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Prevalence of tick borne-encephalitis virus (TBEV) and phylogeographic structure of its vector *Ixodes ricinus*

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This thesis is worth 60 study points.

Abstract

Ixodes ricinus is the most common vector in Europe and it is the primary vector for tick-borne encephalitis virus (TBEV) in Norway. Tick-borne encephalitis (TBE) cases have been reported yearly from Norway since the first case occurred in 1997. The aim of present study was to investigate the prevalence of TBEV and the phylogeographic structure of its vector *I. ricinus* ticks from several parts of Norway.

A total of 1595 ticks were screened for TBEV from two sites of Southern Norway. This region is known for one of the highest number of TBE cases in the country. Ticks were collected by flagging in May and June 2015. A subpopulation of 1480 nymphs and 115 adult ticks were investigated for TBEV by real time polymerase chain reaction (PCR) amplification of the envelope gene. Positive samples from real-time PCR were confirmed by pyrosequencing. The estimated prevalence of TBEV was 0.41% and 2.17% among nymphs and adults from Vanse in Vest-Agder County and 0.69% and 1.4% among nymphs and adults from Bjønnes in Telemark County.

A total of 33 ticks including 30 nymphs (pools of ten) and 3 adults was collected in 2014 from different parts of Norway. Complete mitochondrial genome of six samples (three pools of nymphs and 3 adults) successfully sequenced from Next generation sequencing (NGS). Six sequences from this study and 724 previously published mitochondrial DNA sequences were used for reconstruction of phylogenetic trees with Maximum likelihood (ML) and Bayesian method. The phylogenetic trees showed partial or lack of phylogeographic structure in Norwegian *I. ricinus* ticks.

This study confirmed prevalence of TBEV from two sites in southern part of Norway and there was low or lack of phylogeographic structure in its vector *I. ricinus*.

Abbreviations and terms

Amplification: In which multiple copies of a specific DNA segment are made.

Annealing: PCR step in which the primer adheres to the DNA strand (template) when the temperature is lowered to about 50-65 °C.

Bootstrapping: Is a procedure where a random subset of the data was re-run for the phylogenetic analysis, and the reported value is the percentage of bootstrap replicates in which the node showed up.

Bp: base pairs.

Clade: a group of organism classified together on the basis of homologous feature traced to common ancestor.

Ct-value: Threshold cycle is defined as the number of cycles required for the fluorescent signal to cross the threshold.

Co-feeding: Feeding of ticks on the same host at the same time and transferring infective agents from one to the other.

COI& II: Cytochrome oxidase I and II are subunits of three mitochondrial DNA (mtDNA) encoded subunits (COI, COII, COII) belonging to the respiratory complex IV. COI is used as a DNA bar code to identify animal species.

Cyt B: Cytochrome B gene is a component of the respiratory chain complex III in the mitochondria of eukaryotic cells. Cytochrome b is commonly used as a region of mitochondrial DNA for determining phylogenetic relationships between organisms, due to its sequence variability.

CR: Control region gene is a non-coding region of mitochondrial DNA. It is a polymorphic region and controls RNA and DNA synthesis

Denaturation: PCR step in which double stranded DNA turns into two single strands by heating the reaction to 94-98 °C.

DNA: Deoxyribonucleic acid- is a genetic material with two complementary strands of four nucleotides (Adenine (A), Cytosine (C), Thymine (T) and Guanine (G)), deoxyribose (pentose sugar) and phosphate groups.

DNA-polymerase: A short synthetic thermo-stable enzyme that catalyzes the synthesis of a new DNA strand from the template with the use of dNTPs.

DNTPs: Deoxy ribonucleotides- building block of the DNA strand consisting of various combinations the four deoxy ribonucleotides; dATP, dCTP, dGTP and dTTP.

D-loop: Is synonymously used as control region, where control region includes the D-loop along with adjacent transcription promoter regions.

Gene: A basic unit of heredity present on the chromosome that encodes function.

Genome: Complete set of DNA or RNA, including all genes of one organism.

Haplotype: a group of genes in an organism inherited together from a single parent.

MSIS: National surveillance of infectious diseases in Norway.

mtDNA: Mitochondrial DNA is DNA located on mitochondria which is cellular organelles within eukaryotic cells often used to determine phylogenetic relationships between organism due to rapid evolution of its gene sequences.

NGS: Next generation sequencing **-** Massively parallel sequencing of DNA sequences by clonal amplification of template DNA.

Oligonucleotides: Short synthetic strand of DNA or RNA (primers and probes).

ORF: Open reading frame is a part of DNA that contain no stop codon and it has a stretch of codons that can be translated.

Prevalence: Proportion of individuals in a population at a given time.

Pool: Particular number of organisms (arthropods) analyzed together in a tube, for example nymphs in a group of ten.

Primers: short synthetic segment of DNA that is complementary to a given DNA sequence, DNA polymerase initiates replication from the primer.

Probe: Short synthetic single stranded DNA that binds to the primers and emits fluorescent light when polymerase dissociates from the reporter gene after downstream amplification of PCR primers. Used in real-time PCR to detect the formation of PCR-product.

Polytomy: A section of a phylogeny in which the evolutionary relationships cannot be fully resolved to dichotomies in phylogenetic trees.

Real-time PCR: or, quantitative polymerase chain reaction (qPCR) is used to quantitatively measure the PCR product after each cycle. A probe is used to detect the emission of fluorescence when the template is amplified.

RT-PCR: Reverse transcription PCR- is used to synthesize the complementary DNA from RNA by reverse transcriptase enzyme.

Sequencing: DNA sequencing is the process in which the precise order of nucleotides in a strand of DNA molecule is determined.

12s: 12S ribosomal RNA- is a component of the small subunit of the mitochondrial ribosome which is essential for protein synthesis.

TNA: Total nucleic acid – A complex organic substance that are building blocks of DNA and RNA, whose molecules consist of many nucleotides linked in a long chain.

Transcription: Synthesis of RNA from DNA

TBE: Tick-borne encephalitis is an infection of central nervous system due to TBEV.

TBEV: Tick-borne encephalitis virus is a single stranded RNA virus belonging to genus Flavivirus.

TBEV-Eu: European subtype of tick-borne encephalitis virus.

TBEV-Fe: Far-eastern subtype of tick-borne encephalitis virus.

TBEV-Sib: Siberian subtype of tick-borne encephalitis virus.

Taxa: is a group of one or more populations of an organism.

Whole genome sequencing: Process of determining the complete DNA sequence of an organism's genome at a single time.

Contents

Foreword

This Master thesis is written as a part of a Master degree at the Department of Environmental and Health studies at the University College of Southeast Norway. The current study is a part of two projects; ScandTick Innovation (Interreg V project ID 20200422) and Barentsregion project B1412. ScandTick Innovation focuses on tick-borne diseases in the øksregion in Scandinavia; one work package was designed for surveillance and analysis of the prevalence of various tick pathogens like TBEV and Lyme borreliosis. Screening of TBEV from the southern Norway is included in this package. The whole genome sequencing of *I. ricinus* was part of B1412 Barentsregion project financed by the Norwegian Ministry of Health and Care Services, Norway.

I would like to express my thanks and appreciations to my supervisors, Åshild Kristine Andreassen and John Pettersson for giving me this great opportunity to work on this challenging project and their useful guidance, insightful comments, and considerable encouragements to complete this thesis. The door to Åshild office was always open whenever I had a question about my research work or writing.

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I would like to say thanks to my family especially my parents for their support, love, care and prayers. Thanks to my dear elder brother Waseem Ahmad for your unconditional love. Without your support, I cannot manage anything properly. I am blessed to have a brother like you, one who always help me by every aspect. Thanks to my dear nephew and sister in law for being part of life. Thanks to my only dear elder sister and youngest brother for your emotional support, I miss our interesting and long-lasting chats.

Olso/10.10.2017

Nosheen kiran

Aim of study

Tick-borne encephalitis (TBE) is a common viral infection in Europe with many cases reported every year. The causative agent, TBE virus (TBEV), attacks the central nervous system of the host with potentially fatal outcome in humans. The castor bean tick*, Ixodes ricinus*, is the main vector of TBEV in Europe.

This master thesis is part of the ScandTick Innovation-EU Interreg V project ID 20200422 and Barentregion project B1412 and it was conducted at the Norwegian Institute of Public health (FHI). TBEV are known to cause disease in Norway since 1997. TBE cases has been reported from Southern Norway according to the Norwegian Surveillance System for Communicable Diseases (MSIS) but no human cases are known from northern, eastern and western part of Norway. However, ticks collected from the coastal line of these regions do carry TBEV. Could the lack of human cases be caused by sequence variants of TBEV in Norway?

The aim of our study was to find possible genetic diversity of TBEV from ticks and human patients samples and also to investigate the phylogeographic structure of its vector *I. ricinus*. Six positive tick samples from different locations of country were sequenced with Illumina Hi-seq in search for TBEV. However, no TBEV sequence could be recovered, only sequences of *I. ricinus*. The aim of the current study was therefore redefined to investigate the prevalence of TBEV in ticks and phylogeographic structure of *I. ricinus* ticks from Norway.

Part 1: Prevalence of TBEV from Southern Norway within the yearly surveillance program at FHI and Scandtick Innovation project.

- Tick samples were collected from two sites Bjønnes (Telemark) and Vanse (Vest-Agder).
- Vanse S18, was chosen due to previous knowledge of TBEV.
- Bjønnes T14, was chosen to investigate the prevalence of TBEV in a new location with reported TBE-case according to MSIS.

Part 2: Phylogeographic structure of *I. ricinus* from Norway

- Samples were collected from six localities within Akershus (A1), Buskerud (B1), Hordaland (H3), Møre og Romsdal (MR4) and Vest-Agder counties (S5, S10). Complete genome Sequences of *I. ricinus* from these sites were analyzed with the maximum likelihood and Bayesian method for phylogeographic structure of *I. ricinus* ticks.

Null-Hypothesis and alternative hypothesis for second part of study is

- There is no population structure among *I. ricinus* ticks in Norway compared to Europe.
- There is population structure among *I. ricinus* ticks in Norway compared to Europe.

1. Introduction

1.1. *Ixodes ricinus*

Ticks are obligate hematophagous ectoparasites of most terrestrial vertebrates which have a significant impact on the public health and rural economy in many parts of the world (Jongejan & Uilenberg, 2004). Ticks form a sub-order (Ixodida) in the order Parasitiformes of the sublass Acari. (Figure 1-1). This sub order consists of two major families of ticks: The hard ticks (Ixodidae) the soft ticks (Argasidae) (Gu et al., 2014; Sonenshine, 1991). *Ixodes ricinus* is a hard tick, also known as the castor bean or sheep tick (Parola & Raoult, 2001; Paulauskas et al., 2006). It belongs to a group of closely related and recently divergent hard ticks group known as the *I. ricinus* complex. The group consist of 14 species distributed in different geographic regions of the world (Xu, Fang, Keirans, & Durden, 2003).

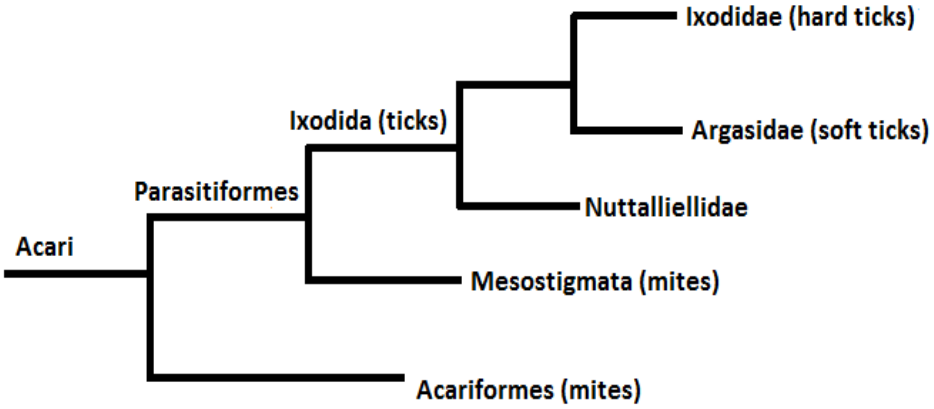

***Fig 1-1**: General Phylogeny of ticks (ixodida)retriever from wikepedia .*

I. ricinus is the most common vector in Europe infesting a large community of hosts (e.g mammals, birds, reptiles) and it is an important vector of microparasites (Parola & Raoult, 2001). As a vector, it is responsible for transmission of various infectious pathogens that cause disease in humans and animals such as Lyme borreliosis, tick-borne encephalitis, ehrlichiosis, and babesiosis (Stanek, 2008).

The *I. ricinus* life cycle has three active life stages and each stage require a blood meal to molt into the next stage or to reproduce (Milne, 1943). Larvae do not move over large

distances and remain aggregated within their environment whilst waiting for a host. Once they find a host and may be dispersed through host movement during feeding, before they develop and molt to the nymphal stage. This feeding process is repeated during different life stages until the adult stage. Distribution of feeding ticks particularly by birds and large mammals, is therefore crucial for the short- and long-range dispersal (Medlock et al., 2013).

I. ricinus is distributed from Scandinavia to western Russia and south to the Atlas mountains in Northern Africa (A Estrada-Peña, Bouattour, Camicas, & Walker, 2004). In Norway, *I. ricinus* is the most commonly encountered tick species although total of 14 different species of ticks have been identified in the country (Jore et al., 2011; Mehl, 1983). The geographical distribution of *I. ricinus* in Norway are along the coastline of Norway up to 66^{o}N (Mehl, 1983; Tambs-Lyche, 1943). However, Jenkins et al found a few ticks attached to cats and dogs in the region north of Brønnøy at 66^{o}N (Jenkins et al., 2012)(Soleng A, 2017 submitted paper).

1.2. Tick-borne encephalitis

Tick-borne encephalitis (TBE) is a disease caused by tick-borne encephalitis virus (TBEV). It is a potentially fatal disease syndrome in humans and some mammals (Gritsun, Lashkevich, & Gould, 2003). TBE is endemic in Northern and central Europe, Russia, China and Japan (Lindquist & Vapalahti; Jochen Süss, 2011; Wu, Na, Wei, Zhu, & Peng, 2013). During the last two decades, 1990-2009, almost 170,000 human TBE clinical cases were reported from Europe and Russia (Jochen Süss, 2011).

About 70% of the reported TBE infections are without symptoms in humans (Gritsun et al., 2003). Virulence and disease symptoms shows characteristic differences relative to virus subtypes. TBE caused by the European virus subtype usually presents as meningitis, encephalitis or meningoencephalitis and clinical symptoms of infections are biphasic in approximately two-thirds of patients. Treatment is usually based on the symptomatic measures (Kaiser, 2008).

1.3. Tick Borne encephalitis virus

TBEV belongs to the genus *Flavivirus* within the family *Flaviviridae* (Kunze, 2016). The TBEV genome contains a approximately 11 KB positive sense, single stranded RNA

genome (Valarcher et al., 2015). The genomic RNA encodes a polyprotein of about 3400 amino acids that is cleaved into three structural proteins designated, capsid (C) membrane(M) and envelope (E) and seven non-structural genes designated, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Lindenbach & Rice, 2001) (Figure 1-2).

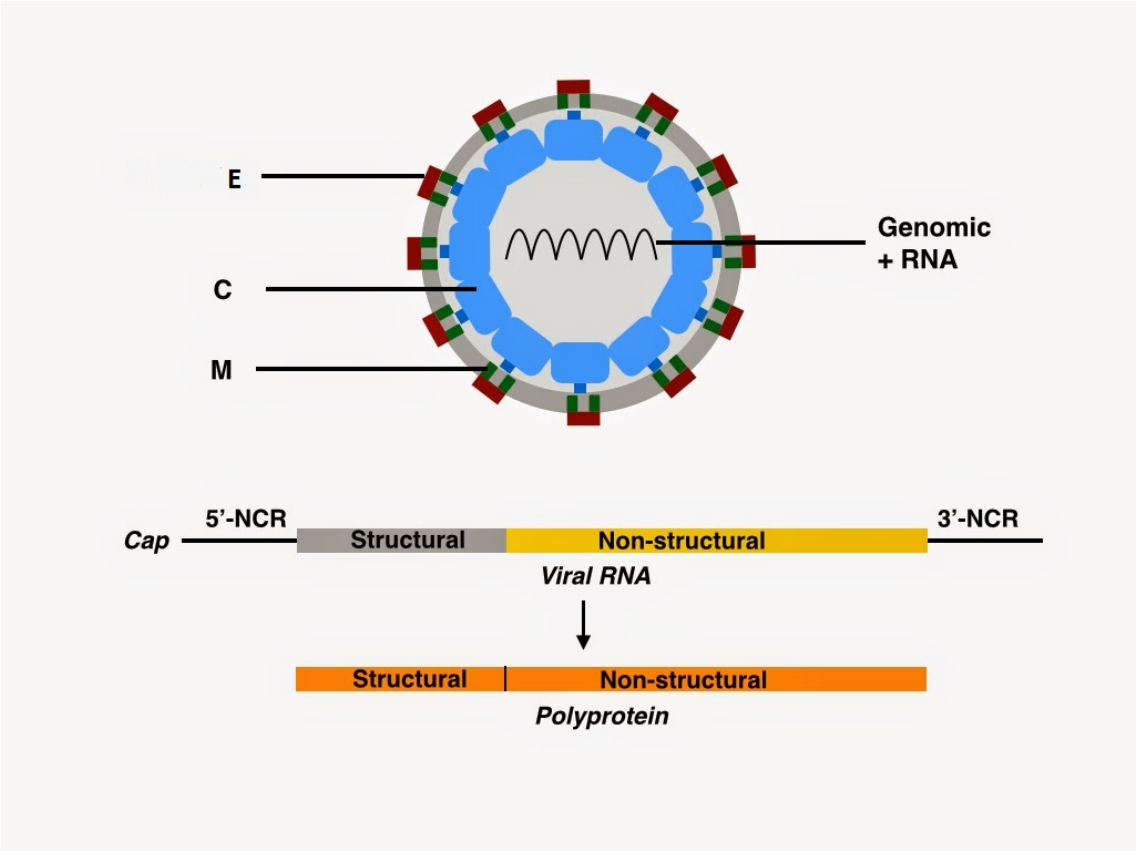

***Figure 1-2**: Structure of tick-borne encephalitis virus retrieved from virology tidbit blogspot .*

TBEV is transmitted mainly by *I. ricinus or I. persulcatus* ticks to humans or domesticated animals or rarely through consumption of unpasteurised milk products (Valarcher et al., 2015). TBEV is maintained in a cycle between ticks and wild animals (Gritsun et al., 2003). The virus chronically infects ticks for the duration of their life cycle and can transmit it transovarial and through mating between ticks (Chunikhin, Stefuktina, Korolev, Reshetnikov, & Khozinskaia, 1982; Kaiser, 2008). Ticks can become infected by feeding on infected animals, by co-feeding or by transstadial transmission at any three stages (larvae, nymphs and adults) of their life cycle (Kaiser, 2008). Host animals of ticks such as small rodents, large mammals and migratory birds are important for transmission and distribution of virus (Blaškovič, 1967; Jaenson, Hjertqvist, Bergström, & Lundkvist, 2012; Labuda & Nuttall, 2005).

TBEV is divided into three subtypes named after their geographic distribution: the include European (TBEV-EU), Siberian-(TBEV-Sib), and Far Eastern-(TBEV-FE) TBEV subtypes (Gritsun et al., 2003). Ticks usually act as both vectors and reservoirs for TBEV (Lindquist & Vapalahti). *I. ricinus* is the dominant vector in Europe while *I. persulcatus* is the main vector in Eastern Europe and Asia. European subtypes of the virus is mainly vectored by *I. ricinus* while *I. persulcatus* is a vector for the Siberian and Far Eastern subtypes (Kaiser, 2008). The TBEV-EU subtype has been isolated from most European countries while all three subtypes are reported to co-circulate in some regions such as the Baltic countries and Finland (Lundkvist et al., 2001). The TBEV-EU subtype is present in Norway (Andreassen et al., 2012; Paulsen et al., 2015).

Phylogenetic studies indicate that TBEV-Sib and TBEV-FE form separate lineages, sharing a common ancestor. The two subtypes probably evolved thousands of years earlier than the TBEV-EU (Tonteri et al., 2013). Diversity of these two subtypes is much higher than the TBEV-EU subtype. TBEV-EU does not show clear geographic clustering (Jochen Süss, 2011).

In Norway, a total of 106 TBE cases have been reported according to the Norwegian Surveillance System for Communicable Diseases (MSIS). TBE cases have been reported from coastal areas in Vestfold, Telemark, Aust-Agder and Vest-Agder (MSIS). TBEV was recently detected in *I. ricinus* nymphs in these three counties and also in østfold (Andreassen et al., 2012; Larsen et al., 2014). TBE IgG antibodies has been reported in blood donors from Østfold County (Larsen et al., 2014). The prevalence of TBEV in *I. ricinus* from northwestern Norway including Møre og Romsdal and Sør-Trøndelag counties was 0.41% in nymphs and 3.08% in adults (Paulsen et al., 2015). In a more recent study the prevalence of TBEV in Nord-Trøndelag and Nordland county was found much higher in adult ticks (personal communication).

1.4. Phylogeographic structure of *I. ricinus* ticks

Understanding the genetic variability of vector population gives information for the taxonomic status of the vector species, the spatial limits of populations and the nature of gene flow among populations (McCoy, 2008). Furthermore, a better understanding of the genetic variability of the vector population is essential to develop effective methods against the vector-borne diseases transmitted by the ticks, through vector control or anti-

ticks vaccine (Dai et al., 2009; Gillet et al., 2009; Philipp et al., 1997). *I. ricinus* is the first ticks species that have been used to study tick population genetics (Paulauskas, Galdikaitė-Brazienė, Radzijevskaja, Aleksandravičienė, & Galdikas, 2016).

The population genetic structure of I*. ricinus* originating from different European regions was studied with the use of mitochondrial DNA (mtDNA) performed by Casati and co-workers (2008). The authors reported lack of population genetic structure among *I. ricinus* from Europe (Casati, Bernasconi, Gern, & Piffaretti, 2008). The *I. ricinus* samples from geographically widely distributed areas such as great Britain and Latvia showed genetic variation based on the multilocus mtDNA (Dinnis et al., 2014). Mitochondrial DNA is particularly well suited as a genetic marker for phylogenetic studies due to high number of copies per cell, the strictly maternal inheritance and lack of recombination (Shao & Barker, 2007).

I. ricinus is frequently the object of research in Norway because of its medical importance (Paulauskas et al., 2006). *I. ricinus* is a principal vector in transmission of Lyme disease bacteria (Nygård, Brantsæter, & Mehl, 2005), and tick borne encephalitis (TBE) virus in humans (MSIS) in that country. Previous studies of *I. ricinus* in Norway has mainly focused on the distribution (Mehl, 1983; Tambs-Lyche, 1943) and, the presence of pathogens and epidemiology of diseases (Asghar et al., 2014; Kjelland, Stuen, Skarpaas, & Slettan, 2010; Paulsen et al., 2015). Considering the genetic structure of the species *I. ricinus* in Norway, the only data are based on Randomly amplified polymorphic DNA (RAPD) (Paulauskas et al., 2006), on control region and on cytochrome B genes of mitochondrial DNA (Røed, Kvie, Hasle, Gilbert, & Leinaas, 2016). A better understanding of the population genetic structure and variability of a parasitic population is potentially important to understand the dispersal and transmission of the pathogens (Kanduma et al., 2016).

1.5. Molecular phylogenetic study

Molecular Phylogenetic is a research field of bioinformatics where an organism is studied at the molecular level to gather information about the phylogenetic relationships between different organisms (Dowell, 2008).

The molecular approach is now preferably used as compared to morphological characters to investigate the evolution and relationship among genes and organisms, molecular information, such as nucleotide or amino acid sequences and Restriction length

polymorphism (RFLP) used to infer the phylogenetic relationships. In order to create the relationships between genes and gene fragments phylogenetic analysis infer the common history between them. For this reason, homologous sites in the sequences are aligned in such a way that these sites form columns in the alignment (Salemi, Lemey, & Vandamme, 2009) (Figure 1-3).

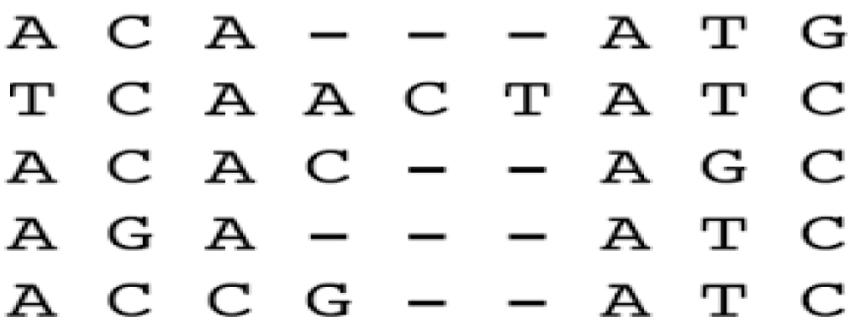

***Figure1-3**: Alignment of multiple sequences taken from python for Bioinformatics blogspot.*

Reconstructing the phylogeny from nucleotide or amino acid alignment depend on the phylogenetic constructing methods. One of phylogeny reconstructing method, Maximum likelihood (ML) methods uses the statistical criteria which consider the probability that a tree give rise to the aligned sequences or observed data according to the specific evolutionary model (Yang & Rannala, 2012). Bayesian method is another way of reconstructing a tree, where Bayesian approach employ the concept of likelihood, but by targeting a probability distribution of the tree. It requires a prior belief, which formalized as a prior distribution on the model parameters, i.e. substitution model parameters, branch length, and tree topology (Yang & Rannala, 2012).

The phylogenetic relationship is usually represented in the form of a binary tree, where the structure of the tree illustrates the possible relationships between ancestor and descendant (Polanski & Kimmel, 2007). A phylogenetic tree is a graph which is composed of nodes and branches where any two nodes are joined together by a unique path (Figure 1-4).

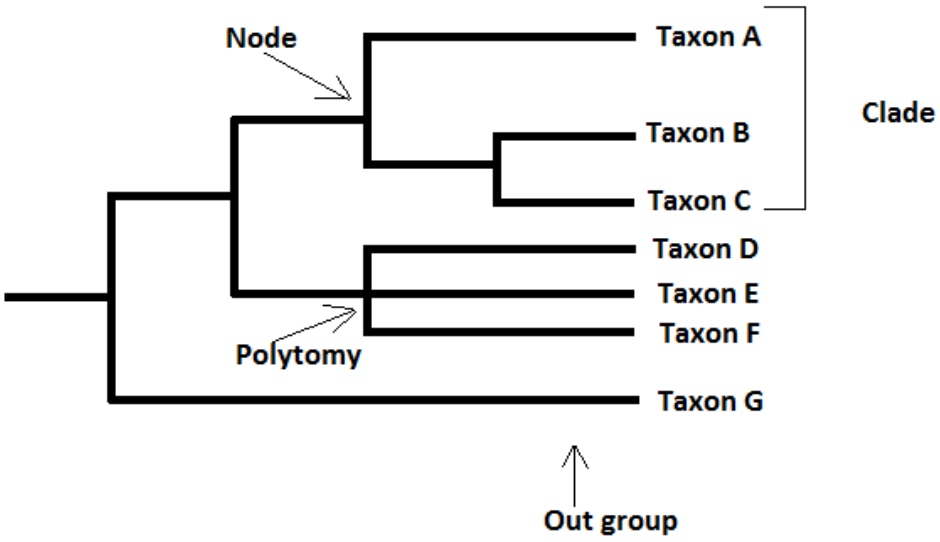

***Figure 1-4**: illustrate the general phylogenetic tree*

Branches and nodes describe the ancestor-descent relationship among the taxonomic unit in the phylogenetic tree. External nodes are also called leaves or tips of the tree. External nodes are referred to as operational or extant taxonomic units in the phylogenetic tree. Internal nodes are also called ancestral taxonomic units. Topology represents the branching pattern in the phylogenetic tree.

2. Materials and methods

2.1. Collection of ticks

The materials in the master thesis were *I. ricinus* nymphs and adults collected from the vegetation in different parts of Norway (Figure 2-1, Table 2-1). The ticks were collected by the Norwegian Institute of Public Health in 2014 and 2015. To study the prevalence of TBEV in *I. ricinus* ticks, questing ticks were collected from Vest-Agder (S18) and Telemark (T4) in 2015 (Red marks in figure 2.1). While ticks collected in 2014 from Akershus (A1), Buskerud (B1), Hordaland (H3), Møre og Romsdal and Vest-Agder (S5,S10) were used for the study of phylogenetic relationship of *I. ricinus* from Norway (Blue marks in figure 2.1).

