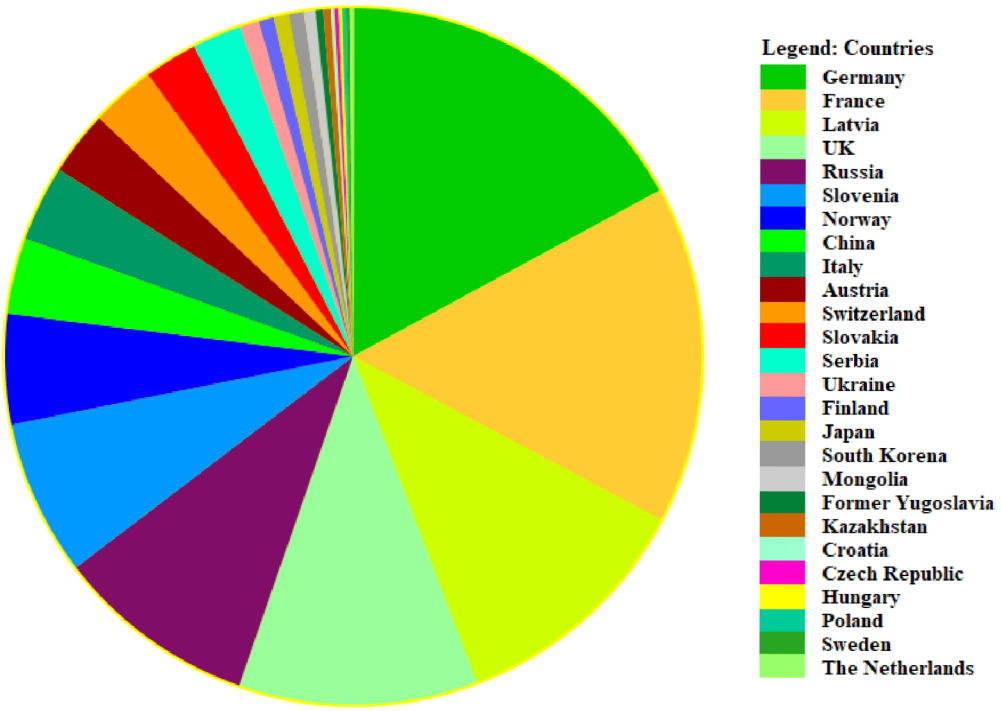
a–86 nymphs in total, b–9 males in total, c–7 females in total

Appendix 10. Sequenced samples for all 8 housekeeping genes of this study with given ST numbers.

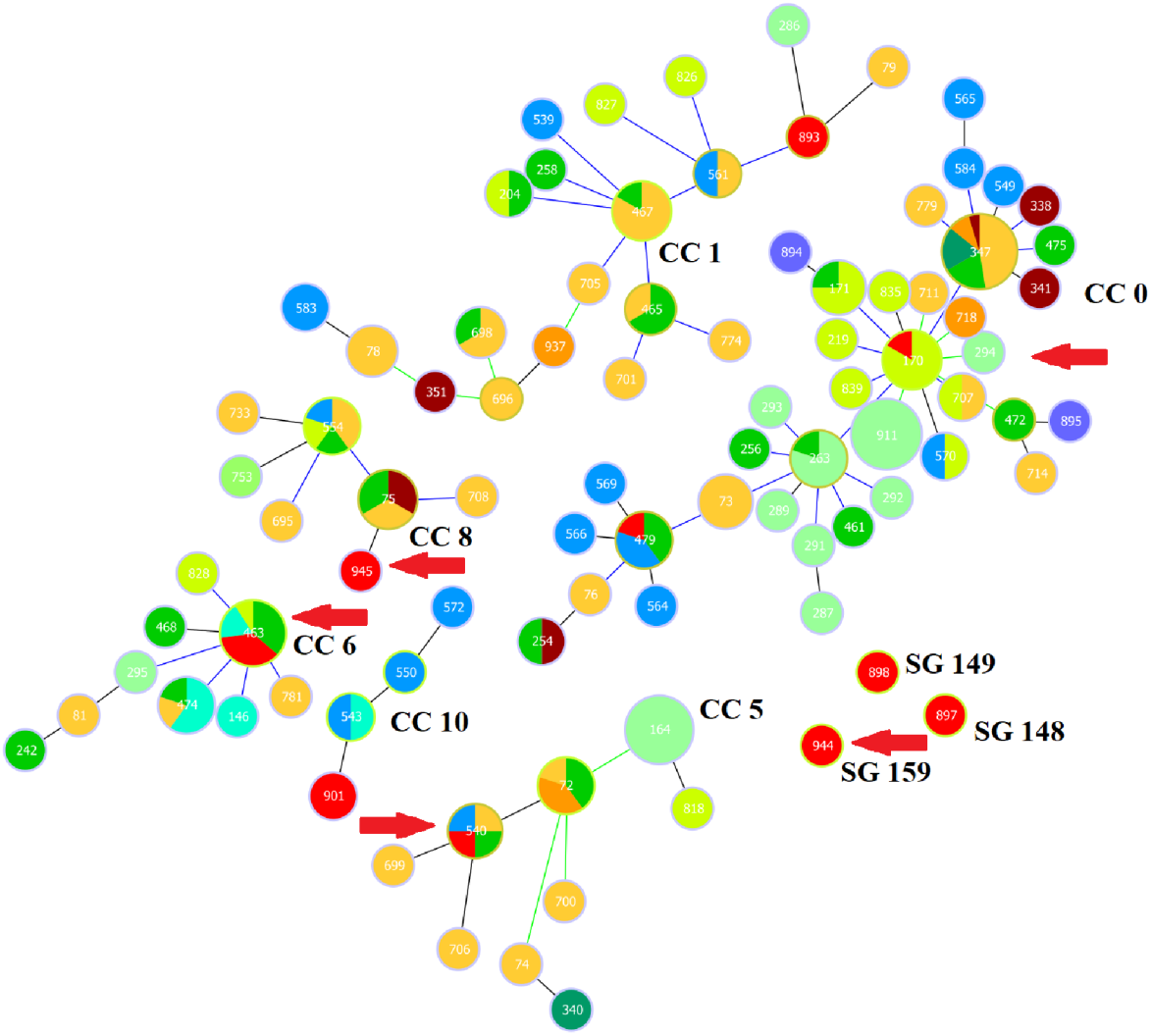
+genopecies	isolate	region	stage / host	clpA	clpX	nifS	pepX	pyr	recG	rplB	uvrA	ST
B. afzelii	MaN25417/12	Malacky	nymph	37	24	24	31	22	92	23	28	170
	KM3517/14	Košice	male	35	24	24	30	21	27	23	41	540
	VN30417/24	Vrbovce	nymph	109	82	24	85	119	27	23	29	944
	VN30417/16	Vrbovce	nymph	36	24	23	31	22	30	23	30	945
	ZSM25417/4	Bratislava	male	38	24	25	32	90	29	24	28	463
	ZSF25417/70	Bratislava	female	38	24	25	32	90	29	29	28	463
	ZSF25417/52	Bratislava	female	38	24	25	32	90	29	24	28	463
	ZSN25417/85	Bratislava	nymph	38	24	25	32	90	29	24	28	463
B. burgdorferi **sensu stricto**	KN3517/75	Košice	nymph	15	9	12	8	1	11	8	16	24
	KF3517/148	Košice	female	14	1	11	1	1	1	1	10	20
	KM3517/172	Košice	male	14	1	11	1	1	1	1	10	20
B. garinii	MaN25417/11	Malacky	nymph	43	28	30	90	87	36	28	34	180
	MaN25417/86	Malacky	nymph	112	80	78	99	81	39	79	87	246
	MaN25417/14	Malacky	nymph	42	27	29	38	29	36	27	33	86
	MaN111017/2	Malacky	nymph	99	77	81	91	88	84	82	33	245
	KF3517/53	Košice	female	42	27	29	38	29	36	27	33	86
	KF3517/45	Košice	female	46	76	29	43	34	42	31	37	243
	KF3517/47	Košice	female	95	29	31	40	31	37	29	35	953
	KM3517/22	Košice	male	95	74	34	96	89	78	77	85	251
	ZSF25417/69	Bratislava	female	44	29	31	40	31	87	80	77	185
	KM3517/19	Košice	male	95	74	34	96	89	78	77	85	251
B. miyamotoi	MaN25417/14	Malacky	nymph	201	167	152	174	185	192	161	66	635
	DNQ107	Drienovec	nymph	201	167	152	174	185	296	161	66	956
	1DIVN4	Drienovec	nymph / *Turdus merula* ♂	201	167	152	174	185	296	161	66	956

