COMPARISON OF THE TICKS AND TICK-BORNE BACTERIA OF SMALL MAMMALS IN WESTERN CANADA

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Abstract

Ticks are important vectors of pathogenic agents that cause disease in humans, domestic animals, and wildlife. They are also hosts for a variety of bacterial endosymbionts. However, little is known about the microbial diversity of many tick species, particularly those species that parasitize small mammals in western Canada. In this thesis, I used a combined morphological and molecular approach to identify, to the species-level, ticks that parasitized small mammals from three localities in Saskatchewan and British Columbia. The genetic diversity and phylogenetic relationships of these tick species was also examined. Comparisons were also made of the composition and diversity of bacteria within individuals of each tick species. Questions relating to the biology, systematics, and vector ecology of the vole tick (*Ixodes angustus*), the rotund tick (*Ixodes kingi*), the sculptured tick (*Ixodes sculptus*) and the Rocky Mountain wood tick (*Dermacentor andersoni*) were also addressed. The results of my thesis work revealed that *I. kingi* and *I. sculptus* were the most encountered tick species on northern pocket gophers (*Thomomys talpoides*) and Richardson's ground squirrels (*Spermophilus richardsonii*), respectively, in Saskatchewan, while *I. angustus* was the most abundant tick on red-backed voles (*Clethrionomys gapperi*) in Kootenay National Park (British Columbia). At least 40 genera of bacteria were detected in the four tick species; however, there were significant differences in the composition of the bacteria among tick species. Two novel species of *Rickettsia* and three putative new species of *Rickettsiella* were also discovered. The findings of this thesis make an important contribution to our understanding of the evolution and ecology of ticks and tick-borne bacteria.

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Chapter 1. General Introduction

1.1 Background

Arthropods belong to the largest and most diverse of the animal phyla. They account for approximately 80% of all known animals and occur in practically every environment (i.e., marine, freshwater, terrestrial and aerial). Many arthropods have adopted a parasitic mode of existence. Haematophagous (i.e., blood-feeding) arthropods, which include a diverse range of insects (e.g., mosquitoes, black flies, tsetse flies, sand flies, triatomine bugs, fleas and lice) and arachnids (e.g., mites and ticks) (Philip & Burgdorfer, 1961; Balashov, 1984; Spielman & James, 1990; Hubálek & Rudolf, 2011), can directly impact the health of their hosts (Tatchell, 1969; Steelman, 1976; Goddard, 1999; Samuel, 2004; Kaufman *et al.,* 2011). For example, infections by large numbers of winter ticks (*Dermacentor albipictus*) on young moose result in skin irritations, reduced stores of visceral fat, anemia, secondary infections (i.e., bacterial and fungal) and reduced growth of the host (Samuel, 2004; Musante *et al.,* 2007). Haematophagous arthropods are also of medical and/or veterinary importance because of their indirect effects on hosts, acting as vectors that transmit disease-causing agents (e.g., bacteria, viruses, protozoa) from one host to another (Steelman, 1976; Sonenshine & Mather, 1994; Azad & Beard, 1998; Gubler, 1998; Hill *et al.,* 2005; Anderson & Magnarelli, 2008).

Understanding the ecology of vector-borne diseases requires detailed information on the vectors, their hosts and the pathogens they transmit to their hosts. This includes knowledge of the distributional ranges of the vectors and their hosts, the influence of environmental factors on survival and reproduction, and the interactions among all three groups of organisms (or 'agents', in the case of viruses). These interactions form the basis for epidemiological triangles, which are

public health models that are developed to help describe and understand these complex interrelationships (Nuttall *et al.,* 2000; Comrie, 2007; Eisen, 2008; Randolph, 2010). An epidemiological triangle can be applied to a variety of circumstances and organisms, and consists of host, vector, and agent (e.g., bacteria, virus, protozoa), many of which can be influenced by environmental changes (e.g., global warming). Change in different abiotic components of the environment can cause the distribution of vectors to shift, potentially resulting in new vector-host interactions and/or an increased risk of infection by vector-borne pathogens to hosts in areas where previously there was little or no risk of exposure to these pathogens (Patz *et al.,* 2000; Comrie, 2007). For example, the distributional ranges of the tick *Ixodes scapularis* (vector) and its bacterial pathogens (e.g., *Borrelia burgdorferi* and *Anaplasma phagocytophilum*), and the mosquito *Anopheles gambiae* (vector) and its protozoan parasites (e.g., *Plasmodium falciparum*), are changing as a consequence of temperature changes associated with global warming (Lindsay *et al.,* 1998; Githeco *et al.,* 2000; Ogden *et al.,* 2008).

Vector-borne bacteria depend upon their vector for survival, reproduction and transmission between vertebrate hosts. Some bacterial species may be generalists (i.e., use several species of vector), while others may be specialists (i.e., adapted to a single species of vector). The ability of a bacterial species to survive in a vector may also be influenced by its interactions with other bacteria (e.g., Beard *et al.,* 1993; Clay *et al.,* 2006; Clay *et al.,* 2008; Jones *et al.,* 2009). The fitness of their arthropod vectors is also influenced by the host species and/or interactions with other parasites (e.g., Price *et al.,* 1986; Alto *et al.,* 2008; Vale & Little, 2009; Wolinska & King, 2009; Brunner *et al.,* 2011). Therefore, the relative specificity of vectors for hosts, and the specificity of pathogenic agents for vectors and/or hosts determine the strength of the relationships of organisms involved in an epidemiological triangle (Nuttall *et al.,*

2000; Eldridge, 2002; Tseng, 2006). The complexity of these relationships are further complicated by the presence of bacterial endosymbionts. These can be defined as microorganisms with no defined pathogenicity that form long-term associations with their hosts (Klepzig *et al.,* 2009). Some of these endosymbionts are closely related to disease-causing pathogens, and use similar mechanisms as their pathogenic relatives to infect their hosts (Burgdorfer *et al.,* 1981; Kugeler *et al.,* 2005; Dale & Moran, 2006; Liu *et al.,* 2013). Some bacterial endosymbionts can be vertically transmitted from one generation to the next, while others can be transmitted to a new host through horizontal transmission (Fine, 1975; Randolph, 1998; Dale & Moran, 2006; Perlman *et al.,* 2006). The same modes of transmission can also be applied to pathogenic bacteria (Jones *et al.,* 1987; Randolph *et al.,* 1996; Howell, 2007; Baldridge *et al.,* 2009). Ticks are known to harbor an abundant and diverse collection of endosymbiotic bacteria (e.g., *Coxiella-, Francisella-* and *Rickettsia*-like organisms) (e.g., Dale & Moran, 2006; Dergousoff *et al.,* 2009; Dergousoff & Chilton, 2010; Dergousoff & Chilton, 2012; Ahantarig *et al.,* 2013) in addition to a diverse range of pathogenic bacteria (e.g., Sonenshine & Mather, 1994; Azad & Beard, 1998; Parola & Raoult, 2001). Bacterial endosymbionts can affect the transmission of pathogenic agents (Burgdorfer *et al.,* 1981; Clay *et al.,* 2006). For example, studies on the Rocky Mountain wood tick (*Dermacentor andersoni*), a common North American tick, showed that infection by the pathogenic bacteria *Rickettsia rickettsii* is prevented by an "interference phenomenon" when ticks are already infected with the endosymbiont *R. peacockii* (Burgdorfer *et al.,* 1981). Macaluso *et al.* (2002) also demonstrated this phenomenon in the American dog tick (*D. variabilis*); which, when infected with *R. montanensis*, could not maintain a secondary infection of *R. rhipicephali*.

In North America, ixodid ticks are the most important vectors of human and animal disease-causing agents (Parola & Raoult, 2001; Jongejan & Uilenberg, 2004; Dantas-Torres *et al*., 2012). Diseases transmitted by tick-borne bacteria include Lyme borreliosis, Rocky Mountain spotted fever, tularemia, ehrlichiosis and anaplasmosis (Parola & Raoult, 2001; Jongejan & Uilenberg, 2004; Dantas-Torres *et al*., 2012). Ticks can also act as vectors of protozoan parasites (e.g., *Babesia*, the causative agent of babesiosis) and viruses, such as tick-borne encephalitis virus, Powassan encephalitis virus and Colorado tick fever virus (Parola & Raoult, 2001; Jongejan & Uilenberg, 2004; Dantas-Torres *et al*., 2012). Some tick species are known vectors for multiple pathogens (Parola & Raoult, 2001; Holman *et al*., 2004; Mixson *et al*., 2006; Harrus *et al*., 2011). For example, in some parts of its distributional range, *D. andersoni* is a vector of *R. rickettsii*, *Francisella tularensis* and *Anaplasma marginale*, the bacteria responsible for Rocky Mountain spotted fever, tularemia, and bovine anaplasmosis, respectively (Burgdorfer, 1975; Foley & Nieto, 2010; Kocan *et al*., 2010). In addition, individual ticks can be infected simultaneously with more than one type of pathogen (Levin & Fish, 2000; Rolain *et al*., 2005; Clay *et al*., 2006; Swanson *et al*., 2006; Jones *et al*., 2009). For instance, in the northeastern United States, blacklegged ticks (*I. scapularis*) have been shown to be co-infected with *B. burgdorferi, A. phagocytophilum* and *Babesia* (Steiner *et al*., 2008), the causative agents of Lyme borreliosis, human granulocytic anaplasmosis, and babesiosis, respectively (Homer *et al*., 2000; Wormser *et al*., 2006; Bakken & Dumler, 2008; Marques, 2010).

Ixodid ticks (family Ixodidae) are divided into two groups, the Prostriata and the Metastriata (Gregson, 1956; Sonenshine, 1991), based on several morphological characters; such as, differences in the scapula, scutum, idiosoma, gnathosomes, spiracular plate, and the presence

or absence of eyes and/or festoons (Gregson, 1956; Sonenshine, 1991). *Ixodes*, the sole members of the Prostriata, are characterized by long mouthparts and the absence of eyes and festoons (Gregson, 1956; Sonenshine, 1991). The presence of an anal groove anterior to the anal pore is the most prominent feature that distinguishes *Ixodes* from all other ixodid ticks (Gregson, 1956; Sonenshine, 1991). Of the 702 species of ixodid ticks known throughout the world, at least 80 species occur in North America (Merten & Durden, 2000; Kolonin, 2007; Guglielmone *et al.,* 2010), and comprise five genera. Of these, species of four genera, *Ixodes, Dermacentor, Amblyomma* and *Rhipicephalus,* are the most important vectors of pathogenic agents to humans and animals (Sonenshine, 1991; Lane, 1994; Gage *et al.,* 1995; Allan, 2001; Parola & Raoult, 2001; Dantas-Torres, 2008).

A key component of any ecological or evolutionary study on ticks requires the accurate identification of individuals of all life cycle stages (i.e., larvae, nymphs and adults) to the species level. However, difficulties can arise in the unequivocal species identification of ticks, particularly when morphologically similar species occur in sympatry, and parasitize the same host species. Furthermore, it is often difficult to identify immature stages of ticks (i.e., larvae and nymphs) to the species-level based on morphological examination, particularly those that are engorged with blood (Andrews *et al*., 1992; Anderson *et al*., 2004). Therefore, alternative approaches are needed to identify larval and nymphal ticks, and to confirm morphological identification of adult ticks to the species level. As a supplement to morphological identification, a variety of genetic markers and molecular approaches are valuable tools to distinguish among tick species, particularly for engorged larvae, where it is often more difficult to determine species identity based on morphological examination alone. The DNA sequences of several mitochondrial (mt) DNA genes and nuclear DNA regions have been used as the targets for tick

identification. These include the first and second internal transcribed spacers (ITS-1 and ITS-2) of the nuclear ribosomal (r) DNA (Zahler *et al.,* 1995; Poucher *et al.,* 1999; Dergousoff & Chilton, 2007; Mtambo *et al.,* 2007; Tian *et al.,* 2011) and the mt small (12S) and large (16S) rRNA genes (Beati & Keirans, 2001; Anderson *et al.,* 2004; Guglielmone *et al.,* 2006; Mtambo *et al.,* 2007; Anstead & Chilton, 2011; Tian *et al.,* 2011). The mt 16S rRNA gene is the most frequently used of these target regions. These target regions have been used in PCR-based assays for species identification. For example, PCR-single strand conformation polymorphism (SSCP) analysis combined with DNA sequencing of the ITS-2 rDNA, provided a reliable method to distinguish among three species of *Dermacentor* (i.e., *D. andersoni*, *D. variabilis* and *D. albipictus*) in Canada (Dergousoff & Chilton 2007). SSCP involves the separation of PCR amplicons based on the conformation (i.e., secondary structure) of single-stranded DNA in a non-denaturing gel, and can be used to differentially display genetic variation between DNA sequences that are 150-450 base pairs (bp) in size, and that differ by one or more nucleotides (Gasser *et al.,* 2006).

There are at least 26 species of ixodid tick (i.e., 20 *Ixodes* spp., 3 *Dermacentor* spp., 2 *Haemaphysalis* spp. and 1 *Rhipicephalus* sp.) that occur in Canada (Gregson, 1956; Wilkinson, 1967; Linquist *et al.,* 1999; Ogden *et al.,* 2009), several of which are of medical and/or veterinary importance (Table 1) (Gregson, 1956; Ogden *et al.,* 2009). Fifteen species of *Ixodes* and three species of *Dermacentor* have been recorded on numerous small mammal host species, including ground squirrels, prairie dogs, mice, voles, shrews, and pocket gophers (Robbins & Keirans, 1992; Sorenson & Moses, 1998; Allan, 2001; Salkeld *et al.,* 2006; Kolonin, 2007; Dergousoff & Chilton, 2007; Anstead & Chilton, 2011). All 18 species also occur south of the Canadian-United States border (Gregson, 1956; Durden & Keirans, 1996; Allan, 2001;

Lubelczyk *et al*. 2007). Small mammals are widespread and abundant in nature, and many act as reservoirs or amplifying hosts of bacteria (Durden, 2006; Mills & Childs, 1998). Important zoonotic tick-borne diseases for which small mammals are known reservoir hosts include: tick-borne encephalitis, Powassan encephalitis, Colorado tick fever, Lyme disease, Rocky Mountain spotted fever, tularemia and human babesiosis (Kruse *et al*., 2004; Davis *et al*., 2005; Durden, 2006; Meerburg *et al*., 2009; Hill & Brown, 2011). Many tick species are dependent on their rodent hosts for survival and this intimate relationship between ticks and their small mammal hosts are hypothesized to promote the maintenance and spread of these zoonotic diseases (Kruse *et al*., 2004; Davis *et al*., 2005; Durden, 2006; Hill & Brown, 2011).

Small mammals are very important hosts for ticks throughout the world, with at least 87 tick species having been documented as parasites of rodents (Horak *et al*., 2002; Kolonin, 2007). Although insectivores (e.g., shrews, hedgehogs and moles) are not as frequently parasitized as rodents (e.g., pocket gophers, ground squirrels, mice and voles), ticks of the genera *Dermacentor* and *Ixodes* have been found to parasitize small mammals from both orders in the Nearctic ecozone (Burachynsky & Galloway, 1985; Kollars *et al*., 2000; Durden, 2006; Kolonin, 2007; Dergousoff, 2011). However, limited data exists on *Ixodes* ticks that parasitize small mammals (e.g., pocket gophers and ground squirrels) in western Canada.

The northern pocket gopher, *Thomomys talpoides,* which comprises a large number of subspecies, has a broad distributional range in North America that includes the northern parts of central and western United States, some mountainous valleys of British Columbia in Canada, and the Canadian prairie provinces of Alberta, Saskatchewan, and Manitoba (Hall & Kelson, 1959). Fossorial in nature, *T. talpoides* is geographically isolated from other populations; except at "contact zones" where hybridization occasionally occurs (Vaughan & Hansen, 1964; Hafner *et*

al., 1983). The northern pocket gopher (*Thomomys talpoides*) is known to forage above ground for short distances in the summer, even though they have a primarily subterranean (i.e., burrowing) life-style (Hansen & Reid, 1973). Although there is information as to which ticks (i.e., *Ixodes* and *Dermacentor* spp.) parasitize pocket gophers (Cooley & Kohls, 1945; Miller & Ward, 1960; Gregson, 1971; Allan, 2001; Salkeld *et al*., 2006), these records are limited to certain parts of the distributional range of *T. talpoides*.

Richardson's ground squirrels (*Spermophilus richardsonii*) are abundant in the prairie regions of southern Alberta, Saskatchewan, southwestern Manitoba, northern Montana, North Dakota, South Dakota and Minnesota (Michener & Koeppl, 1985; Kays & Wilson, 2002). These small mammals are recognized reservoir hosts for pathogenic agents; having been implicated in a human fatality from plague in Alberta, where a mink rancher became infected from skinning mink that had been fed local ground squirrels during an epizootic plague (Gibbons & Humphreys, 1941) and the death of a boy from California after being bitten by an infected *S. richardsonii* (Wherry, 1908). Richardson's ground squirrels are key enzootic reservoirs for Colorado tick fever virus (Bowen *et al*., 1981) and have also been pinpointed as the source of a *Bartonella washoensis* infection in man (Kosoy *et al*., 2003). Although Richardson's ground squirrels are common across the prairies of southern Canada and the northern United States, there is a lack of detailed information on the ecology and population genetics of some of the tick species that parasitize these mammals.

Shrews, voles and mice have also been implicated as disease reservoirs (Mather *et al*., 1989; Bey *et al*., 1995; Schmidt & Ostfeld, 2001; LoGiudice *et al*., 2003). The white-footed mouse (*Peromyscus leucopus*) is the principal natural reservoir for *B. burgdorferi* (Mather *et al*., 1989; Schmidt & Ostfeld, 2001), and shrews are considered "rescue hosts" (Ostfeld & Keesing,

2000; Gilbert *et al*., 2001), as they are capable of sustaining a high disease risk by maintaining the spirochete in the community when mouse density is low (LoGiudice *et al*., 2003). Short-tailed shrews (*Blarina brevicauda*) and *Sorex* shrews have the highest vector potential after chipmunks (*Tamias striatus*) and the white-footed mouse for *B. burgdorferi* (LoGiudice *et al*., 2003; Hamer *et al*., 2010). Red-backed voles (*Clethrionomys gapperi*) have also been shown to be suitable reservoir hosts for this spirochete after being experimentally inoculated with *B. burgdorferi* (Bey *et al*., 1995). Common shrews (*Sorex araneus*), masked shrews (*Sorex cinereus*), field voles (*Microtus agrestis*) and short-tailed shrews (*Blarina brevicauda*) have proven to be strong reservoir hosts for *A. phagocytophilum* (Bown *et al*., 2011; Keesing *et al*., 2012), and *S. araneus* and *M. agrestis* have been shown to harbor *Babesia microti* and/or tick-borne encephalitis virus (Bakhvalova *et al*., 2006; Bown *et al*., 2011). Many small mammals have high vector potential as they have high reservoir competence, provide meals for many ticks, and can occur at high densities (LoGiudice *et al*., 2003). Of the tick species reported in Canada, one third occur in British Columbia (B.C.), many of which parasitize shrews, voles and mice (Gregson, 1956; Wilkinson, 1967; Sonenshine 1991). Despite this, there is a lack of knowledge on the host preferences and bacterial community structures of many ticks found in B.C. Two species, the soft tick, *Ornithodoros hermsi,* and the western blacklegged tick, *I. pacificus,* are known to act as vectors for tick-borne relapsing fever and Lyme disease, respectively (Banerjee *et al*., 1998; Lane *et al*., 1991; Cimolai & Cimolai, 2008). In addition, several studies have been conducted on *I. angustus* (Damrow *et al*., 1989; Banerjee *et al*., 1994; Eisen *et al*., 2006) and *D. andersoni* (Gregson, 1957; Schmitt *et al*., 1969; Bowen *et al*., 1981; McLean *et al*., 1993; Scoles *et al*., 2006) in British Columbia, because of their role as vectors of tick-borne diseases.

Five of the common tick species that parasitize rodents and other small mammals in western Canada are *Dermacentor andersoni, D. variabilis, Ixodes angustus, I. kingi* and *I. sculptus*. The Rocky Mountain wood tick, *D. andersoni,* occurs throughout parts of the western United States; ranging from western Nebraska and South Dakota, westward to the Cascades and Sierra Nevada Mountains, and from northern New Mexico and Arizona, northward into Canada (Bishopp & Trembley, 1945; Gregson 1956; Kocan, 1986; Merten & Durden, 2000; James *et al.,* 2006). In Canada, *D. andersoni* has been reported from southern British Columbia eastward into Alberta and extending into Saskatchewan (Bishopp & Trembley 1945, Gregson 1956; Wilkinson 1967; Dergousoff *et al.,* 2013). Different life cycle stages of *D. andersoni* prefer to parasitize different species of vertebrate host. Adults utilize medium-sized to large mammals, including raccoons, skunks, horses, cattle, mule deer, dogs, cats and humans (Gregson, 1956; James *et al.,* 2006). However, *D. andersoni* immatures prefer small mammal hosts including deer mice, western jumping mice, chipmunks, meadow voles and western bushy-tailed rats (Gregson, 1956; Dergousoff, 2011).

The American dog tick, *D. variabilis,* occurs throughout much of the eastern and central United States, extending into parts of California, Idaho, Oregon, and Mexico (Bishopp & Trembley 1945; Gregson 1956; Wilkinson 1967; Stout *et al.,* 1971; Sonenshine 1979; Merten & Durden, 2000; Rand *et al.,* 2007). Its distributional range in Canada extends from central Saskatchewan eastward to southern Manitoba and Ontario, and can also be found in Nova Scotia (Gregson 1956; Wilkinson 1967; Dodds *et al.,* 1969; Garvie *et al.,* 1978; Campbell & MacKay 1979; Burachynsky & Galloway 1985; Dergousoff *et al.,* 2013). Different life cycle stages of *D. variabilis* are also found on different hosts. Adults utilize the same medium-sized to large mammals as *D. andersoni* (i.e., raccons, skunks, horses, cattle, mule deer, dogs, cats and

humans) (Gregson, 1956; Kollars, 1996; Kollars *et al.,* 2000), whereas *D. variabilis* immatures parasitize small mammal hosts including deer mice, meadow jumping mice, western jumping mice, white-footed mice, marsh rice rats, pine voles, meadow voles, southern red-backed voles, eastern chipmunks, thirteen-lined ground squirrels, raccoons, Virginia opossums and eastern cottontail rabbits (Burachynsky & Galloway, 1985; Kollars *et al.,* 2000; Dergousoff, 2011).

The rotund tick, *Ixodes kingi*, is a common parasite of rodents (i.e., murids, heteromyids, geomyids and sciurids), as well as other vertebrates, including wildlife (lagomorphs and carnivores), domestic animals (dogs and cats), and humans in western North America (Cooley & Kohls, 1945; Allred *et al.,* 1960; Miller & Ward, 1960; Hearle, 1938; Allan, 2001; Bishopp & Trembley, 1945; Gregson, 1971; Salkeld *et al.,* 2006). The distribution of *I. kingi* includes southern Saskatchewan, Alberta and British Columbia, Montana, North Dakota, Nebraska, Wyoming, Colorado, New Mexico, Texas, Arizona, Oregon, Idaho, Nevada, and Utah (Gregson, 1971). A study by Gregson (1971) reported that there were differences in the types of hosts used by *I. kingi* in different geographical regions. On the western slopes of the Rocky Mountains, pocket gophers (*Thomomys* spp.), kangaroo rats (*Dipodomys* spp.) and mice (*Peromyscus* spp.) were the hosts most commonly used, whereas east of the Rocky Mountains the principal hosts were carnivores and sciurid rodents (e.g., *Spermophilus, Urocitellus* and *Cynomys* species).

The sculptured tick, *I. sculptus,* has a wide distribution throughout North America and can be found in Illinois, Michigan, and Louisiana westward in the United States as well as extending northwards into Canada (Bishopp & Trembley, 1945; Cooley & Kohls, 1945; Gregson, 1956; Durden & Keirans, 1996; Allan, 2001; Salkeld *et al.,* 2006). Based on existing literature, this tick species has a preference for Richardson's ground squirrels as hosts (Brown, 1944; Brown & Kohls, 1950; Burgess, 1955) but it has been reported on a diverse range of

mammals (e.g., ground dwelling sciurids, rodents, carnivores, lagomorphs, cats, dogs, goats and humans) (Hixson, 1932; Bishopp & Trembley, 1945; Allred *et al*., 1960; Hilton & Mahrt, 1971; Salkeld *et al*., 2006; Kolonin, 2007).

The geographical distribution of the vole tick, *I. angustus,* is unusual compared to those of *I. kingi* and *I. sculptus*, in that it occurs in western North America (i.e., but only in British Columbia and Alberta in Canada), as well as Russia and Japan (Gregson, 1956; Robbins & Keirans, 1992). This tick species has a preference for red-backed voles (*C. gapperi*) as hosts in Alberta (Sorensen & Moses, 1998), but has been reported from more than 90 species of mammals that includes sciurids, lagomorphs, cats, dogs and humans (Bishopp & Trembley, 1945; Cooley, 1946; Spencer, 1963; Robbins & Keirans, 1992; Peavey *et al*, 2000; Kolonin, 2007).

Therefore, parts of the distributional ranges of *I. kingi*, *I. sculptus* and *I. angustus* overlap one another in western Canada. They also use a similar range of hosts, but they exhibit different host preferences (e.g., red-backed voles for *I. angustus*). The distributional ranges and host usage of all three *Ixodes* species also overlap those of the Rocky Mountain wood tick, *D. andersoni* and/or the American dog tick, *D. variabilis* (Bishopp & Trembley 1945; Gregson, 1956; Wilkinson 1967; Sonenshine, 1979). Given that these tick species are all found on small mammals and have overlapping distributional ranges, then one interesting question that could be addressed is do these ticks have similar bacterial communities? This same question would also apply to other tick species and ectoparasitic arthropods (e.g., fleas, lice, and mites) that parasitize small mammals in western Canada.

There have been studies on the bacterial communities of ticks in the United States that have focused on species of medical and/or veterinary importance, such as *I. scapularis* (Moreno

et al., 2006), the southern cattle tick, *Rhipicephalus microplus* (Andreotti *et al*., 2011) and the lone star tick, *Amblyomma americanum* (Clay et al., 2008; Heise *et al*., 2010). Several studies have also been made on specific bacteria found in populations of *D. andersoni* and *D. variabilis* from the United States (Bell *et al*., 1963; Feng *et al*., 1980; Stich, 1993; Gage *et al*., 1994; Grindle *et al*., 2003; Goethert & Telford III, 2010), and near their northern distributional limits in western Canada (Dergousoff *et al*., 2009; Dergousoff & Chilton, 2010; Dergousoff & Chilton, 2011; Dergousoff & Chilton, 2012; Dergousoff & Chilton, 2013). There is, however, limited published data on the bacterial diversity in Canadian populations of other tick species, even though some species are known vectors of disease-causing agents. For example, *I. sculptus* is a vector of Colorado tick fever (CDC, 1976), *I. kingi* is a vector of *Coxiella burnetii*, the causative agent of Q fever, and *F. tularensis* (Sidwell *et al*., 1964; Thorpe *et al*., 1965), and *I. angustus* has been implicated in the spread of Lyme disease in the Pacific Northwest (Damrow *et al*., 1989; Banerjee *et al*., 1994; Eisen *et al*., 2006), and is also a known vector of the bacterial pathogen, *Babesia microti* (Fay & Rausch, 1969; Goethert *et al*., 2006). Therefore, *I. kingi, I. sculptus* and *I. angustus* are important vectors of pathogenic agents that cause health problems for humans, domestic animals and wildlife. However, most of the published information on the ecology and bacteria of these and related tick species in western Canada are based on studies of these parasites in other regions of North America (e.g., Cooley & Kohls, 1938; McKiel *et al*., 1967; Gregson, 1971; Azad & Beard, 1998; Scoles *et al*., 2005; Salkeld *et al*., 2006).

1.2 **Research objectives**

The overall aim of my PhD research was to compare the composition of bacterial communities in tick species that parasitize small mammals from different habitats and

geographical areas in western Canada. Two study sites in Saskatchewan (i.e., Clavet and Beechy) were selected based on their close proximity to sites (i.e., Blackstrap and Saskatchewan Landing Provincial Park) where the ticks of small mammals have been examined previously (Dergousoff, 2011). The study site near Clavet is located approximately 20 km north from Blackstrap, where *D. variabilis* larvae and nymphs were collected on shrews, mice and voles (Dergousoff, 2011). The site at Beechy is located approximately 37 km north-east from Saskatchewan Landing Provincial Park, where *D. andersoni* and *D. variabilis* occur in sympatry (Dergousoff & Chilton, 2007; Dergousoff *et al.,* 2013) and immature stages of these species parasitize shrews, voles and mice (Dergousoff, 2011). However, the focus of this thesis was on the ticks (and their bacterial communities) of pocket gophers and ground squirrels. In addition, there was the opportunity to examine the bacterial communities in ticks collected from voles, shrews and mice in Kootenay National Park (provided by Dr. Y-T Hwang; Fish and Wildlife Branch, Saskatchewan Ministry of Environment).

