Studies on Genetic Diversity of Spotted Fever Group Rickettsiae in Ixodid Ticks in Japan

（日本産マダニが保有する紅斑熱群リケッチアの遺伝的多様性に関する研究）

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Notes

The contents of chapter I have been submitted in Scientific Reports.

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ABBREVIATIONS

16S rRNA	16S ribosomal RNA
%	percentage
µL	microliter
AG	ancestral group
bp	base pair
°C	degree Celsius
DDBJ	DNA data bank of Japan
DNA	deoxyribonucleic acid
DMEM	Dulbecco's Modified Eagle Medium
JSF	Japanese spotted fever
gltA	citrate synthase gene
htrA	17-kDa common antigen gene
kDa	kilo Dalton
min	minute
No.	number
nM	nanomolar
ompA	outer membrane protein A gene
ompB	outer membrane protein B gene
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
RNA	ribonucleic acid
sca	surface cell antigen gene
SFG	spotted fever group
sec	second
TG	typhus group
TRG	transitional group
USA	The United States of America
V	voltage

GENERAL INTRODUCTION

The term *Rickettsiae* represents a group of microorganisms in the genus *Rickettsia* within the family Rickettsiaceae in the order Rickettsiales that belongs to the class Alphaproteobacteria (Raoult and Roux, 1997). The genus *Rickettsia* was named after Howard Taylor Ricketts, who died from murine typhus during the study of *Rickettsiae*. Rickettsiosis is one of the oldest vector-borne infectious diseases as it was evident as early as the end of 16th century (Raoult and Roux, 1997). Originally, *Rickettsia* was considered as a virus or an unknown organism because of the small size and variable morphology, and it was not easy to identify them under the light microscope (Wolbach, 1919; Parola et al., 2005). *Rickettsia* is a short rod-shaped with 0.3 to 0.5 × 0.8 to 2.0 µm in size and has a typical Gram-negative cell wall on the outside of a thin cytoplasmic membrane. Within the host cell, *Rickettsia* is surrounded by a slime layer or little capsular material built up by lipopolysaccharide, characterized as a halo around the bacterium when imaged by electron microscopy (Winkler, 1990; Parola et al., 2005; Raoult et al., 2005).

The members of the genus *Rickettsia* are divided into four main groups: the spotted fever group (SFG), typhus group (TG), transitional group (TRG), and ancestral group (AG) (Gillespie et al., 2007). SFG and AG rickettsiae are mainly associated with ticks, while TG and TRG rickettsiae are associated with other arthropods such as lice, fleas, and mites. TG is composed of *Rickettsia typhi* and *Rickettsia prowazekii*, while TRG is composed of *Rickettsia akari*, *Rickettsia australis*, and *Rickettsia felis*. Among the tick-borne rickettsiae, AG includes *Rickettsia bellii* and *Rickettsia canadensis*. More than 25 species of tick-borne rickettsiae that have been validated so far belong to SFG. Furthermore, the members

of SFG rickettsiae have been increasing as many new species have been proposed recently (Karpathy et al., 2016; Abdad et al., 2017; Dall'Agnol et al., 2017; Lee et al., 2017; Moreira-Soto et al., 2017).

More than a century ago, the taxonomy of *Rickettsia* has been based on the comparative studies of morphology, antigenic and physiological characters (Fournier and Raoult, 2009). However, the studies of morphological and physiological variation are inadequate to classify all the members of the genus *Rickettsia*. Phylogenetic analysis inferred from the sequences of the 16S ribosomal RNA (rRNA) gene enabled the reclassification of rickettsiae (Weisburg et al., 1991; Raoult and Roux, 1997). Additionally, the outer membrane protein family genes such as outer membrane protein A gene (*ompA*), outer membrane protein B gene (*ompB*) and surface cell antigen-4 gene (*sca4*) have been used to further refine the classification of rickettsiae on the basis of their sequence similarities between rickettsial species (Blanc et al., 2005; Fournier and Raoult, 2009; Phan et al., 2011). The *ompA* is specific to SFG rickettsiae but not found in all the members of genus *Rickettsia* (Fournier et al., 2003), while the *ompB* is retained in both SFG and TG group rickettsiae and has functions associated with invasion and adhesion to the host cells (Roux and Raoult, 2000; Chan et al., 2010). Moreover, the citrate synthase gene (*gltA*) encoding an enzyme essential in the central metabolic pathways of the rickettsiae and 17-kDa common antigen gene (*htrA*) are useful to characterise species of SFG rickettsiae because their sequences are relatively conserved within the same species and sequence data of representative rickettsial species are available in the database (Fournier et al., 2003; Labruna et al., 2004).

In Asia, several pathogenic SFG rickettsiae are known to exist. For instance, *R. sibirica*, the etiological agent of Siberian tick typhus was reported from

Russia, Mongolia, and China (Mediannikov et al., 2004; Shpynov et al., 2006). Thai tick typhus caused by *R. honei* was reported from Thai-Myanmar border and Nepal (Parola et al., 2003; Murphy et al., 2011). Indian tick typhus caused by *R. conorii* was reported from India and Mongolia (Parola et al., 2001; Rolain et al., 2003). *Candidatus* Rickettsia kellyi, *R. raoultii* and *R. monacensis* have been associated with unknown spotted fever in India, China and South Korea, respectively (Rolain et al, 2006; Jia et al., 2014; Kim et al., 2017). *R. felis*, the causative agent of flea-borne spotted fever has been reported in Vietnam and Laos (Phongmany et al., 2006).

So far four pathogenic SFG rickettsiae, including *R. japonica, Rickettsia heilongjiangensis*, *Rickettsia helvetica*, and *Rickettsia tamurae* are known to be endemic in Japan (Mahara, 1997; Noji et al., 2005; Ando et al., 2010; Imaoka et al., 2011). Human infected with *Rickettsia* organisms typically has high fever, headache, and skin eruption. In general, infected humans develop febrile reaction for 1 to 2 weeks and the period can vary depending on the virulence of the organisms (Mahara, 1997). In severe cases, swelling of liver and spleen, cardiomegaly and nervous signs may occur. Death may be due to pericarditis or the result of cytolytic toxin (Mahara, 1997; Imaoka et al., 2011).

Even though the genetic detection and classification of *Rickettsia* could be achieved by PCR-based molecular techniques, the pathogenicity to vertebrate animals is not well understood in most of the rickettsial species, especially those newly found in ticks. The lines of evidence showed some of the *Rickettsia* without any known vertebrate pathogenicity have symbiotic association with their arthropod hosts (Fournier and Raoult, 2009). In some cases, rickettsiae play roles as endosymbionts presumably with some benefit to host ticks, but this is not always

true (Perlman et al., 2006). In the natural environment, ticks become infected with rickettsiae when they feed on hosts carrying the bacteria or vertically through transovarial and transstadial transmission (Raoult and Roux, 1997) (Figure 1). The presence of *Rickettsiae* in the eggs of tick, indicating potential of vertical transmission, is usually an indication of symbiotic interactions between *Rickettsia* and ticks. In general, horizontal transmission of bacteria across different host lineages tends to exacerbate the virulence of bacteria to their hosts, whereas vertical transmission through host generations tends to attenuate their virulence, potentially leading to commensalism and ultimately to mutualism (Dale and Moran, 2006). At present, the nature of endosymbionts of ticks including rickettsiae and their relationships to ticks remain poorly understood.

Ticks are obligate blood-sucking arthropods found in tropical and temperate regions of the world (Parola et al., 2005). In Japan, tick fauna is composed of seven genera: *Argas*, *Ornithodoros*, *Amblyomma*, *Dermacentor*, *Haemaphysalis*, *Ixodes* and *Rhipicephalus*, and 47 different tick species are reported so far (Nakao et al., 1992; Nakao and Ito, 2014; Kwak, 2018). Out of 47 tick species, 20 species are reported to bite humans (Yamauchi et al., 2010; Seishima et al., 2000; Nakamura-Uchiyama et al., 2000; Ando et al., 2010). According to the Japanese Infectious Agents Surveillance Report, rickettsiosis has counted the highest number of cases among all tick-borne bacterial diseases in Japan (Yamaji et al., 2018). Despite the availability of effective antibiotic treatment, fatal cases of rickettsiosis have continued to be reported annually. The mortality rate of some rickettsial diseases such as Japanese spotted fever (JSF) is still indicating 0.91% (National Institute of Infectious Diseases 2017), emphasizing a public health importance of *Rickettsia*. However, little is known about the

prevalence of *Rickettsia* species in hard ticks and bacterial endosymbionts of questing ticks in Japan. It is highly possible that several unrecognised *Rickettsia* species are expected to exist in Japan. The data obtained from this study will provide information on the genetic diversity of rickettsial organisms which may include previously unrecognised rickettsial agents as well as the geographical distribution of known rickettsial pathogens in Japan.

In the first chapter of this thesis, a national wide cross-sectional survey of ixodid ticks in Japan was conducted to evaluate the relationship between SFG rickettsiae and their vector ticks. In addition, the multiple gene analysis was performed to provide the detail comprehensive taxonomy and phylogeny of *Rickettsia*. In the second chapter, isolation of SFG rickettsiae and tick endosymbionts was attempted from ticks using arthropods cells. Further genetic classification of *Rickettsia* species and tick symbionts was conducted.

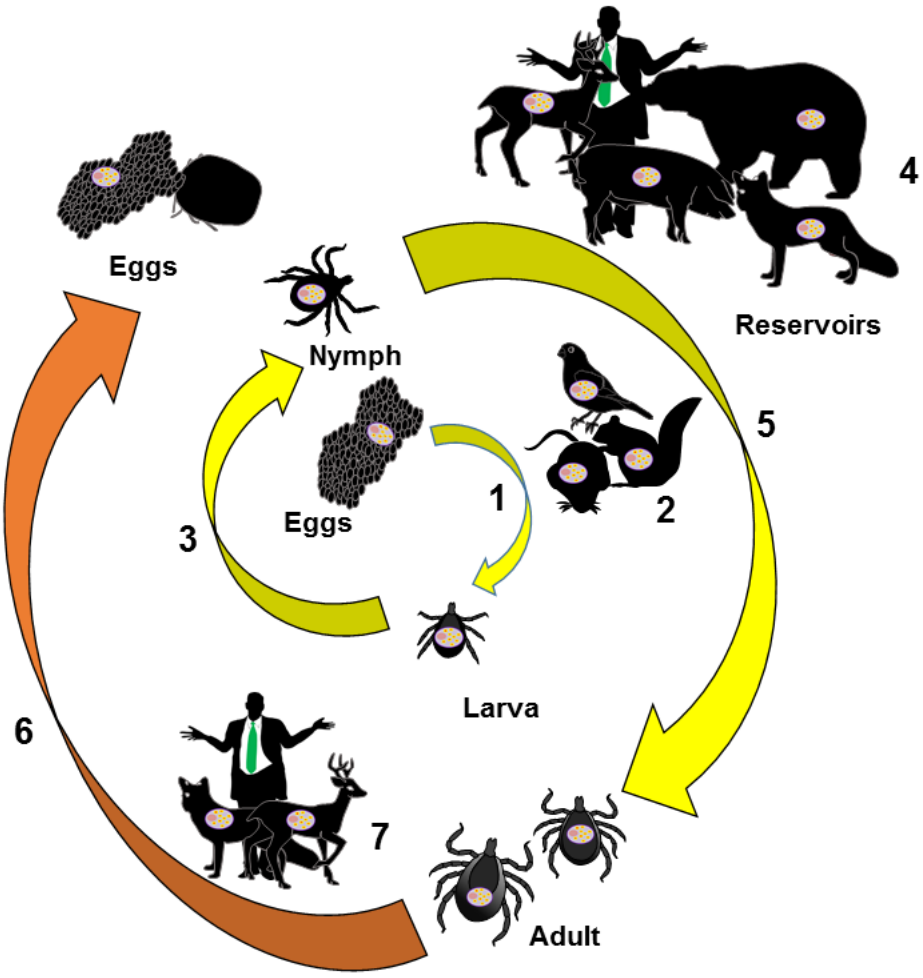

Figure 1. Life cycle of ixodid ticks and transmission of rickettsiae.

Yellow arrows indicate transstadial transmission of rickettsiae and orange arrow indicates transovarial transmission of rickettsiae. (1) Tick eggs hatch into larvae. (2) Larvae feed on small animals. (3) Larvae moult into nymphs. (4) Nymphs feed on animals and humans. (5) Nymphs moult into adult ticks. (6) Oviposition by engorged female. (7) Adult ticks feed on animals or bite humans.

CHAPTER I

Diversity of spotted fever group rickettsiae and their association with host ticks in Japan

1 INTRODUCTION

In Japan, *Rickettsia japonica* was the first Spotted fever group (SFG) *Rickettsia* discovered in 1984 as the causative agent of Japanese spotted fever (JSF) (Uchida et al., 1986; Mahara, 1997). Since then, several other SFG rickettsiae, namely *Rickettsia heilongjiangensis*, *Rickettsia helvetica*, and *Rickettsia tamurae* have been recognised as etiological agents of human diseases (Noji et al., 2005; Ando et al., 2010; Imaoka et al., 2011). SFG rickettsiae with unknown pathogenicity, such as *Rickettsia asiatica* and *Candidatus* R. tarsevichiae, have also been reported (Fujita et al., 2006; Inokuma et al., 2007). In addition, several studies conducted in Japan have documented the presence of other *Rickettsia* species/genotypes in animals and questing ticks (Baba et al., 2013; Gaowa et al., 2013; Someya et al., 2015). However, in most cases, only single or a limited number of genes have been analysed, making it difficult to generate an overview of the genetic diversity of SFG rickettsiae, since multiple gene sequencing are recommended in the classification of rickettsial isolates (Fournier et al., 2003).

The relationship between SFG rickettsiae and their vector tick species has been studied previously. It is evident that some SFG rickettsiae, such as *Rickettsia rickettsii*, are associated with several different tick species in different genera, while others, such as *R. conorii*, are linked to specific tick species (Socolovschi et al., 2009). In Japan, *R. japonica* is considered to be in the former group since it has been recorded from wide range of tick species including *Dermacentor taiwanensis*, *Haemaphysalis hystricis*, *Haemaphysalis cornigera*, *Haemaphysalis longicornis*, *Haemaphysalis flava*, *Haemaphysalis formosensis*, *Haemaphysalis megaspinosa*, and *Ixodes ovatus* (Ando and Fujita, 2013). On the other hand, vector tick species

of other rickettsiae, such as *R. asiatica* and *R. heilongjiangensis*, which are respectively transmitted by *I. ovatus* and *H. concinna*, seem to be limited (Fujita et al., 2006; Ando et al., 2010).

The aim of this chapter was to understand the overall diversity of SFG rickettsiae and their vector tick species in Japan. By collecting questing ticks at more than 100 different sampling sites across Japan, a nationwide cross-sectional study for SFG rickettsiae was conducted. The samples included 19 different tick species covering most of the commonly found species in Japan. Our results indicate that there exist more SFG rickettsiae genotypes than previously known. The information on the relationship between SFG rickettsiae and vector ticks is useful for further characterisation of each rickettsiae member in more detail.

2 MATERIALS AND METHODS

2.1 Sample collection

Ticks were collected by flagging a flannel cloth over the vegetation during the period of tick activity (between April 2013 and March 2016) at 114 different sampling sites in 12 different prefectures. The sampling sites were categorised into geographical blocks: Hokkaido (Hokkaido prefecture), Tohoku (Yamagata and Fukushima prefectures), Chubu (Nagano and Shizuoka prefectures), Kansai (Mie, Nara, and Wakayama prefectures), Kyushu (Kumamoto, Miyazaki, and Kagoshima prefectures), and Okinawa (Okinawa prefecture) (Figure I-1). All field-collected

ticks were transferred to small petri dishes and preserved in an incubator at 16°C until use.

2.2 Tick species identification

Tick species were identified morphologically using standard keys under a stereomicroscope (Yamaguti et al., 1971; Nakao et al., 1992). When more than 10 ticks with the same species and stage/sex were collected from the same sampling sites, a maximum of 10 individual ticks were analysed per species, stage/sex and site. A total of 2,189 individuals (103 nymphs and 2,086 adults) in four genera were examined in this study. These included one species in the genus *Amblyomma* (*A. testudinarium*, n = 85), one species in the genus *Dermacentor (D. taiwainensis*, n = 12), 10 species in the genus *Haemaphysalis* (*H. concinna*, n = 7; *H. cornigera*, n = 1; *H. flava*, n = 128; *H. formosensis*, n = 253*; H. japonica*, n = 78; *H. hystricis*, n = 64; *H. kitaokai*, n = 74; *H. longicornis*, n = 86; *H. megaspinosa*, n = 201; and *H. yeni*, n = 1) and 7 species in the genus *Ixodes* (*I. monospinosus*, n = 58; *I. nipponensis*, n = 5; *I. ovatus*, n = 652; *I. pavlovskyi*, n = 33; *I. persulcatus*, n = 446; *I. tanuki*, n = 2; and *I. turdus*, n = 3). Out of 2,189 ticks, 975, 1,111, and 103 were male, female, and nymph, respectively.

2.3 DNA extraction

Ticks were individually washed with 70% ethanol followed by washing with sterile PBS twice, then homogenised in 100 μL of high glucose Dulbecco's Modified Eagle Medium (DMEM, Gibco, Life Technologies) by using Micro Smash MS100 (TOMY, Tokyo, Japan) for 30 sec at 3,000 rpm as described previously (Nakao et al., 2011). DNA was extracted from 50 μL of the tick

homogenate using a blackPREP Tick DNA/RNA Kit (Analytikjena, Germany) according to the manufacturer's instructions, while the other half was kept at -80°C for future bacterial isolation.

2.4 Real-time PCR

All samples were first screened for citrate synthase gene (*gltA*) using real-time PCR to detect SFG and TG rickettsiae as described previously (Stenos et al., 2005). The primers and probes used are shown in Table I-1. Reactions were performed in a 20 μL of reaction mixture containing 10 μL of THUNDERBIRD Probe qPCR Mix (Toyobo, Osaka, Japan), 300 nM of each primer, 200 nM of probe, 5.0 μL of template DNA, and distilled water. The reaction was carried out in a C1000 Thermal Cycler with a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) at conditions of 50°C for 3 min, 95°C for 1 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min. Each run included a negative control and serially diluted plasmid standards (10^6, 10^4, and 10^2 copies/reaction) as described previously (Nakao et al., 2013).

2.5 Conventional PCR

All the samples that were positive for *gltA* by real-time PCR were further characterised by conventional PCR targeting an approximately 580 bp sequence of the *gltA* gene using the primers *gltA*–Fc and *gltA*–Rc (Table I-1) (Gaowa et al., 2013). The PCR was carried out in a 25 μL reaction mixture containing 12.5 μL of 2×KAPA blood PCR Kit (KAPA Bio systems, USA), 200 nM of each primer, 2.0 μL of DNA template, and sterile water. The reactions were performed at 95°C for 5 min; followed by 45 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 40

sec; and 72°C for 5 min. PCR products were electrophoresed at 100 V in a 1.2% agarose gel for 25 min. DNA from the *R. japonica* YH strain and sterile water were included in each PCR run as positive and negative controls, respectively.

For the selected samples from each *gltA* genotype (n = 57), additional PCR assays were conducted based on five genes: the 190-kDa outer membrane protein A (*ompA*), 120-kDa outer membrane protein B (*ompB*), surface cell antigen-4 (*sca4*), 17-kDa common antigen (*htrA*), and 16S rRNA. The primer sets used for each assay are shown in Table I-1 (Regnery et al., 1991; Roux and Raoult, 2000; Sekeyova et al., 2001; Labruna et al., 2004; Anstead and Chilton, 2013). PCR conditions were the same as mentioned above except for the annealing temperature (48°C for *ompA* and *ompB* PCRs, 52°C for 16S rRNA and *htrA* PCRs, and 50°C for *sca4* PCR).

2.6 Sequencing

The amplified PCR products were purified using a Wizard® SV Gel and PCR Clean-Up System Kit (Promega, USA). Sanger sequencing was conducted using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and an ABI Prism 3130xl Genetic Analyzer Kit (Applied Biosystems) according to the manufacturers' instructions. The sequences data were assembled using ATGC software version 6.0.4 (GENETYX, Tokyo, Japan). The sequences obtained were submitted to the DNA Data Bank of Japan (DDBJ) (http://www.ddbj.nig.ac.jp) under accession numbers (*gltA*: LC379427-LC379443; *ompA*: LC379461-LC379465; *ompB*: LC379466-LC379476; *htrA*: LC379444-LC379460; *sca4*: LC379477-LC379482; 16S rRNA: LC379483-LC379494).

2.7 Phylogenetic analysis

The nucleotide sequences obtained were aligned with representative sequences of known rickettsial species available on GenBank using ClustalW 1.6 as implemented in MEGA 7 (Kumar et al., 2016). After manual modification of the alignments, phylogenetic trees were constructed using the maximum likelihood method using Kimura 2-parameter with bootstrap tests of 1,000 replicates via MEGA. *R. bellii* was included as an outgroup for the bases of the trees for *gltA*, *ompB*, and *htrA*, while *R. typhi*, *R. akari*, and *Ehrlichia chaffeensis* were used as outgroups for *sca4*, *ompA*, 16S rRNA, respectively. In order to generate a phylogenetic tree of tick species that was positive for *Rickettsia* spp., partial nucleotide sequences of mitochondrial 16S rRNA gene obtained from GenBank were used.