new allele new sequence type

Appendix 11. Legend of countries in goeBURST analyses for *B. afzelii* samples (created in Phyloviz 2, edited in GIMP version 2.10.30).

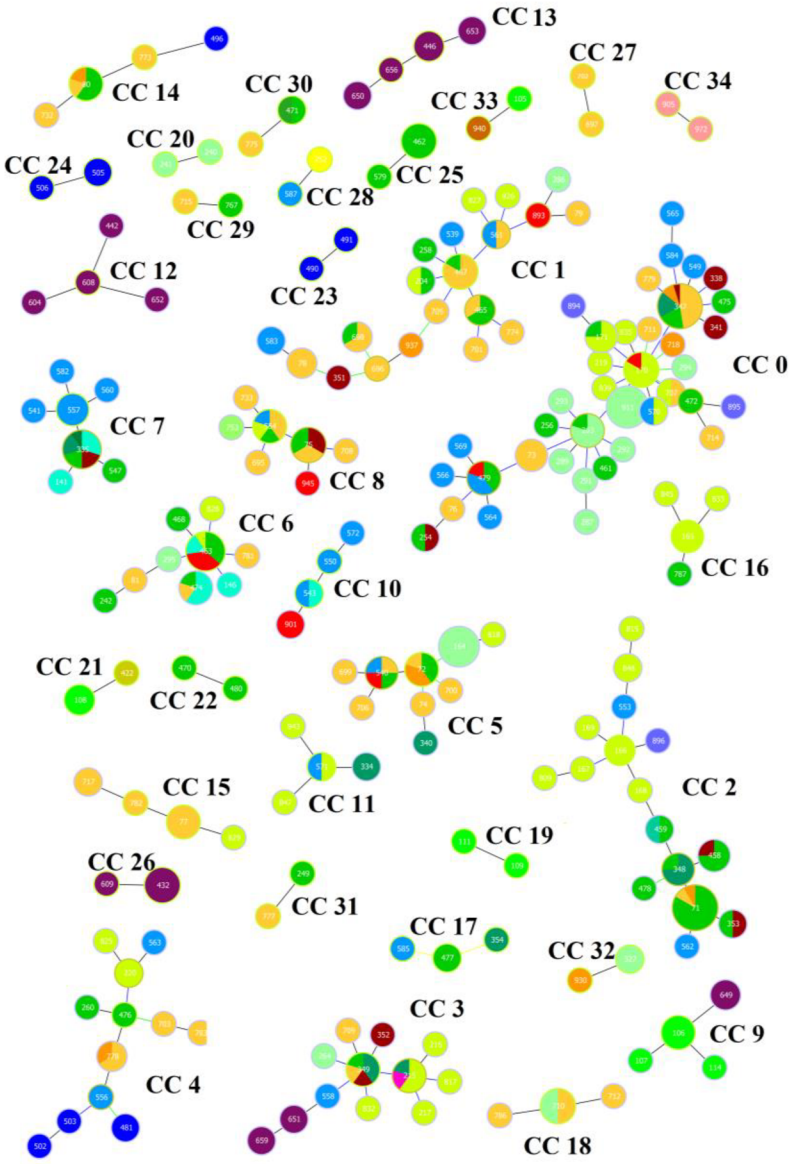


Appendix 12. Population structure of *B. afzelii* genotypes including STs from Slovakia at SLV level using goeBURST algorithm. The scheme includes all isolates of *B. afzelii* of Slovakia (including previously deposited isolates to the database) (created in Phyloviz 2, edited in GIMP version 2.10.30).