All ticks were collected by flagging the vegetation with a white flannel towel. The towel was flagged over the meadow landscapes of the forest and both sides of the towel were used to harvest ticks. Ticks attached to the cloth were removed with forceps and put in the microtubes that were kept on crushed ice during transport to the laboratory. Adult ticks were separated in the laboratory by gender and kept in individual tubes and, nymphs were placed as pools of ten in each tube. All tubes were labeled with the name of collection site, date of collection, and stored dry at -80°C until further analysis.

Figure 2-1: Collection of ticks from different parts of Norway highlighted with red and blue markers. Red highlighted markers are sites which is used for the screening of TBEV in this study, whereas the blue mark represent the collection site of ticks used in phylogenetic study of I. ricinus ticks.

Table 2.1: Collection of ticks from different parts of Norway.

Collection site	*UTM- Coordinate	Date of collection	Total nymphs	Total adults	
				♀	♂
Hordaland, Skånevik H3	32V 331780E 6625890N	28.06.2014	10	0	0
Akershus, Brønnøya A2	32V 0586436E 6636726N	15.09.2014	10	0	0
Vest-Agder, Kilen S5	32V 0411861E 6430610N	11.06.2014	10	0	0
Vest-Agder, Hille S10V	32V 403111E 6430689N	13.06.2014	0	1	0
Møre og Romsdal, Kanestraum MR4V	32V 455393E 6992055N	28.05.2014	0	1	0
Buskerud, Tofte B1V	32V 585617E 6600445N	23.05.2014	0	1	0
Telemark, Bjønnes T4	32V 544428E 6547035N	04.06.2015	740	30	39
Vest-Agder, Vanse S18	32V 364685 E 6443574N	27.05.2015	740	21	25

*UTM-coordinate (Universal Transverse Mercator)

2.2. Nucleic acid extraction from ticks

Total nucleic acid (TNA) and total RNA was extracted from adults and nymphs with the use of total nucleic acid High-Performance Isolation and RNAeasy mini kit respectively. Extraction methods were automated in MagNa Pure (both kit and instrument from Roche Diagnostic GmbH, 82372 Penzberg, Germany) and QIAcube instrument (both kit and

instrument from QIAGEN inc., Valencia, CA, USA) according to Paulsen et al. 2015 and Andreassen et al. 2012.

Sterile loops were used to transfer pools of ten nymphs or individual adults from storage tubes to Fast prep® 2ml tubes containing six steel beads. RLT lysis buffer of 400 µl in nymphs and 350 µl in adults was added for homogenization and lysis of the tick samples. Nymphs were homogenized in the FastPrep-24 5G homogenizer (MP Biomedical Life sciences, CA, USA) for 60 seconds at a speed of 4.0 m/s whereas homogenization of adults require additional 45 seconds. Cells and tissues were disrupted by rapid agitation in the presence of beads and lysis buffer after homogenization. β -mercaptoethanol (β-ME) was already added in RLT lysis buffer which immediately inactivate the RNases to ensure purification of intact RNA. The homogenate was transferred to new tubes and centrifuged for 5 minutes at 14000 rpm (revolution per minute) and debris was settled as a pellet while the supernatant was recovered for the extraction of total RNA and TNA. The supernatant from nymphs was added in new tubes and then transferred to an automated extraction machine QIAcube (QIAGEN inc., Valencia, CA, and the USA) and following the manufacturers' protocol (Purification of RNA form animal tissues and cells, two elution steps). High quality RNA is obtained from silica membranes in this method and there are four main steps in the process: lysis, binding, washing, and elution of samples. Elution volume of our samples was 60 µl after the elution step. Three kinds of buffer are involved in RNA extraction: Buffer RPE with added Ethanol is used as washing buffer to remove traces of salts, Buffer RW contains a guanidine salt along ethanol that efficiently removes biomolecules (protein, fatty acid and carbohydrates) which are non-specifically bound to the silica membrane. The last buffer RLT, including high concentration of guanidine isothiocycanate which supports the binding of RNA to the silica membrane.

The supernatant for adults was transferred to a sample tray, and then placed in the automated MagNA pure LC 2.0 instrument (Roche Diagnostic GmbH, 82372 Penzberg, Germany). Magnetic beads are used for the isolation of TNA from the samples in MagNA Pure (Roche Diagnostic GmbH, 82372 Penzberg, Germany. Homogenization of adult ticks was done with the RLT lysis buffer from RNeasy mini kit because lysis buffer from MagNA pure caused lot of fuming during crushing of ticks. The sample volume was 200 µl and elution volume was 60 µl.

2.3. Detection of TBEV in ticks

Detection of European TBEV strain from Norwegian *I. ricinus* was done by two step real-time PCR using an in house real-time PCR method that was established by Andreassen *et al.,* (2012).

2.3.1. Preparation of cDNA with RT-PCR

Five µl of total (approximately 500 ng) extracted TNA or RNA was reversibly transcribed to cDNA by High-capacity cDNA Reverse Transcription System kit with random hexamer primers, MultiScribe™ reverse transcriptase and RNase inhibitor (Applied Biosystems, Foster city, CA, USA).

RNA was reversibly transcribed into the cDNA in a 2720 thermal cycler according to manufacturers' protocol as described by Andreassen *et al.,* 2012. The cDNA was analyzed next day and then stored at -80°C until to further analysis.

2.3.2. Amplification of TBEV with in-house real time PCR

The presence of TBEV was examined from cDNA by an in-house real-time PCR (Andreassen et al., 2012). A pair of primers and probes were used to generate an average length of 54 base pairs for the European TBEV strain (Table 2.2).

Table 2.2: List of primers and probes used the in real-time PCR for detection of TBEV.

Primer name	Sequences (5′→3′)	Genome position	GenBank accession number
TBE 320F	GGGAGCGCAAAACTGGAA	1662-1680	U27495
TBE 373R	TGAGGAGCCCCAAATTCAAC	1696-1715	U27495
TBE 339 probe	(FAM)-AACGCAGAAAGAC-(BHQ1)	1681-1693	U27495

The positve control used in each run was cultivated from human patient sample (kindly supplied by Dr Christian Beuret, Spiez lab Switzerland). The positive control contains 50 000 virus particles/ µl of RNA. This was diluted from 10^{-3} to 10^{-7} and transcribe into

cDNA. RNAase free water (QIAGEN Inc., Valencia, CA, USA) used as negative control in each run.

To amplify each fragment, a mixture of 3µl cDNA and 22µl mix (total volume 25 µl) was prepared, 0.25 µM primers (TBEV 320F & 373R), 0.3 µM probe (TBEV 339), 5 mM $MgCl_2$, 0.2X dNTPs, 1 X AB Buffer (appendix 6), 0.19 Units Pt-Taq (Invitrogen Life Technology, Inc., Carlsbad, CA, USA) with 15.81 µl RNAase-free water was incubated for 2 min at 95°C and then used in a cycling reaction (48 cycles of 95°C for 15s, 60°C for 45s, and 72 °C for 30 seconds) in rotor gene 6000 real-time PCR machine (Qiagen).

2.3.3. Pyrosequencing to confirm TBEV prevalence in ticks

Positive samples from real-time PCR were confirmed by pyrosequencing according to Andreassen *et al.,* (2012). The Reverse primer, TBE 373 R, was biotinylated in the real-time PCR reaction for later use in pyrosequencing for efficient synthesis of single stranded oligo's using streptavidin-coated sepharose beads.

Pyrosequencing was performed according to the manufacturer's instructions using Pyrogold SQA reagents (QIAGEN, Germany) and SQA analysis in (PyroMark Q24) system (QIAGEN, Germany). Master mix was prepared by adding Binding Buffer, streptavidin-coated sepharose beads and RNase free water. Primer mix was prepared by adding annealing buffer and sequencing primers (appendix7 b,c). 60 µl and 20 µl of master mix and PCR product was added in each pre-labeled strip respectively. PCR tubes were then kept on shaking for 10 min. 25 µl of primer mix with forward primer (TBE 320 F) was added in Pyromark Q24 sequencing plate. PCR product was cleaned after shaking with the use of water, NAOH and washing Buffer. After processing, the washing device was cleaned with sterile water for 20 seconds to avoid cross-contamination in the next run. Samples from the master mix tubes was transferred into the washing device by putting device for 1 min to make sure the complete removal of all samples from PCR product tubes. Meanwhile it was shaken gently to prevent surface tension in it. In this step, the PCR product binds to special filters in the washing devise. The washing device was later placed into 70% ethanol and NAOH for 5 seconds, where NAOH denatures the double stranded DNA into single stranded. Finally, the washing device was put into the washing buffer for 10 seconds to clean and neutralize the PCR product. Then the PCR product was transferred into the primer mix a pyrosequencing plate to elute the samples from filtrates

and leave it for 5 minutes. Pyrosequencing plates were then heated for 2 minutes at 80°C and further cooled by leaving it in room temperature.

Pyromark cartridge was placed in PyroMark Q24 (Qiagen, Germany) machine together with pyrosequencing wells after adding enzyme, substrate and nucleotides (ATCG) in the cartridge. Finally, the sequence obtained from pyrosequencing was compared with the standard.

2.4. Prevalence calculation formula

Prevalence of TBEV was calculated with minimum infection rate (MIR) and estimated pooled prevalence (EPP) as described by Andreassen *et al.,* (2012). Prevalence estimates were based only on confirmed positive samples.

MIR was calculated by using the following formula and expressed as percentage:

MIR = (x/(mk))100%,

Where, x = the number of positive pools

m = the number of pools tested

k = pool size (k = 1 for adult ticks, k = 10 for nymphs),

This method assumes that a positive pool is infected by a single individual. The EPP method provides an estimate of the uncertainty within the confidence interval associated with the prevalence estimations. The method gives prevalence estimation with a 95% confidence limit and accuracy of ± 0.4 and calculated using an online pool prevalence calculator from Epitool epidemiological calculator (http://epitools.ausvet.com.au/content.php?page=PPFreq1).

2.5. Next-generation sequencing data analysis

I.ricinus collected from Hordaland (H3, Skånevik), Akershus (A2, Brønnøya), Vest-Agder (S5, Kilen & S10, Hille), Møre og Romsdal (MR4, Kanestraum) and Buskerud (B1, Tofte) in 2014 were selected for whole genome sequencing. Extraction and sequencing were done previously by students and employees at the in Norwegian health Institute of public health (FHI) according to Andreassen *et al.,* (2102). Samples (RNA and TNA) from these six sites after confirmation from pyrosequencing was selected for next-generation sequencing (NGS). Preparation of NGS libraries of all samples was performed by the staff at FHI. RNA and TNA extracts were treated with Turbo DNA-free™ kit (Thermo fisher,

Carlsbad, USA) to remove DNA and Ribo-Zero Gold kit (Kapa Biosystems, Wilmington, Massachusetts) to remove redundant rRNA according to the manufacturer instructions. Kapa stranded RNA-Seq kit (Kapa Biosystems, Wilmington, Massachusetts) was used for library preparation which involves fragmentation of RNA, synthesis of cDNA and addition of specific adaptors to the end of the fragments according to the manufacturer instructions. Fragments were then amplified with the use of standard hexamer primers. The concentration and size of each library were measured and final libraries were sequenced on a Hiseq 2500 NGS platform (Kapa Biosystems, Wilmington, Massachusetts) at the Norwegian Sequencing Centre (http://www.sequencing.uio.no/).

Specific adaptors added to the ends of cDNA fragments that have sequence complementary to the oligonucleotides of the flow cell in the cartridge. The flow cell consists of two types of oligonucleotides that will hybridize to the complementary sequences of the DNA fragments and synthesis the complementary strand. Template strand will be removed and the free end of newly synthesized strand will create bridge structure by binding to the other complementary oligonucleotide of the flow cell. Each strand will clonally be amplified by bridge amplification and produce millions of clusters on the flow cell. After clonal amplification clusters is converted in to the single strand with removal of the reverse strand. Sequencing started with extension of sequencing primers and addition of each nucleotide detected with emission of specific fluorescence signal. Sequencing of the second strand will follow same method and difference is only cleavage of forward strand and sequencing of reverse strand.

The resulting sequence reads of all six samples were quality checked, trimmed for adaptors with trim galore (www.bioinformatics.babraham.ac.uk/projects/trim_galore/ and mapped against the complete mitochondrial genome of reference KF197136.1 using Bowtie 2 (Langdon, 2015). Quality check, trimming and mapping of reads was done by staff at FHI.

2.5.1. Sequences alignment

Norwegian *I. ricinus* complete mitochondrial genomes (A1, B1, H3, MR4, S5 and S10) from this study and 724 sequences including 25 complete genomes and 696 individual genes (Appendix 1) retrieved from NCBI (https://www.ncbi.nlm.nih.gov) was compared for reconstruction of alignment with Mafft (Katoh & Standley, 2013) and visualized in AliView

alignment software (Larsson, 2014). Three sequences of *I. persulcatus* form NCBI were used as an outgroup.

Five individual gene sequences cytochrome oxidase I (COI), cytochrome oxidase II (COII), cytochrome B, ribosomal 12s (12S) and control region (CR) from Casati et al., (2016) were combined and analyzed with complete mtDNA genome while these five individual genes in addition to some more genes sequences were also analyzed separately. Two individual gene (Cyt B and CR) sequences from Røed *et al.,* (2106) were mainly used for analyzing *I. ricinus* that are geographically separated from each other in Europe. So, these two gene were analyzed in combined form of these two genes with complete mtDNA genome and also analyzed as individual gene sequences.

Three kind of alignment files were made for construction of phylogenetic trees. One file includes only complete mtDNA sequences. The second alignment included concatenated sequences where 5 single gene sequences (COI, COII, CR, 12S, and Cyt B) of each isolate from Casati *et al.,* (2008) and 2 gene sequences (CR, Cyt B) of each isolate from Røed *et al.,* (2016) were merged as one sequence after alignment and phylogenetic trees were constructed by concatenated sequences with complete mtDNA sequences. The third alignment consisted of five individual gene sequences.

To select the best fitting nucleotide substitution models for each alignment, Jmodeltest2 (Darriba, Taboada, Doallo, & Posada, 2012) and Mega software (Guindon & Gascuel, 2003) was employed and models were evaluated according to the Akaike Information Criterion (AIC) and Bayesian information Criterion (BIC) (Table 2-2).

Table 2-2: Model specification for Bayesian inferences.

Model	Number of substitution type (nst)	Rates	Substitution model specification
General time reversible (GTR)	Nst =6	Invgamma	GTR+I+G
General time reversible (GTR)	Nst= 6	Gamma	GTR+G
Hasegawa, Kishino and Yano (HKY)	Nst= 2	Propinv	HKY+I
Hasegawa, Kishino and Yano (HKY)	Nst= 2	Gamma	HKY+G

2.5.2. Phylogenetic analyses

Phylogenetic analysis of complete mitochondrial genomes of Norwegian *I. ricinus* with downloaded sequences was performed using the ML and Bayesian method.

2.5.2.1. Maximum likelihood analysis

All three alignments were used further for the phylogenetic analysis. One of the phyloegentic analyses, ML, was conducted with the Seaview 64 bits software (Gouy, Guindon, & Gascuel, 2010) using phyml. Base frequencies, the shape of the gamma distribution, across-site rate was estimated according to the model parameters.

Tree topology was optimized and started the tree with BioNJ with tree improvement setting using the best of sub-trees pruning and regrafting (SPR) and Nearest Neighbor interchange (NNI). The reliability of internal branches was assessed with 1000 bootstrap replicates.

Bootstrapping is a procedure where a random subset of the data is used and re-run in the phylogenetic analysis. The reported value is the percentage of bootstrap replicates in which the node showed up. This value was used to estimate confidences levels of clades within a phylogenetic tree. Thus, 100 means that the node is well-supported and a

bootstrap support between 75% and 95% is considered reasonably good, anything below 75% is a very poor support. The value of bootstrap support less than 50% is not considered for any use in phylogenetic tree.

2.5.2.2. Bayesian analysis

Bayesian phylogenetic trees was constructed based on alignments with complete genomes and partial genes using Mr Bayes v 3.2.2 (Huelsenbeck & Ronquist, 2001). Two parallel analyses were run for twenty million generations. All analyses were initiated with a random starting tree and trees were sampled every 1000 generations. Each analysis composed of one cold and three incrementally heated chains. The stationary state of the Markov chain was determined by the log-likelihood scores of samples against generation time. Evaluation of convergence done by discarding the burn-in samples and remaining samples were retained for generating consensus trees. It also calculates mean, variance and 95% credibility intervals. Tree topology of each sample incorporates branch length and substitution model parameter values.

The phylogenetic tree from Bayesian inferences were interpreted with the posterior probability values. The posterior probability refers to probability conditional on the data, the model, and the prior values. This value represents phylogenetic accuracy or probability that a clade is correctly resolved. Confidence limit of Bayesian phylogenetic consisted of values above 0.90 is well supported and less than 0.70 is poorly supported.

3. Result

3.1. TBEV prevalence in ticks

A total of 1480 pooled nymphs and 115 single adult ticks were analysed from Vest-Agder and Telemark (Table 2-1). TBEV was detected in nymphs and adults from both sites and positives from the real-time PCR were confirmed by the pyrosequencing. From pyrosequencing, different dilution of the standards (from 10^{-3} to 10^{-7}) were positive and the sequence similarity between standard and positive pools for Norwegian Eu strain was detected between 70-80% (Appendix 7). The overall estimated prevalence of TBEV in nymphs was 0.6% and 1.7% in adult ticks.

TBEV was detected from Vanse in Vest-Agder County where four pools of nymphs were positives by real-time PCR and three of them were confirmed by pyrosequencing. This site gives an estimated pooled prevalence (EPP) and a minimum infection rate (MIR) of 0.41% and 0.40%. Further, TBEV was detected in one adult tick from Vanse by real-time PCR and was confirmed from pyrosequencing. The prevalence was 2.17% in adults from this site (Table 3-1).

TBEV was also detected from a new site, Bjønnes in Telemark. Six pools of nymphs were positives by real-time PCR of which 5 were confirmed by pyrosequencing. The EPP was 0.69% and the MIR was 0.67% from this site. One of the adults from Bjønnes was also confirmed positive for TBEV by pyrosequencing with prevalence of 1.4% (Table 3-1).

***Table 3-1**: Prevalence of TBEV in I. ricnus ticks from Vest-Agder and Telemark.*

Location	Number of tick samples		Positives from real-time PCR		Positives from pyrosequencing		Prevalence % (EPP, MIR)	
	Nymphs[a]	Adults	Nymphs	Adults	Nymphs	Adults	Nymphs[b]	Adults[c]
Vanse, Vest-Agder S18	74	46	4	1	3	1	0.41% 0.40 %	2.17%
Bjønnes, Telemark T4	74	69	6	1	5	1	0.69% 0.67%	1.4%
Total	148	115	10	2	8	2	0.6%	1.7%

a Nymphs were analysed in pools of ten. **b** Nymphs prevalence with EPP and MIR. **c** Adults prevalence with MIR.

3.2. Population genetic structure of *I. ricinus* ticks

A total of 33 ticks including 3 adults and 3 pools of ten nymphs in each was successfully amplified and sequenced by Illumina Hi-seq. The sequence reads from Illumina Hi-seq was followed in assembly of approximately 14650 bp of complete mtDNA. The phylogenetic position of the six sequences from Norway were inferred from ML and Bayesian phylogenetic trees.

A total of 20 phylogenetic trees were made from complete, complete and merged, and single genes of mtDNA sequences with Bayesian and ML method; it included 2 phylogenetic trees from complete mitochondrial DNA, 4 from complete and merged gene sequences, and 14 phylogenetic trees from single gene sequences.

3.2.1. Phylogenetic tree of complete mtDNA genome

The 34 complete mitochondrial sequences including 28 from previously published (Appendix 1 a) and 6 from this study were used to reconstruct the evolutionary history using ML and Bayesian method. Complete phylogenetic trees from both methods showed partial geographic structure in *I. ricinus* from Norway. Phylogenetic trees consist of one clade for most of Norwegian sequences.

In the ML phylogenetic tree (Fig 3-1), our sequences (A2, B1, MR4, H3, S5, and S10) formed a well-supported (Bootstrap support = 96%) clade together with Italian *I. ricinus*

tick sequences where sequences A2 is basal to the remaining Norwegian (B1, MR4, H3, S5 and S10) and Italian sequences. The clade is split up in two sub-clades, where the majority of Norwegian sequences (H3, S5, S10 and MR4) form one clade and one Norwegian sequence (B1) forms a clade with the Italian sequences.

The Bayesian phylogenetic tree (Appendix 9, a) had similar structure for Norwegian sequences compared to the ML tree.

3.2.2. Phylogenetic trees of complete and concatenated sequences

A total of four phylogenetic tree were constructed with ML and Bayesian method using complete and combined partial genes of mtDNA.

3.2.2.1. Phylogenetic tree of complete and merged five genes

Bayesian and ML phylogenetic trees were constructed using 28 complete, 26 concatenated mitochondrial DNA sequences (each of them consist of five merged individual genes) from earlier published data (Appendix 1 b), and six Norwegian sequences from this study. Both trees showed that there is shallow phylogeographic structure for *I. ricinus* from Norway compared to the Europe. One of clade consisted of only Norwegian sequences together with the Danish *I. ricinus* sequences and reaming sequences comes together with rest of different European *I. ricinus* sequences in phylogenetic trees.

In the ML phylogenetic tree (Fig 3-2), *I. ricinus* ticks from Norway formed a well-supported (bootstrap value 96 %) clade with sequences from the Switzerland, Denmark, Italy and Finland and where sequence A2 is basal to the all these sequences. The clade is divided further into two sub-clades, where the majority of Norwegian sequences form one clade with Danish *I. ricinus* sequence and one single sequence B1 comes together with Italian sequences IR_8 and IR_8_6 in another clade.

The Bayesian phylogenetic tree (appendix 9,b) followed similar structure for Norwegian sequences as compared to the ML tree.

3.2.2.2. Phylogenetic trees of complete and merged two genes

The phylogeographical pattern of *I. ricinus* from different geographical regions was investigated by adding some more sequences from Finland, Norway and Great Britain (Appendix 1 c). ML and Bayesian phylogenetic tree consist of 217 concatenated

sequences from Røed *et al.,* 2016 in addition to concatenated sequences (26 sequences) from Casati *et al.,* 2008, complete mitochondrial sequences (28 sequences) and 6 Norwegian *I. ricinus* sequences from this study.

The ML and Bayesian phylogenetic trees display lack of phylogeographic structure where most of our Norwegian sequences formed one clade (Appendix 9, c & d). In Both phylogenetic trees sequences H3, S5 and S10 comes together in one clade with bootstrap value 75% and posterior probability value 0.69 respectively. Furthermore, Sequences B1 appeared with the Italian sequences particularly with IR_8 in Bayesian phylogenetic tree that was supported with 1.00 posterior probability value. Remaining Norwegian sequences from this study comes with different European sequences.

3.2.3. Phylogenetic trees of single gene sequences

Separate phylogenetic trees of five individual genes were also reconstructed with ML and Bayesian methods. Phylogenetic trees of all five genes also showed lack of phylogeographic structure for Norwegian *I. ricinus* ticks. A total of 200 additional single genes (Appendix 1 d, e, f, and g) with single gene sequences from complete mtDNA (28) and single gene sequences from Casati *et al.,* (2008) & Røed *et al.,* (2016) were used for the phylogenetic analysis of Norwegian *I. ricinus* ticks.

3.2.3.1. Phylogenetic trees of Cytochrome B

Phylogenetic trees of cyt B gene reconstructed from ML and Bayesian methods consist of 64 sequences including 26 cytochrome B gene sequences from Casati *et al.,* (2008), 34 Cyt B genes sequences from complete genome sequences, 4 genes sequences from Paulauskas *et al., (*2016) (Appendix d), and 6 sequences from current study.

Phylogenetic tree from geographic separated *I. ricinus* sequences: Cytochrome B gene sequence were used again for reconstruction of phylogenetic tree in order to interpret the phylogeographic structure among the *I. ricinus* sequence that are geographically separated by barriers (Seas and mountains). So, ML and Bayesian trees were constructed with the use of 26 cytochrome B gene sequences from Casati *et al.,* (2008), 248 cytochrome B gene sequences from Røed *et al.,* (2016), 28 Cytochrome B gene sequences from the complete mtDNA sequences, and 6 Norwegian sequences from this study.

Four phylogenetic trees of Cyt B gene including two ML (Appendix 9, e), (Appendix 9, g) and two Bayesian phylogenetic tree (Appendix 9, f), (Appendix 9, h) showed lack of phylogeographic structure for Norwegian *I. ricinus* ticks, where Our Norwegian sequences were not fully resolved and appeared with different European sequences with no or poorly supported bootstrap and posterior probability values.

3.2.3.2. Phylogenetic trees of Control region gene

The ML and Bayesian phylogenetic tree consisted of 28 CR gene sequence from complete mtDNA, 26 from Casati *et al.,* (2008) and 44 Paulauskas *et al.,* (2016) (Appendix 1 e) sequences, and 6 Norwegian sequences of current study.

The ML tree (Appendix 9, I) and Bayesian phylogenetic tree (Appendix 9, J) indicated the lack of phylogeographic structure for Norwegian *I. ricinus* sequences. Both phylogenetic tree consisted of Norwegian *I. ricinus* sequences without or less supported bootstrap and posterior probability values together with the different European *I. ricinus* sequences. Norwegian sequences S5 and S10 consisted of longer branch length in both phylogenetic trees.

Phylogenetic trees of geographically separated *I. ricinus* sequences: The phylogenetic tree reconstructed from ML and Bayesian methods consisted of 236 sequences from Røed *et al.* (2016), 28 gene sequences of CR region from complete mtDNA, 26 gene sequences from Casati *et al.* (2008) and 6 Norwegian sequences from this study.

Phylogenetic trees of the control region reconstructed from the Bayesian and ML method and both produced similar structure for Norwegian *I. ricinus* sequences (Appendix 9, k & j). Both phylogenetic trees lack geographic structure for Norwegian *I. ricinus* sequences and all six sequences (A2, B1, H3, S10 and S5) were appeared with different European sequences. While S5 and S10 Norwegian sequences consisted of longer branch length in both phylogenetic trees. All Norwegian sequences were supported with less or no bootstrap and posterior probability values in the ML and Bayesian phylogenetic trees.

3.2.3.3. Phylogenetic trees of ribosomal 12s gene

ML (Appendix 9, m) and Bayesian (Appendix 9, n) phylogenetic tree of a ribosomal 12s gene consisted of total 60 sequences including 34 ribosomal 12s gene sequences from complete mtDNA, and 26 ribosomal 12s gene sequences from Casati *et al.,* (2008).

In ML phylogenetic tree, Norwegian sequences formed unsupported (bootstrap value 56%) clade with most of European *I. ricinus* sequences. The clade consists of further two sub-clades, where majority of Norwegian together (H3, MR4, S5 and S10) with Danish sequences comes in one sub-clade. The sub-clade nested sequences B1 together with Finland and Switzerland sequence with poor supported bootstrap value 63%, while sequence A2 appeared with Italian and Slovakian sequence with no support value.

The Bayesian phylogenetic tree showed that our Norwegian sequences formed two moderately supported (posterior probability 0.76) sub-clades and a majority of the Norwegian sequences appeared in one clade (posterior probability value 0.87) with Danish sequences, while Akershus (A3) and Buskerud (B1) appeared in other clade (posterior probability value 0.03) together with sequences originating from other regions of Europe.

3.2.3.4. Phylogenetic tree of cytochrome oxidase II gene

Phylogenetic trees of COII included 90 *I. ricinus* sequences. ML and Bayesian phylogenetic trees were made using 34 COII gene sequences from complete mtDNA, 26 COII gene sequences from Casati et al., (2008) and 30 additional COII gene sequences from Porretta et al. (2013) (appendix 1 f), 6 Norwegian sequences from current study.

The ML phylogenetic tree (Appendix 9, o) indicated lack of geographical pattern for Norwegian *I. ricinus* sequences and sequences were not fully resolved. Branches were supported with poorly bootstrap values.