3 RESULTS

3.1 Detection of SFG rickettsiae by real-time PCR for *gltA*

Out of 2,189 ticks, 373 (17.0 %) samples were positive for *Rickettsia* spp. by *gltA* real-time PCR (Table I-2). Among the 19 different tick species, seven tick species, namely *D. taiwainensis*, *H. concinna*, *H. cornigera*, *H. yeni*, *I. pavlovskyi*, *I. tanuki*, and *I. turdus*, were negative for rickettsiae infection. The highest infection rate was observed in *I. nipponensis* (80.0%), followed by *H. longicornis* (62.8%), *I. monospinosus* (58.6%), *H. hystricis* (57.8%), *I. persulcatus* (34.8%), *A.*

testudinarium (23.5%), *H. megaspinosa* (17.4%), *H. flava* (10.2%), *H. japonica* (5.1%), *H. kitaokai* (4.1%), *H. formosensis* (2.8%), and *I. ovatus* (1.1%).

3.2 *gltA* genotyping

Out of 373 samples that tested positive for rickettsiae by real-time PCR for *gltA*, 352 samples yielded amplicons by conventional PCR for *gltA*, while 21 samples did not (Table I-2). All the amplicons were successfully sequenced, which resulted in 15 different *gltA* genotypes (Figure I-2 and Table I-3). In the present study, the *gltA* genotype is defined as a *gltA* sequence type that is different from the others even by a single nucleotide. All *gltA* genotypes (G1, G2, G6, G7, G9, G11, G12, G14, and G15) detected in the genus *Haemaphysalis* were clustered in the same clade, while five genotypes (G3, G4, G5, G10, and G13) obtained from the genus *Ixodes* were allocated to three different clusters while only one genotype (G8) was linked with the genus *Amblyomma* (Figure I-3). A total of 13 genotypes were detected in only one single tick species, while two genotypes (G5 and G11) were detected in two different tick species: G5 was recovered from *I. persulcatus* and *I. monospinosus*, and G11 was from *H. japonica* and *H. flava* (Figure I-3).

3.3 Geographic information on *gltA* genotypes and host ticks

Table I-3 represents the relationship between *gltA* genotypes and their geographical origins. Out of 15 genotypes, 11 genotypes (G1, G2, G3, G4, G5, G6, G8, G10, G11, G12, and G15) were detected from multiple geographical regions. The other 4 genotypes (G7, G9, G13, and G14) were detected in only one single tick species from a single region. G7 and G14 were detected in *H. formosensis* from Kyusyu region, while G9 and G13 were in *H. kitaokai* from Kansai region and *I.*

ovatus from Tohoku region, respectively (Figure I-1). The present study employed *H. formosensis* and *H. kitaokai* from three different regions and *I. ovatus* collected from five different regions (Table I-3).

3.4 Multiple genes sequencing

To further characterise *Rickettsia* spp. based on five other genes (*ompA, ompB, sca4, htrA*, and 16S rRNA), PCR analyses were conducted on the selected samples of each *gltA* genotype. A total of 57 samples were employed in this analysis. We selected more than two samples from each genotype except for G7 which was found in only one sample (Table I-4). The samples with higher rickettsial burden were selected based on the results of *gltA* real-time PCR. The mean rickettsial burden in the template DNA ranged from 2.3E+2 to 2.1E+4 copies/μL (Table I-4). The *htrA* gene was successfully amplified and sequenced for all *gltA* genotypes (Table I-4). Although 16S rRNA PCR gave amplicons in all *gltA* genotypes, the following sequencing analysis revealed that rickettsial 16S rRNA gene sequences were obtained in only 12 *gltA* genotypes. The *ompB, ompA* and *sca4* genes were amplified and sequenced in 11, five and six different *gltA* genotypes, respectively. All genes were successfully sequenced in two *gltA* genotypes (G6 and G7). Four genes were successfully amplified in six *gltA* genotypes (G1, G2, G5, G8, G10, and G11), and three genes were amplified in four *gltA* genotypes (G3, G4, G9 and G13). Only the *htrA* gene was amplified in three *gltA* genotypes (G12, G14, and G15) (Table I-4). The sequencing analysis of the amplified products revealed that there were no sequence differences in any of the genes in the samples with the same *gltA* genotypes. The sequence types obtained from each *gltA* genotype were different from each other.

3.5 Species classification of SFG rickettsiae

Phylogenetic trees inferred from *ompA*, *ompB*, *sca4*, *htrA*, and 16S rRNA analysis are shown in Figure I-4, Figure I-5, Figure I-6, Figure I-7 and Figure I-8, respectively. G4 and G5 formed a distinct cluster with *R. helvetica* in all trees when sequences were available and thus were identified as *R. helvetica*. Being supported by more than three trees, G13, G10, G8, and G3 were identified as *R. asiatica*, *R. monacensis* (former *Rickettsia* sp. In56), and *R. tamurae*, and *Candidatus* R. tarasevichiae, respectively (Table I-5). The other nine *gltA* genotypes could not be classified into specific species due to a lack of consensus between the trees and/or absence of sequences from previously validated rickettsial species in the same phylogenetic cluster.

4 DISCUSSION

The present chapter included a total of 2,189 individual ticks collected at 114 different sampling sites in six regions of Japan for the screening of SFG rickettsiae. Our nationwide sampling enabled us to collect as many as 19 different tick species from four genera, most of which were common tick species prevalent in Japan. A first screening test using *gltA* real-time PCR revealed that 17.0% (373 out of 2,189) of the ticks were infected with SFG rickettsiae. This infection rate was comparative to the results of an earlier study where 21.9% (181 out of 827) of the ticks, including 10 different species collected from central (Shizuoka, Mie, and Wakayama prefectures) and southern (Kagoshima, Nagasaki, and Okinawa

prefectures) parts of Japan, were positive for SFG rickettsiae (Gaowa et al., 2013). Another nationwide survey conducted in 5 prefectures (Chiba, Hokkaido, Kochi, Tokushima, and Toyama prefectures) including JSF-endemic areas reported an overall positive rate for SFG rickettsiae to be 25.8% (186 out of 722) in 10 different tick species (Ishikura et al., 2003).

Partial sequences of the *gltA* gene of SFG rickettsiae were determined by conventional PCR, which was previously designed to characterise SFG rickettsiae in Japan (Gaowa et al., 2013). Based on the sequences of the *gltA* gene obtained from 352 ticks, the SFG rickettsiae detected in the present study were provisionally divided into 15 genotypes (Figure I-2). In the molecular classification of SFG rickettsiae, the analysis of multiple genes commonly used by other researchers is a prerequisite (Fournier et al., 2003). Therefore, further analyses to obtain the sequences of five additional genes, *ompA*, *ompB*, *sca4*, *htrA*, and 16S rRNA, were conducted. These efforts lead to the identification of four validated rickettsial species, namely *R. asiatica*, *R. helvetica*, *R. monacensis*, and *R. tamurae*, and the provisional species *Candidatus* R. tarasevichiae (Figure I-4, Figure I-5, Figure I-6, Figure I-7 and Figure I-8).

Prior to this study, there was no official report of the presence of *R. monacensis* in Japan. A recent study indicated that *Rickettsia* sp. In56, a rickettsial stain reported from ticks in Japan (Ishikura et al., 2003), might be a synonymous of *R. monacensis* (Kim et al., 2013). Although several isolates of *Rickettsia* sp. In56 have been obtained from Japanese ticks (Fujita et al., 2013), lack of their sequence information prevents a direct comparison between *Rickettsia* sp. In56 and *R. monacensis* reported elsewhere. Nevertheless, the sequence analysis of multiple genes (*gltA*, *ompA*, *ompB*, *htrA*, and 16S rRNA) conducted in the present study

confirmed the presence of *R. monacensis* in Japan (Figure I-2, Figure I-4, Figure I-5, Figure I-7 and Figure I-8). *R. monacensis* was initially isolated from *I. ricinus* collected from the English Garden in Germany using ISE6 cells (Simser et al., 2002) and has been detected from the same tick species in Europe and neighbouring countries (Sréter-Lancz et al., 2005; Milhano et al., 2010; Špitalská et al., 2014; Venclikova et al., 2014; Biernat et al., 2016). *I. nipponensis* and *I. sinensis* are considered as main vectors of *R. monacensis* in China and Korea, respectively (Ye et al., 2014; Shin et al., 2013). In our study, *R. monacensis* was detected from four *I. nipponensis* samples collected in the Tohoku and Kansai regions, while none of the other tick species carried *R. monacensis* (Figure I-3 and Table I-3). These results may suggest the relatively wide distribution of *R. monacensis* and a strong association of *R. monacensis* with *I. nipponensis* in Japan. This SFG rickettsiae caused Mediterranean spotted fever-like symptoms in humans in several countries (Jado et al., 2007; Madeddu et al., 2012). More recently, the agent was isolated from the blood of a patient with an acute febrile illness in Korea (Kim et al., 2017). Thus, clinicians should be aware of *R. monacensis* as a possible cause of non-JSF rickettsiosis in Japan.

Although SFG rickettsiae with each prospective *gltA* genotype were analysed in further detail by sequencing five additional rickettsial genes, *ompA*, *ompB*, *sca4*, *htrA*, and 16S rRNA, the amplification was not successful for some genes (Table I-4). The *ompA* and *sca4* genes were amplified only from one third of the tested *gltA* genotypes. Considering the relatively high rickettsial abundance in tested samples (Table I-4). PCR failure is either because some of the SFG rickettsiae lack these genes as shown in TG rickettsiae that do not possess *ompA* gene (Ngwamidiba et al., 2006), or because there are nucleotide mismatches in the primer

annealing sites. PCR failures of variable genes such as *ompA*, *ompB*, and *sca4* are common issues in the genetic characterisation of SFG rickettsiae (Ngwamidiba et al., 2006; Nakao et al., 2013). Thus further attempts including the development of universal primers and/or bacterial isolation followed by whole genome sequencing are required to determine the phylogenetic positions of uncharacterised *Rickettsia* spp.

In a previous nationwide survey of SFG rickettsiae conducted in Japan, Gaowa et al. (2013) classified the detected rickettsiae (n = 181) into five groups (Group 1–5) based on the *gltA* sequences (Gaowa et al., 2013). Groups 1 and 2 were respectively identified as *R. japonica* and *R. tamurae*, whereas groups 3, 4, and 5, showing high sequence similarity with *Rickettsia* sp. LON-13, *R. raoultii*, and *Candidatus* R. principis, respectively, were not classified as validated rickettsial species (Gaowa et al., 2013). In agreement with their report, *gltA* sequences corresponding to groups 3 (G6), 4 (G2), and 5 (G1, G11, G12, G14, and G15) were detected (Figure I-2). Unfortunately, limited information is available about these uncharacterised *Rickettsia* spp. In our study, G6 and G2 were respectively detected in *H. longicornis* and *H. hystricis* with high infection rates (62.8% and 57.8%, respectively) (Table I-3), warranting further studies on the effect of these infections for the survival and reproductive fitness of their hosts.

Two *gltA* genotypes (G7 and G9) were allocated into distinct clusters from *Rickettsia* spp. previously reported from Japan (Figure I-2). G7 and G9 showed the highest *gltA* sequence similarity with *Rickettsia* spp. reported from Kenya (KT257873) and Hungary (EU853834), respectively. *Rickettsia* sp. reported from Kenya was detected in *Rhipicephalus maculatus* (Mwamuye et al., 2017), while one that from Hungary was detected in *H. inermis* and was provisionally named as

Candidatus R. hungarica (Hornok et al., 2010). Since the sequences of other genes were not available from those *Rickettsia* spp., it was difficult to evaluate the degree of genetic relatedness in more detail. Nonetheless, the presence of closely related species in two geographically remote areas may indicate the worldwide distribution of these poorly characterised SFG rickettsiae. Since the present study provided the sequences of multiple genes of those rickettsiae, the information is useful in the classification of SFG rickettsiae.

In the present study, a strong association between rickettsial genotypes and their host tick species was detected, where 13 out of 15 *gltA* genotypes were detected in only one single tick species (Figure I-3 and Table I-4). Furthermore, there was minimal geographical restriction for the 11 *gltA* genotypes that were recovered from multiple geographical regions (Table I-3). These observations may indicate that most of the SFG rickettsiae species are found in ticks but not in vertebrate hosts in the natural environment. However, further examinations are needed to confirm this hypothesis by observing transstadial and transovarial transmission of these SFG rickettsiae in ticks. The effect of these rickettsial infections on tick physiology and reproduction remains to be elucidated.

Although the sampling was conducted at several JSF-endemic areas in Mie, Kagoshima, and Kumamoto prefectures, none of the ticks were infected with *R. japonica*. Considering the low level of genomic plasticity within *R. japonica* isolates (Akter et al., 2017), it was hardly expected that a real-time PCR assay of *gltA* would result in false-negatives. The positive rate of *R. japonica* infection in the questing ticks was as low as 0.86% (18 out of 2,099), even in endemic areas as is the case in Shimane prefecture (Tabara et al., 2011). Collectively, the failure in the detection of *R. japonica* might be partly attributed to the sample selection

procedure with which only a maximum of 10 individual ticks per species, stage/sex, and site were tested for SFG rickettsiae infection. Therefore, it should be noted that the present study might not fully disclose the diversity of SFG rickettsiae in Japan, which warrants further investigations by employing a larger number of samples.

5 SUMMARY

Spotted fever group (SFG) rickettsiae are obligate intracellular Gram-negative bacteria mainly associated with ticks. In Japan, since the discovery of *R. japonica* as the causative agent of Japanses spotted fever (JSF), five other SFG rickettsiae, namely *Rickettsia asiatica*, *Rickettsia heilongjiangensis*, *Rickettsia helvetica*, *Rickettsia tamurae*, and *Candidatus* R. tarsevichiae have been reported. Additionally, previous studies have indicated the presence of other *Rickettsia* species/genotypes in animals and questing ticks; however, their phylogenetic position and pathogenic potential are poorly understood. To understand the overall diversity of SFG rickettsiae and associated tick species in Japan, a nationwide cross-sectional survey was conducted on ticks collected from 114 different sites in 12 prefectures. Out of 2,189 individuals (19 tick species in 4 genera), 373 (17.0%) samples were positive for *Rickettsia* spp. as ascertained by real-time PCR amplification of *gltA* gene. Conventional PCR and sequencing analyses of *gltA* indicated the presence of 15 different genotypes of SFG rickettsiae. Further characterisation based on the analysis of five additional genes, *ompA*, *ompB*, *sca4*, *htrA*, and 16S rRNA, led to the identification of *R. asiatica*, *R. helvetica*, *R. monacensis* (formerly reported as *Rickettsia* sp. In56 in Japan), *R. tamurae*, and *Candidatus* R. tarasevichiae. Furthermore, several uncharacterised *Rickettsia* spp. including ones showing high similarities with those designated as novel *Rickettsia* spp. detected in geographically remote countries such as Kenya and Hungary were discovered. A strong association between rickettsial genotypes and their host tick species was observed, while there was little association between rickettsial genotypes and their geographical origins. These observations may indicate that

most of the SFG rickettsiae have a limited host range and are maintained in certain ticks in the natural environment. Further investigations on the potential roles of these SFG rickettsiae on ticks are required to understand the mechanisms underlying widespread existence of genetically variable rickettsiae in ticks. It is also of importance to further evaluate pathogenic potential of these SFG rickettsiae to humans and animals.

Table I-1. Primers uesd in the present study.

Primer	Sequence (5'-3')	Target gene	Annealing temperature (°C)	Amplicon size (bp)	Reference
CS-F	TCGCAAATGTTCACGGTACTTT	citrate synthase gene (*gltA*)	60	74	Steno et al., 2005
CS-R	TCGTGCATTTCTTTCCATTGTG				
CS-P	TGCAATAGCAAGAACCGTAGGCTGGATG				
gltA_Fc	CGAACTTACCGCTATTAGAATG	citrate synthase gene (*gltA*)	55	580	Gaowa et al., 2013
gltA_Rc	CTTTAAGAGCGATAGCTTCAAG				
Rr.190.70p	ATGGCGAATATTTCTCCAAAA	outer membrane A gene (*ompA*)	48	542	Regnery et al., 1991
Rr.190.602n	AGTGCAGCATTCGCTCCCCCT				
120_2788	AAACAATAATCAAGGTACTGT	outer membrane B gene (*ompB*)	48	816	Roux and Raoult, 2000
120_3599	TACTTCCGGTTACAGCAAAGT				
D1f	ATGAGTAAAGACGGTAACCT	surface cell antigen-4 (*sca4*)	50	928	Sekeyova et al., 2001
D928r	AAGCTATTGCGTCATCTCCG				
sca4_seq1	GCCGGCTATTTCTATTGATTC*				This study
sca4_seq2	TGCAAGCGATCTTAGAGCAA*				This study
17k_5	GCTTTACAAAATTCTAAAAACCATATA	17-kDa common antigen gene (*htrA*)	52	550	Labruna et al., 2004
17k_3	TGTCTATCAATTCACAACTTGCC				
Rick_16S_F3	ATCAGTACGGAATAACTTTTA	16S ribosomal RNA gene (16S rRNA)	52	1328	Anstead et al., 2013
Rick_16S_F4	TGCCTCTTGCGTTAGCTCAC				
rrs_seq1	AGGCCTTCATCACTCACTCG*				This study
rrs_seq2	CTACACGCGTGCTACAATGG*				

*The primers were used for sequencing.

Table I-2. Detection of spotted fever group rickettsiae by real-time and conventional PCR for *gltA* gene.

Tick species	No. tested (Female/ Male/ Nymph)	Real-time PCR	Conventional PCR
		No. of positive (Female/ Male/ Nymph) (%)	No. of positive (Female/ Male/ Nymph) (%)
A. testudinarium	85 (3/0/82)	20 (1/0/19) (23.5)	16 (1/0/15) (18.8)
D. taiwainensis	12 (7/5/0)	0	0
H. concinna	7 (2/5/0)	0	0
H. cornigera	1 (1/0/0)	0	0
H. flava	128 (59/65/4)	13 (7/5/1) (10.2)	11 (6/4/1) (8.6)
H. formosensis	253 (130/122/1)	7 (2/5/0) (2.8)	7 (2/5/0) (2.8)
H. japonica	78 (50/25/3)	4 (2/2/0) (5.1)	4 (2/2/0) (5.1)
H. hystricis	64 (42/21/1)	37 (24/13/0) (57.8)	36 (23/13/0) (56.3)
H. kitaokai	74 (37/36/1)	3 (0/2/1) (4.1)	3 (1/1/1) (4.1)
H. longicornis	86 (56/26/4)	54 (31/22/1) (62.8)	54 (31/22/1) (62.8)
H. megaspinosa	201 (106/92/3)	35 (21/14/0) (17.4)	27 (16/11/0) (13.4)
H. yeni	1 (1/0/0)	0	0
I. monospinosus	58 (38/20/0)	34 (20/14/0) (58.6)	34 (20/14/0) (58.6)
I. nipponensis	5 (0/5/0)	4 (0/4/0) (80)	4 (0/4/0) (80)
I. ovatus	652 (339/313/0)	7 (4/3/0) (1.1)	7 (4/3/0) (1.1)
I. pavlovskyi	33 (16/17/0)	0	0
I. persulcatus	446 (220/222/4)	155 (87/68/0) (34.8)	150 (82/68/0) (33.6)
I. tanuki	2 (1/1/0)	0	0
I. turdus	3 (3/0/0)	0	0
Total	2,189 (1,111/975/103)	373 (199/152/22) (17.0)	352 (187/147/18) (16.1)

Table I-3. Host ticks and geographic origin of 15 *gltA* genotypes of spotted fever group rickettsiae.

gltA genotype	Tick species	No. of positive / No. tested (%)						
		Hokkaido	Tohoku	Chubu	Kansai	Kyushu	Okinawa	Total
G1	*H. megaspinosa*	5/94 (5.3)	0/2 (0)	-	14/97 (14.4)	8/8 (100)	-	27/201 (13.4)
G2	*H. hystricis*	-	-	-	5/8 (62.5)	31/53 (58.5)	0/3 (0)	36/64 (56.3)
G3	*I. persulcatus*	44/376 (11.7)	2/51 (3.9)	0/11 (0)	0/8 (0)	-	-	46/446 (10.3)
G4	*I. persulcatus*	96/376 (25.5)	0/51 (0)	1/11 (9.1)	0/8 (0)	-	-	97/446 (21.7)
G5	*I. persulcatus*	7/376 (18.6)	0/51 (0)	0/11 (0)	0/8 (0)	-	-	7/446 (1.6)
G5	*I. monospinosus*	-	34/58 (58.6)	-	-	-	-	34/58 (58.6)
G6	*H. longicornis*	0/4 (0)	0/2 (0)	5/5 (100)	49/61 (80.3)	0/14 (0)	-	54/86 (62.8)
G7	*H. formosensis*	-	-	-	0/34 (0)	1/216 (0.5)	0/3 (0)	1/253 (0.4)
G8	*A. testudinarium*	-	-	-	11/64 (17.2)	4/20 (20.0)	1/1 (100)	16/85 (18.8)
G9	*H. kitaokai*	-	-	-	2/43 (4.7)	0/14 (0)	0/17 (0)	2/74 (2.7)
G10	*I. nipponensis*	-	2/3 (66.7)	-	2/2 (100)	-	-	4/5 (80.0)
G11	*H. japonica*	2/49 (4.1)	2/27 (7.4)	-	0/2 (0)	-	-	4/78 (5.1)
G11	*H. flava*	-	3/28 (10.7)	-	4/71 (5.6)	1/29 (3.4)	-	8/128 (6.3)
G12	*H. flava*	-	1/28 (3.6)	-	2/71 (2.8)	0/29 (0)	-	3/128 (2.3)
G13	*I. ovatus*	0/463 (0)	7/163 (4.3)	0/10 (0)	0/15 (0)	0/1 (0)	-	7/652 (1.1)
G14	*H. formosensis*	-	-	-	0/34 (0)	2/216 (0.9)	0/3 (0)	2/253 (0.8)
G15	*H. formosensis*	-	-	-	1/34 (2.9)	3/216 (1.4)	0/3 (0)	4/253 (1.6)

-, This tick species was not collected in the region.