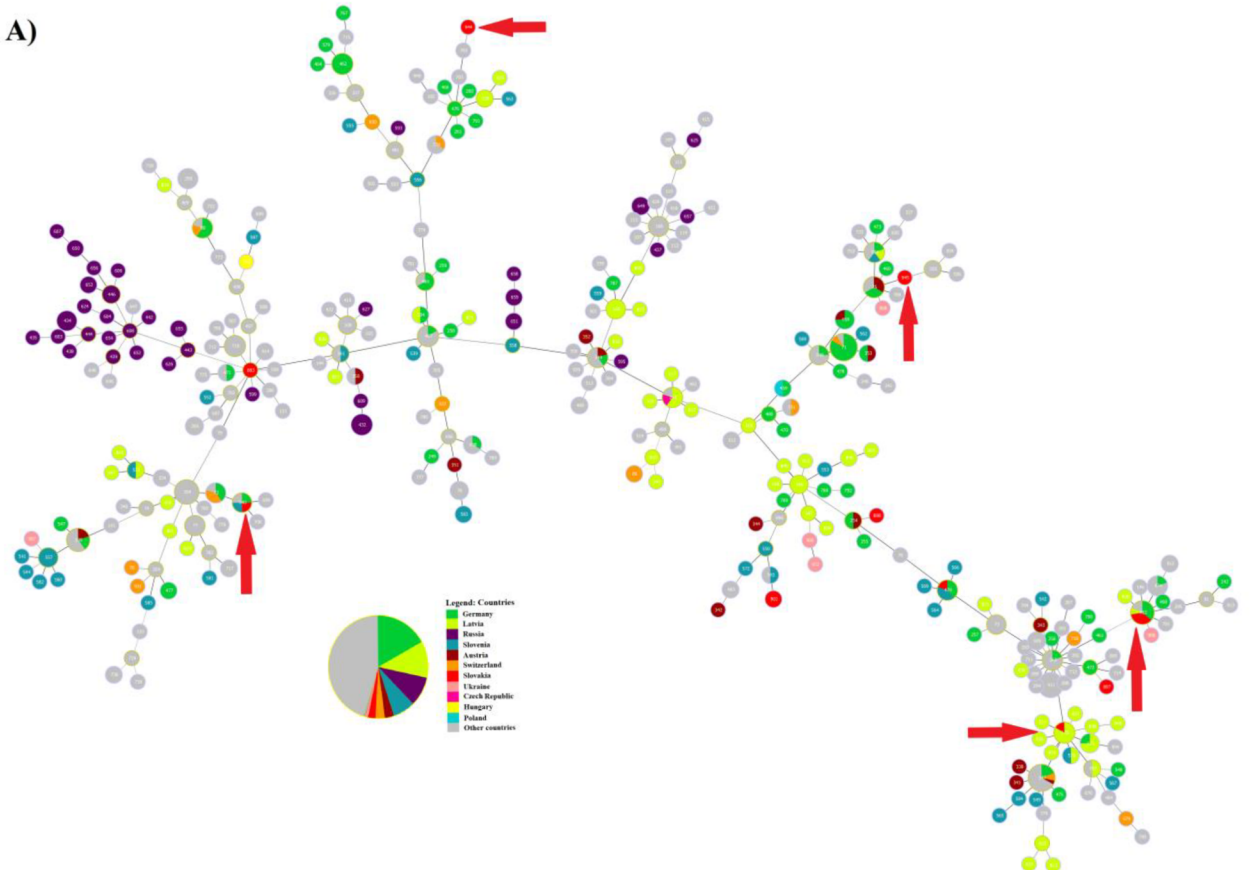
The links between nodes are of black, blue or green colour. Black colour displays link drawn without recourse to tiebreak rules. Blue-coloured links are of tiebreak rule 1 (number of SLVs). Links drawn in green are using tiebreak rule 2 (number of DLVs). Node size indicates the number of samples characterised by specific ST (number in a node). Node edge is coloured in light blue, light green or dark green, which reflect a common node, group founder or sub-group founder, respectively. Node colours reflect the origin (country) of the genotype assigned in MLST database. Red coloured nodes reflect Slovakia. Red arrows point to STs found in this study. CC- clonal complex, SG- singleton

Appendix 13. Population structure of *B. afzelii* isolates from the MLST database (including isolates of our study) at SLV level using goeBURST algorithm (created in Phyloviz 2, edited in GIMP version 2.10.30).