The Bayesian phylogenetic tree (Appendix 9, p) showed our sequences (A1, B1, MR4, H3, S5, and S10) forms a un-supported (posterior probability value 0.01) clade together with Italian, German, Danish and Finnish *I. ricinus* sequences and majority of our Norwegian sequences are basal to the most of these sequences. The clade is split up in two sub-clades, where most of Norwegian sequences (A2, MR4, H3, S5 and S10) form one clade with German and Danish sequences and one Norwegian sequence (B1) forms a clade with the Italian sequences.

3.2.3.5. Phylogenetic tree of cytochrome oxidase I gene

ML and Bayesian phylogenetic trees were from 34 COI gene sequences from complete mtDNA, 26 COI gene sequences from Casati et al., (2008) and 30 additional COII gene sequences (appendix 1 g).

In ML phylogenetic tree (Appendix 9, q), our most of sequences (A2, H3, S5, S10 and MR4) formed a poorly supported (bootstrap value 19%) clade with the Serbia, Danish and German *I. ricinus* sequence. Only one sequences B1 formed a clade together with Italian and Ireland sequences.

The Bayesian phylogenetic tree (Appendix 9, r) structure is similar for Norwegian sequences to the ML phylogenetic tree.

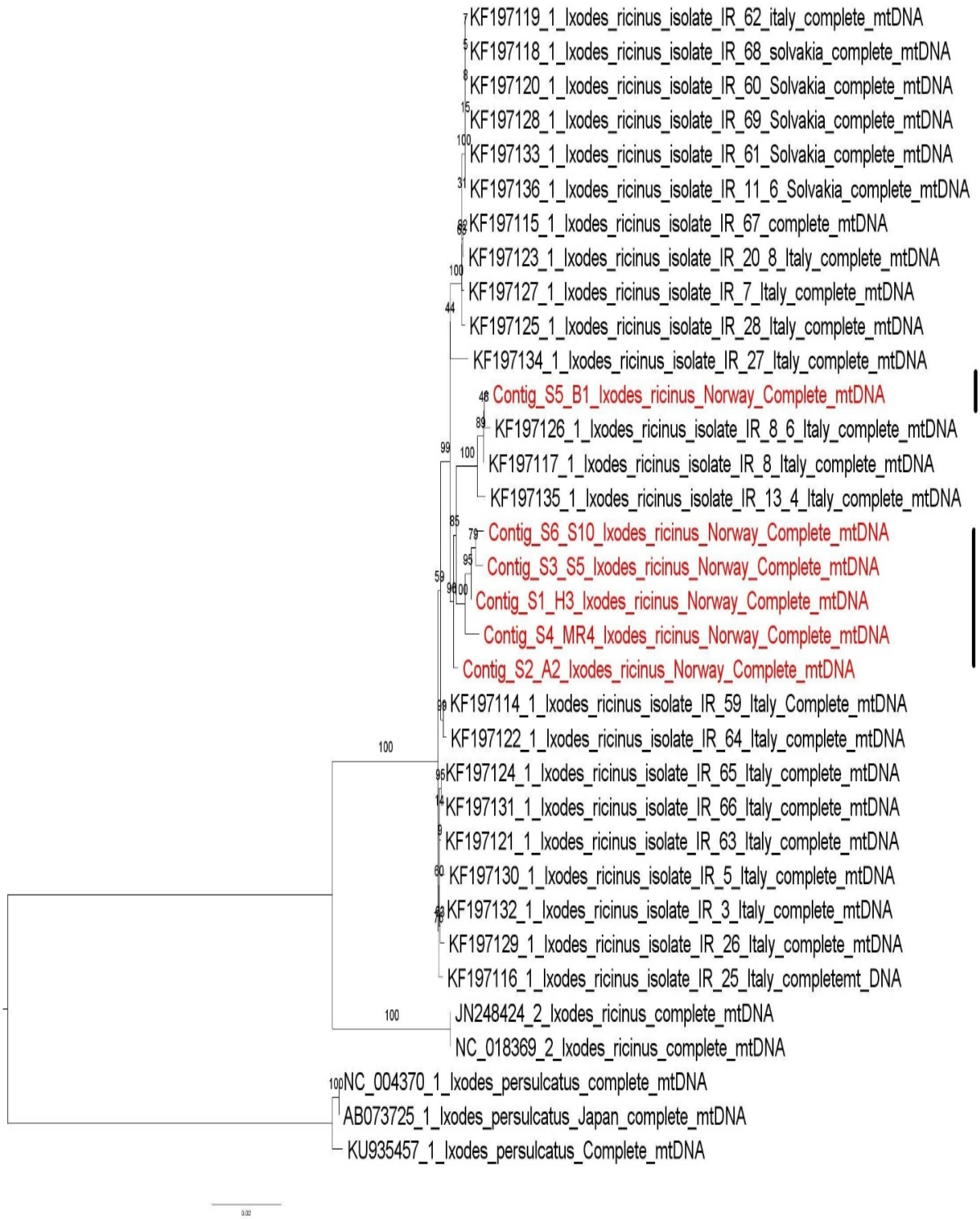

Fig 3-1: shows ML phylogenetic trees of I. ricinus based on complete mitochondrial DNA alignment (ca[a]. 14650bp) under the GTR+I+G model. The Norwegian strains are indicated in red color. Bootstrap support values are based on 1000 replicates are indicated above branches. The tree was rooted with I. persulcatuss.

[a]*ca. caliber scale indicating number of base pars in Aliview software.*

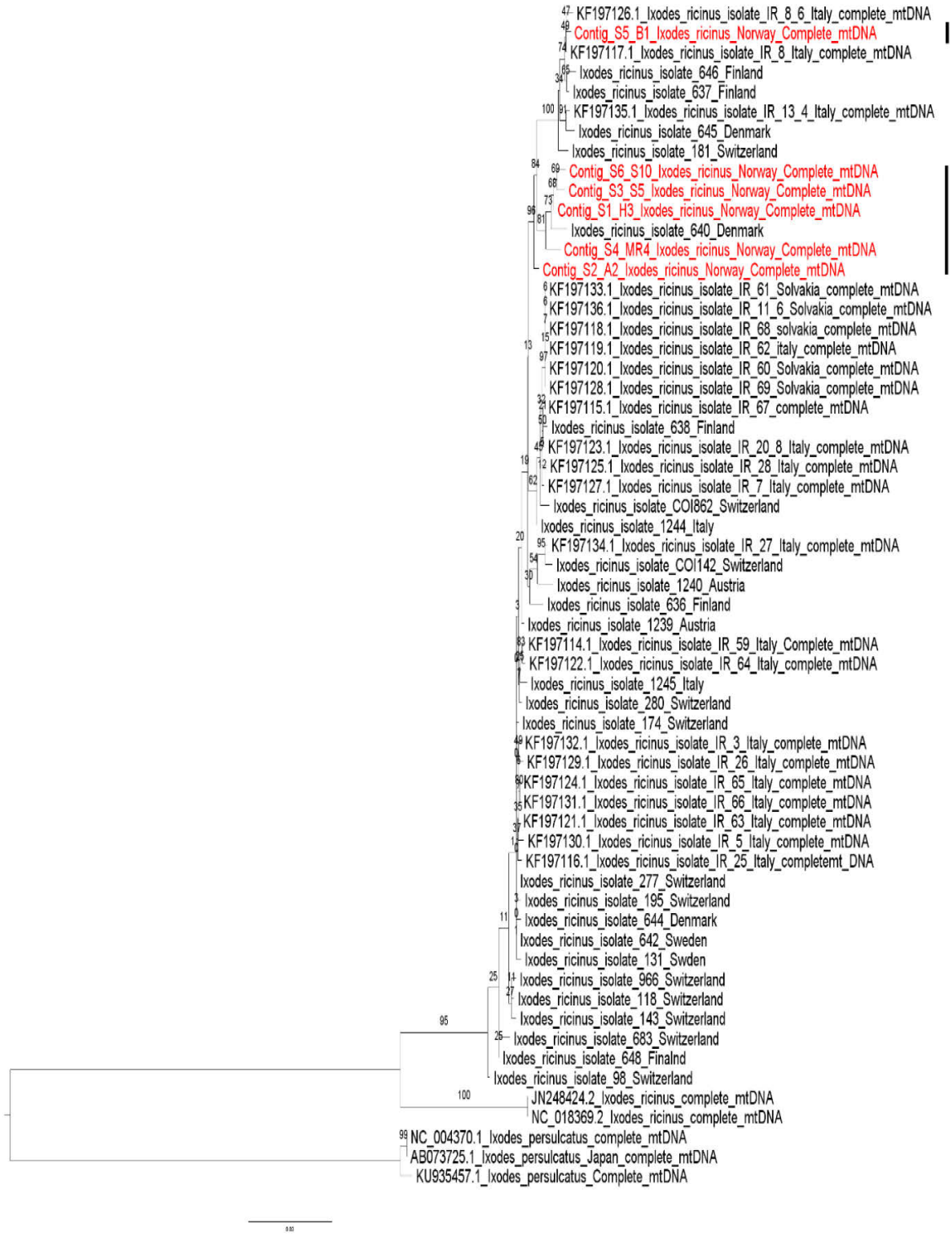

Figure 3-2 indicate ML analysis of I. ricinus ticks based on complete mitochondrial DNA alignment (ca. 14650bp) with merged five individual genes including a control region, CYT B, COI, COII and 12s under the HKY+I+G model. The Norwegian sequences are highlighted in the red color. Bootstrap support values are based on 1000 replicates are indicated above branches. The tree was rooted with I. persulcatus.

4. Discussion

4.1. Prevalence of TBEV in Norway

In this study, a total of 1595 ticks from southern part (Vest-Agder and Telemark) of Norway were analysed for prevalence of TBEV, and TBEV was detected in ticks from both sites. The overall estimated prevalence of TBEV in nymphs and adult *I. ricinus* from these sites were 0.6% and 1.7% respectively. The prevalence found in these sites is in agreement with findings in endemic areas in Europe Scandinavia and Norway. The prevalence of TBEV in *I. ricinus* ranged from 0.1% to 5% in Europe, 0.28% from Scandinavia and 0.53% from Norway (Andreassen et al., 2012; Paulsen et al., 2015; Pettersson, Golovljova, Vene, & Jaenson, 2014). The prevalence of TBEV is higher in adult ticks as compared to nymphs from both sites. Generally, the prevalence of TBEV is higher in adult ticks than in nymphs (Pettersson et al., 2014; J. Süss, Schrader, Abel, Voigt, & Schosser, 1999). This is most likely due to fact that adult ticks have an extra blood meal in their life cycle compared to nymphs. Adults are therefore likely to have encountered virus twice (Pettersson et al., 2014; J. Süss et al., 1999).

The prevalence of TBEV in ticks from Vanse in Vest-Agder County (2015) was 0.41% in nymphs and 2.17% in adults. In a previous master thesis of Benedikte Nevjen Pedersen from the same location, the TBEV prevalence in nymphs and adults were 0.14% and 0.78% respectively. Our result showed relatively higher prevalence of TBEV in adults and nymphs compared to the previously reported from Vanse, Vest-Agder County. In Norway, TBE is a notifiable disease in southern parts of the country since 1994, 1–14 cases have been reported each year (MSIS). Most cases are reported from the coastal areas in the Agder counties of the south of Norway. A total of 33 TBE cases have been reported from this county (MSIS). Skarpaas and coworkers found the first human TBE case from Aust-Agder County (Skarpaas et al., 2006). Our result confirms the presence of TBEV at Vanse, Vest-Agder County.

TBEV have been detected in ticks (unpublished data) and human cases are reported by MSIS from Bjønnes, Telemark county. The collection site was selected close to the area where the patient was believed to be infected. The current study detected TBEV at Bjønnes, Telemark County, with a prevalence in nymphs and adult ticks of 0.69% and

1.4%, respectively. Our result showed similar prevalence of TBEV in adults at this site with previous study where adults had a TBEV prevalence of 1.43% However, previously there were no TBEV positives detected from nymphs (Unpublished data) in Bjønnes. This may be due to a smaller number of nymphs analysed from this site as compared to current study (unpublished data). The prevalence of TBEV in nymphs from this site was higher than previously detected in southern part of Norway, where the detected EPP were 0.53% (Andreassen et al., 2012) and 0.14% (Larsen et al., 2014). It is well known that the abundance of nymphs is positively connected with the incidence of human-tick borne disease (Stafford, Cartter, Magnarelli, Ertel, & Mshar, 1998).

Previous studies have proposed that TBEV is distributed in a patchwork pattern, is probably due to climatic conditions like temperature, moisture, and vegetation (Gritsun et al., 2003), virus prevalence, vector and host relationships and other factors within a specific geographical zone (Burri, Bastic, Maeder, Patalas, & Gern, 2011).

Prevalence of TBEV in ticks varies between the sites (Andreassen et al., 2012) and within the sites (unpublished data) and it is also varies from year to year (J. Süss et al., 1999). Low level of moisture promotes disappearance of virus from ticks and the TBEV prevalence in ticks may be reduced in dry summers (Andreassen et al., 2012). Early spring and favourable temperature for ticks may increase the TBEV prevalence (Randolph & Sumilo, 2007).

TBEV circulates in a triangle of interaction between virus, vector tick and tick host. Hence, it is able to persist in a given habitat over long periods of time (Patricia A Nuttall, 1999).

Ticks acquire infection while feeding on an infected competent reservoir host (a species capable of transmitting infection) or directly from another tick during the co-feeding (Pettersson et al., 2014). When infected ticks quest for another host they might transmit the pathogen, and then they may any susceptible host by feeding on them (Agustín Estrada-Peña, Ayllón, & de la Fuente, 2012). Hosts preferences of ticks are also important for infection distribution. Small mammals like rodents act as main transmission hosts for ticks (Jochen Süss, 2011).

The present study shows a relative high prevalence of TBEV in ticks from two sites within the Telemark and Vest-Agder counties, as compared to previous studies from these two sites. There is a continues need of research on the risk of TBE infection from endemic and non-endemic locations. This is important for providing updated information to health

authorities, micro biological laboratories and general practitioners for clinical diagnosis, vaccine recommendations, and risk management.

4.2. Phylogeographic structure of *I. ricinus* Ticks

Population genetic structure is essential for understanding of parasite and host dispersal, the evolution of host specificity (Boulinier et al., 2001; McCoy et al., 2003), epidemiology and evolutionary dynamic of the disease and the vector. The current study is the first that investigated phylogeographic structure of Norwegian *I. ricinus* ticks based on complete mtDNA sequences. We sequenced and analysed complete mtDNA of *I. ricinus* from six locations from south to north followed by construction of phylogenetic trees.

All six sequences were tested for the phylogeographic structure of *I. ricinus* ticks from Norway in relation to the Europe *I. ricinus*. Phylogenetic trees of complete, concatenated sequences, and single sequences of mtDNA with ML and Bayesian method shows, there is partial or lack of phylogeographic structure among *I. ricinus* in relation to the European *I. ricinus*.

Phylogenetic tree from five merged sequences and 12s single gene showed partial geographic structure. Where one of the sub-clades has only Norwegian sequences together with a Danish sequence. This may indicate that there is some structure relevant to Scandinavia. At the same time the other sub-clade indicates that there are occasional introductions from other regions of Europe.

Phylogenetic trees from complete and merged 2 gene sequences showed partial phylogeographic structure for some of sequences from western Norway. Where sequences from Vest-Agder (S10, and S5), Hordaland (H3), Møre og Romsdal (MR4) formed one distinct clade compared to other localities. Phylogenetic tree of control region showed longer branch length for these three sequences. Complete genome consists of lot of missing base pairs in the control region for these three *I. ricinus* sequences. This may indicate the existence of partial structure for Norwegian sequences from this part of country. While Most of human TBE cases have been reported from different localities of southern part of Norway (MSIS) (Skarpaas et al., 2006).

Our results confirm the findings of Casati *et al*., (2008) and Noureddine *et al.,* (2011). The study of Casati *et al.*, (2008) reported lack of phylogeographic structure based on

mitochondrial genes (Cyt B, 12SrDNA, COI, COII and highly polymorphic control Region) among 26 ticks collected from six European countries. The study showed that the number of substitution was low in all five mtDNA markers within the tested *I. ricinus* sequences, suggesting that there was no correlation between identified haplotypes and their geographic origin (Casati et al., 2008). Noureddin et al. (2011) investigated genetic differences between *I. ricinus* in Eurasia and North Africa based on the analysis of six mtDNA genes and four nuclear genes. They reported the absence of genetic structure among *I. ricinus* ticks sampled from Europe, this contrasted with samples from North-Africa that formed a divergent clade (Noureddine, Chauvin, & Plantard, 2011).

Other studies based on allozyme data (Delaye, Béati, Aeschlimann, Renaud, & De Meeûs, 1997) and microsatellite markers (De Meeûs et al., 2002) have given similar results. Delay et al (1997) concluded a less reliable population genetics due to low variability of allozymes in *I. ricinus* collected from the Northwest Switzerland. De Meeus et al found no genetic differentiation within the *I. ricinus* belonging to different parts of the Switzerland separated by the Alps (De Meeûs et al., 2002).

The homogeneity of the *I. ricinus* ticks can be explained by the two factors. The first one is host dependent movement of the ticks. Movement of ticks over longer distance depends on the host because ticks have limited mobility when they are not attached to the host. Therefore, the rates and patterns of gene flow are determined by host movements during infestation(Araya-Anchetta, Busch, Scoles, & Wagner, 2015; Carroll & Schmidtmann, 1996). Hosts with the greatest potential for dispersing ticks include large mammals and birds (highly mobile often over large distances) may promote homogeneity among closely related population (Casati et al., 2008). Ticks associated with a mobile host might not actually disperse over longer distances if feeding times are short. However, *I. ricinus* is a hard tick and hard ticks (Ixodidae) feed slowly over a period of days or weeks (Anderson & Magnarelli, 2008; Barker & Murrell, 2003; Sonenshine, 1993). Furthermore, human transportation of hosts, particularly cattle and dogs can be a significant factor that affects gene flow at a regional scale or even leads to ticks dispersing between continents (Araya-Anchetta et al., 2015).

Second factor is the recent rapid expansion of *I. ricinus* ticks in Europe. Expansion of population increases the retention of new mutations (Ćakić et al., 2014; Watterson, 1984) and produces number of haplotypes with one or few mutations (Ćakić et al., 2014; Slatkin

& Hudson, 1991). On the base of the low number of substitutions within its members, Xu et al., (2003) hypothesised that *I. ricinus* complex is the most recently evolved group of ticks in the genus *Ixodes.*

Moreover, lack of genetic difference between ticks can be explained by ecological factors. Habitat conditions of ticks remain very strict and are dependent on environmental conditions such as relative humidity, temperature and vegetation. Indeed, despite their discontinuous geographic distribution thus *I. ricinus* ticks live in a stringent but similar environment, even though they can colonies more than 300 different avian and mammalian wild species (Casati et al., 2008; Paulauskas et al., 2016).

Røed et al., (2008) study (Røed et al., 2016) reported distinct population from Great Britain, continental Europe and Western Norway based on the two (CR and Cyt B) mtDNA genes. Our study showed partial geographic structure based on the complete mtDNA. Partial or absence of geographical structure in current study may be due to differences of use of whole mtDNA as genetic marker instead of two individual genes (Røed et al., 2016).

Our study provides the first use of complete mtDNA of *I. ricinus* in Norway. This work is first attempt to investigate phylogeographic structure based on complete mtDNA genome and to determine the phylogenetic relationship among the studied Norwegian *I. ricinus* in relation to Europe. Study confirmed low or lack of phylogeographic structure compared to the European *I. ricinus* sequences. Phylogenetic study based on complete mtDNA genome can resolve better phylogenetic relationship compared to individual genes because it provide information from whole genome instead of one part of genome. There is need for further research involving complete mtDNA as molecular marker for specific identification and characterization of ticks. The information about the genetic variability of disease vector is potentially important to understand the epidemiology and evolutionary relationship of disease and vector.

5. Conclusion

This study reported prevalence of TBEV collected from southern Norway and population genetic structure of its vector *I. ricinus*. We found a prevalence of 0.41% and 2.17% among adult and nymphs ticks from Vanse in Vest-Agder County and prevalence of 0.69% and 1.4% among nymphs and adults from Bjønnes in Telemark County. TBEV is most likely endemic in the southern part of Norway, especially in Vest-agder and Telemark counties. Our data indicate confirmed TBEV in these sites and update information for prevalence TBEV in endemic sites that can be useful for diagnostics and vaccine recommendations. Additional studies are needed to elucidate the distribution of TBEV in Norway.

This is first study from Norway that reported partial or lack of phylogeographic structure of TBEV vector with the use of complete genome of mtDNA. There is no distinct geographical structure for Norwegian *I. ricinus* compared to European *I. ricinus*. Geographically separated areas, like Great Britain and Norway, which are separated by seas, mountain ranges, fjords may not act as geographical barriers and ticks can be dispersed effectively over long distance via mammals and birds. This kind of information needed further studies that might be a useful approach to increase our understanding of the epidemiology of the pathogens.

References/bibliography

Anderson, J. F., & Magnarelli, L. A. (2008). Biology of Ticks. *Infectious Disease Clinics of North America, 22*(2), 195-215. doi:http://dx.doi.org/10.1016/j.idc.2007.12.006

Andreassen, A., Jore, S., Cuber, P., Dudman, S., Tengs, T., Isaksen, K., . . . Vainio, K. (2012). Prevalence of tick borne encephalitis virus in tick nymphs in relation to climatic factors on the southern coast of Norway. *Parasites & Vectors, 5*(1), 177. doi:10.1186/1756-3305-5-177

Araya-Anchetta, A., Busch, J. D., Scoles, G. A., & Wagner, D. M. (2015). Thirty years of tick population genetics: A comprehensive review. *Infection, Genetics and Evolution, 29*, 164-179.

Asghar, N., Lindblom, P., Melik, W., Lindqvist, R., Haglund, M., Forsberg, P., . . . Johansson, M. (2014). Tick-borne encephalitis virus sequenced directly from questing and blood-feeding ticks reveals quasispecies variance. *PLoS One, 9*(7), e103264.

Barker, S. C., & Murrell, A. (2003). Phylogeny, evolution and historical zoogeography of ticks: a review of recent progress. In F. Jongejan & W. R. Kaufman (Eds.), *Ticks and Tick-Borne Pathogens: Proceedings of the 4th International Conference on Ticks and Tick-Borne Pathogens The Banff Centre Banff, Alberta, Canada 21–26 July 2002* (pp. 55-68). Dordrecht: Springer Netherlands.

Blaškovič, D. (1967). The public health importance of tick-borne encephalitis in Europe. *Bulletin of the World Health Organization, 36*(Suppl 1), 5-13.

Burri, C., Bastic, V., Maeder, G., Patalas, E., & Gern, L. (2011). Microclimate and the Zoonotic Cycle of Tick-Borne Encephalitis Virus in Switzerland. *Journal of Medical Entomology, 48*(3), 615-627. doi:10.1603/ME10180

Ćakić, S., Mojsilović, M., Mihaljica, D., Milutinović, M., Petrović, A., & Tomanović, S. (2014). Molecular characterization of COI gene of Ixodes ricinus (Linnaeus, 1758) from Serbia. *Archives of Biological Sciences, 66*(2), 683-690.

Carroll, J., & Schmidtmann, E. (1996). Dispersal of blacklegged tick (Acari: Ixodidae) nymphs and adults at the woods–pasture interface. *Journal of Medical Entomology, 33*(4), 554-558.

Casati, S., Bernasconi, M. V., Gern, L., & Piffaretti, J. C. (2008). Assessment of intraspecific mtDNA variability of European Ixodes ricinus sensu stricto (Acari: Ixodidae). *Infection, Genetics and Evolution, 8*(2), 152-158. doi:http://dx.doi.org/10.1016/j.meegid.2007.11.007

Chunikhin, S., Stefuktina, L., Korolev, M., Reshetnikov, I., & Khozinskaia, G. (1982). Sexual transmission of the tick-borne encephalitis virus in ixodid ticks (Ixodidae). *Parazitologiia, 17*(3), 214-217.

Dai, J., Wang, P., Adusumilli, S., Booth, C. J., Narasimhan, S., Anguita, J., & Fikrig, E. (2009). Antibodies against a tick protein, Salp15, protect mice from the Lyme disease agent. *Cell host & microbe, 6*(5), 482-492. doi:10.1016/j.chom.2009.10.006

Darriba, D., Taboada, G. L., Doallo, R., & Posada, D. (2012). jModelTest 2: more models, new heuristics and high-performance computing. *Nature methods, 9*(8), 772-772. doi:10.1038/nmeth.2109

De Meeûs, T., Béati, L., Delaye, C., Aeschlimann, A., Renaud, F., & Ross, K. (2002). Sex-biased genetic structure in the vector of Lyme disease, Ixodes ricinus. *Evolution, 56*(9), 1802-1807.

Delaye, C., Béati, L., Aeschlimann, A., Renaud, F., & De Meeûs, T. (1997). Population genetic structure of Ixodes ricinus in Switzerland from allozymic data: No evidence of divergence between nearby sites. *International journal for parasitology, 27*(7), 769-773. doi:http://dx.doi.org/10.1016/S0020-7519(97)00040-4

Dinnis, R. E., Seelig, F., Bormane, A., Donaghy, M., Vollmer, S. A., Feil, E. J., . . . Margos, G. (2014). Multilocus sequence typing using mitochondrial genes (mtMLST) reveals geographic population structure of Ixodes ricinus ticks. *Ticks and Tick-borne Diseases, 5*(2), 152-160. doi:http://doi.org/10.1016/j.ttbdis.2013.10.001

Dowell, K. (2008). Molecular Phylogenetics: An introduction to computational methods and tools for analyzing evolutionary relationships. *Math, 500*, 18.

Estrada-Peña, A., Ayllón, N., & de la Fuente, J. (2012). Impact of Climate Trends on Tick-Borne Pathogen Transmission. *Frontiers in Physiology, 3*, 64. doi:10.3389/fphys.2012.00064

Estrada-Peña, A., Bouattour, A., Camicas, J., & Walker, A. (2004). *Ticks of domestic animals in the Mediterranean region* (Vol. 131).

Gillet, L., Schroeder, H., Mast, J., Thirion, M., Renauld, J.-C., Dewals, B., & Vanderplasschen, A. (2009). Anchoring tick salivary anti-complement proteins IRAC I and IRAC II to membrane increases their immunogenicity. *Veterinary Research, 40*(5), 51. doi:10.1051/vetres/2009034

Gouy, M., Guindon, S., & Gascuel, O. (2010). SeaView Version 4: A Multiplatform Graphical User Interface for Sequence Alignment and Phylogenetic Tree Building. *Mol Biol Evol, 27*(2), 221-224. doi:10.1093/molbev/msp259

Gritsun, T. S., Lashkevich, V. A., & Gould, E. A. (2003). Tick-borne encephalitis. *Antiviral Research, 57*(1), 129-146. doi:http://dx.doi.org/10.1016/S0166-3542(02)00206-1

Gu, X.-B., Liu, G.-H., Song, H.-Q., Liu, T.-Y., Yang, G.-Y., & Zhu, X.-Q. (2014). The complete mitochondrial genome of the scab mite Psoroptes cuniculi (Arthropoda: Arachnida) provides insights into Acari phylogeny. *Parasites & Vectors, 7*(1), 340. doi:10.1186/1756-3305-7-340

Guindon, S., & Gascuel, O. (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol, 52*. doi:10.1080/10635150390235520

Huelsenbeck, J. P., & Ronquist, F. (2001). MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics, 17*(8), 754-755.

Jaenson, T. G. T., Hjertqvist, M., Bergström, T., & Lundkvist, Å. (2012). Why is tick-borne encephalitis increasing? A review of the key factors causing the increasing incidence of human TBE in Sweden(a). *Parasites & Vectors, 5*, 184-184. doi:10.1186/1756-3305-5-184

Jenkins, A., Hvidsten, D., Matussek, A., Lindgren, P.-E., Stuen, S., & Kristiansen, B.-E. (2012). Borrelia burgdorferi sensu lato in Ixodes ricinus ticks from Norway: evaluation of a PCR test targeting the chromosomal flaB gene. *Experimental and Applied Acarology, 58*(4), 431-439.

Jongejan, F., & Uilenberg, G. (2004). The global importance of ticks. *Parasitology, 129*(S1), S3-S14.

Jore, S., Viljugrein, H., Hofshagen, M., Brun-Hansen, H., Kristoffersen, A. B., Nygård, K., . . . Ytrehus, B. (2011). Multi-source analysis reveals latitudinal and altitudinal shifts in range of Ixodes ricinus at its northern distribution limit. *Parasites & Vectors, 4*(1), 84. doi:10.1186/1756-3305-4-84

Kaiser, R. (2008). Tick-borne encephalitis. *Infectious disease clinics of North America, 22*(3), 561-575.

