Comenius University in Bratislava

Faculty of Natural Sciences

Genetic variability of tick-borne pathogens and their interactions

Dissertation thesis

Bratislava 2017 **Mgr. Ivana Baráková**

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Study program: Molecular biology

Field of study: 1502, 4.2.3. Molecular biology

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Univerzita Komenského v Bratislave

Prírodovedecká fakulta

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ZADANIE ZÁVEREČNEJ PRÁCE

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Študijný program: molekulárna biológia (Jednoodborové štúdium, doktorandské III. st., externá forma)
Študijný odbor: molekulárna biológia
Typ záverečnej práce: dizertačná
Jazyk záverečnej práce: anglický
Sekundárny jazyk: slovenský
Názov: Genetic variability of tick-borne pathogens and their interactions
Genetická variabilita kliešťami prenášaných patogénov a ich interakcie

Cieľ: Cieľom projektu je štúdium výskytu, genetickej variability a vzájomných interakcií vynárajúcich sa kliešťami prenášaných bakteriálnych patogénov (Anaplasma, Rickettsia, Babesia a Borrelia) v modelových lokalitách Talianska. Pre štúdium prevalencie patogénov budú použité metódy založené na PCR a RTPCR. Genetická variabilita pozitívnych vzoriek bude sledovaná metódou MLST (Multilocus sequence typing). Pre objasnenie cirkulácie jednotlivých genotypov v prírodných ohniskách bude sledovaná ich prítomnosť v kliešťoch z vegetácie, ale aj v rezervoárových hostiteľoch a kliešťoch cicajúcich na týchto hostiteľoch.

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Dátum zadania: 15.09.2012

Dátum schválenia: 11.04.2012 prof. RNDr. Ján Turňa, CSc. garant študijného programu

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THESIS ASSIGNMENT

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Study programme:	Molecular Biology (Single degree study, Ph.D. III. deg., external form)
Field of Study:	Molecular Biology
Type of Thesis:	Dissertation thesis
Language of Thesis:	English
Secondary language:	Slovak

Title: Genetic variability of tick-borne pathogens and their interactions

Aim: The aim of the thesis is to study the occurrence, genetic variability and mutual interactions of emerging tick-borne pathogens (Anaplasma, Rickettsia, Babesia and Borrelia) in model sites in Italy. Methods such as PCR and RTPCR will be used for pathogens detection and MLST (Multilocus sequence typing) will be used for genotyping of positive samples. To clarify the circulation of individual genotypes in natural foci, the prevalence of these pathogens will be monitored in ticks from vegetation, ticks detached from hosts and in reservoir hosts.

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Acknowledgements

Firstly I would like to thank both my supervisors, MVDr. Markéta Derdáková PhD and Dr. Annapaola Rizzoli; they are inspiring women in science and in life. I had the opportunity to learn so much from them in the past few years and for that, I'm very grateful. They gave me outstanding support, valuable research guidance and put trust in me, they were the two pillars supporting me through my PhD, that I know I could count on. They both inspired me with their enthusiasm, love, and passion towards the scientific research. MVDr. Markéta Derdáková despite various commitments as a Department leader helped me with opening the black box of *Anaplasma phagocytophilum*. Dr. Annapaola Rizzoli despite various commitments as the director of Fondazione Edmund Mach always found time for me to help me with the epidemiology of various tick-borne pathogens in Trentino. Finally, I would like to thank the Edmund Mach Foundation and the European Commission for the financial support of my PhD grant funded under EDENext (EU grant FP7-261504) and for partial financing support the Slovak Research and Development Agency (grant number APVV–14–0274) and SAIA, n.o the National Scholarship program of Slovak Republic, for supporting me in my last year of my PhD study.

This study needed complex interactions of people with various expertises to get to the presented results; therefore I would like to include all the thanks that are necessary. I would like to thank Dr. Heidi C. Hauffe, the head of our department for always willing to help with anything, personal or work related, to Roberto Rosà, PhD with his help on statistical analysis and modeling, Fausta Rosso for helping with detection of pathogens from questing ticks and detection of *A. phagocytophilum* in rodent tissue, Dr. Gabriela Margos for helping with the MLST protocols, Giovanna Carpi for guidance on *A. phagocytophilum* issue and providing us ticks from north part of USA, thanks to Margherita Collini, Valentina Tagliapietra, Daniele Arnoldi, Adam Konečný, Emma Gillingham and everyone helping with the ticks, blood and tissue collection and trapping of vertebrate hosts, ornithologist Dott. Franco Rizzolli for helping with ticks collection from birds, Dr Claudio Ramponi for providing us ticks from patients and veterinarians Dr. Danielli, Dr. Zampiccoli, Dr. Zammplini for providing us the ticks from dogs. Thanks to Associazione Cacciatori del Trentino and its guardians and hunters for the collaboration. Thanks to our technicians Matteo Girardi and Chiara Rossi that were always happy to help when needed. Thanks to Liina Voutilainen and docent Tarja Sironen for

providing us ticks from Finland and Lorenza Beati for proving us ticks from South part of USA. Thanks to my PhD colleagues in Slovakia Michal Chvostáč and Tatiana Vaculʼová for their collaboration and for sharing our ups and downs during our study.

Finally thanks to my family, for their support and encouragement. Thanks to my mama bear for taking care of the administration of my PhD while I was abroad and always having my back and my dad for answering all of my annoying questions and inspiring me from my childhood with his passion towards science.

Last but not least, I would like to thank my husband for always encouraging and believing in me in everything I do in life, for his unconditional love and support, for leaving everything behind and moving with me to Italy, without him this would not be possible.

Abstrakt

Epidemiologicky najvýznamnejším kliešťom v Európe je *Ixodes ricinus*. Tento druh kliešťa prenáša široké spektrum pôvodcov zoonóz, vrátane kliešťovej encefalitídy, baktérií, *Anaplasma phagocytophilum, Rickettsia* spp., spirochét z komplexu *Borrelia burgdorferi* sensu lato a protozoá *Babesia* spp. Na vypracovanie predbežných odhadov rizík novo vyskytujúcich sa zoonóz vo Valle dei Laghi v Taliansku sme sledovali výskyt patogénov v kliešťoch v juvenilných a adultných štádiách. Najvyššia prevalencia 11.4% bolo detegovaná pre *A. phagocytophilum* a *Rickettsia* spp. v kliešťoch odobratých z voľne žijúcich kopytníkov. Pre *Babesia* spp. a *B. burgdorferi* s.l. bola najvyššia prevalencia detegovaná u kliešťov odobratých z vtákov, 7.7% a 34.6% respektívne. Ďalšími analýzami sme presne určili genetickú variabilitu detegovaných patogénov. V nami sledovanej lokalite, Valle dei Laghi sme detegovali široké spektrum kliešťami prenášaných patogénov, a to: *A. phagocytophilum, R. helvetica, B. afzelii, B. garinii, B. lusitaniae* a *B. valaisiana* ako aj patogény, ktoré neboli predtým v provincii Trentino detegované; *R. monacensis, R. raoultii, B. venatorum, B. microti, B. capreoli* and *B. turdi*. V prírodných ohniskách predstavujú voľne žijúce cicavce významný článok v cirkulácii kliešťami prenášaných patogénov. Mnohé z nich sú nielen dôležitými hostiteľmi kliešťov, ale aj rezervoármi samotných patogénnych agens. Na presné určenie rezervoárovej kompetencie hostiteľov by mali byť vykonané xenodiagnostické analýzy, tie však sú ekonomicky a eticky náročné. Vzhľadom na to, prenos niektorých kliešťami prenášaných patogénov nie je doteraz celkom objasnený. Prítomnosť vyššie spomenutých patogénov sme zaznamenali v cicajúcich kliešťoch v larválnom štádiu, aby sme poskytli relevantnú informáciu o potenciálnej kapacite rezervoárových hostiteľov v danej oblasti. Potvrdili sme význam voľne žijúcich kopytníkov ako rezervoárových hostiteľov pre *A. phagocytophilum*, vtákov ako rezervoárových hostiteľov pre *B. garinii, B. valaisiana, B. turdi* a *B. lusitaniae* a hlodavce pre *B. afzelii*. Na upresnenie významu vnútrodruhovej genetickej variability v ekológii *A. phagocytophilum* sme vyšetrili kliešte z vegetácie, cicajúce kliešte odobraté z rôznych hostiteľov, ako aj krv a tkanivo z hlodavcov z Talianska, dodatočne sme vyšetrili kliešte z vegetácie ako aj cicajúce kliešte z hlodavcov z USA (severnej a južnej časti) a Fínska. Na základe fylogenetických analýz dvoch genetických markerov sme potvrdili existenciu dvoch enzootických cyklov, genotypov *A. phagocytophilum* v Európe. Okrem fylogenetickej analýzy *A. phagocytophilum* sme sa zamerali aj na druhy *B. burgdorferi* s.l.

cirkulujúce v danej oblasti. Jednotlivé genotypy boli identifikované pomocou molekulárnych markerov intergénového medzerníka a štyroch konzervatívnych génov používaných pri MLST analýzach. Pomocou týchto metodík sme zaznamenali prítomnosť pre Taliansko nového druhu *B. turdi*. Na záver, vďaka medzinárodnej spolupráci, piatich rôznych krajín sme určili akarologické riziko kliešťami prenášaných ochorení v Európe. Využitím geografických informačných systémov, ktoré zahŕňali klimatické a environmentálne údaje spolu so štatistickým modelovaním sme určili nielen vhodný biotop pre kliešte *I. ricinus,* ale aj krajiny a typy biotopov, ktoré majú väčšiu pravdepodobnosť prenosu infekcií.

Kľučové slová: kliešťami prenášané patogény, *Anaplasma phagocytophilum, Rickettsia* druhy, *Babesia* druhy, *Borrelia burgdorferi* sensu lato, genetická variabilita, ekologická variabilita

Abstract

In Europe, sheep tick *Ixodes ricinus* is epidemiologically the most important vector. This tick species transmits a wide variety of zoonotic agents including Tick-borne encephalitis virus, bacteria, *Anaplasma phagocytophilum*, *Rickettsia* spp., *Borrelia burgdorfer*i sensu lato spirochetes and protozoa *Babesia* spp. For estimating the risk of emerging zoonoses in Valle dei Laghi, Italy, we detected the presence of pathogens in nymphs and adult ticks, an important indicator of the hazard of infection. The highest prevalence of *A. phagocytophilum* and *Rickettsia* spp. were detected in ticks feeding on wild ungulates with 11.4% prevalence for both pathogens. For *Babesia* spp. and *B. burgdorferi* s.l. the highest prevalence was detected in ticks detached from birds, with 7.7% and 34.6% of positive ticks, respectively. Moreover, we analysed the intraspecific genetic variability. We were able to detect a wide range of tick-borne pathogens, such as *A. phagocytophilum*, *R. helvetica*, *B. afzelii*, *B. garinii*, *B. luisitaniae* and *B. valaisiana* as well as pathogens not detected in Trentino before, such as; *R. monacensis*, *R. raoultii*, *B. venatorum*, *B. microti*, *B. capreoli* and *B. turdi*. In natural foci, wildlife vertebrates represent a significant element in circulation of tick-borne pathogens. Many of them are not only important hosts of ticks, but also reservoirs of pathogenic agents themselves. In order to determine the reservoir competence of the host, xenodiagnostic analyses must be performed however they are economically and ethically demanding. Therefore the ecology and transmission cycles of some tick-borne pathogens are still not completely understood. For this purpose we screened larval ticks detached from various hosts to provide relevant information on the potential reservoir capacity of a host. We have confirmed the importance of wild ungulates as reservoir hosts for *A. phagocytophilum*, birds as reservoir hosts for *B. garinii*, *B. valaisiana*, *B. turdi* and *B. lusitaniae* and rodents for *B. afzelii*. In order to clarify the importance of intra-specific genetic variability of *A. phagocytophilum*, we examined questing ticks, ticks detached from various hosts, blood and tissue from rodents in Italy and in questing ticks and engorged ticks detached from rodents from USA (South and North of USA) and Finland. Phylogenetic analyses of two loci revealed two distinct enzootic cycles of *A. phagocytophilum* in Europe. In addition to phylogenetic analysis of *A. phagocytophilum*, we focused also on *B. burgdorferi* s.l. genospecies, circulating in a given area. Individual genotypes were identified by molecular markers of the intergenic spacer and the four conserved genes used in MLST analysis. By use of these

phylogenetic analyses, we were able to detect a new genospecies, *B. turdi* in Italy. Finally, by collaboration with partners from five different countries, we were able to determine acarological hazard of tick-borne pathogens in Europe. Remote sensing that included climatic and environmental data, together with statistical modeling revealed not only preferable habitat of *I. ricinus* but also countries and habitat types, that are more likely to experience higher transmission rate of tick-borne agents.

Keywords: tick-borne pathogens, *Anaplasma phagocytophilum*, *Rickettsia* spp., *Babesia* spp., *Borrelia burgdorferi* sensu lato, genetic variability, ecologic variability

Preface

This dissertation thesis with title ‘’Genetic variability of tick-borne pathogens and their interactions’’ focuses on rising occurrence of tick-borne zoonoses in Europe, which are directly linked to more complex phenomena such as climate change, ecological and socio-economical changes. Outbreaks of vector borne diseases represent a challenge at global scale and therefore their monitoring, prediction and prevention became a priority for public health. Furthermore, tick and tick borne-diseases are among the best model to be studied for developing an integrated control strategy under the ‘One Health’ concept.

This molecular-ecological study refers to a series of tick-borne pathogens of relevance to public health in Europe, such as *Anaplasma phagocytophilum*, *Rickettsia* spp., *Babesia* spp. and *Borrelia burgdorferi* sensu lato.

Firstly, this study provides an epidemiological update on the list of tick-borne pathogens circulating within the province of Trento, Italy. Few studies have been done up to date in Valle dei Laghi area, revealing the presence of several tick-borne pathogens. The collection of a large number of samples was implemented with the help of various technicians and researcher of the FEM personnel, which speeded up the process. On the other hand molecular detection of four pathogens in various sample types was highly demanding but successfully in term of results obtained.

While screening the adults and nymphal ticks for the presence of tick-borne agents is a good indicator of hazard in the area, detection of pathogens in larval ticks can contribute to elucidate the complete life cycle of infectious diseases. We were able to identify new wildlife host for some pathogens and for those where transovarial transmission is known, we emphasize the need for further experimental studies.

Next, we studied the genetic variability, of the causative agent of tick-borne fever and human granulocytic anaplasmosis, *A. phagocytophilum* to understand differences in the ecology between Europe and USA. While the pathogenicity in humans as well as animals is well described in USA, the transmission cycle in EU seems to be more complex. Only a few studies were done on this topic in Europe. We were able to obtain large positive sample size to construct phylogenetic analyses of two genetic markers. Our results brought a light in a black box of *A. phagocytophilum* in Europe. We were able to show the presence of two distinct enzootic cycles of this pathogen and confirm presence of the pathogenic strain. This part was

challenging in analyzing a large amount of samples from feeding and questing ticks, blood samples from rodents and ear biopsies from rodents.

Furthermore, we wanted more closely to analyze *B. burgdorferi* s.l. using the multilocus sequence typing (MLST), which is used for evolutionary, epidemiological and population genetics. However we were not able to obtain the results for all 8 housekeeping genes due to a small amount of DNA.

And finally, our work contributed to understand and predict the acarological hazard in Europe as an early warning tool. This part was done in collaboration with five different countries and the expertise of statistical analysis in FEM. Statistical models which included environmental and climatic variables were used to detect a relationship between total nymphs counts and relative density of infected nymphs in all studied countries. Remote sensing was used to identify the predictor of the acarological hazard in Europe.

Contents

Abbreviations

CRASPs - *complement regulatory-acquiring surface proteins*

DEBONEL - Dermacentor-borne necrosis

DIN – density of infected nymphs

GST - glutathione s-transferase

KPI - Kunitz protease inhibitor

EM - erythema migrans

GST - glutathione S-transferase

HGA - human granulocytic anaplasmosis

IGS- 5S-23S rDNA intergenic spacer region

LB - Lyme borreliosis

LD - Lyme diseases

LST - Land surface temperature

MAC - membrane attack complex

MLST/MLSA - multilocus sequence typing/analysis

MODIS - Moderate Resolution Imaging Spectroradiometer

NDVI - Normalized Difference Vegetation Index

NDWI - Normalized Difference Water Index

NGS - Next-generation sequencing

PBS - Phosphate-buffered saline

PCR-RFLP - polymerase chain reaction-restriction fragment length polymorphism

PCR-SSCP - single strand conformation polymorphism

qPCR - quantitative PCR

RE - restriction enzyme

RF - relapsing fever

RLB - reverse line blot

SFG - spotted fever group

SSCP - single-strand conformation polymorphism

ST - sequence type

TG - typhus group

TIBOLA - tick-borne lyphadenopathy

1 Introduction

Ticks are ectoparasites and the second most important vectors of infectious disease agents after mosquitoes. They transmit a great variety of viruses, bacteria and parasites to wildlife animals, domestic animals, and humans.

Circulation of these pathogens among the tick, vector and its hosts is complex and usually only partially known. Most of these agents show considerable intraspecific heterogeneity that is associated with their pathogenicity, specific vectors and reservoir hosts. The identification of these microorganisms is mostly based on the molecular analyses since they don't have special biochemical characteristics and are often hard to cultivate. Therefore, the exact molecular typing and identification are crucial to better understand the ecology, epidemiology and clinical symptomatic of the tick-borne diseases.

One health

Many rising health issues of humans are linked to the health of animals and the environment. More than half of the infectious diseases originate from animals (Taylor et al., 2001). One health became a complex discipline where scientific-health and environmentally related disciplines collaborate together on a local, national and global scale, to learn about the spread of these zoonoses in the environment.

A big part of One health focuses on spread and incidence of tick-borne pathogens. Various factors are responsible for the rise of pathogens in the past decade, specifically climate change, urban changes in the environment and human mobility. These changes have a further impact on the density of reservoir hosts and vectors, and their spread into new geographic areas (Cunningham, 2005, Blancou, 2005). In terms of impact on domestic animals and human health the tick-borne diseases caused by Tick-borne encephalitis virus, *Borrelia burgdorferi* s.l., *Anaplasma* spp., *Babesia* spp., and *Rickettsia* spp. transmitted by *I. ricinus* tick are of the highest concern in Europe and are becoming of increasing public health relevance even in urban and peri-urban areas (Randolph, 2009; Rizzoli et al., 2014).

1.1 Biology and ecology of selected vector-borne infections in Europe

1.1.1 Order Rickettsiales, family Anaplasmataceae

Rickettsiales are small alphaproteobacteria adapted to intracellular lifestyle as a plant mutualist, plant and animal pathogens or endosymbionts. This very diverse group of intracellular bacteria has undergone through reorganization in the past years and consists now of two families: the Anaplasmataceae and Rickettsiaceae (Dumler et al., 2001).

Anaplasmataceae family encompasses genera *Anaplasma*, *Ehrlichia*, *Neorickettsia* and *Wolbachia* (Figure 1.) (Dumler et al., 2001; Rikihisa et al., 2003). These genera consist of intracellular, obligate, gram-negative microorganisms that reside in the cell vacuoles and form characteristic colonies, called morulae. While *Anaplasma*, *Ehrlichia* and *Neorickettsia* are parasites of mammals, *Wolbachiae* are endosymbiont microflora of ticks, insects and some nematodes (Kozek et al., 2007).

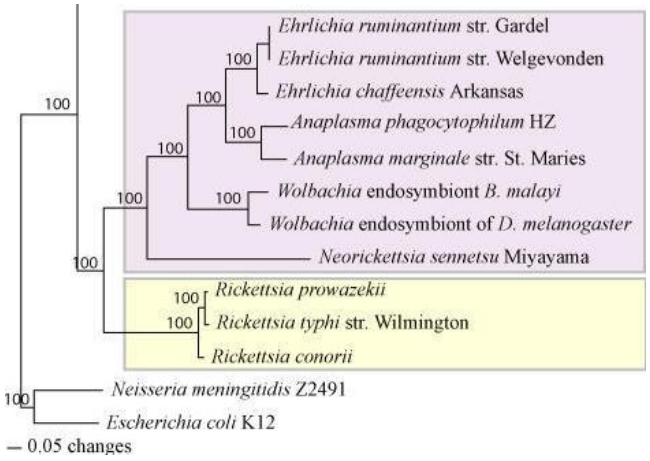

Figure 1. Phylogenetic tree of *Rickettsiales* contains two main families: Anaplasmataceae and Rickettsiaceae (Hotopp et al. 2006).

1.1.1.1. Genus *Anaplasma*

In the recent years, reorganization and reclassification of this genus was done using different molecular markers (Dumler et al. 2001). Three unique entities *Ehrlichia phagocytophil*a, *Ehrlichia equi* and human granulocytic anaplasmosis agent (HGA) have been grouped as a new entity ***A. phagocytophilum***. It infects neutrophils and is associated with

diseases of ruminants, horses, dogs and humans, causing fever, flu-like symptoms, abortuses and lower production in farm animals.

The high degree of clinical diversity is attributed to a circulation of heterogeneous genetic variants that are adapted to specific reservoir host and specific vector tick (de la Fuente et al., 2005, Bown et al., 2009, Blaňárová et al., 2014, Baráková et al., 2014). While some strains are involved in human and animal diseases, some strains are involved only in diseases affecting animals or are not pathogenic (Massung et al., 2002, 2005). ***Anaplasma marginale*** is transmitted by several tick species (*Rhipicephalus microplus*, *Rhipicephalus annulatus and Dermacentor andersoni*) and has a global impact on animals' health (Scoles et al. 2007). It is considered as the most pathogenic species of this genus resulting in significant morbidity and mortality of cattle population causing bovine anaplasmosis (Kocan et al., 2003).

On the other hand, ***Anaplasma centrale,*** which is very closely related to *A. marginale,* infects erythrocytes of ruminants and causes mild anemia. It is thought to be less pathogenic than *A. marginale* (Kuttler, 1984; Kocan et al., 2003). Even though these two species have differences in virulence, antigenically and molecularly they are related. ***Anaplasma platys*** cause canine infectious cyclic thrombocytopenia in dogs, however; new studies from Brazil showed possible infection in cats (Lima et al., 2010). ***Anaplasma ovis*** infects erythrocytes and causes low-level persistent infection, infecting erythrocytes of sheep, goats and wild ruminants (de la Fuente et al., 2007a).

1.1.1.1.1 Morphology of *A. phagocytophilum*

A. phagocytophilum is gram-negative alfa-proteobacterium usually varying in size between 0.4 to 1.3 μm. It contains two membranes from which the outer membrane is ruffled, causing irregular periplasmatic space (Rikihisa et al., 1997). The membrane is a crucial structure for the host-pathogen interactions including proteins responsible for localization within the vacuoles of a host cell. In infected cells, it can form reticulate or dense-cored cells. As other members of the Anaplasmataceae family *A. phagocytophilum* replicates in membrane-bounded vacuoles also called inclusions. After replication, it is released from the vacuoles and can infect other neutrophils in the vertebrate host. *A. phagocytophilum* mostly infects mature neutrophils but it is able to infect bone marrow progenitors and endothelial cells rather than peripheral tissues (Rikihisa, 2010). In the blood stream, it is mostly found in the

structure of intravacuolar microcolonies (morulae) rather than in stage of single cells. Morulae are usually 1.5 to 2.5 μm in diameter but can be as large as 6 μm. These intravascular microcolonies usually contain either only reticulate cells or only dense-cored cells and rarely both cell types (Popov at al., 1998). Due to the thin peptidoglycan cell wall and lipid-rich outer membrane in gram-negative bacteria gram staining is not suitable for visualizing intracellular bacteria. Other methods such as Diff-Quick are used instead (Figure 2.). Diff-Quick is a method based on air-dried prior to alcohol fixation easily allowing detection of the microbiological agents such as bacteria and fungi (Brouqui and Raoult, 1992).

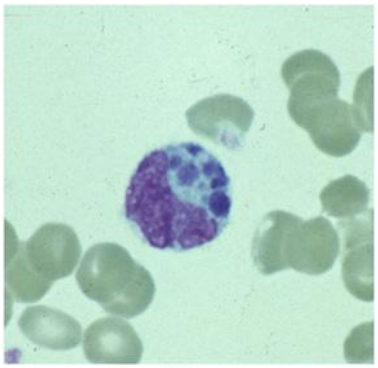

Figure 2. Infected granulocytes with *A. phagocytophilum*.
(http://www.cdc.gov/anaplasmosis/symptoms/)(4/11/2013)

1.1.1.1.2 Genome of *A. phagocytophilum*

The genome of *A. phagocytophilum* is in size 14.7 Mb containing approximately 12% of repetitive sequences from which around 1300 are open reading frames. These open frames are coding mostly housekeeping genes (Dunning et al., 2006). The function of these genes was discovered by tilling microarray method which is based on hybridization of labeled DNA to probes fixed onto a solid surface. Genes, paralogs, and operons were discovered by this technique (Nelson et al., 2008). *A. phagocytophilum* genome is equipped with genes for glycolysis pathway, genes for synthesis of all nucleotides, mostly vitamins and cofactors but only four amino acids (Dunning et al., 2006). The lack of genes for lipopolysaccharides and peptidoglycan makes pathogen sensitive to mechanical stress. Moreover, *A. phagocytophilum* is not able to produce cholesterol which is necessary for growth, stability, survival, and therefore it abducts it from host cell (Rikihisa, 2010).

1.1.1.1.3 Biology

A. phagocytophilum is transmitted in tick transstadially; transovarial transmission has not been detected (Dumler et al., 2005). After the tick is attached to reservoir host and the blood meal is initiated, hemoglobin and other proteins, also as microbes, are accumulated in midgut contend of the tick. The pathogen has to persist in the midgut of a tick until molting and following feeding, therefore it is thought to be the most important tissue for survival and proliferation of the pathogen. Continuing further from the midgut to salivary glands is crucial for the pathogen since it has to face the tick immune system (Hajdušek et al., 2013).

The immune system of the tick has two levels; cellular and molecular level. On the cellular level, tick shields against microbial infection with phagocytosis, encapsulation and nodulation. On the molecular level, it shields by using pattern-recognition proteins, effectors molecules such as lectins, complement-related molecules and antimicrobial peptides (Kopacek et al., 2010). After escaping the immune system of a tick the bacteria are accumulated in salivary glands from which they are spat back to the wound after 24-48 hours (Katavolos et al., 1998). From there it infects the host cells by phagocytosis and inhibits phagolysosome fusion. The bacterium grows within the membrane-bound phagosome and it is released by cell lysis (Figure 3.). After the cell lysis, the bacteria can infect other leukocytes (Parola and Raoult, 2001).

Uninfected ticks that feed on infected animal become infected and then after molting can transmit the infectious agent to another mammal host. After infected tick feeds on uninfected mammal host, around 100 of *msp2* pseudogenes (coding for outer membrane proteins) are activated in one cazet of *A. phagocytophilum* genome. Products of this genes cause silencing of host immune system that enables the host immunity to respond to the undesired organism. This action causes the permanent infection of mammalian hosts. Transstadial transmission and activation of many outer membrane proteins are crucial for the survival of this pathogen (Rejmanek et al., 2012). Therefore, genes coding outer membrane proteins which are interacting with host immune system evolve more rapidly due to the selective pressure. Amplifying these genes can give epidemiologists a better picture of the variability of *Anaplasma* strains circulating in different hosts (de la Fuente et al., 2007; Carpi et al., 2009).

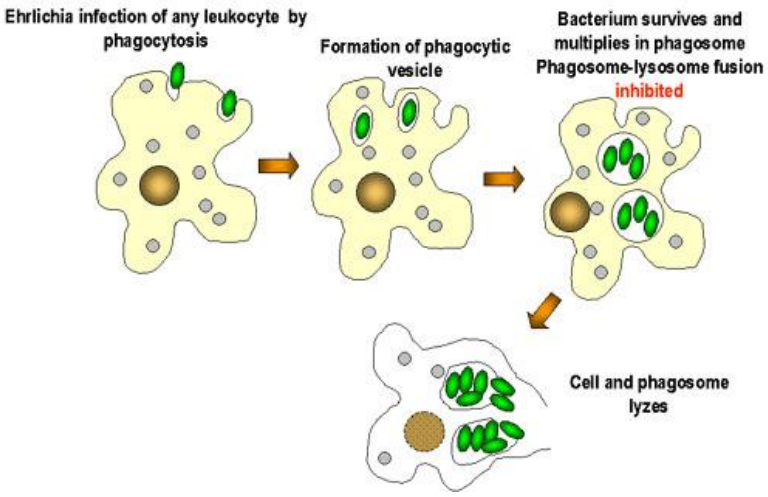

Figure 3. *A. phagocytophilum* infection of leukocyte. http://pathmicro.med.sc.edu/mayer/rick3.jpg (7-11-2013)

1.1.1.1.4 Ecology

In Europe, *A. phagocytophilum* is transmitted mostly by *I. ricinus* (Strle, 2004). Occasional vector of some genotypes associated with rodents is probably *Ixodes trianguliceps* (Birula 1895), (Bown et al. 2009) *Ixodes pacificus* and *Ixodes dentatus* are vectors in south and southwest of USA (Cooley et Kohls, 1943), *Ixodes scapularis* (Say, 1821) in North America and *Ixodes persculatus* (Schulze 1930) in Asia (Cao et al., 2000; Massung and Slater, 2003).