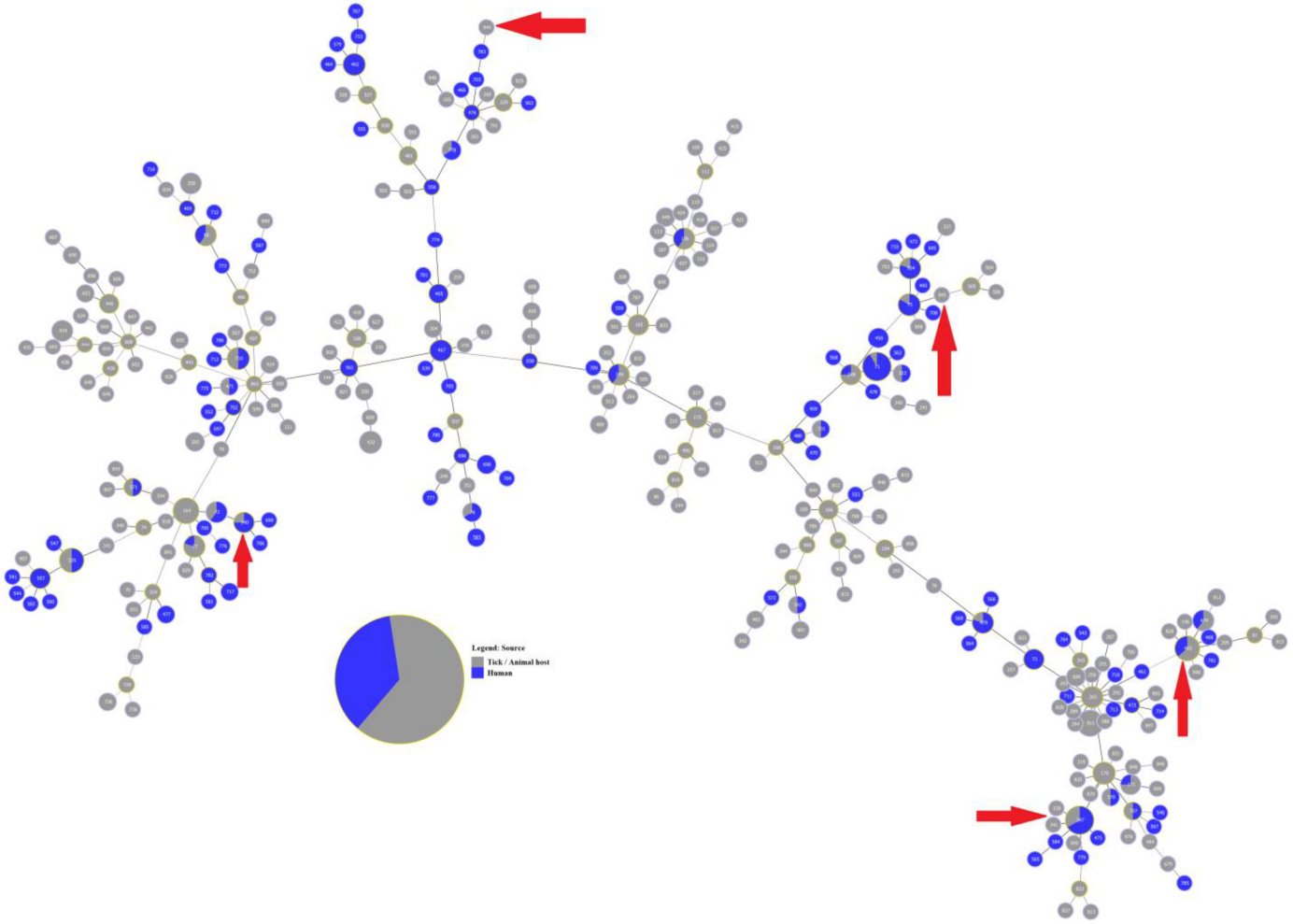


Colours of individual links reflect following data: black- without recourse to tiebreak rules, blue- based on tiebreak rule 1 (number of SLVs), yellow- based on tiebreak rule 4 or 5 (frequency found on the data set and ST number, respectively), green- based on tiebreak rule 2 (number of DLVs). Node size- reflects the number of samples characterised by specific ST. Edges of nodes are coloured in light green (group founder), dark green (sub-group founder) or light blue (common node). Node colours indicate the country of origin of the sample assigned in MLST database. SGs are not shown in the scheme. CC- clonal complex

Appendix 14. Minimum-spanning tree of *B. afzelii* STs (algorithm: goeBURST Full MST, maximum level of locus variants (7)) from the MLST datatabe (including isolates of our study) with highlighted nodes occurring in Central and Eastern Europe (A) and a scheme of *B. afzelii* genotypes found in human patients suffering from LD (B) (created in Phyloviz 2, edited in GIMP version 2.10.30).

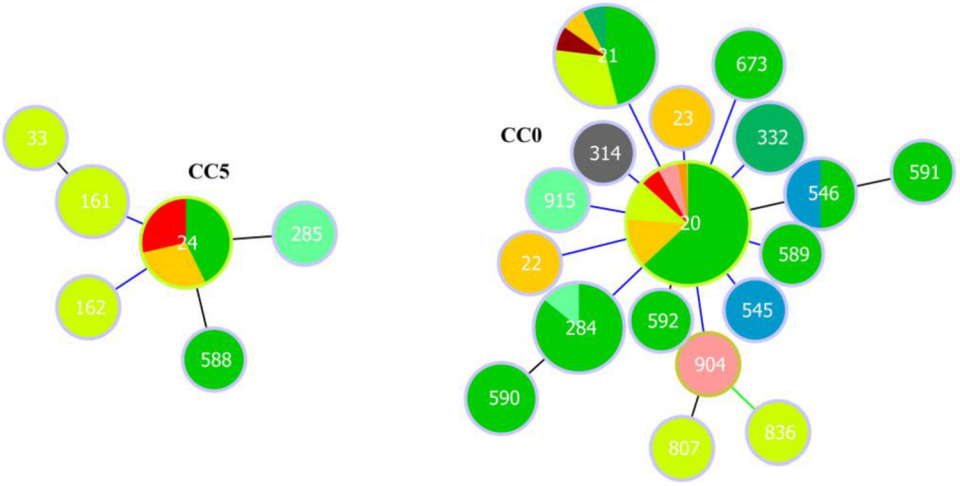


B)



