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Prevalence and phylogeny of *Borrelia burgdorferi* s.l.-infected ticks in central and southern Sweden

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Abstract
Since monitoring efforts began in the 1980s, reported cases of Lyme Borreliosis have been increasing throughout Sweden. Prevalence studies seek to measure changes in the amount of *Borrelia* found in collected ticks. In this study, nearly 900 adult ticks were collected in central and southern Sweden and tested for the presence of *Borrelia burgdorferi* s.l. in hopes of both determining the prevalence of Lyme Borreliosis in Sweden's ticks as well as of using DNA sequence data to determine the path of movement of the disease, based on phylogenetic relationships. No conclusive results were found but efforts continue to finish this research.

Introduction
Lyme Borreliosis (LB) yields at least ten thousand reported cases in Sweden annually and gives one of the highest incidence rates of the disease per population of any European country (Lindgren 2006). The disease is caused by the spirochete *Borrelia burgdorferi* s.l. and is known to be transmitted primarily through the bite of an *Ixodes ricinus* tick (Jaenson *et al.* 2009; Burri *et al.* 2007). Symptoms vary but may include a ring-shaped rash surrounding the tick bite (erythema migrans), achiness or fatigue, fever, and other flu-like symptoms (Smittskyddsinstitutet 2008; Center for Disease Control 2009). In the absence of erythema migrans, diagnosis is typically based on the probability of tick exposure within the month previous to the clinical visit but may be verified with blood tests in advanced cases. Treatment with doxycycline is effective in reducing the effect of the bacteria in the bloodstream over four weeks of prophylactic therapy. However, if the disease is left untreated or caught when symptoms include central nervous system complications, antibiotics such as penicillin or ceftriaxone are recommended (Wormser *et al.* 2006).

Climate change, increased time spent in tick-dense environments (partly attributed to an increase in tick-friendly environments, such as woodland areas), and increased public and clinical awareness of the disease are some of the factors proposed to play a role in the rise of reported Lyme Borreliosis cases in Sweden (Parola *et al.* 2001).

Despite an increase in reported Lyme Borreliosis cases, little is known about the prevalence of *B. burgdorferi* s.l. in *I. ricinus* ticks in Sweden in general (Jaenson *et al.* 2009). Furthermore, few studies have addressed the relationship of different *B. burgdorferi* s.l. populations at different sample locations in Sweden, although studies examining the relationship between European and American populations of the spirochete have emerged in recent years (Marconi *et al.* 1992; Foretz *et al.* 1997; Bunikis *et al.* 2004; Margos *et al.* 2008). Therefore, the purpose of this study is to provide answers to these questions using adult ticks collected from May to September 2008.

Materials and Methods
TICK COLLECTION. Drag sampling using a white flannel cloth of surface area 1 m^2 was used to collect host-seeking *I. ricinus* ticks at 25 locations in central and southern Sweden. The cloth was drawn along the ground for 10 meters, flipped, and all ticks were removed and placed in separate tubes for nymphs, adult females, and adult males. All tubes were stored at -20°C.

TICK PREPARATION. In the P3-Lab at Smittskyddsinstitutet, Stockholm, the tissue-lyser was used to lyse 1 tick in 400 µl PBS in a 2 ml tube. All lysed ticks were stored on ice and the supernatant removed to a new 1.5 ml tube containing 800 µl TriPure Isolation Reagent (Roche Applied Science, Indianapolis,