Table I-4. Results of PCR amplification for the *ompA*, *ompB*, *htrA*, *sca4* and 16S rRNA genes.

gltA genotype	Tick species	No. tested	Mean rickettsial burden (copies/µl)*	PCR amplification				
				ompA	*ompB*	*sca4*	*htrA*	16S rRNA
G1	*H. megaspinosa*	2	7.9E+3	-	+	+	+	+
G2	*H. hystricis*	2	1.1E+4	-	+	+	+	+
G3	*I. persulcatus*	4	8.7E+3	+	-	-	+	+
G4	*I. persulcatus*	3	8.4E+3	-	+	-	+	+
G5	*I. persulcatus*	6	1.3E+3	-	-	-	+	-
G5	*I. monospinosus*	6	2.3E+2	-	+	+	+	+
G6	*H. longicornis*	2	2.4E+3	+	+	+	+	+
G7	*H. formosensis*	1	1.0E+4	+	+	+	+	+
G8	*A. testudinarium*	3	2.1E+4	+	+	-	+	+
G9	*H. kitaokai*	2	1.6E+4	-	+	-	+	+
G10	*I. nipponensis*	2	3.0E+3	+	+	-	+	+
G11	*H. japonica*	3	2.6E+3	-	+	+	+	+
G11	*H. flava*	7	1.8E+3	-	-	-	+	-
G12	*H. flava*	3	1.2E+3	-	-	-	+	-
G13	*I. ovatus*	5	2.5E+3	-	+	-	+	+
G14	*H. formosensis*	3	3.6E+3	-	-	-	+	-
G15	*H. formosensis*	3	1.3E+3	-	-	-	+	-

+, Amplified; -, Not amplified.

*The mean copy number of rickettsial *gltA* gene in the template DNA was calculated by *gltA* real-time PCR.

Table I-5. Classification of SFG rickettsiae and their related tick species.

gltA genotype	*Rickettsia* species	Related tick species
G1	Uncharacterised *Rickettsia* sp.	*H. megaspinosa*
G2	Uncharacterised *Rickettsia* sp.	*H. hystricis*
G3	*Candidatus* R. tarasevichiae	*I. persulcatus*
G4	*Rickettsia helvetica*	*I. persulcatus*
G5	*Rickettsia helvetica*	*I. persulcatus*
G5	*Rickettsia helvetica*	*I. monospinosus*
G6	Uncharacterised *Rickettsia* sp.	*H. longicornis*
G7	Uncharacterised *Rickettsia* sp.	*H. formosensis*
G8	*Rickettsia tamurae*	*A. testudinarium*
G9	Uncharacterised *Rickettsia* sp.	*H. kitaokai*
G10	*Rickettsia monacensis*	*I. nipponensis*
G11	Uncharacterised *Rickettsia* sp.	*H. japonica*
G11	Uncharacterised *Rickettsia* sp.	*H. flava*
G12	Uncharacterised *Rickettsia* sp.	*H. flava*
G13	*Rickettsia asiatica*	*I. ovatus*
G14	Uncharacterised *Rickettsia* sp.	*H. formosensis*
G15	Uncharacterised *Rickettsia* sp.	*H. formosensis*

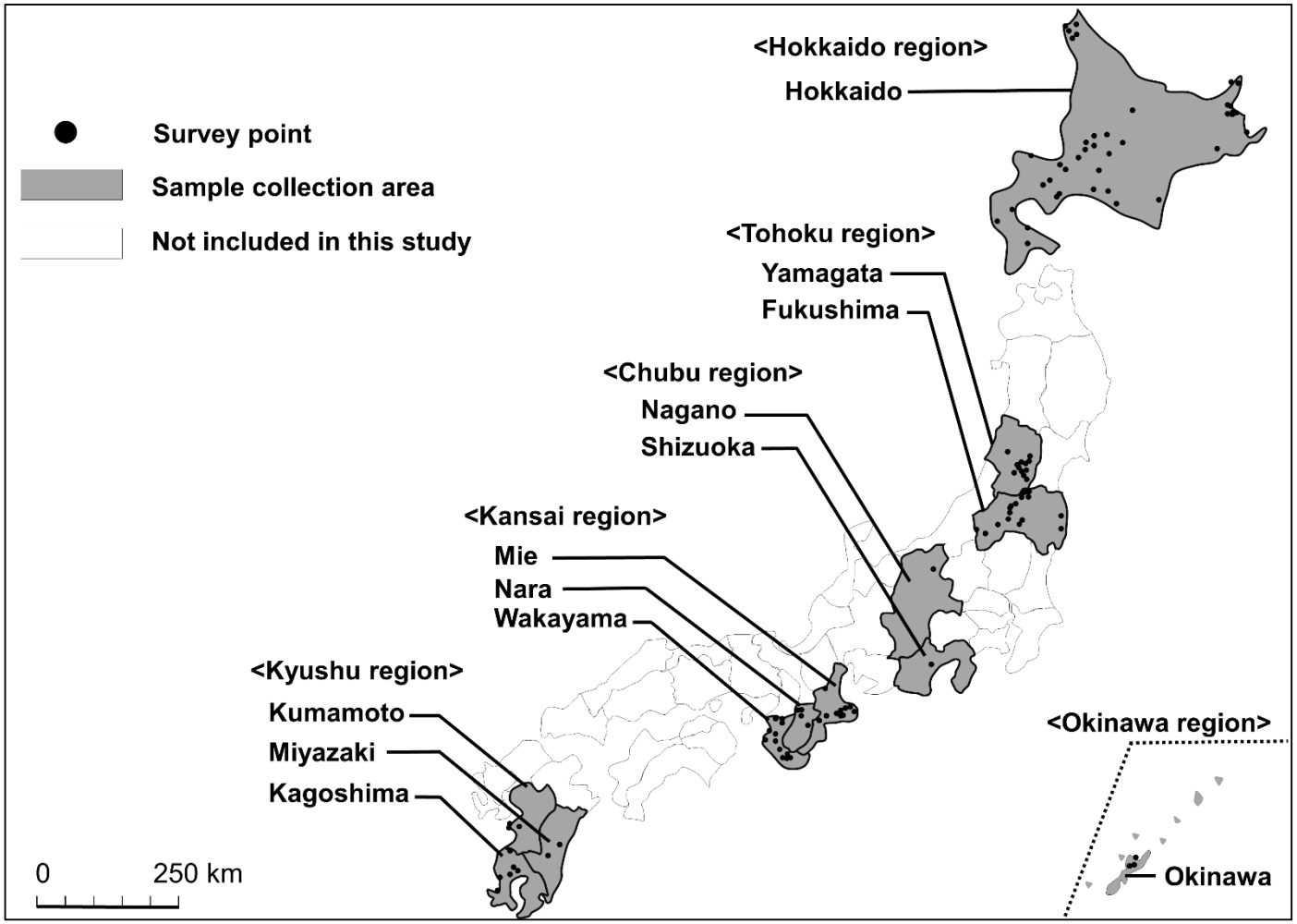

Figure I-1. A map of the 114 sample collection sites in this study.

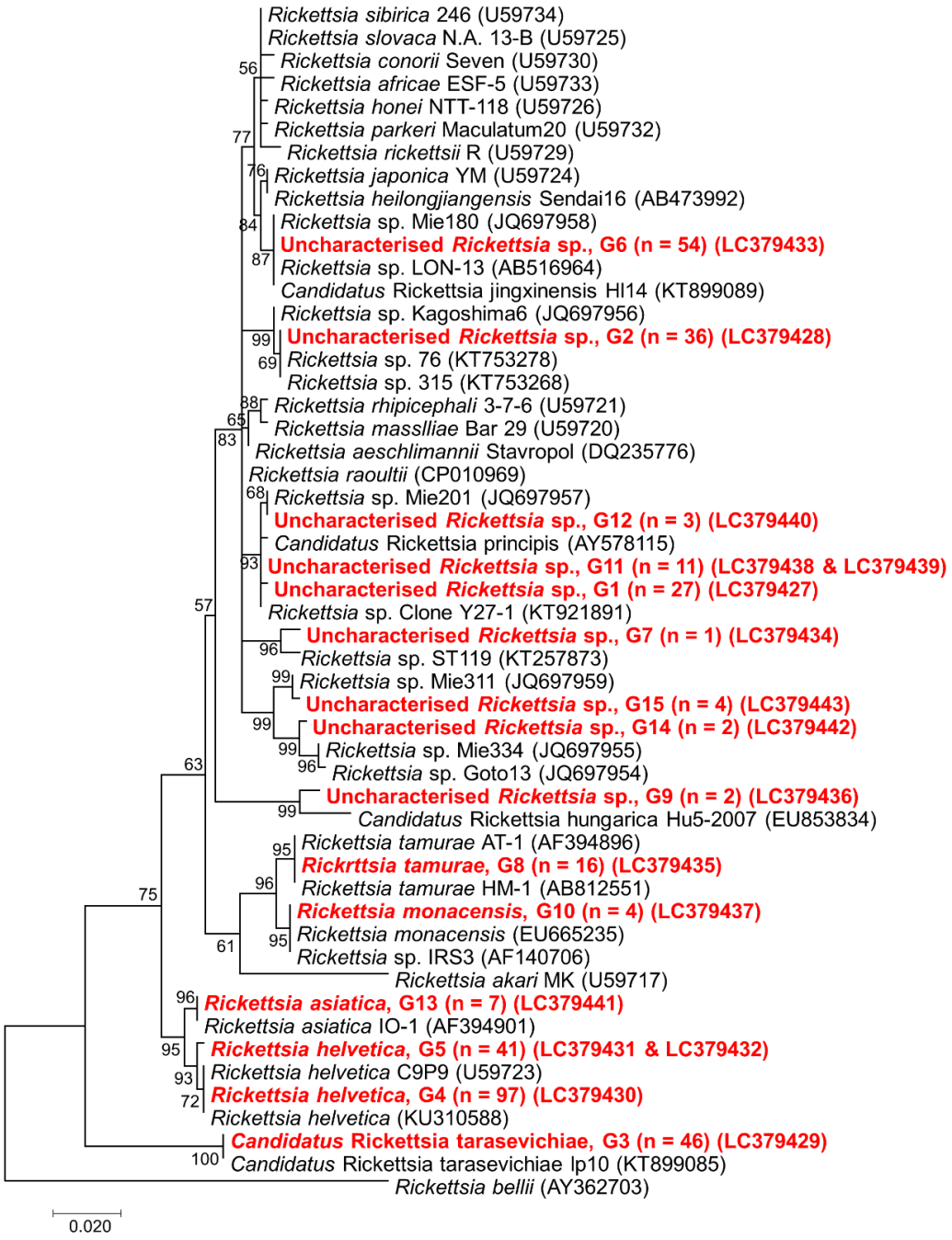

Figure I-2. A phylogenetic tree of spotted fever group rickettsiae based on the *gltA* gene sequences. The analysis was performed using a maximum likelihood method with the Kimura two-parameter model. All bootstrap values from 1,000 replications are shown on the interior branch nodes. The sequences detected in this study are indicated in red. The number of samples positive for each genotype is indicated in the parentheses.

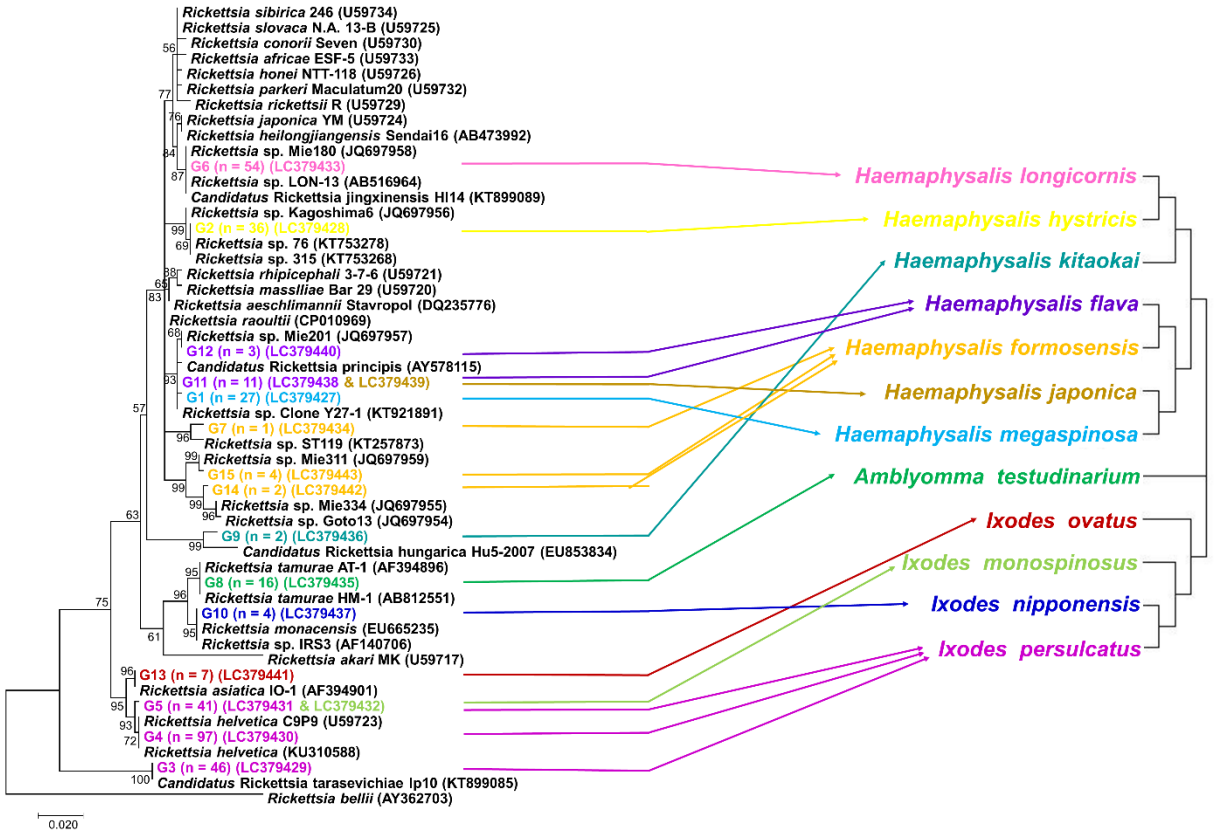

Figure I-3. Relationship between spotted fever group rickettsiae and their related tick species. The left side of the tree shows a phylogenetic tree of spotted fever group rickettsiae based on *gltA* gene sequence. The right side of phylogenetic tree represents a simplified tick phylogeny consisting 12 tick species. The colour of the arrows indicates the link between rickettsial genotypes and different tick species.

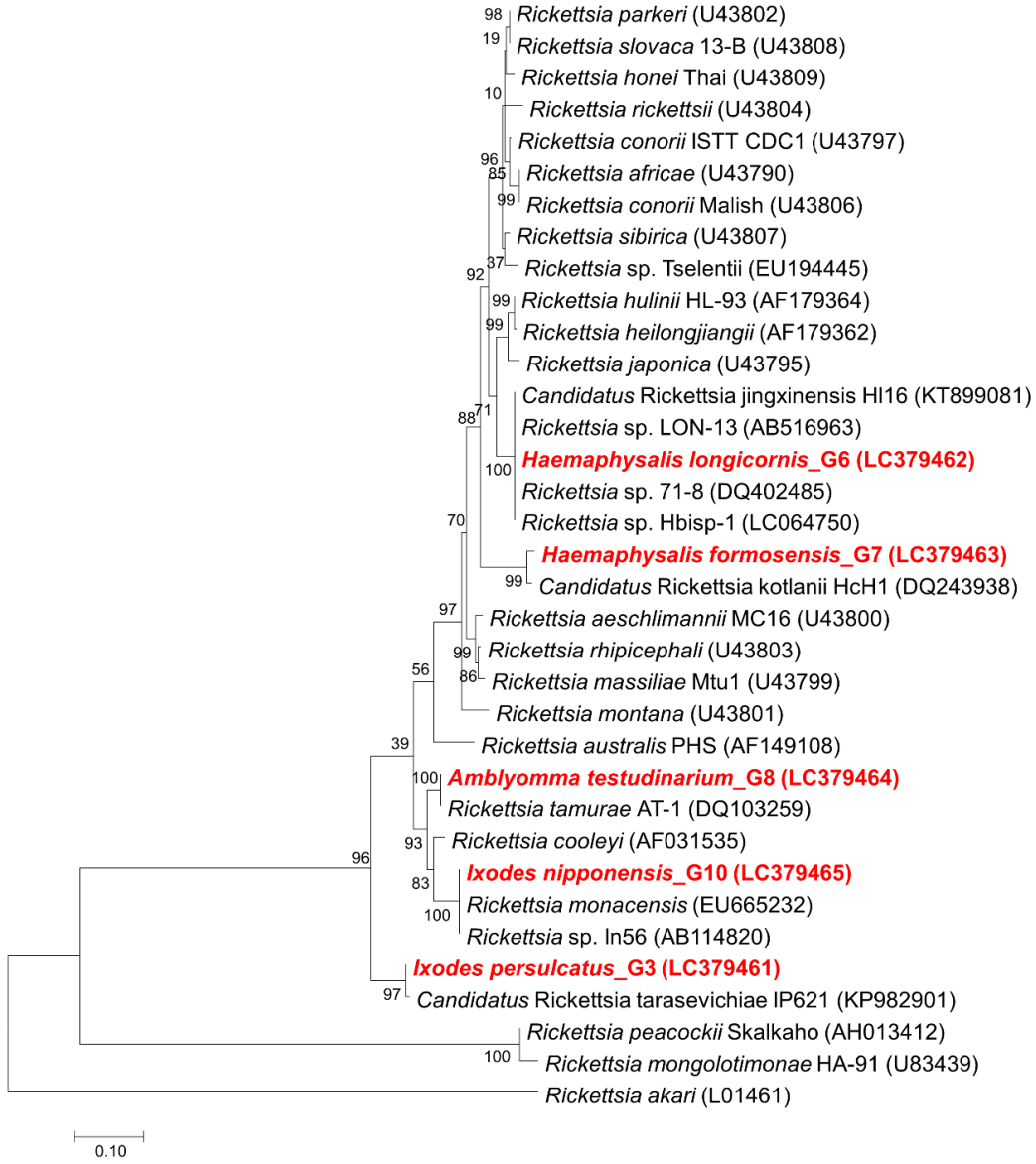

Figure I-4. Phylogenetic tree based on the sequences of the *ompA* gene of spotted fever group rickettsiae. The analyses were performed using a maximum likelihood method with the Kimura two-parameter model. All bootstrap values from 1,000 replications are shown on the interior branch nodes. The sequences obtained in this study are shown in red.

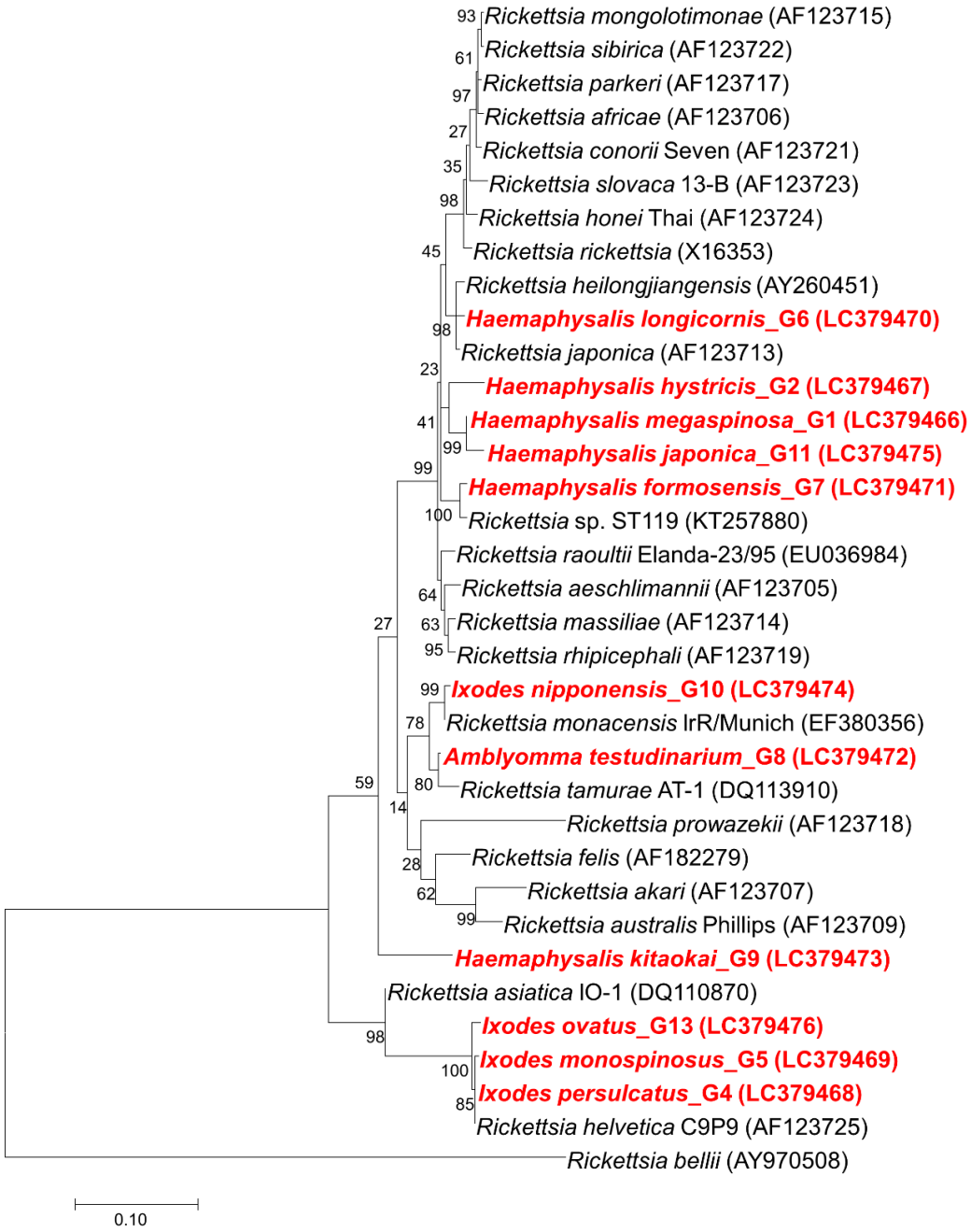

Figure I-5. Phylogenetic tree based on the sequences of the *ompB* gene of spotted fever group rickettsiae. The analyses were performed using a maximum likelihood method with the Kimura two-parameter model. All bootstrap values from 1,000 replications are shown on the interior branch nodes. The sequences obtained in this study are shown in red.