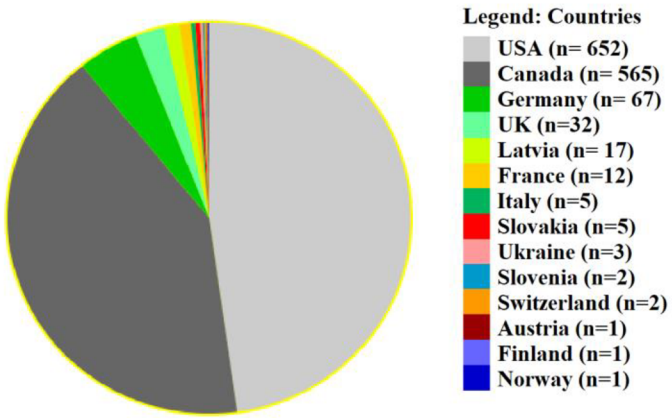
The structure of trees is identical as in the above-mentioned schemes (Appendix 11 and 12). A) Coloured parts within the nodes reflect the number of samples for the given countries. B) Blue colour within nodes is showing the number of samples obtained from human patients of LB for the given STs. A – B) Size of nodes reflects the number of samples characterised by specific ST. The links between nodes are of black (less difference between nodes) or grey (much difference between nodes) colour. Node edges are of dark green (sub-group founder) and light blue (common node) colour. Red arrows are showing STs found in this study.

Appendix 15. Population structure of *B. burgdorferi* s.s genotypes including STs from Slovakia (including isolates of our study) at SLV level using goeBURST algorithm (created in Phyloviz 2).



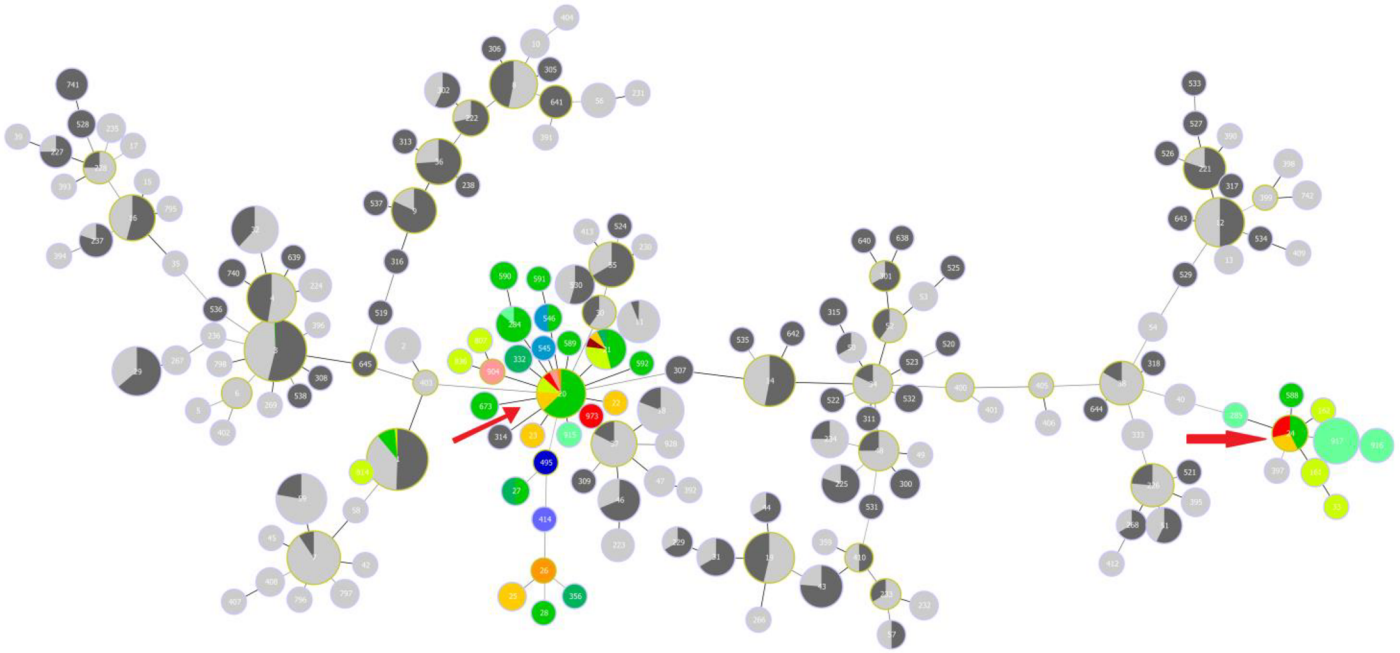
The links between nodes are of black, blue or green colour. Black colour displays link drawn without recourse to tiebreak rules. Blue-coloured links are of tiebreak rule 1 (number of SLVs). Links drawn in green are using tiebreak rule 2 (number of DLVs). Node size indicates the number of samples characterised by specific ST (number in a node). Node edge is coloured in light blue, light green or dark green, which reflect a common node, group founder or sub-group founder, respectively. Node colours reflect the origin (country) of the genotype assigned in MLST database. Legend of countries is shown in Appendix 16. Red coloured nodes reflect STs found in this study. CC- clonal complex, SG- singleton

Appendix 16. Legend of countries in goeBURST analyses for *B. burgdorferi* s.s. samples (created in Phyloviz 2, edited in GIMP version 2.10.30).