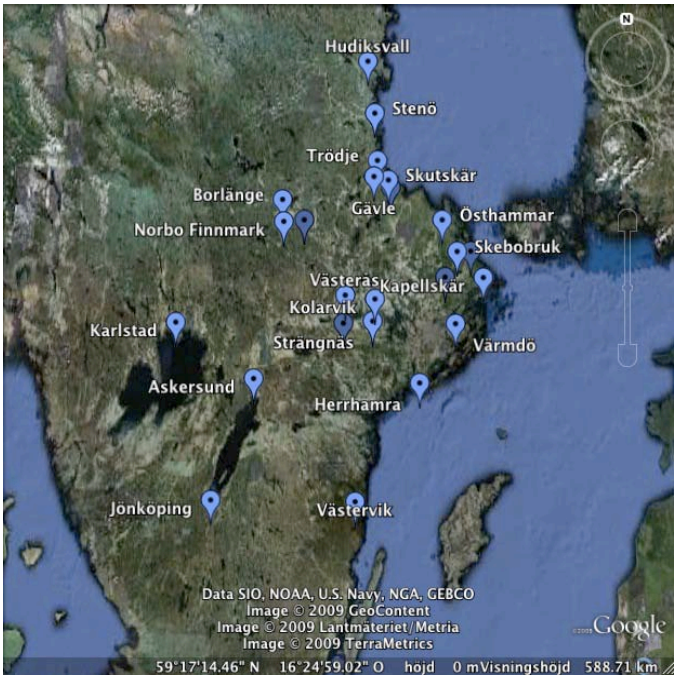

Figure 1. Tick sample locations across southern and central Sweden.

USA) and 2 µl glycogen. The remaining lysate was moved to another new 1.5 ml tube to serve as a spare and all tubes were stored at -70°C until further use.

RNA/DNA SEPARATION. The prepared samples were removed from -70°C and incubated for 5 minutes at room temperature. Two hundred microliters chloroform was added to each sample and they were vortexed for 15 seconds before incubating for 15 minutes at room temperature. The samples were then centrifuged for 15 minutes at 4°C and 14 000 RPM. The upper RNA-containing phase was moved to another new tube already containing 600 µl isopropanol, inverted several times, and kept at -70°C until further use. The remaining tubes (from centrifugation and now containing DNA-TriPure/glycogen solution) were moved to -20°C until further use.

DNA EXTRACTION. Frozen prepared samples were incubated for 5 minutes at room temperature on trays of 18 samples at a time. Three hundred microliters EtOH (95% EtOH for samples 1-500, 99.5% EtOH for samples 501-988) were added to each sample. The samples were vortexed for 2 seconds and precipitated for 15 minutes at room temperature before being centrifuged for 5 minutes at +4°C and 8000 RPM. The supernatant was discarded and 800 µl of 0.1 M sodium citrate in 10% EtOH was added to each sample. The samples were again vortexed for 2 seconds and centrifuged for 5 minutes at +4°C and 8000 RPM. The supernatant was discarded and the samples allowed to air-dry with open lids until completely dry (for at least 60 minutes). Once dry, the DNA pellet of each sample was dissolved in 50 µl 8 mM NaOH + 5 µl 0.1 M HEPES. Twenty microliters of each sample was relocated into separate 1.5 ml tubes for *Rickettsia* analysis by a different lab and the remaining DNA-solution was stored at -20°C.

BORRELIA BURGDORFERI S.L. SCREENING. A nested reverse transcriptase Polymerase Chain Reaction (PCR; GeneAmp PCR System 9700, Applied Biosystems, Carlsbad, USA) was performed on all extracts using protocol provided by J. Bunikis *et al.* (Bunikis *et al.* 2004). Primers from the same article were used. The first PCR targeted the chromosomal intergenic spacer (IGS) region between the 16S rRNA (*rrs*) gene and the first 23S rRNA (*rrlA*) gene of *Borrelia burgdorferi* s.l. using primers F and R. Autoclaved water was used as the negative control and a *B. burgdorferi* s.s. B31 sample provided by Tobias Jakobsson of Sven Bergström's laboratory (Umea Universitet, Umeå, Sweden) was diluted in 300 µl 1xTE buffer for use as the positive control.

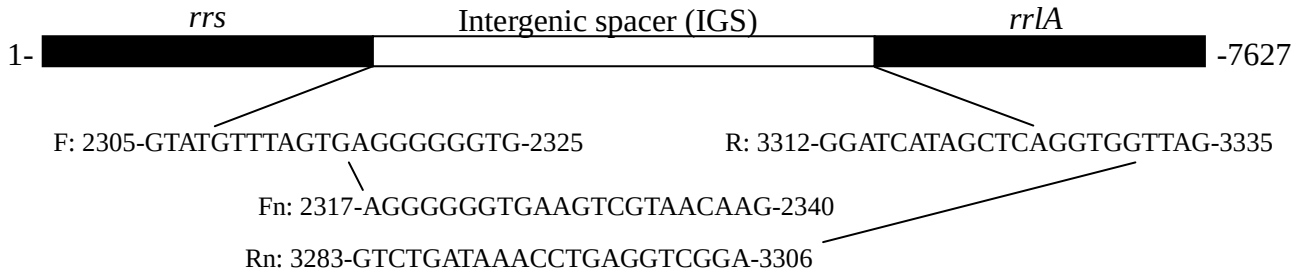

Figure 2. Partial B. burgdorferi *rDNA operon with relative locations of first PCR primers (F, R) and second PCR primers (Fn, Rn) included.*