In this thesis, I tested the hypothesis that each tick species had a unique community of bacteria, the members of which were not shared by other species, even when they occurred in sympatry and parasitized the same species of small mammal host. My thesis work also examined the specificity of relationships (i.e., tick specificity of bacteria). Therefore, the first objective of my thesis was to identify, to the species-level, ticks feeding on pocket gophers (*Thomomys talpoides*) near Clavet, Saskatchewan **(Chapter 2**), Richardson's ground squirrels (*Spermophilus richardsonii*) from Beechy, Saskatchewan (**Chapter 3**), and voles, shrews, mice and ground squirrels from Kootenay National Park, British Columbia (**Chapter 4**). Given the difficulties of distinguishing among larvae and nymphs of morphologically similar species, an important objective of my thesis work was to use molecular methods to identify individual ticks. Initially,

the D3 expansion segment and flanking core regions (=$D3^{+}$) of the nuclear large subunit ribosomal RNA (28S rRNA) gene, which has been shown previously to be phylogenetically informative and useful in population studies for *Ixodes* (McLain, 2001; McLain *et al*., 2001), was examined as a potential genetic marker for the species-level identification of ticks on small mammals and to infer their evolutionary relationships **(Chapter 5)**. Then, the sequences of the mt 16S rRNA gene, which have been used in population genetic studies of several species of *Ixodes* (Caporale *et al*., 1995; Norris *et al*., 1999; Qiu *et al*., 2002), were used as genetic markers in PCR-based assays to identify ticks (**Chapters 2-4**). The evolutionary relationships and population genetics of the *Ixodes* species found on the small mammals was then explored using sequence alignments of the 16S rRNA gene based on the predicted secondary structures of the gene for each species of tick **(Chapter 6)**. Once the identification of all ticks was achieved, it was then possible to address the following question, what tick species parasitize these small mammal hosts? Determining the different host associations of ticks also has important implications for the understanding of how tick-borne microorganisms are maintained in nature, and if these small mammals are acting as reservoir hosts for different bacteria. Another important question that needed to be answered was, do ticks of different species parasitize the same host individual at the same time? The answer to this question is important with respect to determining whether there exists the potential for cross-transmission of pathogenic bacteria from one tick species to the other. Hence, an interesting question that needed to be addressed was, is the specificity of relationships between tick and bacteria, and among bacterial communities within ticks, dependent on the vertebrate host or the tick species? Therefore the focus of the second part of my thesis work was to detect and identify the bacterial genera present within individual ticks of different species, life cycle stages and from different collection localities **(Chapter 7-10)**.

Two of the bacterial genera that were found in several species of tick and from multiple collection locations were characterized further to determine if different tick species were infected with the same species of bacteria **(Chapter 8-10)**.

1.3 **Anticipated significance of research**

Programs aimed at controlling ticks and tick-borne diseases require detailed knowledge of the ecology of the vectors, and a reliable method to distinguish vectors from species that are not vectors of disease(s). Given that the distribution of some tick species are expanding into new areas (e.g., Ogden *et al.,* 2006; Dergousoff *et al.,* 2013) and that the incidence of tick-borne diseases is increasing (Kilpatrick & Randolph, 2012), it is becoming increasingly important to be able to identify these vectors of disease. Furthermore, despite the fact that ticks are proficient in transmitting a wide range of pathogens, including bacteria, rickettsiae, spirochetes, protozoa and viruses (de la Fuente *et al*., 2008; Sparagano *et al.,* 1999), there is still an absence of knowledge concerning the relationship shared between bacteria and many tick species. It is therefore crucial to identify the members of the total bacterial content in ticks, and to determine if they are of medical or veterinary significance.

The findings from my PhD research will fill gaps in our knowledge concerning the ecology of ixodid ticks that parasitize small mammals in western Canada. More importantly, the information obtained from my research will provide valuable insights into the bacterial community structure of these ticks, as well as the detection and identification of the endosymbiotic and pathogenic bacteria these ticks may harbor. My findings will also provide insight into the relative specificity of bacteria for their tick hosts (vectors). Although the majority of studies that have examined the ecology of tick-borne bacteria have examined only one species of tick, or one species of bacteria found within a single tick species; an important and

novel aspect of my thesis is an examination of the bacterial communities of multiple tick species, some of which occur on the same species of small mammal host, and in some cases, the same host individual.

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Chapter 2. Ticks feeding on northern pocket gophers (*Thomomys talpoides*) in central Saskatchewan, and the unexpected detection of *Ixodes scapularis* larvae[1]

2.1 Abstract

Morphological examination of ticks feeding on northern pocket gophers, *Thomomys talpoides*, near Clavet (Saskatchewan, Canada) revealed the presence of two genera, *Ixodes* and *Dermacentor*. All adult ticks collected were identified as *I. kingi*. Single strand conformation polymorphism (SSCP) analyses and DNA sequencing of the mitochondrial 16S rRNA gene confirmed the species identity of most *Ixodes* immatures as *I. kingi* (2 nymphs and 82 larvae), and the *Dermacentor* immatures as *D. variabilis* (1 nymph and 1 larva) and *D. andersoni* (3 larvae). Six *Ixodes* larvae feeding on three *T. talpoides* individuals were identified as four different 16S haplotypes of *I. scapularis*, which was unexpected because there are no known established populations of this species in Saskatchewan. However, flagging for questing ticks and further examination of the ticks feeding on *T. talpoides* in two subsequent years failed to detect the presence of *I. scapularis* near Clavet, suggesting that there is no established population of *I. scapularis* in this area. Nonetheless, since *I. scapularis* is a vector of pathogenic agents, passive and active surveillance needs to be conducted in Saskatchewan on an ongoing basis to determine if this tick species and its associated pathogens become established within the province.

[1] Part of this chapter was reprinted from:
Anstead CA, Chilton NB. 2011. Ticks feeding on northern pocket gophers (*Thomomys talpoides*) in central Saskatchewan and the unexpected detection of *Ixodes scapularis* larvae. J. Vector Ecol. **36**:355-360, with permission from Wiley Journals.

2.2 **Introduction**

Ticks are important vectors of human and animal pathogens in North America. For example, the blacklegged tick, *Ixodes scapularis*, is a vector of *Borrelia burgdorferi*, the causative agent of Lyme borreliosis in the midwest and northeastern U.S.A. (Thompson *et al.,* 2001; Bacon *et al.,* 2008). Lyme borreliosis is also an emerging vector-borne disease in Canada (Ogden *et al.,* 2008, 2009), given that several populations of *I. scapularis* have recently established in southern Ontario, Nova Scotia, southeastern Manitoba and New Brunswick (Ogden *et al.,* 2009). *Ixodes scapularis* is also a vector of *Anaplasma phagocytophilum*, the causative agent of human granulocytic anaplasmosis (Thompson *et al.,* 2001). Another two common tick species, the Rocky Mountain wood tick, *Dermacentor andersoni*, and the American dog tick, *D. variabilis,* are vectors of *Anaplasma marginale*, the bacterium that causes bovine anaplasmosis in North America (Kocan *et al.,* 2010). They are also vectors for *Rickettsia rickettsii* and *Francisella tularensis*, bacteria that are responsible for Rocky Mountain spotted fever and tularemia, respectively (Burgdorfer, 1975; Foley & Nieto, 2010). All three of these tick species, as well as a number of other tick species in North America, use a variety of rodents (e.g., mice, voles, shrews, ground squirrels and pocket gophers) as hosts (Wilkinson, 1967; Keirans *et al.,* 1996; Allan, 2001; Salkeld *et al.,* 2006), some of which are important reservoirs for tick-borne pathogens (Allan, 2001; Oliver *et al.,* 2006; Foley & Nieto, 2010).

The northern pocket gopher, *Thomomys talpoides*, which comprises a number of subspecies, has a broad distributional range in North America that includes the northern parts of central and western U.S.A., some mountainous valleys of British Columbia in Canada, and the Canadian prairie provinces of Alberta, Saskatchewan and Manitoba (Hall & Kelson 1959). Although there is information as to which tick species (i.e., *Ixodes* and *Dermacentor* spp.)

parasitize pocket gophers (Cooley & Kohls, 1945; Miller & Ward, 1960; Gregson, 1971; Allan, 2001; Salkeld *et al.,* 2006), these records are limited to certain parts of the geographical range of *T. talpoides*. In some cases, the species identity of larval ticks feeding on pocket gophers could not be determined by morphological examination (e.g. Miller & Ward, 1960).

Molecular approaches, using a variety of genetic markers, have been shown to be useful in the identification of individual ticks, and for examining the population genetics and phylogenetic relationships of different tick species (Norris *et al.,* 1996; Qiu *et al.,* 2002; Guglielmone *et al.,* 2006; Dergousoff & Chilton, 2007; Patterson *et al.,* 2009; Krakowetz *et al.,* 2010, 2011). In the present study, molecular tools were used to identify, to the species level, ticks feeding on *T. talpoides* from a locality in central Saskatchewan. We report the unexpected detection of *I. scapularis* larvae on *T. talpoides* and discuss the implications of this finding.

2.3 **Materials and Methods**

Northern pocket gophers (*T. talpoides*) were kill-trapped periodically between spring and early autumn (May-October) in 2007 on an acreage situated eight km southwest of Clavet in Saskatchewan (51.9519N, 106.4473W) using Victor® BlackBox gopher traps (model #0635). This site was composed of mostly mixed grass prairie, with occasional shrub cover. A large slough was located adjacent to the property. Each *T. talpoides* was placed separately into a sealed metal container and transported to the laboratory where they were transferred into individual plastic bags and stored at -20ºC.

Ectoparasites were removed from the body and fur of thawed hosts using fine forceps, and the ticks were identified morphologically to genus (Clifford *et al.,* 1961; Keirans & Litwak, 1989), as part of an undergraduate parasitology laboratory exercise. Ticks were then stored in

70% ethanol for future molecular examination. The four adult ticks collected were all identified as *Ixodes kingi* Bishopp, 1911 using the morphological key of Keirans and Litwak (1989). Many of the engorged immature ticks were identified as belonging to the genus *Ixodes* based on the presence of an anal groove (see Kleinjan & Lane, 2008); however, the species identity of most these individuals could not be determined unequivocally. Therefore, a molecular approach was used to determine the species identity of all ticks feeding on *T. talpoides*.

The mitochondrial (mt) 16S rRNA gene was used as the target to determine the species identity of each tick, and to examine the magnitude of genetic variation among the four *I. kingi* adults, and 98 putative *I. kingi* immatures feeding on *T. talpoides*. This gene was selected because it has been used as a genetic marker to examine the population structure of *I. scapularis* (e.g., Norris *et al.,* 1996; Qiu *et al.,* 2002; Krakowetz *et al.,* 2011), and of the phylogenetic relationships of species within the genus *Ixodes* (e.g., Guglielmone *et al.,* 2006). Genomic DNA (gDNA) was extracted and purified from the legs of adults and the complete bodies of each larva and nymph using the DNeasy Blood and Tissue Kit (Qiagen) as described by Dergousoff and Chilton (2007). Part (~ 410 bp) of the mt 16S rDNA was amplified by PCR using the primers 16S-1 (5'-CCACAGCAATTTAAAAAATCATTGAGCAG-3') and 16S+1 (5'-CCGGTCTGAACTCAGATCAAGT-3') (Norris *et al.,* 1996) and the conditions described previously by Krakowetz *et al.* (2010). All amplicons were subjected to single strand conformation polymorphism (SSCP) analyses using the methodology of Krakowetz *et al.* (2010). DNA sequencing was performed on column-purified (MinElute PCR Purification kit, Qiagen) amplicons of representative individuals of the different SSCP banding patterns. BLAST searches (GenBank) were performed on sequences to determine the identity of each sample. Given that there was no sequence data for *I. kingi* on GenBank, the species identity of the putative *I. kingi*

immatures was confirmed by comparing their mt 16S rDNA sequences to those of the morphologically identified *I. kingi* adults. This approach was feasible since mt DNA sequences are maternally inherited, and different species of *Ixodes* have different mt 16S rDNA sequences (Guglielmone *et al.,* 2006). Nucleotide sequence data have been deposited in GenBank under the accession numbers FR854227-FR854232.

2.4 **Results**

Morphological examination of the ticks collected from eight of 27 *T. talpoides* revealed the presence of two genera, *Ixodes* and *Dermacentor*. Of the 102 ticks identified morphologically as *Ixodes*, amplicons of the mt 16S rDNA were produced for a total of 94 individuals. There were no differences in the size (~ 450 bp) of amplicons on agarose gels (data not shown). A comparison of the SSCP profiles of the 94 individuals revealed the presence of more than one banding pattern (Figure 2.1). Five different sequence types of the mt 16S rDNA were obtained for representative samples of the different banding patterns. There was no genetic variation in the 413 bp fragment of the 16S sequences of one male and three female *I. kingi*. There were 82 larval and 2 nymphal *Ixodes* with the same SSCP banding patterns as the *I. kingi* adults. The sequences of 14 of these larvae were also identical to the sequences of the four adult *I. kingi*. However, there were another six larvae from those three hosts with different SSCP banding patterns to those of the *I. kingi* adults. These six larvae also had different 16S sequences when compared to those of *I. kingi* (Table 2.1). A BLAST search of the six sequences (405-406 bp) revealed that they were identical or genetically most similar to the 16S sequences of *I. scapularis*. The species identity of one of these larvae (Tick #CA32) was examined further by

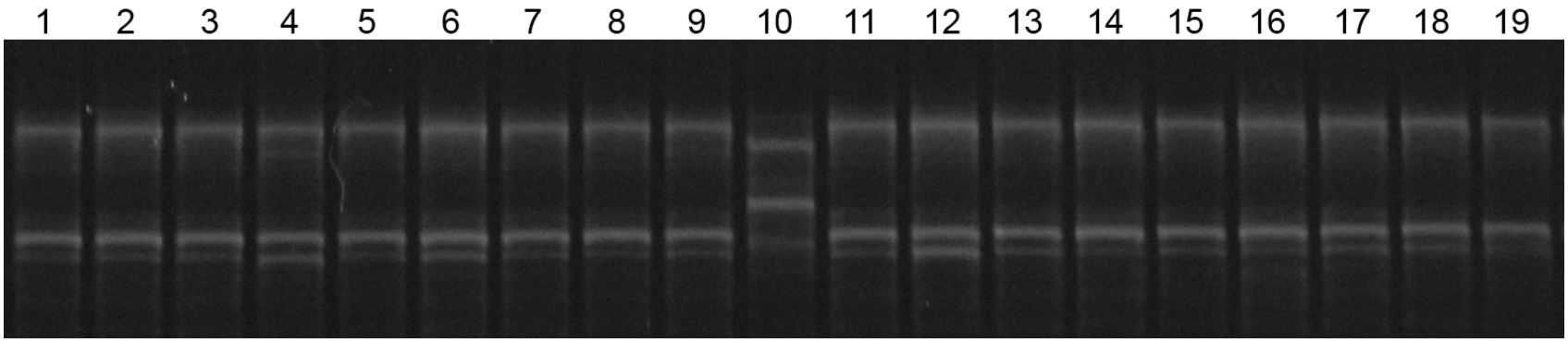

Fig. 2.1 SSCP profiles of mitochondrial 16S rDNA amplicons for representative specimens of larval *I. kingi* (lanes 1-9 and 11-19) and *I. scapularis* (lane 10).

Species	Nucleotide position																																																																	
	15	32	33	57	60	61	87	93	107	108	112	114	120	127	129	142	158	161	162	163	164	165	166	167	168	173	174	175	176	177	178	181	182	183	186	187	188	189	190	198	205	209	222	232	233	234	237	238	239	241	242	245	246	247	251	252	253	262	266	267	278	280	320	322	323	330	337
*I. scapularis**	A	A	T	A	A	A	A	A	T	A	C	A	A	A	T	A	T	T	G	T	T	A	A	T	A	A	A	A	T	T	T	-	G	T	T	T	A	A	-	A	C	A	G	A	G	T	T	-	A	-	-	-	-	-	-	-	-	T	G	C	A	T	A	G	G	A	G
Tick #CA32	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	-	.	-	-	-	-	-	-	-	-	.	.	.	.	.	.	.	.	.	.
Tick #CA35	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	-	.	-	-	-	-	-	-	-	-	.	.	.	.	.	.	.	.	.	.
Tick #CA36	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	-	.	-	-	-	-	-	-	-	-	.	.	.	.	.	.	.	.	.	.
Tick #CA24	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	-	.	-	-	-	-	-	-	-	-	.	.	.	.	.	.	.	.	.	.
Tick #CAA2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	-	.	T	.	.	.	.	.	.	-	.	-	-	-	-	-	-	-	-	.	.	.	.	.	.	.	.	.	.
Tick #CAA58	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	-	.	.	.	.	.	A	.	.	-	.	-	-	-	-	-	-	-	-	.	.	.	.	.	.	.	.	.	.
I. kingi[1]	G	G	C	G	T	T	T	G	.	-	A	G	T	T	A	.	A	A	A	A	A	T	T	A	T	-	-	G	G	G	G	C	C	C	A	A	G	T	T	G	T	T	A	G	T	G	A	G	G	T	G	T	A	T	T	T	T	A	A	T	T	A	T	A	A	G	A

* *I. scapularis* haplotype F (Qiu et al., 2002) accession no. AF308918
[1] based on the sequences of 18 individuals (4 adults and 14 larvae)

Table 2.1 Variable nucleotide positions in the aligned mitochondrial 16S rDNA sequences of the *Ixodes* specimens examined in the present study. A dot indicates the same nucleotide as in the sequence of *I. scapularis*.

sequencing the second internal transcribed spacer (ITS-2) rDNA. The 677 bp ITS-2 sequence of this individual was 99.9% similar (i.e., 1 bp difference) to that of an ITS-2 sequence of *I. scapularis* (i.e., accession number X63868). Three of the six *I. scapularis* larvae from *T. talpoides* had the same 16S sequence as haplotype F of Qiu *et al.* (2002), while the other three individuals each had a unique haplotype that differed from haplotype F individuals at one or two alignment positions (Table 2.1). The four variable sequence differences among the *I. scapularis* individuals represented two purine transitions, one pyrimidine transition, and one indel. A comparison of the aligned 16S sequences (416 bp) of *I. scapularis* and *I. kingi* revealed 64 (15%) nucleotide differences: 17 purine transitions, 3 pyrimidine transitions, 30 transversions and 14 indels (Table 2.1).

A comparison of the mt 16S rDNA sequences of the *Dermacentor* specimens with sequence data on GenBank revealed that they represented one *D. variabilis* nymph, one *D. variabilis* larva and three *D. andersoni* larvae. The *D. variabilis* nymph and larva had the same 16S sequences as those of haplotypes 1 and 7, respectively (accession numbers FN665376 and FN665382), as defined by Krakowetz *et al.* (2010). Each *D. andersoni* larva had a different mt 16S rDNA sequence (Table 2.2). The 16S sequences of two individuals were identical to those of haplotypes P and T (accession numbers FM955611 and FM955615, respectively), while the third had a unique haplotype compared to those of *D. andersoni* from two populations in Saskatchewan and Alberta (Patterson *et al.*, 2009).

	Nucleotide position							
	1	1	1	1	1	1	1	
	5	4	6	7	7	8	9	9
	1	7	3	6	8	0	3	8
Hap P*	G	C	A	T	T	T	T	A
Tick #CA31 (=Hap P)	.	.	.	.	.	.	.	.
Hap Q*	.	.	G	A	-	.	C	G
Hap R*	.	.	.	A	A	A	.	.
Hap S*	.	.	.	A	-	.	.	.
Hap T*	A	.	.	A	-	.	.	.
Tick #CAA96(=Hap T)	A	.	.	A	-	.	.	.
Tick #CAA47(≠Hap P-T)	A	A	.	A	-	.	.	.

* haplotypes P-T from Patterson et al. (2009)

Table 2.2 Variable nucleotide positions in the aligned mitochondrial 16S rDNA sequences of the three *D. andersoni* individuals feeding on northern pocket gophers. A dot indicates the same nucleotide as in the sequence of Hap P.

2.5 **Discussion**

In the present study, 107 ticks representing four tick species were collected from *T. talpoides* near Clavet, Saskatchewan. A small proportion (5%) of these were *D. variabilis* and *D. andersoni*. The presence of two *D. variabilis* immatures (one nymph and one larva) on *T. talpoides* near Clavet was not unusual given that we have collected questing adults at this locality. However, immatures of *D. variabilis* are more commonly found feeding on other smaller rodents (e.g., *Clethrionomys gapperi, Microtus pennsylvanicus* and *Peromyscus maniculatus*) in this region (Dergousoff, 2011). In contrast, the discovery of three *D. andersoni* larvae near Clavet, each representing a different 16S haplotype and feeding on a different host individual, was surprising because this species has not been recorded previously this far east in Saskatchewan (see Wilkinson, 1967). Although the distributional range of *D. andersoni* has expanded eastwards in Saskatchewan since the 1970's, which may have implications for transmission of tick-borne pathogens to livestock and humans, the closest known population of *D. andersoni* to the study site near Clavet is situated ~70 km to the southeast (Dergousoff, 2011). A population of *D. andersoni* has not yet been established at our study site because no *D. andersoni* immatures have been subsequently collected from *T. talpoides*, and flagging vegetation for questing adults has only revealed the presence of *D. variabilis*, and not *D. andersoni*.

The majority of the ticks collected from *T. talpoides* were identified as *I. kingi* based on morphological and molecular analyses. This tick species has been recorded from 40 species of rodent (including *Thomomys* spp.), four species of lagomorph, 17 species of carnivore, domestic dogs and cats, and humans in North America (Cooley & Kohls, 1945; Miller & Ward, 1960; Gregson, 1971; Salkeld *et al.,* 2006). However, in the study by Miller and Ward (1960) on the

ectoparasites of pocket gophers from Colorado, *I. sculptus* but not *I. kingi* were collected from *T. talpoides*, whereas both these tick species were collected from Botta's pocket gopher, *T. bottae*. In the present study, all life cycle stages of *I. kingi* were found on *T. talpoides* near Clavet, including adults of both sexes, whereas no *I. sculptus* were detected on northern pocket gophers. Larvae accounted for 93% of the *I. kingi* collected. Gregson (1971) reported that there were differences in the types of hosts used by *I. kingi* in different geographical regions. On the western slopes of the Rocky Mountains, pocket gophers (*Thomomys* spp.), kangaroo rats (*Dipodomys* spp.) and mice (*Peromyscus* spp.) were the hosts most commonly used, whereas east of the Rocky Mountains the principal hosts were sciurid rodents (e.g., *Spermophilus, Urocitellus* and *Cynomys* spp.) and carnivores (Gregson, 1971). Although the presence of *I. kingi* on *T. talpoides* from central Saskatchewan (i.e., east of the Rocky Mountains) is not consistent with the findings of Gregson (1971), there were no records of *I. kingi* occurring within Saskatchewan in the paper by Gregson (1971). Additional studies are needed to determine if sciurid rodents, such as 13-lined ground squirrels (*S. tridecemlineatus*) and Richardson's ground squirrels (*S. richardsonii*), which occur in the Clavet area, are common hosts for *I. kingi*.

Gregson (1971) also noted morphological differences between *I. kingi* from western populations and those in the eastern populations, which may be a reflection of evolutionary divergence (Oliver *et al.,* 1974). Genetic studies of *I. scapularis* (e.g., Norris *et al.,* 1996; Qiu *et al.,* 2002; Krakowetz *et al.,* 2011) and *Dermacentor* spp. (e.g., Patterson *et al.,* 2009; Krakowetz *et al.,* 2010) in North America using the mt 16S rRNA gene reported the presence of multiple haplotypes (i.e., genetic variants) within tick populations. In the present study, no genetic variation was detected in the 16S gene of *I. kingi* individuals based on SSCP analyses and DNA sequencing, which may be a consequence of sampling ticks from hosts over a relatively small

area. Therefore, the usefulness of the mt 16S rRNA gene as a population genetic marker for *I. kingi* needs to be assessed further using individuals from different geographical localities, including both sides of the Rocky Mountains.

The SSCP banding patterns and DNA sequences of the 16S rDNA of *I. kingi* were distinct from those of the *D. variabilis* and *D. andersoni* found on *T. talpoides*. The SSCP analyses also revealed that the banding patterns of six *Ixodes* larvae, collected from three hosts, were distinct from those of *I. kingi*. A comparison of the mt 16S rDNA sequences of these six individuals revealed that they were *I. scapularis*. Half of these individuals were of haplotype F, which is consistent with studies on haplotype frequencies in populations of this tick in the U.S.A. (e.g., Qiu *et al*., 2002) and in Canada (Krakowetz *et al*., 2011). The species identity of one individual was further verified by its ITS-2 rDNA sequence. The presence of *I. scapularis* larvae feeding on three *T. talpoides* individuals near Clavet was totally unexpected because there appears to be no previous published reports of *T. talpoides* as a host for *I. scapularis* larvae, and there are no known established populations of *I. scapularis* in Saskatchewan. Although there have been genetic studies conducted on *I. scapularis* adults in our laboratory (Krakowetz *et al*., 2011), the results of the molecular work of the present study are not the consequence of a potential contamination of gDNA. We know this because the 16S rDNA sequences of two of the six larvae were different to those of all *I. scapularis* adults examined previously.

Numerous adventitious (i.e., introduced) ticks have been recorded from Saskatchewan in the west to Newfoundland in the east (Ogden *et al*., 2006a), however, only a small number of *I. scapularis* populations have become established in Canada thus far (Ogden *et al*., 2009). A population of *I. scapularis* is considered established at a given locality when larvae, nymphs and adults have all been collected while feeding on resident animals or questing in the environment

for at least two consecutive years (Ogden *et al*., 2008). Therefore, on this basis, there is no evidence for an established population of *I. scapularis* near Clavet because only larvae (i.e., no nymphs or adults) of *I. scapularis* were found feeding on resident mammals in 2007. In addition, subsequent trapping of *T. talpoides* and flagging for questing ticks in 2009 and 2010 failed to detect the presence of any life cycle stage of *I. scapularis*. Although deer mice (*P. maniculatus*), a common host of *I. scapularis* immatures (e.g., Oliver *et al*., 2006), were not trapped at this specific locality, they have been trapped from a nearby area (i.e., Blackstrap Lake, situated 18 km to the south), but were not found to be parasitized by any species of *Ixodes* (Dergousoff, 2011). The absence of *I. scapularis* from the study site since 2007 suggests that individuals of this species were unable to successfully complete their life cycle. Populations of *I. scapularis* may be unable to establish in new areas because of a combination of factors: the incremental risk of mortality at each life cycle stage (i.e., from egg to adult), unfavorable climatic conditions and habitat types, a relative low abundance of suitable hosts and a small number of colonizing adult individuals (Lindsay *et al*., 1995, 1998).

Given the maternal inheritance of mt DNA, the detection of four different 16S haplotypes among the six *I. scapularis* larvae indicates that they are the progeny of at least four adult females. Each of these females would have been mated by a conspecific male, fed on a suitable host and laid viable eggs; some of which hatched successfully. Although only a small number of *I. scapularis* adults have been collected from Saskatchewan by passive surveillance (Ogden *et al*., 2006a; Chilton *et al*., unpublished data), three *I. scapularis* females were collected in 2008 from two dogs on a single property located 29 km north of the study site near Clavet. Another *I. scapularis* female had also been collected from the same property a year earlier (Chilton *et al*., unpublished data). It is possible therefore, for multiple adult ticks to have been present at our

study site. These adult ticks were probably introduced into the area as immature stages because migratory passerines are known to carry *I. scapularis* larvae and nymphs from the U.S.A. into Canada each spring (Ogden *et al.,* 2008). Fed larvae and nymphs dispersed by birds would have had to molt to the next life stage (nymphs and adults, respectively) prior to finding suitable hosts on which to feed. Questing nymphs may have used *T. talpoides* and other species of resident small rodent (i.e., shrews, mice and voles) as hosts, while adults may have used white-tailed deer (*Odocoileus virginianus*). These large mammals are the preferred hosts of adult *I. scapularis* (Keirans *et al.,* 1996) and are common in the area around Clavet. At Long Point in southern Ontario, the life cycle of *I. scapularis* may take three or four years to complete, and involves overwintering by all active life cycle stages, including fed females (Lindsay *et al.,* 1995, 1998). Females then lay eggs in late March to mid-April and larvae emerge from eggs in late July or early August (Lindsay *et al.,* 1995, 1998). If the timing of larval emergence at the site near Clavet was similar to that for ticks at Long Point, then questing larvae could have encountered *T. talpoides* during the summer months. This is possible because *T. talpoides* are known to forage above ground in summer, even though they have primarily a subterranean (i.e., burrowing) life-style (Hansen & Reid, 1973). Nevertheless, the presence of *I. scapularis* larvae feeding on *T. talpoides* near Clavet, followed by absence of individuals of any life cycle stage, either in the environment or on hosts, suggests a failed colonization attempt. Other failed colonization attempts by *I. scapularis* have also been seen at sites in Manitoba and Nova Scotia (L.R. Lindsay, pers. comm.).