n-number of samples

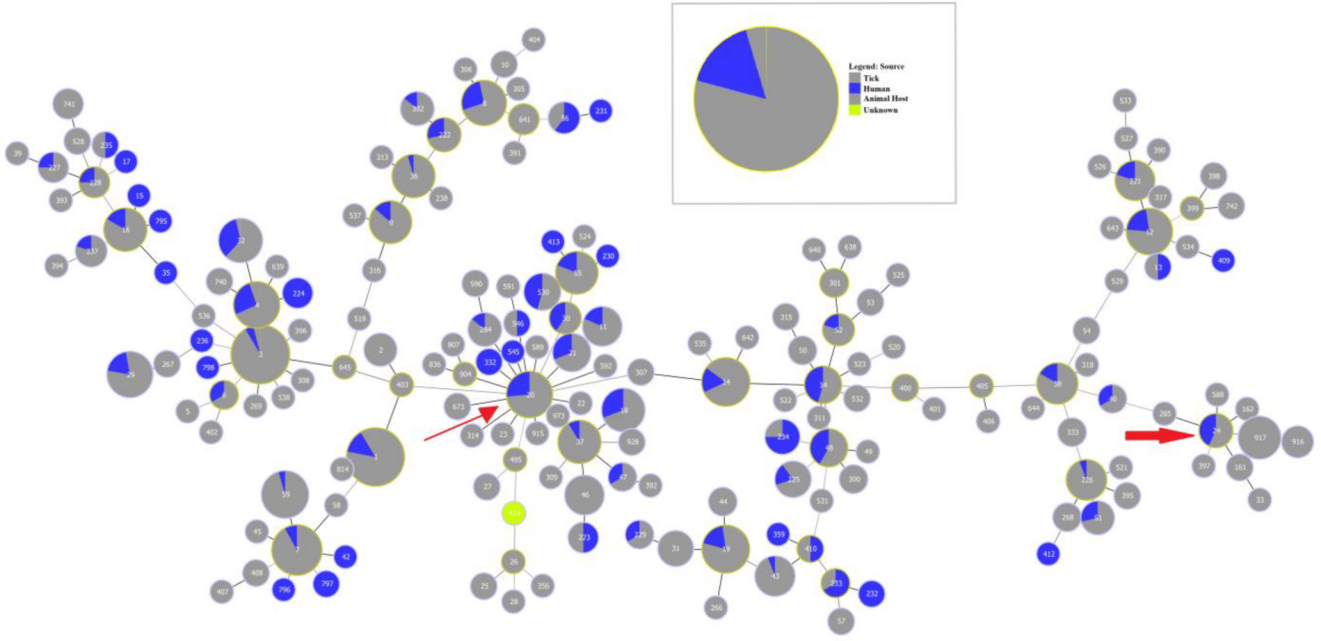
Appendix 17. Minimum-spanning tree of *B. burgdorferi* s.s. genotypes of the MLST database (including isolates of our study) (created in Phyloviz 2, edited in GIMP version 2.10.30).



The goeBURST Full MST algorithm was build up at the maximum level of locus variants, i.e., 8 levels. The number of samples assigned to specific ST is visualised by node size. The links between nodes are in range of black or grey colour. Black-coloured links reflect fewer

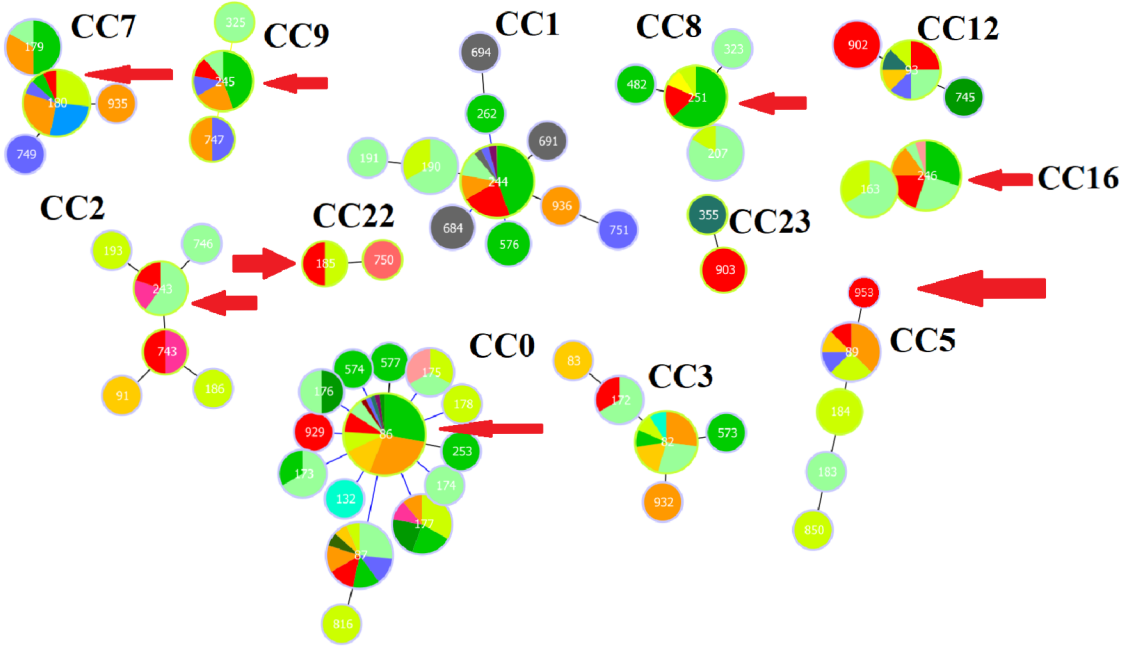
differences between nodes in comparison to grey-coloured links. Colour of node edge indicates: common node (blue) and sub-group founder. (n)-number of samples deposited in the MLST database. Red arrows are showing STs found in this study.

Appendix 18. Minimum-spanning tree of *B. burgdorferi* s.s. MLST profiles (including samples of our study) in association with the human source of the pathogen (created in Phyloviz 2, edited in GIMP version 2.10.30).