The prevalence of *A. phagocytophilum* in ticks differs geographically and depends on the presence of competent reservoir hosts. In Europe, the prevalence in *I. ricinus* ticks ranges from 0.4-66.7% (Blanco and Oteo, 2002). In Italy, the overall prevalence is 9.9% in *I. ricinus* ticks and 5.1% in the studied hosts (Mantelli et al., 2006; Carpi et al., 2009; Aureli et al., 2012), in Germany it ranged from 1% to 4.5% (Hartelt et al.2004, Silaghi et al. 2008), in Slovakia from 2% up to 30% (Derdáková et al. 2011; Kočianová et al. 2008a), in Norway 4.5% (Radzijevskaja et al., 2008), in Sweden 1.7%-22.1% (Severinnson et al., 2010), in Spain 5.6% (Barandika et al., 2008) and in Denmark 23.6% (Skarphedinssona at al., 2007).

A. phagocytophilum causes nonspecific febrile illness of humans, known as human granulocytic anaplasmosis (HGA) and the tick-borne fever in domestic animals (Chen et al.,

1994; Stuen et al., 2013). HGA is spread worldwide with a different number of cases in different countries. Since 90‘s in the USA, the HGA has become one of the most important tick-borne diseases with reported 2389 cases in 2012 (Adams et al., 2014). In Europe incidence of HGA is much lower, around 100 cases have been described up to date (Lotric-Furlan et al., 1998; Van Dobbenburgh et al., 1999; Oteo et al., 2000; Misic-Majerus et al., 2000; Kristensen et al., 2001; Tylewska-Wierzbanowska et al., 2001), most of which were documented in Slovenia (Lotric-Furlan et al., 2006) and Scandinavia (Bjoërsdorff et al., 1999). From the first report of HGA in 1994, there is visible exponential growth in a number of cases, probably due to improved surveillance (Bakken et al., 1994; Cochez et al., 2011; Edouard et al., 2012). As a result, there has been a growing attention to this tick-borne disease in Europe.

1.1.1.1.5 Genetic variability

Even though *A. phagocytophilum* was reclassified as a single organism (Dumler et al. 2001), there is growing evidence based on clinical and host tropism diversity of this bacterium suggesting specific association of different strains and with its pathogenicity to different hosts as well as vectors (Massung et al. 2002, Keesing et al. 2012, Mantelli et al. 2006, Carpi et al. 2009). According to phylogenetic trees based on sequences of coding outer membrane proteins, such as *msp4*, *groEL*, *ankA* (Carpi et al., 2009; Bown et al., 2009; de la Fuente et al., 2005) show that these strains are adapted to different natural cycles involving different reservoir hosts or vectors (Bown et al., 2009; Blaňarová et al., 2014, Baráková et al., 2014). Different studies support the theory where the genetic variation of this bacterium contributes to the zoonotic potential of the pathogen (de la Fuente et al., 2005). Thus the ecology of *A. phagocytophilum* seems to be more complex than it was thought before.

A. phagocytophilum was detected in US, Asia and nearly in all European countries (Massung et al., 2005; Stuen, 2007; Reichard et al., 2009; Woldehiwet, 2010; Gaowa et al., 2012; Jin et al., 2012). The epidemiology and ecology of *A. phagocytophilum* in the US and Europe are quite different. The vectors in the US include *I. scapularis* in the East and Midwest and *I. pacificus* in the Western part of the US. Two different variants Ap-ha and Ap1 based on the analysis of 16S rRNA have been described. Ap - ha variant is maintained (Courtney et al., 2003) in small and medium-sized mammals such as the white-footed mouse (*Peromyscus leucopus*), the raccoon (*Procyon lotor*), and the gray squirrel (*Sciurus carolinensis*) and is able

to cause the infection in humans (Liz et al., 2002; Levin et al., 2002, Massung et al. 2003). On the other hand, Ap-variant 1 is maintained in white-tailed deer as reservoir hosts and is not pathogenic for humans (Massung et al. 2005).

In Europe, the ecology of *A. phagocytophilum* is more complex due to its greater intraspecific variability. Even though there are less human cases documented than in the US, *A. phagocytophilum* infects domestic animals and causes tick-borne pasture fever which presents a large financial burden to the industry (Brodie et al., 1986). Amplification of 16S rRNA in Europe is not as informative as it was in US. To describe the genetic variability of *A. phagocytophilum*, more variable genes were used for the phylogenetic analyses (Liz et al., 2002; de la Fuente et al., 2005). More helpful were genes coding outer membrane proteins, which are more variable due to the contact with host immune system. By amplifying genes coding outer membrane proteins such as *msp4*, *groEL*, *ankA* two main clades were distinguished. In one clade there were strains found in humans, horses, dogs and sheep and in the other clade there were strains found in roe deer (de la Fuente et al., 2005). In the new studies carried out by (Bown et al., 2009, Blaňárová et al. 2014) it is suggested that even though the main vector transmitting *A. phagocytophilum* in Europe is *I. ricinus*, there is a possibility of another important vector *I. trianguliceps* that is involved in the transmission. In areas where *I.ricinus* is in low abundance, *I. triangulice*ps is thought to fulfill the transmission of certain strains (Bown et al., 2009, Blaňárová et al. 2014). These studies indicate two different enzootic cycles; the first one involving *I. trianguliceps* as a vector of strains obtained from rodents and the second one involving *I. ricinus* as a vector of strains obtained in roe deer.

1.1.1.1.6 Vectors

The most widespread tick in Europe is *I. ricinus* which is also called European sheep tick (Parola and Raoult, 2001). This tick species belongs to *I. ricinus* complex which includes other similar species differing in their geographical distribution: taiga tick – *I. persulcatus* found in northeastern Europe (Silber et al., 1946), a deer tick - *I. scapularis* mostly found in Eastern and Southeastern of United States and Canada (Massung and Slater, 2003) and finally *Ixodes pacificus* is found in western coast of North America (Courtney et al., 2003). *A. phagocytophilum* as many other tick-borne diseases agents are primarily transmitted by this tick species. There are also known other tick species contributing to the spread of this

pathogen such as *I. trianguliceps* (Bown et al., 2009) and *Dermacentor marginatus* (Bonnet et al., 2013).

1.1.1.1.7 Reservoir hosts

Since this pathogen lacks transovarial transmission, the spread of *A. phagocytophilum* depends on competent reservoir hosts and ticks to maintain the agent in nature (Rar and Golovljova, 2011). This highlights the essential role of wildlife as reservoirs of *A. phagocytophilum* in its transmission.

From free living ruminants, *A. phagocytophilum* was detected in goats, sheep, roe deer, red deer, wild boar (Ogden, 2003; Silaghi et al., 2011; Carpi, 2009; Michalik et al., 2012). Some studies implicated that rodents such as wood mice (*Apodemus sylvaticus*), yellow-necked shrew (*Apodemus flavicollis*), field voles (*Microtus agrestis*), and bank voles (*Clethrionomys glareolus*) can also be important reservoir hosts of certain strains of *A. phagocytophilum* (Bown et al., 2009; Štefančíková et al. 2008). Even though their life cycle is shorter, they develop low levels of the bacterium (Levin et al, 2004). This pathogen can also infect domestic animals such as cats, dogs, horses and cattle (Bjoersdorff, 1999; Egenvall, 1997, Engvall et al., 2002, Torina et al., 2010). Humans can be infected by this pathogen but cannot transmit the infection further, therefore are dead end host for *A. phagocytophilum*.

1.1.1.1.8 Tick interactions with *A. phagocytophilum*

Almost all species of the genus *Anaplasma* are transmitted by Ixodid ticks. The transmission cycle was mostly studied for *A. marginale* (Kocan et al., 1986, 1992a, b, 2008a). During the blood meal infected red blood cells, neutrophils are released infecting the tick midgut cells. In the next feeding, the bacterium infects other tissue including salivary glands. The transfer of the bacteria from the midgut to salivary glands requires infection of hemocytes. After bacteria amplifies in salivary glands it further travels into the saliva, which is also injected with the infection into the host during the next blood meal (Liu et al., 2011). The regulation of the infection depends on some ticks genes/proteins (de La Fuente et al., 2007b; Kocan et al., 2008; Zivkovic et al., 2009, Sultana et al., 2010; Villar et al., 2010). It includes glutathione S-transferase (GST) which is mostly responsible for cellular detoxication and

signaling (Oakley, 2011), salivary selenoproteins which function as antioxidants (Reeves and Hoffmann, 2009), ATPase proteins which are important for the acidification of variety of intracellular organelles (Nelson, 2003), and ubiquitin molecules important for destruction and recycling of proteins in proteasome (Ferrandon et al., 2007).

1.1.1.1.9 Clinical manifestation

A. phagocytophilum causes granulocytic anaplasmosis not only in humans (Petrovec et al. 1997, Psaroulaki et al., 2009, Sumption et al., 1995) but also in domestic animals such as horses (Bjoersdorff 1990, Engvall et al. 1996, Egenvall 2002), dogs (Engvall et al. 1996, Egenvall 2002, Lester et al. 2005, Poitout et al. 2005), cats (Bjoersdorff et al. 1999), and cattle (Engvall et al. 1996).

In the early 1990s the first case of HGA occurred in Michigan and Wisconsin in the US. These general signs of this febrile illness were recorded: a headache, malaise, and shaking chills, anorexia, nausea, arthralgias and coughing (Carrade et al., 2009). Leucopenia, thrombocytopenia, elevations in transaminases were the signs in the diagnosis of this illness. From life-threatening complications, there are known acute respiratory problems, acute renal failure, and hemodynamic collapse (Ismail et al., 2010). Anaplasmosis doesn't usually affect central nervous system; there are just 1% of cases developing meningoencephalitis. Conversely, the peripheral nervous system can be under the risk causing brachial plexopathy, cranial nerve palsies, demyelinating polyneuropathy, and bilateral facial nerve palsy (Ismail et al., 2010).

In domestic animals, symptoms include fever, fatigue, inappetence, lethargy, lameness, gastrointestinal and central nervous system signs (Rikihisa 1991, Greig et al. 1996, Egenvall et al. 1997, Engvall and Egenvall 2002).

1.1.2 Order Rickettsiales, family Rickettsiaceae

The second family forming order Rickettsiales is family Rickettsiaceae. Rickettsiaceae are a heterogenous group of small obligatory intracellular bacteria compiled by 3 genera: *Rickettsia*, *Orientia* and *Wolbachia*. They are gram-negative bacteria sensitive to environmental exposure, inhabiting arthropods (fleas, lice and ticks) but are able to cause

diseases in vertebrate hosts. The pathogen is transmitted by feces or during the blood meal of the vector (Fournier and Raoult 2009).

1.1.2.1 Genus *Rickettsia*

Rickettsia is a genus of gram-negative, intracellular, pleomorphic bacteria in various shapes. It can form spherical shape cocci (0.1 μm in diameter), rods (1–4 μm long) or thread-like (10 μm long) shape. Bacteria from this genus cannot live in artificial nutrient conditions which is why they depend entirely on growth and replication within cytoplasm of the eukaryotic host cell.

Rickettsia is a diverse group that contains many species. In recent years a new species have been discovered in Europe, some of which are pathogenic to humans. *Rickettsia* have been divided into two groups based on the clinical signs: spotted fever group and typhus group.

1.1.2.1.1 Ecology

The spotted fever group (SFG) contains about 20 different species of which 14 are well described pathogens (Raoult and Roux, 1997). They are distributed around the world and their occurrence is bound to the distribution of tick vector species in the area. SFG *Rickettsia* occurs mostly in Europe (Figure 4.) but some species have also been found in the United States, Asia, Australia, Japan and South Africa (Billings et al., 1998; Kollars et al., 2001; McBride et al., 2007; Kodama et al., 2003; Jensenius et al., 2003). Some of the significant species from this group are *Rickettsia conorii*, *Rickettsia rickettsii*, *Rickettsia sibirica*, *Rickettsia australis*, *Rickettsia japonica*. In addition to *Rickettsia helvetica*, *Rickettsia slovaca* and *Rickettsia massiliae* are found in Europe (Hofmann-Lehmann et al., 2016; Michelet et al, 2016; Chisu et al., 2016).

The typhus group is presented mostly in Asia-Pacific region where they are endemic (Lee et al., 2003). In the group of typhus causing disease are included *Rickettsia typhi* and *Rickettsia prowazekii*. *R. typhi* has been known to cause murine typhus since 16-th century. It was responsible for the death of 3 million people during the World War I (Walker, 1988). *R. prowazekii* has different clinical manifestations around different geographic areas. It can

manifest itself as epidemic typhus in South America and Africa (Perine et al., 1992), as sporadic typhus in the United States and as recrudescent typhus worldwide (McDade et al., 1980).

In Europe, the most common *Rickettsia* species was thought to be *R. conorii* responsible for Mediterranean spotted fever in humans. Since 1998, 4604 clinical cases with 33 deaths have been detected in Italy (Ciceroni et al., 2006). Until recently it was considered the single *Rickettsia* species able to infect humans in this region. Yet, in recent years there have been a number of new *Rickettsia* species pathogenic for humans discovered in Italy. Besides the known pathogens such as *R. helvetica* and *R. slovaca* there the occurrence of *R. monacensis*, *Rickettsia* spp. in the northern, southern and central region have been reported (Lo et al., 2004). In the alpine zone the presence of species with high homology to *R. limoniae* has also been described (Floris et al., 2008).

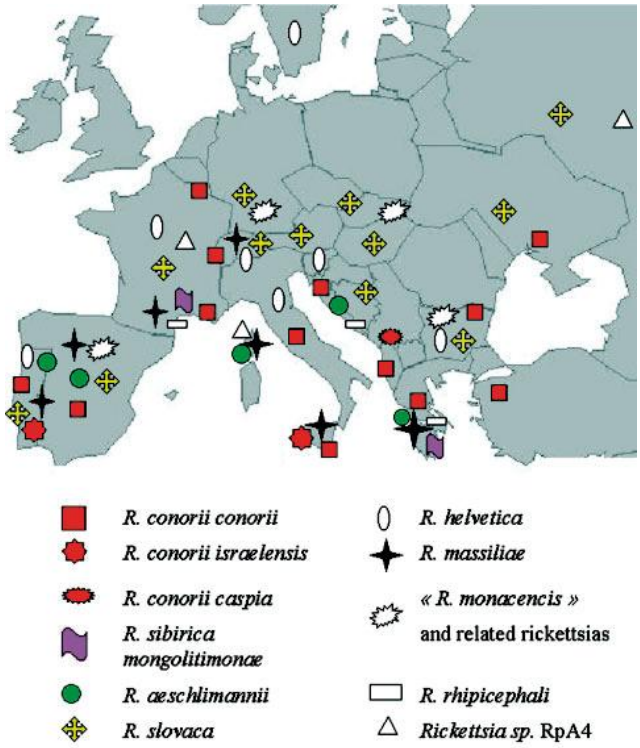

Figure 4. Distribution of *Rickettsia* species in Europe (Map from Parola et al. 2005). Colored symbols represent *Rickettsia* species pathogenic to humans and white symbols are *Rickettsia* species with possible pathogenicity.

1.1.2.1.2 *Rickettsia helvetica*

R. helvetica was firstly isolated from *I. ricinus* tick in Switzerland in 1979 and after 14 years it was validated as new species of SFG (Beati et al., 1993). The genome of this species is longer than the genome of other rickettsiae from SFG, reaching the size of 1.397 Mb (Roux and Raoult, 1993).

To date *R. helvetica* have been found in European countries such as Switzerland, France, Sweden, Slovenia, Portugal, Slovakia (Beati et al., 1993; Nilsson et al., 1997; Christova et al., 2003; Sanogo et al., 2003; Brouqui et al., 2003; Špitalská et al., 2008). The first detection of *R. helvetica* in Italy was documented by Beninati in 2002. In northern Italy, the prevalence of *R. helvetica* in questing *I. ricinus* ticks is in the range from 1.5% to 13.1% and in feeding ticks is around 22% (Corrain et al., 2012; Capelli et al., 2012; Maioli et al., 2012).

R. helvetica is a member of SFG originally not considered as pathogenic to humans until the documentation of mild, self-limited disease in seropositive patients (Nilsson et al., 1999, 2010). Serologic studies recorded 1.9%–12.5% prevalence of *R. helvetica* antibodies in patients from France, Italy, Denmark and Sweden (Fournier et al., 2000; Beninati et al., 2002; Elfving et al., 2008; Nielsen et al., 2004). Few species from SFG including *R. helvetica* were assumed to be associated with meningitis in human (Araki et al., 2002; Glaser et al., 2010). Moreover, *R. helvetica* could be involved in chronic inflammatory cells that can form as nodules in multiple organs causing sarcoidosis diseases (Nilsson et al., 2002).

1.1.2.1.3 *R. monacensis*

R. monacensis was identified as an etiologic agent of a Mediterranean spotted fever (MSF)-like illness with a couple of cases reported in Spain and Italy (Jado et al., 2007; Madeddu et al., 2012). Up to date, it has been detected in 18 European countries with 0.5%-34.6% prevalence in *I. ricinus* (Gargili et al., 2012; Silaghi et al., 2011). A presence of this pathogen in Italy has been reported so far only in the central part, with 13% prevalence in questing *I. ricinus* and 9.1% prevalence in Ixodid ticks detached from animal hosts (Corrain et al., 2012; Maioli et al., 2012).

1.1.2.1.4 *R. raoultii*

Several rickettsial genotypes, DnS14, DnS28, and RpA4, have been detected in 1999 (Rydkina et al., 1999) and shown to belong to unique species named *R. raoultii* in 2008 (Mediannikov et al., 2008). Subsequently, *R. raoultii* has been reported in *Dermacentor* spp. ticks throughout Europe (Boldiš et al., 2010; Chmielewski et al., 2009; Márquez, 2008; Tijsse-Klasen et al., 2011). In Italy, it has been detected only in Tuscany (central Italy), and Puglia and Basilicata (southern Italy) in adult *D. marginatus* feeding on wild boars (Otranto et al., 2014; Selmi et al., 2009).

1.1.2.1.5 Vectors

Rickettsia spp. are transmitted by arthropod vectors including ticks, mites, fleas, and lice (Glaser et al., 2010). Bacteria from the spotted fever group are mostly transmitted by tick vectors, whereas members of the typhus group are transmitted by the faeces of lice and fleas (Socolovschi et al., 2009; Azad et al., 1998). The major tick vectors in Europe are ixodid species, which are naturally infected with rickettsial species. Not all *Rickettsia* spp. are pathogenic and the genus includes species that belong to the natural endosymbiotic microflora of *Ixodes* ticks. *Rickettsia* spp. can be transmitted transovarially and transstadially (Raoult and Roux, 1997). During transovarial transmission the infection is passed from female adult tick to next generation of larvae. In transstadial transmission, the infection is passed from egg to larvae to nymph to adult tick while feeding. Established efficient transovarial transmission of this pathogen means that this pathogen hardly depends on its vertebrate reservoir host and *I. ricinus* plays a role in transmission cycle as a vector and reservoir (Sprong et al., 2009).

1.1.2.1.6 Reservoir hosts

To prove reservoir status, complex laboratory xenodiagnostic experiments have to be carried out on wildlife animals, however, they are economically and ethically challenging. Xenodiagnoses confirmed vole, mouse, rat, rabbit, hare and squirrel to be a reservoir for *R. rickettsii* (Burgdorfer et al., 1966; Burgdorfer et al., 1962) however not many studies have been done for *R. helvetica*, *R. monacensis*, *R. slovaca* and *R. raoultii*. Burri et al. (2014) using

xenodiagnostic experiments showed that infected rodents were not able to transmit *R. helvetica* or *R. monacensis* to *I. ricinus* larvae.

1.1.2.1.7 Tick interactions with *Rickettsia* spp.

Ticks can maintain or become infected with ricketsiae through transovarial transmission, blood feeding on the infected vertebrate host and through co-feeding next to rickettsia-infected ticks on noninfected vertebrate host (Macaluso and Azad, 2005). Lysosome and endosomes are involved during the blood digestion in the midgut; where it is believed that *Rickettsia* escapes the digestion enzymes during the delay of formation of the secondary lysosome (Munderloh and Kurtti, 1995). The mechanism of tick-rickettsia interactions is not well understood. However, using the light microscopy and molecular techniques, the actively dividing *Rickettsia* were spotted in midgut, salivary glands, and epithelium network after only one week of infection. The other tissues such as ovaries and midgut have not been infected. Due to this finding, it is predicted that *Rickettsia* may infect the salivary glands independently to midgut (Baldridge et al., 2007).

Through comparison of tick gene expression uninfected and SFG rickettsia-infected cells, few tick immune factors were discovered that are differently expressed during the infection. Few of them have been identified in tick midgut, where the immune peptides respond to the presence of rickettsial pathogens. Tick immune molecules varisin (defensin 1, 2) (Ceraul et al., 2007), Kunitz protease inhibitor (KPI) and glutathione s-transferase (GST) (Ceraul et al., 2008, 2011) were identified to respond to the infection. These tick immune molecules were observed to be upregulated during the rickettsial infection. KPI seems to limit rickettsial infection due to the direct contact with rickettsiae in midguts (Ceraul et al., 2008, 2011).

1.1.2.1.8 Clinical manifestation

Some of *Rickettsia* spp. are able to infect humans, such as *R. prowazekii*, *R. rickettsii*, *R. conorii*, *R. slovaca* and *R. helvetica* (Walker, 2007). *Rickettsia* can have different clinical manifestation depending on species involved in the infection. For example, *R. prowazekii* causes epidemic typhus, *R. rickettsii* causes Rocky Mountain spotted fever and *R. conorii* is

the causative agent of Mediterranean spotted fever (Parola et al., 2005). Usually, species from SFG have similar symptoms of the disease including fever, headache, hypotension, encephalitis, acute renal failure and respiratory distress. A rash is also presented in most cases. Unlike in typhus group (TG), in SFG the rash firstly occurs at limbs and then spreads to the rest of the body. *R. slovaca* has distinctive features, for example, there is no rash present and enlarged lymph nodes occur. *R. slovaca* causes a disease called DEBONEL (Dermacentor-borne necrosis) or TIBOLA (tick-borne lymphadenopathy) (Lakos, 1997). If the infection caused by rickettsial species from SFG is untreated, it can become life threatening. Species from SFG, in this case, may cause infection of central nervous system and there is evidence of association of *R. rickettsii*, *R. conorii* and *R. japonica* with meningitis (Araki et al., 2002; Glaser et al., 2010).

1.1.3 Order Piroplasmida, family Babesiidae

Piroplasmida are protozoan parasites of the phylum Apicomplexa (Levine et al., 1980) (Figure 5). They are intraerythrocytic eukaryote microorganisms that can be in a round or pyriform shape. These are found within erythrocytes or endothelial cells of vertebrates. Unlike other blood sucking parasites, they contain only single membrane. This order contains parasites from family Babesiidae and Theileriidae.

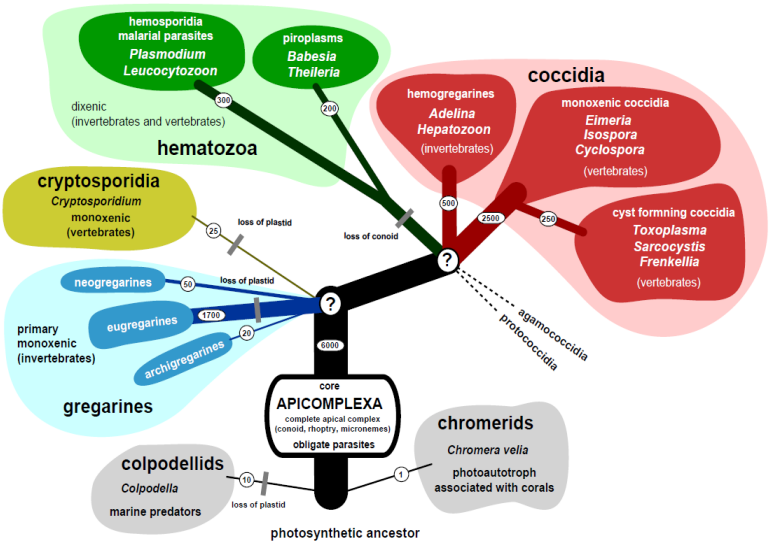

Figure 5. Apicomplexa as an ancestor of free groups: gregarines, coccidia and hematozoa. Group of hematozoa includes piroplasma (*Babesia*, *Thelleria*) and a group of hemosporida and malarial parasites (plasmodium, leucocytozoon) (http://tolweb.org/Apicomplexa; Slapeta, 2011).

The family Babesiidae consists of two genera *Babesia* and *Theileria*. They can be distinguished by the absence of pre-erythrocytic cycle in *Babesia* and the absence of transovarial transmission in *Theileria* (Kakoma and Mehlhorn, 1993; Riek, 1968; Telford, 1993).

1.1.3.1 Genus *Babesia*

Babesia are protozoan parasites that cause hemolytic diseases called Babesiosis with the cosmopolitan distribution. Until now there have been over 100 species recognized based on phylogenetic analysis of 18s rRNA gene (Schnittger et al., 2012). Even though 18S rRNA gene was used for phylogenetic analysis in past the years other more variable genes have been recently analyzed (Schnittger et al., 2012). Phylogenetic tree based on *hsp70* gene sequences generated trees that largely corresponded to 18S rRNA but were able to identify one new clade; clade VI (Figure 6). Based on *hsp70* sequences six clades of *Babesia* strains have been established: 1. *B. microti*, *B. leo*, *B. feli*, 2. *B. duncani*, *B. lengua*, *B. poelea* 3. Western USA Theilerid-like group, 4. *B. equi*, *B. bicornis*, 5. Theileria group that includes all Theileria species from Bovinae, 6. *B. divergense*, *B. canis*, *B. gibsoni*, *B. bovis*, *B. ovis* (Schnittger et al., 2012).

Clade one is often referred to as *B. microti* clade and consists of strains able to infect rodents and feline. In rodents, *B. microti* and *B. rodhaini* are involved in infection while the infection in felines is initiated by *B. leo* and *B. felis* parasites. Clade two represents strains such as *B. duncani*, strains infecting mule deer, bighorn sheep and dogs. Clade three is poorly supported in the phylogenetic tree and contains *Theileria youngi*, *Theileria bicornis* and *Cytauxzoon spp*. Clade four is well supported and contains *B. bicornis* and *T. equi*. Clade five consists of *Theileria* spp. that infects mostly ungulates. Clade six is known as the carnivore-rodent clade, infecting mostly canine (*B. gibsoni*, *B. canis*, *B. rossi* and *B. vogeli*) bear, cougar, raccoon and wild rodents (Schnittger et al., 2012).