Although there are no known established populations of *I. scapularis* in Saskatchewan, it has been predicted that by the 2020's, environmental conditions may become suitable for this species to become established in the province as a consequence of climate change (Ogden *et al.,*

2006b). Given the discovery of *I. scapularis* larvae feeding on *T. talpoides* at one locality in Saskatchewan, the occasional occurrence of adventitious ticks, and the fact that animal and/or human pathogens (e.g., *B. burgdorferi*) can establish following the formation of resident *I. scapularis* populations in Canada (e.g., Ogden *et al.,* 2010), it is essential that passive and active surveillance be conducted within Saskatchewan on an ongoing basis to assess the potential risk of human exposure to pathogens. The findings of the present study also highlight the value of PCR-based techniques (e.g., SSCP in combination with DNA sequencing) to distinguish among tick species, particularly for engorged larvae, where it is often more difficult to determine species identity based on morphological examination alone, and/or to verify the species identity of immature ticks collected by passive and active surveillance.

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Chapter 3. Ticks (Acari: Ixodidae) on Richardson's ground squirrels (*Spermophilus richardsonii*) in southern Saskatchewan, Canada[2]

3.1 Abstract

There is limited detailed information about the tick species that parasitize small mammals in Saskatchewan, Canada. In the present study, morphological and molecular methods were used to determine the species identity of ticks feeding on Richardson's ground squirrels (*Spermophilus richardsonii*) at a site of mixed grassland prairie in southern Saskatchewan. Eighteen (44%) of the 41 *S. richardsonii* collected were parasitized by ticks. The mean intensity of ticks per host was 6.4, with a range of 1-67 ticks per host. The 116 ticks collected were identified morphologically as either *Ixodes* sp. (n = 4, adults, 28 nymphs and 44 larvae) or *Dermacentor andersoni* (n = 20 adults and 20 nymphs). The adult *Ixodes* were further identified as *I. sculptus* (n = 3) and *I. kingi* (n = 1). The combined results of the PCR-single strand conformation polymorphism and DNA sequence analyses of the mitochondrial 16S rRNA gene revealed that a majority (92%) of the larval and nymphal *Ixodes* feeding on *S. richardsonii* were *I. sculptus*. Detection of three tick species, which are known vectors of disease-causing agents, on the same host has important implications in understanding the ecology of vector-borne diseases, and provides an opportunity to examine fundamental questions regarding the structure and composition of their bacterial communities.

[2] Part of this chapter was reprinted from:
Anstead CA, Wallace SB, Chilton NB. 2013. Ticks (Acari: Ixodidae) on Richardson's ground squirrels (*Urocitellus richardsonii*) in southern Saskatchewan, Canada. Mol. Cell. Probes. *submitted.

3.2 **Introduction**

Richardson's ground squirrels (*Spermophilus richardsonii*) are abundant in the prairie regions of southern Alberta, Saskatchewan, southwestern Manitoba, northern Montana, North Dakota, South Dakota and Minnesota (Michener & Koeppl, 1985; Kays & Wilson, 2002). They are often considered agricultural pests because of their ability to cause damage to crops (Marsh, 1998; Johnson-Nistler *et al*., 2005; Proulx & MacKenzie, 2012). The mounds and burrow entrances of Richardson's ground squirrels in pastures also pose hazards to livestock (Lindsay & Galloway, 1997; Marsh, 1998). Although rodenticides are used to control populations of *S. richardsonii*, their natural predators include long-tailed weasels, raccoons, striped skunks, American badgers, red foxes, and birds of prey (Michener & Koeppl, 1985; Proulx & MacKenzie, 2012). Richardson's ground squirrels are known hosts for the Colorado tick fever virus, and act as reservoirs of this virus for tick vectors (e.g., *Dermacentor andersoni*) (Bowen *et al*., 1981). They are also known hosts for *Francisella tularensis* (Wobeser *et al*., 2009), the causative agent of tularemia, and *Bartonella* (Jardine *et al*., 2006). Some of the ectoparasites that occur on *S. richardsonii* are also vectors of pathogenic bacteria. For example, the fleas *Neopsylla inopina* and *Rhadinopsylla fraterna*, which are commonly found on Richardson's ground squirrels (Lindsay & Galloway, 1997), have been shown to carry *Yersinia pestis,* the ethiological agent of plague (Anderson & Williams, 1997; Ubico *et al*., 1988; Wobeser *et al*., 2009). Other ectoparasites of *S. richardsonii* include lice, mites and ticks (Brown & Roy, 1943; Burgess, 1955; Hilton & Mahrt, 1971).

The tick species reported from Richardson's ground squirrels include *Ixodes sculptus, I. kingi, I. marmotae, D. andersoni* and *Haemaphysalis leporispalustris* (Hixson, 1932; Bishopp & Trembley, 1945; Cooley & Kohls, 1945; Brown & Kohls, 1950; Burgess, 1955; Gregson, 1956;

Allred *et al*., 1960; Wilkinson, 1967; Salkeld *et al*., 2006), some of which are known vectors of pathogens to humans or domestic animals (Sidwell *et al*., 1964; Thorpe *et al*., 1965; CDC, 1976). For example, *D. andersoni* is an important vector of *F. tularensis, Rickettsia rickettsii* and *Anaplasma marginale* (Burgdorfer, 1975; Foley & Nieto, 2010; Kocan *et al*., 2010). However, most of the published information on the ecology and bacteria of the tick species that parasitize *S. richardsonii* are based on studies of these parasites on other host species (e.g., Cooley & Kohls, 1938; McKiel *et al*., 1967; Gregson, 1971; Azad & Beard, 1998; Scoles *et al*., 2005; Salkeld *et al*., 2006). In addition, little is known of the genetic variability of these tick species, except for *D. andersoni* and *I. kingi* (Gregson, 1971; Scoles *et al*., 2005; Patterson *et al*., 2009; Anstead & Chilton, 2011).

In the present study, morphological and molecular methods were used to identify ticks feeding on *S. richardsonii* from Beechy in southwestern Saskatchewan. Genetic variation among individual ticks of each species collected on *S. richardsonii* was also examined. The study site is located approximately 23 miles north-east of Saskatchewan Landing Provincial Park where ticks have tested positive for *F. tularensis* (Gordon *et al*., 1983) and *Anaplasma bovis* (Dergousoff & Chilton, 2011), and where the sera of dogs and cats near the park were positive for *F. tularensis*, *Y. pestis* and *R. rickettsii* (Leighton *et al*., 2001). It also represents a site within the overlap zone between *D. andersoni* and *D. variabilis* in Saskatchewan (Dergousoff *et al*., 2013).

3.3 **Materials and Methods**

Richardson's ground squirrels (n = 41) were collected during the summer months (June and July) of 2009 and 2010 from a cattle farm situated on the outskirts of the village of Beechy, Saskatchewan (50.8833N, 107.3833W). This site is primarily mixed grass prairie, with rolling hills, and occasional shrub cover. Ticks were removed from the body and fur of hosts, and fixed in 70% ethanol. Each tick was examined by light microscopy and identified to genus using morphological characters (Brinton *et al*., 1965; Keirans & Litwak, 1989). As the species identity of many engorged larval and nymphal ticks could not be determined unequivocally, all but three larvae and three nymphs were identified using molecular tools. The six ticks not subjected to molecular analyses were kept as voucher specimens that have been stored in the Biology Department at the University of Saskatchewan.

Genomic (g) DNA was extracted and purified from the complete body of each tick (Dergousoff & Chilton, 2007; Anstead & Chilton, 2013). Part of the mitochondrial (mt) 16S rRNA gene (400-450 bp) was amplified by PCR from the tick gDNA using primers 16S-1 (5'-CCACAGCAATTTAAAAAATCATTGAGCAG-3') and 16S+1 (5'-CCGGTCTGAACTCAGATCAAGT-3') (Norris *et al*., 1996) and the conditions described previously (Krakowetz *et al*., 2010). This gene was used as the genetic marker because other studies have demonstrated its usefulness for species-level identification and/or population genetics studies on ixodid ticks (e.g., Caporale *et al*., 1995; Norris *et al*., 1996; Norris *et al*., 1997; Anstead & Chilton, 2011; Krakowetz *et al*., 2011; Tian *et al*., 2011). The PCR products were subjected to electrophoresis on SYBR® Safe-stained 1.5% agarose-TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) gels and their banding patterns were visualized by UV transillumination. The 16S amplicons were then compared using single strand conformation

polymorphism (SSCP) analyses (Dergousoff & Chilton, 2007; Krakowetz *et al*., 2010) to determine if there were differences in DNA sequence among ticks. This mutation scanning technique differentially displays genetic variation between DNA sequences that are 150-450 base pairs (bp) in size, and that differ by one or more nucleotides (Gasser *et al*., 2006). The 16S amplicons of adults of known identity were included on SSCP gels as controls to determine the morphological identity of immature ticks. Amplicons, representing all the different SSCP banding patterns, were purified (Dergousoff & Chilton, 2012) and subjected to DNA sequencing using primers 16S+1 and 16S-1 in separate reactions. BLAST searches (GenBank) were performed on the sequence data. Nucleotide sequence data have been deposited in GenBank under the accession numbers HF968622-HF968629.

3.4 Results

Eighteen (44%) of the 41 *S. richardsonii* collected were parasitized by ticks. The number of ticks per host ranged from 1-67 (Fig. 3.1), and the mean intensity of infection was 6.4 ticks per host. Morphological examination of the 116 ticks (i.e., 24 adults, 48 nymphs, 44 larvae) collected (Table 3.1) revealed that they belonged to two genera: *Ixodes* and *Dermacentor*. Twenty adult ticks (18 females and 2 males) were identified as *D. andersoni*, while four adult female ticks were identified as either *I. sculptus* (n = 3) or *I. kingi* (n = 1) based on the shape of the palps and the posterior margin of the basis capitulum (see Keirans & Litwak, 1989). All ticks produced amplicons of approximately 450 bp on TBE-agarose gels (except for the gDNA of 1 female *I. sculptus* that failed to amplify). No amplicons were produced from the negative (i.e., no gDNA) samples. A comparison of the SSCP profiles of the 20 *D. andersoni* adults revealed that there were four different banding patterns (Fig. 3.2), each of which corresponded to a different

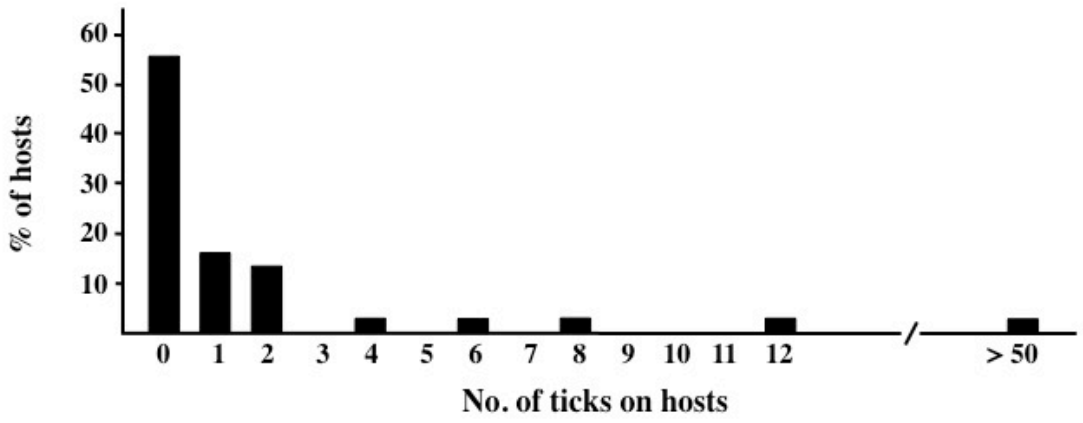

Fig. 3.1 The prevalence of *I. sculptus* on Richardson's ground squirrels.

Tick species	adults	nymphs	larvae
	No.	No.	No.
Ixodes sculptus (n=70)	3	21	40
Ixodes kingi (n=6)	1	4	1
Dermacentor andersoni (n=40)	20	20	0
Total	24	45	41

Table 3.1 The number of larval, nymphal and adult ticks collected from Richardson's ground squirrels (*Spermophilus richardsonii*) at Beechy (Saskatchewan, Canada). An additional three nymphs and three larvae were kept as vouchers, and identified to genus-level only.

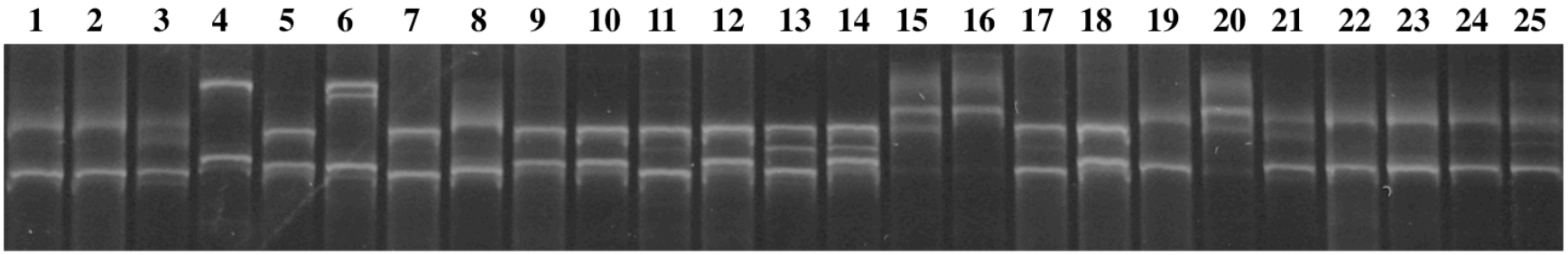

Fig. 3.2 SSCP banding patterns of representative 16S rDNA amplicons of *Ixodes sculptus* haplotype CAH1 (lanes 1-3, 8, 19 & 21-25), *I. kingi* haplotype CAH2 (lane 4), *I. kingi* haplotype CAH1 (lane 6), *D. andersoni* haplotype P (lanes 5, 9, 10, 12, 14 & 18), *D. andersoni* haplotype S (lanes 7, 11, 13 & 17) and *D. andersoni* haplotype R (lanes 15, 16 & 20).

sequence type (i.e., haplotype). The SSCP banding patterns (i.e., profiles) of six adults were identical to one another and their corresponding DNA sequences (404 bp) were identical to those of *D. andersoni* individuals of haplotype P (accession number FM955611) as defined by Patterson *et al.* (2009). The other *D. andersoni* adults had SSCP profiles and corresponding DNA sequences to those of *D. andersoni* individuals of haplotypes R (n = 6), S (n = 5) or T (n = 3) (accession numbers FM955613, FM955614 & FM955615, respectively). The single *I. kingi* female had a unique SSCP profile when compared to those of the *D. andersoni* and *I. sculptus* adults. The 413 bp sequence of the 16S gene for this individual was identical to that of *I. kingi* (accession number HF912422) from near Clavet, Saskatchewan. The SSCP profiles and partial mt 16S rDNA sequences (413 bp) for the two *I. sculptus* females were identical to each other. A BLAST search revealed that the 16S sequences of these two *I. sculptus* females differed by 4.5% (i.e., 11 bp) when compared to a partial (243 bp) 16S rDNA sequence (accession number U95903) of an *I. sculptus* from Fort Collins, Colorado (Norris *et al.*, 1999). The 16S rDNA sequences of *I. sculptus* and *I. kingi* adults from Beechy differed from one another at 21 nucleotide positions when compared over an alignment length of 413 bp (Table 3.2).

All of the larval ticks (n = 44) and approximately half of nymphs (n = 28) were identified morphologically as *Ixodes* sp. based on the presence of an anal groove located anterior to the anal pore (Durden & Keirans, 1996; Kleinjan & Lane, 2008). Some of the nymphs were identified as either *I. sculptus* or *I. kingi* based on the shape of the posterior margin of the basis capitulum and the size of the internal spur on coxa I (see Durden & Keirans, 1996). Twenty nymphs (n = 20) were identified morphologically as *Dermacentor* sp. based on the possession of an anal groove that was located posterior to the anal pore (Brinton *et al.*, 1965). However, the

Haplotype	Alignment position :																				
	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	2	2	3
	7	0	2	6	7	7	7	7	7	7	7	8	8	8	8	8	9	9	0	2	5	7	8
	0	8	0	2	2	3	4	5	7	8	9	4	5	6	7	9	0	4	4	7	0	1	4
I. kingi KH-1	A	–	T	A	T	A	–	–	G	G	G	C	C	C	T	A	–	G	G	A	A	T	G
I. kingi KH-2	.	T	.	.	.	.	–	–	.	.	.	.	.	.	.	.	–	.	.	.	.	.	.
I. kingi KH-3	.	–	.	.	.	G	–	–	.	.	.	.	.	.	.	.	–	.	.	.	.	.	.
I. sculptus SH-1	T	–	A	T	–	.	C	C	A	A	A	T	T	T	A	–	T	A	A	T	–	C	A

Table 3.2 Variable nucleotides in the aligned mitochondrial 16S rDNA sequences of three haplotypes of *I. kingi* from two Saskatchewan locations (KH-1 to KH-3), and one haplotype of *I. sculptus* from Beechy, Saskatchewan (SH-1). A dot indicates the same nucleotide as in the sequence of haplotype CAH-1.

species identity of a majority of the larvae and some of the nymphs could not be determined unequivocally because the ticks were fully engorged with blood and lymph.

Amplicons of the 16S rRNA gene were obtained for a total of 35 larvae and 45 nymphs, whereas no PCR-products were obtained for six larval *Ixodes*. A comparison of the 16S SSCP banding patterns of the larval ticks revealed that 24 individuals had an identical profile to that of the *I. sculptus* adults. The 16S sequences (421 bp) of 8 of these larvae were identical to the 16S sequence of *I. sculptus* adults (Table 3.2). The other larval tick had a unique SSCP profile when compared to the adults of *I. sculptus*, *I. kingi* and *D. andersoni*. The 16S sequence (421 bp) of this tick differed by a single nucleotide when compared to the DNA sequences of the *I. kingi* adult (Table 3.2).

A comparison of the 16S SSCP banding patterns of the nymphs revealed that 11 were identical in profile to *D. andersoni* adults of haplotype P, five were identical to *D. andersoni* adults of haplotype R, while four had the same profile as *D. andersoni* adults of haplotype S (Fig. 3.2). DNA sequencing of representative amplicons (n = 18) confirmed that there were no differences in 16S rDNA sequences of *D. andersoni* nymphs and adults with the same SSCP profile. Another 21 nymphs had identical SSCP profiles to those of the *I. sculptus* adults. The 16S rRNA gene sequences of 5 putative *I. sculptus* nymphs were identical to those of the two *I. sculptus* adults from Beechy. The 16S amplicons of another three nymphs had identical SSCP profiles and DNA sequences to those of the *I. kingi* adult. However, one nymph had a unique 16S SSCP profile when compared to those of all other ticks examined (Fig. 3.2). The partial (421 bp) 16S rDNA sequence of this nymph differed at one alignment position when compared to the DNA sequence of the *I. kingi* adult (Table 3.2).

3.5 **Discussion**

Richardson's ground squirrels are common across the prairies of southern Canada and the northern U.S.A., yet there is a lack of detailed information on the ecology and population genetics of some of the tick species that parasitize these mammals. In the present study, 41 *S. richardsonii* were collected from Beechy in southwestern Saskatchewan, 44% of which were parasitized by ticks. The majority of these hosts were parasitized by one (n = 7) or two (n = 6) ticks; however, 67 ticks were found feeding on one *S. richardsonii*. This aggregation of ticks on Richardson's ground squirrels is similar to that of other species of ticks that parasitize small mammals, such as *Ixodes scapularis* and *I. ricinus*, where the distribution pattern of ticks among individuals of a host population has important implications for the spread of vector-borne diseases (Brunner & Ostfeld, 2008; Harrison & Bennett, 2012).

Programs aimed at controlling ticks and tick-borne diseases require detailed knowledge of the ecology of the vectors, and a reliable method to distinguish vectors from species that are not vectors of disease(s). However, it is sometimes difficult to morphologically distinguish among ticks of closely related species (Andrews *et al*., 1992; Anderson *et al*., 2004; Dergousoff & Chilton, 2007). Another problem in identifying ticks to the species level, particularly larval and nymphal stages, is that specimens are occasionally damaged upon removal from a host, making it difficult to see the diagnostic morphological features (e.g., Salkeld *et al*., 2006). Therefore, molecular approaches have been used in the identification of ticks (e.g., Dergousoff & Chilton, 2007; Anstead & Chilton, 2011). In the present study, adult ticks were identified to the species level by morphological examination, and their PCR-SSCP banding patterns and DNA sequences of the mt 16S rRNA gene were determined. This combined PCR-SSCP and DNA sequencing approach was then used to determine the species identity of the larval and nymphal

ticks using the 16S rDNA amplicons of adult ticks as controls. The results of the morphological and molecular analyses showed that adults of three tick species, *I. sculptus*, *I. kingi* and *D. andersoni*, occurred on *S. richardsonii* at Beechy. The SSCP banding patterns and DNA sequences of the 16S rDNA of adults and immature ticks (i.e., larvae and nymphs) of all three species were different from one another. Hence, PCR-SSCP of 16S rDNA is a cost effective and reliable method for distinguishing among ticks of different species, irrespective of their life cycle stage, or the number of ticks to be examined.

Over half of the ticks collected from *S. richardsonii* at Beechy were identified as *I. sculptus*. All three feeding life cycle stages (i.e., larvae, nymphs and adults) of this tick species were found feeding on *S. richardsonii*. In addition, at least one *I. sculptus* was found feeding on all of the 18 Richardson's ground squirrels parasitized by ticks. This tick species has a wide distribution throughout North America (Bishopp & Trembley, 1945; Cooley & Kohls, 1945; Gregson, 1956; Durden & Keirans, 1996; Allan, 2001) and, based on existing literature, has a preference for *S. richardsonii* as hosts (Brown, 1944; Brown & Kohls, 1950; Burgess, 1955); although it has been reported on a diverse range of mammals (e.g., ground dwelling sciurids, rodents, carnivores, lagomorphs, cats, dogs, goats and humans) (Hixson, 1932; Bishopp & Trembley, 1945; Allred *et al*., 1960; Hilton & Mahrt, 1971; Salkeld *et al*., 2006; Kolonin, 2007). No genetic variation in the mt 16S rRNA gene was detected among *I. sculptus* individuals from different hosts; however, this finding may be a consequence of sampling ticks from a host population over a relatively small area. Therefore, 16S sequence data are needed for individuals from many other localities to assess the magnitude of genetic variation in *I. sculptus*. The only other 16S data available for *I. sculptus* is a 243 bp sequence for one tick collected from Fort Collins, Colorado (Norris *et al*., 1999). The sequence of this *I. sculptus* individual (accession

number U95903) differed at 11 alignment positions when compared to mt 16S rDNA sequences of *I. sculptus* from Beechy. The significance of this difference needs to be explored further.

A few *I. kingi* (i.e., 1 adult, 4 nymphs and 1 larva) were present on four *S. richardsonii*. Several studies have previously reported the presence of *I. kingi* on Richardson's ground squirrels (Hearle, 1938; Allred *et al.,* 1960), in addition to a wide variety of other mammals (Cooley & Kohls, 1945; Miller & Ward, 1960; Gregson, 1971; Salkeld *et al.,* 2006; Anstead & Chilton, 2011). The results of a previous study on *I. kingi* revealed that there was no variation in the sequence of the mt 16S rRNA gene among 88 individuals (i.e., larvae, nymphs and adults) feeding on northern pocket gophers (*Thomomys talpoides*) near Clavet, Saskatchewan (Anstead & Chilton, 2011). This is equivalent to the lack of genetic variation detected among *I. sculptus* individuals feeding on *S. richardsonii*. However, in the present study, three different 16S SSCP banding patterns were detected among the six *I. kingi* individuals. The 16S DNA sequences of four (67%) individuals (i.e., 1 adult, 3 nymphs) were identical to those of *I. kingi* collected from *T. talpoides* near Clavet (Anstead & Chilton, 2011), a location situated approximately 215 km northeast of Beechy. The other two individuals each had a different haplotype and differed in 16S rDNA sequence at one nucleotide position when compared to the sequences of other *I. kingi* individuals.

Two of the 18 *S. richardsonii* were also infested with nymphs and adults of *D. andersoni*. In contrast to the lack of genetic variation in the mt 16S rRNA gene of the *I. sculptus* collected from *S. richardsonii*, four different SSCP banding patterns were detected among the *D. andersoni* individuals, each of which corresponded to a different sequence type (i.e., haplotype). Three of the four haplotypes (i.e., P, R and S) have been previously reported in questing *D. andersoni* adults from Saskatchewan Landing Provincial Park (Patterson *et al*., 2009), a locality

situated 23 miles southwest of Beechy. Individuals of haplotype P represented the most abundant haplotype at Saskatchewan Landing Provincial Park (Patterson *et al*., 2009) and Beechy (55% and 43%, respectively). The fourth 16S haplotype (T) detected among *D. andersoni* individuals from Beechy has been reported previously in *D. andersoni* from Lethbridge, Alberta (Patterson *et al*., 2009).

No *D. andersoni* larvae were found on any of the *S. richardsonii* collected at Beechy. It is possible that *D. andersoni* larvae prefer to feed on other mammalian hosts (e.g., voles, shrews and mice; Gregson, 1956; James *et al*., 2006; Dergousoff *et al*., 2013) or alternatively, *D. andersoni* larvae do not actively feed on Richardson's ground squirrels during the months (i.e., June and July) when the hosts were collected. Dergousoff (2011) did not find *D. andersoni* larvae on small mammals (i.e., shrews, voles and mice) at Saskatchewan Landing Provincial Park between April and early July. They suggested that *D. andersoni* larvae may not be active until late July or August at this locality. There is a record of a *D. andersoni* larva on *S. richardsonii* in early July from Swift Current in southern Saskatchewan (Gregson, 1956). However, *D. andersoni* larvae have been shown to feed on small mammals in field enclosures during July and August in Kamloops within southern British Columbia (Wilkinson, 1968). There are significant differences in the climatic conditions experienced by ticks on the prairies in southern Saskatchewan and those in the montane regions of southern British Columbia (Wilkinson, 1967). As a consequence, the peak activity period of *D. andersoni* adults on hosts in Canadian prairies occurs one month after that of adult ticks in the montane regions of British Columbia (Wilkinson, 1967). Therefore, the peak seasonal activity periods of *D. andersoni* larvae on hosts in the two geographical areas are also likely to be different. Additional studies

are needed to establish the seasonal activity patterns of *D. andersoni* on hosts at localities, such as Beechy, situated near the northeastern distributional limit of this tick species.

Another interesting finding was the lack of any life cycle stage of *D. variabilis* on Richardson's ground squirrels. Both *D. variabilis* and *D. andersoni* have largely allopatric distributions in Canada except for a large overlap zone (~200 km wide) in central Saskatchewan, that includes Beechy and Saskatchewan Landing Provincial Park (Dergousoff *et al*., 2013). The absence of *D. variabilis* on *S. richardsonii* may not be unexpected because, as far as we aware, this tick species has not been reported feeding on Richardson's ground squirrels. In Saskatchewan, the larvae and nymphs of *D. variabilis* have been reported from smaller mammals, such as voles, shrews and mice (Dergousoff *et al*., 2013).

Although *D. andersoni* is an important vector of human and/or animal pathogens (Burgdorfer, 1975; Foley & Nieto, 2010; Kocan *et al*., 2010), *I. sculptus* and *I. kingi* have also been implicated as vectors of pathogenic agents. For example, *I. sculptus* is a vector of Colorado tick fever (CDC, 1976), while *I. kingi* is a vector of *Coxiella burnetii*, the causative agent of Q fever, and *F. tularensis* (Sidwell *et al*., 1964; Thorpe *et al*., 1965). Detection of these three tick species on the same host, all of which are vectors of pathogenic agents, provides an opportunity to examine fundamental ecological and evolutionary questions relating to the structure and composition of bacteria in ticks.