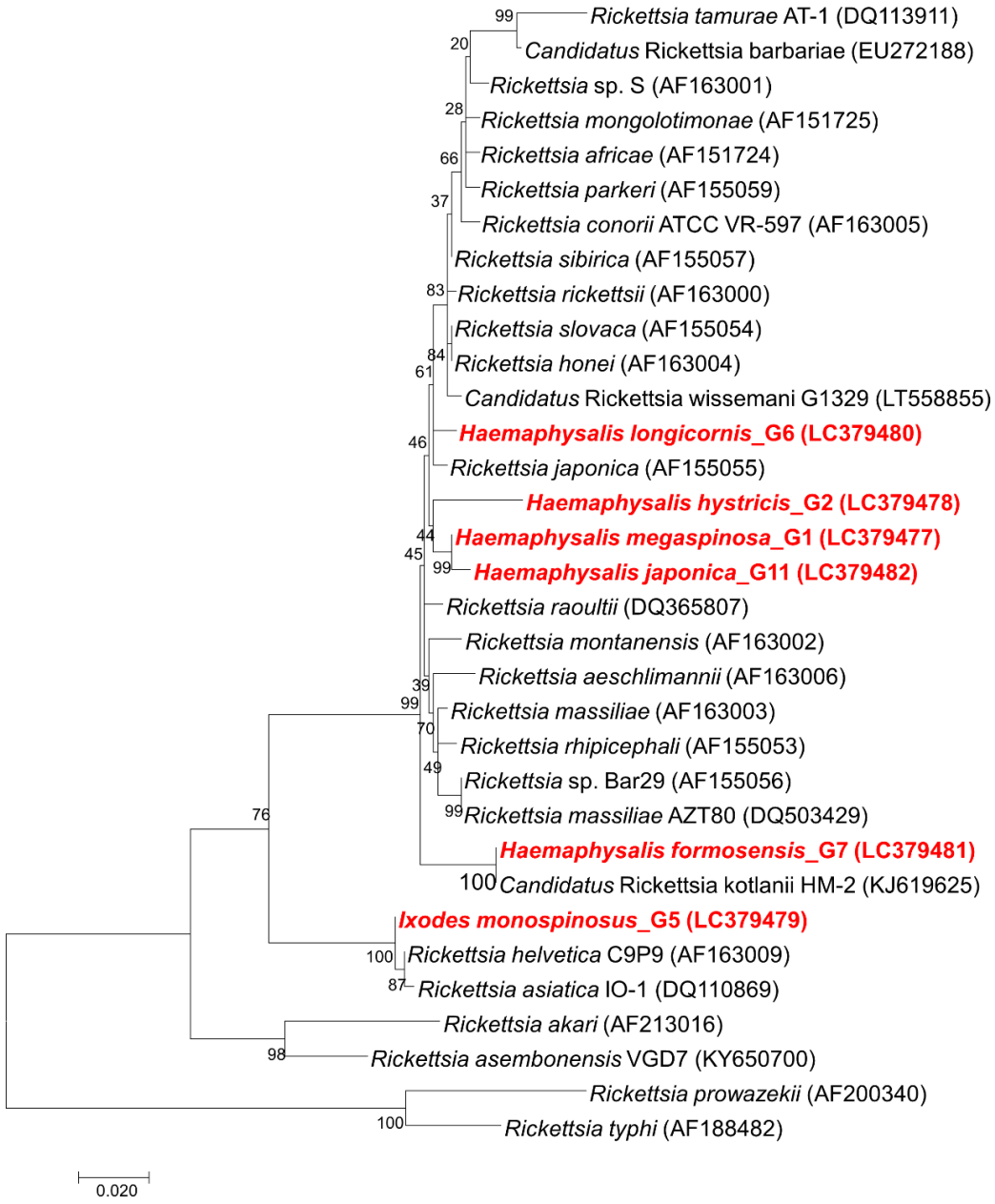

Figure I-6. Phylogenetic tree based on the sequences of the *sca4* gene of spotted fever group rickettsiae. The analyses were performed using a maximum likelihood method with the Kimura two-parameter model. All bootstrap values from 1,000 replications are shown on the interior branch nodes. The sequences obtained in this study are shown in red.

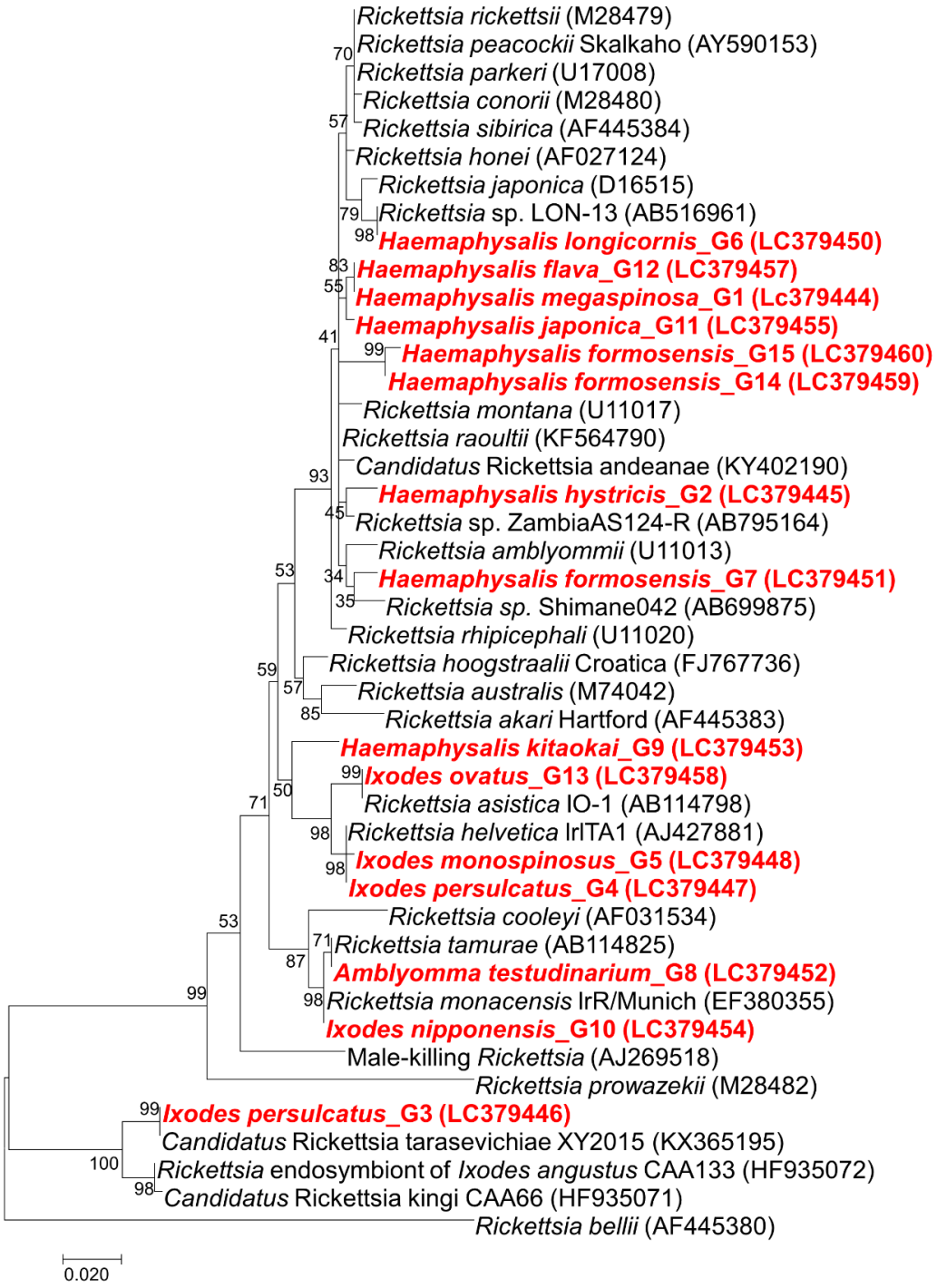

Figure I-7. Phylogenetic tree based on the sequences of the *htrA* gene of spotted fever group rickettsiae. The analyses were performed using a maximum likelihood method with the Kimura two-parameter model. All bootstrap values from 1,000 replications are shown on the interior branch nodes. The sequences obtained in this study are shown in red.

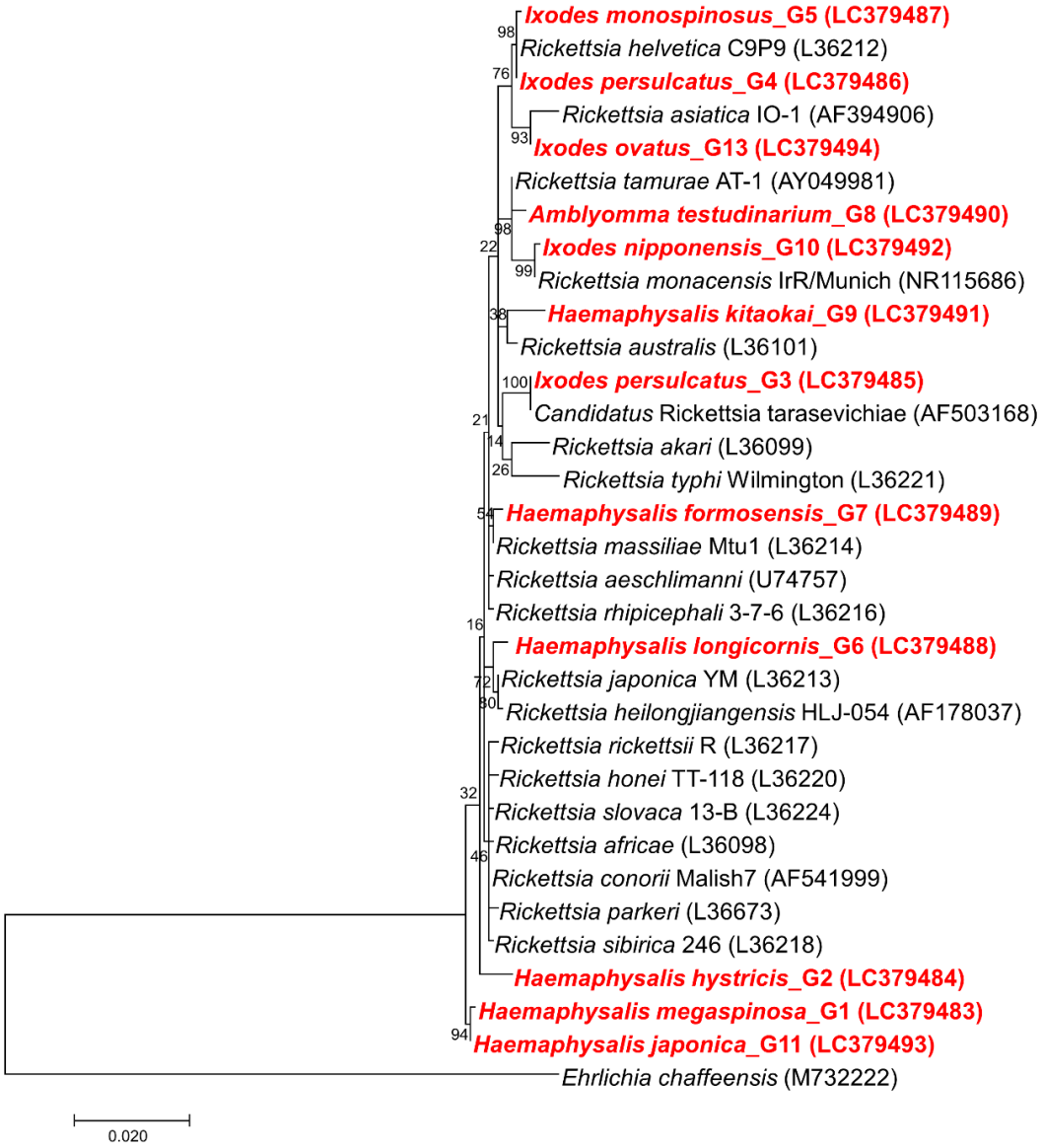

Figure I-8. Phylogenetic tree based on the sequences of the 16S rRNA coding gene of spotted fever group rickettsiae. The analyses were performed using a maximum likelihood method with the Kimura two-parameter model. All bootstrap values from 1,000 replications are shown on the interior branch nodes. The sequences obtained in this study are shown in red.

CHAPTER II

Isolation of *Rickettsia, Rickettsiella,* and *Spiroplasma* from questing ticks in Japan using arthropod cells

1 INTRODUCTION

Ticks are important vectors among blood sucking ectoparasites that transmit various zoonotic pathogens to humans and animals through their bite. Ticks harbour not only pathogenic microorganisms of veterinary and medical importance (Jongejan and Uilenberg, 2004) but also several endosymbionts of the genera *Coxiella*, *Francisella*, and *Rickettsia* (Paddock et al., 2004; Ahantarig et al., 2013). The recent development of deep sequencing technologies has enabled high-throughput screening of pathogens and symbionts in ticks and expanded our knowledge on the diversity of microorganisms harboured by ticks (Nakao et al., 2013; Qiu et al., 2014; Kurilshikov et al., 2015). Although these studies have led to the discovery of several previously unexpected or poorly characterised microorganisms, it is challenging to evaluate their roles in ticks and their pathogenic potential to animals solely based on their partial genome sequences.

In Japan, several tick-borne human diseases have been recognised. Until the recent emergence of severe fever with thrombocytopenia syndrome (SFTS) (Takahashi et al., 2014) and the re-emergence of tick-borne encephalitis (TBE) (Yoshii et al., 2017), most cases of tick-borne human diseases have been associated with bacterial infections. In particular, Japanese spotted fever (JSF) caused by *Rickettsia japonica* is the most common tick-borne human diseases with hundreds of cases ported annually (National Institute of Infectious Diseases 2017). Several other rickettsioses caused by *Rickettsia heilongjiangensis*, *Rickettsia helvetica*, and *Rickettsia tamurae* have also been reported to date (Noji et al., 2005; Ando et al., 2010; Imaoka et al., 2011). The etiological agents of these rickettsial diseases were isolated from patients and ticks primarily using L929 mouse fibroblast cells (Uchida

et al., 1992; Fournier et al., 2002; Fujita et al., 2006; Mahara, 2006; Ando et al., 2010; Andoh et al., 2014). However, none of these studies have used arthropod cells for isolation of tick-borne pathogens.

At present, tick cell lines are indispensable tools to study the interaction between ticks and tick-borne microorganisms including pathogens and symbionts *in vitro* (Bell-Sakyi et al., 2007; Bell-Sakyi et al., 2012). The cells have also been successfully used for isolating and propagating a number of tick-borne microorganisms (Bell-Sakyi et al., 2007; Bell-Sakyi et al., 2015). For example, the previously unculturable *Borrelia lonestari* was isolated and propagated for the first time in a tick cell line (Varela et al., 2004). Similarly, other tick-borne pathogens from genera such as *Anaplasma* and *Ehrlichia* have been successfully cultivated and maintained in tick cell lines (Munderloh et al., 2003; Zweygarth et al., 2013).

The main goal of this chapter was to isolate and characterise tick-borne microorganisms from field-collected ticks using two arthropod cell lines derived from *Ixodes scapularis* embryo (ISE6) and *Aedes albopictus* larvae (C6/36). To further characterize these isolates, four rickettisal genes (16SrRNA, *gltA*, *opmA* and *ompB*) of rickettsiae were amplified and sequenced. As a result, five rickettsial genotypes including four previously validated rickettsial species, one uncharacterised rickettsial genotype and two tick endosymbionts, *Rickettsiella* and *Spiroplasma* were isolated in an arthropod cell lines.

2 MATERIALS AND METHODS

2.1 Tick samples

This study employed unfed ticks collected at 11 different prefectures by a flagging method between 2013 and 2015 (Table II-1). Tick species were identified morphologically under a stereomicroscope using standard keys (Yamaguti et al., 1971; Nakao et al.,1992). The samples included fifteen different tick species; *Amblyomma testudinarium* (n = 10), *Dermacentor taiwanensis* (n = 3), *Haemaphysalis concinna* (n = 3), *H. flava* (n = 8), *H. formosensis* (n = 15), *H. hystricis* (n = 24), *H. japonica* (n = 9), *H. kitaokai* (n = 3), *H. longicornis* (n = 18), *H. megaspinosa* (n = 27), *Ixodes monospinosus* (n = 7), *I. nipponensis* (n = 2), *I. ovatus* (n = 9), *I. pavlovskyi* (n = 2) *and I. persulcatus* (n = 30). After identifying tick species, ticks were washed with 70% ethanol and sterile PBS, then homogenised in 100 µl of high-glucose Dulbecco's Modified Eagle medium (DMEM Gibco, Life Technologies, Carlsbad, CA, USA) by using Micro Smash MS100 (TOMY, Tokyo, Japan). Half of the homogenate was subjected to DNA extraction using a blackPREP Tick DNA/RNA Kit (Analytikjena, Germany), while the other half was kept at -80°C and used for this study. A total of 170 tick homogenates including 158 from single unfed tick (adult or nymph) and 12 from nymphal pools (5 to 24 nymphs/pool) were included in the present study.

2.2 Maintenance of cell lines

ISE6 cells, originally reported by Kurtti et al. (1996) and received from the CEH Institute of Virology and Environmental Microbiology (Oxford, UK), were grown in L-15B medium supplemented with 10% foetal bovine serum, and 5%

tryptose phosphate broth (Sigma-Aldrich, St. Louis, MO, USA) at 32°C as described previously (Munderloh and Kurtti, 1989), except that 0.1% bovine lipoprotein concentrate was not included in the culture medium. C6/36 cells, purchased from the American Type Culture Collection (No. CRL-1660), were grown in Minimum Essential Medium (MEM, Gibco) supplemented with 10% foetal bovine serum, 2% MEM Non-essential amino acids (Gibco), 1% Sodium Pyruvate 100 mM (Gibco), and 1% L-glutamine (Gibco) at 28°C in a humidified atmosphere of 5% CO_2 in air.

2.3 Co-culture with tick homogenates

ISE6 and C6/36 cells were seeded in 24-well culture plates and incubated overnight. On the following day, 5 µl of each tick homogenate was inoculated into separate wells of both cell lines. Culture medium was changed every three days for C6/36 cells and once a week for ISE6 cells. At 2 weeks post-inoculation (pi), 100 µl of culture suspension was passaged into new wells containing uninfected cells. Second and third passages were conducted in the same way as first at 4 and 6 weeks pi, respectively. At 8 weeks pi, the experiment was terminated. All the bacterial isolates obtained in this study were preserved at -80°C for downstream analysis. Cell morphology was observed daily under an inverted microscope to detect cytopathic effects presumably caused by bacterial infections. When contamination of fungi or environmental bacteria was observed, the contaminated wells were sterilised with 10% hypochlorous acid for more than 10 min to prevent the spread of contamination to the neighbouring wells.

2.4 PCR

When the cells showed sign of bacterial infection, DNA was extracted from 100 µl of parent culture suspension and/or first subculture using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) following the manufacturer's instructions. In addition, cell suspensions from all wells at 4 and 8 weeks pi (from first and third subcultures, respectively) were subjected to DNA extraction to detect possible bacterial infection whether or not morphological changes were seen in cells. PCR was conducted using the primers, fD1 and Rp2 to amplify eubacterial 16S ribosomal DNA (rDNA) (Weisburg et al., 1991). In order to characterise rickettsial isolates, three additional genes were amplified: citrate synthase gene (*gltA*) (Gaowa et al., 2013), 190-kDa outer membrane gene (*ompA*) (Regnery et al., 1991) and 120-kDa outer membrane protein gene (*ompB*) (Roux and Raoult, 2000). All PCR reactions were conducted in a 25 µl-reaction mixture containing 2.5 µl of 10 × KOD Plus Neo PCR Buffer, 0.5 µl of a high-fidelity KOD-Plus-Neo DNA polymerase (Toyobo), 200 nM of each primer, and 1.0 µl of template DNA. PCR conditions were as follows: 40 cycles of denaturation (94°C, 15 sec), annealing (55°C for *gltA*, *ompA*, and 16S rDNA and 48°C for *ompB*, 30 sec) and extension (68°C, 30 sec for *gltA*, *ompA*, and *ompB* and 90 sec for 16S rDNA). For some samples, we conducted TA-cloning using the pGEM-T vector (Promega, Madison, WI) as described previously (Nakao et al., 2013). The experimental procedures were approved by the Hokkaido University Safety Committee on Genetic Recombination Experiments (No. 2017-046). All the primer information is available in Table II-2. All negative control containing sterile water instead of template DNA was included in all PCR assays.