Kanduma, E. G., Mwacharo, J. M., Githaka, N. W., Kinyanjui, P. W., Njuguna, J. N., Kamau, L. M., . . . Bishop, R. P. (2016). Analyses of mitochondrial genes reveal two sympatric but genetically divergent lineages of Rhipicephalus appendiculatus in Kenya. *Parasites & Vectors, 9*, 353. doi:10.1186/s13071-016-1631-1

Katoh, K., & Standley, D. M. (2013). MAFFT Multiple Sequence Alignment Software Version 7: Improvements in Performance and Usability. *Mol Biol Evol, 30*(4), 772-780. doi:10.1093/molbev/mst010

Kjelland, V., Stuen, S., Skarpaas, T., & Slettan, A. (2010). Borrelia burgdorferi sensu lato in Ixodes ricinus ticks collected from migratory birds in Southern Norway. *Acta Veterinaria Scandinavica, 52*(1), 59.

Kunze, U. (2016). The International Scientific Working Group on Tick-Borne Encephalitis (ISW TBE): Review of 17 years of activity and commitment. *Ticks and Tick-borne Diseases, 7*(3), 399-404. doi:https://doi.org/10.1016/j.ttbdis.2015.12.018

Labuda, M., & Nuttall, P. A. (2005). Tick-borne viruses. *Parasitology, 129*(S1), S221-S245. doi:10.1017/S0031182004005220

Langdon, W. B. (2015). Performance of genetic programming optimised Bowtie2 on genome comparison and analytic testing (GCAT) benchmarks. *BioData mining, 8*(1), 1.

Larsen, A. L., Kanestrøm, A., Bjørland, M., Andreassen, Å., Soleng, A., Vene, S., & Dudman, S. G. (2014). Detection of specific IgG antibodies in blood donors and tick-borne encephalitis virus in ticks within a non-endemic area in southeast Norway. *Scandinavian Journal of Infectious Diseases, 46*(3), 181-184.

Larsson, A. (2014). AliView: a fast and lightweight alignment viewer and editor for large datasets. *Bioinformatics, 30*(22), 3276-3278. doi:10.1093/bioinformatics/btu531

Lindenbach, B. D., & Rice, C. (2001). Flaviviridae: the viruses and their replication. *Fields virology, 1*, 991-1041.

Lindquist, L., & Vapalahti, O. Tick-borne encephalitis. *The Lancet, 371*(9627), 1861-1871. doi:https://doi.org/10.1016/S0140-6736(08)60800-4

Lundkvist, Å., Vene, S., Golovljova, I., Mavtchoutko, V., Forsgren, M., Kalnina, V., & Plyusnin, A. (2001). Characterization of tick-borne encephalitis virus from latvia: Evidence for co-circulation of three distinct subtypes. *Journal of medical virology, 65*(4), 730-735.

McCoy, K. D. (2008). The population genetic structure of vectors and our understanding of disease epidemiology. *Parasite, 15*(3), 444-448.

Medlock, J. M., Hansford, K. M., Bormane, A., Derdakova, M., Estrada-Peña, A., George, J.-C., . . . Van Bortel, W. (2013). Driving forces for changes in geographical distribution of Ixodes ricinus ticks in Europe. *Parasites & Vectors, 6*(1), 1. doi:10.1186/1756-3305-6-1

Mehl, R. (1983). The distribution and host relations of Norwegian ticks (Acari, Ixodides). *Fauna norvegica Series B, 30*.

Milne, A. (1943). The comparison of sheep-tick populations (Ixodes ricinus L.). *Annals of Applied Biology, 30*(3), 240-250. doi:10.1111/j.1744-7348.1943.tb06195.x

Noureddine, R., Chauvin, A., & Plantard, O. (2011). Lack of genetic structure among Eurasian populations of the tick Ixodesricinus contrasts with marked divergence from north-African populations. *International journal for parasitology, 41*(2), 183-192.

Nygård, K., Brantsæter, A. B., & Mehl, R. (2005). Disseminated and chronic Lyme borreliosis in Norway, 1995-2004. *Euro surveillance: bulletin Europeen sur les maladies transmissibles= European communicable disease bulletin, 10*(10), 235-238.

Parola, P., & Raoult, D. (2001). Ticks and Tickborne Bacterial Diseases in Humans: An Emerging Infectious Threat. *Clinical Infectious Diseases, 32*(6), 897-928. doi:10.1086/319347

Paulauskas, A., Galdikaitė-Brazienė, E., Radzijevskaja, J., Aleksandravičienė, A., & Galdikas, M. (2016). Genetic diversity of Ixodes ricinus (Ixodida: Ixodidae) ticks in sympatric and allopatric zones in Baltic countries. *Journal of Vector Ecology, 41*(2), 244-253. doi:10.1111/jvec.12219

Paulauskas, A., Radzijevskaja, J., Rosef, O., Turcinaviciene, J., Ambrasiene, D., & Makareviciute, D. (2006). Genetic variation of ticks (Ixodes ricinus L.) in the Lithuanian and Norwegian populations. *Experimental & Applied Acarology, 40*(3), 259-270. doi:10.1007/s10493-006-9035-0

Paulsen, K. M., Pedersen, B. N., Soleng, A., Okbaldet, Y. B., Pettersson, J. H. O., Dudman, S. G., . . . Andreassen, Å. (2015). Prevalence of tick-borne encephalitis virus in Ixodes ricinus ticks from three islands in north-western Norway. *APMIS, 123*(9), 759-764. doi:10.1111/apm.12412

Pettersson, J. H., Golovljova, I., Vene, S., & Jaenson, T. G. (2014). Prevalence of tick-borne encephalitis virus in Ixodes ricinus ticks in northern Europe with particular reference to Southern Sweden. *Parasit Vectors, 7*, 102. doi:10.1186/1756-3305-7-102

Philipp, M. T., Lobet, Y., Bohm, R. P., Roberts, E. D., Dennis, V. A., Gu, Y., . . . Xu, K. (1997). The outer surface protein A (OspA) vaccine against Lyme disease: efficacy in the rhesus monkey. *Vaccine, 15*(17), 1872-1887. doi:http://dx.doi.org/10.1016/S0264-410X(97)00133-3

Polanski, A., & Kimmel, M. (2007). *Bioinformatics*.

Python for Bioinformatics. (2009). Retrieved from http://telliott99.blogspot.no/2009/10/hidden-markov-models-1.html

Randolph, S. E., & Sumilo, D. (2007). *In Emerging Pests and Vector-borne Disease in Europe*. Wageningen: BGJ Knols.

Røed, K. H., Kvie, K. S., Hasle, G., Gilbert, L., & Leinaas, H. P. (2016). Phylogenetic Lineages and Postglacial Dispersal Dynamics Characterize the Genetic Structure of the Tick, Ixodes ricinus, in Northwest Europe. *PLoS ONE, 11*(12), e0167450.

Salemi, M., Lemey, P., & Vandamme, A.-M. (2009). *The phylogenetic handbook: a practical approach to phylogenetic analysis and hypothesis testing*: Cambridge University Press.

Shao, R., & Barker, S. (2007). Mitochondrial genomes of parasitic arthropods: implications for studies of population genetics and evolution. *Parasitology, 134*(02), 153-167.

Skarpaas, T., Golovljova, I., Vene, S., Ljøstad, U., Sjursen, H., Plyusnin, A., & Lundkvist, A. (2006). Tickborne encephalitis virus, Norway and Denmark. *Emerging Infect Dis, 12*. doi:10.3201/eid1207.051567

Slatkin, M., & Hudson, R. R. (1991). Pairwise comparisons of mitochondrial DNA sequences in stable and exponentially growing populations. *Genetics, 129*(2), 555-562.

Sonenshine, D. E. (1991). Biology of ticks, vol. 1.

Sonenshine, D. E. (1993). Biology of ticks, vol. 2.

Stafford, K. C., Cartter, M. L., Magnarelli, L. A., Ertel, S.-H., & Mshar, P. A. (1998). Temporal Correlations between Tick Abundance and Prevalence of Ticks Infected with Borrelia burgdorferi and Increasing Incidence of Lyme Disease. *Journal of Clinical Microbiology, 36*(5), 1240-1244.

Stanek, G. (2008). Pandora's Box: pathogens in Ixodes ricinus ticks in Central Europe. *Wiener klinische Wochenschrift, 121*(21-22), 673-683.

Süss, J. (2011). Tick-borne encephalitis 2010: Epidemiology, risk areas, and virus strains in Europe and Asia—An overview. *Ticks and Tick-borne Diseases, 2*(1), 2-15. doi:https://doi.org/10.1016/j.ttbdis.2010.10.007

Süss, J., Schrader, C., Abel, U., Voigt, W. P., & Schosser, R. (1999). Annual and seasonal variation of tick-borne encephalitis virus (TBEV) prevalence in ticks in selected hot spot areas in Germany using a nRT-PCR: results from 1997 and 1998. *Zentralbl Bakteriol, 289*. doi:10.1016/s0934-8840(99)80010-3

Tambs-Lyche, H. (1943). Ixodes ricinus og piroplasmosen i Norge (Meddelelse fra Bergens Museums zoologiske avdeling). *Norsk veterinærtidsskrift, 55*.

Ticks. (2017). Retrieved from https://en.wikipedia.org/wiki/Tick

Tonteri, E., Kipar, A., Voutilainen, L., Vene, S., Vaheri, A., Vapalahti, O., & Lundkvist, Å. (2013). The three subtypes of tick-borne encephalitis virus induce encephalitis in a natural host, the bank vole (Myodes glareolus). *PLoS One, 8*(12), e81214.

Valarcher, J., Hagglund, S., Juremalm, M., Blomqvist, G., Renstrom, L., Zohari, S., . . . Chirico, J. (2015). Tick-borne encephalitis. *Rev Sci Tech OIE, 34*, 453-466.

Virology tidbits. (2014). Retrieved from http://virologytidbits.blogspot.no/2014/07/japanese-encephalitis-virus-jev-er.html

Watterson, G. A. (1984). Allele frequencies after a bottleneck. *Theoretical Population Biology, 26*(3), 387-407. doi:http://dx.doi.org/10.1016/0040-5809(84)90042-X

Wu, X.-B., Na, R.-H., Wei, S.-S., Zhu, J.-S., & Peng, H.-J. (2013). Distribution of tick-borne diseases in China. *Parasites & Vectors, 6*(1), 119. doi:10.1186/1756-3305-6-119

Xu, G., Fang, Q. Q., Keirans, J. E., & Durden, L. A. (2003). Molecular phylogenetic analyses indicate that the Ixodes ricinus complex is a paraphyletic group. *Journal of Parasitology, 89*(3), 452-457.

Yang, Z., & Rannala, B. (2012). Molecular phylogenetics: principles and practice. *Nature Reviews Genetics, 13*(5), 303-314.

Appendix

Appendix 1: Total number of sequences used in the phylogenetic trees

Table a: Total number of complete mitochondrial sequences used for the phylogenetic analysis of I. ricinus ticks.

Sample name	Country	Accession no. of complete mtDNA	Gender
S10	Norway	This study	Adult
H3	Norway	This study	Pool of ten nymphs
MR4	Norway	This study	Adult
A2	Norway	This study	Pool of ten nymphs
S5	Norway	This study	Pool of ten nymphs
B1	Norway	This study	Adult
IR_59	Italy	KF197114.1	Female (Adult)
IR_67	NA	KF197115.1	NA
IR_25	Italy	KF197116.1	Female (Adult)
IR_8	Italy	KF197117.1	Female (Adult)
IR_62	Italy	KF197119.1	Female (Adult)
IR_63	Italy	KF197121.1	Female (Adult)
IR_64	Italy	KF197122.1	Female (Adult
IR_20_8	Italy	KF197123.1	Female (Adult)
IR_65	Italy	KF197124.1	Female (Adult)
IR_28	Italy	KF197125.1	Female (Adult)
IR_8_6	Italy	KF197126.1	Male (Adult)
IR_7	Italy	KF197127.1	Female (Adult)
IR_26	Italy	KF197129.1	Female (Adult)
IR_66	Italy	KF197131.1	Female (Adult)
IR_3	Italy	KF197132.1	Female (Adult)
IR_27	Italy	KF197134.1	Female (Adult)
IR_13_4	Italy	KF197135.1	Male (Adult)
IR_5	Italy	KF197130.1	Female (Adult)
IR_68	Solvakia	KF197118.1	Female (Adult)
IR_60	Solvakia	KF197120.1	NA (Adult)
IR_69	Slovakia	KF197128.1	Female (Adult)

IR_61	Slovakia	KF197133.1	Female (Adult)
IR_11_6	Slovakia	KF197136.1	Female (Adult)
NA	Italy	NC_018369.2	NA
NA	Italy	JN248424.2	Female (Adult)

Table b: Sampling name, country name and GeneBank accession number for Cyt B, CR, 12S, COII and COI gene of mtDNA in Ixodus ricinus.

Species name	Country	Genbank accession numbers					
		Cyt B	CR	12s	COII	COI	References
98	Switzerland	AY945395	AY945473	AY945499	AY945421	AY945447	Casati *et al.,* 2008
118	Switzerland	AY945370	AY945448	AY945474	AY945396	AY945422	Casati *et al.,* 2008
131	Sweden	AY945375	AY945453	AY945479	AY945401	AY945427	Casati *et al.,* 2008
142	Switzerland	AY945376	AY945454	AY945480	AY945402	AY945428	Casati *et al.,* 2008
143	Switzerland	AY945377	AY945455	AY945481	AY945403	AY945429	Casati *et al.,* 2008
174	Switzerland	AY945378	AY945456	AY945482	AY945404	AY945430	Casati *et al.,* 2008
181	Switzerland	AY945379	AY945457	AY945483	AY945405	AY945431	Casati *et al.,* 2008
195	Switzerland	AY945380	AY945458	AY945484	AY945406	AY945432	Casati *et al.,* 2008
277	Switzerland	AY945381	AY945459	AY945485	AY945407	AY945433	Casati *et al.,* 2008
280	Switzerland	AY945382	AY945460	AY945486	AY945408	AY945434	Casati *et al.,* 2008
636	Finland	AY945383	AY945461	AY945487	AY945409	AY945435	Casati *et al.,* 2008
637	Finland	AY945384	AY945462	AY945488	AY945410	AY945436	Casati *et al.,* 2008

638	Finland	AY945385	AY945463	AY945489	AY945411	AY945437	Casati *et al.,* 2008
640	Denmark	AY945386	AY945464	AY945490	AY945412	AY945438	Casati *et al.,* 2008
642	Sweden	AY945387	AY945465	AY945491	AY945413	AY945439	Casati *et al.,* 2008
644	Denmark	AY945388	AY945466	AY945492	AY945414	AY945440	Casati *et al.,* 2008
645	Denmark	AY945389	AY945467	AY945493	AY945415	AY945441	Casati *et al.,* 2008
646	Finland	AY945390	AY945468	AY945494	AY945416	AY945442	Casati *et al.,* 2008
648	Finland	AY945391	AY945469	AY945495	AY945417	AY945443	Casati *et al.,* 2008
683	Switzerland	AY945392	AY945470	AY945496	AY945418	AY945444	Casati *et al.,* 2008
862	Switzerland	AY945393	AY945471	AY945497	AY945419	AY945445	Casati *et al.,* 2008
966	Switzerland	AY945394	AY945472	AY945498	AY945420	AY945446	Casati *et al.,* 2008
1239	Austria	AY945371	AY945449	AY945475	AY945397	AY945423	Casati *et al.,* 2008
1240	Austria	AY945372	AY945450	AY945476	AY945398	AY945424	Casati *et al.,* 2008
1244	Italy	AY945373	AY945451	AY945477	AY945399	AY945425	Casati *et al.,* 2008
1245	Italy	AY945374	AY945452	AY945478	AY945400	AY945426	Casati *et al.,* 2008

Table c: Sampling name, country name and GeneBank accession number for CR, and Cyt B, gene of mtDNA in Ixodus ricinus.

Sample name	Country	Gene bank accession number		
		Control 12S	Cyt B	References
HIT348	Norway	KY025862	KY067031	Røed *et al.,* 2016
HIT349	Norway		KY067032	Røed *et al.,* 2016
HIT350	Norway	KY025863		Røed *et al.,* 2016
HIT351	Norway	KY025864	KY067033	Røed *et al.,* 2016
HIT352	Norway	KY025865		Røed *et al.,* 2016
HIT353	Norway	KY025866	KY067034	Røed *et al.,* 2016
HIT354	Norway	KY025867	KY067035	Røed *et al.,* 2016
HIT355	Norway		KY067036	Røed *et al.,* 2016
HIT356	Norway	KY025868	KY067037	Røed *et al.,* 2016
HIT357	Norway	KY025869		Røed *et al.,* 2016
HIT359	Norway	KY025870	KY067038	Røed *et al.,* 2016
HIT360	Norway	KY025871	KY067039	Røed *et al.,* 2016
HIT361	Norway	KY025872	KY067040	Røed *et al.,* 2016
HIT362	Norway	KY025873	KY067041	Røed *et al.,* 2016
HIT363	Norway	KY025874	KY067042	Røed *et al.,* 2016
HIT364	Norway	KY025875	KY067043	Røed *et al.,* 2016
HIT365	Norway	KY025876	KY067044	Røed *et al.,* 2016
HIT366	Norway	KY025877	KY067045	Røed *et al.,* 2016
HIT367	Norway	KY025878	KY067046	Røed *et al.,* 2016
HIT368	Norway	KY025879	KY067047	Røed *et al.,* 2016
HIT369	Norway	KY025880	KY067048	Røed *et al.,* 2016
HIT370	Norway	KY025881	KY067049	Røed *et al.,* 2016
HAR324	Norway		KY067010	Røed *et al.,* 2016
HAR325	Norway	KY025843	KY067011	Røed *et al.,* 2016
HAR326	Norway	KY025844	KY067012	Røed *et al.,* 2016
HAR327	Norway	KY025845	KY067013	Røed *et al.,* 2016
HAR328	Norway		KY067014	Røed *et al.,* 2016
HAR329	Norway	KY025846	KY067015	Røed *et al.,* 2016
HAR331	Norway		KY067016	Røed *et al.,* 2016
HAR332	Norway	KY025847	KY067017	Røed *et al.,* 2016

HAR333	Norway	KY025848	KY067018	Røed *et al.,* 2016
HAR334	Norway	KY025849	KY067019	Røed *et al.,* 2016
HAR335	Norway	KY025850	KY067020	Røed *et al.,* 2016
HAR336	Norway	KY025851	KY067021	Røed *et al.,* 2016
HAR337	Norway	KY025852	KY067022	Røed *et al.,* 2016
HAR338	Norway	KY025853	KY067023	Røed *et al.,* 2016
HAR339	Norway	KY025854	KY067024	Røed *et al.,* 2016
HAR340	Norway	KY025855	KY067025	Røed *et al.,* 2016
HAR341	Norway	KY025856	KY067026	Røed *et al.,* 2016
HAR342	Norway	KY025857	KY067027	Røed *et al.,* 2016
HAR343	Norway	KY025858	KY067028	Røed *et al.,* 2016
HAR344	Norway	KY025859		Røed *et al.,* 2016
HAR345	Norway	KY025860		Røed *et al.,* 2016
HAR346	Norway		KY067029	Røed *et al.,* 2016
GAU300	Norway	KY025822	KY066987	Røed *et al.,* 2016
GAU301	Norway	KY025823	KY066988	Røed *et al.,* 2016
GAU302	Norway	KY025824	KY066989	Røed *et al.,* 2016
GAU303	Norway	KY025825	KY066990	Røed *et al.,* 2016
GAU304	Norway	KY025826	KY066991	Røed *et al.,* 2016
GAU305	Norway	KY025827	KY066992	Røed *et al.,* 2016
GAU306	Norway	KY025828	KY066993	Røed *et al.,* 2016
GAU307	Norway	KY025829	KY066994	Røed *et al.,* 2016
GAU308	Norway	KY025830	KY066995	Røed *et al.,* 2016
GAU309	Norway	KY025831	KY066996	Røed *et al.,* 2016
GAU310	Norway	KY025832	KY066997	Røed *et al.,* 2016
GAU311	Norway	KY025833	KY066998	Røed *et al.,* 2016
GAU312	Norway	KY025834	KY066999	Røed *et al.,* 2016
GAU313	Norway	KY025835	KY067000	Røed *et al.,* 2016
GAU314	Norway	KY025836	KY067001	Røed *et al.,* 2016
GAU315	Norway	KY025837	KY067002	Røed *et al.,* 2016
GAU316	Norway	KY025838	KY067003	Røed *et al.,* 2016
GAU317	Norway	KY025839	KY067004	Røed *et al.,* 2016
GAU318	Norway		KY067005	Røed *et al.,* 2016
GAU319	Norway	KY025840	KY067006	Røed *et al.,* 2016

GAU320	Norway		KY067007	Røed *et al.,* 2016
GAU321	Norway	KY025841	KY067008	Røed *et al.,* 2016
GAU322	Norway	KY025842	KY067009	Røed *et al.,* 2016
Ask101	Norway	KY025630	KY066777	Røed *et al.,* 2016
Ask102	Norway	KY025631	KY066778	Røed *et al.,* 2016
Ask103	Norway	KY025632	KY066779	Røed *et al.,* 2016
Ask104	Norway	KY025633	KY066780	Røed *et al.,* 2016
Ask105	Norway	KY025634	KY066781	Røed *et al.,* 2016
Ask106	Norway	KY025635	KY066782	Røed *et al.,* 2016
Ask107	Norway	KY025636	KY066783	Røed *et al.,* 2016
Ask108	Norway	KY025637	KY066784	Røed *et al.,* 2016
Ask109	Norway	KY025638	KY066785	Røed *et al.,* 2016
Ask110	Norway	KY025639	KY066786	Røed *et al.,* 2016
ASK111	Norway	KY025640		Røed *et al.,* 2016
Ask112	Norway	KY025641	KY066787	Røed *et al.,* 2016
ASK114	Norway	KY025642	KY066788	Røed *et al.,* 2016
Ask115	Norway	KY025643	KY066789	Røed *et al.,* 2016
Ask116	Norway	KY025644	KY066790	Røed *et al.,* 2016
Ask117	Norway	KY025646	KY066791	Røed *et al.,* 2016
Ask118	Norway	KY025647	KY066792	Røed *et al.,* 2016
Ask119	Norway	KY025648	KY066793	Røed *et al.,* 2016
Ask120	Norway	KY025645	KY066794	Røed *et al.,* 2016
Ask121	Norway	KY025649	KY066795	Røed *et al.,* 2016
Ask122	Norway	KY025650	KY066796	Røed *et al.,* 2016
Ask123	Norway	KY025651	KY066797	Røed *et al.,* 2016
Ask124	Norway	KY025652	KY066798	Røed *et al.,* 2016
Ask125	Norway	KY025653	KY066799	Røed *et al.,* 2016
LIS182	Norway		KY066887	Røed *et al.,* 2016
LIS183	Norway	KY025732	KY066888	Røed *et al.,* 2016
LIS185	Norway	KY025733	KY066889	Røed *et al.,* 2016
LIS186	Norway	KY025734		Røed *et al.,* 2016
LIS187	Norway	KY025735	KY066890	Røed *et al.,* 2016
LIS188	Norway	KY025736	KY066891	Røed *et al.,* 2016
LIS189	Norway	KY025737		Røed *et al.,* 2016

LIS190	Norway	KY025738	KY066892	Røed *et al.,* 2016
LIS191	Norway	KY025739	KY066893	Røed *et al.,* 2016
LIS192	Norway	KY025740	KY066894	Røed *et al.,* 2016
LIS193	Norway	KY025741	KY066895	Røed *et al.,* 2016
LIS194	Norway	KY025742	KY066896	Røed *et al.,* 2016
LIS195	Norway	KY025744	KY066897	Røed *et al.,* 2016
LIS196	Norway	KY025745	KY066898	Røed *et al.,* 2016
LIS197	Norway	KY025743	KY066899	Røed *et al.,* 2016
LIS198	Norway	KY025746	KY066900	Røed *et al.,* 2016
LIS199	Norway	KY025747	KY066901	Røed *et al.,* 2016
LIS200	Norway	KY025749	KY066902	Røed *et al.,* 2016
LIS201	Norway	KY025748	KY066903	Røed *et al.,* 2016
LIS202	Norway		KY066904	Røed *et al.,* 2016
JOM202	Norway	KY025595	KY066744	Røed *et al.,* 2016
JOM203	Norway	KY025597	KY066746	Røed *et al.,* 2016
JON201	Norway		KY066742	Røed *et al.,* 2016
JON202	Norway	KY025596	KY066743	Røed *et al.,* 2016
JON203	Norway	KY025598	KY066745	Røed *et al.,* 2016
JON204	Norway	KY025599	KY066747	Røed *et al.,* 2016
JON205	Norway	KY025600	KY066748	Røed *et al.,* 2016
JON207	Norway	KY025601	KY066749	Røed *et al.,* 2016
JON208	Norway		KY066750	Røed *et al.,* 2016
JON209	Norway	KY025602	KY066751	Røed *et al.,* 2016
JON210	Norway	KY025603	KY066752	Røed *et al.,* 2016
JON211	Norway	KY025604	KY066753	Røed *et al.,* 2016
JON212	Norway	KY025605		Røed *et al.,* 2016
JON213	Norway	KY025606	KY066754	Røed *et al.,* 2016
JON214	Norway	KY025607	KY066755	Røed *et al.,* 2016
OS_OF1	Norway	KY025608		Røed *et al.,* 2016
OS_OF2	Norway	KY025609	KY066756	Røed *et al.,* 2016
OS_OF3	Norway	KY025610	KY066757	Røed *et al.,* 2016
OS_OF4	Norway	KY025611	KY066758	Røed *et al.,* 2016
OS_OF5	Norway	KY025612	KY066759	Røed *et al.,* 2016
OS_OF6	Norway	KY025613	KY066760	Røed *et al.,* 2016

OS_OF7	Norway	KY025614	KY066761	Røed *et al.,* 2016
OS_OF8	Norway	KY025615	KY066762	Røed *et al.,* 2016
OS_OF9	Norway	KY025616	KY066763	Røed *et al.,* 2016
OS_OM1	Norway	KY025620	KY066767	Røed *et al.,* 2016
OS_OM2	Norway	KY025621	KY066768	Røed *et al.,* 2016
OS_OM3	Norway	KY025622	KY066769	Røed *et al.,* 2016
OS_OM4	Norway		KY066770	Røed *et al.,* 2016
OS_OM5	Norway	KY025623	KY066771	Røed *et al.,* 2016
OS_OM7	Norway	KY025624	KY066772	Røed *et al.,* 2016
OS_OM8	Norway	KY025625		Røed *et al.,* 2016
OS_OM9	Norway	KY025626	KY066773	Røed *et al.,* 2016
OSOF10	Norway	KY025617	KY066764	Røed *et al.,* 2016
OSOF11	Norway	KY025618	KY066765	Røed *et al.,* 2016
OSOF12	Norway	KY025619	KY066766	Røed *et al.,* 2016
OSOM10	Norway	KY025627	KY066774	Røed *et al.,* 2016
OSOM11	Norway	KY025628	KY066775	Røed *et al.,* 2016
OSOM12	Norway	KY025629	KY066776	Røed *et al.,* 2016
STO203	Norway	KY025750	KY066905	Røed *et al.,* 2016
STO204	Norway	KY025751	KY066906	Røed *et al.,* 2016
STO205	Norway	KY025752	KY066907	Røed *et al.,* 2016
STO206	Norway		KY066908	Røed *et al.,* 2016
STO207	Norway		KY066909	Røed *et al.,* 2016
STO208	Norway	KY025753	KY066910	Røed *et al.,* 2016
STO209	Norway	KY025754	KY066911	Røed *et al.,* 2016
STO210	Norway	KY025755	KY066912	Røed *et al.,* 2016
STO211	Norway		KY066913	Røed *et al.,* 2016
STO212	Norway	KY025756	KY066914	Røed *et al.,* 2016
STO213	Norway	KY025757	KY066915	Røed *et al.,* 2016
STO214	Norway	KY025758	KY066916	Røed *et al.,* 2016
STO215	Norway		KY066917	Røed *et al.,* 2016
STO216	Norway	KY025759	KY066918	Røed *et al.,* 2016
STO217	Norway	KY025761	KY066919	Røed *et al.,* 2016
STO218	Norway		KY066920	Røed *et al.,* 2016
STO219	Norway	KY025760	KY066921	Røed *et al.,* 2016