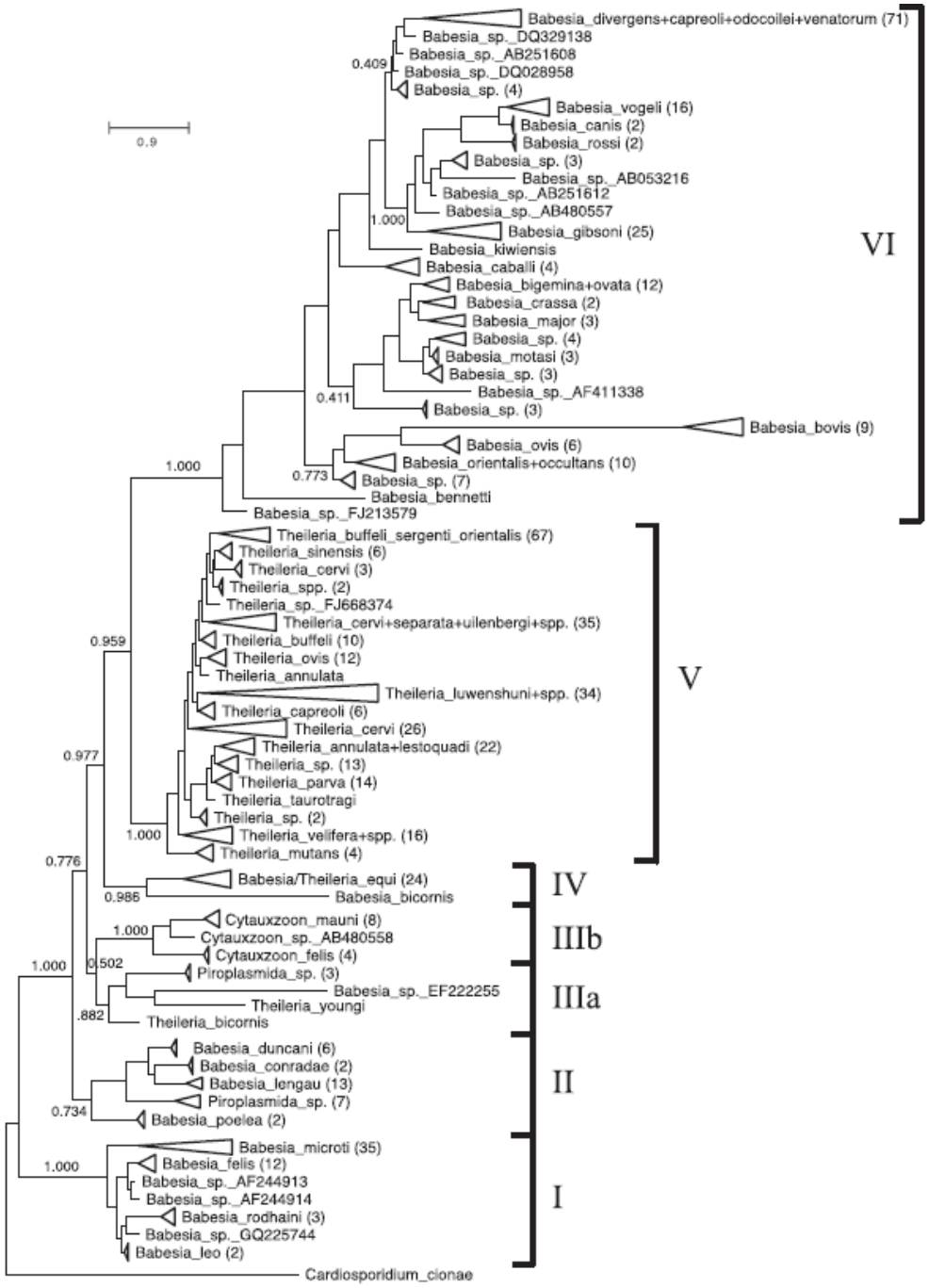

Figure 6. Phylogenetic analysis of *Babesia* strains based on *hsp70* sequences (figure from Schnittger et al., 2012).

1.1.3.1.1 Morphology and biology of *Babesia*

Babesia invades erythrocytes of mammalian hosts such as cattle, sheep, goats, horses, dogs, cats, rodents and humans. Not all *Babesia* can be transmitted transovarially (Imes et al., 2011). After tick attaches to the host, *babesia* sporozoites are inoculated and able to invade the erythrocytes of the host. After transforming from sporozoites into trophozoites they divide into merozoites. Merozoites then infect new red blood cells and increase rapid intracellular multiplication. The organism in infected red blood cells varies from 1.0 – 0.5 μm in small *babesia* (*B. bovis*, *B. divergens* and *B. microti*) to 2 – 5 μm in large *babesia* (such as *Babesia bigemina*) (Homer et al., 2000).

1.1.3.1.2 Ecology

Babesia are one of the most common blood parasites in the world affecting three types of host groups: humans, domestic animals and wildlife animals. Strains that are able to infect humans are *B. microti*, *B. divergens*, *B. venatorum* (EU1-3) (Gray et al., 2010). Best understood ecology in *Babesia* spp. is the one of *B. microti*, which is mostly found in the US and also in Europe and *B. divergens* strain mostly found in Europe. Both strains can cause human babesiosis but have different enzootic cycles. It is known that *B. microti* is transmitted by tick *I. scapularis* in US and by *I. ricinus* in Europe in tick-rodent enzootic cycle where humans are dead-end hosts (Acosta et al., 2013; Foppa et al., 2002; Sebek et al., 1977). *B. divergens* is circulating in *I. ricinus* - cattle enzootic cycle and causes bovine babesiosis (Zint et al., 2003). In the past years, new strain *B. venatorum* (EU1-3) was found to circulate in *I. ricinus* - deer enzootic cycle and infecting also humans (Herwaldt et al., 2003; Haselbarth et al., 2007). Babesiosis in domestic animals is caused by a variety of strains: *B. bovis*, *B. bigemina*, occurring in tropical and subtropical parts of the world are causing infection in bovines. While in Europe bovine babesiosis is caused mostly by *B. divergens*. In small domestic ruminants such as oats, sheep the babesial infection caused by *B. ovis* and, *B. motasi* mostly in Southern Europe, some African and Asian countries. Canine babesiosis is caused by *B. canis*. *B. canis* is transmitted by *Dermacentor* spp. ticks and so far has only been detected in Europe (Schnittger at al., 2012).

The *Babesia* has been detected mostly in warmer areas of Europe but new data shows their spread also to the northern Europe such as Norway. Prevalence of *B. venatorum* in questing ticks has been reported in Norway 0.9% (Øines, 2012), in Italy 0.85%, (Cassini et al., 2010) in Estonia 1.4%, (Katargina et al., 2011) in Poland 1.6% (Cieniuch et al., 2009), Slovenia 2.2% (Duh et al., 2005) and Netherland 0.9% (Wiellinga et al., 2009). The reservoir hosts are ruminants and the infection prevalence differs between different regions of Europe. In Switzerland, the prevalence in domestic and wild ruminants is 2% (Hilpertshauser et al., 2006), Slovenia 21.6% (Duh et al., 2005) and France 23% (Bonnet et al., 2007). Prevalence of closely related *B. divergens* in questing ticks is similar to the prevalence of *B. venatorum* (Skotarczak and Cichocka, 2001; Øines et al., 2012). However in Austria, this pathogen is more frequent and the prevalence rate is up to 51% (Blaschitz et al., 2008). Similarly, the prevalence of *B. venatorum* in wild cervid in Slovenia is up to 54.9% (Duh et al., 2005), 17% in Ireland (Zintl et al., 2011) and low in Belgium 0.7% (Lempereur et al., 2012). *B. microti* is present in questing *I. ricinus* ticks through Europe in lower prevalence rates than previously mentioned *Babesia* pathogens. The lowest prevalence of 0.1% was detected in Switzerland, followed by prevalence in Netherland, Poland, Italy, Hungary, Russia, Czech Republic and with the highest rate in Germany (9.6%) (Yabsley and Shock, 2012). *B. canis* which is transmitted by *Dermacentor reticulatus* is mostly prevalent in Southern Europe but new studies show spread of this pathogen also further to northern areas of Europe such as Netherlands and Norway (Matjilaa et al., 2005; Øines et al., 2010).

1.1.3.1.3 Vectors

The main vectors for *Babesia* spp. are ixodid ticks. So far six different genera of ixodid ticks have been demonstrated as experimental or natural vectors of diverse *Babesia spp*. (Schein et al., 1981; Spielman, 1976; Telford et al., 1993). *B. divergens, B. venatorum and B. microti* in Europe are transmitted by *I. ricinus*. In Europe, there are known also other tick species which contribute in the transmission of Babesiae, such as Rhipicephalus ticks. Rhipicephalus ticks are involved in the transmission of bovine babesiosis infection agent: *B. bovis*, *B. bigemina* and agent infecting small ruminants: *B. ovis* and *B. motasi*. An essential role in the transmission of *B. canis* to dogs is played by *Dermacentor reticulatus* (Schnittger at al., 2012).

1.1.3.1.4 Reservoir hosts

Babesia are known to infect a big range of mammalian hosts. Almost every mammal that is able to obtain infection is a potential reservoir host for this pathogen (Telford et al., 1993). The most common reservoir hosts are from order Rodentia, Artiodactyla and also some bird species (Zint et al., 2003; Schnittger at al., 2012; Telford et al., 1993; Levine, 1971). *B. microti* is able to infect hosts from various rodent species to humans, while genera of mice (*Apodemus* and *Sicista*) and voles (*Eothenomys*, *Lagurus*, and *Myodes*) are the most common one (Beck et al., 2011; Duh et al., 2003; Rar et al., 2011). In contrast, *B. divergens* is maintained in environment in cattle and is also able to infect humans (Cantu et al., 2007), primates (chimpanzees and rhesus monkeys) (Garnham, 1959) and game and ruminants (roe deer, fallow deer, red deer, mouflon and sheep) (Enigk and Friedhoff, 1962; Carcy et al., 2006, Wirtgen et al., 2012).

1.1.3.1.5 Tick interactions with *Babesia*

The long-term co-evolution of ticks and pathogens has evolved into mutual tolerance where ticks have developed defense mechanisms to control infection and regulate mutual interactions. Up to date only few tick genes responsible for the regulation of *Babesia* infection in ticks have been discovered. One of them, longicin, which has an anti-microbial and anti-fungal function, was reported to inhibit proliferation of *B. equi* (Tsuji and Fujisaki, 2007). The similar function has longipain whose expression is upregulated during the blood feeding. Longipain is cysteine protease which increases acquisition and transovarial transmission (Tsuji et al., 2008). RNAi silencing of three genes, coding TROSPA a midgut receptor, serum amyloid A (homolog of vertebrate acute-phase protein reacting to inflammation) and calreticulin (intracellular protein with many functions) (Wang et al., 2012), have been shown to have significant role in the tick-Babesia interactions (Antunes et al., 2012). After silencing of these genes a significant reduction of *B. bigemina* in *R. annulatus* and *R. microplus* was observed (Antunes et al., 2012).

Premature gametes in infected red blood cells attack the midgut of the tick during the blood meal. After transforming into mature gametocytes they fuse and transfer into zygotes, which invade the midgut cells. In midgets, they evolve into prolonged kinetes and further

infect other tissues such as ovaries where the asexual reproduction takes place. Produced sporokinetes ensure the transmission to the next generation (transovarial transmission). Finally, in infected salivary glands, sporokinetes undergo final amplification evolving into sporozoites, which invade tick saliva. This transstadial transmission stage is crucial for the invasion of the host (Chauvin et al., 2009; Florin-Christensen and Schnittger, 2009).

1.1.3.1.6 Clinical manifestation

A manifestation of the disease can range from subclinical illness to a fatal fulminating disease. So far reported human cases are mostly from the USA and Europe. The number has recently risen because of expanded medical awareness. While in USA couple of hundred cases has been reported caused by *B. microti*, in Europe so far only 30 cases caused by *B. divergens* have been documented (Gray and Weiss, 2008; Zintl et al., 2003). Human babesiosis caused by *B. divergens* is presented by symptoms such as hemoglobinuria, high fever, shaking chills, headaches, myalgia, vomiting and diarrhea. Furthermore, respiratory, cardiac, renal, or hepatic failure has also been documented. There have also been reported cases with life-threatening complications such as acute respiratory failure, disseminated intravascular coagulation, congestive heart failure, coma, and renal failure. Similar symptoms have been recorded in patients infected with *B. venatorum* but in milder manifestations (Gray et al., 2010).

Bovine babesiosis infecting cattle has a wide economic impact in the world. Infection in cattle results mostly in hemolytic anemia as well as heart failure presented by vasodilatation, hypotension, increased capillary permeability, edema, vascular collapse, coagulation disorders, endothelial damage and circulatory stasis (Wright et al. 1989).

Domestic animals such as dogs are also liable to infection resulting in canine babesiosis. Dogs develop clinical signs such as hemoglobinuria, hemolytic anemia, fever, lethargy, anorexia, jaundice and vomiting (Zahler et al., 2000) and can also develop neurological abnormalities and gastrointestinal disturbance (Tarello, 2003).

1.1.4 Order Spirochaetales, family Spirochaetaceae

Spirochaetales are phylum of bacteria with helically coiled cells in length from 5 to 250 μm and diameters around from 0.1 to 0.6 μm. They are distinguished from other bacteria

by flagella that allow the movement in the environment. Order Spirochaetales contains three families: Spirochaetaceae, Leptospiraceae, and Spirillaceae.

Family Spirochaetaceae consists of two genera: *Borrelia* and *Treponema*. *Treponema* are responsible for diseases such as syphilis, bejel, yaws, and rabbits while the group of *Borrelia* is causing Lyme borreliosis (LB) and Relapsing fever (RF).

1.1.4.1 Genus *Borrelia*

Genus *Borrelia* was recently reclassified to two separate genera: an emended genus *Borrelia*, containing the causative agents of relapsing fever and novel genus Borreliella gen. nov., containing the causative agents of Lyme disease (Adeolu et al., 2014). However recent article by Margos et al. (2017) points out that there is an inadequate evidence to support the division of this genus, therefore in this thesis further I will refer to *B. burgdorferi* s.l. complex. *Borrelia* genus consists of more than 40 known genospecies where *Borrelia* causing RF includes species such as *B. dauttonii*, *B. parkeri* and *B. hermsii*. Relapsing fever *Borrelia* are distributed mostly in tropical areas and are transmitted by soft (argasid) ticks (Barbour, 2005). Lyme disease is caused by pathogenic species from *B. burgdorferi* s.l. complex. This complex includes 21 known genospecies that in natural foci circulate between hard ticks from the genus *Ixodes* and vertebrate reservoir hosts (Margos et al., 2011; Ivanová et al., 2014).

1.1.4.1.1 Morphology and biology of *Borrelia burgdorferi* sensu lato

B. burgdorferi s.l. are helically shaped bacteria with the length 10-30 μm. Their wave-like shape is due to the endoflagella located in the periplasmic space (Figure 7.). A unique genome consists of a linear chromosome and 21 plasmids (12 linear and 9 circular). Linear chromosome of *B. burgdorferi* s.s. B31 is of size 910 725 base pairs (bp). *Borrelia* are gram-negative bacteria due to double membrane (Fraser et al., 1997), even though they have some characteristics of gram-positive bacteria such as 28.6% of G+C bases in chromosome, and 23.1-32.3% in plasmids, fluid outer membrane and lack of lipopolysaccharides (Barbour and Hayes, 1986; Takayama et al., 1987).

B. burgdorferi s.l. survives in midgut of tick vector. Following the feeding of the tick on the host, *Borrelia* migrates through the midgut wall and haemocell to salivary glands. Outer

surface lipoproteins located on the plasmids are involved in the transmission of *B. burgdorferi* s.l. from tick to the host (Zhang et al. 2011). For example, outer surface protein A (OspA) interacts with tick gut receptor TROSPA which facilitates *Borrelia* to colonize the tick gut (Pal et al., 2004). On its surface, OspA possesses a receptor for plasminogen of a host organism. After the tick starts to feed on the host, plasminogen changes into plasmin, which facilitates *Borrelia* migration through the midgut wall and haemocell (Coleman et al., 1997). The tick must be attached to the host for at least 24 hours before transmission of *Borrelia* starts. The interaction between the tick-*Borrelia*-host is still poorly understood.

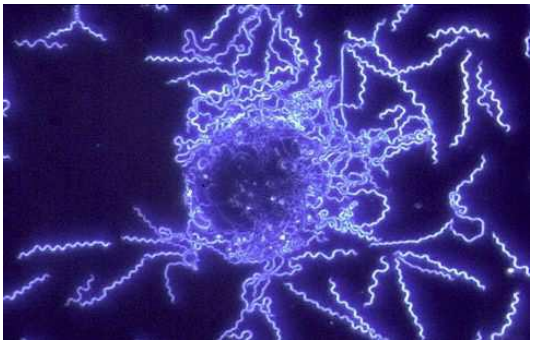

Figure 7. *B. burgdorferi s. l.* under fluorescence (http://www.microbelibrary.org/images/jnelson/images/borrelia-an.jpg)

1.1.4.1.2 Ecology of *Borrelia burgdorferi* s.l

Lyme disease is the most frequent tick-borne disease in northern hemisphere (Strle and Stantic-Pavlinic, 1996; Orloski et al., 2000; Hubálek et al., 2009, Ivanova et al., 2014). In European countries, 85 000 cases are reported every year. This number is probably underestimated since many cases go undiagnosed. In USA, 30 000 cases are documented each year (Cook et al., 2014). Geographic distribution of spirochetes from the *B. burgdorferi* s.l. complex depends on the distribution of the vector tick. In Europe, the transmission is through the ixodid ticks: *I. ricinus* and *I. persulcatus*.

Many species of *B. burgdorferi* s.l. have been isolated from different vertebrate hosts. For a long period of time only *B. burgdorferi* s.s., *B. afzelii* and *B. garinii* were considered pathogenic for humans. Recently another 18 species from *B. burgdorferi* s.l. complex have been discovered, containing species that have been reported pathogenic for humans and

species with unknown pathogenicity (Table 1.). In the group of species with no pathogenicity reported are included: *B. americana*, *B. andersonii*, *B. californiensis*, *B. carolinensis*, *B. japonica*, *B. tanukii*, *B. turdi*, *B. sinica*, and *B. yangtze*. Species with pathogenic potential include: *B. afzelii*, *B. bavariensis*, *B. bissettii*, *B. burgdorferi* s.s., *B. garinii*, *B. kurtenbachii*, *B. lusitaniae*, *B. spielmanii*, and *B. valaisiana* (Rudenko et al., 2011).

Table 1. *B. burgdorferi* s.l. species, their vectors, host/reservoirs and geographic distribution (figure from Rudenko et al., 2011).

***Borrelia* species**	**Vector**	**Hosts/reservoirs**	**Geographical distribution**
B. afzelii	*I. ricinus I. persulcatus*	Rodents	Asia, Europe
B. americana	*I. pacificus, I. minor*	Birds	United States
B. andersonii	*I. dentatus*	Cotton tail rabbit	United States
B. bavariensis	*I. ricinus*	Rodents	Europe
B. bissettii	*I. ricinus, I. scapularis, I. pacificus, I. minor*	Rodents	Europe, United States
B. burgdorferi s. s.	*I. ricinus, I. scapularis, I. pacificus*	Rodents, birds, lizards, big mammals	Europe, United States
B. californiensis	*I. pacificus, I. jellisonii, I. spinipalpis*	Kangaroo rat, mule deer	United States
B. carolinensis	*I. minor*	Rodents, birds	United States
B. garinii	*I. ricinus, I. persulcatus, I. hexagonus, I. nipponensis*	Birds, lizards, rodents	Asia, Europe
B. japonica	*I. ovatus*	Rodents	Japan
B. kurtenbachii	*I. scapularis*	Rodents	Europe, United States
B. lusitaniae	*I. ricinus*	Rodents, lizards	Europe, north Africa
B. sinica	*I. ovatus*	Rodents	China
B. tanukii	*I. tanuki*	Unknown (possibly dogs and cats)	Japan

B. turdi	*I. turdus*	Birds	Japan
B. spielmanii	*I. ricinus*	Rodents	Europe
B. valaisiana	*I. ricinus, I. granulatus*	Birds, lizards	Asia, Europe
B. yangtze	*Haemaphysalis longicornis, I. granulatus*	Rodents	China
genomospecies 2	*I. pacificus*	Unknown	United States
B. finlandensis	*I. ricinus*	Unknown	Europe

B. burgdorferi s.l. has been detected in most European countries. The prevalence differs from country to country, while the overall mean prevalence of *Borrelia* in ticks in Europe is 13.7% with a higher infectious rate in adult ticks than in nymphs. Regarding mean representation of *Borrelia* genospecies, *B. afzelii* and *B. garinii* have similar mean prevalence 38% and 33% respectively, while *B. burgdorferi* sensu strict and *B. valaisiana* were detected in 18% and 19% of infected *I. ricinus* among Europe and lowest mean prevalence was detected for *B. lusitaniae*, 7% (Rauter and Hartung, 2005).

1.1.4.1.3 Vectors

I. ricinus and *I. persulcatus* are responsible for the transmission of *B. burgdorferi* s. l. complex in Europe and Asia. In North America *I. scapularis* and on the West coast of America *I. pacificus* are fulfilling the role of a vector (Rudenko et al., 2011). Also, other tick species were reported to be infected with *Borrelia* strains but there is no evidence so far that they are effective in transmission of Lyme disease.

The most important stage relative to the transmission is the nymphal stage. Nymphs get infected as larvae feeding on small hosts, which are competent for *Borrelia* spirochetes. Transmission occurs mostly transstadially, while the transovarial transmission is very rare (Humair and Gern, 2000; Richter et al., 2012). Co-feeding transmission is also possible but it is considered negligible in comparison to other routes of infection (Gern and Reis, 1996)

1.1.4.1.4 Reservoir hosts

Borrelia species have specific association to their reservoir hosts. This is given by the response of the host complement system to different *Borrelia* genospecies (Kurtenbach et al., 1998). *B. afzelii* is maintained in small rodents while *B. valaisiana* and *B. garinii* prefers birds as reservoir hosts (Hanincová et al., 2003 a, b; Kurtenbach et al., 2002). *B. burgdorferi* s.s can be gained from both rodents and birds (Kurtenbach et al., 1998). The most important rodent reservoir hosts are: wood mouse (*Apodemus sylvaticus*), yellow-necked field mouse (*Apodemus flavicollis*), black striped mouse (*Apodemus agrarius*), bank vole (*Clethrionomys glareolus*) and meadow vole (*Microtus agrestis*) (Kurtenbach et al., 1998; Tälleklint and Jaenson, 1994). Also shrews may serve as reservoirs: European water shrew *Neomys fodiens*, Eurasian pygmy shrew *Sorex minut*us, Eurasian common shrew *Sorex araneus* and European hedgehog *Erinaceus europeus* (Gern et al., 1997). In Central Europe birds such as blackbirds (*Turdus merula*) and song thrushes (*Turdus philomelos*) are an important reservoir hosts for *B. valaisiana* and *B. garinii* (Tarageľová et al., 2008).

1.1.4.1.5 Tick interactions with *Borrelia*

After the tick starts to feed on an infected host, spirochetes remain in the tick midgut where expression of Osps (OspA, OspB) and other molecules (Dps, BptA, La7) take place (Schwan and Piesman, 2002). During the important stage of *Borrelia* colonization of the midgut, expression of OspA is important for the successful transmission of the parasite. OspA is able to shield the molecules of the host immune system, which were injected simultaneously with the spirochetes (Battisti et al., 2008). It allows adherence of the parasite to the tick midgut cells through the receptor known as TROSPA and plays a significant role in the pathogen persistence. TROSPA which is a tick receptor for OspA is localized in the midgut controlling colonization of the vector. Suppression of TROSPA expression led to reduced borrelial transmission from tick to the host (Pal et al., 2004). During the blood meal spirochetes amplify in the midgut of the tick. However, after the molting the number of spirochetes decreases as a result of adverse environment in the midgut caused by digestion processes. This causes a peeling of the bacteria from midgut epitel. Number of bacteria is then decreased by defensins (Piesman et al., 1990). The process of how the spirochetes survive

these conditions is unknown. After the molted infected tick starts to feed again, the spirochetes are exposed to the active complement in the blood from the host. Spirochetes that are resistant to the host complement will survive and invade the haemolymph and salivary glands with a help of OspC (Schwan et al., 1995; Pal et al., 2004).

Complement is a main part of the vertebrate immune system constructed of more than 30 proteins. The complex is activated by the presence of microorganisms, leading to a sequence of events on the surface of the pathogen that helps to destroy the pathogen and to eliminate the infection. Complement intervenes in two pathways; innate (natural) and adaptive immune response. Innate immune response involves antibodies, which are a direct response to the presence of antigen of the pathogen. The adaptive immune response is on the other hand activated directly on a surface of the cell without an involvement of the antigens. Both pathways involve activation of complement proteins C1 to C9 (Figure 8.). Gradual proteolysis of the complement complex of C1-C9 lysis leads to a creation of active smaller proteins and peptides. While the small proteins attached to the microorganism, the peptides diffuse away and act as a chemoattractant factor and an inflammatory paracrine (Kurtenbach et al., 2002).

Borrelia that are sensitive to the complement will be negatively selected in the tick midgut and will be unable to further infect other hosts. Resistance and sensitivity of different *Borrelia* genospecies to the different host complement determines the variability of reservoir hosts. The resistance of *Borrelia* to complement is established by at least five outer membrane proteins including OspE, which specifically bound to the complement protein, factor H and reconnect (factor H like protein) (Stevenson et al., 2002). A complex of these proteins is called CRASPs (*complement regulatory-acquiring surface proteins*), which enable the construction of membrane attack complex (MAC) (Kraiczy et al., 2001).

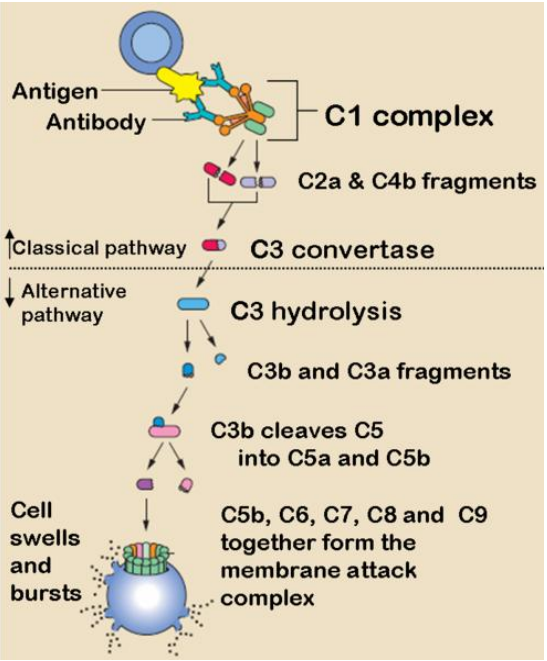

Figure 8. Classical and alternative pathway of complement activation. (http://en.wikipedia.org/wiki/Alternative_complement_pathway)

1.1.4.1.6 Clinical manifestation

Lyme disease is a multisystemic disease and can affect many organs such as skin, muscles, nervous system, heart, and joints. Clinical manifestation can be divided into 3 stages, early localized, disseminated and chronic infection. In the first stage of early localization within few days after infection 60% of patients involve erythema migrans as skin rash in an area of the tick bite. Other nonspecific signs can also be observed, such as a headache, fatigue, muscle ache and fever (Steere, 1989). If untreated after few weeks, the infection spreads to organs and tissues and causes multiple erythema migrants or invades the nervous system or joints. Pathogen intervenes muscles, nervous system, heart, and joints and manifests into neuroborreliosis and Lyme arthritis (Nadelman & Wormser, 1998; Steere, 1989). In the last stage, if untreated, *Borrelia* can hide especially in joints or skin and cause chronic infection that persists few years. A manifestation of chronic Lyme disease involves erosion of cartilage and bones, dysfunction of the nervous system involving problems with memory, concentration, muscle pain, paralyzation or acrodermatitis chronica atrophicans (Asbrink et al., 1984; Nadelman and Wormser, 1998; Steere, 1989).

As other tick-borne diseases also Lyme disease is thought to have a distinct clinical manifestation according to the represented species. *B. afzelii* and *B. garinii* are the most common species in Europe (Gern, 2009). Infection with *B. afzelii* manifests into skin form of Lyme borreliosis (LB) acrodermatitis chronica athrophicans while *B. garinii* and *B. bavariensis* are associated with neurological manifestation and *B. burgdorferi* s.s. with the arthritis. However, these associations are not strict and different symptomatic within different species can be present (Ornstein et al., 2001).

1.2 Ticks as a vectors of pathogens

Most tick species display a preference for feeding on specific wild animals while just a limited number usually feed on domestic animals and humans. These few species adapted to feed on livestock or humans become efficient bridge vectors of pathogenes among wild animals and domestic animals and humans.

These arthropods belong to the order Ixodida which contains three main families Ixodidae "hard ticks", Argasidae "soft ticks" and Nuttalliellidae. These hard and soft ticks differ anatomically and in their life cycle. While Ixodid ticks feed on variety of vertebrate hosts once in every life stage for a long period of time, Argasid ticks feed for a short time usually on one host species. The most common hard ticks are *Dermacentor*, *Haemaphysalis*, *Rhipicephalus* and *Ixodes* (Soneshine, 1991). Epidemiologically the most important tick species in Europe is *I. ricinus*.