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Chapter 4 Ticks (Acari: Ixodidae) on small mammals in Kootenay National Park, British Columbia, Canada[3]

4.1 Abstract

Two hundred and ninety one ticks (185 larvae, 72 nymphs, and 34 adults) were removed from 153 small mammals comprising six species collected in Verdant Forest, Numa Forest and Marble Canyon within Kootenay National Park, British Columbia, Canada. Morphological examination and molecular analyses (i.e., PCR-SSCP and DNA sequencing of the mitochondrial 16S rRNA gene) of the ticks revealed that most individuals were *Ixodes angustus*. All life cycle stages of *I. angustus* were found primarily on southern red-backed voles, *Clethrionomys gapperi* (Vigors). Two *Dermacentor andersoni* females were also found on these small mammals. The results of the molecular analyses also revealed that there were three 16S haplotypes of *I. angustus* and two 16S haplotypes of *D. andersoni*. A comparison of available sequence data suggests genetic divergence between *I. angustus* near the western and eastern limits of the species distributional range in North America. Additional studies are needed to determine if there are genetic differences between *I. angustus* from North America, Japan, and Russia, and whether there is geographical variation in the ability of ticks to transmit pathogens to their mammalian hosts.

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4.2 **Introduction**

Hard ticks (Acari: Ixodidae) are important vectors of pathogenic agents (i.e., bacteria, viruses and protozoa) to humans, domestic animals and/or wildlife throughout the world (Parola & Raoult, 2001; Jongejan & Uilenberg, 2004; Dantas-Torres *et al*., 2012). Some pathogenic bacteria (e.g., *Ehrlichia chaffeensis*) are very tick species-specific, whereas others (e.g., *Rickettsia rickettsii*) use several tick species as vectors (Parola & Raoult, 2001; Dantas-Torres *et al*., 2012). There are at least 26 species of ixodid tick (i.e., 20 *Ixodes* spp., 3 *Dermacentor* spp., 2 *Haemaphysalis* spp. and 1 *Rhipicephalus* spp.) that occur in Canada (Gregson 1956; Wilkinson, 1967; Linquist *et al*., 1999; Ogden *et al*., 2009), several of which are of medical and/or veterinary importance (Gregson 1956; Ogden *et al*., 2009). All 26 species also occur south of the Canadian-United States border (Gregson 1956; Durden & Keirans, 1996; Allan, 2001; Lubelczyk *et al*. 2007). Some of these species, such as *Ixodes scapularis* Say, *I. muris* Bishopp and Smith, *Haemaphysalis leporispalustris* (Packard) and *H. chordeilis* (Packard), are frequently transported into Canada on migratory passerine birds that travel northwards during their spring migration (Scott *et al*., 2001; Morshed *et al*., 2005; Ogden *et al*., 2008; Scott *et al*., 2012). Numerous other ticks, such as *Amblyomma americanum* (Linnaeus), *A. imitator* Kohls, *A. inornatum* (Banks), *A. longirostre* (Koch), *A. maculatum* Koch, *A. sabanerae* Stoll, *Ixodes affinis* Neumann, *I. baergi* Cooley and Kohls, *I. brunneus* Koch and *I. dentatus* Marx, have also been collected from migratory passerine birds that have travelled into Canada from the United States, Mexico, Central and South America (Scott *et al*., 2001; Morshed *et al*., 2005; Ogden *et al*., 2008; Scott *et al*., 2012). However, these species have not yet established populations in Canada.

At least one third of the tick species reported in Canada occur in British Columbia (BC). These species include *Dermacentor andersoni* Stiles, *D. albipictus* (Packard), *I. angustus*

Neumann, *I. auritulus* Neumann, *I. hearlei* Gregson, *I. kingi* Bishopp, *I. marmotae* Cooley and Kohls, *I. ochotonae* Gregson, *I. pacificus* Cooley and Kohls, *I. rugosus* Bishopp, *I. sculptus* Neumann, *I. signatus* Birula, *I. soricis* Gregson, *I. spinipalpis* Hadwen and Nuttall, *I. texanus* Banks, *I. uriae* White, *H. chordeilis* and *H. leporispalustris* (Parker *et al*., 1931; Hearle, 1938; Gregson, 1956; Wilkinson, 1967; Sonenshine, 1991; Sorensen & Moses, 1998; Kain *et al*., 1999; Allan, 2001). Some of these species are known vectors of human and animal pathogens. For example, *Ixodes pacificus* and *I. angustus* have been implicated in the spread of *Borrelia burgdorferi*, the causative agent of Lyme borreliosis, in the Pacific Northwest (Damrow *et al*., 1989; Banerjee *et al*., 1994; Kain *et al*., 1999). Another common species, *D. andersoni*, is a vector of *Rickettsia rickettsii*, *Francisella tularensis* and *Anaplasma marginale*, the bacteria responsible for Rocky Mountain spotted fever, tularemia, and bovine anaplasmosis, respectively (Burgdorfer, 1975; Foley & Nieto, 2010; Kocan *et al*., 2010).

Tick species diversity in BC is greater than that of most other western Canadian provinces (Gregson, 1956). This has been attributed to the milder and more diverse climates providing suitable conditions for a more diverse vertebrate fauna (i.e., potential tick hosts) and a better chance for some tick species to survive and successfully complete their life cycle (Gregson, 1956). Approximately half of BC is covered by forest (Brown *et al*., 1997), providing a range of habitats for small mammals (e.g., shrews, voles, mice, and ground squirrels); the hosts used by many of the tick species that occur in the province (Robbins & Keirans, 1992; Durden & Keirans, 1996; Allan, 2001; Kolonin, 2007). For example, stands of trembling aspen (*Populus tremuloides*) amongst species of coniferous trees within the forests of BC, such as those that occur within the Kootenay National Park in the south-western region of the Canadian Rocky Mountains (White *et al*., 1998; Hallett & Walker, 2000), provide increased habitat quality (i.e.,

representing "hotspots") for small mammals (Oaten & Larsen, 2008).

Understanding the ecology of vector-borne diseases requires detailed information of the vectors (e.g., ticks), their hosts (e.g., small mammals) and the pathogens (e.g., bacteria) transmitted by the vectors to hosts. This includes knowledge of the distributional range of the vectors and their hosts, the interactions among all three groups of organisms, and the influence of environmental factors on their survival and reproduction. A key component of any surveillance program targeting the control of vector-borne diseases requires the accurate identification of individual ticks to the species level. However, it is sometimes difficult to unequivocally identify ticks to the species level by morphological examination, especially if they have been damaged upon removal from a host, or are fully engorged with blood (Andrews *et al*., 1992; Anderson *et al*., 2004). This is often the case for identifying larval and nymphal ticks (Andrews *et al*., 1992; Anderson *et al*., 2004). As a consequence, PCR-based approaches, using a variety of genetic markers, have been shown to be useful in the identification of ticks (Wesson *et al*., 1993; Dergousoff & Chilton, 2007; Anstead *et al*., 2011; Tian *et al*., 2011). In some studies, PCR-single strand conformation polymorphism (SSCP) analyses, in combination with DNA sequencing, have been used to distinguish among individual ticks of different species (e.g., Dergousoff & Chilton, 2007; Anstead & Chilton, 2011) and to determine the magnitude of intraspecific variation in DNA sequence among ticks (e.g., Ketchum *et al*., 2009; Patterson *et al*., 2009; Krakowetz *et al*., 2010). SSCP is a sensitive and powerful molecular tool that visually displays different sequence types that differ by one or more nucleotides in amplicons of ~100-500 base pairs (bp) in size (Hiss *et al*., 1994; Gasser *et al*., 2006). Several target regions, such as the sequences of the mitochondrial (mt) 12S and 16S ribosomal (r) RNA genes, and the nuclear second internal transcribed spacer (ITS-2) of rDNA, have been used to distinguish among tick

species (Caporale *et al*., 1995; Norris *et al*., 1997; Barker, 1998; Chitimia *et al*., 2009; Anstead & Chilton, 2011; Tian *et al*., 2011). These target regions have also been used to examine the population genetics and phylogenetic relationships of ticks (Black & Piesman, 1994; Norris *et al*., 1996; Fukunaga *et al*., 2000; Murrell *et al*., 2001; Guglielmone *et al*., 2006; Leo *et al*., 2010; Krakowetz *et al*., 2011).

In the present study, morphological examination and PCR-based methods (i.e., SSCP and DNA sequencing) using the mt 16S rRNA gene were used to identify, to the species level, ticks feeding on small mammals within Kootenay National Park (KNP). This study area was selected because it represents a "hotspot" for small mammals (Oaten & Larsen, 2008) and has been the focus of another study (Hwang *et al*., 2010) that examined the effects of natural forest fires on the abundance and diversity of small mammals and their endoparasites.

4.3 **Materials and Methods**

A total of 153 small mammals were trapped in Verdant Forest, Numa Forest and Marble Canyon within KNP (50°68'N, 115°93'W), during the summer months of 2005, 2006 and 2007 (Table 4.1), as described in Hwang *et al*. (2010). All ticks were removed from hosts and fixed in 70% ethanol for morphological examination and molecular characterization. All adult ticks and many of the immatures (i.e., larvae and nymphs) were first identified to the genus level based on the positioning of the anal groove and the presence/absence of eyes and festoons (Durden & Keirans, 1996; Kleinjan & Lane, 2008). The size and shape of the hypostome, coxal spurs, spiracular plate and goblet cells were then used to identify individual adult ticks to the species

Mammal species	2005					2006				
	No. of hosts:		No. of ticks:			No. of hosts:		No. of ticks:		
	Collected	Infested (%)	A*	N*	L	Collected	Infested (%)	A	N	L
Clethrionomys gapperi (southern red-backed vole)	36	15 (42)	11	31	68	27	11 (41)	4	16	5
Microtus longicaudus (long-tailed vole)	8	5 (63)	3	-	1	14	0 (0)	-	-	-
Phenacomys intermedius (western heather vole)	1	0 (0)	-	-	-	0	-	-	-	-
Sorex cinereus (masked shrew)	0	-	-	-	-	0	-	-	-	-
Peromyscus maniculatus (deer mouse)	13	0 (0)	-	-	-	19	1 (5)	0	1	1
Spermophilus lateralis (golden-mantled ground squirrel)	3	0 (0)	-	-	-	4	2 (50)	2	0	0
Total	61	20 (33)	14	31	69	64	14 (22)	6	17	6

*A = adults, N = nymphs, and L = larvae

Mammal species	2007				
	No. of hosts:		No. of ticks:		
	Collected	Infested (%)	A	N	L
Clethrionomys gapperi (southern red-backed vole)	21	17 (81)	13	19	106
Microtus longicaudus (long-tailed vole)	2	1 (50)	0	1	0
Phenacomys intermedius (western heather vole)	3	2 (67)	1	4	3
Sorex cinereus (masked shrew)	1	1 (100)	0	0	1
Peromyscus maniculatus (deer mouse)	0	-	-	-	-
Spermophilus lateralis (golden-mantled ground squirrel)	0	-	-	-	-
Total	28	21 (75)	14	24	110

Table 4.1 The number of mammals collected from Kootenay National Park (British Columbia, Canada) during the summer months of 2005, 2006, and 2007, the proportions of individuals infected by ticks, and the number of ticks removed from mammals.

level (Brinton *et al*., 1965; Keirans & Litwak, 1989). However, the species identity for many immatures, particularly those engorged with blood, could not be determined unequivocally. Therefore, PCR-based techniques were used to determine the species identity of all ticks, except for six individuals that were used for scanning electron microscopy, and another eight ticks (i.e., two larvae, three nymphs, one male and two female *Ixodes* sp.) that were kept as voucher specimens (stored in the Biology Department at the University of Saskatchewan). The six *Ixodes* sp. (i.e., three larvae, one nymph and two adult females) examined by scanning electron microscopy were dehydrated in a graded ethanol series, transferred to acetone and critical point dried using carbon dioxide. The ticks were mounted on stubs, sputter coated with gold and examined in a Philips 505 scanning electron microscope (SEM). For the two adult females, several legs were removed for molecular analyses prior to SEM.

Total genomic DNA (gDNA) was extracted and purified from the complete bodies of each tick using the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany). Ticks were placed individually in 1.5ml micropestle tubes into which 180μl of ATL buffer (QIAGEN) was added. Ticks were crushed using disposable micro-pestles attached to a cordless drill. After the ticks were fully homogenized, 20μl of proteinase K (QIAGEN) was added to the samples and incubated at 55°C in a heat block for approximately 18 hours. The gDNA was purified according to the protocol provided in the DNeasy Blood & Tissue Kit; however, the elution process was performed twice using 50μl of AE buffer (QIAGEN) and both eluates (100μl) were pooled into a single tube. Part of the mt 16S rRNA gene (400-450 bp) was amplified from the tick gDNA using primers 16S-1 (5'-CCACAGCAATTTAAAAAATCATTGAGCAG-3') and 16S+1 (5'-CCGGTCTGAACTCAGATCAAGT-3') (Norris *et al*., 1996) and the conditions described previously (Krakowetz *et al*., 2010). Amplicons were subjected to electrophoresis on SYBR®

Safe-stained 1.5% agarose-TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) gels and their banding patterns were visualized by UV transillumination. SSCP analyses (Gasser *et al*., 2006) were then performed on all amplicons to pre-screen for genetic variation before selecting representative samples for DNA sequencing. Twelve amplicons, representing all the different SSCP banding patterns, were purified (Dergousoff & Chilton, 2012) and subjected to DNA sequencing using primers 16S+1 and 16S-1 in separate reactions. BLAST searches (GenBank) were performed on the sequence data to determine the species identity of individual ticks. Nucleotide sequence data have been deposited in GenBank under the accession numbers HF912727- HF912731.

The DNA sequences of the different 16S rDNA haplotypes of *I. angustus* from KNP were aligned manually with those of *I. angustus* from the eastern U.S.A. (Accession numbers U14140 and U14140: Caporale *et al*., 1995), and *I. kingi* (Accession number HF912422: Anstead & Chilton, 2013); the latter of which was used as an outgroup in the phylogenetic analyses. The computer program PAUP (Swofford, 2003) was used to perform the phylogenetic analyses on the sequence data using both the neighbor joining (NJ) and maximum parsimony (MP) methods. For the MP analyses, characters were treated as unordered and were equally weighted; alignment gaps were treated as 'missing' characters. Exhaustive searches with TBR branch swapping were used to infer the shortest trees. The length, consistency index excluding uninformative characters, and the retention index of the most parsimonious tree were recorded. Bootstrap analyses (1000 replicates for the NJ analyses and 100 replicates for MP analyses) were conducted to determine the relative support for clades in the consensus tree.

4.4 **Results**

A total of 153 small mammals, representing six species, were trapped in Kootenay National Park (BC) and of these, 55 (36%) were infested with ticks (Table 4.1). Two hundred and ninety one ticks (i.e., 185 larvae, 72 nymphs, and 34 adults) were removed from these small mammals, a large proportion of which (94%, $n = 273$) were found on southern red backed voles, *Clethrionomys gapperi* (Vigors) (Table 4.1). At least 50% of the *C. gapperi* individuals collected ($n = 84$) were parasitized by ticks with a mean intensity of 6.4 (i.e., range of 1-54 ticks per parasitized host).

Two adult female ticks, one from *C. gapperi* and the other from *Spermophilus lateralis* (Say), were identified morphologically as *D. andersoni*. The partial mt 16S rDNA sequence (403 bp) of the *D. andersoni* female from the *C. gapperi* was identical to that of *D. andersoni* haplotype S (GenBank accession number FM955614), whereas the 16S sequence of the second *D. andersoni* female (404 bp), had one additional nucleotide when compared to the sequence of *D. andersoni* individuals of haplotype S.

The other 32 adult ticks (i.e., 1 male and 31 females) collected were identified as *Ixodes angustus* using light microscopy and/or SEM. Adult *I. angustus* were collected from four host species: *C. gapperi*, *S. lateralis, Microtus longicaudus* Merriam, and *Phenacomys intermedius* Merriam (Table 4.1). Amplicons of the 16S rRNA gene were obtained for 27 of the 29 gDNA samples prepared from *I. angustus* females. A comparison of their SSCP profiles revealed that there were three different banding patterns (Fig. 4.1), each of which corresponded to a different sequence type (i.e., haplotype). A total of 23 adults had the SSCP profile of haplotype CAH-1, two had the profile of haplotype CAH-2, and two had the profile of haplotype CAH-3 (Table 4.2).

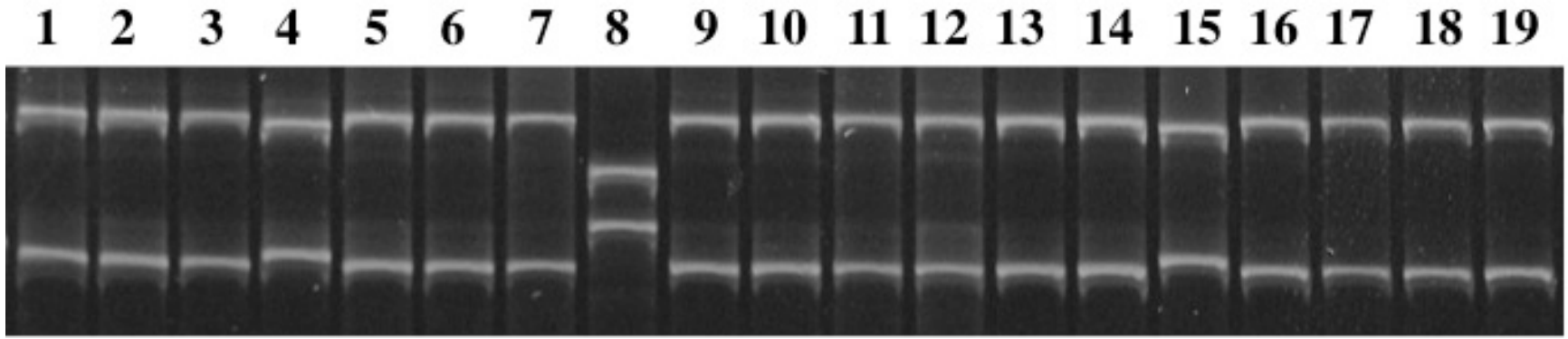

Fig. 4.1 SSCP profiles of representative 16S rDNA amplicons from the total gDNA of *Ixodes angustus* haplotype CAH-1 (lanes 1-3, 5-7, 9-14, 16

Haplotype	Alignment position :																			
						1	1	1	1	1	1	2	2	2	2	2	2	2	2
	3	6	6	6	9	6	6	7	7	7	8	8	0	1	3	3	3	3	4	4
	7	4	8	9	0	7	9	1	4	9	0	8	0	0	3	6	7	9	0	1
I. angustus CAH-1	A	A	G	G	A	T	C	T	A	G	T	T	G	C	G	G	T	A	A	G
I. angustus CAH-2	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
I. angustus CAH-3	.	G	.	.	.	C	.	.	G	.	.	.	.	.	.	.	.	.	G	.
I. angustus ANG-1	G	G	A	A	G	.	T	G	G	A	-	G	A	T	T	A	G	G	.	T
I. angustus ANG-2	G	G	A	A	G	.	T	G	G	A	-	G	A	T	T	A	G	G	.	T

Table 4.2 Variable nucleotides in the aligned mitochondrial 16S rDNA sequences of three haplotypes of *I. angustus* in western Canada (CAH-1 to CAH-3), and two haplotypes of *I. angustus* from the eastern U.S.A. (ANG-1 & ANG-2). A dot indicates the same nucleotide as in the sequence of haplotype CAH-1.

One to four nucleotide differences were detected among the partial 16S rDNA sequences (415 bp) of the five representative samples of three haplotypes of *I. angustus* (i.e., two individuals of haplotypes CAH-1 and CAH-3, and one individual of haplotype CAH-2). The four variable positions in the sequence alignment of the different haplotypes consisted of three purine transitional changes and one pyrimidine transitional change (Table 4.2). Representatives of each haplotype were used on SSCP gels as controls to confirm the morphological identity of all immature ticks collected.

Larval and nymphal ticks were collected from five host species, *C. gapperi*, *M. longicaudus*, *P. intermedius*, *Sorex cinereus* Kerr and *Peromyscus maniculatus* Wagner. Most of the larvae and nymphs were identified morphologically as belonging to the genus *Ixodes* based on the presence of an anal groove that is located anterior to the anal pore, as opposed to that of *Dermacentor* sp., which possess an anal groove posterior to the anal pore (Durden & Keirans, 1996; Kleinjan & Lane, 2008). In addition, some larvae and nymphs were easily identified as *I. angustus* based on the prominent anterior and posterior spurs located at the base of the palps, as well as the shape of the palpal segments (Fig. 4.2). However, some larvae and nymphs were fully engorged and difficult to unequivocally determine their species identity. Amplicons of the 16S gene were obtained for a total of 176 larvae and 68 nymphs, whereas no products were obtained for 4 larvae. A comparison of the 16S SSCP banding patterns revealed that 189 immature ticks (i.e., 133 larvae and 56 nymphs) were identical in profile to *I. angustus* females of haplotype CAH-1, 44 (i.e., 33 larvae and 11 nymphs) had the same profile as *I. angustus* females of haplotype CAH-2, while 10 larvae and one nymph had the same profile as *I. angustus* females of haplotype CAH-3 (Fig. 4.1). DNA sequencing of representative amplicons ($n = 5$) confirmed that

Fig. 4.2 Scanning electron microscope (SEM) photograph of a larval *Ixodes angustus*. Note the prominent spurs at the base of each palp.

there were no differences in 16S rDNA sequences between immature and adult ticks of the same haplotype.

A comparison of the 16S rDNA sequences of the three haplotypes of *I. angustus* with sequence data available on GenBank revealed that each haplotype differed in sequence by 17-18 bp when compared to two sequences of *I. angustus* (i.e., ANG-1 & ANG-2) from the northeastern U.S.A. (Table 4.2). The NJ tree produced from the analysis of the sequence data placed the three haplotypes of *I. angustus* from western Canada in a clade, with statistical support (i.e., bootstrap value of 84%), to the exclusion of *I. angustus* from the eastern U.S.A. (Fig. 4.3). The MP analysis of the same data set (i.e., 13 cladistically informative characters) produced one most-parsimonious tree (length = 46, CI = 0.93 and RI = 0.94), with the same topology as the NJ tree, and strong statistical support (i.e., bootstrap value of 97%) for the clade containing the three haplotypes of *I. angustus* from western Canada (Fig. 4.3).

4.5 **Discussion**

Of the 153 voles, shrews, mice and ground squirrels collected within KNP, 36% were parasitized by ticks. All adult ticks and some of the immature ticks (i.e., larvae and nymphs) were identified to the species level by morphological examination using light microscopy, and in some cases, using SEM. Although SEM is an effective and high-resolution tool for species-level identification, specimens prepared for SEM cannot be subsequently included in molecular-based studies that examine population genetics or the bacterial communities of individual ticks. Given this and the difficulties of identifying some immature ticks by morphological examination, a

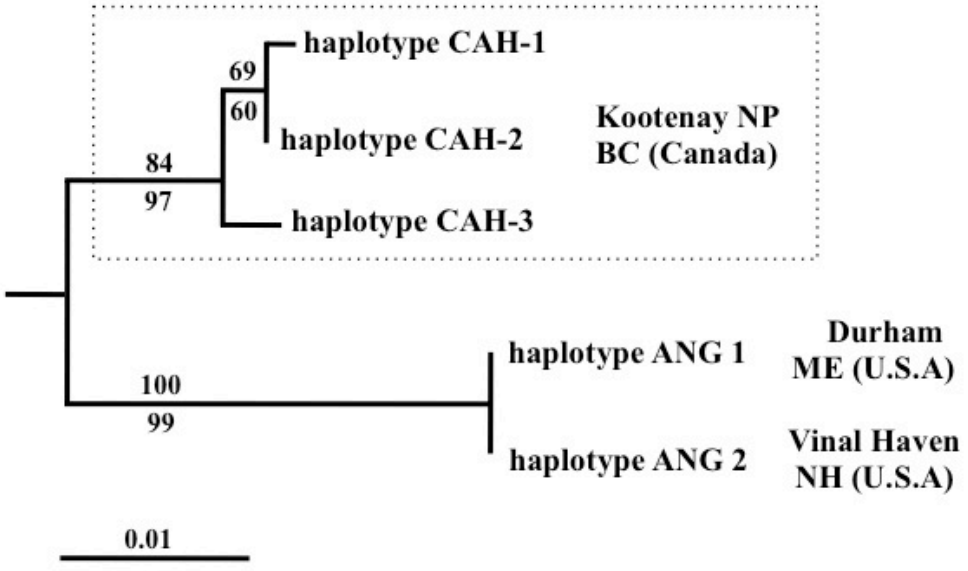

Fig. 4.3 Neighbor-joining tree depicting the relationships of the 16S rRNA gene sequences of the three haplotypes of *Ixodes angustus* from Kootenay National Park (BC) and those from Durham (NH) and Vinalhaven (ME). The scale bar represents the inferred substitutions per nucleotide site. The relative support for clades in the tree produced from the NJ and MP analyses are indicated above and below branches, respectively.

molecular approach, PCR-SSCP combined with DNA sequencing of the mt 16S rRNA gene, was therefore used to unequivocally determine the species identity of ticks.

Most of the ticks feeding on small mammals in KNP were identified as *I. angustus* based on morphological and molecular analyses. All three feeding life cycle stages (i.e., larvae, nymphs and adults) were found on all three species of vole examined: *C. gapperi* (southern red-backed vole), *M. longicaudus* (long-tailed vole) and *P. intermedius* (western heather vole). However, most (94%) of the *I. angustus* were collected on *C. gapperi*, even though this host species comprised only 55% of the small mammals collected from KNP. Furthermore, 51.2% of the *C. gapperi* collected from KNP (n = 84) were parasitized by *I. angustus*. Only a few *I. angustus* were collected from the other species of small mammals: six *M. longicaudus*, two *P. intermedius*, one *S. cinereus* (masked shrew), one *S. lateralis* (golden-mantled ground squirrel) and a single *P. maniculatus* (deer mouse). The strong host preference of *I. angustus* for *C. gapperi* in KNP is similar to that which was reported for *I. angustus* in a mature aspen forest near Lac La Biche in north-central Alberta (Sorensen & Moses, 1998). In the study of Sorensen and Moses (1998), 61% of the *I. angustus* were collected from *C. gapperi*. Other host species used by *I. angustus* near Lac La Biche included *Microtus pennsylvanicus* (meadow voles) and *P. maniculatus* (Sorensen & Moses, 1998). In addition, a significantly higher proportion of *C. gapperi* (34.6%; n = 402) were parasitized by *I. angustus* than were *P. maniculatus* (3.2%; n = 282) (Sorensen & Moses, 1998). Only 1 (3.1%) of 32 deer mice from KNP was parasitized by *I. angustus*, which is equivalent to the prevalence reported by Sorensen and Moses (1998). In contrast, a large proportion (72%) of *I. angustus* on small mammals within two forests in western Oregon (i.e., Neptune State Forest near Yachats and the William L. Finley National Park near Corvallis) were found primarily on *P. maniculatus* and secondarily on shrews (*Sorex vagrans*, *S.*

trowbridgii, and *S pacificus*) (Easton & Goulding, 1974). Although southern red-backed voles probably do not occur at these sites (see Verts & Carraway, 1998), voles (e.g., *Microtus montanus* and *M. townsendii*) do occur in forested areas of western Oregon; however, no individuals were examined for ticks in the study of Easton and Goulding (1974). Nonetheless, *I. angustus* were recovered from the nests of *M. montanus* and *M. townsendii* (Easton & Goulding, 1974), suggesting that voles may represent important hosts for this tick species in western Oregon. Although the *I. angustus* populations in KNP and at Lac La Biche prefer voles to shrews and mice as hosts, the same host preference does not occur for populations of this tick species in other parts of its distributional range where southern red backed voles also occur. For example, Martell *et al*. (1969) found that four species of small mammal were hosts for *I. angustus* in the Tobeatic Wilderness Area (south central Nova Scotia), but that the prevalence was greater (23.9%) for northern short-tailed shrews (*Blarina brevicauda*) than for *S. cinereus* (2.1%), *M. pennsylvanicus* (5.1%) *C. gapperi* (1.7%) and *P. maniculatus* (0%). In addition, Martell *et al*. (1969) examined twice as many southern red-backed voles (n = 59) for ticks than northern short-tailed shrews (n = 21), the latter of which do not occur in north-central Alberta or in the KNP (Brant & Ortí, 2003). Therefore, a number of ecological factors may contribute to the reported differences in host preference in different parts of the geographical range of *I. angustus*.