STO220	Norway	KY025762	KY066922	Røed *et al.,* 2016
STO221	Norway		KY066923	Røed *et al.,* 2016
STO222	Norway	KY025763	KY066924	Røed *et al.,* 2016
STO223	Norway		KY066925	Røed *et al.,* 2016
STO224	Norway		KY066926	Røed *et al.,* 2016
Sto225	Norway		KY066927	Røed *et al.,* 2016
STO226	Norway		KY066928	Røed *et al.,* 2016
STO227	Norway	KY025764	KY066929	Røed *et al.,* 2016
STO228	Norway	KY025765	KY066930	Røed *et al.,* 2016
STO229	Norway	KY025766	KY066931	Røed *et al.,* 2016
STO230	Norway	KY025767	KY066932	Røed *et al.,* 2016
STO231	Norway	KY025768		Røed *et al.,* 2016
STO233	Norway		KY066933	Røed *et al.,* 2016
STO234	Norway		KY066934	Røed *et al.,* 2016
STO235	Norway	KY025769	KY066935	Røed *et al.,* 2016
STO236	Norway	KY025770	KY066936	Røed *et al.,* 2016
STO237	Norway	KY025771		Røed *et al.,* 2016
STO239	Norway	KY025772	KY066937	Røed *et al.,* 2016
STO240	Norway		KY066938	Røed *et al.,* 2016
STO241	Norway	KY025773	KY066939	Røed *et al.,* 2016
STO243	Norway	KY025774	KY066940	Røed *et al.,* 2016
STO244	Norway	KY025775	KY066941	Røed *et al.,* 2016
STO245	Norway	KY025776	KY066942	Røed *et al.,* 2016
FIN126	Finland	KY025713	KY066865	Røed *et al.,* 2016
FIN127	Finland	KY025714	KY066866	Røed *et al.,* 2016
FIN128	Finland	KY025715	KY066867	Røed *et al.,* 2016
FIN129	Finland	KY025716	KY066868	Røed *et al.,* 2016
FIN130	Finland	KY025717	KY066869	Røed *et al.,* 2016
FIN131	Finland	KY025718	KY066870	Røed *et al.,* 2016
FIN132	Finland	KY025719	KY066871	Røed *et al.,* 2016
FIN133	Finland	KY025720		Røed *et al.,* 2016
FIN134	Finland	KY025721	KY066872	Røed *et al.,* 2016
FIN135	Finland	KY025722	KY066873	Røed *et al.,* 2016
FIN136	Finland		KY066874	Røed *et al.,* 2016

FIN137	Finland	KY025723	KY066875	Røed *et al.,* 2016
FIN138	Finland	KY025724	KY066876	Røed *et al.,* 2016
FIN139	Finland	KY025725	KY066877	Røed *et al.,* 2016
FIN140	Finland	KY025726	KY066878	Røed *et al.,* 2016
FIN141	Finland	KY025727	KY066879	Røed *et al.,* 2016
FIN143	Finland		KY066880	Røed *et al.,* 2016
FIN144	Finland	KY025728	KY066881	Røed *et al.,* 2016
FIN145	Finland	KY025730	KY066882	Røed *et al.,* 2016
FIN146	Finland	KY025729	KY066883	Røed *et al.,* 2016
FIN147	Finland	KY025731	KY066884	Røed *et al.,* 2016
FIN148	Finland		KY066885	Røed *et al.,* 2016
FIN149	Finland		KY066886	Røed *et al.,* 2016
UKI420	England	KY025902	KY067071	Røed *et al.,* 2016
UKI421	England	KY025903	KY067072	Røed *et al.,* 2016
UKI422	England	KY025904	KY067073	Røed *et al.,* 2016
UKI423	England	KY025905	KY067074	Røed *et al.,* 2016
UKI424	England		KY067075	Røed *et al.,* 2016
UKI425	England	KY025906		Røed *et al.,* 2016
UKI426	England		KY067076	Røed *et al.,* 2016
UKI428	England	KY025907		Røed *et al.,* 2016
UKI429	England	KY025908		Røed *et al.,* 2016
UKI430	England	KY025909	KY067077	Røed *et al.,* 2016
UKI431	England	KY025910	KY067078	Røed *et al.,* 2016
UKI432	England	KY025911	KY067079	Røed *et al.,* 2016
UKI433	England	KY025912	KY067080	Røed *et al.,* 2016
UKI434	England	KY025913	KY067081	Røed *et al.,* 2016
UKI435	England	KY025914	KY067082	Røed *et al.,* 2016
UKI436	England	KY025915	KY067083	Røed *et al.,* 2016
UKI437	England	KY025916	KY067084	Røed *et al.,* 2016
UKI438	England		KY067085	Røed *et al.,* 2016
UKI439	England	KY025917	KY067086	Røed *et al.,* 2016
UKI440	England	KY025918	KY067087	Røed *et al.,* 2016
UKI441	England	KY025919	KY067088	Røed *et al.,* 2016
UKI442	England	KY025920	KY067089	Røed *et al.,* 2016

UKI443	England		KY067090	Røed *et al.,* 2016
UKD372	England	KY025882	KY067050	Røed *et al.,* 2016
UKD373	England	KY025897	KY067051	Røed *et al.,* 2016
UKD374	England	KY025883	KY067052	Røed *et al.,* 2016
UKD375	England	KY025884	KY067053	Røed *et al.,* 2016
UKD376	England	KY025898	KY067054	Røed *et al.,* 2016
UKD377	England		KY067055	Røed *et al.,* 2016
UKD378	England	KY025885	KY067056	Røed *et al.,* 2016
UKD379	England	KY025886	KY067057	Røed *et al.,* 2016
UKD380	England	KY025887	KY067058	Røed *et al.,* 2016
UKD381	England	KY025888	KY067059	Røed *et al.,* 2016
UKD382	England	KY025889	KY067060	Røed *et al.,* 2016
UKD384	England	KY025890	KY067061	Røed *et al.,* 2016
UKD385	England		KY067062	Røed *et al.,* 2016
UKD386	England	KY025899	KY067063	Røed *et al.,* 2016
UKD387	England	KY025900	KY067064	Røed *et al.,* 2016
UKD388	England	KY025901	KY067065	Røed *et al.,* 2016
UKD389	England	KY025891	KY067066	Røed *et al.,* 2016
UKD390	England	KY025892	KY067067	Røed *et al.,* 2016
UKD391	England	KY025893		Røed *et al.,* 2016
UKD392	England	KY025894	KY067068	Røed *et al.,* 2016
UKD393	England	KY025895	KY067069	Røed *et al.,* 2016
UKD394	England	KY025896	KY067070	Røed *et al.,* 2016
UKM396	England	KY025921	KY067091	Røed *et al.,* 2016
UKM397	England	KY025922	KY067092	Røed *et al.,* 2016
UKM398	England	KY025923	KY067093	Røed *et al.,* 2016
UKM399	England	KY025924	KY067094	Røed *et al.,* 2016
UKM400	England	KY025925		Røed *et al.,* 2016
UKM402	England	KY025926		Røed *et al.,* 2016
UKM405	England	KY025927		Røed *et al.,* 2016
UKM406	England	KY025928		Røed *et al.,* 2016
UKM407	England	KY025929		Røed *et al.,* 2016
UKM408	England	KY025930	KY067095	Røed *et al.,* 2016
UKM409	England	KY025931	KY067096	Røed *et al.,* 2016

UKM410	England	KY025932	KY067097	Røed *et al.,* 2016
UKM411	England	KY025933	KY067098	Røed *et al.,* 2016
UKM412	England		KY067099	Røed *et al.,* 2016
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UKM414	England	KY025935	KY067101	Røed *et al.,* 2016
UKM415	England	KY025936	KY067102	Røed *et al.,* 2016
UKM416	England	KY025937	KY067103	Røed *et al.,* 2016
UKM417	England	KY025938	KY067104	Røed *et al.,* 2016
UKM418	England	KY025939	KY067105	Røed *et al.,* 2016
UKM419	England	KY025940	KY067106	Røed *et al.,* 2016

Table d): Sampling name, country name and GeneBank accession number for Cyt B gene of mtDNA in Ixodus ricinus.

Species Name	Country	Cyb	References
Haplotype CB1	Lithuania	KT070761.1	Paulauskas *et al.,(*2016)
Haplotype CB2	Latvia	KT070762.1	Paulauskas *et al.,(*2016)
Haplotype CB3	Estonia	KT070763.1	Paulauskas *et al.,(*2016)
Haplotype CB4	Estonia	KT070764.1	Paulauskas *et al.,(*2016)

Table e: Sampling name, country name and GeneBank accession number for CR gene of mtDNA in Ixodus ricinus.

Species Name	Country	Accession number CR	References
Haplotype CR 1	Lithuania	KT070717.1	Paulauskas *et al.,*(2016)
Haplotype CR 2	Latvia	KT070718.1	Paulauskas *et al.,*(2016)
Haplotype CR 3	Lithuania	KT070719.1	Paulauskas *et al.,*(2016)
Haplotype CR 4	Latvia	KT070720.1	Paulauskas *et al.,*(2016)
Haplotype CR 5	Latvia	KT070721.1	Paulauskas *et al.,*(2016)
Haplotype CR 6	Latvia	KT070722.1	Paulauskas *et al.,*(2016)
Haplotype CR 7	Latvia	KT070723.1	Paulauskas *et al.,*(2016)
Haplotype CR 8	Lithuania	KT070724.1	Paulauskas *et al.,*(2016)
Haplotype CR 9	Lithuania	KT070725.1	Paulauskas *et al.,*(2016)
Haplotype CR 10	Lithuania	KT070726.1	Paulauskas *et al.,*(2016)
Haplotype CR 11	Lithuania	KT070727.1	Paulauskas *et al.,*(2016)
Haplotype CR 12	Latvia	KT070728.1	Paulauskas *et al.,*(2016)

Haplotype CR 13	Lithuania	KT070729.1	Paulauskas *et al.,*(2016)
Haplotype CR 14	Latvia	KT070730.1	Paulauskas *et al.,*(2016)
Haplotype CR 15	Latvia	KT070731.1	Paulauskas *et al.,*(2016)
Haplotype CR 16	Latvia	KT070732.1	Paulauskas *et al.,*(2016)
Haplotype CR 17	Lithuania	KT070733.1	Paulauskas *et al.,*(2016)
Haplotype CR 18	Latvia	KT070734.1	Paulauskas *et al.,*(2016)
Haplotype CR 19	Latvia	KT070735.1	Paulauskas *et al.,*(2016)
Haplotype CR 20	Estonia	KT070736.1	Paulauskas *et al.,*(2016)
Haplotype CR 21	Lithuania	KT070737.1	Paulauskas *et al.,*(2016)
Haplotype CR 22	Estonia	KT070738.1	Paulauskas *et al.,*(2016)
Haplotype CR 23	Estonia	KT070739.1	Paulauskas *et al.,*(2016)
Haplotype CR 24	Estonia	KT070740.1	Paulauskas *et al.,*(2016)
Haplotype CR 25	Estonia	KT070741.1	Paulauskas *et al.,*(2016)
Haplotype CR 26	Estonia	KT070742.1	Paulauskas *et al.,*(2016)
Haplotype CR 27	Estonia	KT070743.1	Paulauskas *et al.,*(2016)
Haplotype CR 28	Lithuania	KT070744.1	Paulauskas *et al.,*(2016)
Haplotype CR 29	Lithuania	KT070745.1	Paulauskas *et al.,*(2016)
Haplotype CR 30	Estonia	KT070746.1	Paulauskas *et al.,*(2016)
Haplotype CR 31	Estonia	KT070747.1	Paulauskas *et al.,*(2016)
Haplotype CR 32	Estonia	KT070748.1	Paulauskas *et al.,*(2016)
Haplotype CR 33	Estonia	KT070749.1	Paulauskas *et al.,*(2016)
Haplotype CR 34	Estonia	KT070750.1	Paulauskas *et al.,*(2016)
Haplotype CR 35	Lithuania	KT070751.1	Paulauskas *et al.,*(2016)
Haplotype CR 36	Lithuania	KT070752.1	Paulauskas *et al.,*(2016)
Haplotype CR 37	Lithuania	KT070753.1	Paulauskas *et al.,*(2016)
Haplotype CR 38	Lithuania	KT070754.1	Paulauskas *et al.,*(2016)
Haplotype CR 39	Lithuania	KT070755.1	Paulauskas *et al.,*(2016)
Haplotype CR 40	Lithuania	KT070756.1	Paulauskas *et al.,*(2016)
Haplotype CR 41	Lithuania	KT070757.1	Paulauskas *et al.,*(2016)
Haplotype CR 42	Lithuania	KT070758.1	Paulauskas *et al.,*(2016)
Haplotype CR 43	Lithuania	KT070759.1	Paulauskas *et al.,*(2016)
Haplotype CR 44	Lithuania	KT070760.1	Paulauskas *et al.,*(2016)

Table f: Sampling name, country name and GeneBank accession number for COII gene of mtDNA in Ixodus ricinus.

Species	Country	Accession number of COII	References
Isolate Sil3	Turkey	JX141711.1	Porretta *et al.,* (2013)
Isolate Sil2	Turkey	JX141710.1	Porretta *et al.,* (2013)
Isolate Cor2	Italy	JX141709.1	Porretta *et al.,* (2013)
Isolate Pot10	Italy	JX141708.1	Porretta *et al.,* (2013)
Isolate Pot9	Italy	JX141707.1	Porretta *et al.,* (2013)
Isolate Pis4	Italy	JX141706.1	Porretta *et al.,* (2013)
Isolate Par8	Italy	JX141705.1	Porretta *et al.,* (2013)
Isolate Tre4	Italy	JX141704.1	Porretta *et al.,* (2013)
Isolate Tre3	Italy	JX141703.1	Porretta *et al.,* (2013)
Isolate Var3	Italy	JX141702.1	Porretta *et al.,* (2013)
Isolate Dom16	Italy	JX141701.1	Porretta *et al.,* (2013)
Isolate Dom11	Italy	JX141700.1	Porretta *et al.,* (2013)
Isolate Dom6	Italy	JX141699.1	Porretta *et al.,* (2013)
Isolate Dom4	Italy	JX141698.1	Porretta *et al.,* (2013)
Isolate Lug15	Spain	JX141697.1	Porretta *et al.,* (2013)
Isolate Lug12	Spain	JX141696.1	Porretta *et al.,* (2013)
Isolate Lug7	Spain	JX141695.1	Porretta *et al.,* (2013)
Isolate Gar8	France	JX141694.1	Porretta *et al.,* (2013)
Isolate Vil7	France	JX141693.1	Porretta *et al.,* (2013)
Isolate Vil5	France	JX141692.1	Porretta *et al.,* (2013)
Isolate Zur1	Switzerland	JX141691.1	Porretta *et al.,* (2013)
Isolate Mon5	Germany	JX141690.1	Porretta *et al.,* (2013)
Isolate Mon2	Germany	JX141689.1	Porretta *et al.,* (2013)
Isolate Zag3	Croatia	JX141688.1	Porretta *et al.,* (2013)
Isolate Bud4	Hungary	JX141687.1	Porretta *et al.,* (2013)
Isolate Bud1	Hungary	JX141686.1	Porretta *et al.,* (2013)
Isolate Pri10	Czech Republic	JX141685.1	Porretta *et al.,* (2013)
Isolate Pri5	Czech Republic	JX141684.1	Porretta *et al.,* (2013)
Isolate Kau5	Lithuania	JX141683.1	Porretta *et al.,* (2013)

Isolate Kau2	Lithuania	JX141682.1	Porretta *et al.,* (2013)
Isolate Kau1	Lithuania	JX141681.1	Porretta *et al.,* (2013)
Isolate Upp12	Sweden	JX141680.1	Porretta *et al.,* (2013)
Isolate Upp6	Sweden	JX141679.1	Porretta *et al.,* (2013)
Isolate Upp4	Sweden	JX141678.1	Porretta *et al.,* (2013)
Isolate Upp3	Sweden	JX141677.1	Porretta *et al.,* (2013)
Isolate Upp1	Sweden	JX141676.1	Porretta *et al.,* (2013)

Table g: *Sampling name, country name and GeneBank accession number for COI gene of mtDNA in Ixodus ricinus.*

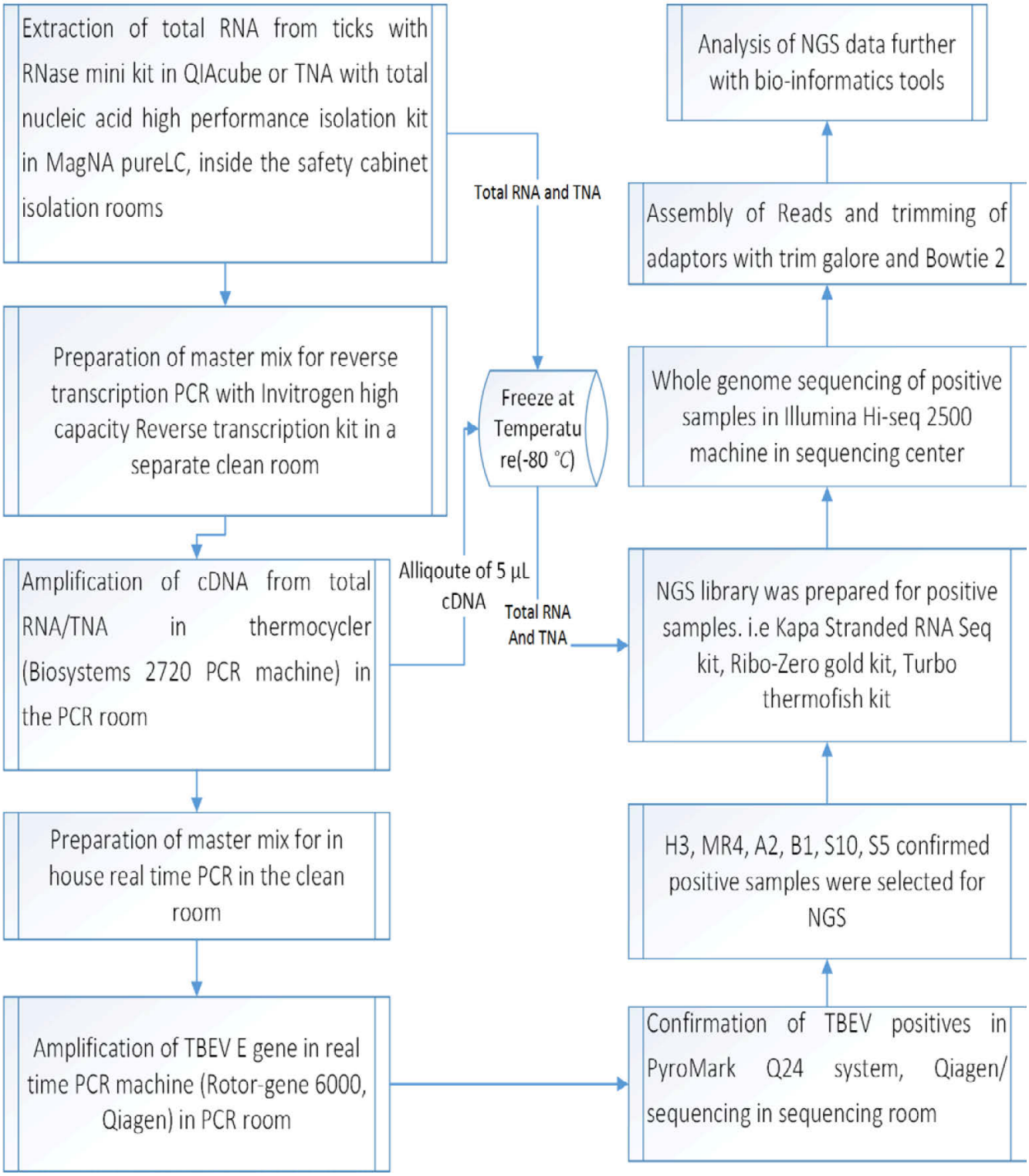
Species name	Country	Accession number of COI	Refrences
isolate Sil7	Turkey	JX141711.1	Porretta *et al.,* (2013)
isolate Sil5	Turkey	JX141710.1	Porretta *et al.,* (2013)
isolate Sil4	Turkey	JX141709.1	Porretta *et al.,* (2013)
isolate Sil1	Turkey	JX141708.1	Porretta *et al.,* (2013)
isolate Bar2	Italy	JX141707.1	Porretta *et al.,* (2013)
isolate Pot1	Italy	JX141706.1	Porretta *et al.,* (2013)
isolate Pis13	Italy	JX141705.1	Porretta *et al.,* (2013)
isolate Pis4	Italy	JX141704.1	Porretta *et al.,* (2013)
isolate Par9	Italy	JX141703.1	Porretta *et al.,* (2013)
isolate Par8	Italy	JX141702.1	Porretta *et al.,* (2013)
isolate Par5	Italy	JX141701.1	Porretta *et al.,* (2013)
isolate Par4	Italy	JX141700.1	Porretta *et al.,* (2013)
isolate Par2	Italy	JX141699.1	Porretta *et al.,* (2013)
isolate Var11	Italy	JX141698.1	Porretta *et al.,* (2013)
isolate Var9	Italy	JX141697.1	Porretta *et al.,* (2013)
isolate Var3	Italy	JX141696.1	Porretta *et al.,* (2013)
isolate Dom1	Italy	JX141695.1	Porretta *et al.,* (2013)
isolate Lug5	Spain	JX141694.1	Porretta *et al.,* (2013)
isolate Lug4	Spain	JX141693.1	Porretta *et al.,* (2013)
isolate Gar7	France	JX141692.1	Porretta *et al.,* (2013)
isolate Vil10	France	JX141691.1	Porretta *et al.,* (2013)
isolate Vil5	France	JX141690.1	Porretta *et al.,* (2013)

isolate Vil3	France	JX141689.1	Porretta *et al.,* (2013)
isolate Vil4	France	JX141688.1	Porretta *et al.,* (2013)
isolate Vil1	France	JX141687.1	Porretta *et al.,* (2013)
isolate Zur13	Switzerland	JX141686.1	Porretta *et al.,* (2013)
isolate Zur11	Switzerland	JX141685.1	Porretta *et al.,* (2013)
isolate Zur9	Switzerland	JX141684.1	Porretta *et al.,* (2013)
isolate Zur5	Switzerland	JX141683.1	Porretta *et al.,* (2013)
isolate Mon5	Germany	JX141682.1	Porretta *et al.,* (2013)
isolate Zag1	Croatia	JX141681.1	Porretta *et al.,* (2013)
isolate Clu9	Romania	JX141680.1	Porretta *et al.,* (2013)
isolate Clu6	Romania	JX141679.1	Porretta *et al.,* (2013)
isolate Clu2	Romania	JX141678.1	Porretta *et al.,* (2013)
isolate Bud5	Hungary	JX141677.1	Porretta *et al.,* (2013)
isolate Pri10	Czech Republic	JX141676.1	Porretta *et al.,* (2013)
isolate Pri9	Czech Republic	JX141675.1	Porretta *et al.,* (2013)
isolate Pri8	Czech Republic	JX141674.1	Porretta *et al.,* (2013)
isolate Pri6	Czech Republic	JX141673.1	Porretta *et al.,* (2013)
isolate Pri1	Czech Republic	JX141672.1	Porretta *et al.,* (2013)
isolate Kau4	Lithuania	JX141671.1	Porretta *et al.,* (2013)
isolate Kau1	Lithuania	JX141670.1	Porretta *et al.,* (2013)
isolate Upp15	Sweden	JX141669.1	Porretta *et al.,* (2013)
isolate Upp14	Sweden	JX141668.1	Porretta *et al.,* (2013)
isolate Upp12	Sweden	JX141667.1	Porretta *et al.,* (2013)
isolate Upp10	Sweden	JX141666.1	Porretta *et al.,* (2013)
isolate Upp7	Sweden	JX141665.1	Porretta *et al.,* (2013)
isolate Upp6	Sweden	JX141664.1	Porretta *et al.,* (2013)
isolate Upp2	Sweden	JX141663.1	Porretta *et al.,* (2013)
isolate Upp1	Sweden	JX141662.1	Porretta *et al.,* (2013)
1	Slovakia	GU074892.1	Noureddine *et al.,* (2011)
2	Slovaki	GU074893.1	Noureddine *et al.,* (2011)
3	Slovakia	GU074894.1	Noureddine *et al.,* (2011)
4	Moldova	GU074895.1	Noureddine *et al.,* (2011)
5	Denmark	GU074896.1	Noureddine *et al.,* (2011)
6	Sweden	GU074897.1	Noureddine *et al.,* (2011)

7	Estonia	GU074898.1	Noureddine *et al.,* (2011)
8	Ireland	GU074899.1	Noureddine *et al.,*(2011)
9	Tunisia	GU074900.1	Noureddine *et al.,* (2011)
10	France	GU074901.1	Noureddine *et al.,* (2011)
11	Algeria	GU074902.1	Noureddine *et al.,* (2011)
12	Iran	GU074903.1	Noureddine *et al.,* (2011)
13	Finland	GU074904.1	Noureddine *et al.,* (2011)
14	Bulgaria	GU074905.1	Noureddine *et al.,* (2011)
15	Morocco	GU074906.1	Noureddine *et al.,* (2011)
16	England	GU074907.1	Noureddine *et al.,* (2011)
17	Hungary	GU074908.1	Noureddine *et al.,* (2011)
18	Germany	GU074909.1	Noureddine *et al.,* (2011)
19	Spain	GU074910.1	Noureddine *et al.,* (2011)
20	Netherland	GU074911.1	Noureddine *et al.,* (2011)
21	France	GU074912.1	Noureddine *et al.,* (2011)
22	France	GU074913.1	Noureddine *et al.,* (2011)
23	France	GU074914.1	Noureddine *et al.,* (2011)
24	France	GU074915.1	Noureddine *et al.,* (2011)
25	France	GU074916.1	Noureddine *et al.,* (2011)
26	France	GU074917.1	Noureddine *et al.,* (2011)
27	France	GU074918.1	Noureddine *et al.,* (2011)
28	France	GU074919.1	Noureddine *et al.,* (2011)
29	France	GU074920.1	Noureddine *et al.,* (2011)
30	France	GU074921.1	Noureddine *et al.,* (2011)
31	France	GU074922.1	Noureddine *et al.,* (2011)
32	France	GU074923.1	Noureddine *et al.,* (2011)
33	France	GU074924.1	Noureddine *et al.,* (2011)
34	France	GU074925.1	Noureddine *et al.,* (2011)
35	France	GU074926.1	Noureddine *et al.,* (2011)
36	France	GU074927.1	Noureddine *et al.,* (2011)
37	France	GU074928.1	Noureddine *et al.,* (2011)
38	France	GU074929.1	Noureddine *et al.,* (2011)
39	France	GU074930.1	Noureddine *et al.,* (2011)
40	France	GU074931.1	Noureddine *et al.,* (2011)

41	France	GU074932.1	Noureddine *et al.,* (2011)
42	France	GU074933.1	Noureddine *et al.,* (2011)
43	France	GU074934.1	Noureddine *et al.,* (2011)
44	France	GU074935.1	Noureddine *et al.,* (2011)
45	France	GU074936.1	Noureddine *et al.,* (2011)
46	France	GU074937.1	Noureddine *et al.,* (2011)
47	France	GU074938.1	Noureddine *et al.,* (2011)
48	France	GU074939.1	Noureddine *et al.,* (2011)
49	France	GU074940.1	Noureddine *et al.,* (2011)
50	France	GU074941.1	Noureddine *et al.,* (2011)
51	France	GU074942.1	Noureddine *et al.,* (2011)
52	France	GU074943.1	Noureddine *et al.,* (2011)
53	France	GU074944.1	Noureddine *et al.,* (2011)
54	France	GU074945.1	Noureddine *et al.,* (2011)
55	France	GU074946.1	Noureddine *et al.,* (2011)
56	France	GU074947.1	Noureddine *et al.,* (2011)
57	France	GU074948.1	Noureddine *et al.,* (2011)
58	France	GU074949.1	Noureddine *et al.,* (2011)
59	France	GU074950.1	Noureddine *et al.,* (2011)
60	France	GU074951.1	Noureddine *et al.,* (2011)
34_12a	Serbia	KC809977.1	Ćakić et al., (2014)
44_12c	Serbia	KC809976.1	Ćakić et al., (2014)
114_12	Serbia	KC809975.1	Ćakić et al., (2014)
164_12d	Serbia	KC809974.1	Ćakić et al., (2014)
164_12e	Serbia	KC809973.1	Ćakić et al., (2014)
167_12a	Serbia	KC809972.1	Ćakić et al., (2014)

Appendix 2: Flow chart for prevalence of TBEV and phylogenetic relationship of ticks



Appendix 3: QIAcube protocol for extraction total RNA

Purification of total RNA from animal tissues and cells with two elution steps was chosen in the QIAcube protocol.