2.5 Real-time PCR

Real-time PCR to detect *gltA* gene of SFG and TG rickettsiae was conducted using the primers and probe shown in Table II-2. Reactions mixtures were prepared using THUNDERBIRD Probe qPCR Mix (Toyobo, Osaka, Japan) and the reactions were carried out in a C1000 Thermal Cycler with a CFX96 Real-Time PCR Detection System (BioRad Laboratories, Hercules, CA) as described in chapter I.

2.6 Sequencing and data analysis

All amplified PCR products were purified using a NucleoSpin Gel and PCR Clean Up Kit (Takara Bio Inc.) and sequenced using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). To sequence 16S rDNA PCR products, sequencing primers were newly designed in the present study (Table II-2). The purified sequencing products were analysed on an ABI Prism 3130xl Genetic Analyzer Kit (Applied Biosystems) according to the manufacturers' instructions. The sequences that were obtained were submitted to the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp) under accession numbers (16S rRNA: LC388759-LC388776; *gltA*: LC388777-LC388788; *ompA*: LC388789-LC388795; and *ompB*: LC388796-LC388807). Sequenced data were aligned using ATGC software version 6.0.4. Phylogenetic analyses were conducted by a maximum likelihood method using MEGA7 version 7.0.18 (Kumar et al., 2016). Bootstrap values were obtained with 1,000 replicates.

3 RESULTS

3.1 Real-time PCR

Among 170 tick homogenates, 114 were tested positive by real-time PCR (Table II-1). Some of the samples were tested by real-time PCR in chapter I. For the bacterial isolation, both rickettsiae-positive and -negative samples were used.

3.2 Isolation results

During the 8 weeks observation period, we confirmed bacterial isolation in 14 and 4 different samples using ISE6 and C6/36 cells, respectively (Table II-3). Ten isolates (9 from ISE6 and 1 from C6/36) were obtained in the parent cultures, while 8 isolates (5 from ISE6 and 3 from C6/36) were obtained in the first subcultures (Figure II-1). The sequencing analysis of the 16S rDNA PCR products indicated that they were previously known tick-borne bacteria in three different genera; *Rickettsia*, *Rickettsiella*, and *Spiroplasma* (Table II-4). Although a further 11 and 4 samples in ISE6 and C6/36 cells, respectively, also showed bacterial growth in the well, sequencing analysis indicated the growth of environmental bacteria such as *Bacillus* spp. *Pseudomonas* spp., and *Mycobacterium* spp. (data not shown). Fungal infections developed in 75 ISE6 and 57 C6/36 wells; the remaining 77 and 98 wells were respectively did not yield any isolates (Table II-3). Although 4 rickettsial and 3 spiroplasmal isolates did not show any cytopathic effects in the infected cells, their infections were detected by PCR conducted at 28 days pi (Figure II-1).

3.3 *Rickettsia* isolation

Twelve isolates of *Rickettsia* (11 from ISE6 and 1 from C6/36) were obtained from different tick homogenates (Table II-3). The amplification of *gltA* and *ompB* genes was successful in all rickettsial isolates, while the *ompA* gene was amplified only from seven isolates (Table II-4). Based on the phylogenetic analysis of each rickettsial gene, the 12 isolates were identified as 4 previously validated species, *R. asiatica* (n = 2), *R. helvetica* (n = 3), *R. monacensis* (n = 2), *R. tamurae* (n = 3) and *Rickettsia* sp. LON, one uncharacterised rickettsial genotype, previously isolated from *H. longicornis* in Japan (Fujita, 2008) (n = 2) (Figure II-2, Figure II-3, Figure II-3, Figure II-4 and Figure II-5). There was a complete correspondence between rickettsial species/genotype and tick species of origin; *R. asiatica*, *R. helvetica*, *R. monacensis*, *R. tamurae*, and *Rickettsia* sp. LON were isolated from *I. ovatus*, *I. persulcatus*, *I. nipponensis*, *A. testudinarium*, and *H. longicornis*, respectively. A cytopathic effect was observed in *R. helvetica*-, *R. monacensis*-, and *R. tamurae*-infected cells at 6, 14 and 10 days pi, while there was no obvious morphological damage observed in *R. asiatica*- and *Rickettsia* sp. LON-infected cells (Figure II-1).

3.4 *Rickettsiella* isolation

Rickettsiella was isolated from a homogenate of *H. concinna* collected in Hokkaido using both ISE6 and C6/36 cells (Table II-1). A cytopathic effect was observed at 6 and 13 days pi in ISE6 and C6/36 cells, respectively (Figure II-1). The sequences of 16S rDNA of *Rickettsiella* obtained from two cell lines (Hcn-412I and Hcn-412C) were identical and showed 100% identity with *Rickettsiella* sp. detected from *Ixodes uriae* from Grimsey Island in Iceland (GenBank No.

KT697673). A phylogenetic analysis showed that our isolates formed a cluster with *Rickettsiella* spp. detected from pea aphids (Figure II-6).

3.5 *Spiroplasma* isolation

Four *Spiroplasma* isolates were obtained (2 each from ISE6 and C6/36) (Table II-3). Since two isolates were obtained from the same tick homogenate using different cell lines, four isolates originated from three tick homogenates: *I. monospinosus*, *I. persulcatus*, and *H. kitaokai* collected from Yamagata, Hokkaido and Fukushima prefectures, respectively (Table II-1). Only one isolate (Ipe-147I) showed a cytopathic effect in ISE6 cells at 23 days pi (Figure II-1). Among 4 isolates, two sequence types of 16S rDNA had two base pair (bp) differences in their sequences. One sequence type was obtained from two isolates (Imo-135I and Imo-135C) from *I. monospinosus* and one isolate (Hki-1033C) from *H. kitaokai*, while the other was from *I. persulcatus* (Ipe-147I). Both sequences showed the highest identities (1443/1444 bp and 1441/1444 bp) with 16S rDNA from *Spiroplasma* sp. detected from *Fannia manicata* (little housefly) (GenBank No. AY569829). In a phylogenetic analysis, these isolates were clustered together with 16S rDNA of two tick-derived *Spiroplasma* isolates; *Spiroplasma ixodetis* (GenBank No. NR104852) obtained from *Ixodes pacificus* and *Spiroplasma* sp. Bratislava 1 (GenBank No. KP967685) obtained from *I. ricinus*, and several *Spiroplasma* spp. detected from various arthropods (Figure II-7).

4 DISCUSSION

Isolation and propagation of *R. monacensis* was firstly achieved using ISE6 cells. This rickettsial agent has not been officially reported in Japan; in a recent nationwide survey where *R. monacensis* infection was found in *I. nipponensis* with high infection rates (2 positive out of 3 in the Tohoku region and 2 positive out of 2 in the Kansai region) (Chapter I). *R. monacensis* was first isolated from *Ixodes ricinus* collected in Germany using ISE6 cells (Simser et al., 2002), where the authors detected a cytopathic effect after the third passage (5 months). In this experiment, a cytopathic effect was observed as early as 14 days after the inoculation in both ISE6 and C6/36 cells (Figure II-1). Although the reason for this difference is not clear, it may suggest that phenotypes under in *vitro* culture conditions are different between *R. monacensis* strains. *R. monacensis* has been associated with a human rickettsiosis presenting Mediterranean spotted fever-like symptoms (Jado et al., 2007; Madeddu et al., 2012; Kim et al., 2017). The present study reconfirmed the presence of this rickettsial agent in Japan and highlighted the necessity for further investigation of the clinical cases it may cause.

In addition to *R. monacensis*, three previously validated rickettsial species, *R. asiatica*, *R. helvetica*, *R. tamurae*, and one uncharacterised genotype, *Rickettsia* sp. LON were isolated using ISE6 cells, among which only *R. asiatica* was also isolated from C6/36 cells (Table II-4). Although the C6/36 cells inoculated with the tick homogenates from which *Rickettsia* were isolated using ISE6 cells were tested for rickettsial infections by PCR at 8 weeks pi, there was no positive amplicons. However, when the isolates of *R. monacensis* and *R. helvetica* obtained from ISE6 culture were inoculated into C6/36 cells in preliminary experiments, their persistent

growth in the cells was observed (data not shown). In fact, *R. monacensis* and *R. helvetica* were isolated from *I. ricinus* in Portugal using C6/36 cells in a previous study (Milhano et al., 2010). These results may indicate that lower success rates of rickettial isolation using C6/36 cells is partly attributed to low bacterial burden in the inocula. It is also possible that propagation in ISE6 cells might help the rickettsial isolates adapt to *in vitro* culture conditions using C6/36.

There are only a few reports on the *in vitro* propagation of *Rickettsiella*. For example, *Rickettsiella grylli* was isolated from the variegated grasshopper, *Zonocerus variegatus*, was cultured in several cell lines derived from different arthropods (Henry et al., 1986). To the best of our knowledge, this is the first report of the successful isolation of *Rickettsiella* spp. from ticks using ISE6 cells and C6/36 cells. Bacteria within the genus *Rickettsiella* are known to be symbionts of many arthropods (Tsuchida et al., 2010; Leclerque et al., 2011; Iasur-Kruh et al., 2013; Łukasik et al., 2013). In ticks, *Rickettsiella* species have been reported in genera *Ixodes* and *Ornithodoros* (Kurtti et al., 2002; Vilcins et al., 2009; Duron et al., 2015; Duron et al., 2016). A recent metagenomic approach based on 16S rDNA amplicons also showed the presence of *Rickettsiella* in the genus *Haemaphysalis* (Khoo et al., 2016). The sequence analysis of 16S rDNA revealed that the *Rickettsiella* sp. isolated from *H. concinna* showed 100% identity with *Rickettsiella* endosymbiont detected in *I. uriae* collected from a seabird in Iceland (Figure II-6). These facts may support the hypothesis that *Rickettsiella* has a wide geographical distribution in ticks and is maintained by horizontal transfer between arthropod species as previously suggested (Duron et al., 2016). However, a more detailed analysis such as whole genome comparison between *Rickettsiella* spp. found in different arthropod hosts is essential to prove this hypothesis. The role of

Rickettsiella in ticks is totally unknown; however, the lines of evidence from other arthropods indicated an effect on the survival of their host arthropods (Tsuchida et al., 2010; Łukasik et al., 2013). The isolate obtained in this chapter might be useful to further investigate potential roles of *Rickettsiella* in ticks.

Four spiroplasmal isolates were obtained from three tick species: *I. monospinosus*, *I. persulcatus*, and *H. kitaokai*. The isolates obtained from *I. monospinosus* and *H. kitaokai* had completely identical 16S rDNA sequence and all spiroplamal isolates in this chapter made one clade with previously reported *Spiroplasma* species which were detected from a variety of arthropod including ticks, ladybirds, plant hoppers, and mealybugs (Figure II-7). These findings may suggest that *Spiroplasma* is maintained by horizontal transfer between different arthropod species as suggested for *Rickettsiella*. This hypothesis should be explored in future studies. Most members of *Spiroplasma* are symbionts in arthropods and some of them are known to be beneficial to their hosts for example, by protecting from fungal or parasitic infections (Łukasik et al., 2013; Xie et al., 2014; Yadav et al., 2018). In some arthropods, *Spiroplasma* species are pathogenic and cause gender-ratio distortions known as a male-killing effect in *Drosophila* (Harumoto et al., 2014). However, a recent study conducted on a nidicolous tick *Ixodes arboricula* did not find any association between female-biased sex ratios and infections of six maternally inherited bacteria including *Spiroplasma* (Van Oosten et al., 2018). Moreover, *Spiroplasma mirum*, an isolate obtained from rabbit tick *Haemaphysalis leporispalustris* in the USA (Tully et al., 1982), was shown to have potentially pathogenic properties; for example, *S. mirum* was virulent for chick embryos and induced cataracts or lethal brain infections when introduced intracerebrally into experimental animals such as suckling rats, rabbits (Tully et al.,

1977). Collectively, further biological characterisation of *Spiroplasma* detected in the present study is necessary to understand their roles in ticks and potential risks for human and animal health.

In chapter II, a number of wells were contaminated with bacterial or fungal infections (79 wells of ISE6 culture and 78 wells of C6/36 culture) (Table II-3), despite the fact that tick surface was washed with 70% ethanol and sterile PBS. This result highlights the necessity of using additional chemicals to sterilize tick surface, especially the ones effective for fungal infections. Another possible option might be to use only internal organs for bacterial isolation by dissecting ticks.

5 SUMMARY

Ticks are blood sucking ectoparasites that transmit zoonotic pathogens to humans and animals. Ticks harbour not only pathogenic microorganisms, but also endosymbionts. Although some tick endosymbionts are known to be essential for the survival of ticks, their roles in ticks remain poorly understood. The main aim of this chapter was to isolate and characterise tick-borne microorganisms from field-collected ticks using two arthropod cell lines derived from *Ixodes scapularis* embryo (ISE6) and *Aedes albopictus* larvae (C6/36). A total of 170 tick homogenates originating from 15 different tick species collected in Japan were inoculated into each cell line. Bacterial growth was confirmed by PCR amplification of 16S ribosomal DNA (rDNA) of eubacteria. During the 8 weeks observation period, bacterial isolation was confirmed in 14 and 4 different samples using ISE6 and C6/36 cells, respectively. The sequencing analysis of the 16S rDNA PCR products indicated that they were previously known tick-borne pathogens/endosymbionts in three different genera; *Rickettsia*, *Rickettsiella*, and *Spiroplasma.* These included 4 previously validated rickettsial species namely *Rickettsia asiatica* (n = 2), *Rickettsia helvetica* (n = 3), *Rickettsia monacensis* (n = 2), and *Rickettsia tamurae* (n = 3) and one uncharacterised genotype *Rickettsia* sp. LON (n = 2). Four isolates of *Spiroplasma* had the highest similarity with previously reported *Spiroplasma* isolates; *Spiroplasma ixodetis* obtained from ticks in North America and *Spiroplasma* sp. Bratislava 1 obtained from *Ixodes ricinus* in Europe, while two isolates of *Rickettsiella* showed 100% identity with *Rickettsiella* sp. detected from *Ixodes uriae* at Grimsey Island in Iceland. To the best of our knowledge, this is the first report on successful isolation of *Rickettsiella* from ticks.

The isolates obtained in this study can be further analysed to evaluate their pathogenic potential to animals and their roles as symbionts in ticks.

Table II-1. Collection detail of ticks used for bacterial isolation using arthropod cells.

Prefecture	Collected year	Tick species	Female	Male	Nymph	Nymphal pool[a]
Hokkaido	2013	*H. concinna*	-	3 (0)[b]	-	-
	2013	*H. japonica*	5 (0)	1 (1)	-	-
	2013 & 2015	*H. megaspinosa*	3 (1)	10 (4)	-	5 (5)
	2013	*I. ovatus*	1 (0)	5 (1)	-	-
	2015	*I. pavlovskyi*	-	2 (0)	-	-
	2013, 2014 & 2015	*I. persulcatus*	20 (18)	8 (5)	-	-
Fukushima	2014	*D. taiwanensis*	-	1 (0)	-	-
	2013	*H. flava*	-	3 (2)	-	1 (1)
	2014	*H. japonica*	1 (1)	1 (1)	-	1 (1)
	2014	*H. kitaokai*	2 (0)	1 (0)	-	-
	2014	*H. megaspinosa*	-	-	-	1 (1)
	2014	*I. monospinosus*	-	1 (1)	-	-
	2014	*I. nipponensis*	-	1 (1)	-	-
	2014	*I. persulcatus*	1 (1)	1 (0)	-	-
Yamagata	2014	*H. flava*	1 (1)	-	-	1 (1)
	2013 & 2014	*I. monospinosus*	5 (5)	1 (1)	-	-
	2013	*I. nipponensis*	-	1 (1)	-	-
	2014	*I. ovatus*	1 (1)	1 (1)	-	-
Tochigi	2014	*H. flava*	-	-	-	1 (1)
Shizuoka	2013	*H. longicornis*	5 (5)	-	-	-
Nara	2014	*H. longicornis*	-	-		1 (1)
	2014	*H. megaspinosa*	-	1	1	
Wakayama	2015	*A. testudinarium*	1 (1)	-	-	-
	2015	*D. taiwanensis*	1 (0)	1 (0)	-	-
	2015	*H. formosensis*	1 (0)	-	-	-
	2013	*H. hystricis*	4 (3)	-	-	-
	2013 & 2015	*H. longicornis*	3 (2)	8 (8)	1 (1)	-
	2015	*H. megaspinosa*	1 (1)	-	-	-
	2015	*I. ovatus*	1 (0)	-	-	-
Kumamoto	2015	*H. formosensis*	4 (1)	3 (2)	-	-
	2015	*H. hystricis*	1 (1)	2 (1)	-	-
Miyazaki	2013	*A. testudinarium*	-	-	6 (3)	-
	2013	*H. flava*	1 (1)	-	-	-
	2013	*H. formosensis*	1 (1)	2 (0)	-	-
	2013	*H. megaspinosa*	2 (2)	1 (1)	-	-
Kagoshima	2015	*A. testudinarium*	-	-	2 (1)	-
	2015	*H. formosensis*	-	4 (1)	-	-
	2015	*H. hystricis*	5 (4)	12 (11)	-	-
	2013 & 2015	*H. megaspinosa*	1 (1)	1 (1)	-	-
Okinawa	2014	*A. testudinarium*	-	-	1 (1)	-
Total			72 (51)	76 (45)	10 (6)	12 (12)

-, This tick species was not collected in the study.

[a]Nymphal pool samples were prepared from a pool of 5 to 24 nymphs.

[b]The number in parentheses indicates the number of samples positive for rickettsiae by *gtlA* real-time PCR.

Table II-2. Oligonucleotide primer pairs and probe used in real-time and conventional polymerase chain reactions and sequencing.

Primer	Primer sequence (5'-3')	Target gene	Target organism(s)	Annealing temperature (°C)	Amplicon size (bp)	Reference
CS-F	TCGCAAATGTTCACGGTACTTT	citrate synthase gene (*gltA*)	Rickettsiae spotted fever group and typhus group	60	74	Stenos et al., 2005
CS-R	TCGTGCATTTCTTTCCATTGTG					
CS-P	TGCAATAGCAAGAACCGTAGGCTGGATG					
gltA_Fc	CGAACTTACCGCTATTAGAATG	citrate synthase gene (*gltA*)	*Rickettsia* spp.	55	580	Gaowa et al., 2013
gltA_Rc	CTTTAAGAGCGATAGCTTCAAG					
Rr.190.70p	ATGGCGAATATTTCTCCAAAA	outer membrane A gene (*ompA*)	*Rickettsia* spp.	55	632	Roux et al., 1996
Rr.190.701n	GTTCCGTTAATGGCAGCATCT					
120-2788	AAACAATAATCAAGGTACTGT	outer membrane B gene (*ompB*)	*Rickettsia* spp.	48	816	Roux and Raoult, 2000
120-3599	TACTTCCGGTTACAGCAAAGT					
fD1	AGAGTTTGATCCTGGCTCAG	16S ribosomal RNA gene (16S rRNA)	Eubacteria	55	about 1500	Weisburg et al., 1991
Rp2	ACGGCTACCTTGTTACGACTT					
rrs_seq1	AGGCCTTCATCACTCACTCG*	16S rRNA of *Rickettsia*	*Rickettsia* spp.			This study
rrs_seq2	CTACACGCGTGCTACAATGG*	16S rRNA of *Rickettsia*, *Spiroplasma* and *Rickettsiella*	*Rickettsia* spp., *Spiroplasma* spp. *and Rickettsiella* spp.			This study
rrs_seq3	CGTGTCTCAGTCCCAATGTG*	16S rRNA of *Spiroplasma*	*Spiroplasma* spp.			This study

*The primers were used for sequencing.

Table II-3. Summary of bacterial isolation from homogenates of ticks collected in Japan using tick (ISE6) and mosquito (C6/36) cell lines.

Isolation result	Cell line	
	ISE6	C6/36
Rickettsia and symbionts	14	4
Bacterial contamination	4	11
Fungal contamination	75	57
No isolate	77	98
Total	170	170

Table II-4. Details of ticks from which bacterial isolates were obtained and the results of real-time and conventional PCRs amplifying bacterial isolates.

ID	Tick species	Stage /Sex	Prefecture	Arthropod cell line		Real-time PCR result[a]	PCR result[b]			
				ISE6	C6/36		16S rRNA	Rickettsial *gltA*	Rickettsial *ompA*	Rickettsial *ompB*
135	*I. monospinosus*	M	Yamagata	Isolated (*Spiroplasma*)	Isolated (*Spiroplasma*)	+	+	NA	NA	NA
141	*I. nipponensis*	M	Yamagata	Isolated (*Rickettsia*)	No isolate	+	+	+	+	+
147	*I. persulcatus*	F	Hokkaido	Isolated (*Spiroplasma*)	Contaminated (*Mycobacterium*)	+	+	NA	NA	NA
202	*I. persulcatus*	M	Hokkaido	Isolated (*Rickettsia*)	No isolate	+	+	+	-	+
309	*I. persulcatus*	M	Hokkaido	Isolated (*Rickettsia*)	No isolate	+	+	+	-	+
318	*I. persulcatus*	M	Hokkaido	Isolated (*Rickettsia*)	No isolate	+	+	+	-	+
412	*H. concinna*	M	Hokkaido	Isolated (*Rickettsiella*)	Isolated (*Rickettsiella*)	-	+	NA	NA	NA
772	*A. testudinarium*	N	Miyazaki	Isolated (*Rickettsia*)	Contaminated (7 dpi)	+	+	+	+	+
774	*A. testudinarium*	N	Miyazaki	Isolated (*Rickettsia*)	No isolate	+	+	+	+	+
1033	*H. kitaokai*	F	Fukushima	No isolate	Isolated (*Spiroplasma*)	-	+	NA	NA	NA
1187	*I. nipponensis*	M	Fukushima	Isolated (*Rickettsia*)	No isolate	+	+	+	+	+
1284	*I. ovatus*	F	Yamagata	Isolated (*Rickettsia*)	Contaminated (*Bacillus*)	+	+	+	-	+
1328	*I. ovatus*	M	Yamagata	Contaminated (2dpi)	Isolated (*Rickettsia*)	+	+	+	-	+
1994	*A. testudinarium*	F	Wakayama	Isolated (*Rickettsia*)	Contaminated (1 dpi)	+	+	+	+	+
2014	*H. longicornis*	M	Wakayama	Isolated (*Rickettsia*)	No isolate	+	+	+	+	+
2019	*H. longicornis*	M	Wakayama	Isolated (*Rickettsia*)	Contaminated (*Williamsia*)	+	+	+	+	+

[a]Screened by genus specific real-time PCR using DNA extracted from the whole ticks. +, positive; -, negative.