In addition to the presence of *I. angustus* on small mammals in KNP, two female ticks, one on *C. gapperi* and the other on *S. lateralis*, were identified both morphologically and genetically as *D. andersoni*. The distributional range of *D. andersoni* in Canada extends from the coastal mountains in British Columbia eastward to central Saskatchewan (Holland, 1940; Wilkinson, 1967; Dergousoff *et al*., 2013). Detection of *D. andersoni* adults on *C. gapperi* and *S. lateralis* in KNP was not unexpected because these are known hosts for this tick species

(Wilkinson, 1967; Clark *et al*., 1970; Whitaker *et al*., 1975; Timm, 1975; Dergousoff *et al*., 2013). The 16S rDNA sequences of the two *D. andersoni* females differed from one another by one nucleotide, a difference that was easily detected when their SSCP banding patterns were compared. The 16S SSCP banding patterns of both *D. andersoni* females were also distinct from those of all the *I. angustus* found within KNP. However, three different 16S haplotypes, which differed in sequence by 1-4 nucleotides, were detected among individuals of *I. angustus*. The majority (78.4%) of *I. angustus* were of one haplotype. There is, however, little information on genetic variation in *I. angustus* despite its broad geographical range that includes North America, Russia and Japan (Robbins & Keirans, 1992). The results of the present study revealed that the 16S rDNA sequences of all *I. angustus* from KNP in western Canada differ markedly (17-18 bp) compared to those of *I. angustus* from eastern U.S.A. (i.e., Durham, New Hampshire and Vinalhaven, Maine), suggesting possible genetic divergence between tick populations at the western and eastern limits of the species distribution in North America. Nonetheless, additional population genetic studies are needed to determine the magnitude of genetic variation among *I. angustus* populations in different parts of the species distributional range. This knowledge will have implications for our understanding of the transmission of vector-borne pathogens.

Although *I. angustus* in KNP demonstrated a preference for southern red-backed voles as hosts, this tick species has been reported from more than 90 species of mammals including sciurids, lagomorphs, cats, dogs and humans (Bishopp & Trembley, 1945; Cooley, 1946; Spencer, 1963; Robbins & Keirans, 1992; Peavey *et al*, 2000; Kolonin, 2007), and has been implicated in the spread of Lyme disease in the Pacific Northwest (Damrow *et al*., 1989; Banerjee *et al*., 1994; Eisen *et al*., 2006). It is also a known vector of the bacterial pathogen, *Babesia microti* (Fay & Rausch, 1969; Goethert *et al*., 2006). The other tick species found on

small mammals in KNP, *D. andersoni*, is also a vector of several human and animal pathogens (Burgdorfer, 1975; Gordon *et al.,* 1983; Foley & Nieto, 2010; Kocan *et al.,* 2010). Therefore, the ability to accurately identify ticks, determine the hosts they parasitize, and establish if they are expanding their distributional ranges, are important components in understanding the ecology of vector-borne diseases.

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Chapter 5 An assessment of genetic differences among ixodid ticks in a locus within the nuclear large subunit ribosomal RNA gene[4]

5.1 Abstract

We examined the usefulness of the D3 domain and flanking core regions (=$D3^+$) of the nuclear large subunit (LSU) ribosomal DNA as a genetic marker for species-level identification and the inference of evolutionary relationships of ixodid ticks. Genetic variation was also examined in relation to the secondary structure of the LSU rDNA. The results revealed a lack of sequence difference in the $D3^+$ among species of *Dermacentor* and among some species of *Ixodes*, demonstrating that this gene region is not suitable as a species marker for all species of ixodid ticks. Of the 45 variable nucleotide positions in the sequence alignment of the $D3^+$, 23 did not alter the secondary structure of the LSU rDNA, because they occurred in unpaired positions, whereas 16 represented partial or full compensatory changes, which maintained the secondary structure. Six deletions in the $D3^+$ sequence of all *Ixodes* species examined resulted in a shorter d4_1 helix compared with that of other tick species. The results of the phylogenetic analyses also showed that the $D3^+$ is of limited value in resolving evolutionary relationships among ixodid ticks. In addition, we also demonstrated that the $D3^+$ of ascomycete fungi could also be amplified along with, or instead of, the $D3^+$ of some tick species depending upon the primers used in PCR. Nonetheless, the $D3^+$ of the fungal contaminants are readily distinguished from the $D3^+$ of ixodid ticks because of a shorter length and the absence of helix d4_1 in the secondary structure of the LSU rDNA.

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5.2 **Introduction**

Ticks are important vectors of human and animal pathogenic agents (e.g., viruses, bacteria and protozoa) in different regions of the world (Estrada-Peña & Jongejan, 1999). The accurate identification of individual ticks to the species level is an important requirement for the establishment of effective programs aimed at controlling and managing tick populations, and for the treatment of diseases caused by tick-borne pathogens. However, it is sometimes difficult to unequivocally identify ticks at all life cycle stages to species because of morphological similarities among closely related species (Andrews *et al*., 1992; Jackson *et al*., 2000; Anderson *et al*., 2004; Andrews *et al*., 2006). This is particularly the case for immature stages (i.e., larvae and nymphs), adult females having fed on hosts, and ticks that are damaged following their removal from a host (Andrews *et al*., 1992; Jackson *et al*., 2000; Anderson *et al*., 2004; Andrews *et al*., 2006). Therefore, the geographical localities from which ticks are collected are sometimes used to aid in their identification. This approach however, can be problematic when morphologically similar species occur in sympatry (Jackson *et al*., 2000). As a consequence, biochemical and molecular techniques have been developed to identify ticks (Andrews *et al*., 1992; Poucher *et al*., 1999; Jackson *et al*., 2000; Anderson *et al*., 2004; Andrews *et al*., 2006).

Several nuclear and mitochondrial DNA genes provide useful genetic markers for the identification of individual ticks to the species-level, irrespective of life cycle stage or their state of engorgement (Poucher *et al*., 1999; Anderson *et al*., 2004; Guglielmone *et al*., 2006; Dergousoff & Chilton, 2007; Mtambo *et al*., 2007). These markers have also been used to study the population genetics of ticks and to infer their evolutionary relationships (Black & Piesman, 1994; Klompen *et al*., 2000; Qiu *et al*., 2002; Guglielmone *et al*., 2006; Dergousoff & Chilton, 2007; Krakowetz *et al*., 2010). The D3 (divergent) domain or expansion segment of the nuclear

large subunit (LSU) ribosomal RNA gene has been used as a marker for phylogenetic studies on a variety of arthropods, including chelicerates (Wheeler & Hayashi, 1998), mites (Maraun *et al*., 2004), and ticks (McLain *et al*., 2001). The D3 domain has also been used to compare blacklegged ticks (*Ixodes scapularis*) from different parts of their distributional range (Qiu *et al*., 2002) and as a species marker for some orbatid mites (Maraun *et al*., 2003). Previous studies of ixodid ticks (McLain, 2001; McLain *et al*., 2001) have demonstrated that there are marked differences in the D3 sequences of six species of *Ixodes*, suggesting that this DNA region is a suitable species marker and that it would be useful for phylogenetic studies on ticks. In the present study, we explored the nature and extent of the sequence variation within and among several species of ixodid tick representing the two major subfamilies, the Prostriatia and Metastriata. Nucleotide alterations in the DNA sequences of the D3 domain and flanking regions of the nuclear LSU rRNA gene (=$D3^{+}$) were also examined in relation to the ribosomal RNA secondary structure. Furthermore, we assessed the utility of this region of the LSU to infer the phylogenetic relationships of ixodid ticks.

5.3 **Materials and Methods**

Total genomic DNA (gDNA) was extracted and purified from 104 individual ticks, representing 10 species (*Ixodes angustus*, *n* = 8; *I. kingi n* = 12; *I. ricinus*, *n* = 4; *I. scapularis*, *n* = 77; *I. sculptus*, *n* = 12; *Dermacentor andersoni*, *n* = 4; *D. albipictus*, *n* = 4; *D. variabilis*, *n* = 7; *D. occidentalis*, *n* = 1; and *Rhipicephalus sanguineus*, *n* = 1), using the methods described previously (Dergousoff & Chilton, 2007). The $D3^{+}$ was amplified from gDNA by PCR using the forward (5'-GTGAATTCACCCGTCTTGAAACAC-3') and reverse primers (5'-GTGGATCCTGAGGGAAACTTCGG-3') of McLain *et al*., (2001). Reactions were performed

in 25 µl volumes containing 1-2 µl of gDNA, 250 µM of each dNTP, 3.5 mM $MgCl_2$, 25 pmol of each primer and 0.5 U of *Taq* polymerase (Biorad). The PCR conditions used were 95°C for 5 min, then 30 cycles of 95°C for 30 sec, 48°C for 30 sec and 74°C for 30 sec, followed by 74°C for 5 min. No template (i.e., negative) controls were included with each set of PCR reactions. Individual amplicons were compared on SYBR-safe (Molecular Probes) stained 1.5% agarose-TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3; EMD Biosciences) gels. In addition, single-strand conformation polymorphism (SSCP) analyses were conducted to screen for genetic variation within species. SSCP was performed using the methods described previously (Gasser *et al*., 2006; Dergousoff & Chilton, 2007; Krakowetz *et al*., 2010).

Representative amplicons were either purified using spin columns (MinElute PCR purification kit, Qiagen) or ExoSap-IT (GE Healthcare), and subjected to automated DNA sequencing using the forward and reverse primers in separate reactions. All DNA sequences were compared with sequence data on GenBank (using BLAST). Sequences were aligned manually. Variable positions in the $D3^+$ sequence alignment were examined in relation to the secondary structure of the LSU rRNA based on the model of Wuyts *et al*., (2001). Phylogenetic analyses were conducted on the sequence data using the neighbour-joining (NJ) and maximum parsimony (MP) methods in PAUP (Swofford, 1999). The $D3^+$ sequence of the holothyroid mite, *Allothyrus* cf. *constrictus* (GenBank accession number AY626629), was used as the outgroup in the analyses. For the MP analysis, gaps were treated as a fifth character state, and all character states were assigned an equal value. The consistency index (CI) excluding uninformative characters and the retention index (RI) were recorded for the most parsimonious trees.

5.4 **Results and Discussion**

The $D3^+$ amplicons produced from the majority of tick gDNA samples were ~380 bp in size on agarose gels; however, smaller-sized amplicons (~330 bp) were produced from the gDNA of six *Ixodes sculptus* and seven *I. kingi* (Fig. 5.1A). In addition, the amplicons of three *I. sculptus* and two *I. kingi* individuals had two bands (~330 and 380 bp) on an agarose gel (Fig. 5.1A). The sequences of the 380 bp amplicons from the *I. sculptus* and *I. kingi* gDNA were 99% similar to the LSU rDNA of a related species, *I. cookei* (accession number AY62631; Klompen *et al.,* 2007). In contrast, a BLAST search of the sequences of the 330 bp amplicons from *I. sculptus* and *I. kingi* revealed that they were identical to the LSU sequences of ascomycete fungi (accession numbers AB470555 and FJ567949, respectively). Amplification of the LSU rDNA of fungal contaminants from invertebrate gDNA samples is often a problem, given the relatively high genetic similarity in the sequences and secondary structure for many regions of the LSU rRNA gene among distantly related organisms, particularly in the core regions of the rDNA (Wuyts *et al.,* 2001). The sequence of the regions flanking the D3 domain of the ascomycete fungus associated with *I. kingi* was 85% similar (i.e., 28 bp differences over 183 alignment positions) to that of *I. kingi*, but only 49% similar for the D3 domain (i.e., 82 bp differences over 160 alignment positions). There were 61 point mutations (37 transitions and 24 transversions) and 49 indels when comparing the $D3^+$ sequences of *I. kingi* and its associated fungus. The relatively shorter D3 domains of the two fungal species compared with those of *I. kingi* and *I. sculptus* were evident based on the absence of stem d4_1 and a reduced d5 stem (Fig. 5.2).

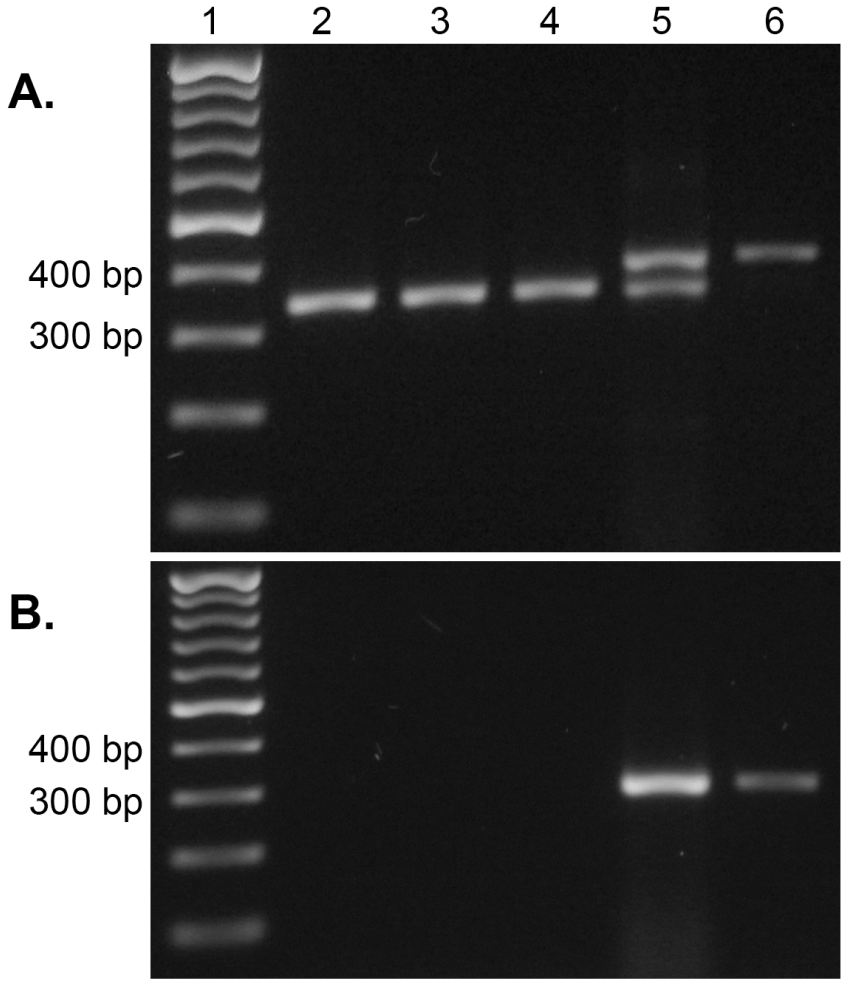

Fig. 5.1 (A) An agarose gel displaying the amplicons produced by PCR from gDNA of individual *I. kingi* (lanes 2 to 6) using primers reported by McLain *et al.,* (2001). Amplicons of ~380 bp and ~330 bp are those of the D3^{+} LSU of ticks and fungi, respectively. (B) Agarose gel of the amplicons produced by PCR of the same gDNA samples, but using primers Tick-28S-C2-F and Tick-d9-D3-R (designed herein). Amplicons of ~300 bp are those of the D3^{+} LSU of ticks, and not fungi. A 100 bp TrackIt™ DNA ladder (Invitrogen) was used as a size standard on both gels (lane 1).

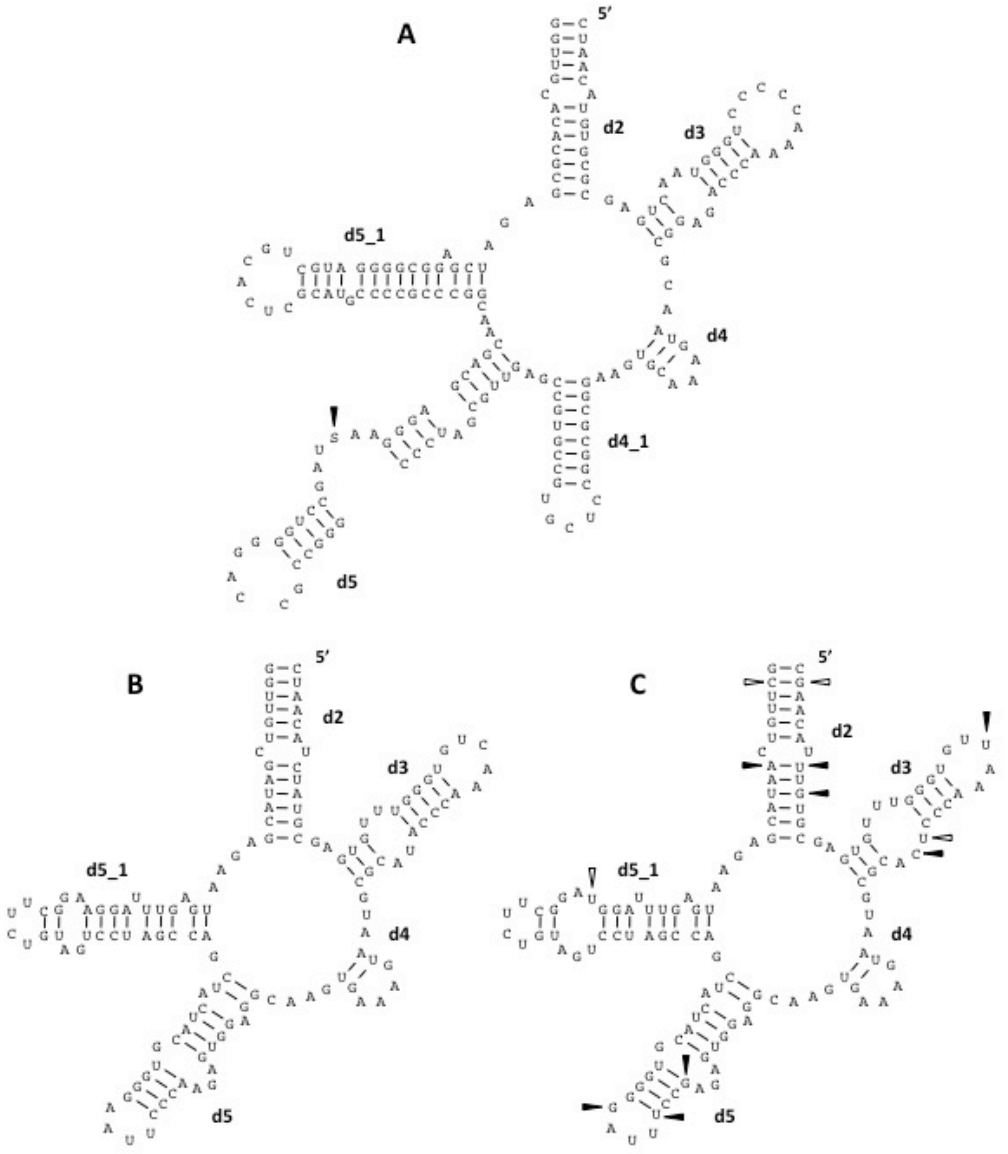

Fig. 5.2 The secondary structures of the D3 region of the LSU rRNA gene for (A) *Ixodes kingi* and *Ixodes sculptus* (solid arrow indicating the interspecific difference), and the ascomycete fungi associated with gDNA samples of (B) *I. kingi* and (C) *I. sculptus*. Helices are numbered (d2 to d5_1) according to the model of Wuyts and co-workers (2001). Closed and open arrows on the secondary structure of the *I. sculptus* associated fungus indicate the transitional and transversional sequence differences (respectively) compared with the *I. kingi* associated fungus.

As a consequence of the fungal contamination in some amplicons, two new primers, Tick-28S-C2-F (5'-GCGGCGAGTAGGTCGGTAACC-3') and Tick-d9-D3-R (5'-ACGTCAGAATCGCTTCGGA-3'), were designed to amplify the D3$^+$ of ticks (and other arthropods), but not the D3$^+$ of fungi. These primers were tested using the same PCR conditions as specified above, except that the annealing temperature was raised to 60 °C. No amplicons were produced for the fungal contaminants present in the tick gDNA (Fig. 5.1B), whereas the positive amplicons were confirmed to be the D3$^+$ of *I. kingi* and *I. sculptus* by DNA sequencing.

There was no evidence of amplification of fungal LSU rDNA from the gDNA of the other tick species following amplification with forward and reverse primers described by McLain *et al.,* (2001) (or with primers Tick-28S-C2-F and Tick-d9-D3-R) based on amplicon size and BLAST searches of the sequence data produced. For *Dermacentor*, no intraspecific variation was detected in the D3$^+$ sequences of *D. variabilis* collected from geographically isolated populations in Saskatchewan, Canada (n = 4) and California, U.S.A. (n = 3), or among multiple individuals of *D. albipictus* and *D. andersoni*. In addition, there were no interspecific differences in D3$^+$ sequences among representative individuals of the four species of *Dermacentor* (Table 5.1). The sequences of these ticks were also identical to that for a *Dermacentor* (indeterminate species) collected from a dog in Ewartsville, Washington, U.S.A. (accession number AY859582; Mallatt & Giribet, 2006). The D3$^+$ sequence determined for a single adult of *R. sanguineus* was identical to that of the published sequence for *R. sanguineus* (accession number AF062986; Wheeler & Hayashi, 1998), except for two transitional changes (i.e., C's compared to Y and T in the sequence with accession number AF062986 at nucleotide positions 111 and 343; Fig. 5.2).

Species	39	41	43	51	52	74	76	77	79	81	83	85	86	88	91	93	107	112	120	122	123	124	125	126	127	131	146	150	151	152	157	161	162	167	168	176	177	179	183	267	294	296	297	316	343
I. kingi	C	C	A	G	A	G	G	G	C	C	T	C	C	T	C	A	G	C	C	G	A	T	C	A	A	A	C	G	T	A	–	–	–	T	A	A	G	T	–	A	C	C	A	T	C
I. sculptus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	–	–	–	.	.	.	.	.	–	.	.	.	.	.	.
I. angustus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	–	–	–	.	.	.	.	.	–	.	.	.	.	.	.
I. cookei[a]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	–	–	–	.	.	.	.	.	–	.	T	.	G	C	T
I. scapularis	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	A	C	.	–	–	–	.	G	C	.	.	–	C	.	.	.	.	.
I. ricinus	.	T	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	G	.	.	.	.	A	C	.	–	–	–	.	G	C	.	.	–	C	.	.	.	.	.
D. albipictus	T	T	G	A	T	–	–	–	.	T	C	–	–	–	T	C	A	A	.	.	.	A	A	.	.	.	T	A	G	G	–	T	C	C	C	.	.	G	–	.	.	T	G	C	.
D. andersoni	T	T	G	A	T	–	–	–	.	T	C	–	–	–	T	C	A	A	.	.	.	A	A	.	.	.	T	A	G	G	–	T	C	C	C	.	.	G	–	.	.	T	G	C	.
D. occidentalis	T	T	G	A	T	–	–	–	.	T	C	–	–	–	T	C	A	A	.	.	.	A	A	.	.	.	T	A	G	G	–	T	C	C	C	.	.	G	–	.	.	T	G	C	.
D. variabilis	T	T	G	A	T	–	–	–	.	T	C	–	–	–	T	C	A	A	.	.	.	A	A	.	.	.	T	A	G	G	–	T	C	C	C	.	.	G	–	.	.	T	G	C	.
R. sanguineus	T	.	G	A	T	–	–	–	.	A	.	–	–	–	T	C	A	A	.	.	.	.	A	.	.	C	T	A	G	G	–	A	C	C	C	.	.	G	–	.	T	.	G	C	.
A. americanum[b]	T	T	G	A	T	–	–	–	T	.	C	–	–	–	.	C	A	–	T	–	–	–	–	–	–	C	T	A	G	G	T	T	T	C	C	.	A	G	A	.	T	.	G	C	.
Outgroup (mite)																																													
Allothyrus[c]	.	G	T	C	.	–	.	A	.	.	G	T	.	–	T	C	.	A	.	.	–	–	–	–	–	C	.	A	A	G	–	–	T	.	T	.	.	A	–	.	.	T	G	.	.

GenBank accession numbers: a = AY62631, b = AF291874 and c = AY626629

Table 5.1 Variable nucleotide positions in the aligned D3[+] sequences of 12 species of ixodid tick (Family Ixodidae). A dot indicates the same nucleotide as in the sequence of *I. kingi*. Nucleotide positions 25-184 are located in the D3 domain, while positions 1-24 and 185-344 are located in the flanking regions (see Fig. 5.4).

The D3^{+} sequences of all five *Ixodes* species (340 bp) determined in the present study were 4 bp longer than those of *R. sanguineus* and the four *Dermacentor* species (i.e., 336 bp). SSCP profiles for 78 *I. scapularis* adults collected from Manitoba and Nova Scotia in Canada (*n* = 1 and 3, respectively) and Minnesota in U.S.A. (*n* = 74) were the same (not shown), but they differed from those of other *Ixodes* species (Fig. 5.3). The lack of variation in the SSCP profiles among *I. scapularis* adults inferred an absence of intraspecific variation in the D3^{+} sequence. This inference was supported by the DNA sequencing results for five representative *I. scapularis* adults. The D3^{+} sequences of these ticks were identical to one another (340 bp), but differed to the D3^{+} sequences of the four *I. ricinus* individuals at a single nucleotide position (i.e., alignment position 88) and at 4-10 nucleotide positions when compared with the other *Ixodes* species (Table 5.1). Except for *I. angustus* and *I. sculptus*, which were identical in sequence to one another, all *Ixodes* species examined had a different D3^{+} sequence. The present sequence results for representatives of two genera, *Ixodes* and *Dermacentor*, show that the D3 region is not a suitable genetic marker for the identification of all ixodid ticks to the species-level.

The magnitude of sequence differences in the D3 domain (i.e., 0-5%, excluding flanking regions) among the five species of *Ixodes* examined herein (i.e., *I. angustus*, *I. kingi*, *I. ricinus*, *I. scapularis* and *I. sculptus*) was significantly less than the 11-32% sequence differences reported previously in studies of *Ixodes* (McLain, 2001; McLain *et al.,* 2001), which also included *I. scapularis* and *I. ricinus*. The sequences determined for *I. scapularis* and *I. ricinus* also differed significantly (i.e., at 15% and 11% of 192 and 188 alignment positions, respectively) to the published sequences for these species (accession numbers AF303987 and AF303988, respectively; McLain, 2001; McLain *et al.,* 2001). Moreover, the D3 and flanking sequences of *I. scapularis* and *I. ricinus* from McLain *et al.,* (2001) differed from one another at 30 of 192

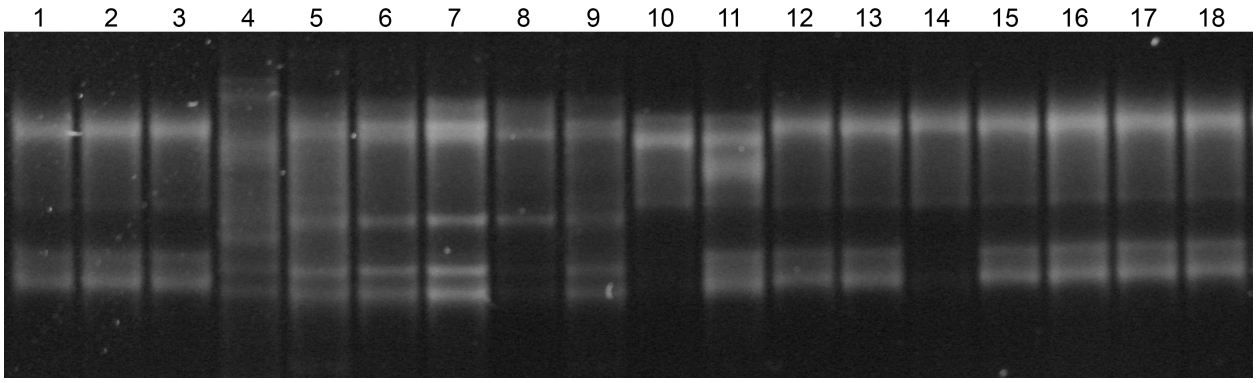

Fig. 5.3 Single-strand conformation polymorphism (SSCP) profiles of the $D3^{+}$ LSU rDNA for individual adults of *Ixodes angustus* (lanes 1-3 and 15-18), *I. ricinus* (lane 4), *I. scapularis* (lanes 5-9), *I. kingi* (lanes 10 and 11) and *I. sculptus* (lanes 12-14).