Extraction of RNA was done according to the following protocol:

- QIAcube machine was turned on.
- Fill 1000 µl filter pipette tips rack into the two tips rack.
- Place the spin column and elution tube on the rotor adaptor and put them into centrifuge in QIAcube machine.
- Add buffer RW1, Buffer RPE, 70% Ethanol and 10 mM Tris pH 8.0, in the reagent bottles and removed their lid placed them in reagent bottles rack.
- Samples are then put in the samples rack (shaker).
- Select RNA with RNeasy Mini procedure from Animal tissues and cells using Two elution steps (2x30 µl) and start it.
- Machine will start extraction and will took approximately 30 mins.

Appendix 4: MagNA Pure LC protocol for extraction of TNA

MagNA Pure LC 2.0 instrument was installed with software v 3.0.12. MagNa Pure LC Total Nucleic Acid Isolation Kit - High Performance Version 8 Was used for isolation of total nucleic acid.

Isolation of total nucleic acid was according to the following protocol:

- Start the instrument and software.
- Choose appropriate protocol: Total NA Hp 200.
- Select number of samples, sample volume (200 µl) and elution volume (60 µl).
-
- Before starting the isolation procedure, fill all Reagent Tubs outside the Instrument with the required amount of reagents.
- Place tips in the tips racks and samples in the sample rack in the machine and start the isolation procedure.
- Isolation of total nucleic acid will finish in approximately the 2 hours.

Appendix 5: Reverse transcription PCR mix

Composition of mix for reverse transcription PCR

Component	Amount µl/ test
10x RT Random Primers	2.0
10x RT Buffer	2.0
Multiscribe^{TM} Reverse Transcriptase	0.8
25x dNTP Mix (100mM)	1.0
RNase Inhibitor	1.0
Nuclease -free H_2O	8.2
Total mix	15 µl

Total Volume = 20 µL = 15 µl RT Mix + 5 µL TNA/RNA

PCR cycling condition

Temperature	Time
25 ^{O} C	10 min
37 ^{O} C	120 min
85 ^{O} C	5 min
4.0 ^{O} C	∞

Appendix 6: Real-time PCR mix for TBEV

Composition of real-time PCR mix for TBEV

Component	Amount µl/ test
10x AB Buffer	2.5
50Mm $Mgcl_2$	2.5
25x dNTP	0.2
25 pmol 320 F	0.25
25 pmol 320 R+ Biotin	0.25
25 pmol 339 probe	0.3
*Pt-taq	0.19
RNase-free H_2O	15.81

Total mix	22 µl

*Platinum ®Taq DNA polymerase enzyme from Invitrogen™

Total volume= 25 µl= 22 µl Mix+ 3 µl cDNA

10X AB Buffer Concentration:

Component	Volume
1 M Tris pH 8.8	75 ml
$(NH4)_2$ SO4	20 ml
Tween 20	0.2 ml
RNase free water	4.8 ml
Total	100 ml

Appendix 7: Pyrosequencing

The pyrosequencing method is based on detecting the activity of DNA polymerase (a DNA synthesizing enzyme) with another chemoluminescent enzyme. In the pyrosequencing reaction, biotinylated single-stranded DNA template attached to Streptavidin Sepharos™beads (GE Healthcare, Little Chalfont, UK) in a solution containing enzymes and substrates. When correct deoxyribonucleotide triphosphate (dNTP) is incorporated in to the DNA by DNA polymerase, this incorporation releases inorganic pyrophosphate (iPP). The iPP is converted into ATP in the presence of sulfurylase and adenosine 5'-phosphosulfate (APS). Then luciferase hydolyse ATP to oxidize the luciferin into oxyluciferin and produced light signal too. Unincorporated nucleotides are degrading by Apyrase in the mix.

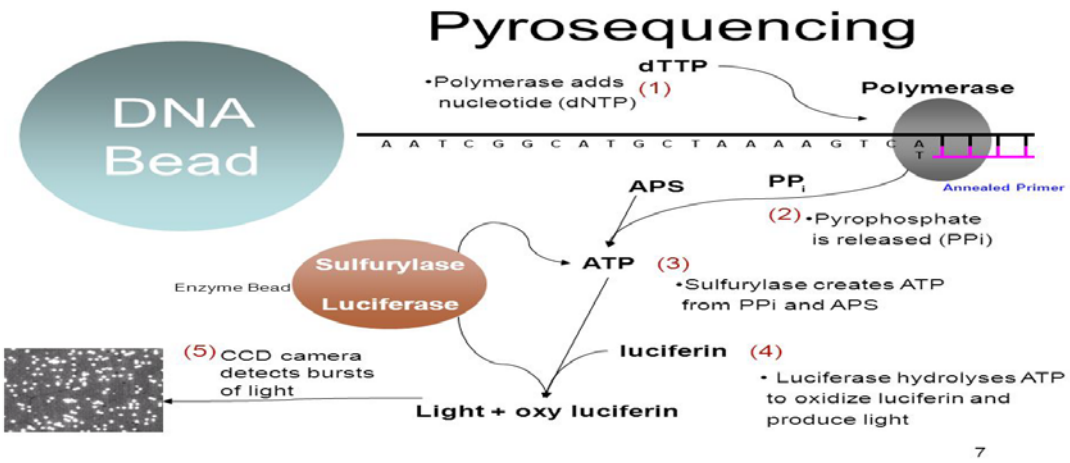


Figure a): Principle of pyrosequencing (http://slideplayer.com/slide/9021471/images/7/).

Pyrosequencing SQA Protocol Using the PyroMark™ Q24 (Biotage)

Reagents

Table b): Master mix

Reagents	Amount 1x µl
RNase free water	18 µl
Binding buffer	40 µl
Streptavidin Sepharos™beads	2 µl

Table c): Primer mix

Reagents	Amount 1x µl
Anealing buffer	24.7 µl
Sequencing primers (25 µM)	0.3 µl

Procedure

Preparation of master mix and primer mix

1. Reagent were brought to the room temperature 4 ºC before making the mix and make sure that Streptavidin Sepharose™ beads re mixed properly.
2. Add 60 µl of the master mix in pre-labbeled strips and remove lid from them. Add 20 µl of PCR products in them inside the safety cabinet. Then kept them in plate shaker for 10 minutes at 1400 0 rpm after closing the lid gently.

3. Prepare primer mix and add 25 µl in Pyro Mark Q24 sequencing plate.

Washing PCR products

1. Place the plastic reagent tray in the designated positions on the Vacuum Prep Workstation and filled wash buffer, denaturation solution (NaOH), 70% ethanol and sterile water tray.
2. Turn on the vacuum machine and placing the device in the sterile water for 20 seconds. This is to check the filter of the vacuum tool if it is working properly. Leave it on in sterile water until it is ready.
3. Set the heating block at 80 ºC for 2 min.
4. Samples and mix was placed in respective position on the Vacuum Prep Workstation right after completion of shaking, the cover is gently removed inside the biosafety cabinet.
5. Turn on the vacuum pump. Drawn up the sample and reagents through the vacuum tool and make sure tubes are empty. The vacuum tool consists of filter probes inside the tips that captures the immobilized amplicons-streptavidin.
6. Transfer the vacuum tool in 70% ethanol tray washed it for 5 seconds. It will allow any unbound amplicon or reagents from the real time PCR reactions to be washed off.
7. Move the Prep tool on NaOH tray for approximately 5 seconds and it will allow denature the double-stranded DNA amplicon. The complementary strand synthesized without biotin primer will be washed away.
8. Transfer the Prep tool in wash buffer for 10 seconds.
9. Lift the perp tool up at 90º and turn off the vacuum pump to allow all liquid drain out. Place perp tool on pyrosequencing plate to elute the samples from filters.
10. The plate is then transfer to the pre-heated block at 80 ºC for 2. This step allows denaturation of template and primer mix.

Prepare the PyroMark Q24 ID and Software

1. Turn on the PC and pyrosequencing machine.
2. Log into PyroMark Q24 user ID.
3. Start SQA-run and Select the appropriate run and click new run.
4. Select the appropriate method Eg. PyroMARK Q24 Method 006.

5. In the 'New Run Setup' highlight all the wells that are to be used and Write sample identification name (plate ID) and load desired assay.

6. Select the 'Tools' tab and choose 'Pre-Run' Information` from drop down menu. It will calculate the amount of the Enzyme (E), substrate (S) and nucleotides (A, C, G and T) that need to be added to the cartridge.

Prepare the cartridge of the PyroMark Q24.

1. Fill out the mixture in the cartridge. Fill out the cartridge containing enzyme, substrate and nucleotides onto the PyroMark Q24 instrument. The amount of Enzyme and substrate and nucleotides were calculated by computer according to the number of samples. Place the cartridge in the machine that have label facing front towards the user.
2. Load the plastic plate containing template and primer into the PyroMark Q24 instrument
3. Select 'run' to start. Substrate peak will ensure that the enzyme and substrate are working well right after starting run.
4. Results generated by selecting the 'SQA Full Reprot' under report tab after the run is completed.

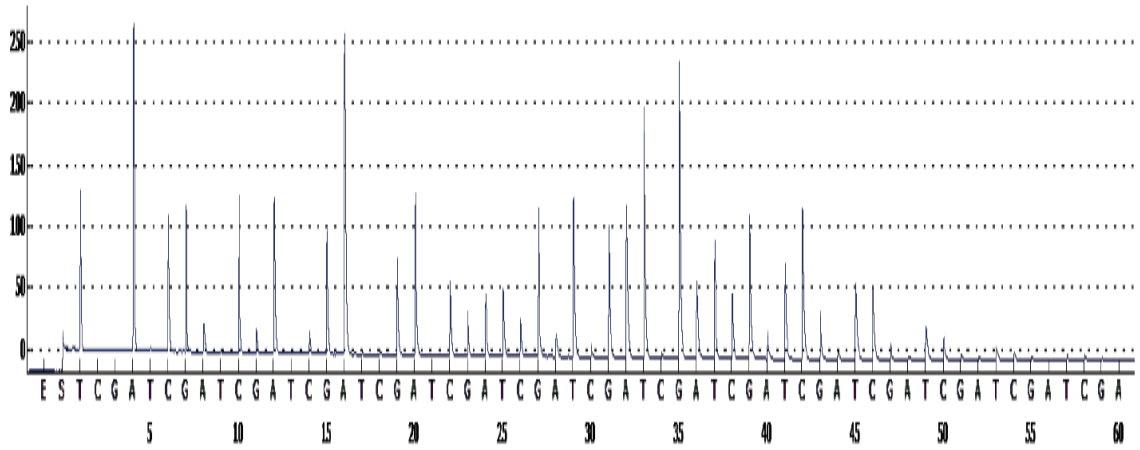
Enzyme and substrate

mix

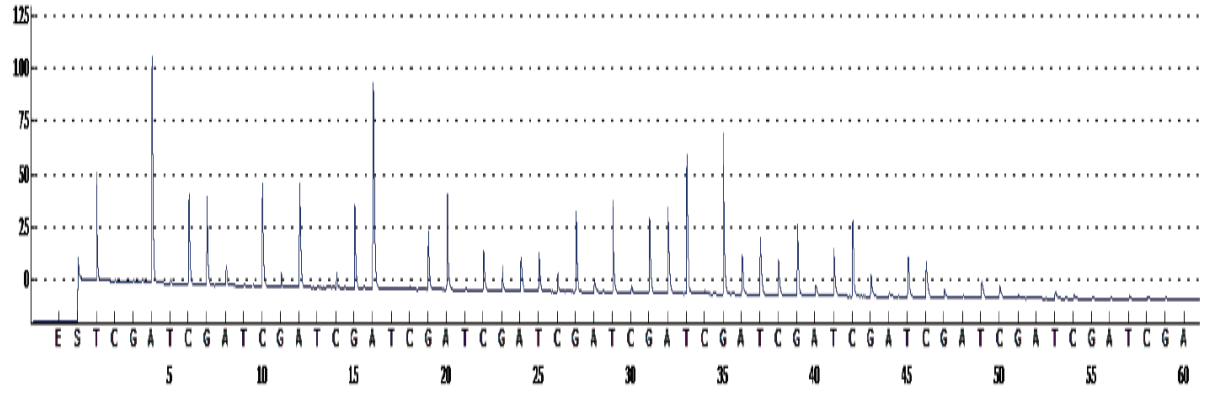
Enzyme mix consist of DNA polymerase, nucleotide, ATP sulpharase, and Luciferase. DNA polymerase incorporated the nucleotides. ATP sulpharase used to transmit pyrophosphate to ATP and produce light while apyrase degrades unbounded nucleotides and ATP. Single stranded binding protein (SSB) in the mix used to destroy secondary structure in the template.

Figure 7a): Images of the Pyrosequencing Analysis

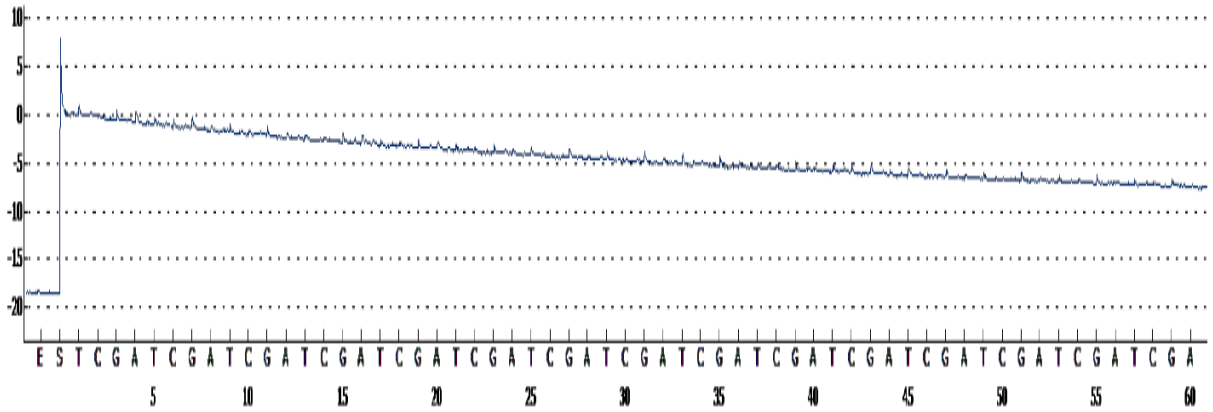
Positive control: Skoupa 10-3 dilution



Positive sample S18-6:



Negative control:



Appendix 8: Phylogenetic inferences

Alignmentof complete verses complete mtDNA, complete verses combined 5 genes verses complete genome, combined 2 genes verses complete genome, and singles verses single genes was done AliView software.

↓

Alignment files were exported Fasta file and Nexus file formats.

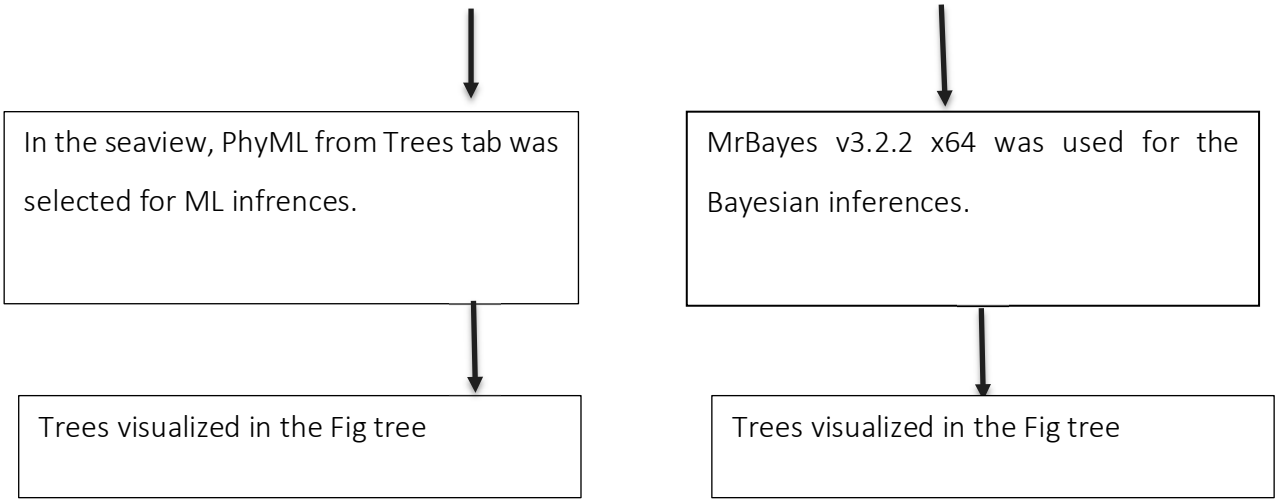
↓

Fasta file was upload in the Mega 7.0.25 and PhyML 3.0 softwares.

↓

After getting model specification, Fasta files were used for the maximum likelihood analysis.

After getting model specification, Nexus block was added in the nexus file according to model specification.



Bayesian Inferences

Nexus file from complete, concatenated and single gene sequences consisted of Nexus block. This block consist of diffent command lines for analysis.

Bayesian phylogenetic analysis follows four steps in MrBayes :

- Reading of Nexus data file
- Setting the evolutionary model
- Runing the analysis
- Summarizing the samples

Figure a): Nexus file format

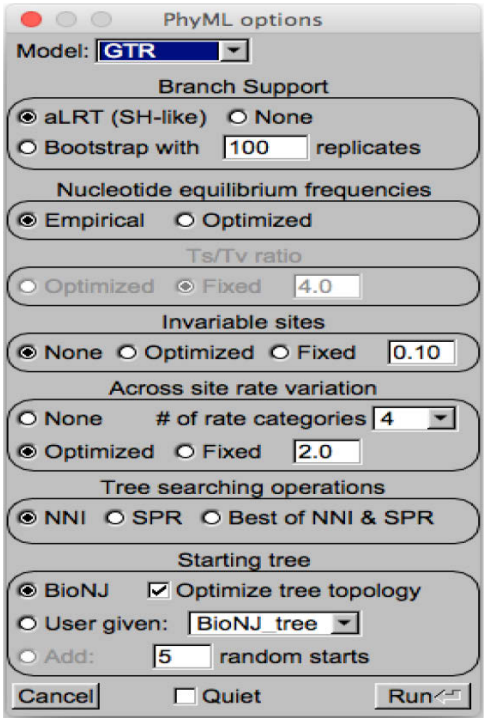
```
#NEXUS
BEGIN DATA;
DIMENSIONS  NTAX=60 NCHAR=315;
FORMAT DATATYPE=DNA GAP=- MISSING=?;
MATRIX
Contig_S6_S10_Ixodes_ricinus_Norway_Complete_mtDNA            atattaatattttactattaaatcctaac---
Contig_S2_A2_Ixodes_ricinus_Norway_Complete_mtDNA             atattaatattttactattaaatcctaacn--
Contig_S5_B1_Ixodes_ricinus_Norway_Complete_mtDNA             atattaatattttactattaaatcctaac----
Contig_S1_H3_Ixodes_ricinus_Norway_Complete_mtDNA             atattaatattttactattaaatcctaac---
Contig_S3_S5_Ixodes_ricinus_Norway_Complete_mtDNA             atattaatattttactattaaatcctaac---
Contig_S4_MR4_Ixodes_ricinus_Norway_Complete_mtDNA             atattaatattttactattaaatcctaac---
KF197124_1_Ixodes_ricinus_isolate_IR_65_Italy_complete_mtDNA     atattaatattttactattaaatcctaac---
KF197118_1_Ixodes_ricinus_isolate_IR_68_solvakia_complete_mtDNA   atattaatattttactattaaatcctaact-
END;
BEGIN SETS;
charset ixodes.mtDNA = 1-1000;
Partition DNA = 1: ixodes.mtDNA;
Set partition = DNA;
[HKYI]
[Lset applyto=(1) nst=2 rates=propinv;
Prset applyto=(1) revmatpr=Dirichlet(1.0,1.0,1.0,1.0,1.0,1.0) statefreqpr=Dirichlet(1.0,1.0,1.0,1.0)
shapepr=Uniform(0.1,50.0) pinvarpr=Uniform(0.0,1.0);
 mcmcp nruns=2 ngen=5000000 printfreq=1000 samplefreq=1000 nchains=4 diagnfreq=10000
burninfrac=0.25 stoprule=no stopval=0.002 temp=0.2 checkpoint=yes checkfreq=500000;
mcmc;]
sumt burnin=0.75 nruns=2 Contype=Allcompat;
END;
```

- Nexus file is imported in the MrBayes by typing `execute´ or exe and pasting the path of file in it. MrBayes will read the file successfully and start the analysis
- lset commands in the nexus block sets the evolutionary model with gamma-distributed rate variation across sites and a proportion of invariable sites. For instance, lset nst=2 rates=gamma it gave suggestion to model that it uses two substitution types (nst=2), and use a gamma distribution to account for different rates at different sites in the sequence.
- Mcmc commands represent the method known as MCMCMC ("Metropolis-coupled Markov chain Monte Carlo") to empirically determine the posterior probability distribution of trees, branch lengths and substitution parameters. MrBayes will start two entirely independent runs from different random trees. In start of the run, the two runs will sample very different trees but when they have reached convergence, the two tree samples should be very similar. The program will compute a measure of how similar the tree-samples. It measure in the form of average standard deviation of split frequencies. Usually, run will continue until the value of split frequencies is less than 0.01.

- MrBayes provides the sumt command to summarize the sampled trees. Before using it, MrBayes to discard the samples as burnin Since the convergence diagnostic used previously to determine when to stop the analysisand then discarded the first 25% (usually but can burn in vlaue can vary) of the samples as burnun in.
- Tree will be print as nexus.con file that will be visualized in the Figtree viewer.

Maximum liklehood inferences

- Fasta alignment file of complete, merged and singel gene sequences were used for the ML analysis.
- Following steps were included for analysis:



- Choose Maximum likelooh from drop down manu of tab Tree in seaview software.
- Model is selected from Model menu.
- Brnach support is calculated with 1000 replicated
- Invariable sites and across site rate variation is selected according to the model specification. “invariable sites “is the “I “in e.g. HKY+I+G and the “across site rate variation “is the gamma distribution. if the models suggest HKY+I+G, then both I and gamma distribution will be selected for analysis.

- Select a “best of SPR and NNI” in the tree searching operation options. Tree rearrangements used heuristic algorithms for searching an optimal tree structure. The simplest tree-rearrangement is known as nearest-neighbor interchange (NNI) (Fig a, it exchanges the connectivity of four subtrees within the main tree. Subtree pruning and regrafting (SPR) (Fig b)more wide-ranging search, it selects and removes a subtree from the main tree and reinserts it elsewhere on the main tree to create a new node.
- Choose BioNJ and optimize option in starting tree bar. Bio Neigbour joing (BioNJ) is a algorithm used for re-constructing phylogenetic trees and compute the lengths of the branches of a tree. In each stage, the two nearest nodes of the tree are defined as neighbours in tree.
- Tree reconstrcution time depened on the number of sequences in each alignment file. Tree file will be visualized in the Fig trees software at the end.

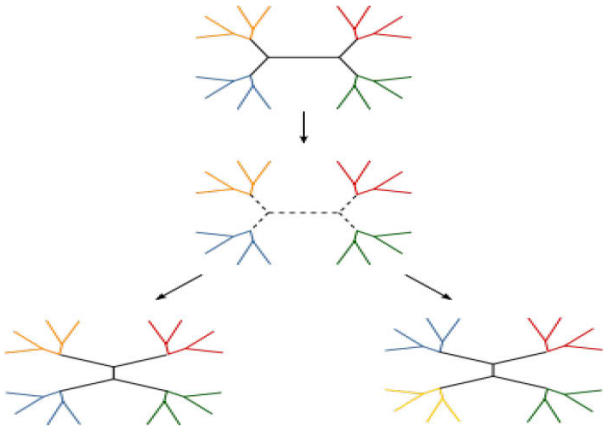


Figure a): NNI (https://en.wikipedia.org/wiki/Tree_rearrangement#/media/File:SPR.svg).

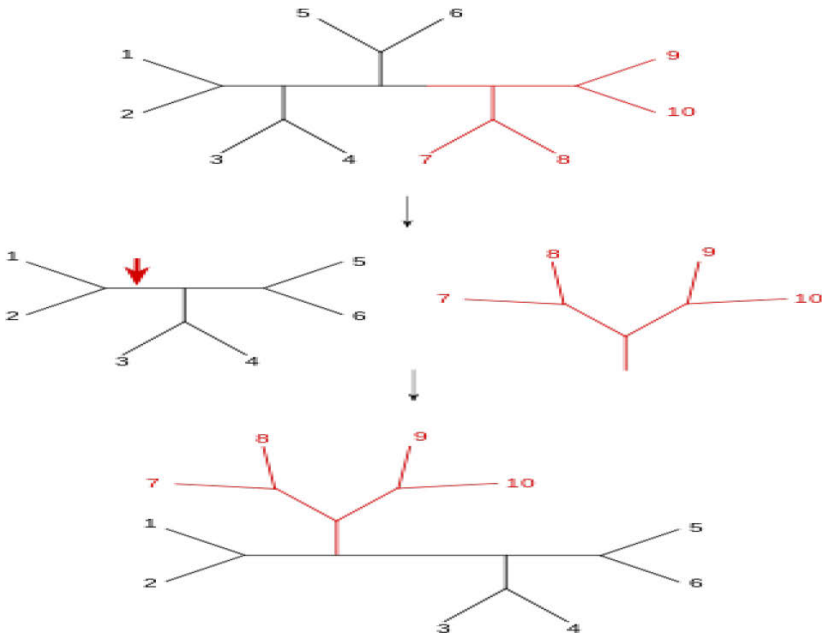


Figure b): SPR (https://en.wikipedia.org/wiki/Tree_rearrangement#/media/File:NNI.svg).