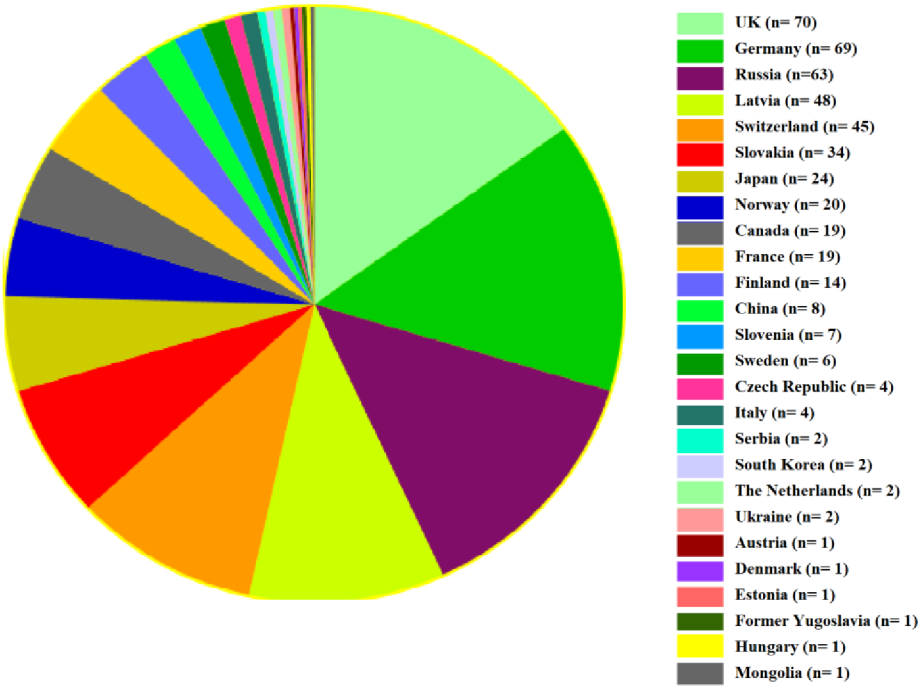
The goeBURST Full MST algorithm was build up at the maximum level of locus variants, i.e., 8 levels. The number of samples assigned to specific ST is visualised by node size. The links between nodes are in range of black or grey colour. Black-coloured links reflect fewer differences between nodes in comparison to grey-coloured links. Colour of node edge indicates: common node (blue) and sub-group founder. Red arrows are showing STs found in this study.

Appendix 19. Population structure (goeBURST algorithm, SLV level) of *B. garinii* STs including samples from Slovakia (previously deposited data + data of our study) (created in Phyloviz 2, edited in GIMP version 2.10.30).



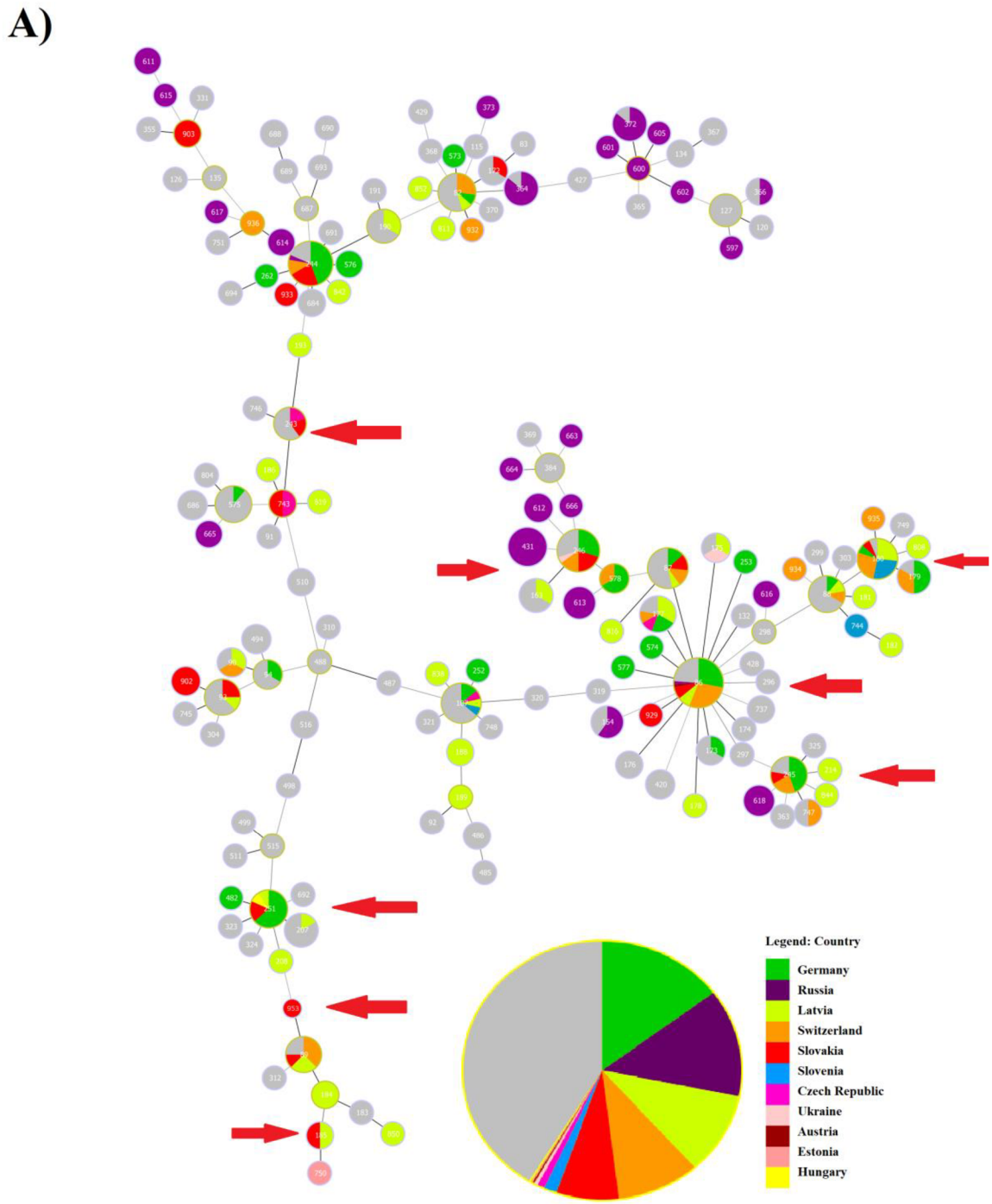
The links between nodes are of black or blue colour. Black colour displays link drawn without recourse to tiebreak rules and blue colour is applied for links using tiebreak rule 1 (number of SLVs). Node size indicates the number of samples characterised by specific ST (number in a node). Node edge is coloured in light blue or light green, which reflect a common node or group founder, respectively. Node colours reflect the origin (country) of the genotype assigned in MLST database. Red arrows point to STs found in this study. SGs are not shown in the scheme. CC-clonal complex, (n)-number of samples deposited in the MLST database.