The second PCR protocol used primers Fn and Rn and was designed to further amplify an increasingly restricted sequence of known *B. burgdorferi* s.l. DNA. An additional negative control using only the PCR cocktail was also used.

Each PCR product from the second PCR was run on a gel to check for the presence of *B. burgdorferi* s.l. A 1.5% agarose gel (150 ml 1xTAE buffer plus 2.2 g agarose) was prepared using standard procedure and gels were run at 149 V with a corresponding positive control, negative control, and DNA ladder each time. A ratio of 2 µl loading dye for every 3µl product was used

(here, 3.3 µl loading dye for 5 µl product). Completed gels were analyzed after exposure to EtBr for at least ten minutes. All positive samples were subjected to a second identical nested reverse transcriptase PCR to confirm that they were positive for *B. burgdorferi*.

Purification of the positive PCR products was performed using Qiagen's QIAquick PCR Purification Kit and its standard protocol for purification using a microcentrifuge (Appendix II). The DNA concentrations for each sample were determined using GeneQuant II and standard protocol (Appendix II).

All purified samples were sent to Macrogen Inc. (Seoul, Korea) for DNA sequencing.

ANALYSIS. Sequences were prepared for alignment using the Staden Package. Alignment was performed using MUSCLE via SeaView with default settings (Edgar, 2004; Galtier *et al.* 1996). The final tree was compiled using Mr.Bayes.

Results

PREVALENCE. A total of 21 adult ticks from 10 different sampling locations were found to be positive for *B. burgdorferi* s.l. by PCR analysis (Figure 3). This gives an overall prevalence of 2.38% (21 of 879 adult ticks) for all sampled locations. Of the 10 locations found to have *Borrelia*-infected *I. ricinus* ticks, Karlstad showed the highest prevalence with 25% (2 of 8) infected, followed by Askersund with 20% (1 of 5) and Hudiksvall with 9.09% (1 of 11). Stenö showed the lowest prevalence at 1.1% (2 of 182). (See Table 1.)

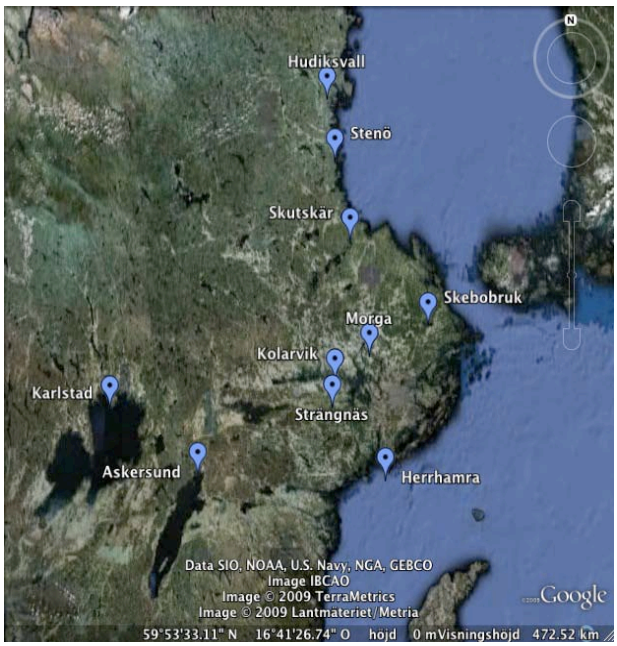

Figure 3. Location of samples shown to be positive by PCR. Of these 10 locations, samples from 4 locations (Karlstad, Herrhamra, Stenö, and Hudiksvall) were able to be considered for phylogenetic analysis.