Appendix 9: Phylogenetic trees

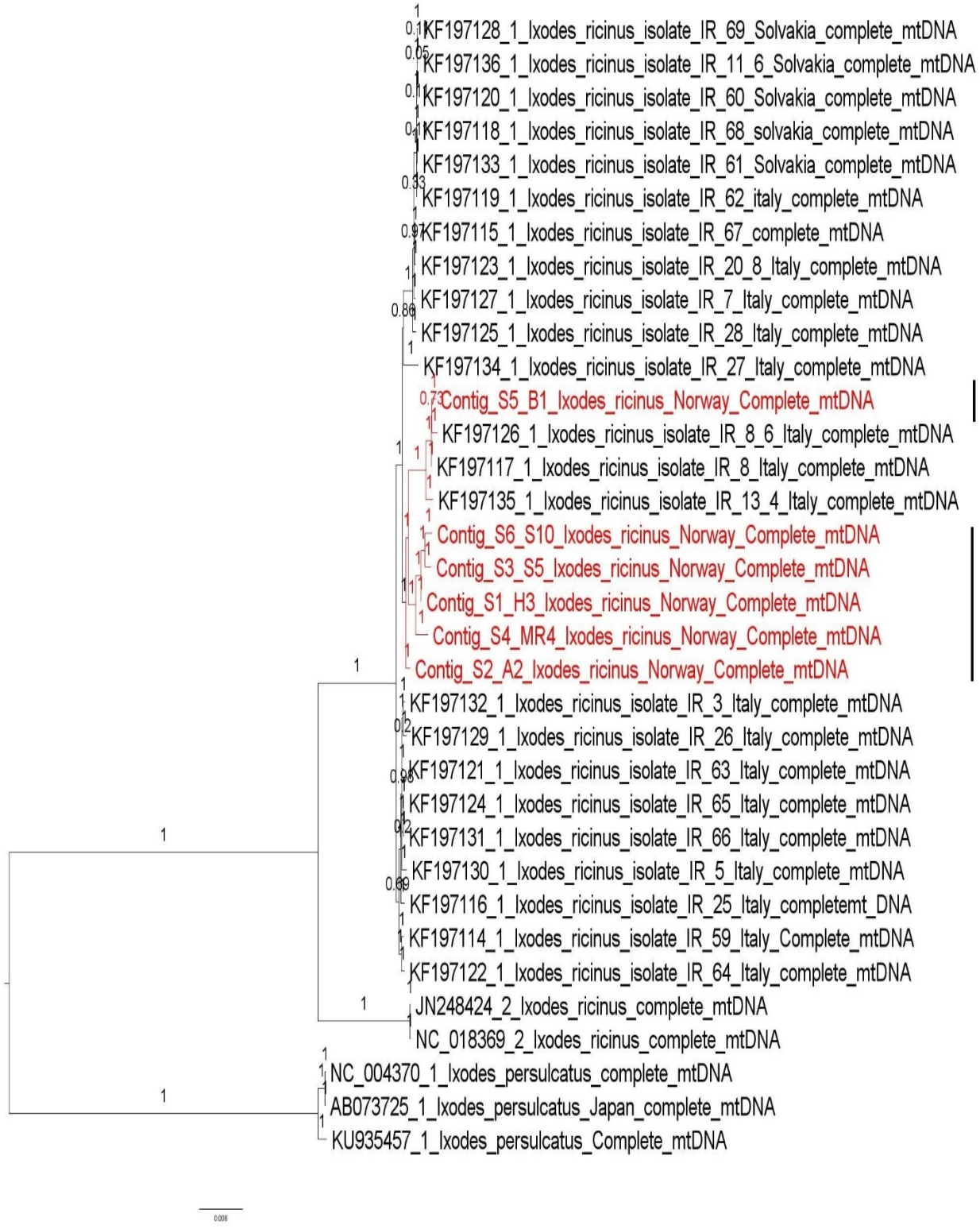


Fig a) Bayesian phylogenetic tree of complete mtDNA for Norwegian I. ricinus. *The estimates are based on complet mtDNA alignment (ca. 14650bp) under the GTR+I+G model. Bayesian posterior probabilities provided at the tree-nodes. The Norwegian strains are indicated in red color. The tree was rooted with I. persulcatus.*

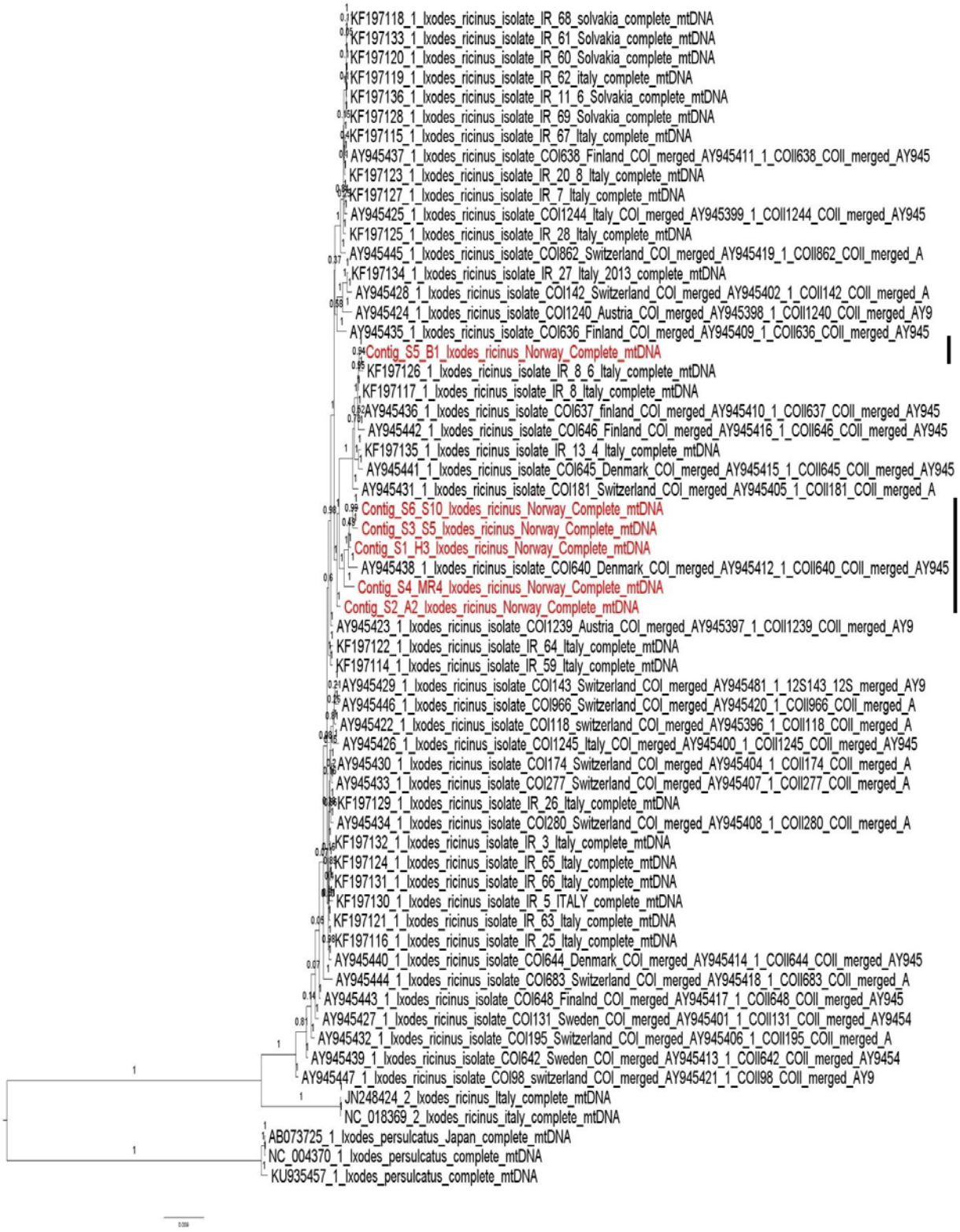


Figure b) Bayesian phylogenetic tree of concatenated (five genes) mtDNA sequences for Norwegian I. ricinus. *The estimates are based on complete mtDNA alignment (ca. 14650bp) with merged five individual genes including a control region, Cyt B, COI, COII and 12s under the HKY+I+G model. Bayesian posterior probabilities provided at the tree-nodes. The Norwegian strains are indicated in red color. The tree was rooted with I. persulcatus.*

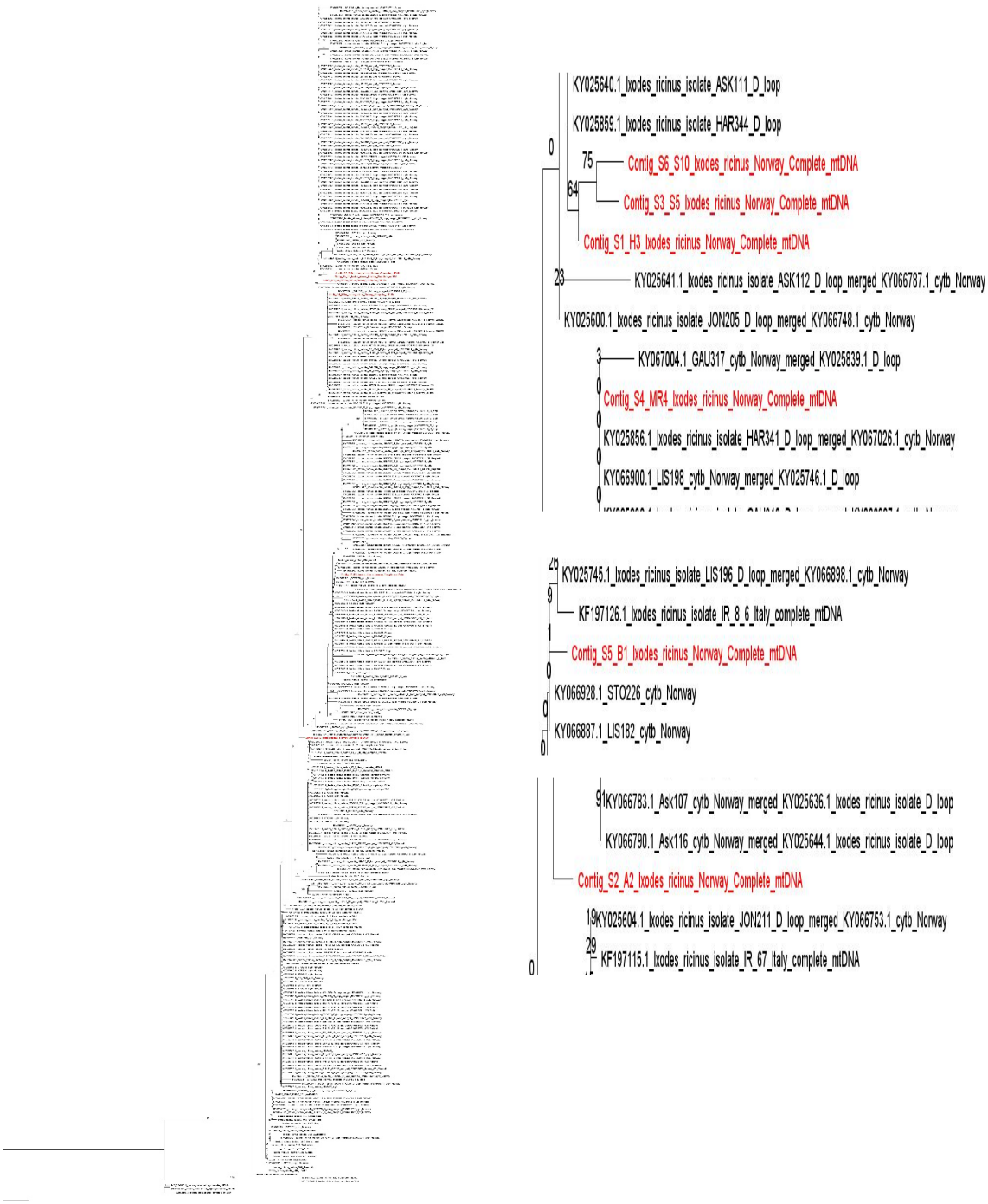


Figure c) Maximum likelihood phylogenetic tree of concatenated (two genes) mtDNA sequences for Norwegian I. ricinus. *The estimates are based on mtDNA alignment (ca. 14650bp) with merged two individual genes including a control region, and Cyt B under the GTR+I+G model. Bootstrap support values are based on 1000 replicates are indicated above branches. The Norwegian strains are indicated in red color and highlighted in the box next to tree. The tree was rooted with I. persulcatus.*

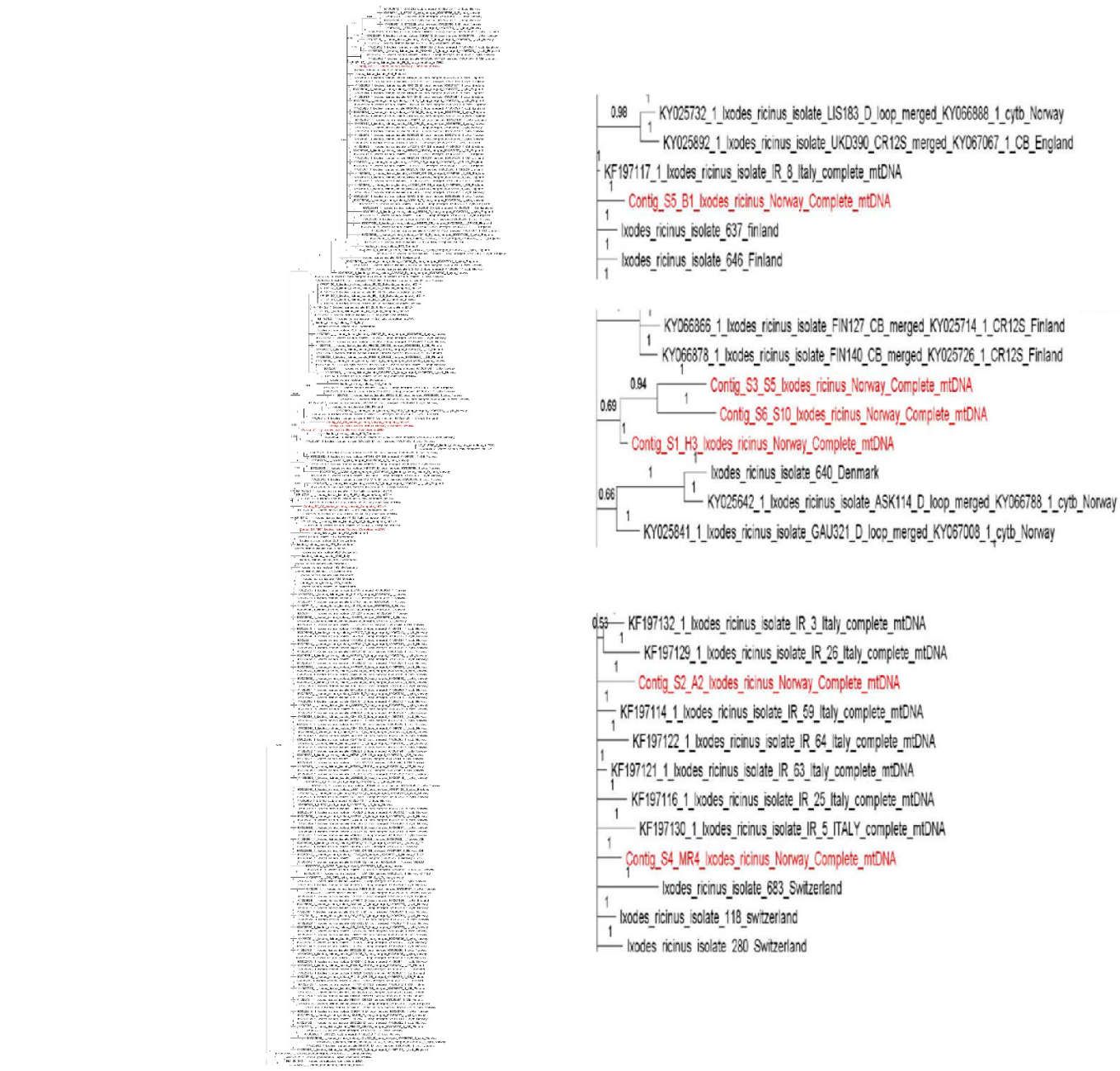


Figure d) Bayesian phylogenetic tree of concatenated (two genes) mtDNA sequences for Norwegian I. ricinus. *The estimates are based on complete mtDNA alignment (ca. 14650bp) with merged two individual genes including a control region, and Cyt B under the GTR+I+G model. Bayesian posterior probabilities provided at the tree-nodes. The Norwegian strains are indicated in red color and highlighted in the box next to tree. The tree was rooted with I. persulcatus.*

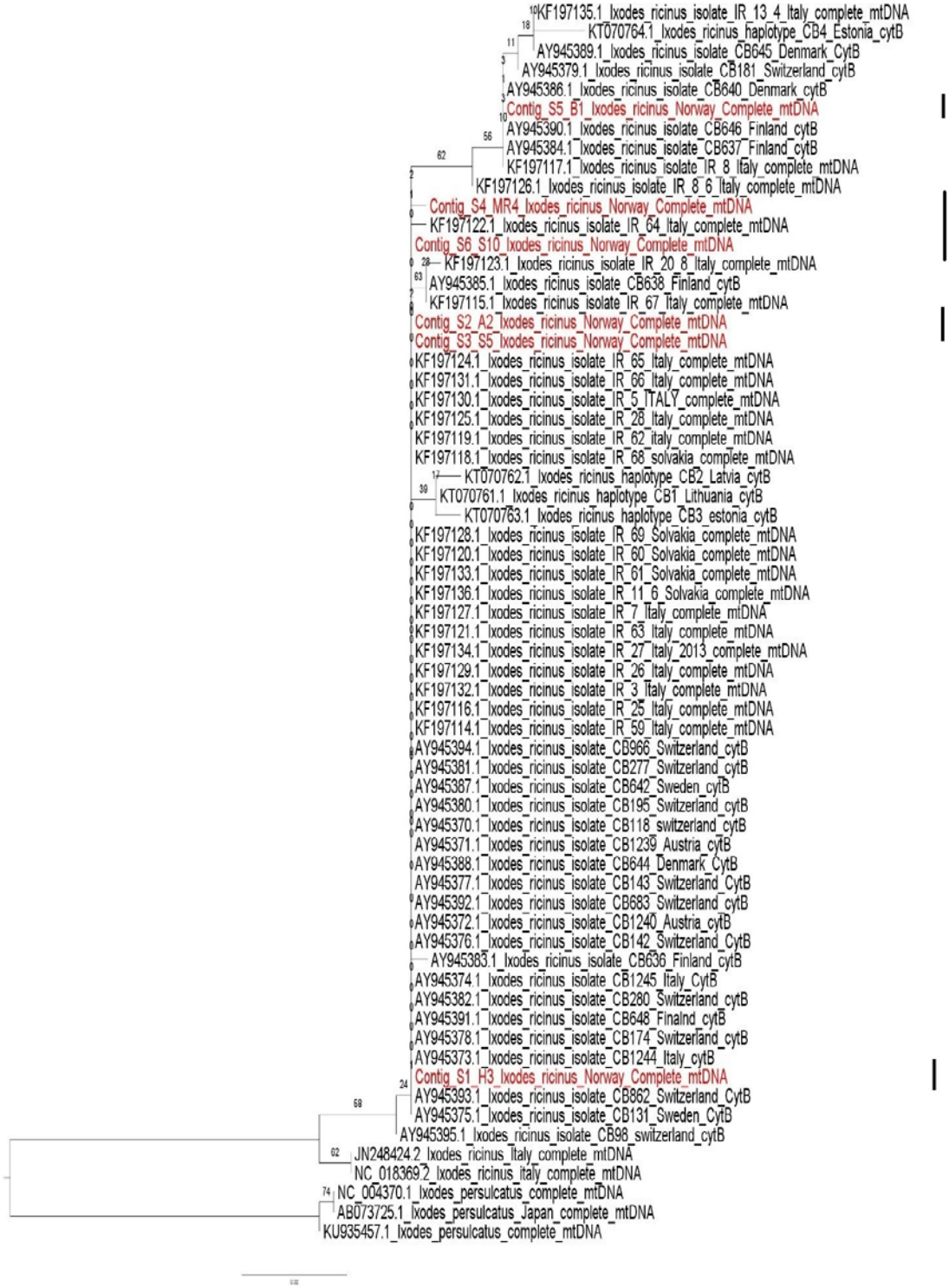


Fig e) Maximum likelihood phylogenetic tree of Cyt B for Norwegian I. ricinus. *The estimates are based on Cyt B mtDNA alignment (ca. 10250-10590 bp) under the HKY+G model. Bootstrap support values are based on 1000 replicates are indicated above branches. The Norwegian strains are indicated in red color. The tree was rooted with I. persulcatus.*

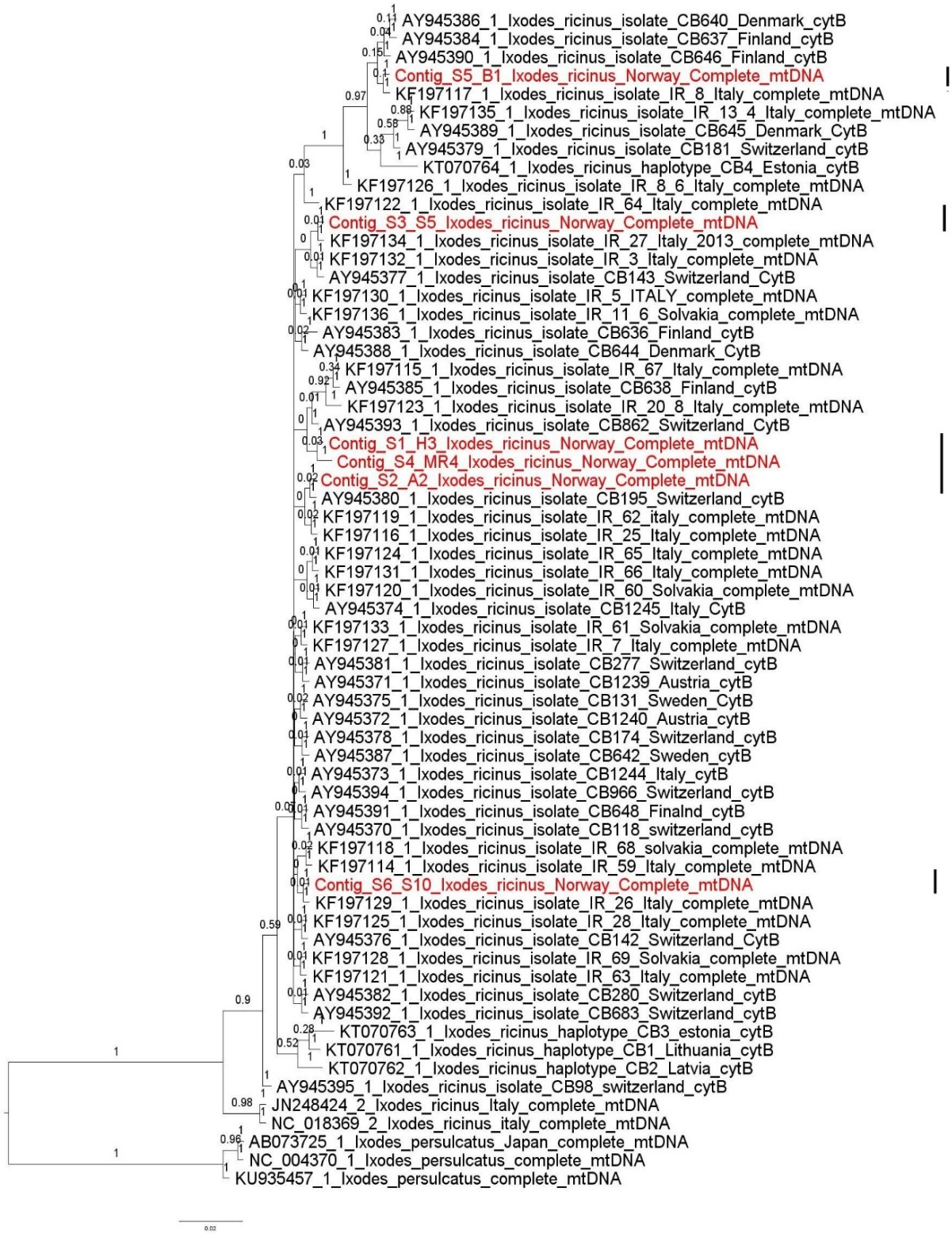


Fig f) Bayesian phylogenetic tree of Cyt B for Norwegian I. ricinus*. The estimates are based on Cyt B mtDNA alignment (ca.10250-10590 bp) under the HKY+G model. The Norwegian sequences are highlighted in the red color. Bayesian posterior probabilities provided at the tree-nodes. The tree was rooted with I. persulcatus.*

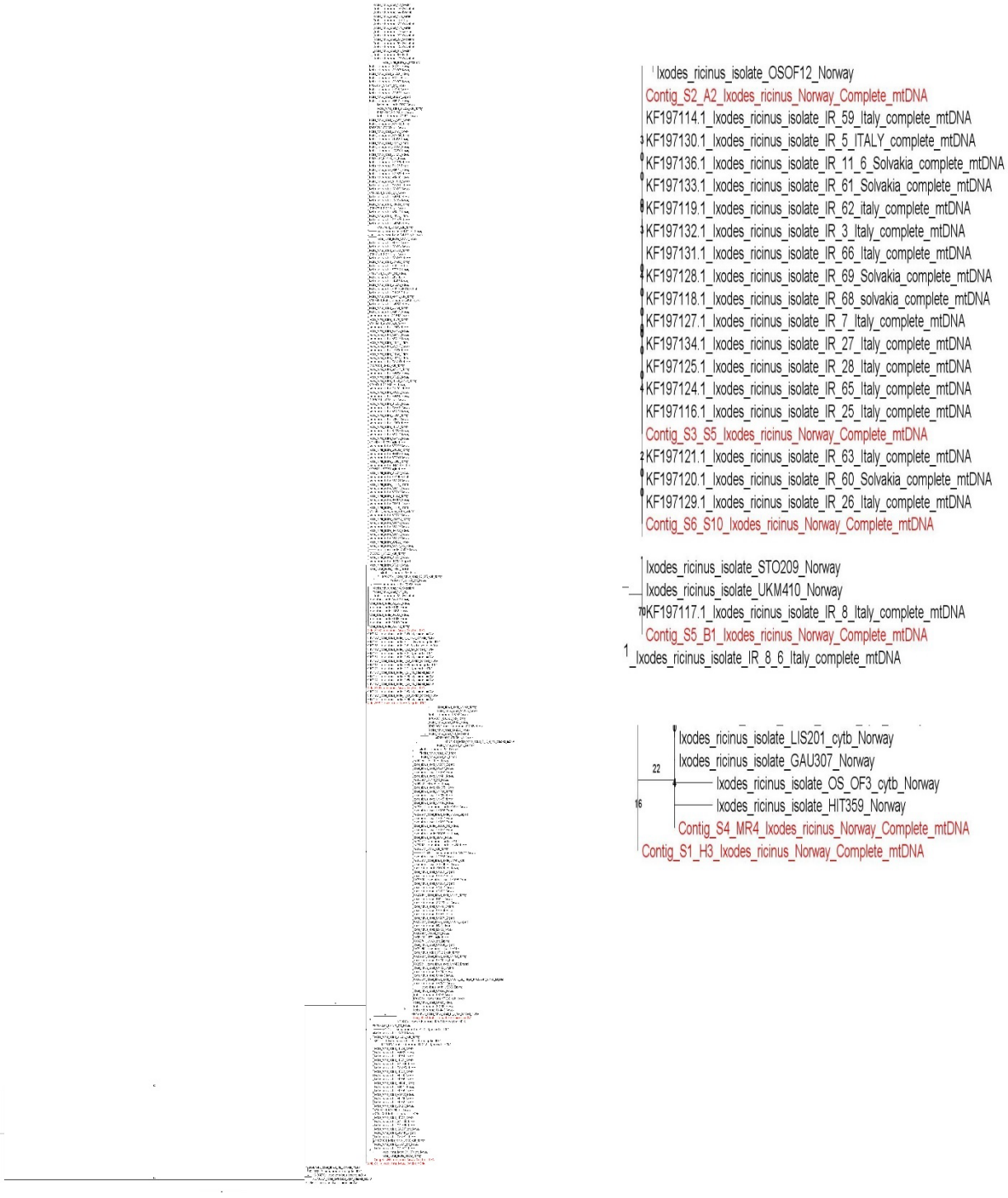


Fig g) Maximum likelihood phylogenetic tree of Cyt B for Norwegian I. ricinus*. The estimates are based on Cyt B mtDNA alignment (ca. 10250-10590 bp) under the GTR+G model. The Norwegian sequences are highlighted in the red color. Bootstrap support values are based on 1000 replicates are indicated above branches. The tree was rooted with I. persulcatus.*

Fig h) Bayesian phylogenetic tree of Cyt B for Norwegian I. ricinus. *The estimates are based on Cyt B mtDNA alignment (ca. 10250-10590 bp) under the GTR+G model. The Norwegian sequences are indicated in the red color and highlighted in box. Bayesian posterior probabilities provided at the tree-nodes. The tree was rooted with I. persulcatus.*

Fig i) Maximum likelihood phylogenetic tree of CR for Norwegian I. ricinus. *The estimates are based on control region mtDNA alignment (ca. 14000-14490 bp) under the GTR+I+G model. Bootstrap support values are based on 1000 replicates are indicated above branches. The Norwegian sequences are highlighted in the red color. The tree was rooted with I. persulcatus.*

Fig j) Bayesian phylogenetic tree of CR for Norwegian I. ricinus. *The estimates are based on CR region mtDNA alignment (ca. 14000-14490 bp) under the GTR+I+G model. The Norwegian sequences are highlighted in the red color. Bayesian posterior probabilities provided at the tree-nodes. The tree was rooted with I. persulcatus.*