[b]PCR using DNA extracted from ISE6 and C6/36 cells. NA, not applicable; dpi, day post inoculation.

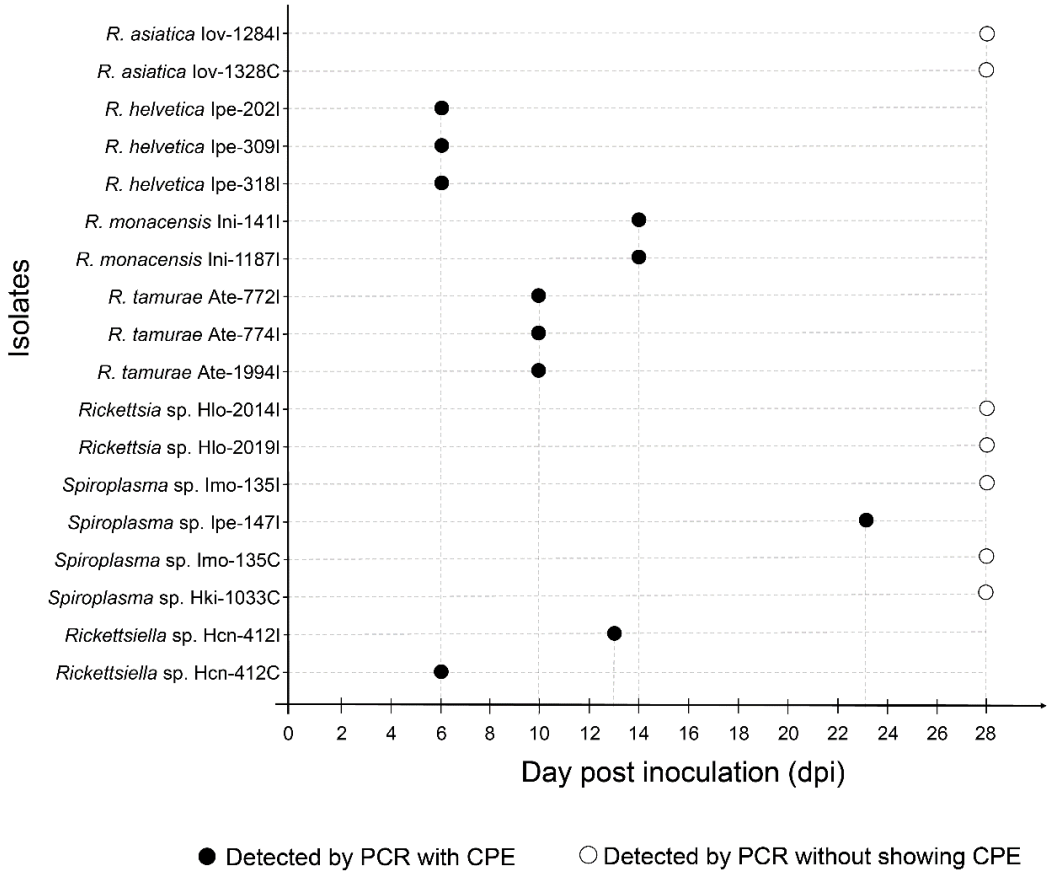

Figure II-1. The time of onset of cytopathic effect observed in each bacterial isolate in ISE6 and C6/36 cells.

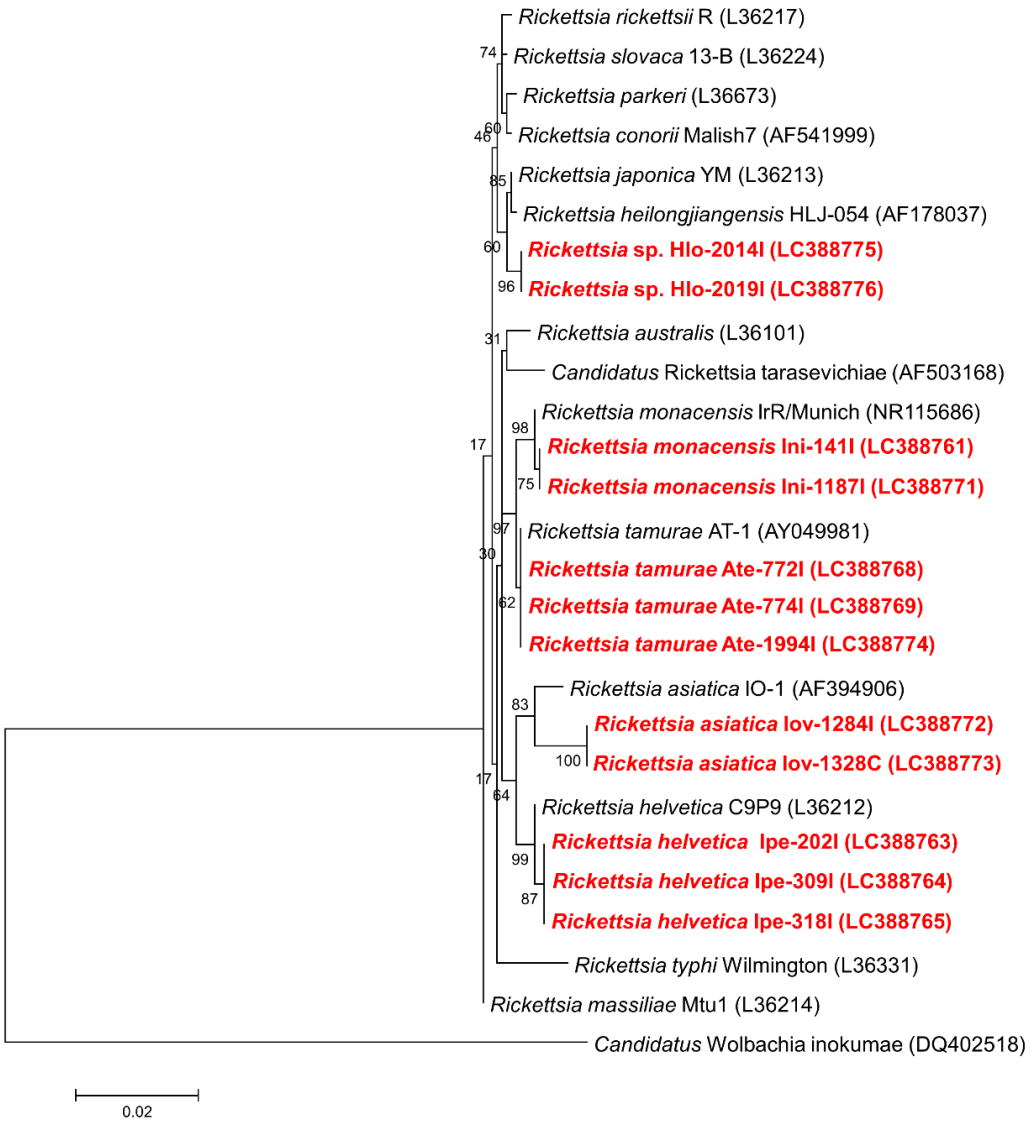

Figure II-2. Phylogenetic tree based on the sequences of 16S rRNA coding gene of *Rickettsia* isolates. The analyses were performed using maximum likelihood method with the Kimura two-parameter model. All bootstrap values from 1,000 replications are shown on the interior branch nodes. The sequences obtained in this study are shown in red.

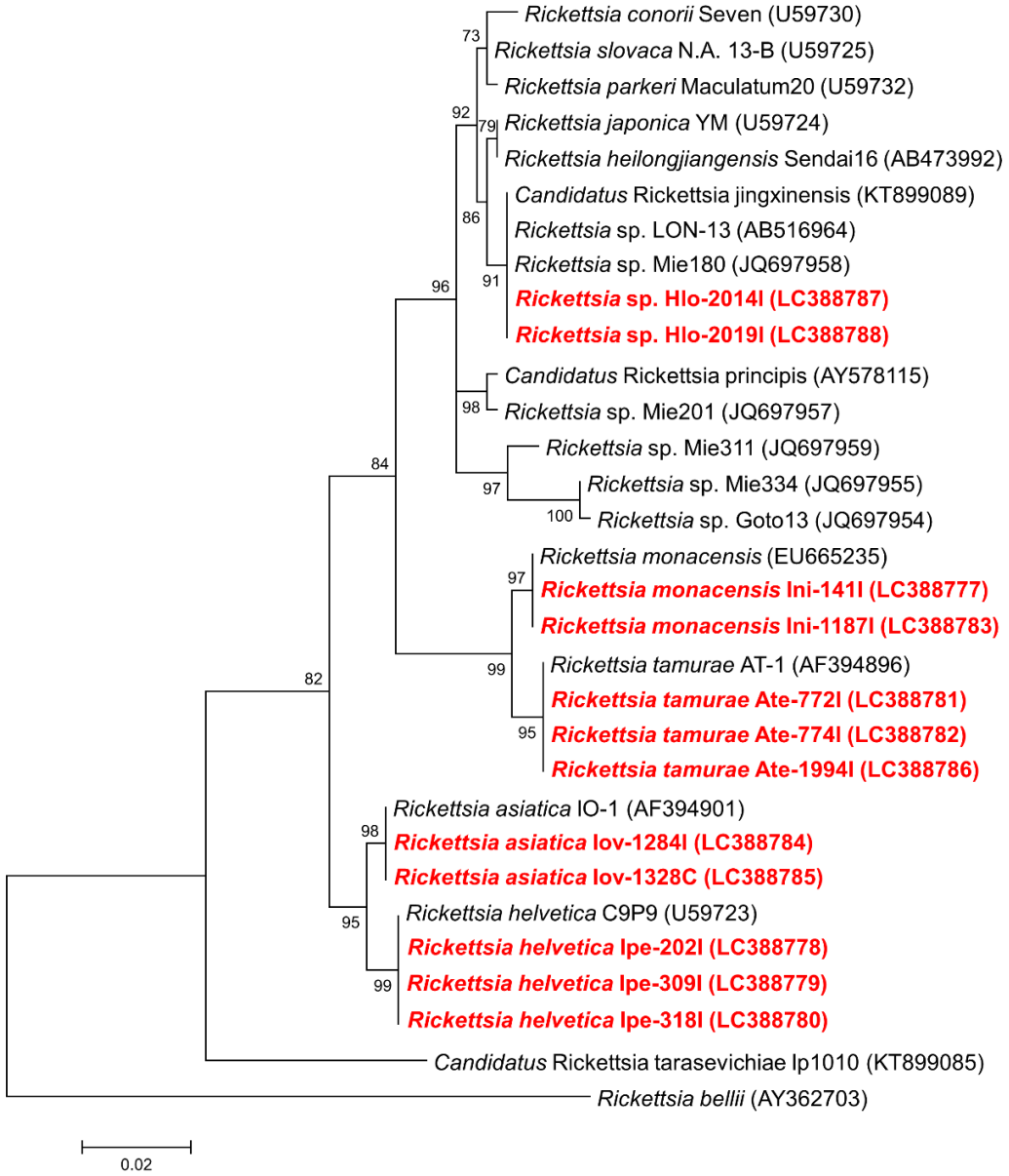

Figure II-3. Phylogenetic tree based on of the sequences of the *gltA* gene of *Rickettsia* isolates. The analyses were performed using maximum likelihood method with the Kimura two-parameter model. All bootstrap values from 1,000 replications are shown on the interior branch nodes. The sequences obtained in this study are shown in red.

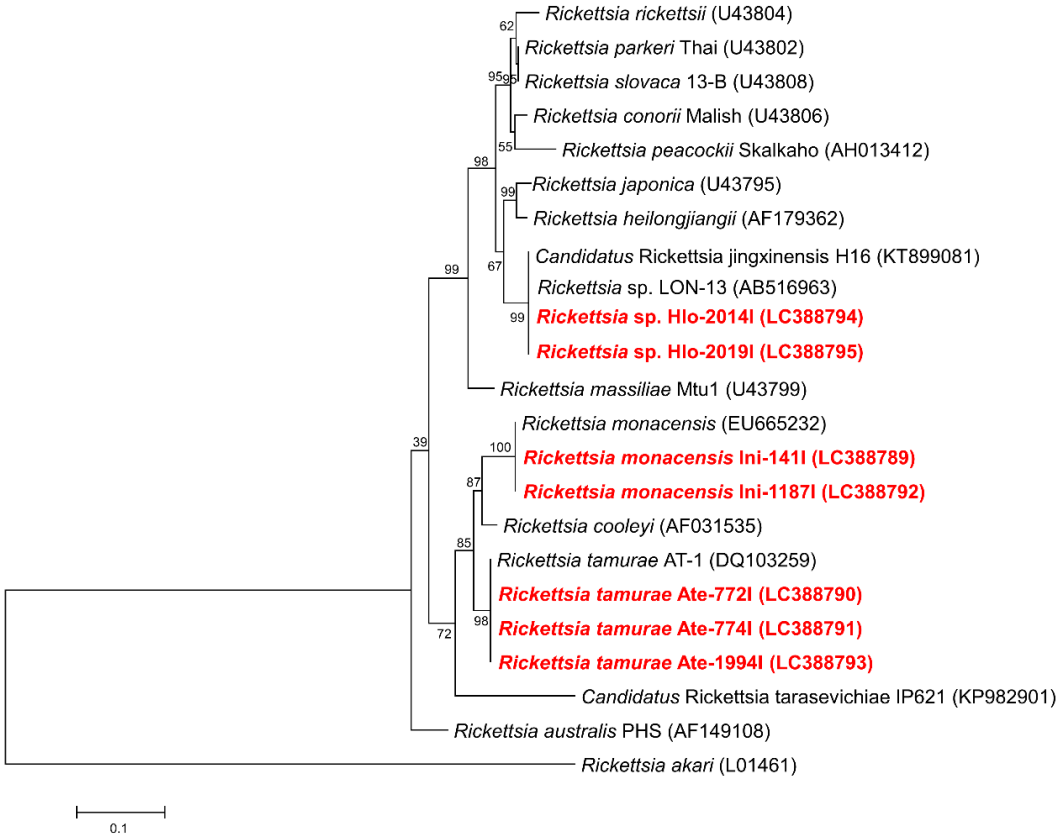

Figure II-4. Phylogenetic tree based on the sequences of *ompA* gene of *Rickettsia* species. The analyses were performed using maximum likelihood method with the Kimura two-parameter model. All bootstrap values from 1,000 replications are shown on the interior branch nodes. The sequences obtained in this study are shown in red.

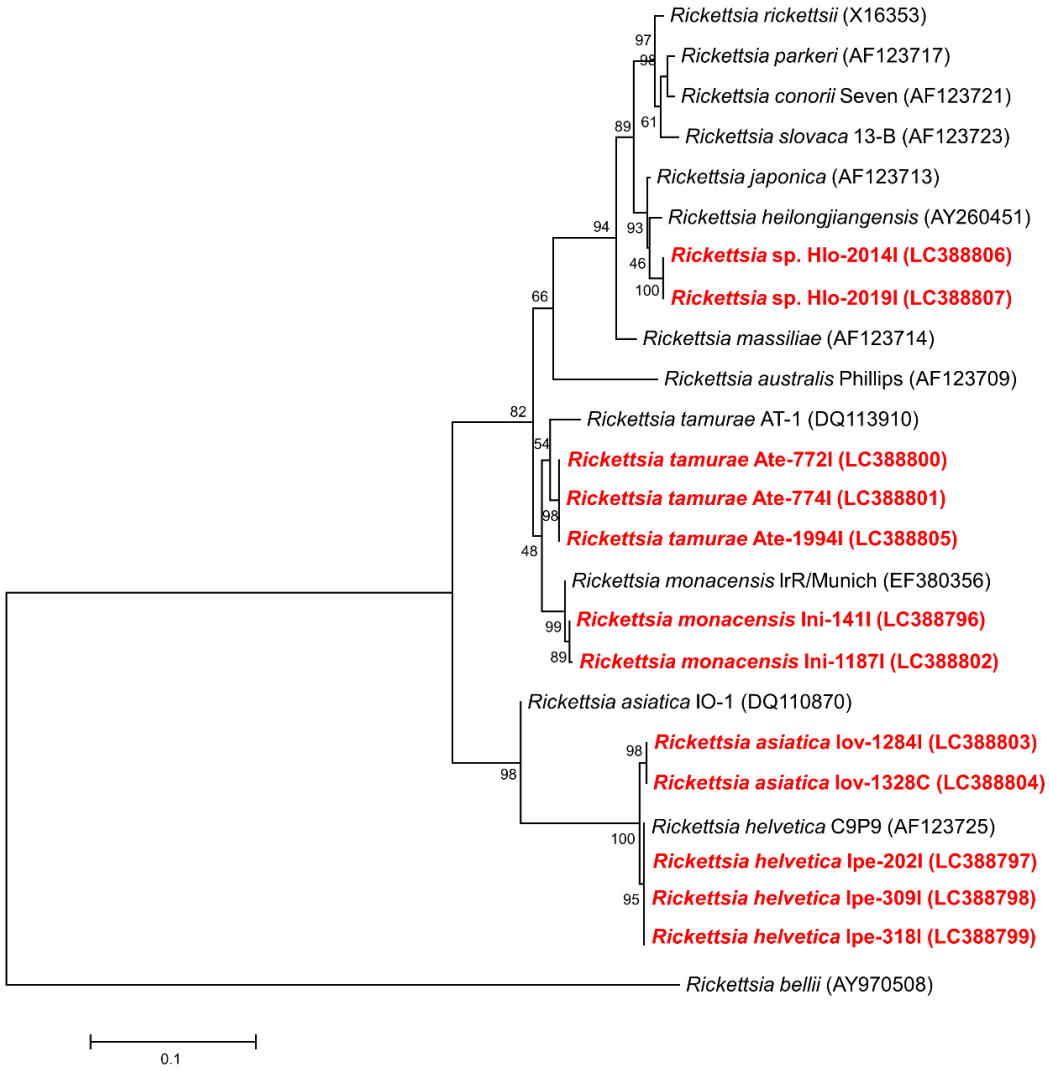

Figure II-5. Phylogenetic tree based on the sequences of *ompB* gene of *Rickettsia* isolates. The analyses were performed using maximum likelihood method with the Kimura two-parameter model. All bootstrap values from 1,000 replications are shown on the interior branch nodes. The sequences obtained in this study are shown in red.

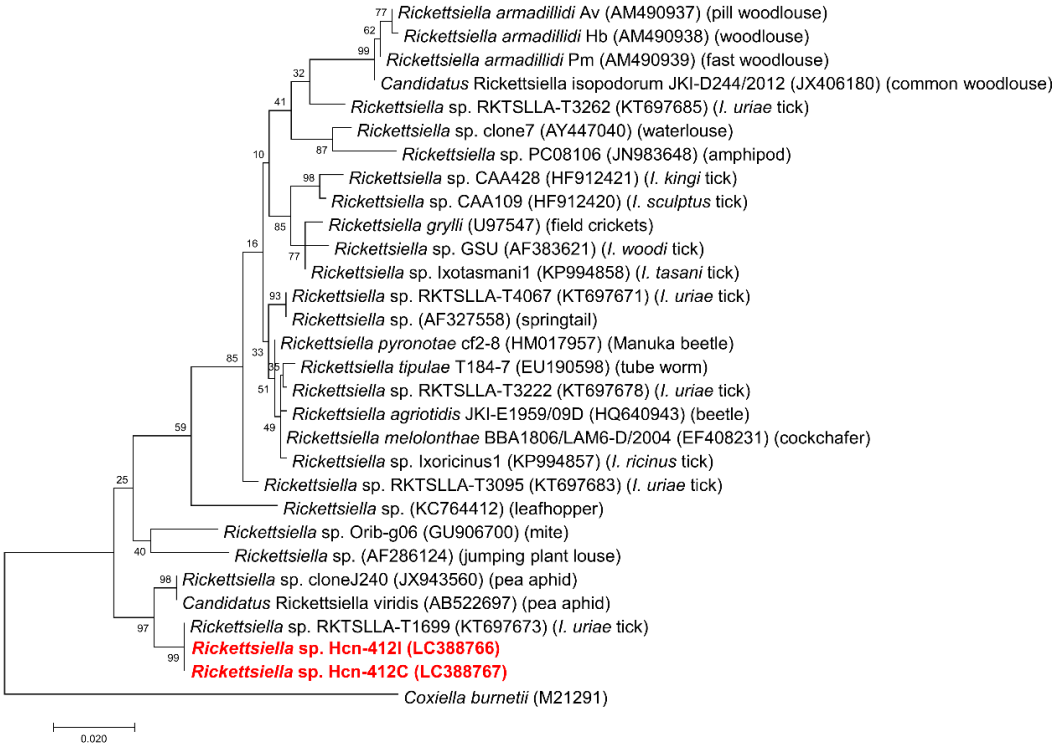

Figure II-6. Phylogenetic tree based on the sequences of 16S rRNA coding gene of *Rickettsiella* isolates. The analyses were performed using maximum likelihood method with the Kimura two-parameter model. All bootstrap values from 1,000 replications are shown on the interior branch nodes. The sequences obtained in this study are shown in red.