Location	Prevalence % (positive/total)
Askersund	20 % (1/5)
Herrhamra	7.04 % (5/71)
Hudiksvall	9.09 % (1/11)
Karlstad	25 % (2/8)
Kolarvik	1.87 % (2/107)
Morga	4.44 % (4/90)
Skebobruk	3.13 % (1/32)
Skutskär	3.85 % (1/26)
Stenö	1.1 % (2/182)
Strängnäs	2.04 % (1/49)

Table 1. Percent prevalence of PCR-positive adult ticks by location. Overall prevalence was 2.38% (21/879); 11 locations yielded no positive results (0%).

PHYLOGENY. Of the 21 cleaned PCR products from adult ticks, only 8 samples provided sufficient data following sequencing to be used in phylogenetic analyses. Of those 8 samples, 1 sample was shown to be most similar to *Ixodes scapularis* (a tick) by BLAST analysis and therefore was excluded from phylogenetic analyses. In total, 7 samples were considered. Figure 4 gives the tree created using sequence data for the aforementioned 7 samples, with Mr.Bayes (2 000 000 generations). Bootstrap values can be found on the branches.

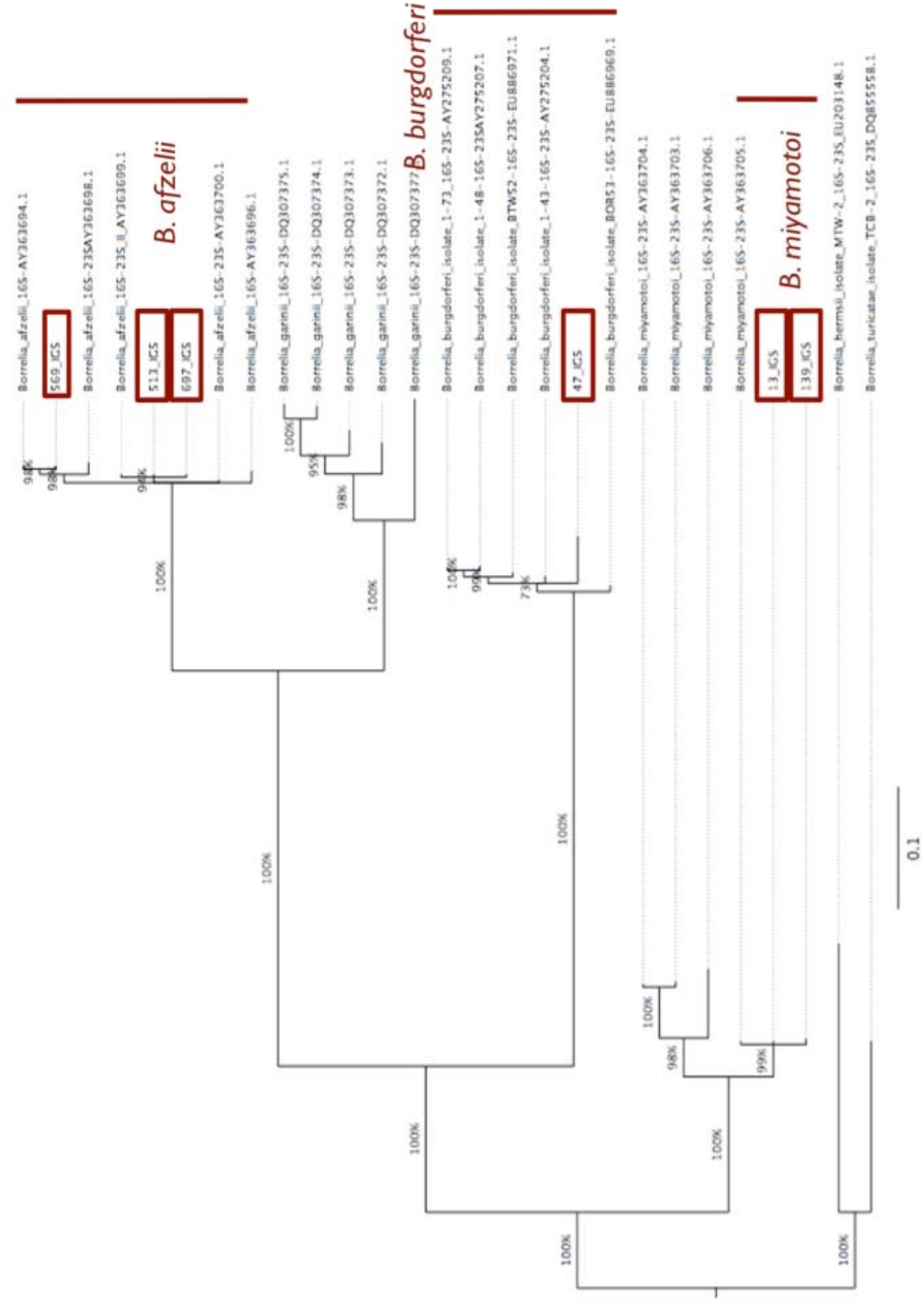

Figure 4. Tree constructed with 7/21 positive samples (boxed in red and labeled according to corresponding Borrelia *species, based on BLAST analysis) using Mr.Bayes (2 000 000 generations). The species* B. hermsii *and* B. turicatae *were used to root the tree.*

Discussion

Current prevalence results. A total of 21 of 879 adult ticks were identified as positive for *Borrelia burdorferi* s.l. by PCR analysis and prepared for sequencing. Of those 21 ticks, 8 ticks provided enough genetic material for sequencing. All DNA sequences obtained were compared to previously published genetic sequences using BLAST analysis and 1 of the sequenced samples was shown to be most similar to *Ixodes scapularis*, a tick. The remaining 7 sequenced samples were used to create a tree in the program Mr.Bayes (2 000 000 generations) and found to be related to either *B. afzelii*, *B. burgdorferi*, or *B. miyamotoi*.

The aims of this study were to determine the prevalence of *B. burgdorferi* in *Ixodes* ticks collected in central and southern Sweden and use sequence analysis to determine the phylogenetic relationships among the *Borrelia* detected. Neither of these aims was accomplished in full, although some positive *Borrelia* samples were found and sequenced. The prevalence was determined to be 21/879, or 2.28%. This is significantly lower than the expected 15-20% prevalence (according to previous studies). Difficulties with the DNA extraction and/or with the PCR procedure may have led to these unexpected findings. Inconsistent results were observed throughout the project, with the PCR seeming to "work" because