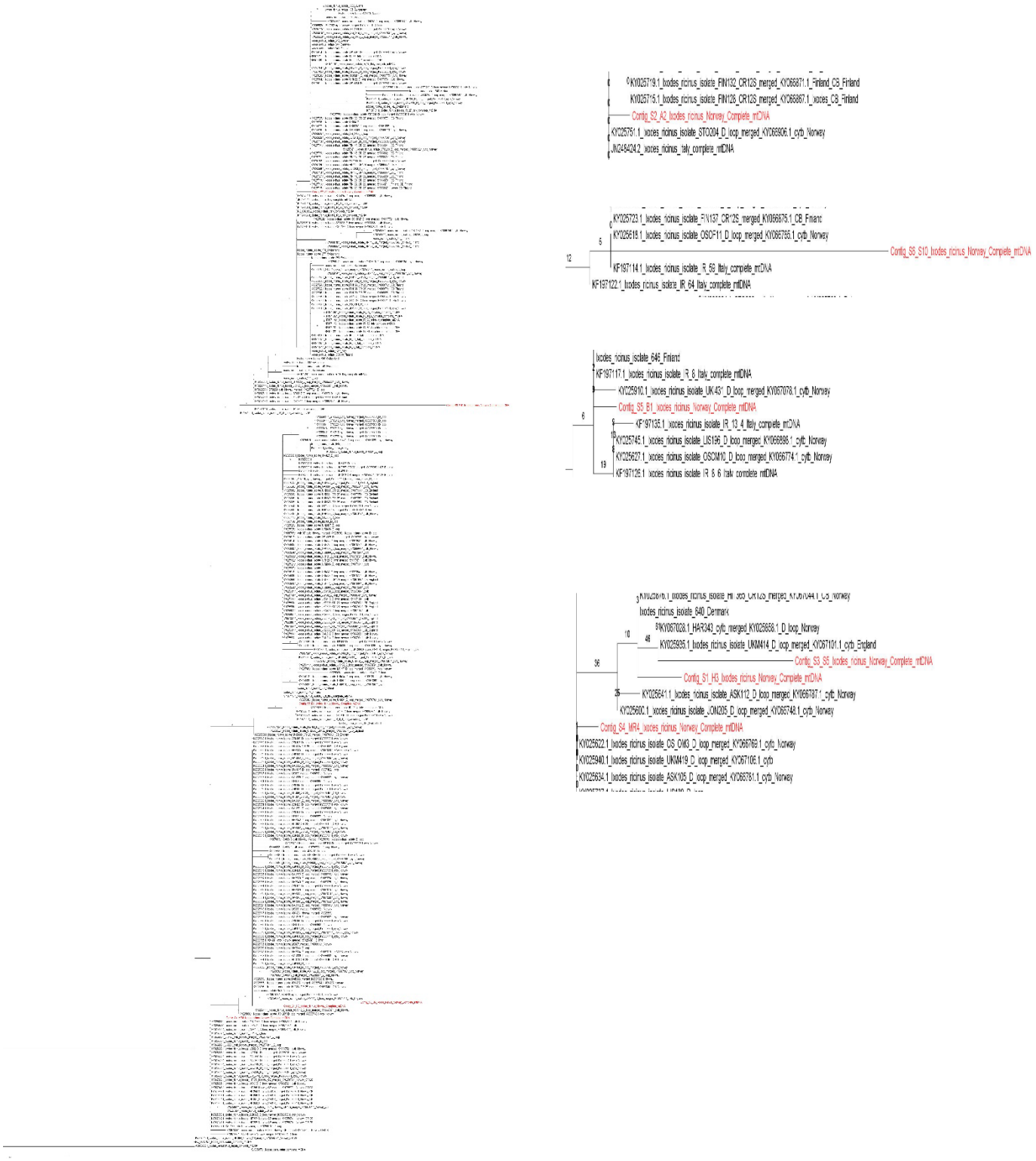


Fig k) Maximum likelihood phylogenetic tree of CR for Norwegian I. ricinus. *The estimates are based on control region mtDNA alignment (ca. 14000-14490 bp) under the GTR+I+G model. Bootstrap support values are based on 1000 replicates are indicated above branches. The Norwegian sequences are highlighted in the red color. The tree was rooted with I. persulcatus.*

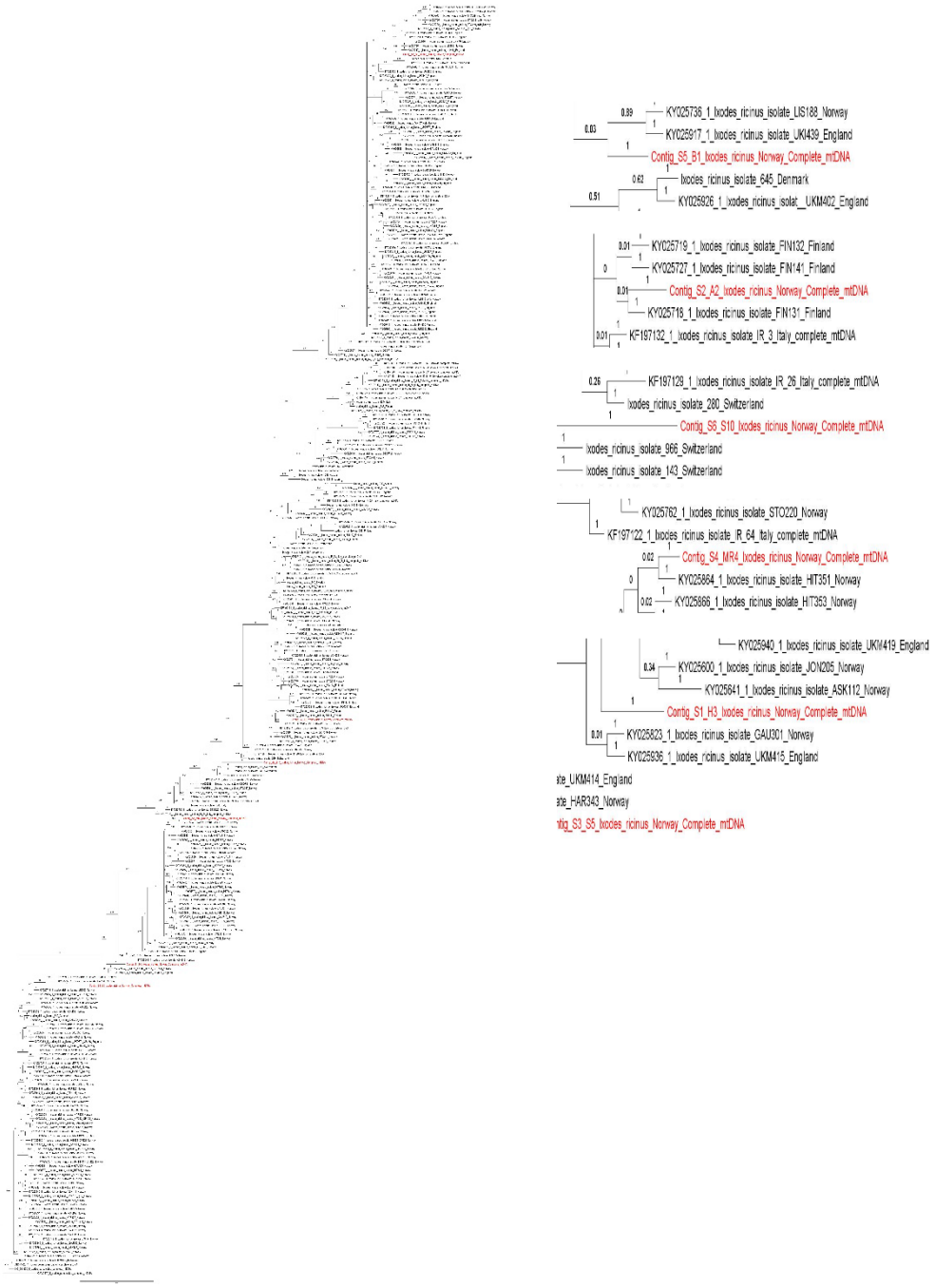


Fig l) Bayesian phylogenetic tree of CR for Norwegian I. ricinus. *The estimates are based on control region mtDNA alignment (ca. 14000-14490 bp) under the GTR+I+G model. The Norwegian sequences are indicated in the red color highlighted in box region. Bayesian posterior probabilities provided at the tree-nodes. The tree was rooted with I. persulcatus.*

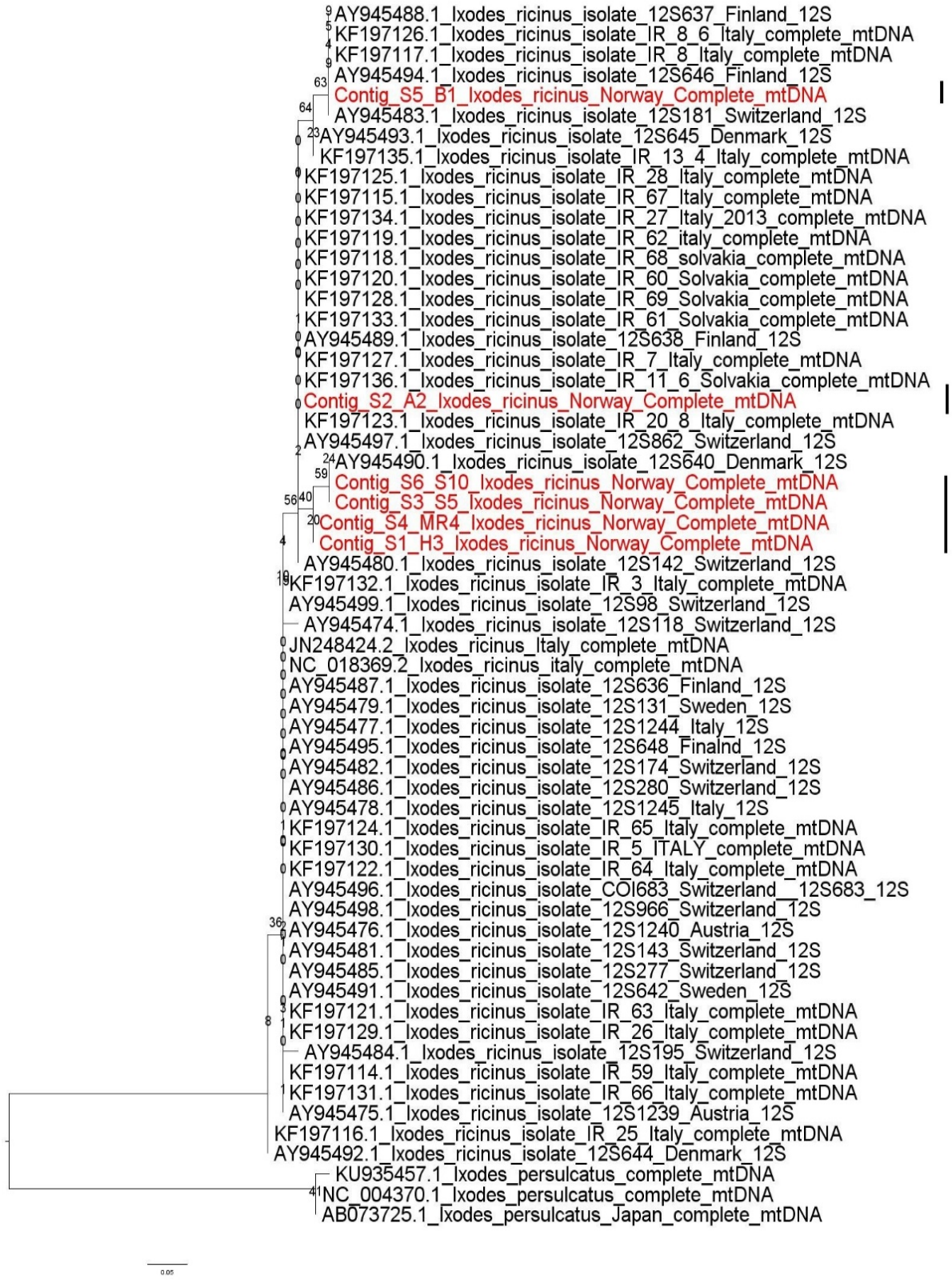


Fig m) ML phylogenetic tree of 12s for Norwegian I. ricinus. *The estimates are based on 12S mtDNA alignment (ca. 13500-13800 bp) under the HKY+I model. Bootstrap support values are based on 1000 replicates are indicated above branches. The Norwegian strains are indicated in red color. The tree was rooted with I. persulcatus.*

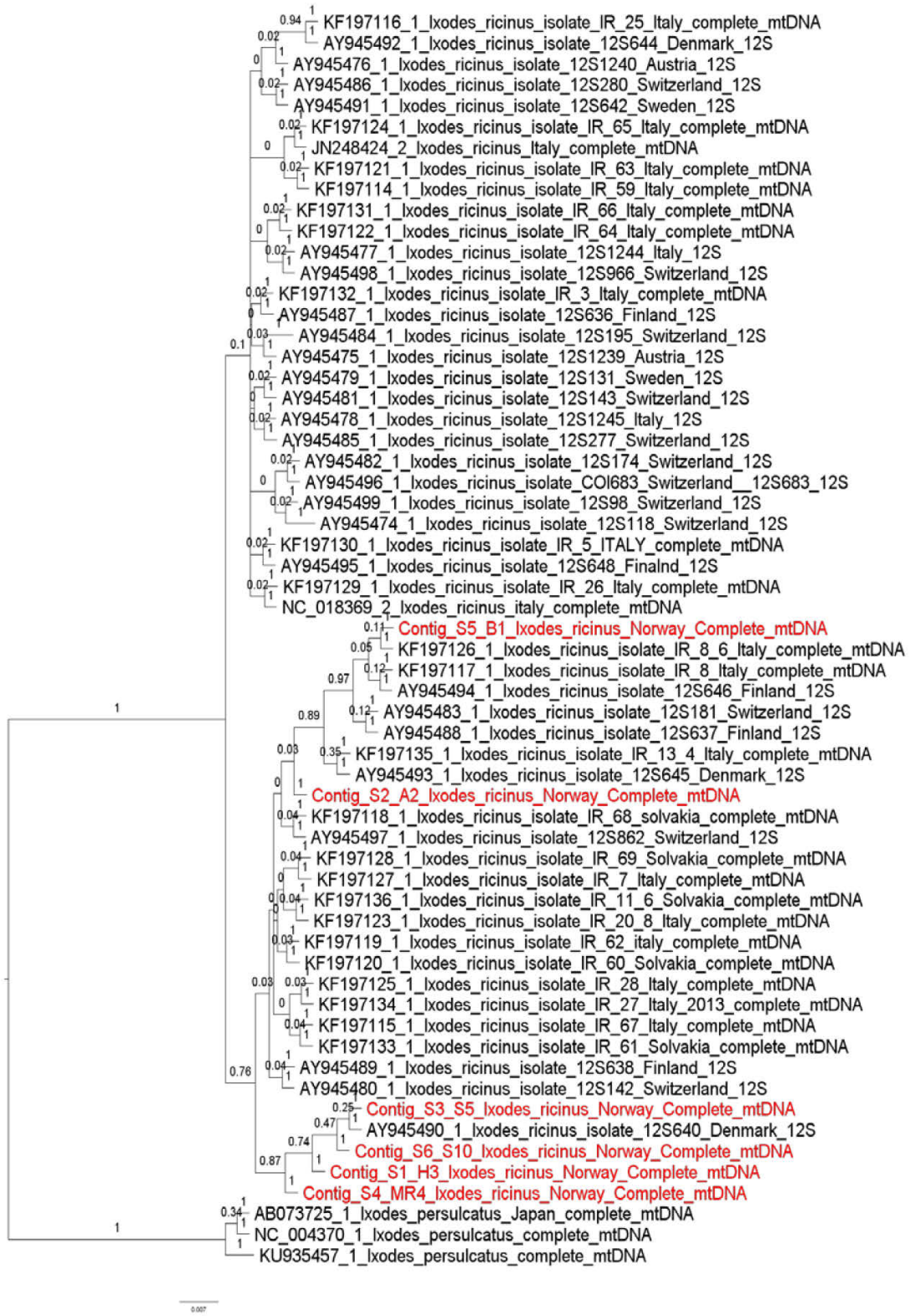


Fig n) Bayesian phylogenetic tree of 12s for Norwegian I. ricinus. *The estimates are based on 12S mtDNA alignment (ca. 13500-13800 bp) under the HKY+I model. The Norwegian strains are indicated in red color. Bayesian posterior probabilities provided at the tree-nodes. The tree was rooted with I. persulcatus.*

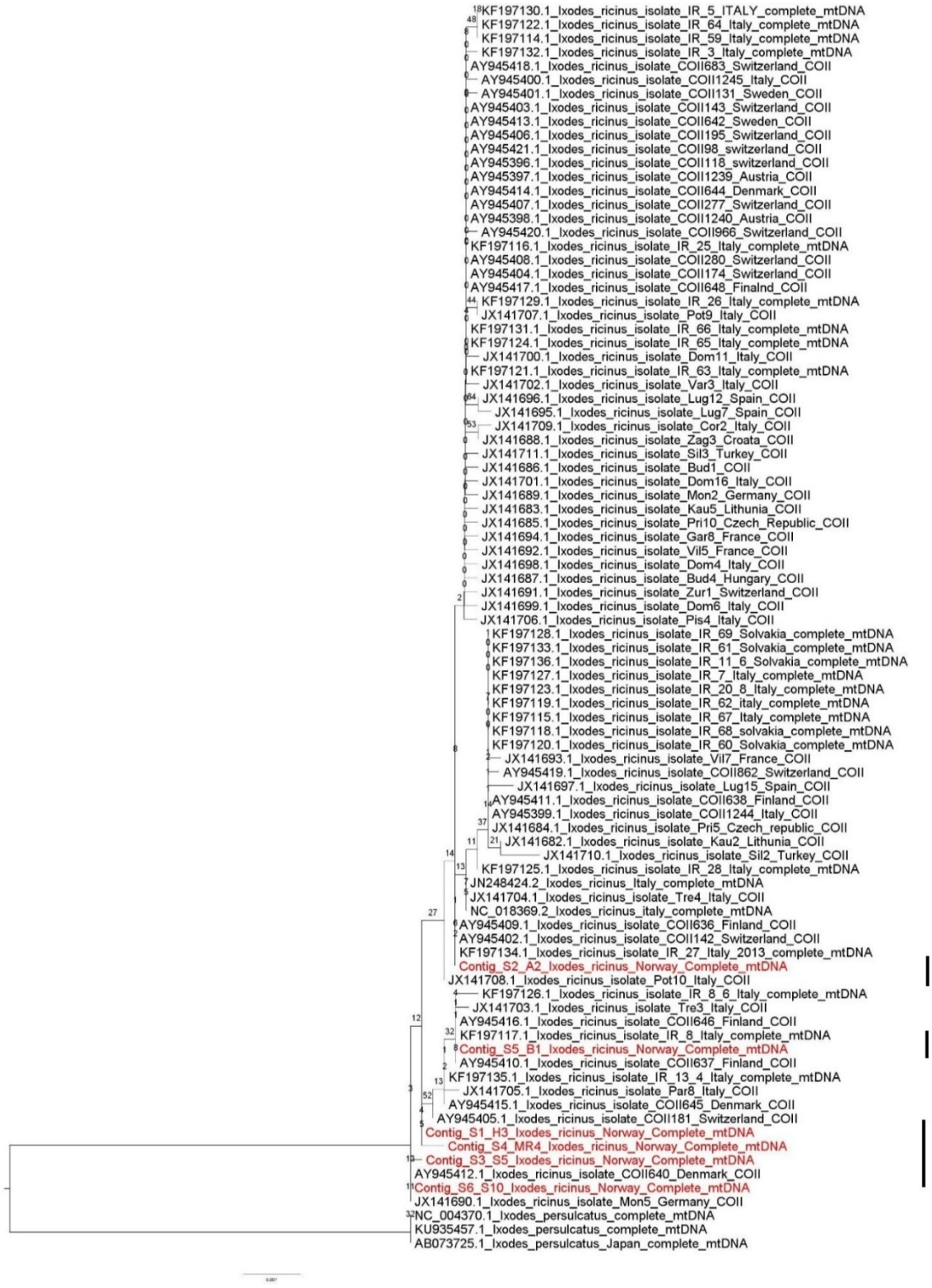


Fig o) ML phylogenetic tree of COII for Norwegian I. ricinus. *The estimates are based on COII mtDNA alignment (ca. 2800-3550 bp) under the GTR+G model. Bootstrap support values are based on 1000 replicates are indicated above branches. The Norwegian strains are indicated in red color. The tree was rooted with I. persulcatus.*

Fig p) Bayesian phylogenetic tree of COII for Norwegian I. ricinus. *The estimates are based on COII mtDNA alignment (ca. 2800-3550 bp) under the GTR+G model. Bayesian posterior probabilities provided at the tree-nodes. The Norwegian strains are highlighted in red color. The tree was rooted with I. persulcatus.*

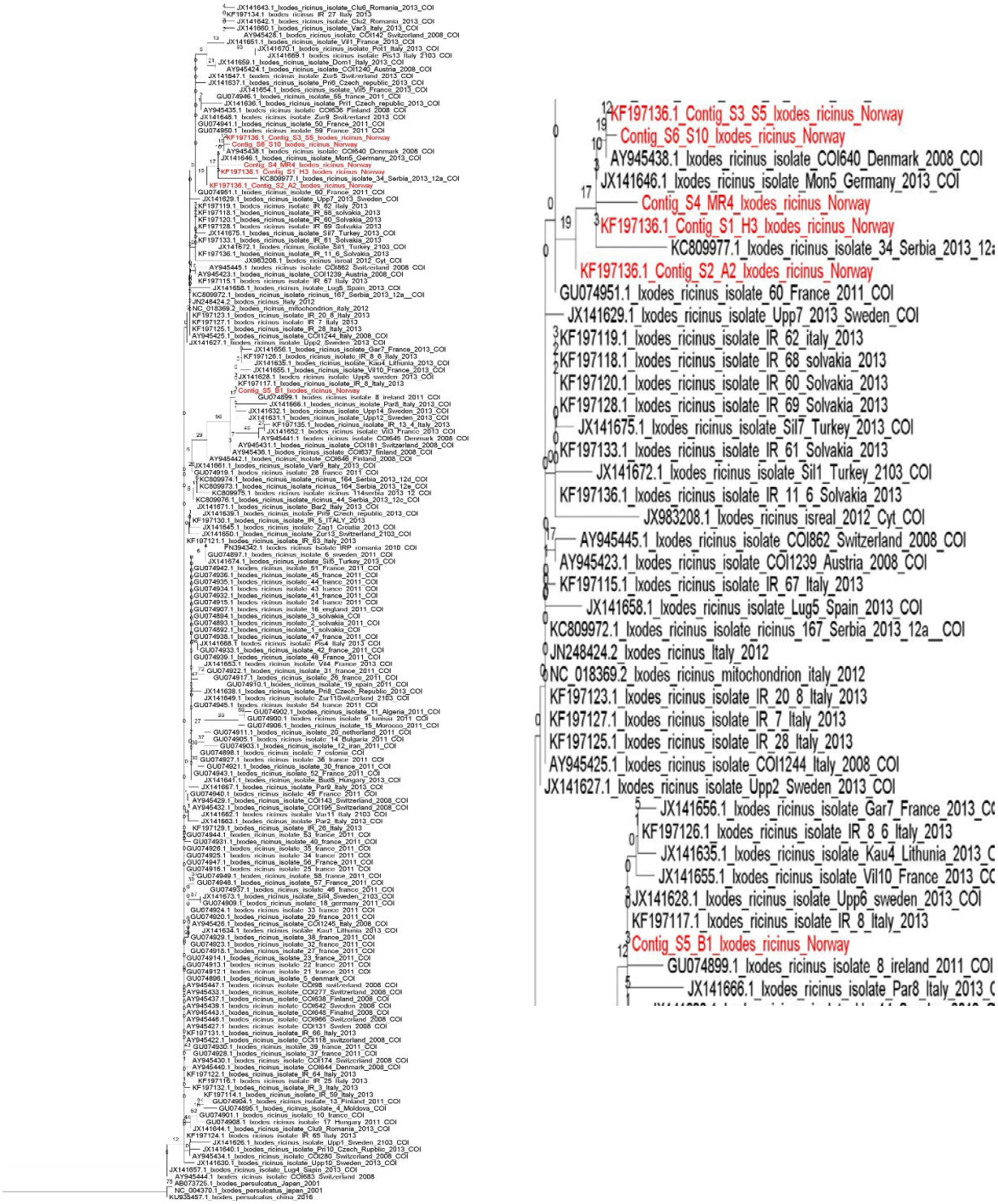


Fig q) ML phylogenetic tree of COI for Norwegian I. ricinus. *The estimates are based on COI mtDNA alignment (ca. 1250-2750 bp) under the GTR+G model. Bootstrap support values are based on 1000 replicate are indicated above branches. The Norwegian strains are indicated in red color highlighted in box next to tree. The tree was rooted with I. persulcatus.*

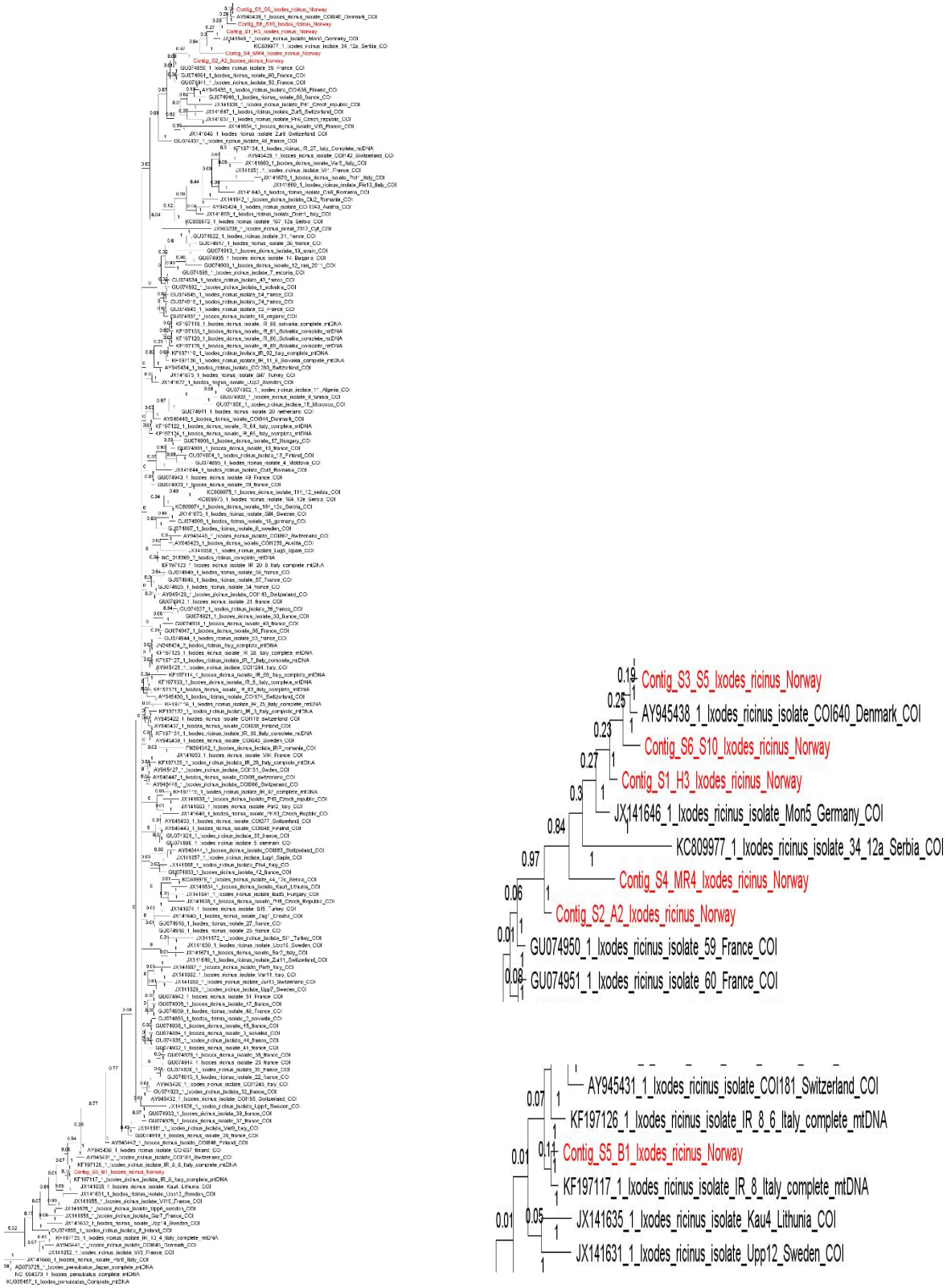


Fig r) Bayesian phylogenetic tree of COI for Norwegian I. ricinus. *The estimates are based on COI sequences alignment (ca. 1250-2750 bp) under the GTR+G model. Bayesian posterior probabilities provided at the tree-nodes. Norwegian sequences are highlighted in the box. The tree was rooted with I. persulcatus.*