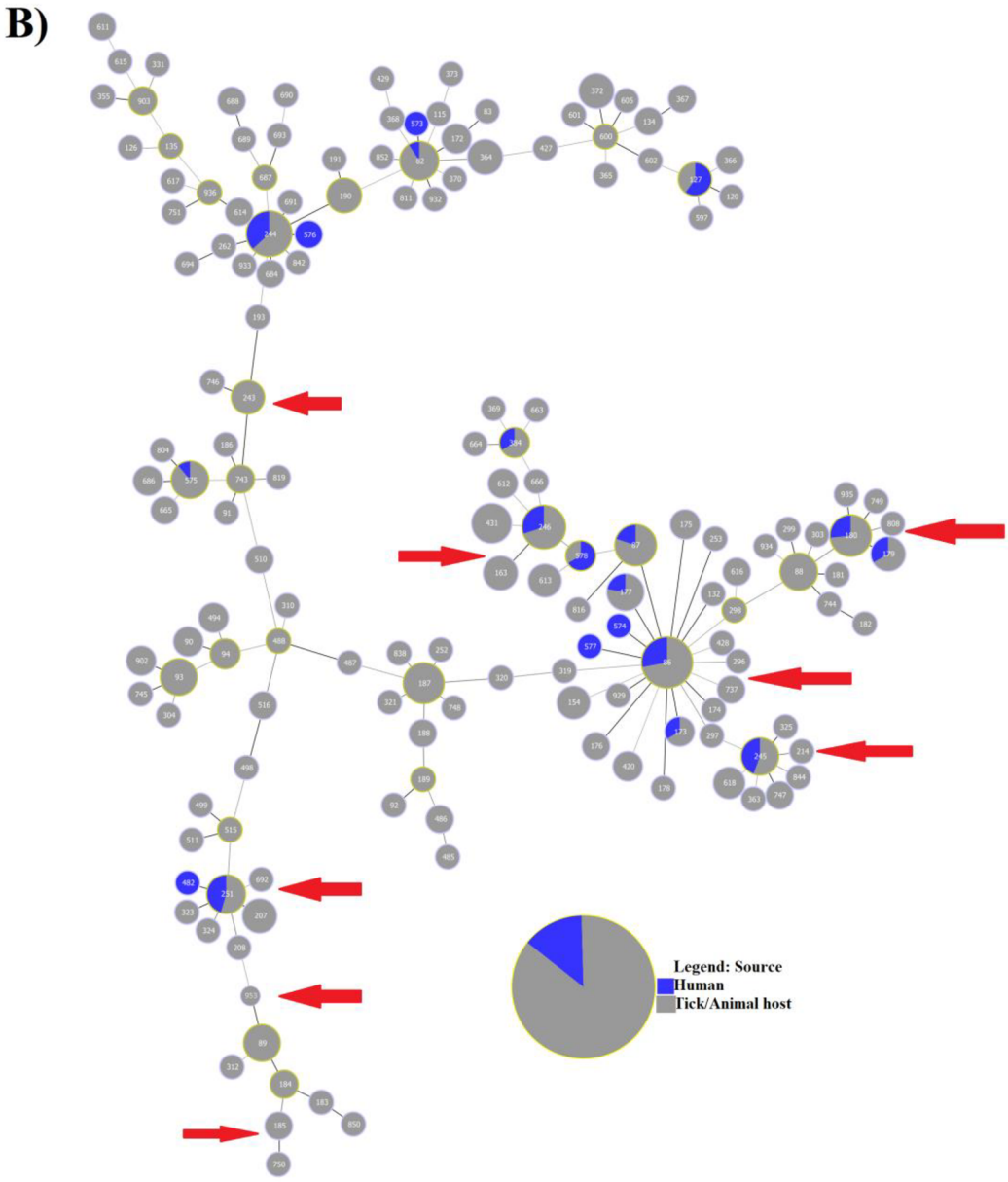
Appendix 20. Legend of countries in goeBURST analyses for *B. garinii* samples (created in Phyloviz 2, edited in GIMP version 2.10.30).



n-number of samples

Appendix 21. Minimum-spanning tree of *B. garinii* STs of the MLST database (algorithm: goeBURST Full MST) with highlighted nodes occurring in Central and Eastern Europe (A) and a scheme of *B. garinii* STs extracted from human patients suffering from LD (B) (including data from Slovakia of our study) (created in Phyloviz 2, edited in GIMP version 2.10.30).





A) Coloured parts within the nodes reflect the number of samples for the given countries.
B) Blue colour within nodes is showing the number of samples obtained from human patients for the given STs. A – B) Size of nodes reflects the number of samples characterised by specific ST. The links between nodes are of black (less difference between nodes) or grey (much difference between nodes) colour. Edges of nodes are coloured in light green (group founder) and light blue (common node). Red arrows are showing STs of this study.