1.2.1 *I. ricinus*

I. ricinus tick which is known as a 'wood-tick' and 'sheep-tick' is the most common tick in Europe. From the epidemiological point of view it is important due to the possibility to transmit bacterial, viral and protozoal infections to wild and domestic animals and also to humans. This species is spread all around Eurasia while the density is affected by biotic and abiotic environmental factors. It occurs in Europe, north Africa and part of Asia, from Great Britain, to north Africa, southwestern of Turkmenistan, north of Iran and western part of Siberia (Filippova, 1977).

I. ricinus is very sensitive to variation of the environmental conditions, because of the requirement of a high degree of relative humidity necessary to complete the life cycle. Therefore, any variation in climatic conditions or habitat structure deriving from changes, for example, in land use, can lead to significant variation in its geographic range of distribution and local abundance (Medlock et al, 2013). Currently this tick species has expanded its distributional range, with invasion at higher altitudes and latitudes in Europe (Figure 9.).

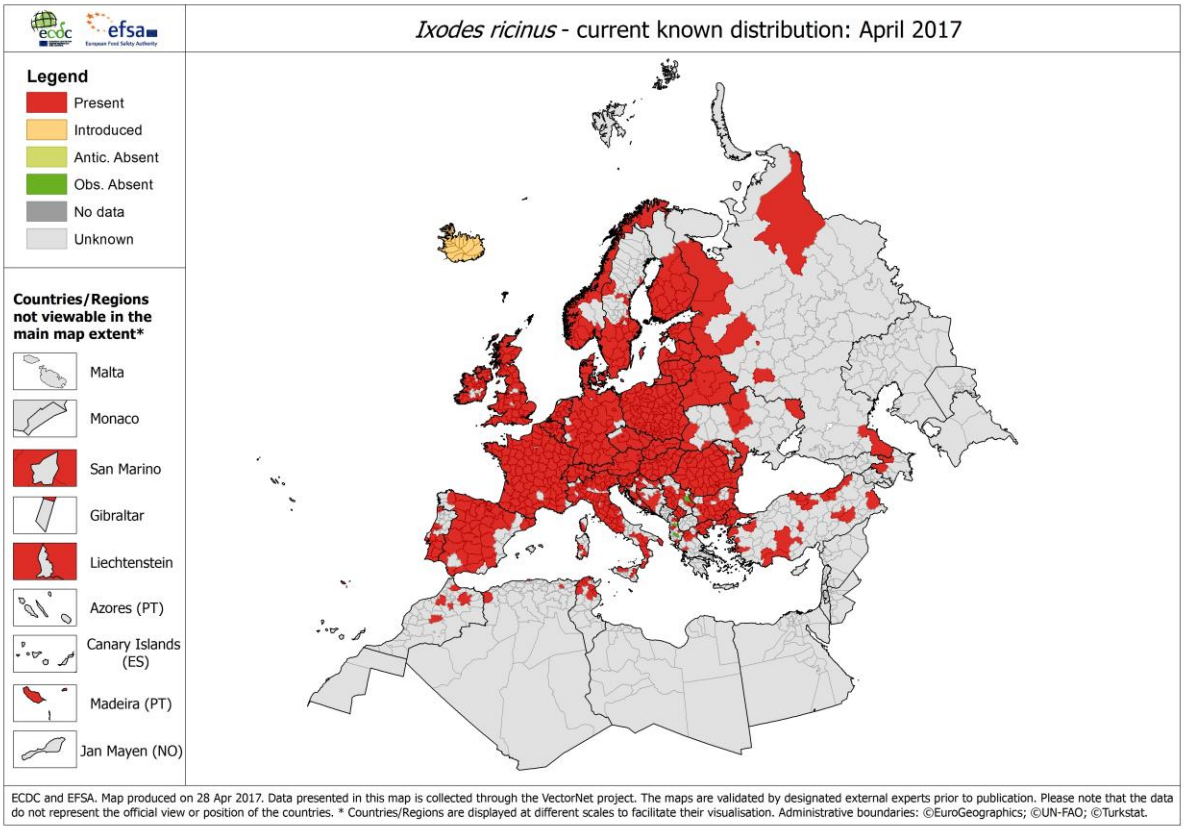

Figure 9. The map shows the current known distribution of the *I. ricinus* tick species in Europe at 'regional' administrative level (NUTS3). This map is based on published historical data and confirmed data provided by experts from the respective countries as part of the VBORNET project supported by ECDC (http://ecdc.europa.eu/en/healthtopics/vectors/vector-maps/Pages/VBORNET-maps-tick-species.aspx (24.5.2017)).

In Italy, the densest areas are hilly and pre-alpine northern areas with a specific climate and high population density of wild animals. Climate is specific with cold winters and with humidity up to 85% in the summer period. As a tick density is increased in the area, the prevalence of tick-borne pathogens has the tendency to increase as well (Rizzoli et al., 2004).

The northern area of Italy is also known for the majority of human cases with LB, TBV and anaplasmosis (Ciceroni et al., 2001; Ruscio et al., 2003; Beltrame et al., 2006, Otranto et al., 2014).

The life cycle of *Ixodes* ticks is usually completed in 1-6 years depending on tick species, climatic condition, and presence of host and vector. Ticks have four life stages: egg, larva, nymph and adult, and in the case of *I. ricinus*, three-host life cycle (Figure 10). Morphology of the tick is in every stage different. While larva has 3 pairs of legs, nymphs and adults have 4 pairs of legs. Nymphs are much smaller than adults, which also have a significant sexual dimorphism (Figure 10.). In all stages, the body of the tick lacks a distinct head, thorax, and abdomen. The front part of the body includes mouthparts, sensory organs, cutting organs, chelicery and pedilpalpae and hyposthome with teeth that enable the tick to attach to the host skin. Ticks are disposed with various sensory organs helping them to sense a presence of the host. The hair-like structures on the body, legs, and mouthparts and a sensory complex containing a cluster of olfactory and gustatory receptors are used for locating the host and communicating with other ticks (Parola and Raoult, 2001).

After every blood meal tick detaches from the host and molts to next life stage. After molting it quests and searches for another host to take another blood meal. This process repeats till the tick molts to the final adult stage. The molting usually takes place on the host. After feeding and mating on the host, engorged female drops and seeks for suitable conditions for egg production. This can take up to one week. After 4-8 weeks in these suitable conditions, the female will produce up to 2000-5000 eggs. The female then dies and the larvae search for a host to attach (Soneshine, 1991) (Figure 11).

Figure 10. Three stages of *I.ricinus*. From left, larva, nymph, adult male, adult female. (http://kleinkamp2010.wordpress.com/category/uncategorized/ (22.1.2013))

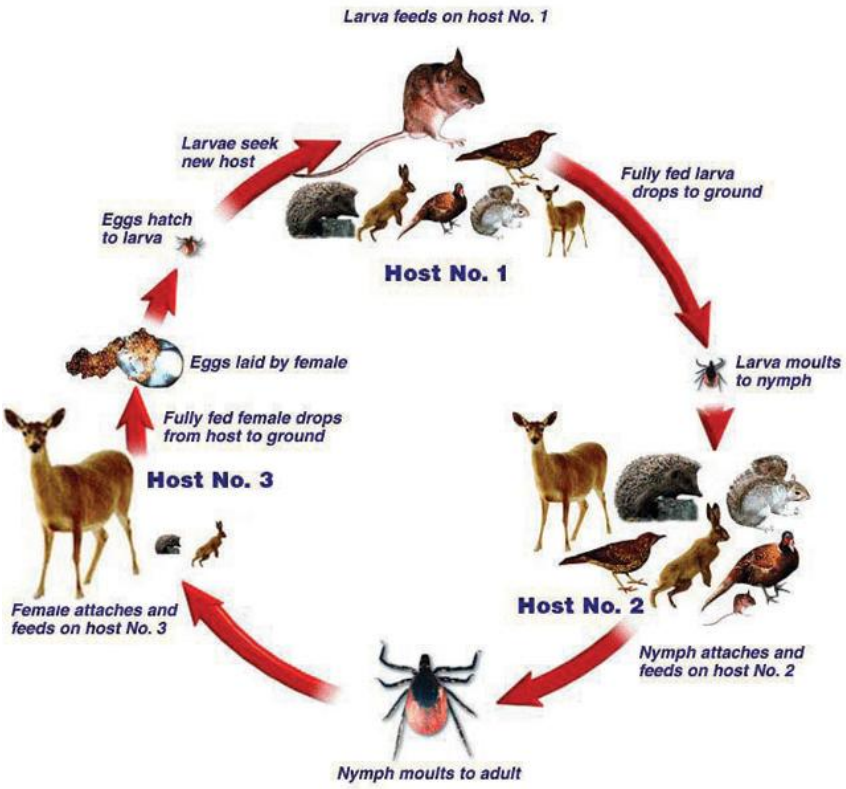

Figure 11. Three-host life cycle of a tick *I. ricinus* (http://www.deerandtick.org/life_cycle.html (22.1.2014)).

1.2.2 Ecology

Different tick species have different behavior in the environment including their phenology, attachment to host and host preference. According to the environmental preference, we can divide ticks into endophilic (*I. trianguliceps, I. hexagonus, Rhipicephalus* spp.) and exophilic ticks (*I. ricinus*, *Dermacentor* spp., *Haemaphysalis* spp.). The endophilic ticks hide in the nests and burrows of their host waiting for their arrival while exophilic ticks prefer open environment like meadows and forests. Once the host is present in near distance the tick becomes highly responsive to the stimulus such as chemical stimulus (CO2, NH3), phenols, humidity, aromatic chemicals, and airborne vibrations and body temperatures. Among exophilic ticks, two host-seeking behaviors can occur. Questing behavior where the questing ticks crawls up the stems of grass and waits for the host to pass by and the active behavior where the tick runs toward the host (Klompen, 2005).

Host preference varies among tick species. While some species are host specific, others feed on a variety of hosts, like *I. ricinus* which feeds on different small and large mammals including birds as well. According to the number of the hosts a tick feeds on during its three-life stages, we can divide them into one-host, two-host or three-host ticks. Ixodid ticks feature are three-host parasites which means, that ticks can feed on different host during all three stages (Soneshine, 1991). The host preference depends on habitat distribution; if the tick is adapted to a specific habitat, it will attach to a host that occurs in that habitat. The preference of tick species to humans also varies. *I. ricinus* in Europe and *I. scapularis* in United States are the most common antropophilic species (Sonenshine, 1991, Hillyard et. al., 1996)

1.3 Acarological hazard

To predict possible new outbreaks of tick-borne pathogens, identifying parameters that form suitable habitat for their common vector *I. ricinus* are in great interest. Data on abundance of questing ticks and their infection with tick-borne pathogens are valuable; however they capture only a small fragment of tick population and infection rate. Therefore for predicting acarological hazard climatic and environmental data that directly influence tick phenology should be taken into account. *I. ricinus* is sensitive to environment conditions, mostly to humidity and temperature. These factors affect its metamorphosis to the next active stage and the questing period (Randolph, 2004). Further vegetation covers as well as availability of vertebrate host are important factors for survival of the vector (Ruiz-Fons et al., 2012).

In the past, some studies focused on predicting tick phenology, they however had their limitations. Either they predict only short term phenology based on ground climate data using simple models or they use correlative (Estrada-Peña, 1999) or modified matrix models (Dobson et al., 2011). Recently Remote sensing imaginary has been proven as a reliable tool for predicting vector habitat (Estrada-Peña et al., 2014c; 2015; 2016), however no studies were done to predict tick-borne infection rate (Rosà et al., 2017 in preparation for Eurosurveillance).

1.4 Identification and typing of microorganisms

There are phenotypic and genotypic molecular techniques for identification and typing of microorganisms mostly differing in sensitivity, time requirements, price and specificity. Phenotypic molecular techniques are based on microscopy, protein analysis and serology while genotypic techniques analyze DNA sequences (Tenover et al., 1997).

1.4.1 Microscopic identification of pathogens

In the past the identification by light microscopy, electron microscopy and fluorescence microscopy were used. While with light microscopy detection of the pathogen in low concentration is not possible, electron microscopy and fluorescence microscopy are more reliable (Oschmann et al., 1999). However, the dark field microscopy has also shown determination of lower ability compared to PCR detection (Strube et al., 2010).

1.4.2 Molecular identification of pathogens

Genetic variability of the pathogens among the species often results in different ecology and host infectivity, therefore identification of different strains is necessary. The application of various molecular methods is needed to give us precise information on their diversity. Most commonly used molecular methods are: DNA-DNA hybridization, species specific PCR, DNA sequencing, real-time PCR (RT-PCR), MLST/MLSA (multilocus sequence typing/analysis), restriction fragment length polymorphism (PCR-RFLP), reverse line blot (RLB), single-strand conformation polymorphism (SSCP) (Margos et al., 2008, 2010; Wang et al., 1999, Wang et al., 2014), next generation sequencing (Vayssier-Taussat et al., 2013) and DNA chip (Kocianová and Barák, 2005, Melničáková et al. 2013, Michelet et al., 2014).

Different molecular approaches are used depending on the scientific question. If we want to track local epidemiology focusing on micro variations among species in one geographic area, methods for identification of high variability of the bacterial genome are used. Methods derived from PCR are mostly applied giving us results on the maximum variation within the population. On the other hand, global epidemiology is focusing on relationships in between the species from one geographic area compared to the isolates from

the rest of the world. Global epidemiology studies are investigating the genome regions and a slow accumulation of variations that tend to be selective neutral. To these approaches we can add MLST/MLSA (Maiden et al., 1998; Margos et al., 2008, 2010).

1.4.2.1 DNA-DNA hybridization and sequencing of 16S gene

DNA-DNA hybridization is method used for distinguishing closely related strains based on the ability to hybridize bacterial genomes. Depending on how well the different genomes hybridized in optimized conditions we can observe overall genetic similarity between isolates. If the DNA-DNA binding values are more than 70% and less than 5 ºC difference in melting temperatures (ΔTm) they are classified as one species. Alternatively, if the DNA-DNA binding values are less than 50% they are with certainty classified as different species (Wayne, 1987).

In new isolates phylogenetic position is established with sequencing of 16S rRNA gene. According to Van Dam isolates with less than 97% identity of 16S rRNA gene are defined as a new species since there are no known isolates among the species with higher diversity (van Dam, 1996).

There is no known theory that would classify species according to the range of common genes between them. 70% criterion was empirically calibrated based on observations of genotypes known species in the early discovery and description of methods (Cohan, 2002).

Information obtained by DNA-DNA hybridization is identical with sequencing of whole genomes or multilocus sequences analyses. Unfortunately, this method has also disadvantages where a high amount of culture is needed, radioactive markers have to be used and it is also time consuming. Hybridization values are also difficult to reproduce and compare, because this method is properly implemented only in few laboratories and there is no database to compare different strains using a codified set of criteria that would allow the inclusion of strains to be analyzed in known or new taxon (Amann et al. 1995).

1.4.2.2 Polymerase chain reaction (PCR) and real-time PCR (RT-PCR)

PCR is a highly specific and sensitive method for detection of pathogens. It requires an isolation of DNA and amplification of target gene of the pathogen. This method is simple and

relatively quick, but sensitivity is questionable according to different results from different laboratories. There can be also an occurrence of false positive samples due to the contamination and false negative samples due to the inhibition for example of heparin and hemoglobin. Therefore optimization and standardization is necessary (Oschmann et al. 1999).

In PCR amplification of target gene or part of a target gene is amplified by specific oligonucleotides. Genes used for PCR amplification are 16S rRNA for *A. phagocytophilum*, *B. burgdorferi* s.l. and *Rickettsia* spp. (Liebisch a kol., 1998; Massung and Slater, 2003), 18S rRNA for *Babesia* spp. (Casati et al. 2006), intergenic spacer 5S-23S rRNA for *B. burgdorferi* s.l. (Rijpkema et al. 1995) and 17 kDa gene for *Rickettsia* spp. (Reye et al., 2010). PCR is usually the first step towards characterization of intragenetic variations.

Real-Time PCR is a great development of PCR technology, which maximizes the potential of this technique. This method follows the general principles of PCR technique. Its key feature is in detecting and measuring of the products that are generated after each cycle of PCR process in ''real time''. RT-PCR unlike PCR requires specific DNA probe composed of oligonucleotides that are labeled with a fluorescent reporter, which permits detection only after hybridization of the probe with its complementary sequence. Possibility of detection of more than one pathogen or different strains among one pathogen at a time gives a great advantage compared to PCR (Sirigireddy and Ganta, 2005). At least three tick-borne agents can be detected at once in multiplex RT-PCR (Chan et al., 2013).

1.4.2.3 Sequencing analysis of DNA

This method is used in different fields such as genetic, evolutionary, taxonomy and epidemiological studies (Stackebrandt a Goebel, 1994). Genetic variations can be observed by direct sequencing of a target gene, which was obtained in single PCR or nested PCR. Nested PCR is usually used for more specific amplification of longer fragments.

Different strains of *Rickettsia* spp., *Babesia* spp., *B. burgdorferi* s.l. can be distinguished by amplification and sequencing of chromosomal or plasmid genes. In some cases, such as ecologically different strains of *A. phagocytophilum* are hardly distinguished by fragments of 16S rRNA due to their low variability. For this approach more variable genes coding outer membrane proteins are used. The most commonly used targets include *groEL*, *msp4* and *ankA* (Liz et al. 2002, de la Fuente et al., 2005, Massung et al. 2000). Genes coding

outer membrane proteins that interact with host immune system have evolved faster due to selective pressure. Therefore they are useful for studies of intragenetic variations among species.

Although *A. phagocytophilum* has been recently reclassified as single bacterial species, some genetic variations were found in *groEL*, *msp2*, *msp4* and *ankA* genes. Genes coding outer membrane proteins *groEL*, *msp2*, *msp4* and *ankA* are more variable and are used to distinguish strains with different infectivity and host preference (Silaghi et al. 2011, Carpi et al. 2009, de la Fuente et al. 2005).

Rickettsia spp. can be detected by amplification of following genes: 16S rRNA, 17kDa, *gltA* and genes coding outer membrane proteins such as *ompA* and *ompB* for more reliable phylogenetic analysis (Stothard and Fuerst, 1995; Anderson and Tzianabos, 1989; Wood et al. 1987; Roux et al. 1996; Gilmore et al. 1991). 16S rRNA in rickettsial species is rearranged. This feature is rare in other bacteria and it was documented only in bacteria with small genomes. 16S rRNA in comparison to other bacteria is located upstream from 23S and 5S that are linked (Andersson et al. 1999). 17000 molecular weight antigen (17 kDa gene) is one of the best characterized loci in both spotted fever and the typhus group rickettsiae. This gene has 94% level of similarity among the genus (Anderson and Tzianabos, 1989).

Markers encoding rRNA are widely used because they are present in every eukaryotic cell. *Babesia* probably contains tree combinations of ribosomal genes in the following order: 18S-ITS1-5, 8S-ITS2-28S where 18S is mostly used for identification of strains among *Babesia* group. 18S gene includes variable regions V1-V9 from which the biggest and the most variable is V4. Another marker used for *Babesia* identification is gene that codes for β-tubulin. The length of its fragment differs between 310-460 bp depending on the identified strain (Skotarczak, 2008).

B. burgdorferi s.l. cluster of rrn usually contains one copy of 16S rRNA (*rrs*) and tandem repeats of 23S rRNA (*rrlA* a *rrlB*) and 5S rRNA (*rrfA* a *rrfB*) (Fukunaga et al. 1992). The cluster of rRNA genes is located in the central part of the linear chromosome and it is arranged in the following order: rrs-rrlA-rrfA-rrlB-rrfB. 16S gene (*rrs*) with high homology among the species (95.3 – 99.6%) or 5S (*rrf*)–23S (*rrl*) intergenic spacer amplicon gene, which is highly variable, are used for reliable species identification (Le Fleche et al., 1997, Postic et al., 1994). Another target gene is *flaB* (Gassmann et al. 1991) that encodes a 41 kDa

flagellin protein FlaB (Ge et al., 1997). It is located on a linear chromosome (Fraser et al., 1997). This gene is used in distinguishing between *Borrelia* from the relapsing fever group and *B. burgdorferi* s.l. complex (Fukunaga and Koreki, 1996).

1.4.2.4. Next generation sequencing (NGS)

Next generation sequencing is term used to refer to high-throughput DNA sequencing technologies. This technology is similar to Sanger sequencing but it differs in the ability to sequence a large number of different DNA sequences in one reaction at considerably low cost.

The basic principle of the method consists of template preparation, sequencing and analysis. First, the starting material is fragmented into libraries of small templates (sequencing library). Afterwards specific synthetic DNA is added at the ends of library fragments which serve as primers for downstream amplification and sequencing. Template generation is also needed for immobilization of DNA fragments by attaching them to solid surfaces or beads. This allows sequencing of millions of distinct fragments in one reaction, generating a high volume of data. This great amount of data presents challenges and opportunities for analysis (Rizzo and Buck, 2012). NGS seem to have endless applications with considerable potential application in both diagnostic and public health microbiology laboratories (Salipante et al., 2013). This method is continuing to grow in popularity and therefore new additional innovative applications will be very likely proposed.

NGS has a potential to identify pathogens, predict antibiotic resistance, obtain information on virulence factors and track the spread of emerging bacteria (Kourani, 2013). It has been already used for identification of pathogens transmitted by *I. ricinus* ticks, causing diseases in humans in Europe. *R. helvetica*, *A. phagocytophilum*, *B. garinii*, *B. afzelii*, *B. burgdorferi* s.s. and C*andidatus* Neoehrlichia mikurensis have been detected by means of NGS method (Vayssier-Taussat et al., 2013).

1.4.2.5 MLST (*multilocus sequence typing*) a MLSA (*multilocus sequence analysis*)

The ability of precise identification of infectious causative agents is essential for monitoring epidemiology and exact diagnostics. However, currently used methods lack reproducibility between laboratories and are unable to quantify the genetic relationships

between isolates (Maiden et al. 1998). MLST/MLSA are methods solving the problem of pure reproducibility and storage of data in the global database.

Most of the MLST/MLSA schemes are based on housekeeping genes that evolve slowly and variations among them are almost neutral (Enright and Spratt, 1999). Despite the fact that the individual housekeeping genes are less polymorphic compared to the hypervariable genes, the combined use of multiple sequencing of housekeeping genes showed distinctive ability and remaining characters for the analysis of evolutionary relatedness (Maiden et al., 1998; Enright and Spratt, 1999). Development of MLST scheme for studying evolutionary, epidemiological and population genetics of *B. burgdorferi* s.l. was done by Margos et al. (2008) (Mechai et al., 2014), of *A. phagocytophilum* is under current work of Winter et al., for *Babesia* spp. MLST was so far designed for *B. bovis* and *B. bigemina* (Guillemi et al. 2013) and for *R. conorii* (Zhu et al. 2005), *R. sibirica* (Fournier et al. 2006).

MLST is a method used for genotypic characterization of prokaryotes by analyzing small allelic variations of (usually 7) housekeeping genes (Maiden et al., 1998). For the analyses, the internal parts of housekeeping genes with the length around 450-500 bp are used. Genes selected for MLST analysis are located next to each other with similar function and this diminishes deviance between the individual genes which were subject to strong selective pressure. Genes should have similar levels of genetic diversity for each gene to provide a similar benefit to phylogenetic analysis and no gene is more dominant (Urwin and Maiden, 2003).

The MLST allele of each gene is assigned with a unique number. The isolates can be characterized by multiplying the total number which is called allelic profile. After all target genes are selected for the analysis and MLST scheme is created. Sequence data, information on strain and allelic profiles are collected in virtual form in the online database. Each unique allelic profile is assigned to a unique number referred to as sequence type (ST) (Urwin and Maiden, 2003).

MLST is a simple method requiring only the amplification of DNA fragments by PCR and sequencing of these fragments (Maiden et al. 1998). It allows a high level of discrimination between isolates using fewer loci. Study of several loci is essential for providing meaningful relationships between strains. It is also important because of the mutation and recombination events in the strains that could provide a misleading result in the

study of a single locus (Maiden et al., 1998). Groups of isolates with identical allelic profiles determine strains or clones and form the basis of genotype classification system. MLST helps to cluster isolates of the major genetic lines within species (Cooper and Feil, 2004).

The original purpose of MLST analysis was to improve the clinical diagnosis, epidemiological monitoring and population studies. Recently the concept of MLST has expanded and currently, it includes analysis of closely related species, called MLSA. MLSA was developed for rapid and robust hierarchical classification of prokaryotic species. It serves as a substitute for DNA-DNA hybridization and is becoming commonly used in bacterial classification (Gevers et al. 2005).

Classification using a MLSA includes a universal set of genes that allow the hierarchical classification of all prokaryotes (Zeigler, 2003). The problem is that the informative genes within genera or families may not be present in more distant taxa. Moreover, the conserved genes, which are also present in distant species, have evolved slowly, and thus do not allow the definition of closely related species. Bacterial identification with a MLSA involves following steps: rRNA sequencing and assessment of an unknown isolate to the group. This further defines genes and oligonucleotides, which should be used in MLSA for inclusion of isolate to a particular species (Gevers et al. 2005).

1.4.2.6 Genotypization by PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) and PCR-SSCP (single strand conformation polymorphism)

1.4.2.6.1 PCR-RFLP

Pathogens such as *Rickettsia, Babesia* genospecies and *B. burgadorferi* s.l. can be besides sequencing of PCR fragments identified also by using PCR-RFLP. Three methods were designed for identification of this microorganism; all depending on a unique structure of rRNA, *rrs*, *rrfA-rrlB* and *rrs-rrlA*. In PCR-RLFP one or more restriction enzymes are used to obtain fragments with different molecular weight, which are separated on a gel. Different profiles of tested samples are the result of substitutions, deletions and insertions in place of restriction. Frequently used gene is 5S-23S rRNA with the length of 250-300bp. Various restriction enzyme RE (for example: *MseI*) gives fragments of a different length typical for different genospecies of *B. burgdorferi* s.l. (Postic et al., 1994, Derdáková et al., 2003).

1.4.2.6.2 PCR-SSCP

The PCR-SSCP has a similar approach as PCR-RFLP. This method uses point mutations in RNA, DNA, cDNA to identify closely related species or distinguished intragenetic variety among species. Conformation of the strain will change according to the difference in primary structure. Denaturation of the fragments followed by electrophoresis in non-denatured conditions will cause differences in ssDNA secondary structure due to nucleotide sequences. The conformation of secondary structure will cause the difference in weight of the strain on the gel. Already very small changes in primary structure will have an effect on the secondary structure of the product. The PCR-SSCP was optimized for 5S-23S to characterize all known species of *B. burgdoreferi* s.l. and also to be able to detect new genospecies such as *B. bavariensis* and *B. spielmanii* (Ondrisková and Derdáková 2013).

1.4.2.7 Detection of bacterial pathogens using DNA chip and quantitative PCR

DNA chip and multiplex PCR are methods used to quickly identify more pathogens in one step. Diagnostic based on DNA chip should allow simultaneous detection of all pathogens for which DNA probes have been implemented such as *B. burgdorferi* s.l., *Coxiella* spp., *Anaplasma* spp., *Francisella* spp., *Rickettsia* spp. and *Candidatus* N. mikurensis. DNA chip method is based on variable regions of different genes depending on the pathogen. Different pathogens can be detected at once by hybridization of amplified pathogen preferred gene to specific oligonucleotide probes attached to oligo-chip (Blaškovič a Barák, 2005, Melničáková et al. 2013).

Quantitative PCR (qPCR) is based on the identification of the hypervariable regions of genes such as 16S rRNA gene. qPCR follows the general principle of PCR but compare to PCR, qPCR is able to simultaneously detect or quantify a targeted DNA molecule during the amplification. qPCR shows the concentration of amplified product in a ‘’real time’’. This method has the advantage of speed, throughput and a high degree of potential automation unfortunately is not able to detect as many pathogens at once as it is with the DNA chip method (Higuchi et al., 1993).

2 Aims of the thesis

One of the main aims of the work was to analyze the eco-epidemiology and genetic variability of four tick-borne pathogens (*A. phagocytophilum*, *Rickettsia* spp., *Babesia* spp. and *B. burgdorferi* s.l.) from ticks detached from various hosts in northern Italy, by the use of PCR and nested PCR. This study has further contributed to clarifying of the many aspects of the transmission cycle of these pathogens. We screened larval ticks detached from a number of wildlife animal species to focus on the role of these hosts as reservoirs of the infections. These results will help to provide epidemiological parameters useful in predicting and assessing the hazard for humans and domestic animals.

The second aim of the work was to analyze the genetic diversity of *A. phagocytophilum* to get the most detailed data on the occurrence and pathogenicity of divergent strains of this bacterium in northern Italy, Finland and USA. Comparison of these strains brings us closer to a better understanding of ecology and potential threat of this pathogen on a global scale.