(22%) alignment positions, whereas only a single nucleotide difference was detected between these two species (Table 5.1). Given the differences in sequence results between studies, two internal primers (forward: 5'-TGAGGCGAATGAAACGCC-3' and reverse: 5'-TCTAGCTAGCTCACGTCG-3') were designed based on the published sequence for *I. scapularis* (i.e., accession number AF303987) by McLain *et al.,* (2001). However, no amplicons were detected on agarose gels following PCR of the gDNA of *I. scapularis* adults using these internal primers together or in combination with forward and reverse primers described by McLain *et al.,* (2001). In addition, BLAST searches of the previously published D3 sequences of *I. scapularis* and *I. ricinus* (McLain *et al.,* 2001) showed very little genetic similarity to the LSU sequences of any arachnid, whereas the $D3^{+}$ sequences determined for these two tick species were 81-87% similar to those of a variety of arachnid species. As a consequence of these findings, we did not include any sequences from McLain *et al.,* (2001) in our determination of the nucleotide alterations in the secondary structure of the tick $D3^{+}$ rRNA (Fig. 5.4) or in the phylogenetic analyses (Fig. 5.5).

The results of the present study revealed that the magnitude of sequence differences in $D3^{+}$ among tick species within a genus (i.e., 0% for *Dermacentor* and 0-3% for *Ixodes*) was less than that among genera (2-11%) (see Table 5.1). This finding is consistent with the reported genetic differences in the D3 region among orbatid mites of the same genus (0-6%; Maraun *et al.,* 2003) and the more substantial sequence differences among genera of orbatid mites (Maraun *et al.,* 2004). There were 45 variable nucleotide positions in the $D3^{+}$ among tick species, 39 of which were detected in the D3 domain. The nucleotide differences represent 25 point mutations (19 transitions and 6 transversions), 11 indels, and 9 multiple changes (i.e., indel, transition and/or transversion). With respect to the secondary structure of the $D3^{+}$ rRNA (Fig. 5.4),

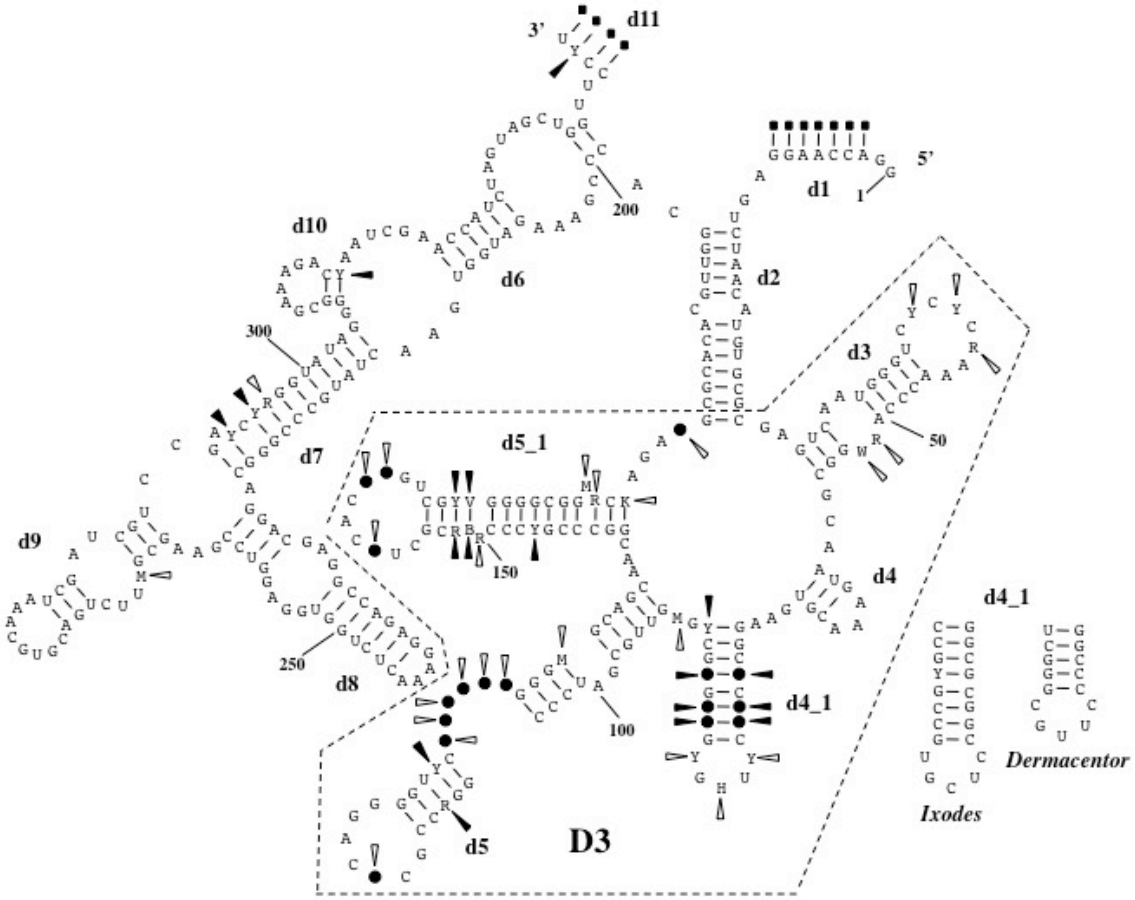

Fig. 5.4 Variable nucleotide positions in the D3 domain and flanking regions ($D3^{+}$) of the LSU rRNA gene for 12 species of ixodid tick (see Table 1). Solid arrows indicate partial or complete compensatory nucleotide alterations that maintain base pairing of stems. Open arrows indicate nucleotide alterations of unpaired positions (e.g., loops and bulges) or partial compensatory nucleotide alterations that do not maintain the base pairing on stems. Solid circles represent indels, while solid squares represent nucleotide positions from other regions of the LSU rDNA that are involved in base pairing with the $D3^{+}$ (Wheeler & Hayashi, 1998). Helices are numbered (d1-d11) according to the model of Wuyts and co-workers (2001).

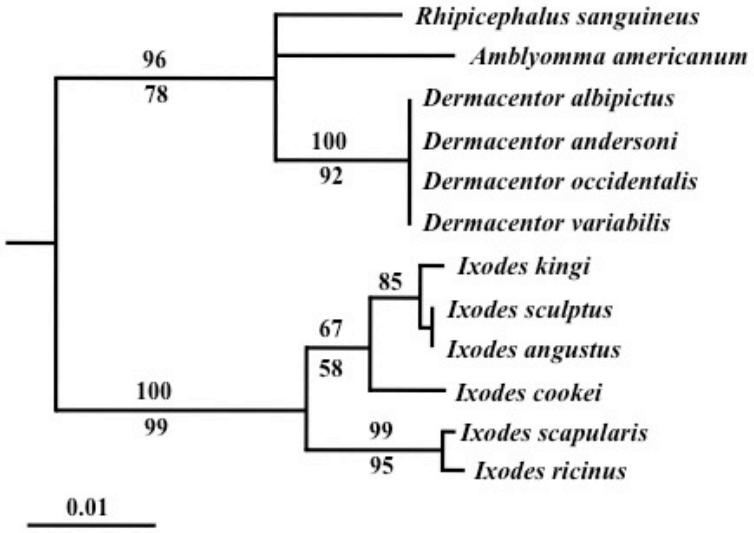

Fig. 5.5 Phylogenetic relationships of the 12 species of ixodid tick inferred from a neighbour-joining (NJ) analysis of sequence data of the $D3^{+}$ of the LSU rRNA gene. Values above and below branches are the bootstrap support (based on 1,000 replications) for NJ and MP analyses, respectively. Sequence data of the mite *Allothyrus* cf. *constrictus* was used as the outgroup for the NJ and MP analyses.

approximately half of the nucleotide alterations (n = 23) occurred in unpaired positions on stems or in the end loops of stems. Eight transitional changes (positions 91, 107, 120, 146, 249, 296, 316 and 343) represented partial compensatory changes which maintained the secondary structure. Nucleotide alterations at four positions (151, 152, 167 and 168) represented complementary changes on both sides of helix d5_1. The six indels (positions 74, 76, 77, 85, 86 and 88) in the sequence of all *Ixodes* species represented full compensatory base pair changes on helix d4_1, resulting in a longer helix for this part of the D3 domain compared with the other tick species (Fig. 5.4).

The phylogenetic tree produced from the NJ analyses of the $D3^{+}$ sequences (Fig. 5.5) resulted in the separation of the 12 tick species into two major groups (i.e., clades); one containing the six species of *Ixodes*, and the other including representatives of the genera *Amblyomma*, *Dermacentor* and *Rhipicephalus*. There was very strong statistical support (i.e., bootstrap values of 96-100%) for each of these clades. Within the genus *Ixodes*, there was strong support (bootstrap value: 99%) for a sister taxon relationship between *I. scapularis* and *I. ricinus*, and for a clade (bootstrap value: 85%) that included *I. kingi*, *I. sculptus* and *I. angustus*. Of the 45 variable positions in the $D3^{+}$ sequences of the 12 ixodid tick species (Table 5.1), 38 were informative in the maximum parsimony (MP) analysis. These analyses produced two equally most parsimonious trees (L = 100, CI = 0.81 and RI = 0.91) (not shown). As in the NJ analyses, there was strong bootstrap support for monophyly of species within the genus *Ixodes*, and for a sister taxon relationship between *I. scapularis* and *I. ricinus*. Both *I. scapularis* and *I. ricinus* belong to the subgenus *Ixodes*, whereas the other four *Ixodes* species belong to different subgenera (i.e., *Ixodiopsis* and *Pholeoixodes*). As in the NJ tree, there was no resolution of the relationships among species of the other genera (i.e., *Dermacentor*, *Amblyomma*, and

Rhipicephalus) in the consensus MP tree. The placement of *Ixodes* into a different clade from the other three genera is consistent with the separation of the Ixodidae into the subfamilies Prostriata and Metastriata (respectively) (Hoogstraal & Aeschlimann, 1982), and the findings of other molecular studies that have examined the evolutionary relationships of ixodid ticks (Black & Piesman, 1994; Klompen *et al*., 2000).

In conclusion, the $D3^{+}$ region of the LSU rDNA is not suitable as a species marker for all species of ixodid ticks because of a lack of sequence differences among some species of *Ixodes*, and among the four species of *Dermacentor* examined in the present study. This gene region however, is of some use for examining the evolutionary relationships of different genera of ixodid ticks.

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Chapter 6: Comparison of the partial sequences and secondary structures of the mitochondrial 16S rRNA gene of *Ixodes angustus*, *I. kingi* and *I. sculptus*

6.1 Abstract

The sequences and secondary structure of the 3' region of the mitochondrial 16S rRNA gene were compared for three species of ixodid tick, *Ixodes angustus, I. kingi* and *I. sculptus* that are commonly found on small mammals in western Canada. The evolutionary relationships of these tick species were examined in relation to other species within the genus using sequence alignments based on the predicted secondary structure. The results revealed that *I. angustus, I. kingi* and *I. sculptus* were easily distinguishable from one another based on differences in their 16S sequences, despite existing intraspecific variation in DNA sequence within a tick species. The secondary structure of this gene was similar for the three tick species because many of the mutational changes in DNA sequence occurred at unpaired positions or represented partial or full compensatory base pair changes. Many of the interspecific differences in DNA sequence occurred within a hypervariable region of the 3' end of the 16S rRNA gene. The results of the phylogenetic analyses showed that *I. angustus, I. kingi* and *I. sculptus* formed a clade that contained members of two subgenera, the *Pholeoixodes* and *Ixodiopsis*. In addition, the results supported the current view that *I. kingi* and *I. sculptus* are more closely related to one another (both members of the *Pholeoixodes*) than either species is to *I. angustus* (a member of the *Ixodiopsis*). One interesting result was that the *I. sculptus* individuals collected from Beechy, Saskatchewan (Canada) did not form a monophyletic clade with a single *I. sculptus* individual collected from Fort Collins, Colorado (U.S.A.). The significance of this finding is discussed.

6.2 **Introduction**

Accurate identification of individual ticks to the species level, irrespective of their life cycle stage, is critical, as some species are vectors of disease causing agents (i.e., bacteria, protozoa and viruses) to humans, domestic animals and wildlife (Parola & Raoult 2001; Swanson *et al.,* 2006). However, it is sometimes difficult to unequivocally identify ticks, particularly engorged individuals, to the species level by morphological examination. Therefore, a variety of PCR-based techniques are now commonly used as alternatives or adjuncts to morphological examination in the species identification of ticks (e.g., Andrews *et al.,* 1992; Poucher *et al.,* 1999; Jackson *et al.,* 2000; Anderson *et al.,* 2004; Mtambo *et al.,* 2007; Anstead *et al.,* 2011). Molecular approaches overcome the difficulties associated with morphological similarities of some closely related species, the engorgement state of ticks, or the lack of morphologically informative characters (e.g., mouthparts) as a consequence of damage or loss when ticks are removed from hosts.

Mitochondrial (mt) DNA genes and nuclear DNA regions have been frequently used as the targets in PCR-based assays for tick identification (e.g., Poucher *et al.,* 1999; Beati & Keirans, 2001; Anderson *et al.,* 2004; Guglielmone *et al.,* 2006; Dergousoff *et al.,* 2007; Mtambo *et al.,* 2007; Anstead *et al.,* 2011; Tian *et al.,* 2011). However, not all genes have the same rate of molecular evolution, and thus differ in their relative use for examining questions relating to the systematics (i.e., taxonomy, nomenclature and phylogeny) of ticks. For example, the D3 domain and flanking core regions of the nuclear 28S (large subunit) rRNA gene has been used as a species marker and/or to infer phylogenetic relationships in some invertebrates (Wheeler & Hayashi, 1998; McLain, 2001; McLain *et al.,* 2001; Maraun *et al.,* 2003; Maraun *et al.,* 2004), whereas this region is not useful to distinguish among all species of *Dermacentor* or

Ixodes, but has potential for examining higher level phylogenetic relationships among genera of ixodid ticks (Chapter 5). In contrast, the 16S rRNA gene, which encodes the mt large subunit (LSU) in animals, has been used extensively to explore phylogenetic relationships of ticks spanning from the family level to the genus level and below (e.g., Black & Piesman 1994; Caporale *et al.,* 1995; Norris *et al.,* 1996, 1997; Klompen *et al.,* 2000; Qiu *et al.,* 2002; Guglielmone *et al.,* 2004, 2006; Krakowetz *et al.,* 2010, 2011; Anstead & Chilton 2011; Tian *et al.,* 2011). The wide applicability of the 16S rRNA gene to address questions at different taxonomic ranks within the Ixodidae suggests different rates of evolutionary change in different parts of this gene, which may be strongly influenced by functional and structural constraints (Lopez *et al.,* 1997; Misof *et al.,* 2002; Smit *et al.,* 2007).

The aim of the present study was to compare the sequences of the 3' region of the 16S rRNA gene among three species of ixodid tick, *Ixodes angustus, I. kingi* and *I. sculptus*, that are commonly found on small mammals in western Canada (Bishopp & Trembley, 1945; Cooley & Kohls, 1945; Brown & Kohls, 1950; Gregson, 1956), and to examine where the mutational changes in DNA sequence both within and among the tick species occur relative to the secondary structure of the mt LSU. The evolutionary relationships of these three tick species to other species within the genus *Ixodes* were also explored using sequence alignments based on the predicted secondary structure.

6.3 **Materials and Methods**

The secondary structures of the 3' end of the mt 16S rRNA gene were determined, based on the secondary structure model of Gutell and co-workers (Gutell & Fox 1988; Gutell *et al.,* 1993; Gutell, 1996), for the different haplotypes of *Ixodes angustus, I. kingi* and *I. sculptus*

described in our recent studies of ticks on small mammals in western Canada (Chapters 2-4). In addition, new sequence data were included in the present study for the mt 16S rRNA gene of six *I. kingi* adults collected from cats and dogs at different localities in Saskatchewan (Table 6.1). Genomic DNA (gDNA) was extracted and purified from the complete bodies of these ticks (Dergousoff & Chilton, 2007; Anstead *et al.,* 2013). The 3' end (413-415 base pairs) of the mt 16S rRNA gene was then amplified from gDNA by PCR using primers 16S-1 (5'-CCACAGCAATTTAAAAAATCATTGAGCAG-3') and 16S+1 (5'-CCGGTCTGAACTCAGATCAAGT-3') (Norris *et al.,* 1996) and the conditions described previously (Krakowetz *et al.,* 2010). Also included were negative (i.e., no gDNA) control samples. Amplicons were either purified using spin columns (MinElute PCR purification kit, Qiagen) or ExoSap-IT (GE Healthcare), and subjected to automated DNA sequencing using primers 16S-1 and 16S+1 in separate reactions.

All sequences were initially aligned manually and then modified based on the secondary structure. Each nucleotide position in the sequence alignment was assigned to one of four structural functions: stem (= nucleotides involved in base pairing), end loop (= unpaired nucleotides at the end of a stem), internal loop (= unpaired nucleotides that occur within a stem) and connecting region (= unpaired nucleotides that link stems). Contingency tests (χ^2) were performed to test if there were any significant differences between the proportion of variable positions among the four structural categories, and between paired and unpaired positions of the secondary structure of the 16S rRNA gene.

Phylogenetic analyses were performed on the aligned sequence data using the neighbour joining (NJ) and maximum parsimony (MP) methods in PAUP (Swofford, 1999). Included in the analyses were sequences for other representatives of the genus *Ixodes* (Table 6.2). For the MP

Sample code	Collection locality	Host	16S haplotype
SK10-1	20 km E of Maple Creek	Dog	KH-1
SK10-164G	Vanguard	Dog	KH-4
SK10-175	Unity	Dog	KH-1
SK10-200	Swift Current	Cat	KH-1
SK10-202	Swift Current	Dog	KH-1
SK12-237	Saskatoon	Cat	KH-1

Table 6.1 The mt 16S rDNA haplotype identities of six *Ixodes kingi* females collected from dogs and cats at different localities within Saskatchewan, Canada.

Species	Subgenus	GenBank Accession nos.
Ixodes angustus	*Ixodiopsis*	HF912727- HF912731
Ixodes woodi	*Ixodiopsis*	AF549843
Ixodes arboricola	*Pholeoixodes*	JF791812
Ixodes banksi	*Pholeoixodes*	U95881
Ixodes cookei	*Pholeoixodes*	U95883
Ixodes dampfi	*Pholeoixodes*	AF549837
Ixodes hexagonus	*Pholeoixodes*	AF549844
Ixodes kingi	*Pholeoixodes*	HF912422, HF968622-HF968624
Ixodes sculptus	*Pholeoixodes*	HF968625
Ixodes acutitarsus	*Ixodes*	AB105167
Ixodes granulatus	*Ixodes*	DQ093309
Ixodes jellisoni	*Ixodes*	AF549849
Ixodes muris	*Ixodes*	U95896
Ixodes ricinus	*Ixodes*	GU074610, JF928513, *
Ixodes pacificus	*Ixodes*	AF549854
Ixodes pavovskyi	*Ixodes*	AF549835
Ixodes persculatus	*Ixodes*	AF549856
Ixodes scapularis	*Ixodes*	AF309023, FR854227-FR854232
Ixodes luciae	*? Ixodes*	AF549851
Ixodes vespertilionis	*Eschatocephalus*	U95910
Ixodes brunneus	*Trichotoixodes*	AF549836
Ixodes frontalis	*Trichotoixodes*	AF549839
Outgroup		
Haemaphysalis cretica		L34308
Rhipicephalus appendiculatus		L34301

* = Anstead, unpublished sequences

Table 6.2 Subgenera classification and GenBank accession numbers of sequences of the mt 16S rRNA gene belonging to *Ixodes* species.

analyses, characters were treated as unordered and were equally weighted, and alignment gaps were treated as 'missing' characters. Heuristic searches with TBR branch swapping were used to infer the shortest trees. The lengths, consistency indices (excluding uninformative characters), and the retention indices of the most parsimonious trees were recorded. The sequences of *Haemaphysalis cretica* and *Rhipicephalus appendiculatus* (Black & Piesman, 1994), members of the Metastriata (i.e., the sister group to the Prostriata; Black & Piesman, 1994), were used as outgroups in the MP analyses. Bootstrap analyses (i.e., 1000 replicates for the NJ analyses and 100 replicates for MP analyses) were conducted to determine the relative support for clades in the consensus trees.

6.4 **Results**

A single band (~ 450 bp) was detected on TBE-agarose gels for the 16S amplicons of the six *I. kingi* females collected from cats and dogs in Saskatchewan. No amplicons were produced from the negative control samples. Two different 16S haplotypes were detected among the six *I. kingi* females (Table 6.1) one of which (haplotype KH-4) has not been previously reported. The sequence of this haplotype differed from those of the other three haplotypes of *I. kingi* from western Canada at 1-2 nucleotide positions (i.e., alignment positions 102, 173 and 184; Fig. 6.1).

The secondary structure of the 3' end of the mt 16S rRNA gene for the different haplotypes of the *I. kingi*, *I. sculptus* and *I. angustus* are shown in Fig. 6.2. The three variable alignment positions in the sequences of the four *I. kingi* haplotypes (Fig. 6.1) represented one indel (i.e., position 102), one purine transitional change (i.e., position 173), and one pyrimidine transitional change (i.e., position 184). The indel occurred in an unpaired region of the secondary

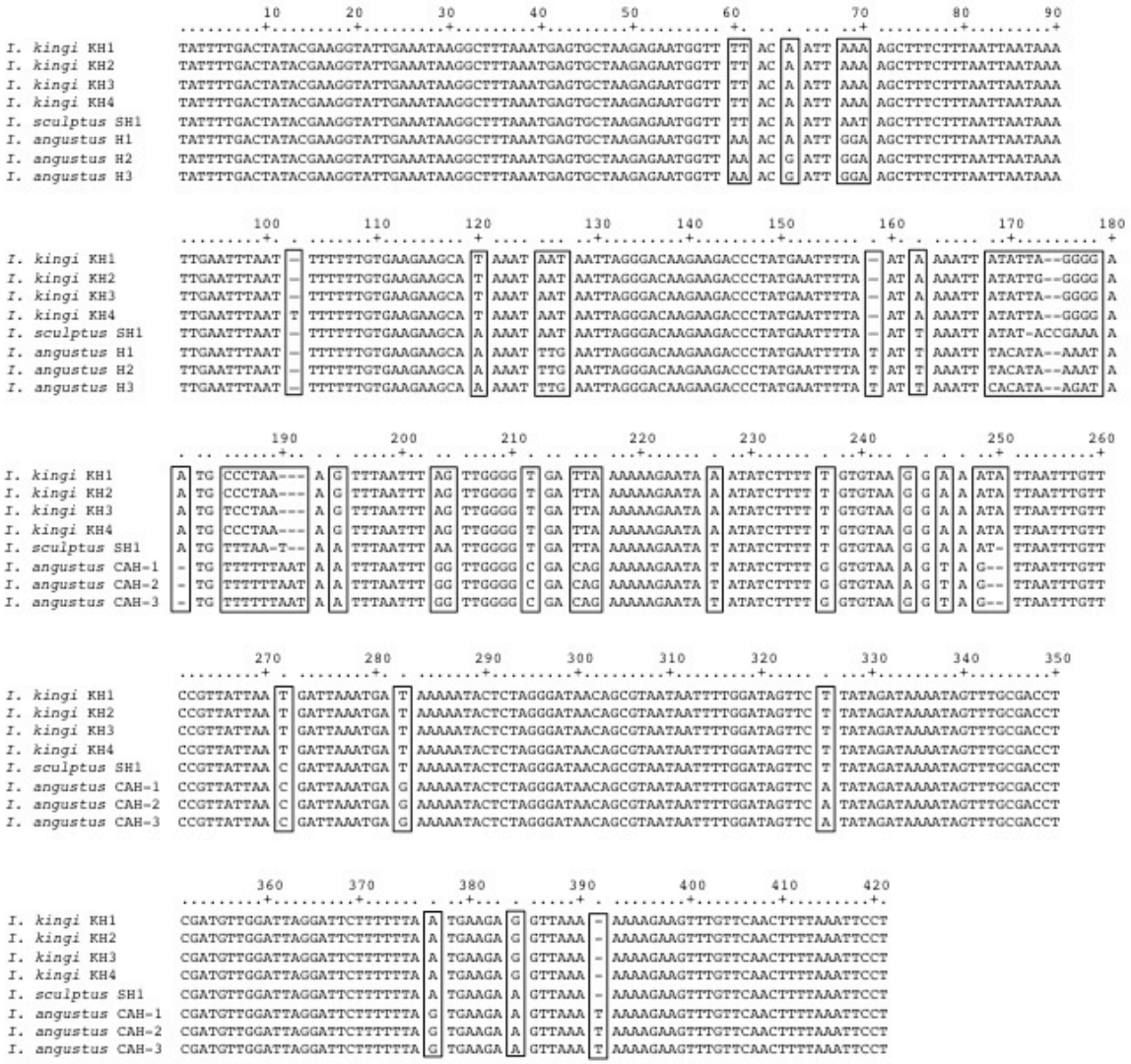

Fig. 6.1 Alignment of the partial mt 16S rRNA gene sequences of the different haplotypes of *Ixodes kingi*, *I. sculptus* and *I. angustus* in western Canada. Boxes indicate the variable nucleotide positions in the aligned sequences.

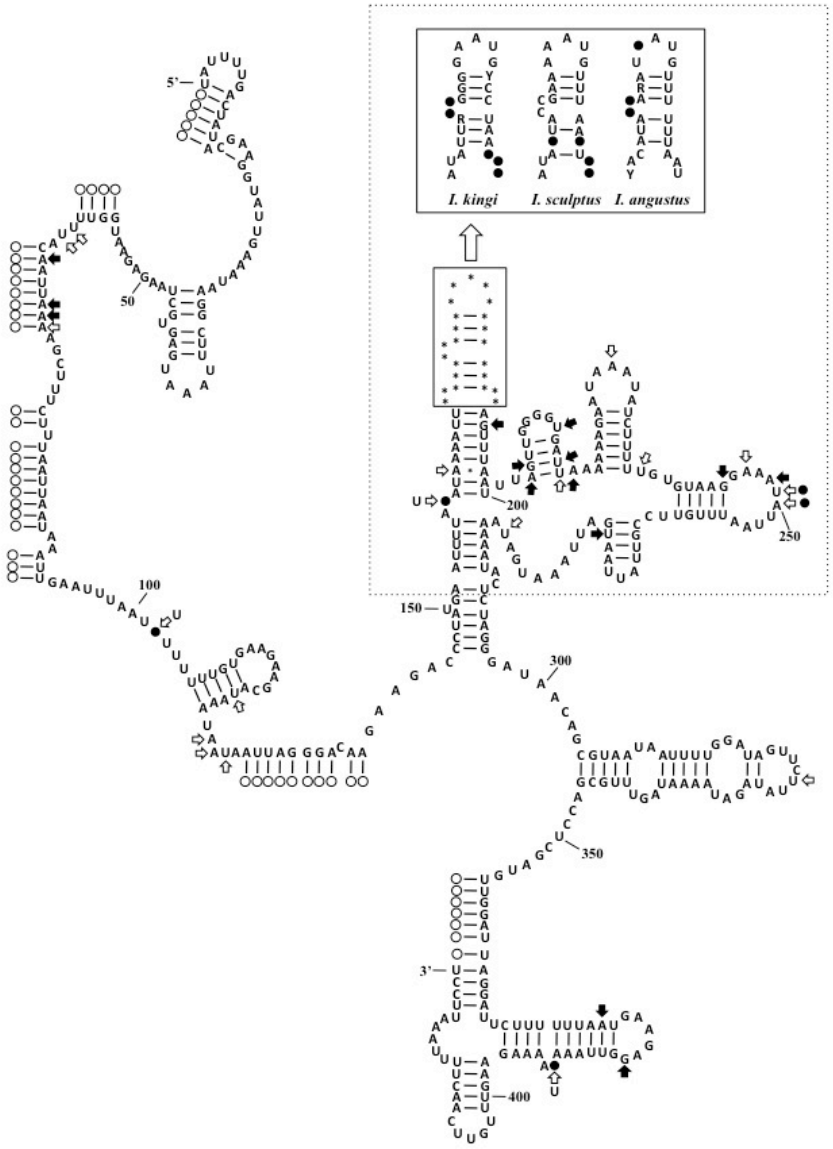

Fig. 6.2 The secondary structure of the 3' end of the mt 16S rRNA gene for haplotype KH-1 of *Ixodes kingi*. Open circles indicate putative nucleotide pairing with other parts of the 16S gene. Solid circles indicate indels whereas the solid and open arrows indicate variable positions (i.e., transitional and transversional changes, respectively) among haplotypes of *I. kingi*, *I. sculptus* and *I. angustus* in western Canada. The dotted box indicates the hypervariable region within the 16S gene. The solid boxed region provides a comparison of the first stem within the hypervariable region for *I. kingi*, *I. sculptus* and *I. angustus*. The secondary structure is based on the model of Guttel and co-workers (Gutell & Fox 1988; Gutell *et al.,* 1993; Gutell, 1996).

structure of the 16S rRNA gene, whereas the two transitional changes represented partial compensatory changes on a stem in the hypervariable region of the 16S gene (Fig. 6.2). Of the four variable positions in the sequences of the three haplotypes of *I. angustus* (i.e., positions 64, 168, 177 and 244; Fig. 6.1), three purine transitional changes represented partial compensatory base pair changes on stems, two of which occurred in the hypervariable region of the 16S gene (Fig. 6.2). The fourth variable position (i.e., alignment position 177; Fig. 6.1) was a pyrimidine transitional change and occurred at an unpaired site in the hypervariable region of the secondary structure (Fig. 6.2).