the positive control showed results, but only in conjunction with an unexpectedly low number of samples that also showed positive results. Basic PCR troubleshooting attempts – including using different machines, buying new primers and new materials, optimizing the protocol to our equipment, and using higher concentrations of original sample – were performed during and after the course of the project work; nothing seemed to provide consistent results. Also, it was observed that the first PCR of the nested PCR procedure yielded a greater number of positive results, which then disappeared during the second PCR. This was unexpected and we are continuing to hypothesize what may have caused this. Due to our inconclusive results, further efforts to complete this research will continue (please see "proposed continuation of study" below).

We have hypothesized several reasons that may account for our difficulties with this study. First, it is possible that the inconsistencies in our results stem from inconsistencies with the amount of DNA in each sample, since each was extracted from homogenized ticks and therefore, a huge amount of material (from both tick and otherwise, not limited to *Borrelia*) was present in the small aliquots used for analysis. These "dirty" original samples could explain why only some samples worked, or why some samples worked only some of the time (cleaner? Greater amount of *Borrelia*? Lucky?). Another reason could be unspecific amplification. During my thesis work in Stockholm, one of the doctoral students, who was trying to check if certain colonies of bacteria were positive for a specific ampicillin-resistance insert, noticed that his PCR amplifications were showing positive results even when the bacteria refused to grow in media containing ampicillin. It was concluded that one reason for this could be unspecific binding, since the bacteria colonies he picked contained a large amount of DNA and some of that may have interacted with the primers to reveal bands which did not indicate what he hoped. It is possible that a similar event is occurring in our experiments, particularly since we observed higher potential positives during the first PCR, which disappear during the second. We also wondered, after observing positive results in the first PCR which disappeared in the second, if the second PCR failed due to primer design; a portion of both forward primers of the nested PCR overlapped, and may cause a higher ratio of forward primer to reverse primer during the second PCR reaction. This could potentially disrupt the outcome of the second PCR procedure.