The third aim of the study was to detect genetic diversity of *B. burgdorferi* sensu lato from ticks by amplifying 5S-23S intergenic spacer and in some cases by amplifying four MLST housekeeping genes. *B. burgdrferi* s.l. genospecies have different clinical manifestations and associations with reservoir host. Exact identification of the genospecies can help to apply proper preventive strategies.

Finally, the fourth aim was to predicting acarological hazard in Europe with data provided from five different countries by use of remote sensing and statistical models.

Target pathogens were:

1. bacterial: *A. phagocytophilum*, *Ricketsia* spp. and *B. burgdorferi* s.l.

2. protozoal: *Babesia* spp.

Target vector was: *I. ricinus* tick (European sheep-tick)

3 Materials and Methods

3.1. Study sites

3.1.1. Study site in Italy

This study was carried out in mixed broad-leaf and coniferous forests in the Valle dei Laghi (from 46°5'N11°6'E southwestern to 45°54'N10°52'E) in the Province of Trento (eastern Italian Alps) where a presence of *I. ricinus* is documented (Figure 12.). More specifically, we carried our study in 12 study sites in the valley (Trento, Lamar, Cavedine, Mt. Bondone, Pietramurata, Covelo, Lasino, Calavino, Cadine, Lundo, San Gioavanni and Arco). The Province of Trento is endemic for several tick-borne pathogens, including TBE virus and *B. burgdorferi* s.l. (Rezza et al., 2015, Rizzoli et al., 2009).

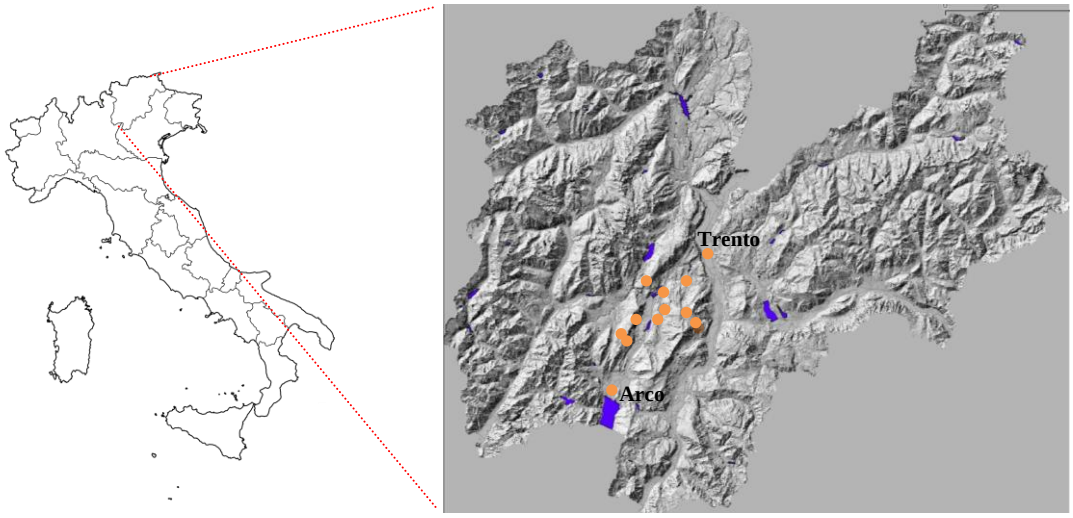

Figure 12. Study sites in northeast Italy, province of Trentino (http://italymap.facts.co/italymapof/italymap.php)

3.1.2. Study site in United States

Samples from North part of USA were collected in sites in Mansfield (41.7885° N, 72.2293° W), Connecticut and Block Island (41.1721° N, 71.5576° W), Rhode Island, USA. Only *I. scapularis* ticks species were present at the sampling sites. Questing ticks were collected from both study sites, while feeding ticks on rodents were analyzed only from Mansfield study site.

Samples from the South part of USA were collected in site in Chattahoochee-Oconee National Forest in Georgia (34.7660° N, 84.1435° W). All samples belong to tick species *I. scapularis*.

3.1.3. Study site in Finland

Samples were collected in South Eastern Finland by researcher of the Luke institute (from 59.8728° N, 23.3971° E to 60.9242° N, 23.3711° E). Only *I. ricinus* ticks were present and collected at the sampling site.

3.1.4. Study sites in five European countries for predicting acarological hazard in Europe

Samples were collected by EDENext partners in five European countries: Italy, Germany, Slovakia, Czech Republic and Hungary within the framework of the EU funded project EDENext.

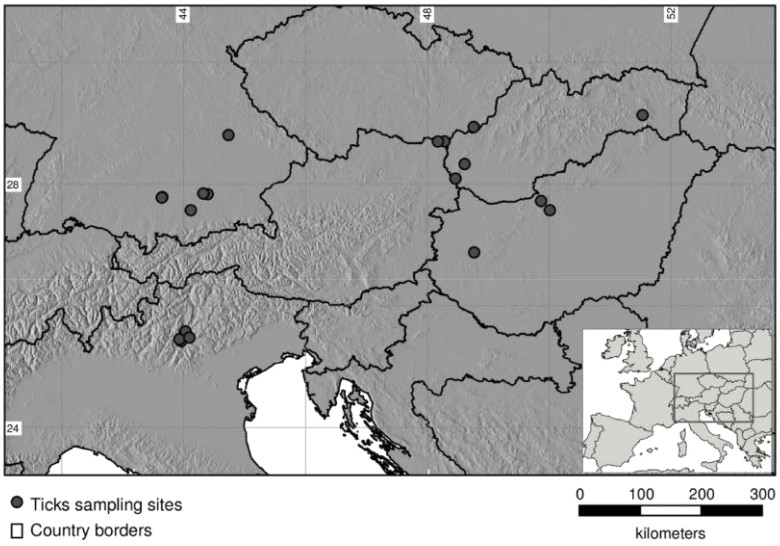

Figure 13. Study sites in five European countries (Figure from Rosa et al., 2017).

3.2. Field sampling

3.2.1. Questing tick sampling

Collection of questing ticks was carried out during the peak of tick population activity in spring from April till June (2011 and 2013) by FEM personal (Valentina Tagliapietra, Daniele Arnoldi, Margherita Collini) and myself (Tagliapietra et al., 2011) in three habitat

types, natural, agricultural and urban. Totally, 1791 questing *I. ricinus* ticks were collected by conventional blanket-dragging method using 1m x 1 m white felt blanket, attached by one side to a wooden pole (Sonenshine, 1993). Every 5 m the blanket was checked for ticks. Ticks were removed from the blanket using forceps sterilized with diluted bleach (10%). They were placed individually in 2 mL and frozen afterward at -80°C, until DNA extraction.

3.2.2. Feeding tick sampling

Ticks feeding on various hosts were collected. More specifically rodents were represented mostly by yellow-necked mice (*Apodemus flavicollis*) and less by bank voles (*Myodes glareolus*) and hazel dormouse (*Muscardinus avellanarius*). Since *A. flavicollis* and *A. sylvaticus* have similar morphology, *A. flavicollis* was confirmed following Michaux et al. (2001). Ticks were collected from several bird species such as, European robin (*Erithacus rubecula*), nightingale (*Luscinia megarhynchos*), great tits (*Parus major*), Eurasian blackcap (*Sylvia atricapilla*), song thrushes (*Turdus philomelos*), Chaffinch (*Fringilla coelebs*), Eurasian jay (*Garrulus glandarius*), dunnock (*Prunella modularis*), blackbirds (*Turdus merula*). Wild large mammals such as roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*) and chamois (*Rupicapra rupicapra*) were also checked for ticks. Ticks from dogs as well as sheep and humans were collected.

3.2.2.1. Trapping of small mammals

Trapping of rodents was carried out by FEM staff (Valentina Tagliapietra, Daniele Arnoldi, Adam Konečný and Margherita Collini) and myself using Ugglan livetraps (model 2, Granhab, Sweden). Trapped rodents were checked for ticks as well blood samples and ear tissues were taken. Trapping session took place during spring-summer 2011, 2012 and 2013. Live traps were placed in linear transects, approximately 10 m apart with variable numbers of traps used per session, 42-47 traps. Live traps contained seeds and potato to provide moisture. Traps were placed in the morning and checked in the evening of the next day. Rodents were released to clear polyethylene bag for the technician to be able to take them firmly by the scruff of the neck. Ticks were gently removed with sterile forceps and placed individually in 2mL Eppendorfs with 1 ml of 70% ethanol. Blood was taken with sterile needle from their eye

without damaging their eye side. Ear biopsy was taken using sterile disposable ear punch needles (Ø 3mm) and samples were stored individually and frozen at -80° until analysis. Rodents were subsequently discharged in the forest were they were captured.

All animal handling procedures and ethical issues were approved by the Provincial Wildlife Management Committee (authorization n. 595 issued on 04.05.2011). Ethical guidelines concerning animal welfare were followed as defined by the European Commission and detailed in the EDENext project.

3.2.2.2. Bird netting

In order to collect ticks from resident birds, we used mist-netting technique during a reproductive period for the bird species. Netting was carried out by me or Margherita Collini (FEM PhD student) with the assistance of ornithologist Dott. Franco Rizzolli, a licensed bird ringer. Mist-nets of a length 120m erected vertically on poles were placed in the boarder of a meadow and forest or wetlands. The areas near to food sources (like orchards and bushes with berries) were taken in consideration in placing the mist-nets as well. Nets were divided into two or more transects and kept for two days. Bird netting started in early mornings when the birds were most active (hunting for food) and mist-nets were checked every hour. During heavy rains or during the hottest hour during midday, the nets were closed. Licensed ornithologist Dott. Franco Rizzolli handled the birds and morphological mesurmants were taken. Ticks were removed from the area around the eyes, ears and beak with sterile forceps and placed into 2 mL vial with 70% ethanol. Ticks from the same animal were placed in same vial.

3.2.2.3. Collection of ticks from large mammals

Ticks from the most common wild ungulates in the area were collected with collaboration of the Trentino Hunter's Association during the autumn hunting season. Ticks were collected by FEM staff, myself, wardens and/or hunting guards that were trained. Collection of ticks was carried out within 24 hours the animal was killed or the wardens removed the forelegs of the animal and stored them on -20°C till they were delivered to FEM. Scanning the forlegs for ticks was carried out under a magnifying lamp, as previously

described by Carpi et al. (2008). Ticks from the same animal were placed in the same 2 mL vial filled with 70% ethanol. Species, sex and age, altitude and location were provided by the Trentino Hunter's Associat.

Ticks from sheep were collected by FEM staff (Valentina Tagliapietra or Margherita Collini) or shepherd. Ticks from dogs were collected by veterinarians in Sarche Calavino (TN), Arco (TN), Trento (Dr. Danielli, Dr. Zampiccoli, Dr. Zammplini). Collection of ticks from humans was carried out by Dott. Claudio Ramponi in Santa Chiara hospital in Trento. All procedures were done using sterile forceps and ticks were placed into vials filled with 70% ethanol and stored at -20 °C until DNA extraction. Ticks detached from the same animal were placed into the same vial.

3.3. Laboratory methods

3.3.1. Morphological identification of ticks

The most common tick in the area is exophilic species *I. ricinus*, widely spread around Europe. While less representative are *I. hexagonus* and *I. trianguliceps*. These specialist and nidicolous tick species usually stay in nest of the host animals, therefore only *I. ricinus* were found while questing. Identification of questing and feeding tick species was done based on their morphology using recognized identification keys (Cringoli et al., 2005; Estrada-Peña et al., 2004), at 40X magnification under a dissecting microscope. Since engorged ticks in larval and nymphal stage are sometimes hardly identified morphologically all tick species were confirmed as well by molecular analysis (see chapter 3.3.3).

3.3.2. DNA extraction

3.3.2.1. DNA extraction from questing ticks and ticks from host (adults, nymphs)

DNA extraction from questing ticks was carried out by FEM staff because it was used for various studies within the EDENext project, while DNA extraction from feeding ticks was carried out by myself. Genomic DNA from feeding and questing ticks (adults and nymphs) was extracted using a commercial DNA extraction kit (Qiagen Dneasy Blood and Tissue kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol.

To avoid contamination from environmental DNA, human DNA and cross-contamination, forceps were sterilized with bleach and ethanol; washing of the ticks and DNA extraction were performed under a biological (UV sterilized) hood; sterile and DNA-free consumables and reagents were used for all methods. Ticks were washed in DNA-free distilled water before proceeding further to eliminate possible surface contaminants. Ticks were placed in 200 µL of RNase DNase free water (Sigma-Aldrich, Saint Louis, USA) in 0.5 mL sterile tubes for 20 s and samples were gently flipped few times. This step was repeated twice.

3.3.2.1.1. Sample homogenization and lysis

For engorged ticks in adult or nymphal stage, pre-lysis step was performed. Each tick was placed in 2mL autoclaved Eppendorf tube with a 5 mm stainless steel bead and 100 µL PBS (Phosphate-buffered saline) solution. Ticks were homogenized using a mixer mill for 3 min at 30 Hz. The homogenate was then transferred to a sterile 1.5 ml Eppendorf tube and 20 µL Proteinase-K 10 mM/mL (Sigma-Aldrich, Saint Louis, USA) and 180 µL Qiagen ATL tissue lysis buffer were added. The homogenate together with the buffer and proteinase were left overnight to digest at 56°C.

3.3.2.1.2. Tissue protocol

Qiagen Dneasy Blood and Tissue kit (Qiagen, Valencia, CA, USA) was used for engorged ticks (adults and nymhps) as well as ear biopsy DNA extraction. Extraction of DNA from ear biopsy was performed by FEM staff within the EDENext project. 1 µL Carrier RNA (1 µg/µL in ATE (Qiagen) was added to the lysate to increase DNA binding to the silica membrane. Purification was performed manually or by using the QIAcube (Qiagen, Inc., Valencia, CA, USA), a robotic workstation for automated purification of DNA, RNA and proteins. Purification was performed according to the manufacturer's protocol. Protocol was adjusted by preheating ATE solution at 56°C and lowering the final volume to 40 µL to increase the final DNA concentration. At a time 11 ticks were proceed with one negative-contamination control. DNA was stored at -20°C till further procedure.

3.3.2.2. DNA extraction from host seeking larvae

DNA extraction from larvae ticks is challenging due to their size and the hard chitinous exoskeleton. Alkaline hydrolysis was described as an efficient method for this purpose (Guy and Stanek, 1991). Its advantages are in cost and the final concentration of DNA. 70% ethanol in 1.5ml eppendorf where ticks were soared was left open, for the ethanol to evaporate at room temperature under the hood. Each tick was squashed in the eppendorf with an end of a tip. We added 100 µl of prepared 1.25% ammonia solution (19 ml sterile water + 1 ml ammonia (Merck)). Closed eppendorfs were incubated at 100°C for 30 min. Further the samples were centrifuged at highest speed for 15 sec. and we let them evaporate to half of their volume. Samples were after placed in freezer at -20°C.

3.3.2.3. DNA extraction from blood samples

DNA from blood was extracted using ReliaPrepTM Blood gDNA Miniprep System (Promega). All steps were carried out according to the manufacturer's protocol, except that: elution was performed with 60 µl of Nuclease-Free Water that was preheated for increases the concentration of the DNA. Around 50 samples were processed at once with at least 5 negative controls added (one negative control per 10 samples). Samples were stored at -20°C.

3.3.3. Molecular identification of ticks

The most common tick species found in the Alps are *I. ricinus*, *I. hexagonus*, *I. trianguliceps, I. canisuga, I. frontalis, Riphicephalus sanguineus* and *Haemaphisalis punctata* (Cringoli et al., 2005). To verify a successful isolation of genomic DNA from ticks, partial 16S gene of length 470 bp was amplified using primers Ir_16S_681-F and Ir_16S_1159-R (Table 2.). Primers for identification of tick species were designed by Collini et al. (2015).

PCR volume of 20 µl included 0.5 µM of each primer, 0.25 mM of each dNTP, 1x HotMaster Taq Buffer, 1.25 U HotMaster Taq (5-Prime), and 1 µL of template DNA. The thermal cycling was performed in a Veriti® Thermal Cycler (Applied Biosystems, Foster City, CA, USA) (Table 3.).

3.3.4. Molecular detection of pathogens

3.3.4.1. Molecular detection of *A. phagocytophilum*

A. phagocytophilum was detected in positive samples of questing ticks, feeding ticks and tissues from rodents by nested PCR by amplifying 16S ribosomal rRNA, while detection in blood samples from rodents was carried out by Real-Time PCR. Nested PCR was performed at a final volume of 20 μL, containing 10 μM of each primer, 10 mM of each dNTP, 10x HotMaster Taq Buffer, 5 U HotMaster Taq (5-Prime), and 2 μL of template DNA (Table 2., Table 3.). As a result, of interspecific variability in positive samples, protein coding genes, *msp4* and *groEL* were amplified using Nested PCR. The Nested PCR for *msp4* and *groEL* contained same reagents and amounts as mentioned above for 16S of *A. phagocytophilum* with an addition to 0.5mg/ml of bovine serum albumin (BSA) in each reaction (Table 3.) and specific primers (Table 2.). All amplified fragments were further sequenced. The Real-Time PCR was performed at a final volume of 20 μL, containing 15 μL of gene expression TaqMan MMX (Applied Biosystems), 10 μM primers ApMsp2f, ApMsp2r (each 2.25 μL) and 10 μM probe ApMsp2p (0.5 μL) (Table 2.). Negative controls for both DNA extraction and PCR amplifications were included in all amplification reactions.

3.3.4.2. Molecular detection of *Rickettsia* spp.

Rickettsia spp. were detected in feeding ticks by amplification of the partial gene for 17 kDa protein by nested PCR. The reaction was carried out at a final volume of 49 μL, containing 10 μM of each primer, 10 mM of each dNTP, 10x Taq 360 Buffer, 0.5 mg/ml BSA, 25mM $MgCl_2$, 5 U AmpliTaq 360 (Applied Biosystem, Foster City, CA, USA), and 1 μL of template DNA (Table 2., Table 3.). *R. helvetica* as well as *R. monacensis*/*R. tamurae* were able to be confirmed by phylogenetic analysis of 17 kDa gene, for confirmation of other species real-time PCR with specific primer or amplification of other genes were necessary. *R. raoultii* was confirmed by real-time PCR using the specific primers Rraou2850F2, Rraou2956R2 and probe Rrao2896P previously described by Jiang et al. (2012) (Table 2.). *R. monacensis* was distinguished from *R. tamurae* by amplifying the *ompA* gene by PCR. For the PCR amplification, a Promega GoTaq® DNA Polymerase protocol was used with 2 μl of DNA in final volume of 20 μl. The ompA gene was amplified from 2 μl of DNA by PCR with

10 pmol of each primer (R-ompA-13-R and R-ompA-554-F) (Table 2.) in a 20 µl volume (25 mM MgCl, 10 mM deoxynucleosidetriphosphate, Promega GoTaq® reaction buffer, 5 U of Promega GoTaq® DNA Polymerase) (Table 3.). Negative controls for both DNA extraction and PCR amplifications were included in all amplification reactions. All amplified fragments were further preceded to sequencing.

3.3.4.3. Molecular detection of *Babesia* spp.

DNA from feeding ticks was used for detection of *Babesia* spp. by PCR. The partial gene of small ribosomal subunit - 18S, specific for *Babesia* species was amplified in positive samples (Table 2., Table 3.). PCR performed for detection of *Babesia* spp. contained components and volume of the components mentioned above in *A. phagocytophilum* detection (see chapter 3.3.4.1). Negative controls for both DNA extraction and PCR amplifications were included in all amplification reactions. All amplified fragments were further preceded to sequencing.

3.3.4.4 Molecular detection of *B. burgdorferi* s.l.

Ticks of all stages, feeding on hosts were further analyzed for occurrence of *B. burgdorferi* s.l. Samples were analyzed using nested PCR for a variable region of the 23S-5S intergenic spacer (IGS). Reaction was carried out at a final volume of 25 µL, containing 10 µM of each primer, 10 mM of each dNTP, 10x Taq 360 Buffer, 5 U AmpliTaq 360 (Applied Biosystem, Foster City, CA, USA), and 5 µL of template DNA (Table 2., Table 3.). Positive samples were used for amplification of 8 housekeeping genes for MLST method by touchdown PCR (genes: *clpA*, *clpX*, *uvrA*, *rplB*, *pepX*, *nifS*, *pyrG*) and nested PCR (*recG* gene) (Table 3.). Apmlification of housekeeping genes was performed in first round in 25 µL volume containing 2.5 µL of DNA and the second round was performed in 30 µL volume with 3 µL of template. Touchdown PCR and nested PCR further contained 10 µM of each primer, 10 mM of each dNTP, 10x HotMaster Taq Buffer, 5 U HotMaster Taq (5-Prime) (Table 2., Table 3.). Negative controls for both DNA extraction and PCR amplifications were included in all amplification reactions. All amplified fragments were further sequenced.

Table 2. List of used primers for tick species identification, pathogen detection and *B. burgdorferi* s.l. genotyping

Organism	*Primers, probs*	*Sekvence (5´-3´)*	*Gene*	*Lenght*	*Reference*
Ticks *Ixodes* **spp.**	Ir_16S_681 F	TTTTAGCAATAAACTTTTCAAG	16S	470 bp	Collini et al., 2015
	Ir_16S_115 R	AATAAAAAATATCATTCTGG			
Anaplasma	ge3a	CACATGCAAGTCGAACGGATTATTC	16S rDNA	932 bp	Massung et al., 1998
phagocytophilum	ge10r	TTCCGTTAAGAAGGATCTAATCTCC			
	ge9f	AACGGATTATTCTTTATAGCTTGCT	nested PCR	546 bp	
	ge2r	GGCAGTATTAAAAGCAGCTCCAGG			
	HS1a	AITGGGCTGGTAITGAAAT	*groEL*	1297 bp	Liz et al., 2002
	HS6a	CCICCIGGIACIAIACCTTC			
	HS43	AT(A/T)GC(A/T)AA(G/A)GAAGCATAG TC	nested PCR		
	HSVR	CTCAACAGCAGCTCTAGTAGC			
	MSPAP5	ATGAATTACAGAGAATTGCTTAGTAG G	*msp4*	849 bp	de La Fuente et al., 2005
	MSPAP3	TTAATTGAAAGCAAATCTTGCTCCTA TG			
	msp4f	CTATTGGYGGNGCYAGAGT	nested PCR	498 bp	
	msp4r	GTTCATCGAAAATTCCGTGGTA			
	ApMSP2F	ATGGAAGGTAGTGTTGGTTATGGTAT T	*msp2*	77 bp	Courtney et al., 2004
	ApMSP2R	TTGGTCTTGAAGCGCTCGTA	RT PCR		
	Probe	FAM-TGGTGCCAGGGTTGAGCTTGAGATTG			
Rickettsia **spp.**	17k1p	TTTACAAAATTCTAAAACCAT	17kDa		Reye et al., 2013
	17k.539n	TCAATTCACAACTTGCCATT			
	Rr17k90p	GCTCTTGCAACTTCTATGTT	nested PCR	480bp	

	R-ompA-13-R	GCAATTCAAAAAGGTCTTAAA	*ompA*		Baráková et al., 2017
	R-ompA-554-F	TTTCCTGTAAGTGTTATCTTTG			
	Rraou2850F2	GTGGTGGTGTTCCTAATACTCC	Real-time PCR	107bp	Jiang et al. 2012
	Rraou2956R2	ACCTAAGTTGTTATAGTCTGTAGTAAAC			
	probe	6-FAM-CGCGATATTGGCACTGTACAGTTAAAGCATCGCG-TAMRA			
***Babesia* spp.**	BJ1	GTCTTGTAATTGGAATGATGG	18S	400-500 bp	Casati et al., 2006
	BN2	TAGTTTATGGTTAGGACTACG			
***B. burgdorferi* s.l.**	nifS-OR	GATATTATTGAATTTCTTTTAAG	touchdown PCR		
	nifS-OF	ATGGATTTCAAACAAATAAAAAGCAA			
	nifS-IR	TCCTGTTGGAGCAAGCATTTTATG		564bp	
	clpA-IF	GACAAAGCTTTTGATATTTTAGATGA	*clpA*		
	clpA-IR	CAAAAAAAACATCAAATTTTCTATCTCT			
	clpA-OF	AAAGATAGATTTCTTCCAGAC	touchdown PCR	579bp	
	clpA-OR	GAATTTCATCTATTAAAAGCTTTCCC			
	pyrG-OF	GATTGCAAGTTCTGAGAATA	*pyrG*		Margos et al., 2008
	pyrG-OR	CAAACATTACGAGCAAATTC			
	pyrG-IF	GATATGGAAAATATTTTATTTATTGAGAC	touchdown PCR	603bp	
	pyrG-IR	AAACCAAGACAAATTCCAAGAAAGG			
	clpX-OF	GCTGCAGAGATGAATGTGCC	*clpX*		
	clpX-OR	GATTGATTTCATATAACTCTTTTG			
	clpX-IF	TTATTCCAAACCTTGCAATCCATTTAA	touchdown PCR	624bp	
	clpX-IR	TGTGCCTGAAGGAACATTTGCTTTC			
	pepX-OF	ACAGAGACTTAAGCTTAGCAG	*pepX*		
	pepX-OR	GTTCCAATGTCAATAGTTTC			

	pepX-IF	TTATTCCAAACCTTGCAATCCATTTAA	touchdown PCR	570bp	
	pepX-IR	TGTGCCTGAAGGAACATTTGCTTTC			
	uvrA-OF	GAAATTTTAAAGGAAATTAAAAGTAGGCTTAA	*uvrA*		
	uvrA-OR	CAAGGAACAAAAACATCTGG			
	uvrA-IF	GCTTAAATTTTTAATTGATGTTGG	touchdown PCR	570bp	
	uvrA-IR	CCTATTGGTTTTTGATTTATTTGAATAA			
	rplB-OF	TGGGTATTAAGACTTATAAGC	*rplB*		
	rplB-OR	GCTGTCCCCAAGGAGACAC			
	rplB-IF	CGCTATAAGACGACTTTATCTTT	touchdown PCR	624bp	
	recG-OF	CCCTTGTTGCCTTGCTTTC	*recG*		
	recG-OR	GAAAGTCCAAAACGCTCAGCATGC			
	recG-IF	CTTTAATTGAAGCTGGATATCT	nested PCR	651bp	
	recG-IR	CAAGTTGCATTTGGACAATC			
	23SN1	CCATAGACTCTTATTACTTTGACCA	5S-23S	380bp	Rijpkema et al., 1995
	23SC1	TAAGCTGACTAATACTAATTACCC			
	23SN2	CCTATGACTCTTATTACTTTGACCA	nested PCR	240 bp	
	5SC2	GAGAGTAGGTTATTGCCAGGG			

Table 3. Termal cycles of PCR, nested PCR and RealTime PCR

Primers	**Iniciation**	**Denaturation**	**Hybridization**	**Polymerization**	**Cycles**	**Fin. polym.**
Ir_16S_681-F, Ir_16S_1159-R	94°C/2min	94°C/30 s	51°C/1 min	65°C/100s	30-35	65°C/10min
ge3a, ge10r	95°C/2min	94°C/30s	54°C/30s	72°C/60s	39	72°C/10min
ge9f, ge2r	95°C/2min	94°C/30s	54°C/30s	72°C/60s	40	72°C/5min
HS1a, HS6a	94°C/2min	94/88°C/1min	48°C/2min.	68°C/90s	3/37	68°C/5min.