There were 54 variable nucleotide positions in the aligned partial 16S rRNA gene sequences between *I. angustus, I. kingi* and *I. sculptus* (Fig. 6.1), 39 of which were located in the hypervariable region (Fig. 6.2). The nucleotide differences represented 40 point mutations (i.e., 15 purine transitions, 6 pyrimidine transitions and 19 transversions), 11 indels, and 3 multiple changes (i.e., indel, transition and/or transversion). A total of 28 (52%) of the mutational changes occurred at unpaired positions in the secondary structure of the 16S rRNA gene (Fig. 6.2), while two indels in the sequence of *I. sculptus* represented a loss of a base pairing in the stem of the hypervariable region. Nine mutational changes resulted in a reduction in the base pairing of stems, primarily in the hypervariable region. A total of 15 mutational changes represented partial or complete compensatory base pair changes that maintained the secondary structure (Fig. 6.2). There was no significant difference ($\chi^2_3 = 1.67$, $P < 0.05$) in the proportion of variable nucleotide sites among the four structural categories of the secondary structure (i.e., stems, end loops, internal loops and connecting regions) of *I. angustus, I. kingi* and *I. sculptus* (Table 6.3). Similarly, there was no significant difference ($\chi^2_1 = 0.48$, $P < 0.05$) in the proportion of paired and unpaired positions that were variable within the secondary structure (Table 6.3).

Structural Category	No. of Positions	No. (%) of variable positions
Unpaired positions		
Connecting region	71	9 (12.7)
Inner loops	41	8 (19.5)
End loops	75	10 (13.3)
Total	187	27 (14.4)
Paired positions		
Stems	223	27 (12.1)

Table 6.3 Summary of the genetic variability within different parts of the secondary structure of the 3' end of the mt 16S rRNA gene for *I. kingi*, *I. sculptus* and *I. angustus*.

The phylogenetic tree produced from the NJ analyses of the 16S sequence data (Fig. 6.3) revealed that *I. angustus, I. kingi* and *I. sculptus* were placed in a clade, with strong statistical support (i.e., bootstrap value of 81%), that contained most of the other members of the subgenera *Pholeoixodes* (i.e., *I. dampfi*, *I. cookei*, *I. banksi* and *I. hexagonus*) and *Ixodiopsis* (i.e., *I. woodi*). Another member of the subgenus *Pholeoixodes*, *I. arboricola*, was not placed within this clade. There was no evidence for the two species within the subgenus *Ixodiopsis* (i.e., *I. angustus* and *I. woodi*) forming a clade to the exclusion of *I. kingi, I. sculptus* and the other members of the subgenus *Pholeoixodes*. Of the 198 variable positions (out of 428 positions; data not shown) in the 16S rDNA sequence alignment, 138 were informative in the maximum parsimony (MP) analyses. These analyses produced over 1000 equally most parsimonious trees with a length of 625, a CI of 0.41 and a RI of 0.73 (consensus tree not shown). In contrast to the NJ analyses, there was little statistical support (i.e., bootstrap support of 57%) for a clade containing members of *Pholeoixodes* and *Ixodiopsis* (Fig. 6.3). In both the NJ and MP analyses, multiple *I. sculptus* individuals collected from Beechy, Saskatchewan in Canada did not form a monophyletic clade with a single *I. sculptus* individual collected from Fort Collins, Colorado in the United States (Fig.6.3).

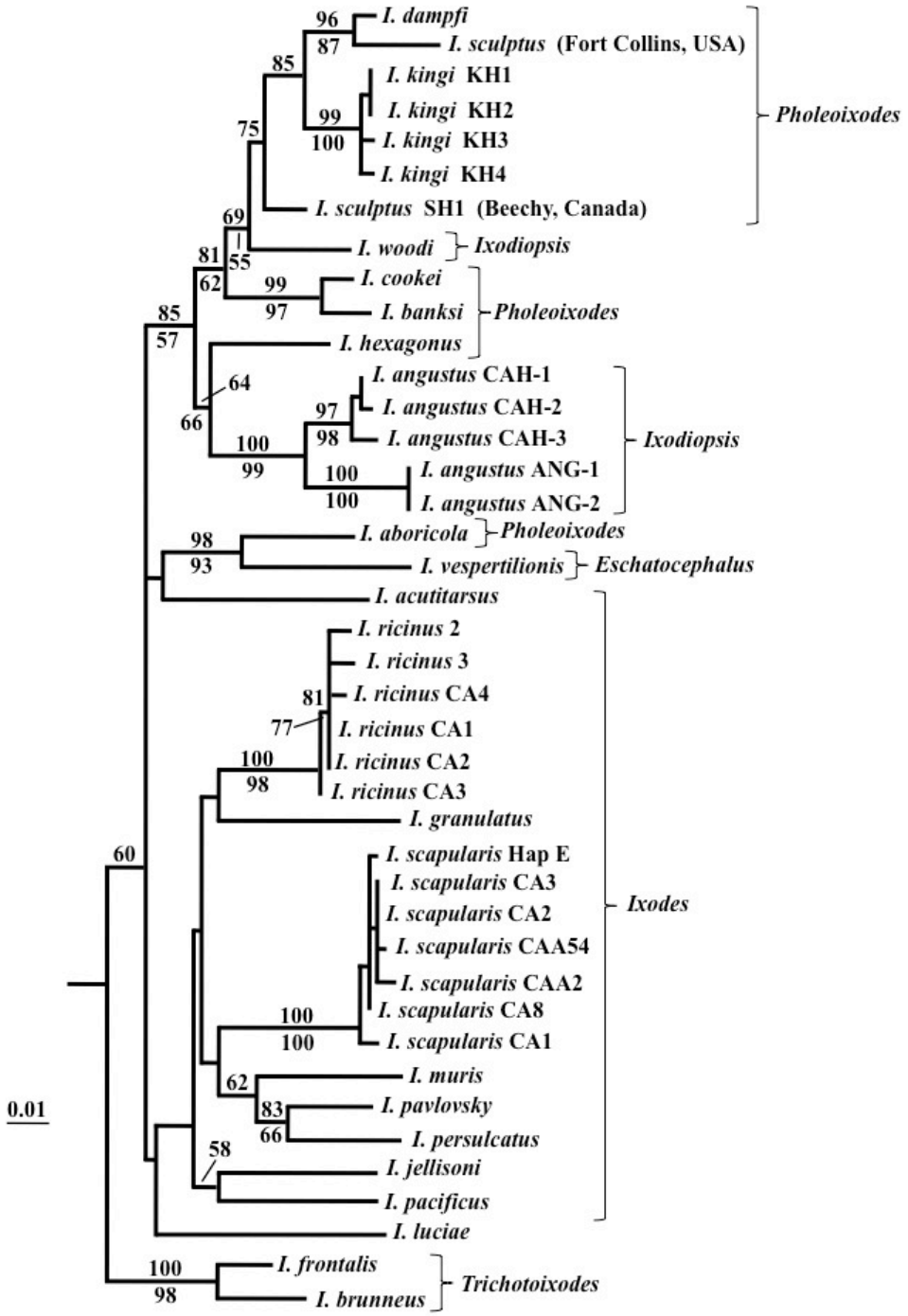

Fig. 6.3 Phylogenetic relationships of the different haplotypes of *I. kingi*, *I. sculptus* and *I. angustus* in western Canada with other species of *Ixodes* inferred from a neighbour-joining (NJ) analysis of sequence data of the 3' end of the mt 16S rRNA gene. Values above and below branches are the bootstrap support values for the NJ and MP analyses, respectively. Also indicated is the subgenus of all species of *Ixodes* included in his study.

6.5 **Discussion**

In the present study, one new mt 16S rDNA haplotype of *I. kingi* was detected among the six engorged females collected from dogs and cats in Saskatchewan. The 16S sequence of this individual from Vanguard differed by 1-3 bp when compared to sequences of the three haplotypes of *I. kingi* individuals feeding on northern pocket gophers (Chapter 2), Richardson's ground squirrels (Chapter 3), and on domestic animals (this Chapter). However, the number of haplotypes detected among *I. kingi* individuals from localities separated by distances of up to 316 km was significantly fewer than that reported for other species of *Ixodes*, such as *I. scapularis* (Trout *et al.,* 2009; Krakowetz *et al.,* 2011). Nonetheless, the 16S rRNA gene may still provide a useful genetic marker to examine the population genetics of *I. kingi* individuals on a broader scale. For instance, Gregson (1971) noted morphological differences between *I. kingi* populations on the western and eastern sides of the Rocky Mountains, which Oliver *et al.* (1974) postulated might be a reflection of evolutionary divergence within the species. Therefore, it would be interesting to determine if the 16S haplotypes of *I. kingi* on the eastern side of the Rocky Mountains (e.g., Saskatchewan) are distinct from those on the western side of the Rocky Mountains (e.g., British Columbia).

The number of 16S haplotypes detected for *I. kingi* was similar to that for *I. angustus*, yet only a single haplotype of *I. sculptus* was found among individuals collected from Beechy, Saskatchewan. However, as indicated previously (Chapter 3), these individuals differed markedly in 16S sequence when compared to the sequence of an *I. sculptus* individual from Fort Collins, Colorado. The phylogenetic analyses conducted in this chapter revealed that the two *I. sculptus* 16S haplotypes did not form a monophyletic clade. There was strong statistical support (bootstrap value of 96%) for a sister taxa relationship between the *I. sculptus* from Fort Collins

and *I. dampfi*, yet there was also statistical support (bootstrap value of 75%) for a sister taxa relationship between the *I. sculptus* from Beechy and *I. kingi*. These analyses suggest that *I. sculptus* may represent a cryptic (i.e., genetically distinct but morphologically similar) species; however, this hypothesis requires further work using significantly larger sample sizes from more sampling localities, together with additional genetic markers.

Unlike the D3 domain and flanking core regions of the nuclear 28S (large subunit) rRNA gene where *I. angustus* and *I. sculptus* had identical sequences (Chapter 5), there were 54 variable positions in the sequence alignment of the 16S gene among *I. angustus, I. kingi* and *I. sculptus* (Fig. 6.1). There were 12 bp differences in the sequences of the four haplotypes of *I. kingi* and *I. sculptus*, 39 bp differences between the haplotypes of *I. angustus* and *I. sculptus,* and 43 bp differences between all haplotypes of *I. angustus* and all of those of *I. kingi*. Thus, individuals of *I. angustus, I. kingi* and *I. sculptus* can easily be distinguished from one another based on their sequences of the 3' end of the mitochondrial 16S rRNA gene, even taking into account the intraspecific sequence variation.

A secondary structure model of the 3' end of the 16S rRNA gene was constructed for each of the three *Ixodes* species based on a comparison to the secondary structures of this gene for other eukaryotes (Gutell & Fox 1988; Gutell *et al.,* 1993; Gutell, 1996). The DNA sequences of the three species were aligned over 410bp based on the secondary structures of the 16S rRNA. A large proportion (72%) of the nucleotide changes that occurred within and among species were situated within what is known as the hypervariable region (i.e., alignment positions 152-291; Figure 6.3 and Table 6.1) of the gene. Mutational changes in DNA sequence among closely related species often occur at unpaired regions in the secondary structure of genes because they have a limited effect on the secondary structure (e.g., Chilton *et al.,* 1998; Chilton *et al.,* 2003).

However, mutational changes that occur on stems (= helices) are more frequently purine (A <-> G) and pyrimidine transitions (A <-> G and C <-> U) that represent partial or complete compensatory base changes that maintain the secondary structure, whereas transversions (e.g., A <-> C) and indels (i.e., insertions / deletions) that occur on stems only maintain the secondary structure if there is a complementary change on the opposite side of that stem. A comparison of the secondary structures of the three species of *Ixodes* revealed that 46% of the 410 alignment positions corresponded to nucleotide positions that were not involved in base pairing. These variable positions occurred in the end loops and inner loops of stems, and the connecting regions between two stems. There were seven variable nucleotide positions in the 16S DNA sequences that corresponded to sites of intraspecific variation (i.e., 3 for *I. kingi* and 4 for *I. angustus*); one indel, four purine transitional changes and two pyrimidine transitional changes. Most of these variable positions represented partial compensatory base pair changes in stems, maintaining the secondary structure; however, two changes occurred at unpaired sites (i.e., 1 indel and 1 pyrimidine transitional change). A comparison of the 16S DNA sequences of *I. kingi*, *I. sculptus* and *I. angustus* revealed 54 variable positions (Table 6.3), 50% of which occurred at unpaired positions. The 54 variable positions represented 11 indels, three multiple changes, 15 purine transitional changes, six pyrimidine transitional changes and 19 transversions. Thus, 41% of the mutations in DNA sequence among the three species represented transitional changes. Thirty-nine of the 54 variable positions occurred in the hypervariable region, with 15 mutational changes representing partial or complete compensatory base pair changes that maintained the secondary structure.

Therefore, the secondary structure of the partial 16S rRNA gene was relatively conserved among the three species of *Ixodes*, except for the hypervariable region (Fig. 6.2). This study

provides information as to where microevolutionary changes are taking place at both the sequence level and relative to the secondary structure of the 16S rRNA gene. Most changes occurred in the stems, representing partial or compensatory changes that did not result in a change of secondary structure. In addition, alignment of sequence data based on secondary structures increases the likelihood that homologous characters (i.e., alignment positions) are being compared in phylogenetic analyses. Thus, sequences of the 16S rRNA gene of other species within the genus *Ixodes*, and the two species used as outgroups for the phylogenetic analyses (i.e., *H. cretica* and *R. appendiculatus*), were aligned with the sequences of *I. kingi*, *I. sculptus* and *I. angustus* based on the secondary structure (Fig 6.2) to infer evolutionary relationships of the three species of *Ixodes* from small mammals in western Canada.

The results of the phylogenetic analyses showed that *I. kingi* and *I. sculptus* from western Canada were more related to each other than either species is to *I. angustus*. This finding is consistent with the placement of *I. sculptus* and *I. kingi* in the subgenus *Pholeoixodes* (Durden & Keirans, 1996), and *I. angustus* in the subgenus *Ixodiopsis* (Robbins & Keirans, 1992; Durden & Keirans, 1996). However, the results of the phylogenetic analyses lead to the placement of *I. woodi* (a member of the subgenus *Ixodiopsis*; Robbins & Keirans, 1992) within a clade that contained members of the subgenus *Pholeoixodes* (i.e., *I. banksi, I. cookei, I. sculptus, I. kingi* and *I. dampfi*; Durden & Keirans, 1996). Similarly, there was weak statistical support for a sister taxa relationship between *I. angustus* and *I. hexagonus*, the latter of which belongs to *Pholeoixodes*. Another member of the genus *Pholeoixodes*, *I. aboricola,* was placed external to the *Pholeoixodes - Ixodiopsis* clade, and was the sister taxon of *I. vespertilionis,* a member of the subgenus *Eschatocephalus*. Therefore, the taxonomic placement of *I. aboricola* within the subgenus *Pholeoixodes* may require re-examination. In addition, it would be useful to further

examine the relationships of *I. angustus*, *I. kingi*, *I. sculptus* and the other members of the subgenera *Ixodiopsis* and *Pholeoixodes* using a larger part of the 16S rRNA gene to gain further resolution of their phylogenetic placements.

In summary, the sequences of the 3' end of the 16S rRNA gene can be used to distinguish among *I. angustus, I. kingi* and *I. sculptus* and is a useful genetic marker for species-level identification within the genus *Ixodes*. Variation in the sequence of this gene region among individuals of the same species also indicates the potential of the 16S rRNA gene for population genetics studies of some of these tick species. Since *I. kingi* and *I. sculptus* are more related to one another than either are to *I. angustus*, and that they all feed on the same hosts as *D. andersoni* (Chapters 2-4), it would be interesting to determine if the composition of the bacterial communities within these tick species are based on their phylogenetic relationships and/or the hosts they parasitize. Several hypotheses could be explored, such as: (1) if each tick species has a unique community of bacteria, then the members of which will not be shared by other species, even when they occur in sympatry and parasitize the same species of small mammal host, or (2) if the vertebrate host determines the bacterial community of the tick species, then the tick bacterial community will be similar in composition to that of the vertebrate host. In Chapter 7, one of the interesting comparisons I will conduct is an examination of the bacterial communities of *I. kingi, I. sculptus* and *D. andersoni* collected from the same geographical area (i.e., Beechy, SK) and on the same hosts (i.e., Richardson's ground squirrels; *Spermophilus richardsonii*).

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Chapter 7 Microbial communities of four tick species (Acari: Ixodidae) parasitizing small mammals in western Canada.

7.1 Abstract

Despite the important medical and veterinary implications of tick-borne pathogens, there remains much to be learned about microbial diversity within ticks. There is limited published data on the bacterial diversity of most tick species in Canada, particularly those that are parasites of small mammals. Therefore, the microbial communities within four commonly occurring tick species that parasitize small mammals in western Canada was explored in the present study. PCR-SSCP analyses combined with DNA sequencing, targeting the prokaryotic 16S rRNA gene, was conducted on 454 ticks (i.e., 268 *Ixodes angustus*, 58 *I. sculptus*, 42 *Dermacentor andersoni* and 86 *I. kingi*) to determine which genera of bacteria were present in each tick. These analyses detected a total of 40 different bacterial genera, some of which (e.g., *Francisella* and *Pasteurella*) were specific to one tick species (i.e., *D. andersoni* and *I. angustus*, respectively). Other genera (e.g., *Rickettsia, Rickettsiella*, *Staphylococcus, Ralstonia*, *Sphingomonas, Stenotrophomonas* and *Pseudomonas*) were found in more than one species of tick. Sequence alignments of short 16S rRNA gene fragments (~200 base pairs) belonging to several of these bacterial genera (e.g., *Rickettsiella, Pseudomonas, Sphingomonas* and *Staphylococccus*) revealed differences in nucleotide composition among bacteria from different tick species; suggesting the possibility of different bacterial species within different species of tick. In contrast, some genera (e.g., *Rickettsia, Ralstonia* and *Stenotrophomonas*) displayed identical short 16S rRNA sequences despite being found in several species of tick. These data raise questions regarding the tick-specificity of the bacteria identified in this study. Further species-level characterization of

these bacterial taxa is required, and additional studies are needed to determine the functional role of the microbes detected in this study for each tick species examined, as well as determine their effects on human and animal health.

7.2 **Introduction**

Ticks are important arthropod vectors of human and animal disease-causing agents (Parola & Raoult, 2001; Jongejan & Uilenberg, 2004; Dantas-Torres *et al.,* 2012). Diseases transmitted by tick-borne pathogens include Lyme borreliosis, Rocky Mountain spotted fever, tularemia, ehrlichiosis, anaplasmosis, babesiosis, tick-borne encephalitis, Powassan encephalitis virus and Colorado Tick Fever virus (Parola & Raoult, 2001; Jongejan & Uilenberg, 2004; Dantas-Torres *et al.,* 2012). New emerging tick-borne diseases (e.g., Southern Tick-Associated Rash Illness [STARI], Panola Mountain *Ehrlichia*) continue to be recognized (Paddock & Yabsley, 2007; Loftis *et al.,* 2008). Ticks also harbor an abundant and diverse collection of endosymbiotic bacteria (e.g., *Coxiella-, Francisella-* and *Rickettsia*-like organisms), which are defined as non-disease causing, symbiotic microorganisms that are found in vertebrate and invertebrate hosts (Dale & Moran, 2006; Ahantarig *et al.,* 2013). Some of these endosymbionts are closely related to disease-causing pathogens, and use similar mechanisms as their pathogenic relatives to infect their hosts (Burgdorfer *et al.,* 1981; Childs & Paddock, 2002; Kugeler *et al.,* 2005; Dale & Moran, 2006; Liu *et al.,* 2013). Yet, despite the important medical and veterinary implications of tick-borne pathogens, there remains much to be learned about tick microbial diversity and the community structure of bacteria, particularly those of many tick species that occur in Canada.

There are approximately 26 species of ixodid tick that occur in Canada (Gregson 1956;

Wilkinson, 1967; Lindquist *et al*., 1999; Ogden *et al*., 2009), many of which occur on small mammals (Wilkinson, 1967; Keirans *et al*., 1996; Allan, 2001; Salkeld *et al*., 2006). The overlapping geographical ranges of many of these tick species (e.g., Dergousoff *et al*., 2013) can result in multiple tick species parasitizing the same host. For example, *Ixodes kingi, I. scapularis, Dermacentor andersoni* and *D. variabilis* were all found on northern pocket gophers near Clavet in Saskatchewan (Chapter 2). Similarly, *I. kingi, I. sculptus* and *D. andersoni* were collected from Richardson's ground squirrels near Beechy in Saskatchewan (Chapter 3), while *I. angustus* and *D. andersoni* were collected from six species of small mammal (i.e., voles, shrews, mice and golden-mantled ground squirrels) in Kootenay National Park, British Columbia (Chapter 4). Several of the tick species that parasitize these small mammals are of medical and/or veterinary importance (Gregson 1956; Ogden *et al*., 2009). For example, *D. andersoni* is a vector of *Rickettsia rickettsii*, *Francisella tularensis* and *Anaplasma marginale*, the bacteria responsible for Rocky Mountain spotted fever, tularemia, and bovine anaplasmosis, respectively (Burgdorfer, 1975; Foley & Nieto, 2010; Kocan *et al*., 2010). In addition, *I. angustus* has recently been implicated in the spread of *Borrelia burgdorferi*, the causative agent of Lyme borreliosis, in the Pacific Northwest (Damrow *et al*., 1989; Banerjee *et al*., 1994). Several studies have been made on the bacteria found within Canadian populations of *D. andersoni* and *D. variabilis* (Scoles, 2004; Dergousoff *et al*., 2009; Dergousoff & Chilton, 2010; Dergousoff & Chilton, 2011; Dergousoff & Chilton, 2012; Dergousoff & Chilton, 2013); however, the bacterial diversity of *I. kingi, I. angustus* and *I. sculptus* has not been examined previously. To our knowledge, there is little information on the structure of bacterial communities of many tick species in Canada, particularly those that parasitize small mammals. Given that *I. kingi* and *I. sculptus* are the most genetically similar (Chapter 6), and can parasitize the same host animals (Chapter 3), it is

possible that they will have the most similar bacterial communities compared to those of *I. angustus* and *D. andersoni* if there is coevolution of bacteria and their tick hosts. This hypothesis is examined in this chapter using molecular approaches.

In the past, bacterial identification relied upon phenotypic identification using traditional methods such as gram staining and culture to characterize individual colonies. With the advent of culture-independent molecular techniques (e.g., PCR, microarrays, pyrosequencing), previous challenges encountered in bacterial identification have been largely overcome. For example, molecular approaches targeting the prokaryotic 16S rRNA gene are both powerful and informative, and make possible the detection, identification and classification of microbes (Amann & Ludwig, 2000; Clarridge, 2004; Rajendhran & Gunasekaran, 2011). Although this gene is highly conserved within a bacterial species, there are nine hypervariable regions (i.e., V1-V9) that demonstrate considerable sequence diversity among different bacteria and can therefore be used for species-level identification (Clarridge, 2004; Chakravorty *et al.,* 2007). Many studies have used PCR-based techniques targeting this prokaryotic gene region to identify the bacterial community structure of a variety of arthropods, including fleas (Murrell *et al.,* 2003; Pornwiroon *et al.,* 2007; Jones *et al.,* 2010), mites (Hoy & Jeyaprakash, 2005), lice (Reed & Hafner, 2002; Murrell *et al.,* 2003) and ticks (Murrell *et al.,* 2003; Schabereiter-Gurtner *et al.,* 2003; Moreno *et al.,* 2006; Clay *et al.,* 2008; Steiner *et al.,* 2008; van Overbeek *et al.,* 2008; Rudolf *et al.,* 2009; Harrus *et al.,* 2010; Heise *et al.,* 2010; Carpi *et al.,* 2011; Tveten & Sjåstad, 2011; Lalzar *et al.,* 2012). These molecular approaches have also been used in studies of the bacterial endosymbionts of ticks (Scoles, 2004; Dergousoff & Chilton, 2009; Dergousoff & Chilton, 2010; Dergousoff & Chilton, 2012; Ahantarig *et al.,* 2013), and have been proven especially important when screening ticks that are potential vectors of disease (Kugeler *et al.,* 2005; Moreno *et al.,*

2006; Steiner *et al*., 2008; Rudolf *et al*., 2009; Tveten & Sjåstad, 2011).

In the past decade, mutation-scanning techniques have been used more and more frequently to characterize the bacterial community structure within a broad range of samples, including those from the environment (Lee *et al*., 1996; Schwieger & Tebbe, 1998; Smalla *et al*., 2007), anaerobic digesters (Delbès *et al*., 2000; Zumstein *et al*., 2000; Leclerc *et al*., 2001; Leclerc *et al*., 2004), methanogenic bioreactors (Hori *et al*., 2006), human blood samples (Turenne *et al*., 2000) and arthropods (Czarnetzki & Tebbe, 2004; Schabereiter-Gurtner *et al*., 2003; Mohr & Tebbe, 2006). Mutation scanning methods, such as denaturing gradient gel electrophoresis (DGGE), and single strand conformation polymorphism (SSCP) analyses have been used to examine the bacterial communities of invertebrates (Schabereiter-Gurtner *et al*., 2003; Czarnetzki & Tebbe, 2004; Mohr & Tebbe, 2006). DGGE is a molecular technique where double-stranded DNA is separated on gels with a denaturing gradient, such that PCR-amplicons migrate differentially based on their sequence composition (Muyzer *et al*., 1993; Schabereiter-Gurtner *et al*., 2003; Hori *et al*., 2006; Smalla *et al*., 2007). SSCP involves the separation of PCR amplicons based on the conformation (i.e., secondary structure) of single-stranded DNA in a non-denaturing gel (Gasser *et al*., 2006). SSCP can be used to differentially display genetic variation between DNA sequences that are 150-450 base pairs (bp) in size, and that differ by one or more nucleotides (Gasser *et al*., 2006). Both of these mutation-scanning techniques provide a fast, effective, and inexpensive way to detect and identify the members of bacterial communities. Therefore, in the present study, PCR-SSCP analyses were used to determine the bacterial genera present within individual ticks, providing insight into what bacterial genera are found within *I. angustus, I. kingi, I. sculptus* and *D. andersoni*, four commonly occurring species that parasitize small mammals in western Canada.

7.3 Materials and Methods

7.3.1 Sample collection

For this study, the bacterial communities within ticks were determined for 454 individuals collected from small mammals in Saskatchewan and British Columbia. These ticks included 268 *Ixodes angustus* collected from 46 red-backed voles (*Clethrionomys gapperi*), three long-tailed voles (*Microtus longicaudus*), two western heather voles (*Phenacomys intermedius*), one masked shrew (*Sorex cinereus*), two golden-mantled ground squirrels (*Spermophilus lateralis*), and one deer mouse (*Peromyscus maniculatus*) that were live-trapped at three sites (i.e., Verdant Forest, Numa Forest and Marble Canyon) within the Kootenay National Park (50°68'N, 115°93'W), British Columbia (Chapter 4). In addition, 58 *I. sculptus*, 39 *Dermacentor andersoni* and one *I. kingi* were collected from 17 Richardson's ground squirrels (*Spermophilus richardsonii*) near Beechy (50°53'N, 107°23'W), Saskatchewan (Chapter 3), while 85 *I. kingi* and three *D. andersoni* were collected from eight northern pocket gophers (*Thomomys talpoides*) 8 km southwest of Clavet (51.9519°N, 106.4473°W), Saskatchewan (Anstead & Chilton, 2011). Ticks were identified to the species-level using morphological and molecular methods (Anstead & Chilton, 2011; Chapter 3; Chapter 4).