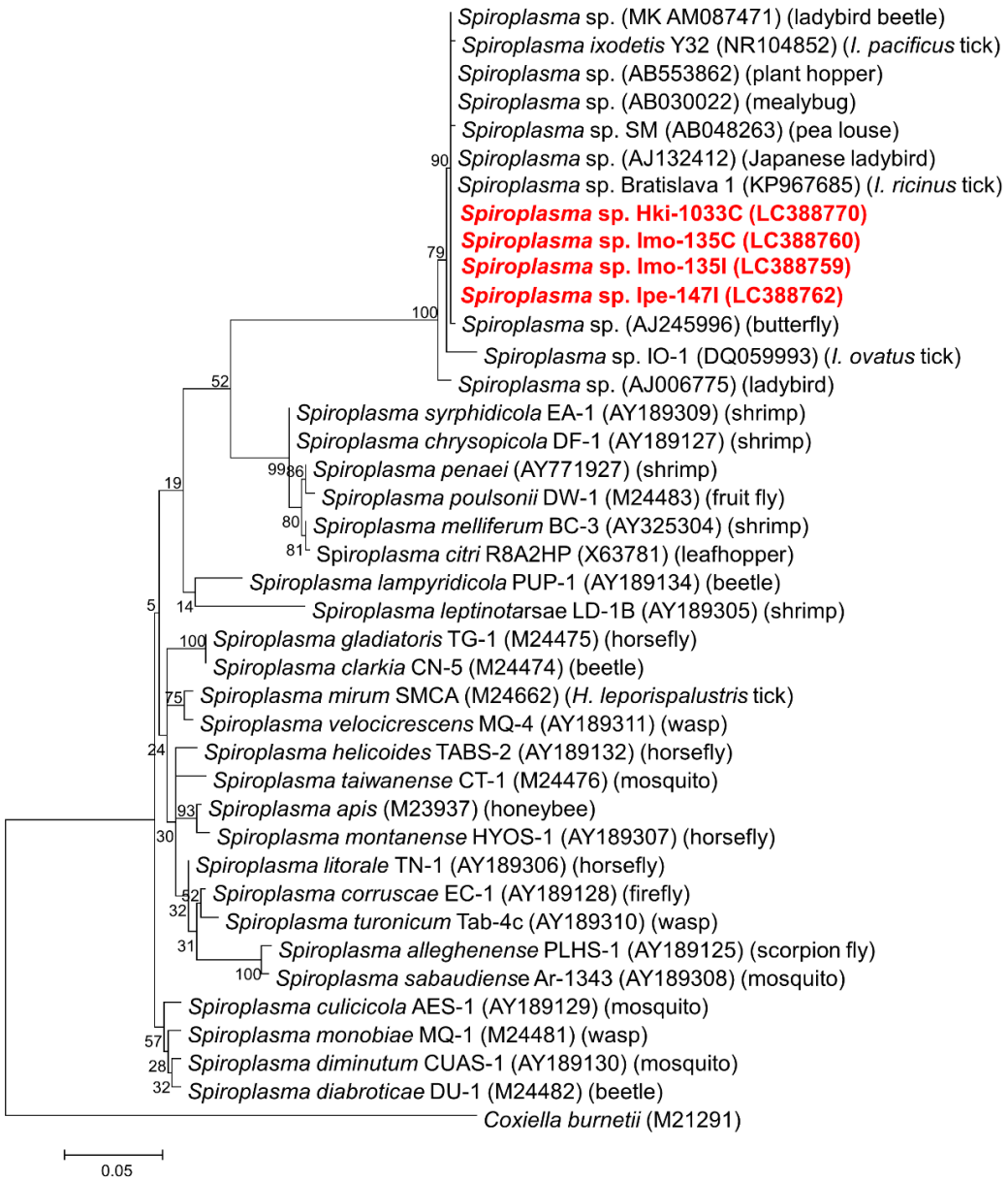

Figure II-7. Phylogenetic tree based on the sequences of 16S rRNA coding gene of *Spiroplasma* isolates. The analyses were performed using maximum likelihood method with the Kimura two-parameter model. All bootstrap values from 1,000 replications are shown on the interior branch nodes. The sequences obtained in this study are shown in red.

GENERAL CONCLUSION

Rickettsiae are obligate intracellular Gram-negative bacteria that cause rickettsioses in humans throughout the world. Ticks harbor most of the members of spotted fever group (SFG) rickettsiae and transmit them to humans and animals. In Japan, *Rickettsia japonica*, a causative agent of Japanese spotted fever (JSF), was firstly identified as a human pathogen. Subsequently, several other rickettsioses caused by *Rickettsia heilongjiangensis*, *Rickettsia helvetica* and *Rickettsia tamurae* have also been reported; however, there is scanty information on the overall diversity of *Rickettsia* species circulating in Japan. This thesis aimed to characterise a wide range of SFG rickettsiae in vector ticks in Japan by analysing multiple rickettsial genes which enables the detailed phylogenetic classification of SFG rickettsiae and to isolate *Rickettsia* using arthropod cell lines.

In chapter I, 19 tick species collected from 114 different sites in 12 prefectures were tested for rickettsial infections. Out of 2,189 individuals, 373 (17.0%) samples were positive for *Rickettsia* spp. as ascertained by real-time PCR amplification of *gltA* gene. Fifteen genotypes of SFG rickettsiae based on the sequences of the *gltA* gene were detected from three tick genera; *Amblyomma*, *Ixodes* and *Haemaphysalis* collected from different regions. There was a strong association between rickettsial genotypes and their host tick species, indicating that most of the SFG rickettsiae are maintained in certain tick species in the natural environment. From the analysis of multiple rickettsial genes, five validated rickettsial species, namely *R. asiatica*, *R. helvetica*, *R. monacensis* (formerly reported as *Rickettsia* sp. In56 in Japan), *R. tamurae*, and *Candidatus* R. tarasevichiae, were identified; however, only the limited number of *ompA*, *ompB*

and *sca4* sequences were obtained from some of the SFG rickettsiae. There is need for further studies of whole genome sequencing to investigate the poorly characterised *Rickettsia*.

In chapter II, arthropod cell lines (C6/36 and ISE6) were employed to isolate tick-borne pathogens including rickettsiae. A total of 170 tick homogenates originating from 15 different tick species collected in Japan were employed. The use of arthropod cell lines led to successful isolation of bacteria in three different genera; *Rickettsia*, *Rickettsiella*, and *Spiroplasma*. These included 4 previously validated rickettsial species such as *R. asiatica*, *R. helvetica*, *R. monacensis*, and *R. tamurae* and one uncharacterised genotype *Rickettsia* sp. LON. Although the technique needs to be improved to reduce contaminations by fungal infections, the use of arthropod cell lines seems promising to expand the knowledge on microorganisms in ticks.

The studies included in this thesis highlight the wide distribution and high frequency of SFG rickettsiae in ixodid ticks and provide basic information essential to understand epidemiology of rickettsiosis in Japan. The genetic information is useful for future development of diagnostic methods specific for pathogenic rickettsiae. The bacterial isolates are important to further analyse their pathogenic potential in vertebrate animals and their roles as symbionts in ticks.

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REFERENCES

1. Abdad MY, Abdallah RA, Karkouri KE, Beye M, Stenos J, Owen H, Unsworth N, Robertson I, Blacksell SD, Nguyen TT, Nappez C, Raoult D, Fenwick S, Fournier PE. *Rickettsia gravesii* sp. nov.: a novel spotted fever group *Rickettsia* in Western Australian *Amblyomma triguttatum triguttatum* ticks. Int J Syst Evol Microbiol 67, 3156-3161, 2017.
2. Ahantarig A, Trinachartvanit W, Baimai V, Grubhoffer L. Hard ticks and their bacterial endosymbionts (or would be pathogens). Folia Microbiol 58, 419-428, 2013.
3. Akter A, Ooka T, Gotoh Y, Yamamoto S, Fujita H, Terasoma F, Kida K, Taira M, Nakadouzono F, Gokuden M, Hirano M, Miyashiro M, Inari K, Shimazu Y, Tabara K, Toyoda A, Yoshimura D, Itoh T, Kitano T, Sato MP, Katsura K, Mondal SI, Ogura Y, Ando S, Hayashi T. Extremely low genomic diversity of *Rickettsia japonica* distributed in Japan. Genome Biol Evol 9, 124-133, 2017.
4. Ando S, Kurosawa M, Sakata A, Fujita H, Sakai K, Sekine M, Katsumi M, Saitou W, Yano Y, Takada N, Takano A, Kawabata H, Hanaoka N, Watanabe H, Kurane I, Kishimoto T. Human *Rickettsia heilongjiangensis* infection, Japan. Emerg Infect Dis 16, 1306-1308, 2010.
5. Andoh M, Ogasawara Y, Sakata A, Ito T, Fujita H, Kawabata H, Ando S. Isolation of the rickettsial agent genetically similar to *Candidatus* Rickettsia kotlanii, from *Haemaphysalis megaspinosa* in Japan. Vector Borne Zoonotic Dis 14, 681-684, 2014.
6. Ando S, Fujita H. Diversity between spotted fever group *Rickettsia* and ticks as vector. Eisei Dobutsu 64, 5-7, 2013.

7. Anstead CA, Chilton NB. A novel *Rickettsia* species detected in vole ticks (*Ixodes angustus*) from Western Canada. Appl Environ Microbiol 79, 7583-7589, 2013.
8. Baba K, Kaneda T, Nishimura H, Sato H. Molecular detection of spotted fever group *Rickettsia* in feral raccoons (*Procyon lotor*) in the Western Part of Japan. J Vet Med Sci 75, 195-197, 2013.
9. Bell-Sakyi L, Kohl A, Bente DA, Fazakerley JK. Tick cell lines for study of Crimean-Congo hemorrhagic fever virus and other arboviruses. Vector Borne Zoonotic Dis 12, 769-781, 2012.
10. Bell-Sakyi L, Palomar AM, Kazimirova M. Isolation and propagation of a *Spiroplasma* sp. from Slovakian *Ixodes ricinus* ticks in *Ixodes* spp. cell lines. Ticks Tick Borne Dis 6, 601-606, 2015.
11. Bell-Sakyi L, Zweygarth E, Blouin EF, Gould EA, Jongejan F. Tick cell lines: tools for tick and tick-borne disease research. Trends Parasitol 23, 450-457, 2007.
12. Biernat B, Stańczak J, Michalik J, Sikora B, Cieniuch S. *Rickettsia helvetica* and *R. monacensis* infections in immature *Ixodes ricinus* ticks derived from sylvatic passerine birds in west-central Poland. Parasitol Res 115, 3469-3477, 2016.
13. Blanc G, Ngwamidiba M, Ogata H, Fournier PE, Claverie JM, Raoult D. Molecular evolution of *Rickettsia* surface antigens: evidence of positive selection. Mol Biol Evol 22, 2073-2083, 2005.
14. Chan YGY, Riley S, Martinez JJ. Adherence to and invasion of host cells by spotted fever group *Rickettsia* species. Front Microbiol 1, 139, 2010.
15. Dale C, Moran NA. Molecular interactions between bacterial symbionts and their hosts. Cell 126, 453-465, 2006.

16. Dall'Agnol B, Souza U, Webster A, Weck B, Stenzel B, Labruna M, Klafke G, Martins JR, Ferreira CAS, Reck J. *Candidatus* Rickettsia asemboensis in *Rhipicephalus sanguineus* ticks, Brazil. Acta Trop 167, 18-20, 2017.
17. Duron O, Noël V, McCoy KD, Bonazzi M, Sidi-Boumedine K, Morel O, Vavre F, Zenner L, Jourdain E, Durand P, Arnathau C, Renaud F, Trape JF, Biguezoton AS, Cremaschi J, Dietrich M, Léger E, Appelgren, Dupraz M, Gómez-Díaz E, Diatta G, Dayo GK, Adakal H, Zoungrana S, Vial L, Chevillon C. The recent evolution of a maternally-inherited endosymbiont of ticks led to the emergence of the Q fever pathogen, *Coxiella burnetii*. PLoS Pathog 11, e1004892, 2015.
18. Duron O, Cremaschi J, McCoy KD. The high diversity and global distribution of the intracellular bacterium *Rickettsiella* in the Polar seabird tick *Ixodes uriae*. Microb Ecol 71, 761-770, 2016.
19. Fournier PE, Dumler JS, Greub G, Zhang J, Wu Y, Raoult D. Gene sequence-based criteria for identification of new *Rickettsia* isolates and description of *Rickettsia heilongjiangensis* sp. nov. J Clin Microbiol 41, 5456-5465, 2003.
20. Fournier PE, Fujita H, Takada N, Raoult D. Genetic identification of rickettsiae isolated from ticks in Japan. J Clin Microbiol 40, 2176-2181, 2002.
21. Fournier PE, Raoult D. Current knowledge on phylogeny and taxonomy of *Rickettsia* spp. Ann NY Acad Sci 1166, 1-11, 2009.
22. Fujita H. Cell culture system for isolation of disease agents: 15 years of experience in Ohara Research laboratory. Annu Rep Ohara Hosp 48, 21-42, 2008.
23. Fujita H, Fournier PE, Takada N, Saito T, Raoult D. *Rickettsia asiatica* sp. nov., isolated in Japan. Int J Syst Evol Microbiol 56, 2365-2368, 2006.

24. Fujita H, Yano Y, Takada N, Ando S, Kawabata H, Fujita N. Tick species and tick-borne rickettsiae confirmed by the year of 2012 in Fukushima prefecture, Japan. Med Entomol Zool 64, 37-41, 2013.
25. Gaowa, Ohashi N, Aochi M, Wuritu D, Wu, Yoshikawa, Y, Kawamori F, Honda T, Fujita H, Takada N, Oikawa Y, Kawabata H, Ando S, Kishimoto T. Rickettsiae in ticks, Japan, 2007–2011. Emerg Infect Dis 19, 338-340, 2013.
26. Gillespie JJ, Beier MS, Rahman MS, Ammerman NC, Shallom JM, Purkayastha A, Sobral BS, Azad AF. Plasmids and rickettsial evolution: insight from *Rickettsia felis*. PLoS One 2, e266, 2007.
27. Harumoto T, Anbutsu H, Fukatsu T. Male-killing *Spiroplasma* induces sex-specific cell death via host apoptotic pathway. PLoS Pathog 10, e1003956, 2014.
28. Henry JE, Streett DA, Oma EA, Goodwin RH. Ultrastructure of an isolate of *Rickettsiella* from the African grasshopper *Zonocerus variegatus*. J Invertebr Pathol 47, 203-213, 1986.
29. Hornok S, Meli ML, Perreten A, Farkas R, Willi B, Beugnet F, Lutz H, Hofmann-Lehmann R. Molecular investigation of hard ticks (Acari: Ixodidae) and fleas (Siphonaptera: Pulicidae) as potential vectors of rickettsial and mycoplasmal agents. Vet Microbiol 140, 98-104, 2010.
30. Iasur-Kruh L, Weintraub PG, Mozes-Daube N, Robinson WE, Perlman SJ, Zchori-Fein E. Novel *Rickettsiella* bacterium in the leafhopper *Orosius albicinctus* (Hemiptera: Cicadellidae). Appl Environ Microbiol 79, 4246-4252, 2013.
31. Imaoka K, Kaneko S, Tabara K, Kusatake K, Morita E. The first human case of *Rickettsia tamurae* infection in Japan. Case Rep Dermatol 3, 68-73, 2011.

32. Inokuma H, Ohashi M, Jilintai, Tanabe S, Miyahara K. Prevalence of tick-borne *Rickettsia* and *Ehrlichia* in *Ixodes persulcatus* and *Ixodes ovatus* in Tokachi District, Eastern Hokkaido, Japan. J Vet Med Sci 69, 661-664, 2007.
33. Ishikura M, Ando S, Shinagawa Y, Matsuura K, Hasegawa S, Nakayama T, Fujita H, Watanabe M. Phylogenetic analysis of spotted fever group rickettsiae based on *gltA*, 17-kDa, and *rOmpA* genes amplified by nested PCR from ticks in Japan. Microbiol Immunol 47, 823-832, 2003.
34. Jado I, Oteo JA, Aldámiz M, Gil H, Escudero R, Ibarra V, Portu J, Portillo A, Lezaun MJ, García-Amil C, Rodríguez-Moreno I, Anda P. *Rickettsia monacensis* and human disease, Spain. Emerg Infect Dis 13, 1405-1407, 2007.
35. Jia N, Zheng YC, Ma L, Huo QB, Ni XB, Jiang BG, Chu YL, Jiang RR, Jiang JF, Cao WC. Human infections with *Rickettsia raoultii*, China. Emerg Infect Dis 20, 866-868, 2014.
36. Jongejan F. Uilenberg G. The global importance of ticks. Parasitology 129, S3-S14, 2004.
37. Karpathy SE, Slater KS, Goldsmith CS, Nicholson WL, Paddock CD. *Rickettsia amblyommatis* sp. nov., a spotted fever group *Rickettsia* associated with multiple species of *Amblyomma* ticks in North, Central and South America. Int J Syst Evol Microbiol 66, 5236-5243, 2016.
38. Khoo JJ, Chen F, Kho KL, Shanizza AIA, Lim FS, Tan KK, Chang LY, Abubakar S. Bacterial community in *Haemaphysalis* ticks of domesticated animals from the Orang Asli communities in Malaysia. Ticks Tick Borne Dis 7, 929-937, 2016.

39. Kim YS, Choi YJ, Lee KM, Ahn KJ, Kim HC, Klein T, Jiang J, Richards A, Park KH, Jang WJ. 2017. First isolation of *Rickettsia monacensis* from a patient in South Korea. Microbiol Immunol 61, 258-263, 2017.
40. Kumar S, Stecher G, Tamura K. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 33, 1870-1874, 2016.
41. Kurilshikov A, Livanova NN, Fomenko NV, Tupikin AE, Rar VA, Kabilov MR, Livanov SG, Tikunova NV. Comparative metagenomic profiling of symbiotic bacterial communities associated with *Ixodes persulcatus*, *Ixodes pavlovskyi* and *Dermacentor reticulatus* ticks. PLoS ONE 10, e0131413, 2015.
42. Kurtti TJ, Munderloh UG, Andreadis TG, Magnarelli LA, Thomas NM. Tick cell culture isolation of an intracellular prokaryote from the tick *Ixodes scapularis*. J Invertebr Pathol 67, 318-321, 1996.
43. Kurtti TJ, Palmer AT, Oliver JH. *Rickettsiella*-like bacteria in *Ixodes woodi* (Acari: Ixodidae). J Med Entomol 39, 534-540, 2002.
44. Kwak ML. A checklist of the ticks (Ascari: Argasidae, Ixodidae) of Japan. Exp Appl Acarol 75, 263-267, 2018.
45. Labruna MB, Whitworth T, Bouyer DH, McBride J, Camargo LMA, Camargo EP, Popov V, Walker DH. *Rickettsia belli* and *Rickettsia amblyommii* in *Amblyomma* ticks from the State of Rondonia, Western Amazon, Brazil. J Med Entomol 41, 1073-1081, 2004.
46. Leclerque A, Kleespies RG, Ritter C, Schuster C. Feiertag S. Genetic and electron-microscopic characterization of *Rickettsiella agriotidis*, a new *Rickettsiella* pathotype associated with wireworm, *Agriotes* sp. (Coleoptera: Elateridae). Curr Microbiol 63, 158-163, 2011.

47. Lee JK, Moraru GM, Stokes JV, Wills RW, Mitchell E, Unz E, Moore-Henderson B, Harper AB, Varela-Stokes AS. *Rickettsia parkeri* and *Candidatus* Rickettsia andeanae in questing *Amblyomma maculatum* (Acari: Ixodidae) from Mississippi. J Med Entomol 54, 476-480, 2017.
48. Łukasik P, Asch MV, Guo H, Ferrari J. Godfray HC. Unrelated facultative endosymbionts protect aphids against a fungal pathogen. Ecol Lett 16, 214-218, 2013.
49. Madeddu G, Mancini F, Caddeo A, Ciervo A, Babudieri S, Maida I, Fiori ML, Rezza G, Mura MS. *Rickettsia monacensis* as cause of Mediterranean spotted fever-like illness, Italy. Emerg Infect Dis 18, 702-704, 2012.
50. Mahara F. Japanese spotted fever: report of 31 cases and review of the literature. Emerg Infect Dis 3, 105-111, 1997.
51. Mahara F. Rickettsioses in Japan and the Far East. Ann N Y Acad Sci 1078, 60-73, 2006.
52. Mediannikov O, Sidelnikov Y, Ivanov L, Mokretsova E, Fournier PE, Tarasevich I, Raoult D. Acute tick-borne rrickettsiosis caused by *Rickettsia sibirica* in the Russian Far East. Emerg Infect Dis 10, 810-817, 2004.
53. Milhano N, de Carvalho IL, Alves AS, Arroube S, Soares J, Rodriguez P, Carolino M, Núncio MS, Piesman J, de Sousa R. Coinfections of *Rickettsia slovaca* and *Rickettsia helvetica* with *Borrelia lusitaniae* in ticks collected in a Safari Park, Portugal. Ticks Tick Borne Dis 1, 172-177, 2010.
54. Moreira-Soto RD, Moreira-Soto A, Corrales-Aguilar E, Calderón-Arguedas O, Troyo A. *Candidatus* Rickettsia nicoyana: A novel *Rickettsia* species isolated from *Ornithodoros knoxjonesi* in Costa Rica. Ticks Tick Borne Dis 8, 532-536, 2017.