HSVR,HS43	94°C/2min	94/88°C/1min	55°C/2min	72°C/90s	3/37	72°C/5min
MSP4AP3,MSP4AP5	95°C/2min	94°C/30s	56°C/10s	72°C/60s	39	72°C/5min
msp4f, msp4r	95°C/2min	94°C/30s	56°C/10s	72°C/60s	40	72°C/5min
ApMSP2F, ApMSP2R,P		50°C/2min	95°C/10min	95°C/15s	40	60°C/1min
BJ1, BN2	94°C/2min	94°C/20s	55°C/20s	65°C/60s	35	65°C/5min
Rr17k 539n, Rr17k 1p	94°C/3min	94°C/30s	56°C/30s	72°C/60s	15	72°C/7min
17k 90p, Rr17k 539n	94°C/3min	94°C/30s	57°C/35s	72°C/70s	35	72°C/7min
ompA-13-R, ompA-554-F	94°C/20s	52°C/20s	57°C/35s	72°C/60s	35	72°C/7min
Rraou2850F2, Rraou2956R2, Rrao2896P	50°C/2min	94°C/2min	94°C/5s	60°C/30s	45-50	
23SN1, 23SC1	94°C/3min	94°C/30s	52°C/30s	72°C/60s	25	72°C/7min
23SN2, 5SC2	94°C/10s	94°C/30s	57°C/30s	72°C/60s	45	72°C/7min
nifS-OR, nifS-OF	95°C/15min	94°C/15s	55°-48°C 30 s /30s	72°C/60s	9/30	72°C /5min
nifS-IR, nifS-OF						
clpA-IF,clpA-IR						
clpA-OF,clpA-OR						
pyrG-OF,pyrG-OR						
pyrG-IF, pyrG-IR						
clpX-OF, clpX-OR						
clpX-IF, clpX-IR						
pepX-OF, pepX-OR						
pepX-IF, pepX-IR						
uvrA-OF, uvrA-OR						
uvrA-IF, uvrA-IR						
rplB-OF, rplB-OR						
rplB-IF, rplB-OR						
recG-OF, recG-OR	95°C/15 min	94°C/15s	55°C/30s	72°C/60s	40	72°C/5min
recG-IF, recG-IR	95°C/15 min	94°C/15s	55°C/30s	72°C/60s	40	72°C/5min

3.3.5. Visualization and sequencing of PCR products

Amplified fragments were detected via capillary electrophoresis using a QIAxcel system (Qiagen, Valencia, CA, USA). Capillary electrophoresis contained DNA High-Resolution Cartridge and QX 15 bp-3 Kb size marker. The analysis was performed with OM500 method and QIAxcel ScreenGel 1.0.2.0. Exo-SAP-IT TM kit (GE Healthcare, Little Chalfont, England) was used for purification of both, forward and reverse strands. Sequencing took place on an ABI 3130 XL using Big Dye Terminator v3.1 (Applied Biosystems, Foster City, CA, USA). Electropherogram was checked and a consensus sequence was created using Sequencer v.5.1. BLASTn search was carried out for species identification.

3.3.6. Phylogenetic analysis

Sequences obtained in our study were submitted to GenBank using Sequin software (https://www.ncbi.nlm.nih.gov/Sequin/). Association numbers of our sequences are listed below (Table 4.). Consensus sequences of *msp4*, *groEL*, 17kDa, 16S rRNA and MLST (*clpX*, *recG*, *pepX* and *pyrG*) were aligned in MEGA 5. Phylogenetic analysis and construction of phylogenetic trees were carried out by Maximum likelihood for each gene (as well as by other method such as Neighbor joining, Minimum evolution and Maximum parsimony, data not shown).

3.3.7. Statistical analysis

3.3.7.1. Tick-borne pathogens detected in feeding ticks

Statistical analyses were performed only for *A. phagocytophilum* and *R. helvetica* due to a low prevalence of other pathogens. A binomial Generalized Linear Model was applied to assess if the infection in ticks was influenced by host species and tick stages (R Development Core Team, 2013). The response variable was the occurrence of the most prevalent tick-borne pathogens detected in ticks (i.e. *A. phagocytophilum* or *R. helvetica*), while explanatory variables were host species and different tick stages (larva, nymph or adult).

3.3.7.2. Tick-borne pathogens detected in questing ticks from five European countries

For statistical analyses, categorical, environmental and climatic predictors (year, country and habitat types, LST, precipitation, NDVI and NDWI) were used to detect a relationship between total nymphs counts and relative density of infected nymphs by Roberto Rossa PhD, FEM. Total nymphs counts was performed with Negative Binomial Generalized Linear models (Bates et al., 2015) and relative density of infected nymphs was performed using Tobit models (Yee 2016). Both full models were constructed after excluding collinear and non-significant variables and including the remaining environmental and climatic variables (Rosà et al., 2017 in preparation for Eurosurveillance).

3.3.8. Climatic and environmental data

Remote sensing and interpolated climatic datasets were used as tools for processing climatic and environmental data. The environmental predictors were determined based on already published literature (Schulze and Jordan 2003; Perret et al. 2004; Barrios et al., 2012; Estrada-Peña et al., 2016). Land surface temperature (LST), Normalized Difference Vegetation Index (NDVI) and Normalized Difference Water Index (NDWI) data were collected with the Moderate Resolution Imaging Spectroradiometer (MODIS) and processed in GRASS GIS 7 (Neteler et al. 2012). The MODIS LST products were reconstructed from 1000 to 250 m resolution and NDVI and NDWI had spatial resolution of 500 m. The environmental and climatic data were aggregated per month and seasonally (3-months moving window from January to June). The climatic data were obtained from ECA&D dataset (European Climate Assessment & Dataset, Version 13.1 available at: http://www.ecad.eu/) as previously described by Haylock et al. (2008) (Rosà et al., 2017 in preparation for Eurosurveillance).

4 Results

4.1. Feeding ticks

A total of 862 feeding *Ixodes* ticks belonging to five species (821 *I. ricinus*, 12 *I. trianguliceps*, 15 *I. hexagonus*, 11 *I. turdus*, 3 *I. frontalis*) with access number (*I. ricinus* [KY319188], *I. trianguliceps* [KY319190], *I. hexagonus* [KY319189]) were collected from various wild and domestic hosts. Specifically, 393 ticks were collected from rodents, 229 from wild ungulates, 112 from birds, 88 from human patients, 27 from dogs and 13 from sheep. The maximum number of larval ticks analyzed from one host was five (Baráková et al., 2017, submitted to Tick and tick borne pathogens).

4.1.1. Ticks collected from rodents

4.1.1.1. Ticks collected from rodents in Italy

We collected and analyzed 393 (4 adults/31 nymphs/358 larval) *Ixodes* ticks belonging to *I. ricinus* (381) and *I. trianguliceps* (12) from 134 rodent individuals. Specifically, 389 ticks were collected from 130 yellow-necked mice (*Apodemus flavicollis*), 3 ticks from 3 bank voles (*Myodes glareolus*) and 1 tick from 1 hazel dormouse (*Muscardinus avellanarius*) (Tables 5., Table 6.).

Among the 31 nymphs (23 *I. ricinus*, 8 *I. trianguliceps*) and 4 adults (*I. trianguliceps*) detached from rodents, two nymphs 5.7% were positive for *A. phagocytophilum*, while no larval ticks tested positive. 8.6% (3/35) of ticks in the mature stage were infected with *Rickettsia* spp., of which two were *R. helvetica* and one from an unidentified *Rickettsia* spp. (Table 6.). From 358 larvae (348 *I. ricinus*, 10 *I. trianguliceps*) detached from rodents, 4.5% (16/358) were tested positive for *Rickettsia* spp., with 56.3% (9/16) belonging to *R. helvetica* and 43.8% (7/16) belonging to *R. monacensis*/*R. tamurae*. *Babesia* spp. was present in 5.7% (2/35) of nymphs. One nymph was positive for *B. venatorum* and one for *B. microti*. Six (1.7%) larvae were infected with *B. venatorum*. Among ticks at nymphal and adult stage, 5.7% (2/35) were infected by *B. afzelii* and further 7.3% (26/358) of larvae detached from these hosts were positive for the same *Borrelia* genospecies (Table 5.). All positive ticks were detached from yellow-necked mice except one nymph from a bank vole (Baráková et al.,

2017, submitted to Tick and tick borne pathogens). All positive samples were identified by PCR or nested PCR and DNA sequencing (for details see chapter 3.3.4).

4.1.1.2. Ticks collected from rodents in the USA

Feeding ticks from rodents were provided by Giovanna Carpi PhD (Yale University) from Northeastern USA (Connecticut and Rhode Island). In total 77 *I. scapularis* larvae were collected from 17 white-footed mice (Peromyscus leucopus) from Mansfield study site. Among 77 *I. scapularis* detached from rodents, 11.7% (9/77) were positive for *A. phagocytophilum*. These samples were used only for detection of *A. phagocytophilum*. All positive samples were identified by real-time PCR, nested PCR and DNA sequencing (for details see chapter 3.3.4).

4.1.1.3. Ticks collected from rodents in Finland

Ticks from rodents from Finland where provided by Liina Voutilainen PhD and Tarja Sironen PhD, docent (Helsinki University). We analyzed 67 feeding *I. ricinus* ticks (26 nymphs, 41 larvae) from *A. flavicolis* and *M. glareolus* for the presence of *A. phagocytophilum* and *Rickettsia* spp. None were positive for *A. phagocytophilum*, while 3% (2/67) of *I. ricinus* tick were positive for *R. helvetica*. Detection of other pathogens (*B. burgdorferi* s.l. and *Babesia* spp.) was carried out in collaboration with Institute of Zoology of the Slovak Academy of Sciences in Bratislava (Mgr. Michal Chvostáč and Mgr. Tatiana Vaculová). All positive samples were identified by nested PCR and DNA sequencing (for details see chapter 3.3.4).

4.1.2. Ticks collected from wild ungulates

We collected and analyzed 229 (17A/27N/185L) *I. ricinus* ticks from 43 wild ungulates (Tables 5., Table 6.). In particular, 191 *I. ricinus* ticks were detached from 34 roe deer (*Capreolus capreolus*), 37 were detached from 8 red deer (*Cervus elaphus*) and one was removed from a chamois (*Rupicapra rupicapra*).

A. phagocytophilum was present in 11.4% (5/44) of adults and nymphs detached from roe deer and red deer (Table 6.), while 5.4% (10/185) of larvae were positive from roe deer

(Table 5.). *Rickettsia* spp. were detected in 11.4% (5/44) of *I. ricinus* adults and nymphs (Table 6.). It was present in 9.7% (18/185) of larval ticks (14 from roe deer, 4 from red deer), with representative species of *R. helvetica* 66.7% (12/18), *R. raoultii* 5.6% (1/18) and *R. monacensis*/*R. tamurae* 27.8% (5/18) (Table 5.). Larvae from roe deer were positive for *R. helvetica* and *R. monacensis*/*R. tamurae*, while larvae detached from red deer were positive for *R. helvetica* and *R. raoultii*. *B. venatorum* was present in 2.3% (1/44) of adults, nymphs (Table 6.) and in 0.5% (1/185) of larvae detached from roe deer (Table 5.). *B. burgdorferi* strain was detected in 1 adult detached from free-living ungulates (Table 6.) (Baráková et al., 2017, submitted to Tick and tick borne pathogens). All positive samples were identified by PCR or nested PCR and DNA sequencing (for details see chapter 3.3.4).

4.1.3. Ticks collected from birds

We collected and analyzed a total of 112 ticks (0A/26N/86L) detached from birds, 98 of them belonging to *I. ricinus*, 11 of ticks belonging to *I. turdus* and 3 ticks belonging to *I. frontalis*. Ticks were detached from 75 birds belonging to eight species. Specifically, 75 ticks were collected from 53 blackbirds (*Turdus merula*), 11 from 8 European robins (*Erithacus rubecula*), 11 from 6 nightingales (*Luscinia megarhynchos*), 8 from 3 great tits (*Parus major*), 2 from 2 song thrushes (*Turdus philomelos*), 2 from 2 dunnock (*Prunella modularis*), 1 from a Eurasian blackcap (*Sylvia atricapilla*), 1 from a Chaffinch (*Fringilla coelebs*) and 1 from a Eurasian jay (*Garrulus glandarius*).

A. phagocytophilum was present in 11.5% (3/26) of nymphs and 5.6% (4/72) of larvae (Tables 5., Table 6.). *R. helvetica* was detected in 3.8% (1/26) of the nymphs and in 6.9% (5/72) of larval ticks. *B. venatorum* was detected in 7.7% (2/26) of the nymphs, while 2.8% (2/72) of larvae were positive for *Babesia* spp. (1 *B. venatorum* and 1 *B. capreoli*). Genospecies of *B. burgdorferi* s.l. *complex* were described by phylogenetic analyses (see Figure 17., Figure 18.). From 26 nymphs, 34.6% (9/26) ticks were positive for *B. burgdorferi* s.l., with representative species, *B. garinii* and *B. afzelii* (Table 6.). Higher prevalence of this pathogen was observed in larvae ticks detached from birds with 43% prevalence including the high variety of genospecies such as, *B. garinii*, *B. valaisiana*, *B. turdi*, *B.afzelii*, *B. lusitaniae* and *B. burgdorferi* strain (Table 5.).

Ticks detached from blackbirds were positive for *Babesia* spp. and *A. phagocytophilum* while *Rickettsia* spp. were recorded in ticks collected from song thrushes and great tits (Baráková et al., 2017, submitted to Tick and tick borne pathogens). All positive samples were identified by PCR or nested PCR and DNA sequencing (for details see chapter 3.3.4).

4.1.4. Ticks collected from humans

In total, 11 out of 88 (25 A, 63 N) *I. ricinus* ticks feeding on humans carried at least one pathogen. *A. phagocytophilum* was detected in 2.3% (2/88) of these ticks. *Rickettsia* spp. was detected in 3.4% (3/88) of ticks, *R. helvetica* in one adult and one nymph, and *R. monacensis*/*R. tamurae* in one nymph (Baráková et al., 2017, submitted to Tick and tick borne pathogens). The most prevalent pathogen was, however, *B. burgdorferii* s.l. with 6.8% (6/88) prevalence. *B. afzelii* occurred in four ticks, while *B. garinii* was detected in two (Table 6.). All positive samples were identified by PCR or nested PCR and DNA sequencing (for details see chapter 3.3.4).

4.1.5. Ticks collected from sheep and dogs

A. phagocytophilum was detected in 2 *I. ricinus* of the 40 ticks (25 *I. ricinus* and 15 *I. hexagonus*) feeding on sheep and dogs, with a prevalence of 7.7% (1/13) in sheep and 3.7% (1/27) in dogs (Baráková et al., 2017, submitted to Tick and tick borne pathogens). *B. burgdorferi* s.l. was detected in 15.4% (2/13) of mature ticks detached from sheep, one nymph was positive for *B. afzelii* and other one for *B. garinii* (Table 6.). All positive samples were identified by nested PCR and DNA sequencing (for details see chapter 3.3.4).

4.1.6. Co-infection in feeding ticks

The co-infection of *A. phagocytophilum* and *R. helvetica* in larvae detached from wild ungulates was 0.5% (1/185). In general, 5.6% (4/72) of larvae detached from birds were co-infected with more than one pathogen. Co-infection with pathogen from different genera among larvae occurred among *A. phagocytophilum* and *B. garinii* in prevalence rate 1.4% (1/72), among *A. phagoctophilum* and *B. valaisiana* in prevalence of 1.4% (1/72), among *R.*

helvetica and *B. garinii* in prevalence 1.4% (1/72) and among *R. helvetica* and *B. valaisiana* in prevalence of 1.4% (1/72). Co-infection of *R. helvetica* and *B. afzelii* occurred among larva detached from rodent, nymph detached from bird and adult tick detached from human. All positive samples were identified by nested PCR and DNA sequencing (for details see chapter 3.3.4).

4.2. *A. phagocytophilum* detection in questing ticks

4.2.1. Questing ticks from Italy

In total 821 questing, *I. ricinus* ticks (155 adults, 666 nymphs) were analyzed for *A. phagocytophilum* by nested PCR and DNA sequencing by Fausta Rosso (FEM). 1.8% (15/821) of *I. ricinus* ticks (7 adults, 8 nymphs) were positive for this pathogen (Table 7.). Further positive samples were used for amplification of *msp4*, *groEL* and phylogenetic analysis (see 3.3.4.1) (Baráková et al., 2014).

4.2.2. Questing ticks from USA

In total 598 questing, *I. scapularis* nymphs from North USA (Mansfield and Block Island study sites) were analyzed for *A. phagocytophilum*. 3.7% (22/598) of them tested positive (Table 7.).

From 277 questing *I. scapularis* from South USA none tested positive for *A. phagocytophilum* (Table 7.). All positive samples were identified by real-time PCR or/and nested PCR and DNA sequencing (for details see chapter 3.3.4.1).

4.2.3. Questing ticks from Finland

From 564 questing *I. ricinus* from South Eastern Finland 0.1% (1/564) was positive for *A. phagocytophilum* and 7.8% (44/564) were positive for *R. helvetica* (Table 7.). All positive samples were identified by nested PCR and DNA sequencing (for details see chapter 3.3.4.1).

Table 4. Accession numbers of 17kDa, *ompA*, 18S rRNA, or 16S rRNA sequences deposited in GenBank. Identical sequences of positive samples for each pathogen from each tick life stage feeding on the same host were deposited as a single representative sequence. Reference sequences are listed below (table from Baráková et al., 2017, submitted to Tick and tick borne pathogens).

Pathogen	gene	Sample type *	Host	N identical sequences	Access number
A. phagocytophilum	16S rRNA	N	rodent	1	**KY319196**
		N	rodent	1	**KY319195**
		A	wild ungulate	3	**KY319198**
		A	wild ungulate	1	**KY319194**
		L	wild ungulate	11	**KY319197**
		N	bird	3	**KY319191**
		L	bird	2	**KY319192**
		N	human	2	**KY319193**
		A	sheep	1	**KY319199**
	Msp4	A	wild ungulate	1	**KF031433**
		A	wild ungulate	1	**KF031432**
		A	wild ungulate	1	**KF031431**
		N	dog	1	**KF031430**
		L	wild ungulate	1	**KF031429**
		N	rodent	1	**KF031428**
		A	wild ungulate	1	**KF031427**
		blood	rodent	1	**KF031426**
		N	rodent	1	**KF031425**
		blood	rodent	1	**KF031424**
		blood	rodent	1	**KF031423**
		blood	rodent	1	**KF031422**
		N	questing	1	**KF031421**
		N	human	1	**KF031420**
		N	questing	1	**KF031419**
		N	questing	1	**KF031418**
		A	questing	1	**KF031417**
		N	questing	1	**KF031416**
		A	questing	1	**KF031415**
		N	questing	1	**KF031414**
		N	human	1	**KF031413**
		N	questing	1	**KF031412**
		A	questing	1	**KF031411**
		A	questing	1	**KF031410**
		N	questing	1	**KF031409**
		N	human	1	**KF031408**

		A	questing	1	**KF031407**
		N	questing	1	**KF031406**
		N	questing	1	**KF031405**
		N	bird	1	**KF031404**
		N	bird	1	**KF031403**
		ear punch	rodent	39	**KX517840**
		N	questing	1	**KF031402**
		N	questing	1	**KF031401**
		N	questing	1	**KF031400**
		N	questing	1	**KF031399**
		N	questing	1	**KF031398**
		A	questing	1	**KF031397**
		A	questing	1	**KF031396**
		A	questing	1	**KF031395**
		L	wild ungulate	1	**KF031394**
		N	bird	1	**KF031393**
	groEL	N	rodent	1	**KF031392**
		A	wild ungulate	1	**KF031391**
		blood	rodent	1	**KF031390**
		A	wild ungulate	1	**KF031389**
		N	rodent	1	**KF031388**
		A	sheep	1	**KF031387**
		A	wild ungulate	1	**KF031386**
		blood	rodent	1	**KF031385**
		blood	rodent	1	**KF031384**
		A	wild ungulate	1	**KF031383**
		N	human	1	**KF031382**
		N	human	1	**KF031381**
		N	human	1	**KF031380**
		ear punch	rodent	38	**KX517839**
R. helvetica		N	rodent	2	**KY319212**
		L	rodent	7	**KY319211**
		A	wild ungulate	3	**KY346828**
	17kDa	N	wild ungulate	2	**KY319214**
		L	wild ungulate	12	**KY319213**
		N	birds	1	**KY319208**
		L	birds	5	**KY319209**
		A	human	1	**KY319210**
R.monacensis/tamurae		L	rodent	4	**KY319217**
	17kDa	L	rodent	1	**KY319216**
		L	rodent	2	**KY319218**
		L	wild ungulate	4	**KY319219**
		N	human	1	**KY319215**

R. monacensis	*ompA*	A	sheep	1	**KY319220**
		L	rodent	3	**KY319223**
		L	rodent	1	**KY319224**
Rickettsia spp.	17kDa	N	rodent	1	**KY319222**
R. raoultii		L	wild ungulate	1	**KY319221**
B. venatorum	18S rRNA	N	rodent	1	**KY319205**
		L	rodent	6	**KY319203**
		N	wild ungulate	1	**KY319206**
		L	wild ungulate	1	**KY319207**
		N	bird	1	**KY319200**
		L	bird	1	**KY319201**
B. microti		N	rodent	1	**KY319204**
B. capreoli		L	bird	1	**KY319202**

*****sample type include, ticks (represented by ticks stage: A-adult, N-nymph, L-larvae), blood samples or ear punches

Table 5. Prevalence of *A. phagocytophilum*, *Rickettsia* spp., *Babesia* spp., and *B. burgdorferi* s.l. in engorged larval *I. ricinus* ticks from wildlife animals (adjusted table from Baráková et al., 2017, submitted to Tick and tick borne pathogens).

Host type	***A. phagocytophilum***	***Rickettsia* spp.**	***Babesia* spp.**	***B. burgdorferi* s.l.**
Rodents	0%	**4.5%** (16/358) [1]	**1.7%** (6/358) [4]	**7.3%** (26/358)[6]
Ungulates	**5.4%** (10/185)	**9.7%** (18/185) [2]	**0.5%** (1/185) [4]	0%
Birds	**5.6%(**4/72)	**6.9%** (5/72) [3]	**2.8%** (2/72) [5]	**43%** (31/72)[7]

[1] 9 *R. helvetica*, 7 *R. monacensis*

[2] 12 *R. helvetica*, 5 *R. monacensis*, 1 *R. raoultii*

[3] 5 *R. helvetica*

[4] 6 *B. venatorum*

[5] 1 *B. venatorum* and 1 *B. capreoli*

[6] 26 *B. afzelii*

[7] 12 *B. garinii*, 11 *B. valaisiana*, 5 *B. turdi*, 1 *B. afzelii*, 1 *B. luisitaniae*, 1 *B. burgdorferi* strain

Table 6. Prevalence of *A. phagocytophilum*, *Rickettsia* spp., *Babesia* spp. and *B. burgdorferi* s.l. in engorged adult and nymphal *I. ricinus* ticks (A/N) from various wild and domestic hosts, as well as humans (adjusted table from Baráková et al., 2017, submitted to Tick and tick borne pathogens).

Host type	Tick species			Number of Ticks	Pathogen prevalence			
	I. ricinus	*I. hexagonus*	*I. trianguliceps*	(A/N)	*A. phagocytophilum*	*Rickettsia* spp.	*Babesia* spp.	*B. burgdorferi* s.l.
Wild rodents	23	0	12	4/31	5.7% (2/35)	8.6% [1] (3/35)	5.7% [5] (2/35)	5.7% (2/35)[8]
Wild ungulates	44	0	0	17/27	11.4% (5/44)	11.4% [2] (5/44)	2.3% [6] (1/44)	3.7% (1/27)[9]
Wild birds	26	0	0	0/26	11.5% (3/26)	3.8% [3] (1/26)	7.7% [7] (2/26)	34.6% (9/26)[10]
Domestic sheep	13	0	0	10/3	7.7% (1/13)	0%	0%	15.4% (2/13)[11]
Domestic dog	12	15	0	7/20	3.7% (1/27)	0%	0%	0%
Humans	88	0	0	25/63	2.3% (2/88)	3.4% [4] (3/88)	0%	6.8% (6/88)[12]

A-adult, N-nymph

[1] 2 nymphs *R. helvetica*; 1 nymph *Rickettsia sp.*

[2] 2 adults and 3 nymphs *R. helvetica*

[3] 1 nymph *R. helvetica*

[4] 1 adult and 1 nymph *R. helvetica*; 1 nymph *R. monacensis*

[5] 1 nymph *B. venatorum*; 1 nymph *B. microti*

[6] 1 nymph *B. venatorum*

[7] 2 nymphs *B. venatorum*

[8] 2 nymphs *B. afzelii*

[9] 1 adult *B. burgdorferi* strain

[10] 4 nymphs *B.garinii*, 3nymphs *B.valaisiana*, 1 nymph *B. luisitaniae*, 1 nymph *B. afzelii*

[11] 1 adult *B. garinii*, 1adult *B. afzelii*

[12] 1 nymph *B.garinii*, 3 nymphs *B. afzelii*, 1 adult *B. garinii*, 1 adult *B. afzelii*

Table 7. Prevalence of *A. phagocytophilum* in questing ticks from Italy, North and South USA and from Finland.

Country	Ticks species	Number of ticks	*A. phagocytophilum*
Italy	*I. ricinus*	821	1.8% (15/821)
North USA	*I. scapularis*	598	3.7% (22/598)
South USA	*I.scapularis*	277	0% (0/277)
Finland	*I. ricinus*	564	0.1% (1/564)

4.3. Blood and tissue from rodents detested for *A. phagocytophilum*

Blood from 1295 rodents and tissue from 964 rodents was tested for presence of *A. phagocytophilum* to describe the role of small mammals in transmission cycles of this pathogen in nature. Only 0.3% (4/1295) of blood samples tested positive (Baráková et al., 2014), while the prevalence among tissue samples was higher, 5.4% (52/964) (Rosso et al., 2017, accepted by journal Parasites & Vectors). Positive blood samples were collected from the bank vole *M. glareolus*, while positive tissues were collected from *M. multiplex*, *A. flavicollis* and *M. glareolus*. All positive samples were used for amplification of *msp4* and *groEL* genes for further phylogenetic analysis (for details see chapter 3.3.4.1)

4.4. Statistical analysis of *A. phagocytophilum* and *R. helvetica* in feeding ticks

Table 8. highlights the results of the GLM model applied to the prevalence of *A. phagocytophilum* in different hosts. The occurrence of this pathogen was influenced by both host species and tick stage. Specifically, *A. phagocytophilum* prevalence was higher in ticks that fed on blackbirds and roe deer in comparison to ticks that fed on yellow-necked mice and humans. Moreover, infection of *A. phagocytophilum* was higher in adult ticks than in larvae.

R. helvetica prevalence was also influenced by host species. Its occurrence was higher in ticks that fed on ungulates than in those that fed on wild mice and humans. No differences

in *R. helvetica* prevalence were detected among different tick stages (Table 9.) (statistical analyses were carried out by Roberto Rossa PhD, FEM) (Baráková et al., 2014).

Table 8. Model coefficients, standard errors and their statistics for *A. phagocytophilum* model. (Reference levels are yellow-necked mouse for host species and adults for tick stage) (Baráková et al., 2014).

Explanatory variables	**Coefficient**	**Std. Error**	**z-value**	**Pr(>|z|)**
(Intercept)	-3.39	0.91	-3.74	<0.001***
host.species.dog	0.77	1.29	0.60	0.55
host.species.roe.deer	2.50	0.77	3.24	<0.01**
host.species.red.deer	1.16	1.29	0.90	0.37
host.species.human	0.25	1.07	0.23	0.82
host.species.sheep	1.08	1.37	0.79	0.43
host.species.blackbird	2.89	0.82	3.51	<0.001***
tick.stage.larvae	-2.05	0.63	-3.24	<0.01**
tick.stage.nymph	-1.02	0.65	-1.58	0.11

*** Very highly significant, ** Highly significant, *Significant

Table 9. Model coefficients, standard errors and their statistics for *R. helvetica* model (Reference levels are yellow-necked mouse for host species and adults for tick stages) (Baráková et al., 2014).