Another area of potential difficulty is the DNA extraction. The original homogenization of the ticks was followed by RNA extraction so that each sample could be examined for the presence of the tick-borne encephalitis (TBE) virus. Therefore, a different procedure was used for DNA extraction than performed in the paper, based on the RNA extraction that had been previously performed. Recent conversations with the company that provided the DNA extraction kit and protocol that was

used revealed that another reason for our difficulties could be that the *Borrelia* DNA was not extracted in all cases. During the project, we had verified that DNA was present in our samples via random sampling but the company representative informed us that perhaps the DNA that was present was largely tick DNA and not bacterial DNA. We do not know a way for us to economically verify this.

Despite an apparent failure of this project, much was learned over its official 10 weeks and beyond. For example, I discovered that science takes time and patience, that even the simplest things can go wrong, and that an organized lab notebook is imperative, among other lessons. Also, our few results also show some exciting findings, such as the presence of *B. miyamotoi* in our samples. *B. miyamatoi* is a species of *Borrelia* that originates in Japan and is considered to enter Sweden via Russia. It has only recently been discovered in Sweden, and any indication of its increased presence would certainly be noteworthy.

Unfortunately, we have insufficient data to begin evaluation of the phylogenetic relationships among *Borrelia* in Sweden, but we hope to obtain that data as we continue this work.

Proposed continuation of study. Due to difficulties with this project work, we have been in contact with Dr. Sven Bergström and members of his laboratory of Umeå University. They have performed similar studies with better success. We will visit their laboratory in the last week of May with about 200 of our samples (50 extracted samples, 150 unisolated/unextracted samples) and perform everything from the beginning, using their materials, their machines, and particularly, their protocol for DNA extraction. If this works, we will repeat their procedure in Uppsala with the remainder of our samples.

If our research does not yield conclusive results in Umeå, there are a few remaining paths we can take. It may be possible to re-extract the bacterial DNA from our extracted samples, possibly using plant extraction kits (which utilize Proteinase K, which I am unsure is part of the Tripure procedure) or by repeating the original procedure. Also, if we were able to extract the DNA from our samples, we could also purify the DNA before performing the nested PCR procedure in hopes of decreasing any unspecific binding while increasing the chance for appropriate amplification with our primers. Also, an effort to design new primers or choose primers from a different region of the *Borrelia* DNA could provide better results as well.

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Appendix I

PCR 1 Protocol:

1 µl DNA-template
2.5 µl 10x PCR buffer (Thermo Fisher Scientific Inc., Indianapolis, USA)
2.5 µl $MgCl_2$ (Thermo Fisher Scientific Inc., Indianapolis, USA)
2.5 µl dNTP-mix
2 µl Primer F (5 µM, Sigma-Aldrich, Stockholm): 5'-GTATGTTTAGTGAGGGGGGTG-3'
2 µl Primer R (5 µM, Sigma-Aldrich, Stockholm): 5'-GGATCATAGCTCAGGTGGTTAG-3'
0.2 µl Taq polymerase (5 U/µl, Thermo Fisher Scientific Indianapolis, USA)
10.8 µl Autoclaved water

Cycler program (IGS-PCR):

94°C, 5 minutes
94°C, 30 seconds
56°C, 30 seconds } x 35
74°C, 2 minutes
74°C, 7 minutes
4°C, indefinitely

PCR 2 Protocol:

3 µl PCR-product
2.5 µl 10x PCR buffer (Thermo Fisher Scientific Inc., Indianapolis, USA)
2.5 µl $MgCl_2$ (Thermo Fisher Scientific Inc., Indianapolis, USA)
2.5 µl dNTP-mix
2 µl Primer Fn (5 µM, Sigma-Aldrich, Stockholm): 5'-AGGGGGGTGAAGTCGTAACAAG-3'
2 µl Primer Rn (5 µM, Sigma-Aldrich, Stockholm): 5'-GTCTGATAAACCTGAGGTCGGA-3'
0.2 µl Taq polymerase (5 U/µl, Thermo Fisher Scientific Inc., Indianapolis, USA)
10.8 µl Autoclaved water

Cycler program (IGS Nested-PCR):

94°C, 5 minutes
94°C, 30 seconds
60°C, 30 seconds } x 39
74°C, 2 minutes
74°C, 7 minutes
4°C, indefinitely

Appendix II

Standard QIAquick PCR Purification Kit Protocol:

One hundred microliters of Buffer PBI was added to each sample and mixed. Each sample was transferred to a QIAquick spin column in a 2 ml collection tube and centrifuged for 60 seconds. The flow-through was discarded and the QIAquick column returned to the same tube before 0.75 ml Buffer PE was added to each sample. Again, the samples were centrifuged for 60 seconds, the flow-through discarded, and the QIAquick spin column returned to the same collection tube before being centrifuged an additional time (also for 60 seconds). Then, the QIAquick spin column was placed in

a new 1.5 ml microcentrifuge tube and 30 µl autoclaved water carefully added to the center of each spin column. After 60 seconds, the samples were centrifuged for an additional 60 seconds and the spin columns discarded; the flow-through contained the purified PCR products.