55. Munderloh UG, Kurtti TJ. Formulation of medium for tick cell culture. Exp Appl Acarol 7, 219-229, 1989.
56. Munderloh UG, Tate CM, Lynch MJ, Howerth EW, Kurtti TJ, Davidson WR. Isolation of an *Anaplasma* sp. organism from white-tailed deer by tick cell culture. J Clin Microbiol 41, 4328-4335, 2003.
57. Murphy H, Renvoisé A, Pandey P, Parola P, Raoult D. *Rickettsia honei* infection in Human, Nepal, Emerg Infect Dis 17, 1865-1867, 2011.
58. Mwamuye MM, Kariuki E, Omondi D, Kabii J, Odongo D, Masiga, D, Villinger J. Novel *Rickettsia* and emergent tick-borne pathogens: A molecular survey of ticks and tick-borne pathogens in Shimba Hills National Reserve, Kenya. Ticks Tick Borne Dis 8, 208-218, 2017.
59. Nakao M, Miyamoto K, Kitaoka S. A new record of *Ixodes pavlovskyi* Pomerantzev from Hokkaido, Japan (Acari: Ixodidae). Eisei Dobutsu 43, 229-234, 1992.
60. Nakao R, Magona JW, Zhou L, Jongejan F, Sugimoto C. Multi-locus sequence typing of *Ehrlichia ruminantium* strains from geographically diverse origins and collected in *Amblyomma variegatum* from Uganda. Parasit Vectors 4, 137, 2011.
61. Nakao R, Qiu Y, Igarashi M, Magona JW, Zhou L, Ito K, Sugimoto C. High prevalence of spotted fever group rickettsiae in *Amblyomma variegatum* from Uganda and their identification using sizes of intergenic spacers. Ticks Tick Borne Dis 4, 506-512, 2013.
62. Nakao M, Ito T. *Haemaphysalis japonica*, *Haemaphysalis jezoensis* and *Haemaphysalis douglasi* (Ascari: Ixodidae): Which tick is distributed in Hokkaido? Med Entomol Zool 65, 33-35, 2014.

63. Nakamura-Uchiyama F, Komuro Y, Yoshii A, Nawa Y. *Amblyomma testudinarium* tick bite: On case of engorged adult and a case of extraoridinary number of larval tick infestation. J Dermatol 27, 774-777, 2000.

64. National Institute of Infectious Diseases. Scrub typhus and Japanese spotted fever in Japan 2007-2016. ISAR (Infectious Agents Surveillance Report) 2017; 38: 109-112. [accessed 2018 June 4]. https://www.niid.go.jp/niid/ja/tsutsugamushi-m/tsutsugamushi-iasrtpc/7324-448t.html (in Japanese).

65. Ngwamidiba M, Blanc G, Raoult D, Fournier PE. Sca1, a previously undescribed paralog from autotransporter protein-encoding genes in *Rickettsia* species. BMC Microbiol 6, 12, 2006.

66. Noji Y, Takada N, Ishiguro F, Fujino S, Aoyama T, Fujita H, Yano Y, Shiomi S, Mitsuto I, Takase K, Haba T, Mabuchi H. The first reported case of spotted fever in Fukui Prefecture, the Northern part of Central Japan. Jpn J Infect Dis 58, 112-114, 2005.

67. Paddock CD, Sumner JW, Comer JA, Zaki SR, Goldsmith CS, Goddard J, McLellan SL, Tamminga CL, Ohl CA. *Rickettsia parkeri*: a newly recognized cause of spotted fever rickettsiosis in the United States. Clin Infect Dis 38, 805-811, 2004.

68. Parola P, Paddock CD, Raoult D. Tick-borne rickettsioses around the world: Emerging diseases challenging old concepts. Clin Microbiol Rev 18, 719-756, 2005.

69. Parola P, Fenollar F, Badiaga S, Brouqui P, Raoult D. First documation of *Rickettsia conorii* infection (Strain Indian Tick Typhus) in a traveler. Emerg Infect Dis 7, 909-910, 2001.

70. Parola P, Miller RS, McDaniel P, Telford SR, Rolain JM, Wongsrichanalai C, Raoult D. Emerging rickettsioses of the Thai-Myanmar border. Emerg Infct Dis 9, 592-595, 2003.

71. Phongmany S, Rolain JM, Phetsouvanh R, Blacksell SD, Soukkhaseum V, Rasachack B, Phiasakha K, Soukkhaseum S, Frichithavong K, Chu V, Keolouangkhot V, Martinez-Aussel B, Chang K, Darasavath Vidamaly S, Parola P, Thammavong C, Heuangvongsy M, Syhavong B, Raoult D, White NJ, Newton PN. Rickettsial infections and fever, Vientiane, Laos. Emerg Infect Dis 12, 256-262, 2006.

72. Perlmam SJ, Hunter MS, Zchori-Fein E. The emerging diversity of *Rickettsia*. Proc R Soc 273, 2097-2106, 2006.

73. Phan JN, Lu, CR, Bender WG, Smoak lll, RM, Zhong J. Molecular detection and identification of *Rickettsia* species in *Ixodes pacificus* in California. Vector Borne Zoonotic Dis 11, 957-961, 2011.

74. Qiu Y, Nakao R, Ohnuma A, Kawamori F, Sugimoto C. Microbial population analysis of the salivary glands of ticks; A possible strategy for the surveillance of bacterial pathogens. PLoS One 9, e103961, 2014.

75. Raoult D, Roux R. Rickettsioses as paradigms of new or emerging infectious diseases. Clin Mirobiol Rev 10, 694-719, 1997.

76. Raoult D, Fournier PE, Eremeeva M, Graves S, Kelly PJ. Oteo JA, Sekeyova, Z, Tamura A, Tarasevich I, Zhang L. Naming of rickettsiae and rickettsial diseases. Ann NY Acad Sci 1063, 1-12, 2005.

77. Regnery RL, Spruill CL, Plikaytis BD. Genotypic identification of rickettsiae and estimation of intraspecies sequence divergence for portions of two rickettsial genes. J Bacteriol 173, 1576-1589, 1991.

78. Rolain JM, S Shpynov S, Raoult D. Spotted fever-group rickettsioses in north Asia. The Lancet 362, 1939, 2003.

79. Rolain JM, Mathai E, Lepidi H, Somashekar HR, Mathew LG, Prakash JAJ, Raoult D. *Candidatus* Rickettsia kellyi , India Emerg Infect Dis 12, 483-485, 2006.

80. Roux V, Raoult D. Phylogenetic analysis of members of the genus *Rickettsia* using the gene encoding the outer-membrane protein rOmpB (*ompB*). Int J Syst Evol Microbiol 50, 1449-1455, 2000.

81. Roux V, Fournier PE, Raoult D. Differentation of spotted fever group rickettsiae by sequencing analysis of restriction fragment length polymorphism of PCR-amplified DNA of the gene encoding the protein rOmpA. J Clin Microbiology 34, 2058-2065, 1996.

82. Seishima M, Izumi T, Oyama Z, Kadosaka T. Tick bite by *Haemaphysalis megaspinos*a-First case. Eur J Dermatol 10, 389-391, 2000.

83. Sekeyova Z, Roux V, Raoult D. Phylogeny of *Rickettsia* spp. inferred by comparing sequences of gene D, which encodes an intracytoplasmic protein. Int J Syst Evol Microbiol 51, 1353-1360, 2001.

84. Shin SH, Seo HJ, Choi YJ, Choi MK, Kim HC, Klein TA, Chong ST, Richards AL, Park KH, Jang WJ. Detection of *Rickettsia monacensis* from *Ixodes nipponensis* collected from rodents in Gyeonggi and Gangwon Provinces, Republic of Korea. Exp Appl Acarol 61, 337-347, 2013.

85. Shpynov SN, Fournier PE, Rudakov NV, Samoilenko IE, Reshetnikova TA, Yastrebov VK, Schaiman MS, Tarasevich IV, Raoult D. Short report: Molecular identification of a collection spotted fever group rickettsiae obtained from patients and ticks from Russia. Am J Trop Med Hyg 74, 440-443, 2006.

86. Simser JA, Palmer AT, Fingerle V, Wilske B, Kurtti TJ, Munderloh UG. *Rickettsia monacensis* sp. nov., a spotted fever group *Rickettsia*, from ticks (*Ixodes ricinus*) collected in a European City Park. Appl Environ Microbiol 68, 4559-4566, 2002.
87. Socolovschi C, Mediannikov O, Raoult D, Parola P. The relationship between spotted fever group rickettsiae and ixodid ticks. Vet Res 40, 34, 2009.
88. Someya A, Ito R, Maeda A, Ikenaga M. Detection of rickettsial DNA in ticks and wild boars in Kyoto City, Japan. J Vet Med Sci 77, 37-43, 2015.
89. Špitalská E, Boldiš V, Derdáková M, Selyemová D, Taragel'ová VR. Rickettsial infection in *Ixodes ricinus* ticks in urban and natural habitats of Slovakia. Ticks Tick Borne Dis 5, 161-165, 2014.
90. Sréter-Lancz Z, Sréter T, Széll Z, Egyed L. Molecular evidence of *Rickettsia helvetic*a and *R. monacensis* infection in *Ixodes ricinus* from Hungary. Ann Trop Med Parasitol 99, 325-330, 2005.
91. Stenos J, Graves SR, Unsworth NB. A highly sensitive and specific real-time PCR assay for the detection of spotted fever and typhus group rickettsiae. Am J Trop Med Hyg 73, 1083-1085, 2005.
92. Tabara K, Kawabata H, Arai S, Itagaki A, Yamauchi T, Katayama T, Fujita H, Takada, N. High incidence of rickettsiosis correlated to prevalence of *Rickettsia japonica* among *Haemaphysalis longicor*nis tick. J Vet Med Sci 73, 507-510, 2011.
93. Takahashi T, Maeda K, Suzuki T, Ishido A, Shigeoka T, Tominaga T, Kamei T, Honda M, Ninomiya D, Sakai T, Senba T, Kaneyuki S, Sakaguchi S, Satoh A, Hosokawa T, Kawabe Y, Kurihara S, Izumikawa K, Kohno S, Azuma T, Suemori K, Yasukawa M, Mizutani T, Omatsu T, Katayama Y, Miyahara M,

Ijuin M, Doi K, Okuda M, Umeki K, Saito T, Fukushima K, Nakajima K, Yoshikawa T, Hideki T, Fukushi S, Fukuma A, Ogata M, Shimojima M, Nakajima N, Nagata N, Katano H, Fukumoto H, Sato Y, Hasegawa H, Yamagishi T, Oishi K, Kurane I, Morikawa S, Saijo M. The first identification and retrospective study of severe fever with thrombocytopenia syndrome in Japan. J Infect Dis 209, 816-827, 2014.

94. Thu MJ, Qiu Y, Matsuno K, Kajihara M, Mori-Kajihara A, Omori R, Monma N, Chiba K, Seto J, Gokuden M, Andoh M, Oosako H, Katakura K, Takada A, Sugimoto C, Isoda N, Nakao R. Diversity of spotted fever group rickettsiae and their association with host ticks in Japan. In press
95. Tsuchida T, Koga R, Horikawa M, Tsunoda T, Maoka T, Matsumoto S, Simon JC, Fukatsu T. Symbiotic bacterium modifies aphid body color. Science 330, 1102-1104, 2010.
96. Tully JG, Whitcomb RF, Clark HF, Williamson DL. Pathogenic mycoplasmas: cultivation and vetebrate pathogenicity of a new *Spiroplasma*. Science 195, 892-894, 1977.
97. Tully JG, Whitcomb RF, Rose DL, Bové JM. *Spiroplasma mirum*, a new species from the rabbit tick (*Haemaphysalis leporispalustris*). Int J Syst Bacteriol 32, 92-100, 1982.
98. Uchida T, Tashiro F, Funato T, Kitamura Y. Isolation of a spotted fever group *Rickettsia* from a patient with febrile exanthematous illness in Shikoku, Japan. Microbiol Immunol 30, 1323-1326, 1986.
99. Uchida T, Uchiyama T, Kumano K, Walker DH. *Rickettsia japonica* sp. nov., the etiological agent of spotted fever group rickettsiosis in Japan. Int J Syst Bacteriol 42, 303-305, 1992.

100. Van Oosten RA, Duron O, Heylen DJA. Sex ratios of the tick *Ixodes arboricola* are strongly female-biased, but there are no indications of sex-distorting bacteria. Ticks Tick Borne Dis 9, 307-313, 2018.

101. Varela AS, Luttrell MP, Howerth EW, Moore VA, Davidson WR, Stallknecht DE, Little SE. First culture isolation of *Borrelia lonestari*, putative agent of southern tick associatedd rash illness. J Clin Microbiol 42, 1163-1169, 2004.

102. Venclikova K, Rudolf I, Mendel J, Betasova L, Hubalek Z. Rickettsiae in questing *Ixodes ricinus* ticks in the Czech Republic. Ticks Tick Borne Dis. 5, 135-138, 2014.

103. Vilcins IM, Old JM, Deane E. Molecular detection of *Rickettsia*, *Coxiella* and *Rickettsiella* DNA in three native Australian tick species. Exp Appl Acarol 49, 229-242, 2009.

104. Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173, 697-703, 1991.

105. Winkler HH. *Rickettsia* species (as organisms). Annu Rev Microbiol 44, 131-153, 1990.

106. Wolbach SB. Studies on Rocky Mountain spotted fever. J Med Res 41, 1-193, 1919.

107. Xie J, Butler S, Sanchez G, Mateos M. Male killing *Spiroplasma* protects *Drosophila melanogaster* against two parasitoid wasps. Heredity 112, 399-408, 2014.

108. Yadav S, Frazer J, Banga A, Pruitt K, Harsh S, Jaenike J, Eleftherianos I. Endosymbiont-based immunity in *Drosophila melanogaster* against parasitic nematode infection. PLoS One 13, e0192183, 2018.

109. Yamaguti N, Tipton VJ, Keegan HL, Toshioka S. Ticks of Japan, Korea and the Ryushu islands. Brigham Young University Science Bulletin, Biological Series 15, 1971.

110. Yamaji K, Aonuma H, Kanuka H. Distribution of tick-borne diseases in Japan: Past patterns and implications for the future. J Infect Chemother 24, 499-504, 2018.

111. Yamauchi T, Fukui Y, Watanabe M, Nakagawa H, Kamimura K. Forty cases of human infestations with hard ticks (Ascari: Ixodidae) in Toyama prefecture, Japan. Med. Entomol. Zool. 61, 133-143, 2010.

112. Ye X, Sun Y, Ju W, Wang X, Cao W, Wu M. Vector competence of the tick *Ixodes sinensis* (Ascari: Ixodidae) for *Rickettsia monacensis*. Parasit Vectors 7, 2-7, 2014.

113. Yoshii K, Song JY, Park SB, Yang J, Schmitt HJ. Tick-borne encephalitis in Japan, Republic of Korea and China. Emerg Microbes Infect 6, e82, 2017.

114. Zweygarth E, Schöl H, Lis K, Cabezas Cruz A, Thiel C, Silaghi C, Ribeiro MF, Passos LM. In vitro culture of a novel genotype of *Ehrlichia* sp. from Brazil. Transbound Emerg Dis 60, 86-92, 2013.

JAPANESE ABSTRACT

リケッチアはグラム陰性の細胞内寄生細菌で、しばしばヒトにおいてリケッチア症の原因となる。多くの紅斑熱群リケッチアはマダニによってヒトや動物に伝播される。本邦においては、*Rickettsia japonica* が日本紅斑熱の病原菌として報告されて以来、*Rickettsia heilongjiangensis*、 *Rickettsia helvetica*、*Rickettsia tamurae* がヒトに病原性をもつ紅斑熱群リケッチアとして検出されてきた。一方で、ヒトへの病原性が不明な複数のリケッチア種および遺伝子型の存在が示唆されており、本邦における紅斑熱群リケッチア種の全容を解明する必要がある。そこで、本研究では国内のマダニが保有する紅斑熱群リケッチアについて複数の遺伝子座の塩基配列解析により遺伝的多様性を評価すること、ならびに節足動物由来細胞を用いて紅斑熱群リケッチアを含むマダニ保有微生物を分離することを目的とした。

第一章では、国内の 114 地点で採集した 19 種のマダニを対象に紅斑熱群リケッチアの遺伝子検出を試みた。リアルタイム PCR による解析の結果、2,189 個体のマダニのうち 373 個体(17.0%)で陽性反応を得た。クエン酸合成酵素遺伝子（*gltA*）の塩基配列に基づいた型別の結果、検出された紅斑熱群リケッチアは 15 の遺伝子型に分別された。リケッチア *gltA* 遺伝子型とマダニ種との間には強い関連性がみられたことから、ほとんどの紅斑熱群リケッチアはそれぞれ特定のマダニ種によって自然界で維持されていることが示唆された。さらに、複数のリケッチア遺伝子座の塩基配

列解析により、*Rickettsia asiatica*, *R. helvetica*, *Rickettsia monacensis* (*Rickettsia* sp. In56), *R. tamurae*, and *Candidatus* Rickettsia tarasevichiae を特定した。一方で、いくつかのリケッチア *gltA* 遺伝子型では、外膜蛋白質 A 遺伝子（*ompA*）など複数の遺伝子で PCR 増幅がみられなかった。今後、全ゲノム解析などにより、これらの紅斑熱群リケッチアの系統学的な位置関係を明らかにする必要がある。

第二章では、節足動物由来細胞（C6/36 および ISE6）を用いて、紅斑熱群リケッチアを含むマダニ保有微生物の分離培養を試みた。15 種のマダニに由来する 170 検体のマダニ乳剤を用いて、節足動物由来細胞との共培養を行った。その結果、16 週間の実験期間において、リケッチア属、リケッチエラ属、スピロプラズマ属の細菌、合計 18 菌株の分離に成功した。得られた分離株の遺伝子解析の結果、4 種のリケッチア属細菌（*R. asiatica*、*R. helvetica*, *R. monacensis*、*R. tamurae*）と 1 種のリケッチア遺伝子型（*Rickettsia* sp. LON）が確認された。節足動物由来細胞は紅斑熱群リケッチアを含む多様なマダニ保有微生物の分離培養に有用であることが示された。

以上の結果から、本邦のマダニには遺伝的に多様な紅斑熱群リケッチアが広汎に分布していることが明らかとなった。得られた成果は、本邦におけるリケッチア症の疫学を理解する上で有益な情報となる。また、本研究で蓄積したリケッチア遺伝子配列情報およびリケッチア分離株は、病原性リケッチアの特異的診断法の確立や哺乳類における病原性の解析に有用な研究資源となる。

ABSTRACT

Rickettsiae are obligate intracellular Gram-negative bacteria that cause rickettsioses in humans throughout the world. Ticks harbour most of the members of spotted fever group (SFG) rickettsiae and transmit them to humans and animals. In Japan, *Rickettsia japonica*, a causative agent of Japanese spotted fever (JSF), was firstly identified as a human pathogen. Several other SFG rickettsiae have also been reported from both animals and ticks; however, there is scanty information on their pathogenic potential and the overall diversity of *Rickettsia* species circulating in Japan. This thesis aimed to characterise a wide range of SFG rickettsiae in vector ticks in Japan by analysing multiple rickettsial genes which enables the detailed phylogenetic classification of SFG rickettsiae and to isolate *Rickettsia* using arthropod cell lines.

In chapter I, a nationwide cross-sectional survey was conducted on questing ticks to understand the overall diversity of SFG rickettsiae in Japan. Out of 2,189 individuals (19 tick species in 4 genera), 373 (17.0%) samples were positive for *Rickettsia* spp. by *gltA* real-time PCR. Conventional PCR and sequencing analyses of *gltA* indicated the presence of 15 different genotypes of SFG rickettsiae. Based on the multiple gene sequence analysis, five *Rickettsia* species, namely *R. asiatica*, *R. helvetica*, *R. monacensis* (formerly reported as *Rickettsia* sp. In56 in Japan), *R.*

tamurae, and *Candidatus* R. tarasevichiae, and several unclassified SFG rickettsiae were identified. A strong association between rickettsial genotypes and their host tick species was observed, while there was little association between rickettsial genotypes and their geographical origins. These observations may indicate that most of the SFG rickettsiae have a limited host range and are maintained in certain ticks in the natural environment.

In chapter II, two arthropod cell lines (ISE6 derived from *Ixodes scapularis* tick and C6/36 derived from *Aedes albopictus* mosquito) were used to isolate microorganisms from questing ticks. A total of 170 tick homogenates were inoculated into each cell line. Bacterial growth was confirmed by PCR amplifying 16S ribosomal DNA (rDNA) of eubacteria. During the 16 weeks of observation period, bacterial isolation was confirmed in 14 and 4 samples using ISE6 and C6/36 cells, respectively. These included 4 previously validated rickettsial species namely *R. asiatica*, *R. helvetica*, *R. monacensis*, and *R. tamurae* and one uncharacterised rickettsial genotype *Rickettsia* sp. LON and two tick symbionts, *Spiroplasma* sp. and *Rickettsiella* sp. The use of arthropod cell lines seems promising to expand the knowledge on microorganisms in ticks.

In conclusion, the present study highlights the wide distribution and high frequency of SFG rickettsiae in ixodid ticks and provides basic information

essential to understand epidemiology of rickettsiosis in Japan. The genetic information obtained from this study is useful for future development of diagnostic methods for *Rickettsia* infections. The bacterial isolates are important to further analyse their pathogenic potential in vertebrate animals and their roles as symbionts in ticks.