Explanatory variables	**Coefficient**	**Std. Error**	**z-value**	**Pr(>|z|)**
(Intercept)	-2.91	0.74	-3.95	<0.001***
host.species.roe.deer	0.85	0.42	1.99	<0.05*
host.species.red.deer	1.34	0.63	2.13	<0.05*
host.species.human	-0.73	0.87	-0.84	0.40
host.species.blackbird	0.28	0.67	0.42	0.67
tick.stage.larvae	-0.68	0.69	-0.98	0.33
tick.stage.nymph	-0.18	0.77	-0.23	0.82

*** Very highly significant, ** Highly significant, *Significant

4.5. Phylogenetic analysis

4.5.1. Phylogenetic analysis of 16S ticks species

To verify tick species we amplified 441 bp long 16S RNA gene sequence from each analyzed tick (see chapter 3.3.3). Each obtained sequence was compared with sequences from GenBank by BLAST search N2.2.13. 821 sequences belonged to tick species *I. ricinus*, 12 sequences belonged to *I. trianguliceps*, 15 sequences belonged to *I. hexagonus* and 14 to *I. turdus*/*I. frontalis*. To distinguish *I. turdus* and *I. frontalis*, four different analyses were used to construct phylogenetic trees (Maximum likelihood, Neighbor joining, Minimum evolution and Maximum parsimony). All four analyses showed the same output (only Maximum likelihood is shown, Figure 14.). Three 16S sequences (ID: 6B866983, SE50313, 6A56609) from our study clustered with *I. frontalis* group. Eleven 16S sequences (ID: SB866902E-5A57830 in phylogenetic tree, Figure 14.) clustered with the main group of *I. turdus* from Japan and USA although within this main group our samples created a subgroup with KP769861 and KP769862 that are described as *I. frontalis* from Portugal. It seems to us that these two samples might have been incorrectly classified as *I. frontalis*. For this purpose we used ALIGN program to confirm that our samples accounted higher homology to AF549838, AB819258, AB819262-AB819264 samples belonging to *I. turdus* from Japan and USA then to KJ414454, AF549839, KP769863 belonging to *I. frontalis* from Belgium, USA and Brazil. We compared as well samples KP769861, KP769862 from Portugal (which are identified as *I. frontalis*) with the *I. turdus* (AF549838) samples from USA, resulting in 96.8% and 97.4% homology. On the other hand, when comparing these samples (KP769861, KP769862) with *I. frontalis* (KJ414454), the homology was lower, 95.7% and 96.3% respectively. Therefore we concluded that samples from Portugal (with which our sample had the highest homology 100% (KP769861) and 99.4% (KP769862) respectively) were mistakenly identified as *I. frontalis*. Homology comparisons, as well as phylogenetic analysis of 16S RNA, conclude that our samples are in fact *I. turdus* and not *I. frontalis*.

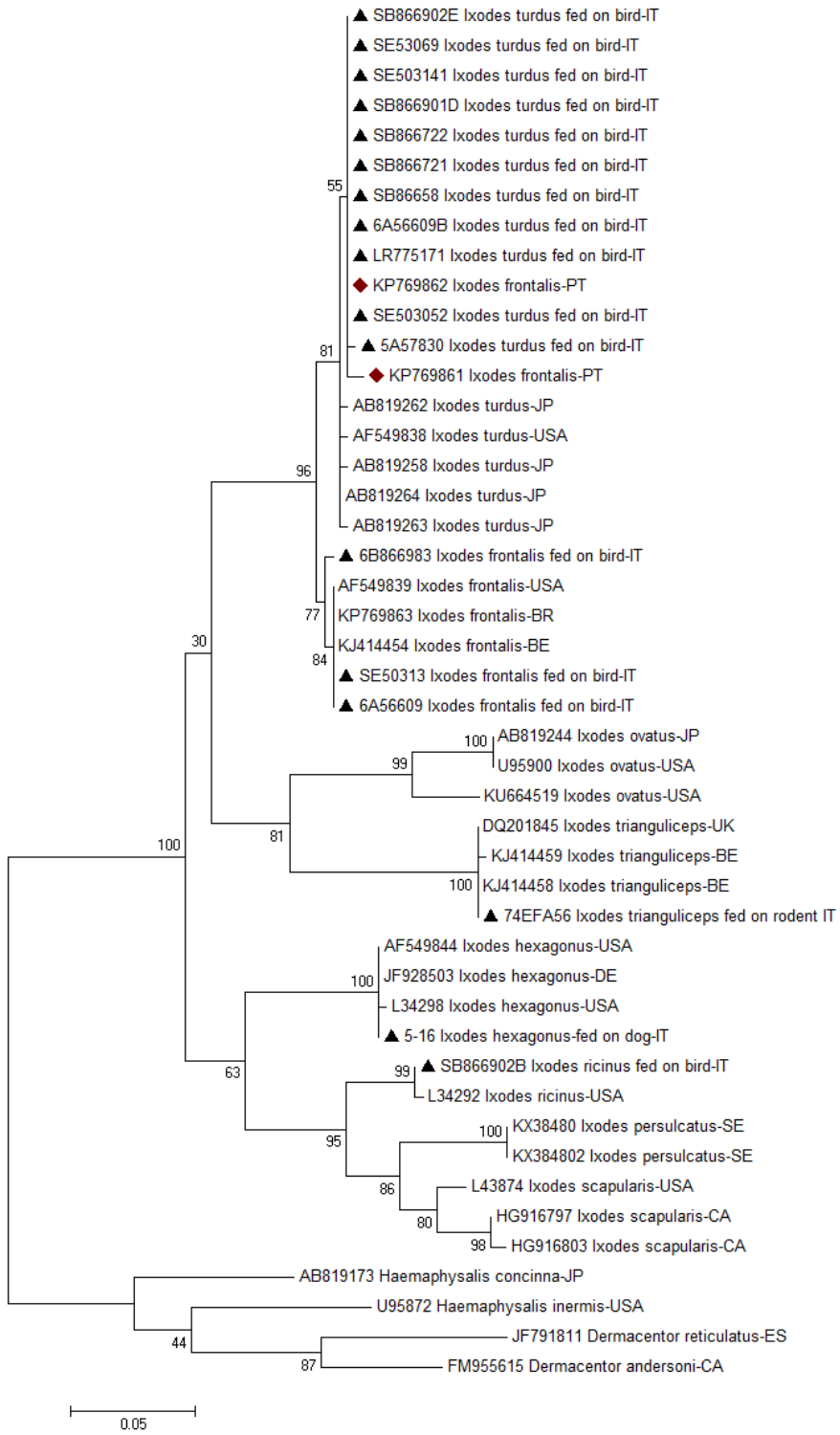

Figure 14. The distance tree inferred by Maximum likelihood analysis using the Tamura-Nei model of 441 bp long 16S gene sequence of tick species. GenBank accession numbers, tick species, two-letter country code are shown in downloaded sequences from NCBI. Our samples further included host

species and host ID. The numbers at the nodes are bootstrap values expressed as percentages of 1000 bootstrap replicates: the bar (0.05) represents the number of mutations per site. The analysis involved 46 nucleotide sequences of 16S. Representative samples of this study are indicated with ▲, two *I. frontalis* samples from Portugal that clustered with our *I. turdus* samples are indicated with ◆ (see discussion).

4.5.2. Enzootic cycle of *A. phagocytophilum* in Italy, USA and Finland

Positive samples for *A. phagocytophilum*, were further used for amplification of variable genes by nested PCR (for details see chapter 3.3.4.1). We obtained 17 *msp4*-IT (KF031433-KF031427, KF031425, KF031421-KF031403) and 16 *groEL*-IT sequences (KF031402-KF031391, KF031389- KF031386, KF031383-KF031380) from questing and feeding *I. ricinus* ticks, 4 *msp4*-IT (association numbers KF031426, KF031424, KF031423, KF031422) and 3 *groEL*-IT (association numbers KF031390, KF031385, KF031384) sequences from rodent blood samples and 39 *msp4*-IT and 38 *groEL*-IT sequences from rodent tissues (representative association number for *msp4*-IT is KX517840 and for *groEL*-IT is KX517839) from Italy (Table 4.). From Finland only one sample tested positive for *A. phagocytophilum*, *msp4*-FI and *groEL*-FI sequence was included in the phylogenetic analysis below (Figure 15., Figure 16.). Further 19 *groEL*-USA sequences from USA were obtained. Phylogenetic trees were constructed with the Maximum likelihood method using the Tamura-Nei model with 1000 bootstrap replicates. The mean nucleotide diversity (π) among the *A. phagocytophilum* sequences was 0.073 (range 0.0–0.161) for *msp4* and 0.034 (range 0.0–0.192) for *groEL*.

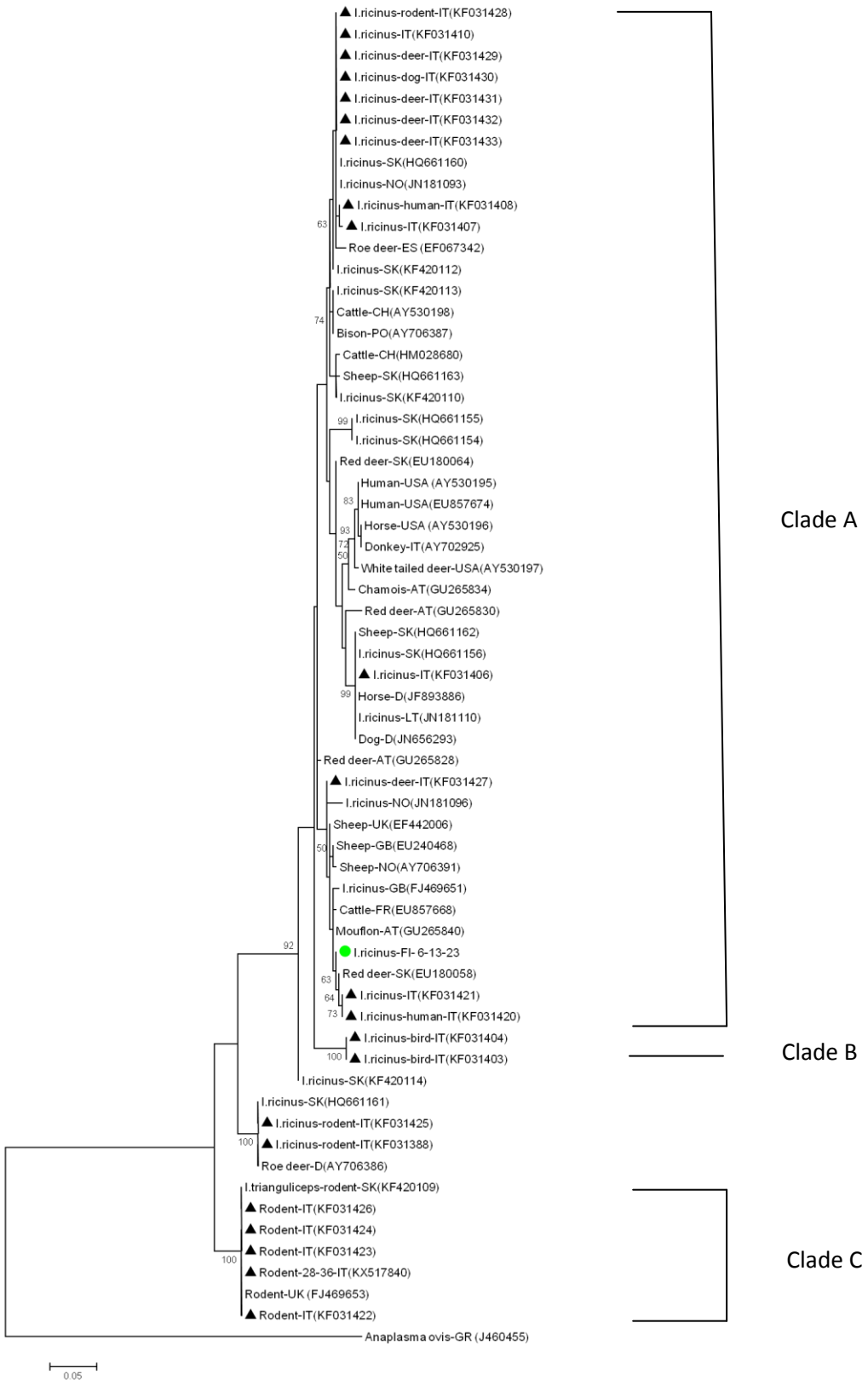

Figure 15. The distance tree inferred by Maximum likelihood analysis using the Tamura-Nei model of 300 bp long *msp4* gene sequence of *A. phagocytophilum*. Host species, two-letter country code and

GenBank accession numbers are shown in sequences. The numbers at the nodes are bootstrap values expressed as percentages of 1000 bootstrap replicates: the bar (0.05) represents the number of mutations per site. The analysis involved 63 nucleotide sequences of *msp4*. Representative samples of this study from Italy are indicated with ▲ and representative sample of this study from Finland is indicated with ●.

Both phylogenetic trees (containing *msp4* sequences and *groEL* sequences) had similar topologies containing 3 main clades, each with different host and vector associations (Figure 15., Figure 16.). Clade A contained sequences from questing *I. ricinus* ticks and engorged ticks from various hosts, such as humans, dogs, free-living ungulates, rodents and sheep. Second clade (clade B) contained sequences only from engorged ticks that fed on birds and clade C contained sequences from rodents, specifically, bank voles (*M. glareolus*), other voles, shrews, and mice (*A. flavicollis*). Among tick species we found *I. persulcatus* and *I. trianguliceps* to belong to this clade (Figure 15., Figure 16.). Specifically, all *groEL*-IT sequences from rodent blood or tissue generated in our study, regardless of rodent host species, were 99% identical to *groEL* sequences of *A. phagocytophilum* extracted from *I. trianguliceps* feeding on voles from Slovakia (KF383233, KF383235) (Blaňarová et al., 2014). The *msp4*-IT representative sequence was 100% identical with that from blood samples of bank voles from the UK (FJ469653), as well as from *I. trianguliceps* feeding on rodents from Slovakia (KF420109) (Bown et al., 2009, Baráková et al., 2014, Blaňarová et al., 2014) (Figure 15., Figure 16.).

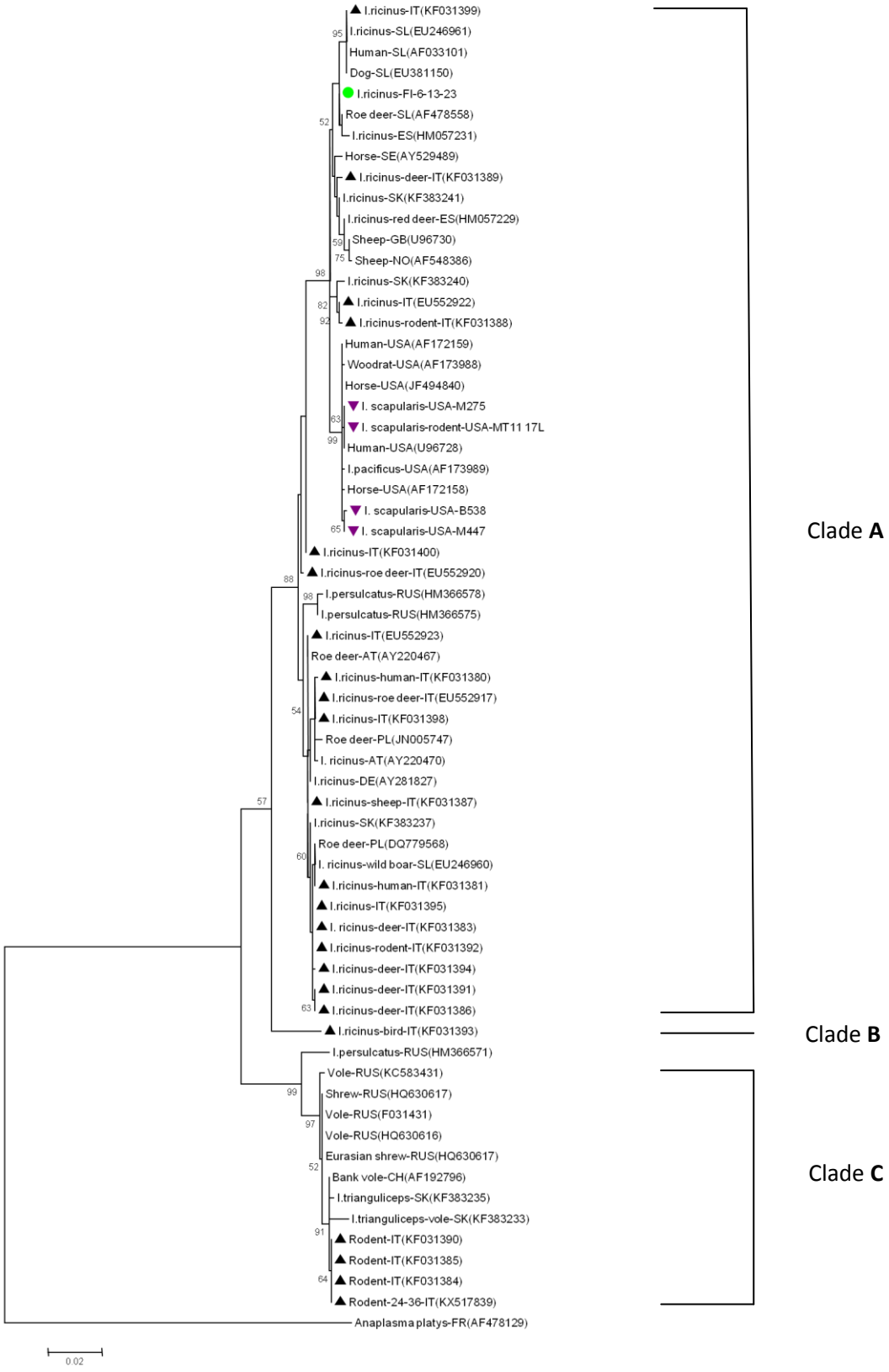

Figure 16. The distance tree inferred by Maximum likelihood analysis using the Tamura-Nei model of 1119 bp long *groEL* gene sequence of *A. phagocytophilum*. Host species, two-letter country code and

GenBank accession numbers are shown in sequences. The numbers at the nodes are bootstrap values expressed as percentages of 1000 bootstrap replicates: the bar (0.02) represents the number of mutations per site. The analysis involved 64 nucleotide sequences of *groEL*. Representative samples of this study from Italy are indicated with ▲ and representative sample of this study from Finland is indicated with ● and representative sample of this study from USA is indicated with ▼.

4.5.3. Identification of *B. burgdorferi* s.l. complex species circulation in northern Italy

To distinguish *B. burgdorferi* s.l. genospecies occurring in northern Italy, IGS representative sequences of positive samples were used to construct phylogenetic tree by Maximum likelihood method (Figure 17.). The ouput of the phylogenetic analysis was sufficient in genotyping of *B. burgdorferi* s.l. genospecies. By the means of this method, we were able to identify a new *borrelia* genospecies in the area, *B. turdi*.

Regarding MLST, we were not able to amplify all 8 housekeeping genes per each positive sample, therefore we could not assign to our positive samples exact sequence type according to MLST database. This was due to lack of amount of DNA to repeat some amplifications, as well as occurrence of mixed-infections in half of the positive samples. Samples with mixed-infection had to be excluded from further analysis. We were able to obtained four genes for 14 positive samples. The internal fragments of four housekeeping genes per sample (*clpX*, *recG*, *pepX* and *pyrG*) were used to create concatenated sequence for each positive sample. The concatenated representative sequences were further aligned with sequences downloaded from *Borrelia* MLST database. Phylogenetic tree was reconstructed by Maximum Likelihood (Figure 18.). The mean distance nucleotide diversity of the concatenated sequence based on *clpX*, *recG*, *pepX* and *pyrG* genes from the MLST analysis was 0.082 and among, *B. afzelii* it was 0.004, *B. burgdorferi* 0.012, *B. garinii* 0.09, *B. luisitaniae* 0.009, *B. valaisiana* 0.029 and *B. turdi* 0.002.

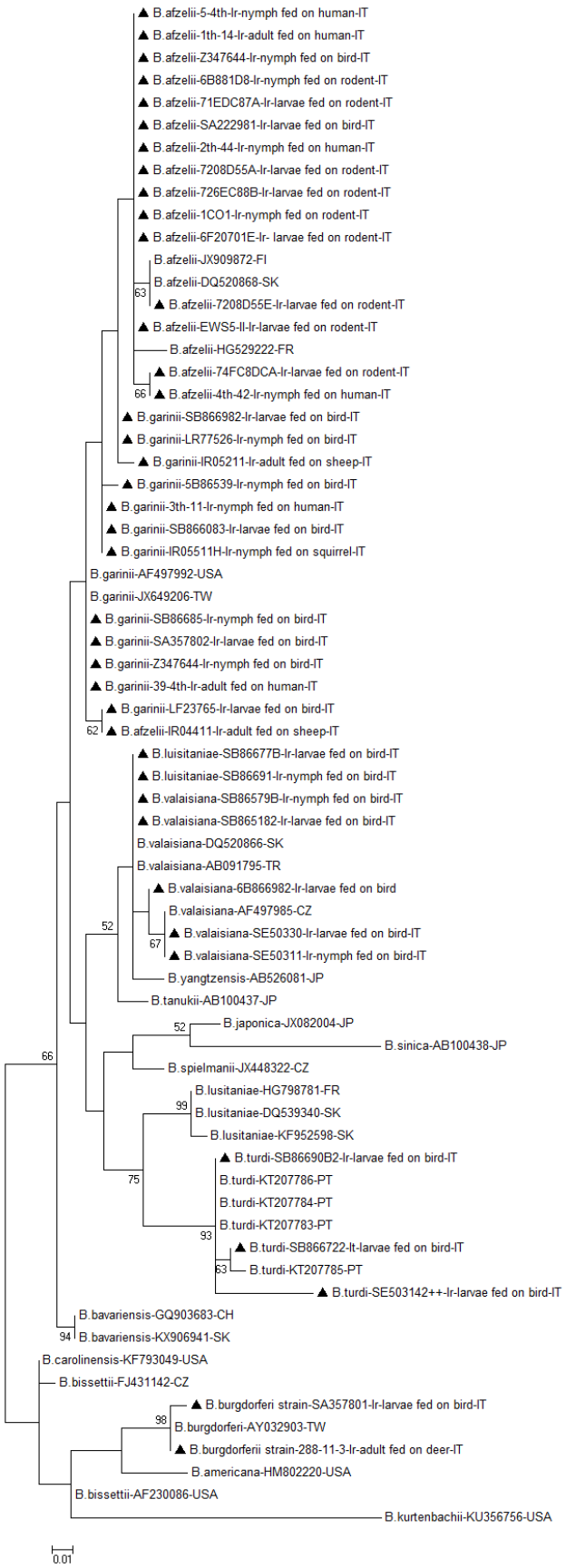

Figure 17. The distance tree in ferred by Maximum likelihood analysis using the Tamura-Nei model of 183 bp long 5S-23S rDNA intergenic spacer region (IGS) of *B. burgdorferi* s.l.. *Borrelia* species, GenBank accession numbers, two-letter country code are shown in downloaded sequences from NCBI. Our samples further included host ID and host species. The numbers at the nodes are bootstrap values expressed as percentages of 1000 bootstrap replicates: the bar (0.01) represents the number of mutations per site. Representative samples of this study are indicated with ▲.

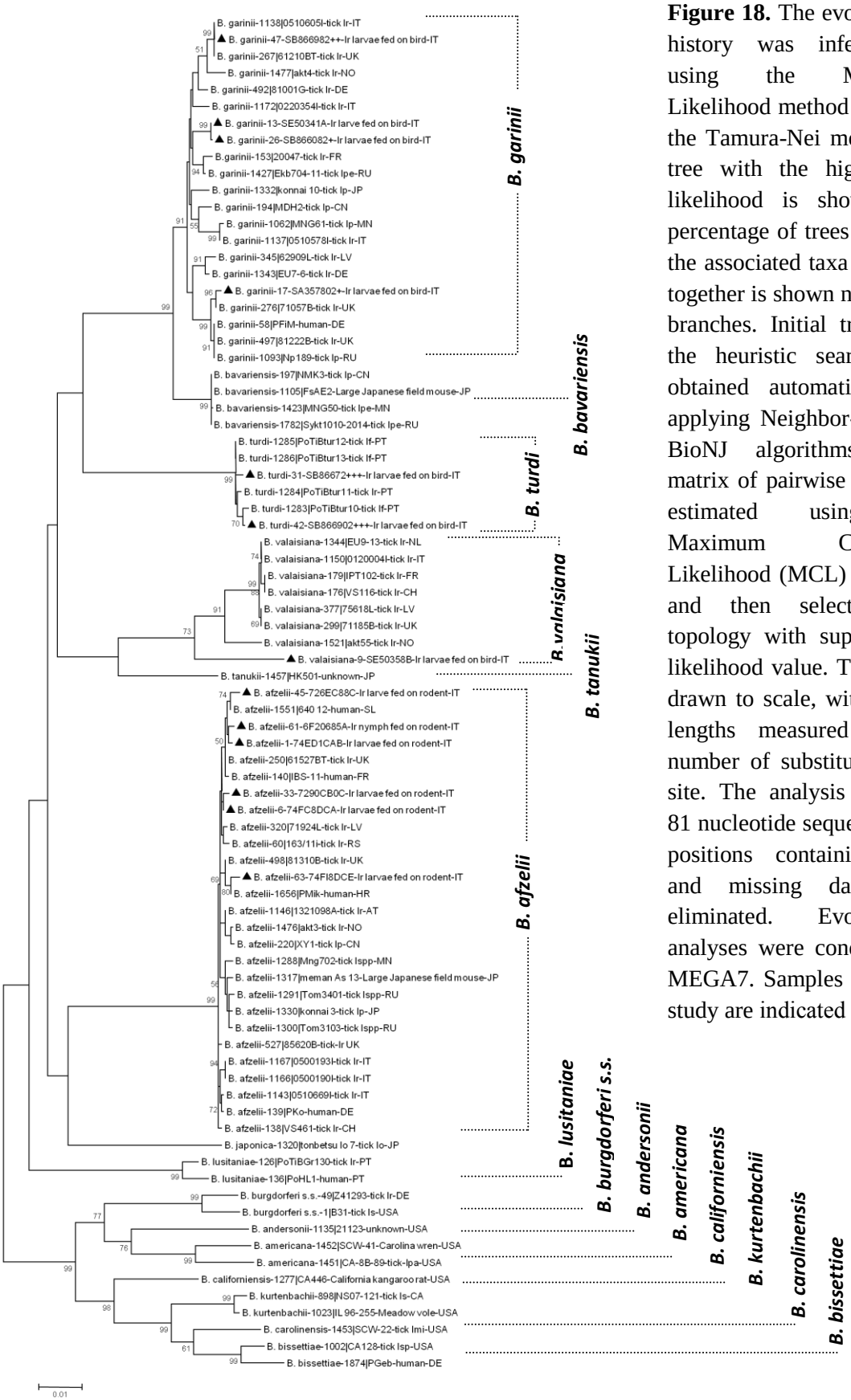

Figure 18. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 81 nucleotide sequences. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7. Samples from this study are indicated with ▲.

4.6. Acarological hazard in Europe

4.6.1. Tick abundance among five EU countries

In the collaboration EDENext which comprises five EU countries (Italy, Slovakia, Germany, Czech Republic and Hungary) the detection of *A. phagocytophilum*, *Rickettsia* spp. and *B. burgdorferi* s.l. in ticks from vegetation was established in three habitat types, natural, agricultural and urban. In total 13291 nymphs were collected by dragging in three years (2011-2013). The highest abundance of nymphs collected from vegetation was observed in Slovakia, while significantly lower abundance was observed in Czech Republic. NDVI had a positive effect on tick abundance (Rosà et al., 2017).

4.6.2. Screening of questing ticks among five EU countries for three pathogens

The highest prevalence in studied countries was detected for *B. burgdorferi* s.l., followed by *Rickettsia* spp. and *A. phagocytophilum*. The density of infected nymphs (DIN) varied significantly for *A. phagocytophilum* and *Rickettsia* spp. among the countries. Highest DIN for *A. phagocytophilum* was observed in Slovakia and highest DIN for *Rickettsia* spp. was observed in Germany and Slovakia. The prevalence of *A. phagocytophilum*, *Rickettsia* spp. and *B. burgdorferi* s.l. in questing ticks from Italy showed overall prevalence 1.6%, 8.9% and 20.9% respectively. For Slovakia, the overall prevalence of these pathogens was 3%, 7.7%, and 12.3% respectively, for Germany overall prevalence was 1.3%, 12.7%, and 20.8% respectively. Further data from Czech republic and Hungary include only overall prevalence for two pathogens, *A. phagocytophilum*, *Rickettsia* spp.. The overall prevalence for these pathogens for the Czech Republic was following, 1.3% and 5.1% respectively and from Hungary, overall prevalence was 4.2% and 5.6%. NDVI had a positive effect on DIN (Rosà et al., 2017).

Table 10. Best models for nymph counts (Negative Binomial GLM) and density of infected nymphs (Tobit GLM) with *A. phagocytophylum*, *B. burgdorferi s.l.* and *Rickettsia* spp. The columns report the estimated coefficients for explanatory variables, their standard errors, z-values (estimate to standard error ratio) and p-value for z-statistic. Reference levels are Czech Republic for country (table from Rosa et al., 2017).