DNA Concentration Determination

A GeneQuant II RNA/DNA Calculator (Pharmacia, Stockholm, Sweden) was used to determine the DNA concentration of each of the cleaned PCR products. Seventy microliters of autoclaved water was used as the reference standard and 3.5 µl cleaned product with 66.5 µl autoclaved water was used for each sample. A path length of 10 mm, wavelength of 320 nm, dilution factor of 1, and dsDNA factor of 50.0 was used for all samples. All samples gave a sufficient DNA concentration (> 6 µg/ml) with the exception of sample 2 (0.0 µg/ml).

Appendix III

Total results on prevalence study, including locations with no positives determined.

Location	Total Adult Ticks	Total (Potential) Positive Adult Ticks	Prevalence (%)
Askersund	5	1	20 %
Borlänge	8	0	0
Eskilstuna	11	0	0
Gävle	1	0	0
Herrhamra	71	5	7.04 %
Hudiksvall	11	1	9.09 %
Jönköping	3	0	
Kapellskär	130	0	
Karlstad	8	2	25 %
Kolarvik	107	2	1.87 %
Morga	90	4	4.44 %
Norbo Finnmark	13	0	0
Rimbo	6	0	0
Skebobruk	32	1	3.13 %
Skutskär	26	1	3.85 %
Stenö	182	2	1.1 %
Strängnäs	49	1	2.04 %
Trödje	4	0	0
Vikmanshyttan	6	0	0
Väddö	17	0	0
Värmdö	9	0	0
Västervik	9	0	0
Västerås	35	0	0
Älvkarleby	21	0	0
Östhammar	25	0	0
TOTAL	*879*	*20*	*2.28 %*

Appendix IV

Project Evaluation

The aims of this project were to determine the prevalence and phylogenetic relationships among *B. burgdorferi* s.l.-infected adult ticks in about 900 samples collected from central and southern Sweden. Ten weeks were provided for DNA extraction, PCR analysis, and sequence analysis of all samples; this was an ambitious but possible goal if only minor problems with the PCR analysis had been experienced. However, since we had a lot of difficulty, neither aim was completed in full by the time of the presentation.

Work on this project continued in the evenings and on the weekends and eventually culminated five months later with a week-long trip to Sven Bergström's laboratory at Umeå University, Umeå, Sweden. Much of the same trouble-shooting that I had performed in Uppsala while completing my thesis work in Stockholm was also performed in Umeå; however, our focus in Umeå was limited to the samples known to positive by sequence analysis. As in Uppsala, it was concluded in Umeå that new primers targeting different regions of DNA should be used, as well as RT-PCR (if it became an affordable option). Work will continue most likely in August. We continue to hope that this project will work, though the Umeå laboratory is concerned that nucleases or something similar may be the problem and therefore that we may not have enough DNA in our samples to identify which are positive for *Borrelia*.

Recommendations for improvement/continuation of this project were provided in full in the discussion portion of this report. In short, I recommend that new primers be used, and possibly multiple sets of primers. This is because the IGS sequences of any *Borrelia*-positive samples may be too variable to be detected using PCR, particularly if DNA concentrations are low or if there is nuclease activity. I believe that using multiple sets of primers targeting different areas of the bacteria's genome would provide the greatest chance of success. If RT-PCR is an affordable option, I also recommend that such analysis be performed first so that which samples to focus on could be determined. However, I am not sure that I would wait with the samples until August in order to do this but rather would try a few of the known *Borrelia*-positive samples with different primers. One final suggestion would be to use tick primers to check to see if any DNA can be detected by PCR in our samples; this is something that I think has been missing from our experiments in the past.