Model	Explanatory variable	Estimate	Std.Error	z value	Pr(>|z|)
Nymphs counts	Intercept	4.463	0.268	16.639	< 0.001***
	NDVI	0.243	0.121	2.003	0.045*
	Country_Germany	1.165	0.338	3.451	< 0.001***
	Country_Hungary	0.921	0.418	2.200	0.028 *
	Country_Italy	1.046	0.384	2.725	0.006 **
	Country_Slovakia	1.647	0.379	4.342	< 0.001***
Anaplasma	(Intercept):1	-0.511	0.706	-0.723	0.469
	(Intercept):2	0.312	0.157	1.990	0.047 *
	NDVI	0.716	0.327	2.187	0.029 *
	Country_Germany	-0.563	0.813	-0.693	0.488
	Country_Hungary	1.509	0.838	1.800	0.072
	Country_Italy	0.832	0.839	0.993	0.321
	Country_Slovakia	1.796	0.731	2.458	0.014 *
	Habitat type_Natural	0.005	0.692	0.007	0.995
	Habitat type_Urban	1.905	0.660	2.888	0.004 **
Rickettsia	(Intercept):1	1.023	0.742	1.378	0.168
	(Intercept):2	0.694	0.1400	4.953	< 0.001***
	NDVI	1.029	0.4055	2.537	0.011 *
	Country_Germany	4.814	1.4321	3.362	< 0.001***
	Country_Hungary	0.984	1.1752	0.837	0.402
	Country_Italy	2.175	1.1340	1.918	0.055
	Country_Slovakia	3.715	1.0108	3.675	< 0.001***
Borrelia	(Intercept):1	6.273	0.7371	8.510	< 0.001***
	(Intercept):2	0.634	0.1699	3.732	< 0.001***
	NDVI	1.999	0.5231	3.823	< 0.001***
	Habitat type_Natural	5.185	1.0839	4.784	< 0.001***
	Habitat type_Urban	-1.492	1.2010	-1.242	0.214

5 Discussion

Spread of tick-borne pathogens and the occurrence of tick-borne zoonoses in Europe became a one health priority over the past two decades (Hofhuis et al., 2006; Hubalek 2009; Rizzoli et al., 2011). It has been documented that it is directly proportional to changing geographical distribution and abundance of their principal vector *I. ricinus* (Medlock et al., 2013). *I. ricinus* is the main vector of tick-borne zoonoses in Europe with wide geographical distribution mostly due to its ecological plasticity. Various biotic and abiotic factors are driving habitat expansion of *I. ricinus* to the northern latitude and higher altitude and therefore affecting the epidemiological dynamics of vector-borne diseases (Jore et al., 2011).

We screened feeding Ixodid ticks collected directly from a range of hosts, including wildlife, some domestic animals and humans, with the aim to show the presence of these pathogens in the area (screening adults and nymphs) and to better understand the role of these hosts in their ecology (screening larval ticks) (Baráková et al., 2017, submitted to Tick and tick borne pathogens).

The ecology of *Ixodes*-transmitted pathogens is often complex and involves several vertebrate hosts that not only play a role as tick blood-meal providers, but in certain cases may also act as reservoirs or amplifiers for the infection (Sprong et al., 2009, Burri et al., 2014 and Yabsle and Shock, 2012). Reservoir capacity varies among species and it is still poorly assessed for a large number of vertebrates since complex laboratory xenodiagnostic experiments involving wildlife hosts must be performed. These experiments are economically and ethically challenging and not feasible for all vertebrate reservoir hosts. Therefore the positive larvae detached from wildlife animals are good indicator of the reservoir competence of the hosts, in cases where transovarial transmission of pathogens in vectors is missing. Identification of the competent reservoir hosts is essential to develop meta-community-based risk assessment estimates for zoonoses and to identify the most appropriate preventive and control options (Estrada-Peña and de la Fuente, 2014; Baráková et al., 2017, submitted to Tick and tick borne pathogens).

A. phagocytophilum is known to be mainly transmitted horizontally. Since transovarial transmission (Hotopp et al., 2006) and transmission by co-feeding (Rar and Golovljova, 2011) seem to be inefficient or absent, infected larvae should get infected while feeding on a bacteriemic host. According to our statistical analysis, there is a significantly higher rate of *A.*

phagocytophilum prevalence in *I. ricinus* larvae detached from blackbirds and roe deer than in ticks removed from other sampled hosts (Table 8.). Our results show 10.7% prevalence of *A. phagocytophilum* in nymphs detached from birds (Baráková et al., 2014) and prevalence of 5.6% in larval ticks, suggesting a possible role of certain bird species (especially blackbirds) as reservoir hosts (Baráková et al., 2017, submitted to Tick and tick borne pathogens). *A. phagocytophilum* is known to be maintained mainly by domestic and wild ruminants. Moreover, it has been reported in dogs, red foxes and bears (Stuen et al., 2013). A 5.4% prevalence of *A. phagocytophilum* in larval *I. ricinus* from roe deer in our study is similar to the findings from northern Italy (Carpi et al., 2009). Our result highlights the potential of roe deer to infect *I. ricinus* larvae and confirms the role of roe deer as a competent reservoir (see also Liz et al., 2002; Silaghi et al., 2011).

Different transmission strategies of *A. phagocytophilum* depending on the host or vector were documented (Severo et al., 2012). The transmission cycle of *A. phagocytophilum* is complex, involving many vertebrates and different species of *Ixodes* ticks. Our phylogenetic analysis of *groEL* and *msp4* show similar topologies with support for three main clades, representing three ecotypes.

The clade A based on our phylogenetic trees has the broadest host range, including human, dogs, sheep, horses, free-living ungulates and questing *I. ricinus* ticks from our study (from Italy and Finland). The *I. ricinus* generalist feeding behavior seems to help exchange ecotype A between various vertebrate hosts easily. In 1 questing *I. ricinus* tick at the nymphal stage we detected a *groEL*-IT sequence (KF031399) identical to a sequence isolated from humans with human granulocytic anaplasmosis in Europe (AF033101). The *msp4*-IT sequence for the same sample (KF031406) belonged to clade A, and contained sequences of a strain found in 96 infected persons in the United States. This suggests that >1 human pathogenic strain now circulates in the investigated area. However, we did not find this strain in any of the host fed ticks analyzed, so the host responsible for maintaining the circulation of this pathogenic strain must be identified before any recommendation for preventive measures can be provided (Baráková et al., 2014). Further clade A includes sequences from *I. scapularis* fed on rodents and questing *I. scapularis* from North part of USA from our study. 0.7% (4/598) of questing *I. scapularis* and 6.5% (5/77) of feeding *I. scapularis* detached from rodents show 100% homology with pathogenic strain from humans in USA.

Clade B according to our phylogenetic analyses contains only sequences from *I. ricinus* fed on birds. The unique bird associated strain was as well identified by Jahfari et al. (2014) and *I. frontalis* was proposed as the vector of this *A. phagocytophilum* strain. However not many studies have been performed on bird associated *A. phagocytophilum* strain, therefore the role of this species remains unclear in the natural maintenance.

Clade C contains only sequences from this study from rodent sera and tissue (from Italy) with 100% homology among them (Baráková et al., 2014 and Rosso et al., 2017, accepted by journal Parasites & Vectors). Our *msp4*-IT sequences from rodents were identical to those from blood samples of bank voles from the UK (FJ469653) and *I. trianguliceps* feeding on rodents from Slovakia (KF420109). The *groEL*-IT sequences from this study from rodents show 99% homology with *I. trianguliceps* feeding on voles from Slovakia (KF383233, KF383235) (Bown et, al. 2009 and Blaňarová et al. 2014). This ecotype C has not been found in any other host or *I. ricinus* tick. The role of the vector for this strain is probably secured by *I. trianguliceps* as proposed by Bown et, al. (2009) and Blaňarová et al. (2014). Moreover our results support the theory that *I. ricinus* is not an efficient vector of the rodent-associated *A. phagocytophilum* ecotype in Europe, since no *A. phagocytophilum* in *I. ricinus* larvae collected from yellow-necked mice, bank voles, or dormice were positive, while *I. ricinus* nymphs from rodents had infection prevalence of 5.7% (Baráková et al., 2014).

Our results show a different ecology of *A. phagocytophilum* in Europe then in USA. In Europe we show two distinct enzootic cycles, one where *I. ricinus* is a vector of *A. phagocytophilum* strain that is able to infect various domestic and wildlife animals as well as human, and the second enzootic cycle where *I. trianguliceps* is a vector of *A. phagocytophilum* strain that has been detected only among rodents. On the other hand in USA, *A. phagocytophilum* strain AP-ha pathogenic for a human is transmitted by rodents and Ap-V1 non-pathogenic strain for human is maintained by white-tailed deer (Courtney et al., 2003; Liz et al., 2002; Levin et al., 2002; Massung et al. 2003,2005).

Sprong et al. (2009) noted *R. helvetica* in the blood of wild rodents, roe deer and wild boar while the presence of this pathogen in blood from several bird species (European robin and dunnock) was confirmed by Hornok et al., (2014). Our results show an infection prevalence of 2.5% in larval ticks from rodents, 6.5% in larval ticks collected from roe deer and 6.9% in larval ticks detached from birds (blackbirds, nightingales and song thrushes)

(Baráková et al., 2017, submitted to Tick and tick borne pathogens). However, in the absence of experimental infection, the role of various hosts in the life cycle of this pathogen cannot be definitively assessed due to efficient transovarial transmission of this pathogen in ticks (Sprong et al., 2009 and Řeháček, 1984). These findings suggest that these hosts may serve as a source of infection and positive larvae may imply that host is able to transmit the infection further. However experimental studies are now needed to clarify the role of these potential reservoir hosts in the maintenance of *R. helvetica* in natural foci (Baráková et al., 2017, submitted to Tick and tick borne pathogens).

We detected *R. monacensis*/*R. tamurae* in larval *I. ricinus* ticks feeding on rodents and wild ungulates. Using the gene for 17 kDa protein we could not exactly type the species, therefore we amplified more variable gene *ompA*. Due to lack of DNA from all of the positive samples we were able to amplify *ompA* only for four samples. The sequences confirmed the presence of *R. monacensis* (Baráková et al., 2017, submitted to Tick and tick borne pathogens). We assume that all positive samples are *R. monacensis*, since *R. tamurae* has not been found in Europe to date and its occurrence has been recorded only in Japan and Thailand, where it is transmitted by *Amblyomma testudinarium* (Fournier et al., 2006). *R. monacensis* was relatively recently identified as an etiologic agent of a Mediterranean spotted fever (MSF)-like illness with a couple of cases reported in Spain and Italy (Jado et al., 2007 and Madeddu et al., 2012). Therefore only a few studies on reservoir competence have been done and no studies so far confirmed transovarial transmission for this pathogen. De Sousa et al. (2012) reported the Madeira wall lizard, *Teira dugesii*, as a reservoir for this pathogen. The xenodiagnostic tests done by Burri et al. (2014) demonstrated that rodents are probably not that important source of *R. monacensis* infection for feeding ticks. We found *R. monacensis* in larvae feeding on rodents; however, thus we assume the possibility of *R. monacensis* transovarial transmission in *I. ricinus* ticks. Further, this study noted 2.7% prevalence in larvae detached from roe deer. Further research on the transovarial transmission and the role of roe deer in the natural cycle of *R. monacensis* is now needed (Baráková et al., 2017, submitted to Tick and tick borne pathogens).

R. raoultii has been identified in 2008 and subsequently it has been reported throughout Europe (Mediannikov et al., 2008; Dautel et al., 2006; Márquez, 2008; Vitorino et al., 2007; Nijhof et al., 2007; Boldiš et al., 2010; Mediannikov et al., 2008; Chmielewski et al.,

2009 and Tijsse-Klasen et al., 2011). In Italy it has been detected only in Tuscany (central Italy), Puglia and Basilicata (southern Italy) in *Dermacentor* spp. ticks (Selmi et al., 2009 and Otranto et al., 2014). Its presence in *I. ricinus* ticks has only been detected in Poland up to date (Reye et al., 2013 and Chmielewski et al., 2009). Due to the recent identification of this pathogen, its ecology in natural foci is relatively unknown. *Dermacentor* ticks are known to be efficient vectors of *R. raoulti* with very high rate (43%-100% depending on tick species) of transovarial transmission (Samoylenko et al., 2009). Nevertheless, nothing is known about transovarial transmission in *I. ricinus* ticks. In our study we report the presence of *R. raoultii* in *I. ricinus* larvae detached from red deer; therefore, further studies should be carried out to better understand the role of deer as a reservoir of this pathogen (Baráková et al., 2017, submitted to Tick and tick borne pathogens).

In Italy, few studies have been carried out on the prevalence of the recently described *B. venatorum.* This pathogen has been recorded in roe deer and has also been associated with mild clinical symptoms in patients in Austria, Italy and Germany (Duh et al., 2005 and Hunfeld et al., 2008). To the best of our knowledge, the occurrence of this pathogen in ticks collected from avian hosts has only been recorded in Norway (Øines et al., 2012). Here, we found *B. venatorum* in nymphs and larvae detached from wild mice, roe deer and great tits.
As Bonnet et al. (2007) showed that transovarial transmission occurs for *B. venatorum* and *B. capreoli*, the role of these vertebrates as reservoirs remains open. In our study, *B. capreoli* was found in one larva detached from a great tit. The role of birds in the ecology of babesiae needed to be further determined (Baráková et al., 2017, submitted to Tick and tick borne pathogens).

B. microti and *B. venatorum* genospecies, both associated with human disease (Hildebrandt et al., 2007 and Hunfeld et al., 2008), were present in nymphs detached from rodents in this study. *B. microti* is a well known human pathogen in the USA while the pathogenic potential of the European strain is still not clear (Hildebrandt et al., 2007; 2013). In the past, *B. microti* was thought to be circulating in Europe in *I. trianguliceps*-rodent enzootic cycle only. The low number of human cases in Europe was thought to be due to the involvement of *I. trianguliceps* and not *I. ricinus* as a vector of this strain. However, other studies support the view that European *B. microti* rodent strain can be transmitted by *I. ricinus* ticks and infection of humans with this pathogen may be a regular occurrence (Duh et al.,

2001; Gray et al., 2002; Blaňárová et al., 2016). Therefore European *B. microti* in *I. ricinus* ticks should be considered a potential health risk in the Province of Trento (Baráková et al., 2017, submitted to Tick and tick borne pathogens).

For the genotyping of *B. burgdorferi* s.l. genospecies we used IGS and four MLST genetic markers. We identified following genospecies among collected ticks; *B. afzelii, B.garinii, B. valaisiana, B. lusitaniae* and new genospecies in Italy, *B. turdi*. The phylogenetic tree of four housekeeping genes showed similar results as phylogenetic tree of IGS sequences. Our results show that both approaches can be used for reliable genotyping of *B. burgdorferi* s.l., however phylogenetic tree from MLST sequences shows more precise separation of *B. afzelii* and *B. garinii*. On other hand genotyping of IGS genes was less time and material consuming.

We observed a higher prevalence of *B. burgdorferi* s.l. among larval ticks than among adult and nymph ticks detached from the hosts. More frequent infection in larvae ticks is common as a result of feeding mostly on rodents, birds and lizards which are reservoirs for this pathogen (Table 11.). Transovarial transmission for *B. burgdorferi* s.l. has not been recorded up to date (Richter et al., 2012) therefore the infected larvae detached from a host are a good indicator of hosts able to acquire and transmit the infection further.

Accordingly to the current knowledge on reservoir competence, *B. afzelii*, *B. burgdorferii* s.s., *B. spielmanii* and *B. bavariensis* are the main *Borrelia* genospecies commonly maintained by rodents. Our study shows 16.7% (12/72) prevalence of *B. afzelii* in larval ticks detached from rodents.

Free living ungulates do not play an important role in the maintenance of *B. burgdorferi* s.l. in nature, even more; they are known to clear the infection from feeding ticks (Kurtenbach et al., 2002). Our results support this since no larval ticks detached from wild ungulates were infected with this pathogen, only one adult *I. ricinus* tick was positive. However transstadial transmission of this pathogen in *I. ricinus* is known, therefore the tick most probably got infected in previous life stage feeding on a different host.

In Europe, birds play an important role as reservoir hosts of two *Borrelia* genospecies: *B. garinii* and *B. valaisiana* (Humair et al., 1998). The reservoir capacity of passerines especially blackbirds was confirmed in various studies across the Europe (Humair et al., 1998; Kurtenbach et al., 1998, Hanincová et al. 2003b, Tarageľová et al. 2008). Our results reveal

the prevalence of *B. burgdorferi* s.l. in ticks detached from blackbirds with 34.6% prevalence among nymphs and 43% among larvae. This provides an indication of the high rate of the exposure of this bird species to the pathogen and the concurrent high capacity of these species to amplify the infection and to infect larvae. In term of genospecies, we identified *B. luisitaniae, B. afzelii, B. garinii, B. valaisiana* and *B. turdi* in larvae detached from birds. It is known that *B. garinii* and *B. valaisiana* are usually associated with birds but *B. afzelii* is most commonly reported in rodents. In our study, one larva and one nymph detached from birds were positive for *B. afzelii*. This finding together with study done by Franke et al. (2010), where the prevalence of *B. afzelii* in ticks detached from birds was interestingly high, provides the opportunity for further research to understand the role of birds in the ecology of this genospecies. Another genospecies not so frequently reported among ticks collected from birds is *B. luisitaniae*. The importance of birds as reservoir hosts of *B. lusitaniae* was previously proposed by study in Switzerland (Poupon et al. 2006) where *B. lusitaniae* was identified in larval *I. ricinus* feeding on migrating passerine birds. Another *Borrelia* genospecies, *B. turdi* is mostly found in Asia where it is transmitted by *I. turdus*, up to date there are no reports of this ticks species in Europe. In this study, we report for the first time *I. turdus* infected with *B. turdi* at this continent. The first evidence of *B. turdi* in Europe was reported in Portugal in *I. frontalis* ticks (Norte et al. 2013). The authors also confirmed the vector competence of *I. ricinus* for this genospecies using xenodiagnostic experiments (Norte et al. 2013). Our study shows the occurrence in naturally feeding *I. ricinus* ticks on birds in central Europe, suggesting *I. ricinus* as ‘’bridging vector’’ that may be able to transmit this bacterium with unknown pathogenicity to alternative hosts such as humans. Our study shows the occurrence of this genospecies in 6.9% of larval ticks collected from birds, highlighting the potential reservoir competence of blackbirds for this pathogen. These findings confirm that birds thought their migration routes can participate in the spread of new *Borrelia* genospecies to the new areas of Europe.

In regard of co-infection in feeding larval ticks, the most frequent co-infection was observed for *R. helvetica* and *B. burgdorferi* s.l. more specifically; *B. afzelii*, *B. garinii*, *B. valaisiana*. Since rodents are not considered as reservoirs of *R. helvetica* and they are an important reservoir of *B. afzelii*, the co-infection could be a result of co-feeding at the same time on the same host. The co-infection with *R. helvetica* and *B. garinii* or *B. valaisiana* may

be a result of feeding on common reservoir host, a bird (Table 11.). Less frequent co-infection was detected between *A. phagocytophilum* and *B. burgdorferi* s.l. (*B. garinii* and *B. valaisiana*) as a result of feeding on a bird, a common reservoir host for these two pathogens. Apart of the *B. burgdorferi* s.l. co-infection with other pathogens, we detected also co-infection among *A. phagocytophilum* and *R. helvetica* in one larva detached from wild ungulates. Wild ungulates are known to be reservoir hosts for both pathogens; therefore the co-infection could occur while feeding on the same host. Further evaluation of infection hazard after a tick bite in Trentino, should take into consideration the diversity of tick-borne pathogens and the occurrence of co-infections detected in this study.

On the note of acarological hazard in Europe, up to date no studies were successful predicting infectious hazard. We used environmental parameters obtained from remote sensing imagery that are relative to *I. ricinus* phenology to describe their correlation with spring abundance of *I. ricinus* as well as with infection prevalence rates of tick-borne pathogens. Our data show positive correlation between vegetation index NDVI and overall abundance of the principal vector *I. ricinus* as well as NDVI and infected nymphs in all studied EU countries (Rosà et al., 2017). Some studies argued that NDVI is not a good predictor of tick abundance (Fryxell et al., 2015) because vegetation water content is arguable (Barrios et al. 2012). However, it was established that tick survival depends on relative humidity in the vegetation cover and further that it correlates with photosynthetic activity which is measured with NDVI (Benefetti and Rossini, 1993). There are also studies confirming correlation between NDVI and tick abundance, however, they were carried out on a small geographical scale and non of them demonstrated their correlation with infection prevalence of tick-borne pathogens (Bisanzio et al. 2008, Alonso-Carne et al., 2015; Estrada-Peña et al. 2016). Our results propose NDVI as a predictor of tick-borne pathogens that can be implicated on a large geographical scale (Rosà et al., 2017 in preparation for Eurosurveillance).

Table 11. Table is constructed of current knowledge as well as data obtained in this study on the presence of pathogens among nymphs, larvae *I. ricinus* detached from various hosts, the presence of these pathogens in hosts, on ability of some host to transmit the infection further and on the transovarial transmission of this pathogens in *I. ricinus* ticks.

Pathogen		**Presence of pathogen in nymph from**	**Presence of pathogen in larvae from**	**Presence of pathogen in host**	**Xenodiagnosis technique**	**Confirmation of transovarial transmission**
***Anaplasma* spp.**	***A. phagocytohpilum***	Birds (**This study**; Lommano et al., 2014) Wild ungulates, Rodents, Sheep, Dog, Human (**This study**)	Birds (**This study**; Lommano et al., 2014) Roe deer (**This study,** Carpi et al., 2009)	Roe deer, red deer, chamois (Silaghi et al., 2011) Sheep, Dog, Cattle, Horses (Stuen et al., 2013)	European bison (Matsumoto et al., 2009) Rodents (Burri et al., 2014)	Has not been proven
***Rickettsia* spp.**	***R. helvetica***	Birds (Lommano et al., 2014) Wild ungulates, Rodents, Birds (**This study**)	Birds (**This study**; Lommano et al., 2014) Wild ungulates (**This study**)	Birds Rodents, roe deer, wild boar (Sprong et al., 2009; Stefanidesova et al., 2008)	Rodents didn't tranlsmitt *R. helvetica* to larvae ticks (Burri et al., 2014)	Exists
	R. monacensis	Birds (Lommano et al., 2014)	Rodent, Wild ungulates (**This study**) Birds (Lommano et al., 2014)	No rodents were infected (Burri et al., 2014)	Rodents didn't tranlsmitt *R. monacensis* to larvae ticks (Burri et al., 2014)	Has not been proven
	R. raoultii	No data	Wild ungulates (**This study**)	No data	No data	Exists (Alberdi et al., 2012)
***Babesia* spp.**	***B. venatorum***	Rodents, Wild ungulates (**This study**) Birds (**This**	Birds, Rodents, Wild ungulates (**This study**)	Roe deer (Andersson et al., 2016)	No data	Exists (Bonnet et al., 2009; Mazyad et al., 2010)

		study, Hasle et al., 2011)				
	B. capreoli	No data	Birds (**This study**)	Roe deer, red deer, chamois (Hoby et al., 2009)	No data	Exists (Nikol'skii and Pozov, 1972)
	B. microti	Rodents (**This study**; Welc-Falęciaka et al., 2008)	Rodents (Welc-Falęciaka et al., 2008)	Rodents (Yabsley and Shock, 2012; Welc-Falęciaka et al., 2008)	Gerbils (Grey et al., 2002)	Not efficiant (Walter and Weber, 1981; Grey et al., 2002; Hersh et al., 2012)
***B. burgdorferi* s.l.**	***B. afzelii***	Birds (**This study**)	Rodents, (**This study,** Hanicova et al., 2003a**)** Birds **(This study,** Franke et al., 2010)	Rodents (Burri et al., 2014)	Rodents (Burri et al., 2014)	Not efficiant (Richter et al., 2012)
	B. garinii	Birds (**This study,** Hanicova et al., 2003b)	Birds (**This study**, Olsen et al., 1995), Rodents (**This study**)	Birds (Humair et al., 1998)	Birds (Humair et al., 1998; Kurtenbach et al., 2002)	
	B. turdi	Birds (Hasle et al., 2011)	Birds (**This study**, Hasle et al., 2011)	Birds (Norte et al., 2013)	Birds (Norte et al., 2013)	
	B. luisitaniae	Birds (**This study**)	Birds (**This study**, Poupon et al., 2006)	Lizards (Dsouli et al., 1998)	Lizard (Dsouli et al., 2006)	
	B. valaisiana	Birds (**This study,** Kurtenbach et al., 2002, Hanicova et al., 2003b)	Birds (**This study,** Hanicova et al., 2003b)	Birds (Kurtenbach et al., 2002)	Birds (Humair et al., 1998)	

Conclusion

This thesis focuses on current knowledge of the occurrence and pathogenicity of tick-borne bacterial and protozoan microorganisms and the role of various wildlife hosts in natural foci. Small mammals, rodents, birds and also wild ungulates serve as hosts for many vectors carrying tick-borne pathogens. The hosts are necessary for the full transmission cycle of *I. ricinus*; moreover reservoir hosts carry on spread of pathogens transmitted by this tick species to humans and domestic animals. Larval ticks prefer small mammals for blood meal, therefore rodents are important hosts for them. According to the host-size restriction, larvae and nymphs are able to suck blood on birds while wild ungulates serve as hosts mostly to nymphs and adults. Rodents are the most important reservoirs due to their replication and maintenance of a broad range of microorganisms. However, their role of reservoirs for various pathogens has to be better understood. Short generation time, high number of offspring and relatively quick sexual maturity usually leads up to overpopulation. Many species subsequently tend to spread and penetrate into new habitats and create a new outbreak for pathogens. Birds, as important reservoirs can also harbor a large diversity of pathogens. Due to the large population size, wide geographic distribution and high mobility, they play a significant role in maintaining and dispersing disease agents at large geographical scales. Ungulates which are considered as ‘’tick reproduction host’’ (Gray, 1998) are important for successful reproduction of adult stages based on which they ensure the density of immature *I. ricinus* ticks (Ruiz-Fons and Gilbert, 2010).

All points mentioned above, point out, that wildlife hosts play an important role in the transmission of tick-borne pathogens and significantly contribute to the maintenance of these organisms in nature. Hence, these hosts were targeted as sentinel of the disease hazard in this study.

In this study, we report evidence of several tick-borne pathogens not previously reported within the province of Trento, the alpine region of northern Italy. Particularly we detected new pathogens to the area, *R. raoultii*, *R. monacensis*, *B. venatorum*, *B. capreoli*, *B. microti* and *B. turdi*. Moreover, for the first time in Europe we confirmed the presence of *I. turdus* which was so far detected only in Asia and Japan. *I. turdus* acts as vector of *B. turdi* with unknown pathogenicity. These newly detected pathogens are considered of rising interest for public health in Italy. Our study provides new insights into the ecology and transmission

cycle of these pathogens by updating list of vertebrate species that might be involved in their circulation in nature. We were able to confirm rodents, wild ungulates and birds as reservoirs for *A. phagocytophilum*, rodents as reservoirs for *B. afzelii* and birds as reservoirs for *B. garinii*. Further we point out the need for experimental studies in future for clarifying free-living ungulates as reservoir for *R. helvetica, R. monacensis* and *R. raoultii*. We also propose birds as reservoir for *B. venatorum*, *B. capreoli, B. lusitaniae*, *B. valaisiana* and *B. turdi* based on our results.

Furthermore, we detected *A. phagocytophilum* in *I. ricinus* nymphs and rodent blood and tissues, however, no larvae *I. ricinus* fed on rodents were positive for this pathogen in Italy or Finland. On the other hand *A. phagocytophilum* was prevalent in larvae *I. scapularis* fed on rodents from USA. Phylogenetic analysis of two genetic loci reveals that *A. phagocytophilum* circulating in rodents from Europe is distinct from *A. phagocytophilum* circulating in rodents in USA. The rodent-associated strain in Europe is probably not pathogenic to humans as is the case in USA. Due to no positive *I. ricinus* larvae detached from rodents in our study and studies carried by Bown et al. (2009) and Pangracova et al. (2014), *I. ricinus* may not be a suitable vector for transmitting rodent-associated strain and other vertebrate species may be responsible for the pathogenic *A. phagocytophilum* genotype.

Further, we have been able to identify *Borrelia* genospecies circulating in the area using phylogenetic analysis constructed from IGS and MLST markers. Both methods have been successful in genotyping, despite the fact that the individual MLST housekeeping genes are less polymorphic than IGS genes. However, the combined use of multiple sequencing of housekeeping genes has shown the ability to analyze evolutionary relatedness and even more it showed more precise separation of *B. afzelii* and *B. garinii*.

Finally, by determining total abundance of questing *I. ricinus* and density of infected nymphs in five EU countries, taking into account environmental and climatic factors we were able to define NDVI as a successful predictor of acarological hazard on a geographical scale.

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