7.3.2 DNA extraction and PCR amplification of prokaryotic 16S rDNA

Total genomic DNA (gDNA) was extracted and purified from the complete body of each tick using the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany), and the modifications described previously (Dergousoff & Chilton, 2007). PCR analyses were conducted on the total gDNA of each tick to test for the presence of prokaryotic 16S rDNA. PCRs were conducted using the general bacterial primers 554f (5'- TCG GAA TTA CTG GGC GTA AA -3') and 802r (5'-

ACT ACC AGG GTA TCT AAT CCT G -3') to amplify a partial fragment (~250bp) of the prokaryotic 16S rRNA gene in the hypervariable V4–V5 region. PCR's were performed in 25μl volumes containing 2.5μl 10X i*Taq* PCR buffer (Bio-Rad), 1.5 mM $MgCl_2$, 200μM of each deoxynucleoside triphosphate (dNTP), 25pmol (1μM) of each primer, 0.5U/μl i*Taq* DNA polymerase (Bio-Rad), and 2μl of gDNA template. A negative control (i.e., without gDNA) sample was included in each set of PCR assays. PCR's were performed in a thermocycler (iCycler; Bio-Rad, Hercules, CA) using the following conditions: 95°C for 5 min, followed by 30 cycles of 95°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec, and a final extension step at 72°C for 5 min. Amplicons were subjected to electrophoresis on SYBR® Safe-stained 1.5% agarose-TBE (89mM Tris, 89mM boric acid, 2mM EDTA, pH 8.3) gels and their banding patterns were visualized by UV transillumination.

7.3.3 **SSCP analysis, band excision and re-amplification of SSCP bands**

All PCR-positive samples were pre-screened for genetic variation using single strand conformation polymorphism (SSCP) analyses (Gasser *et al.,* 2006) following the same protocol as described previously (Dergousoff & Chilton, 2007). Individual DNA bands from representative SSCP banding patterns (n=38 amplicons) were excised using disposable gel-excision pipette tips (Fig. 7.1). The excised bands were homogenized and centrifuged at high power (18,000 rcf) for ten minutes in order for the supernatant to become separated from the SSCP gel residue. PCR analyses were then conducted with primers 554f and 802r and the conditions described above using 2μl of the supernatants as templates. Negative control samples were also included in each set of PCRs. Amplicons were subjected to electrophoresis on SYBR® Safe-stained 1.5% agarose-TBE and visualized by UV transillumination.

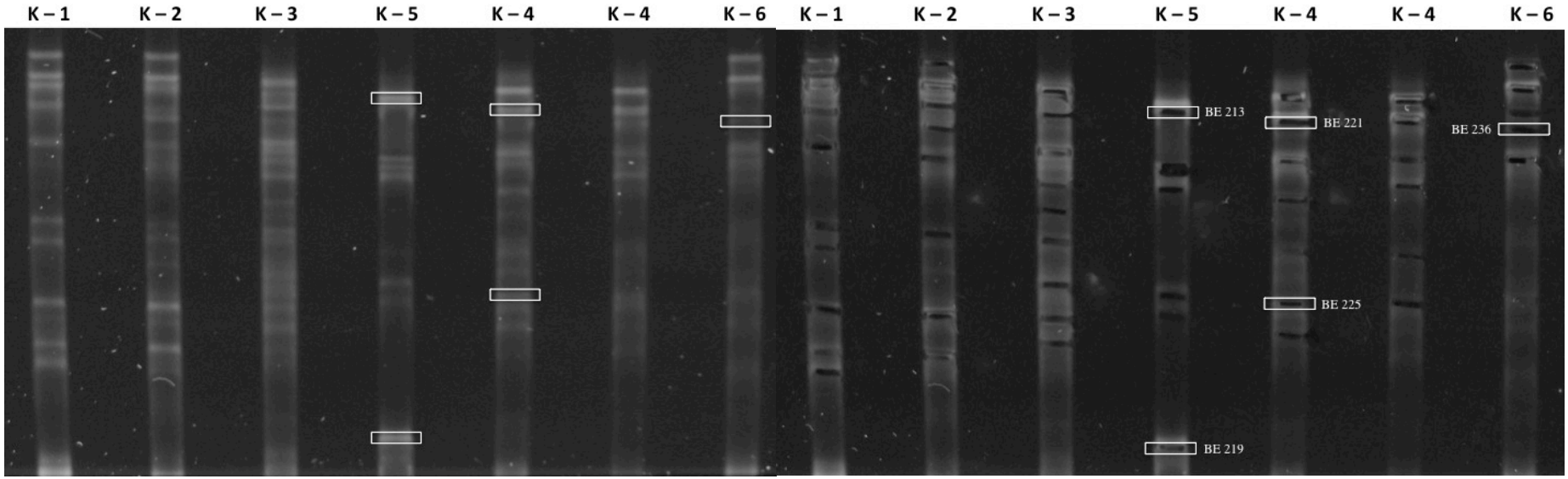
7.3.4 **Sequencing of prokaryotic 16S rDNA**

Amplicons from 345 SSCP bands, excised from 38 different SSCP banding patterns (i.e., profiles), representing 68 representative PCR-positive ticks (i.e., 15 *I. angustus,* 15 *I. sculptus,* 15 *D. andersoni* and 23 *I. kingi*), were purified (Dergousoff & Chilton, 2007) and subjected to automated DNA sequencing using primers 554f and 802r in separate reactions, or just in the forward direction using primer 554f. BLAST searches (GenBank) were performed on sequences to determine the genus-level identity of each amplicon.

7.4 **Results**

7.4.1 **Ticks positive for bacteria**

A total of 268 *I. angustus*, 58 *I. sculptus*, 43 *D. andersoni* and 86 *I. kingi* were each screened for the presence of prokaryotic rDNA using general bacterial primers targeting the prokaryotic 16S rRNA gene (Table 7.1). Two hundred and thirty-five *I. angustus* (15 adults, 53 nymphs and 167 larvae), 30 *I. sculptus* (10 nymphs and 20 larvae), 86 *I. kingi* (5 adults, 2 nymphs and 79 larvae) and 38 *D. andersoni* (19 adults, 16 nymphs and 3 larvae) were PCR-positive for prokaryotic 16S rDNA (Table 7.1). All PCR-positive ticks produced amplicons of approximately 250-bp on TBE-agarose gels and no amplicons were produced from the negative (i.e., no gDNA) samples.



A B

Fig. 7.1 SSCP gel displaying prokaryotic 16S rDNA amplicons produced from the gDNA of *I. kingi*. (A) Gel prior to excision of bands from amplicons displaying six different band profiles (i.e., K-1 – K-6). (B) Gel following band excisions. The identity of the bacterial genera from DNA sequencing analyses of five representative bands that were excised and re-amplified by PCR were: BE 213 = *Staphylococcus* (Fig. 18), BE 219 = *Rickettsia* (Fig. 19), BE 221 = *Pseudomonas* Type 1 (Fig. 16), BE 225 = *Sphingomonas* (Fig. 17) and BE 236 = *Ralstonia* (Fig. 20).

7.4.2 **Diversity of 16S rRNA gene sequences**

A comparison of the SSCP profiles of the bacterial amplicons from 235 *I. angustus* revealed that there were four different banding patterns (Fig. 7.2). The majority of the samples (n=191) shared an identical banding pattern (i.e., profile), whereas 42 samples shared a second profile and the remaining two amplicons each had unique profiles when compared to the rest of the *I. angustus* amplicons. Sequencing of SSCP-band amplicons of representative banding profiles revealed several different microbial genera within the *I. angustus* samples (Table 7.2). A BLAST search of the prokaryotic 16S rDNA sequences showed the majority of *I. angustus* (81.3%) to be infected with one genus of bacteria, whereas 0.8% and 17.9% of individuals were infected with two or three genera of bacteria, respectively (Fig. 7.3). The average number of bacterial genera occurring in *I. angustus* was 1.20 ± 0.05. Further BLAST analyses of sequence data revealed the presence of seven different microbial genera (Fig. 7.4) Almost all (n=233; 99%) of the *I. angustus* were infected with a *Rickettsiella*, and 42 ticks (17.9%) were positive for bacteria belonging to the genus *Rickettsia*. Forty-four (18.7%) of the *I. angustus* had several genera of gammaprotebacteria (i.e., *Pasteurella, Klebsiella* and a bacterial endosymbiont of *Curculio*) and a small percentage of individuals (0.4%) were positive for an unknown betaproteobacterium and an unknown member of the bacterial class Clostridiales.

Eight different SSCP banding patterns were found among the 30 *I. sculptus* samples, with ten amplicons sharing the most common profile. Sequence analyses revealed that the majority of *I. sculptus* (83.4%) were infected with one genus of bacteria, whereas 13.3% and 3.3% of individuals were infected with two or three genera of bacteria, respectively (Fig. 7.5). The average number of bacterial genera infesting *I. sculptus* was 0.62 ± 0.09, as the remaining 28 *I. sculptus* screened did not produce amplicons during PCR analyses. BLAST analyses of

sequences derived from SSCP band cuts outs revealed the presence of seven different microbial genera (Table 7.3), the majority of which belonged to the class Bacilli (Fig. 7.6). A large proportion of *I. sculptus* (n=23; 76.7%) tested positive for 16S rDNA belonging to the genus *Rickettsiella*. A total of five ticks (16.7%) were infected with bacteria of the class Bacilli (i.e., *Lysinibacillus, Geobacillus, Bacillus* and *Staphylococcus*) and another five individuals were infected with betaproteobacteria belonging to the genus *Comamonas*. Finally, a single *I. sculptus* tested positive for prokaryotic 16S rDNA belonging to an unknown species of Actinobacteria.

A total of 14 different SSCP banding patterns were found among the 86 *I. kingi* samples (Fig. 7.7), 13 of which belonged to the 85 *I. kingi* from Clavet (SK) and one belonging to the single *I. kingi* from Beechy (SK). Approximately half (n=45; 53%) of the *I. kingi* amplicons from Clavet shared the same SSCP profile, while another 17 amplicons from the same location (19.8%) shared the second most common profile. Sequence analyses revealed that the majority of *I. kingi* from Clavet (53%) were infected with 11 genera of bacteria, whereas 17.6% and 22.3% of individuals were infected with three or four genera of bacteria, respectively (Fig. 7.8). The remaining *I. kingi* from Clavet were infected with two genera, five genera or seven genera (3.5%, 1.2%, and 2.4%, respectively) of bacteria. The average number of bacterial genera infecting *I. kingi* from this location was 7.54± 0.41. BLAST analyses of SSCP-band sequence data revealed the presence of 27 different microbial genera (Table 7.4), with the majority of genera belonging to the bacterial class Betaproteobacteria (Fig. 7.9). A large proportion of *I. kingi* tested positive for 16S rDNA belonging to the genus *Ralstonia* (n=64; 75.3%), *Pseudomonas* (n=61; 71.8%) and *Stenotrophomonas* (n=54; 63.5%). On the contrary, a single *I. kingi* tested positive for *Rickettsia* 16S rDNA. Bacteria belonging to the classes Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Actinobacteria and Bacilli were

Locality (Coordinates) Tick species	Life cycle stage	No. tested	No. (%) PCR-positive
Kootenay N.P., BC (49º44'N 112º50'W)			
Ixodes angustus	larvae	176	167 (94.9%)
	nymphs	68	53 (77.9%)
	adults	24	15 (62.5%)
Beechy, SK (50°53'N, 107°23'W)			
I. sculptus	larvae	34	20 (58.8%)
	nymphs	21	10 (47.6%)
	adults	3	0 (0%)
I. kingi	adults	1	1 (100%)
D. andersoni	nymphs	20	16 (80%)
	adults	20	19 (95%)
Clavet, SK (51.9519°N, 106.4473°W)			
I. kingi	larvae	79	79 (100%)
	nymphs	2	2 (100%)
	adults	4	4 (100%)
D. andersoni	larvae	3	3 (100%)

Table 7.1 The number of larvae, nymphs and adults of different tick species collected at different localities in western Canada that were PCR-positive for prokaryotic 16S rDNA

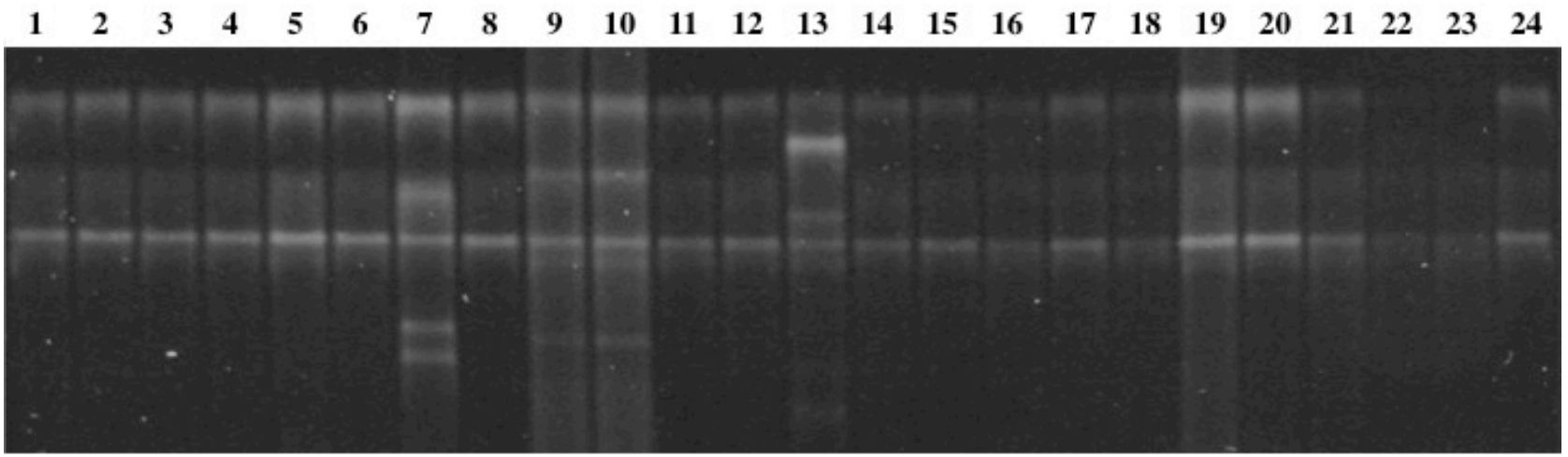


Fig. 7.2 SSCP banding patterns of the prokaryotic 16S rRNA gene for representative amplicons derived from the total gDNA of *Ixodes angustus*. Banding profile A-1 (lanes 1-6, 8-12, 14-24), banding profile A-2 (lane 13), and banding profile A-3 (lane 7).

SSCP profile	Closest related genus	No. positive	Life cycle stage
1	*Rickettsiella*	191	14A, 51N, 126L
2	*Rickettsiella* *Rickettsia* *Curculio* endosymbiont	42	1N, 41L
3	*Pasteurella* *Klebsiella*	1	1A
4	*Clostridiales* betaproteobacteria	1	1N
		Total: 235	

Table 7.2 Identification of bacteria in *Ixodes angustus* obtained by sequence analyses of re-amplified SSCP bands.

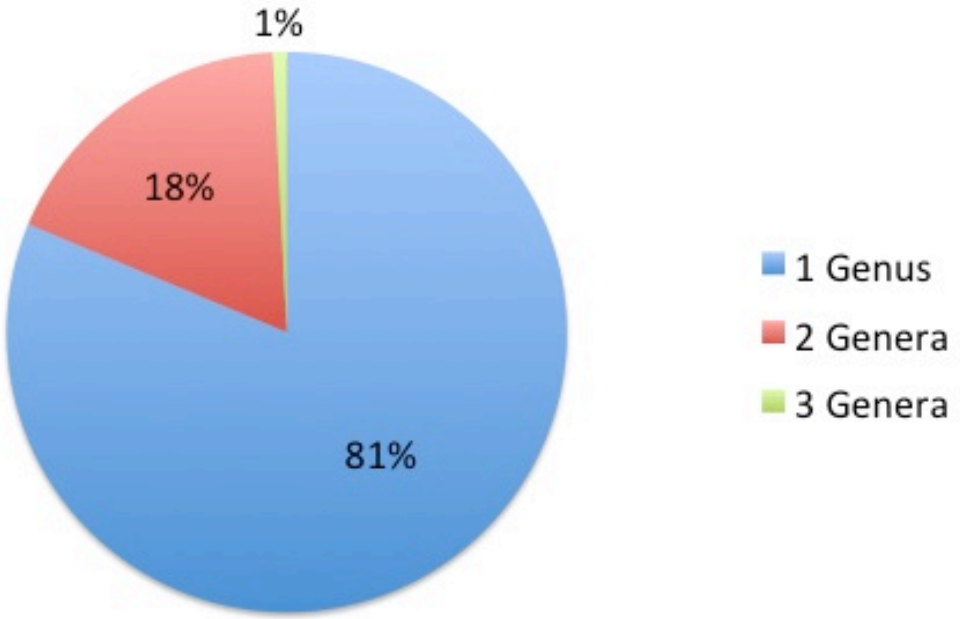


Fig. 7.3 Proportion of *I. angustus* (n = 15A, 53N, 167L) infected with one – three different bacterial genera.

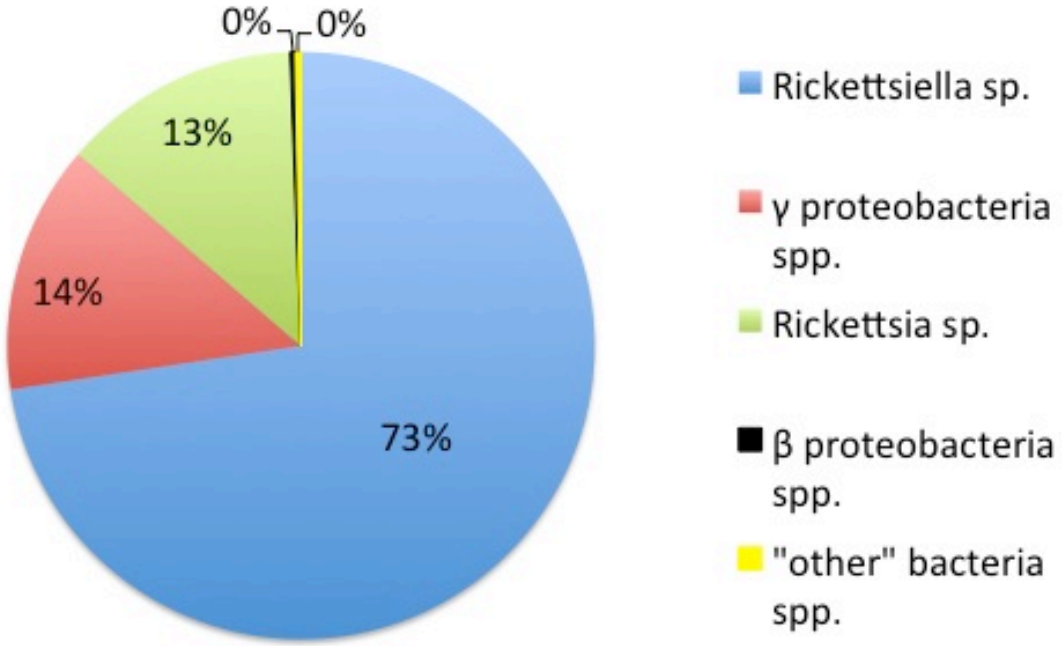


Fig. 7.4 Proportion of the different bacterial genera found in *I. angustus*.

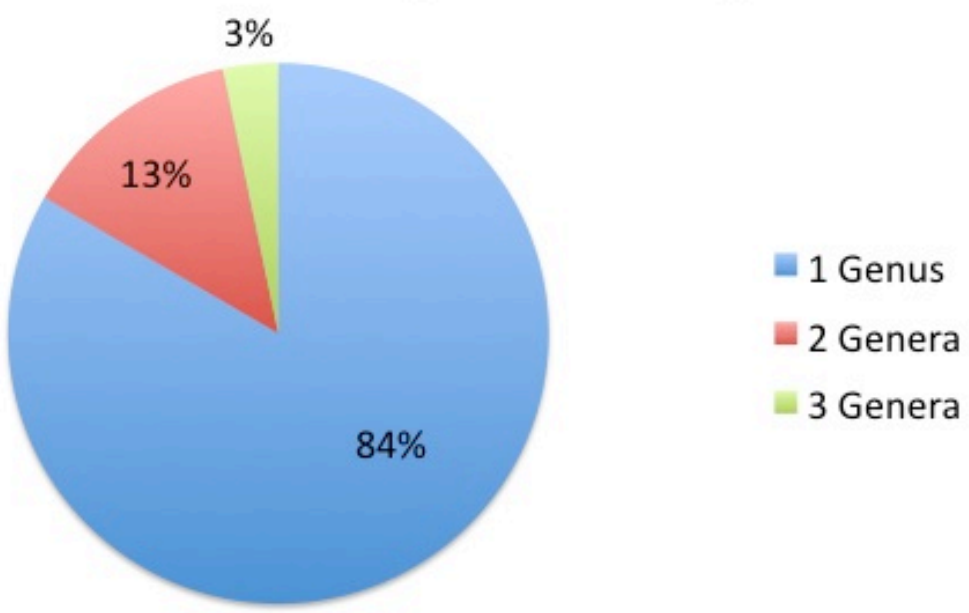


Fig. 7.5 Proportion of *I. sculptus* (n = 10N, 20L) infected with one – three different bacterial genera.

SSCP profile	Closest related genus	No. positive	Life cycle stage
1	*Rickettsiella*	2	1N, 1L
2	*Bacillus* *Geobacillus* *Lysinibacillus*	1	1N
3	*Rickettsiella* *Staphylococcus*	4	4L
4	*Rickettsiella*	6	6L
5	*Comamonas*	5	5L
6	*Rickettsiella*	1	1L
7	*Rickettsiella*	10	7N, 3L
8	*Williamsia*	1	1N
		Total: 30	

Table 7.3 Identification of bacteria in *Ixodes sculptus* obtained by sequence analyses of re-amplified SSCP bands.

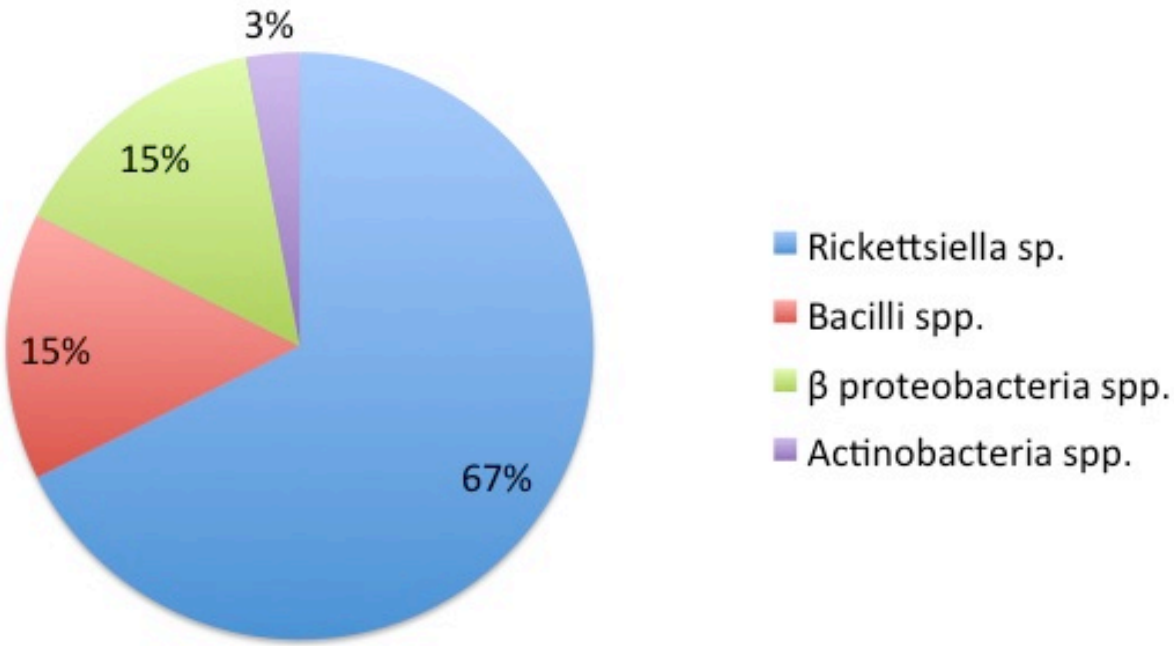


Fig. 7.6 Proportion of the different bacterial genera found in *I. sculptus*.

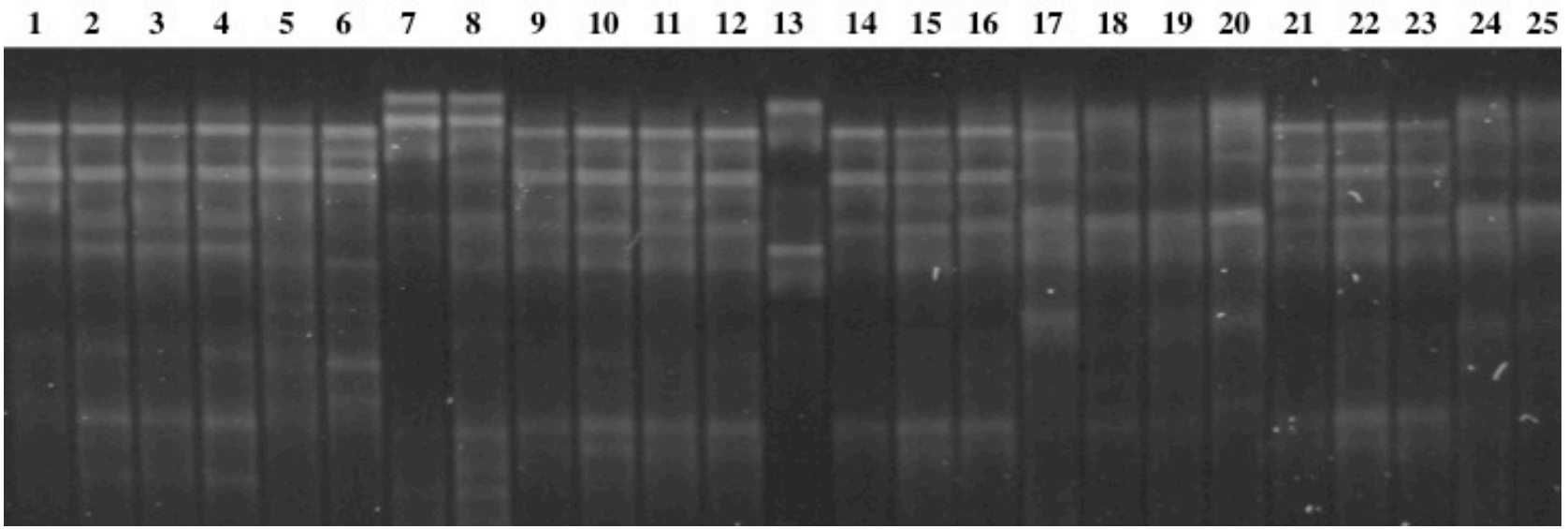


Fig. 7.7 SSCP banding patterns of the prokaryotic 16S rRNA gene for representative amplicons derived from the gDNA of *Ixodes kingi*. Banding profile K-4 (lanes 1-6, 9-12, 14-16, 21-23), banding profile K-5 (lanes 7, 8), banding profile K-6 (lane 17-19), banding profile K-7 (lane 20), banding profile K-8 (lanes 24, 25) and *I. angustus* control (lane 13).

also widespread among the *I. kingi* from Clavet (Table 7.4). The one *I. kingi* collected from *S. richardsonii* from Beechy that tested positive was infected with two genera of prokaryotic 16S rDNA, which BLAST sequence analyses revealed belonged to the genera *Rickettsiella* and *Rickettsia* (Fig. 7.10).

A comparison of the SSCP profiles of the bacterial amplicons from the gDNA of 38 *D. andersoni* revealed that there were ten different banding patterns, seven of which belonged to the 35 *D. andersoni* from Beechy and three different profiles each belonging to the three *D. andersoni* collected from Clavet. Fourteen (40%) *D. andersoni* amplicons from Beechy shared the same SSCP profile, while nine samples (25.7%) shared the second most common banding pattern. A BLAST search of the prokaryotic 16S rDNA sequences showed that 40% of *D. andersoni* collected from Beechy to be infected with seven genera of bacteria, whereas 46% were infected with only one genus of bacteria (Fig. 7.11). The remaining *D. andersoni* from Beechy were infected with two, three or four genera (2.9%, 2.9%, and 8.6%, respectively) of bacteria. The average number of bacterial genera infesting *D. andersoni* from this location was 3.36 ± 0.46. BLAST analyses of the sequences derived from SSCP band cuts outs revealed the presence of 15 different microbial genera (Table 7.5), with the majority of genera belonging to the bacterial class Betaproteobacteria (Fig. 7.12). A proportion of *D. andersoni* tested positive for 16S rDNA belonging to the genus *Ralstonia* (n=19; 54.3%), *Pseudomonas* (n=16; 45.7%) and *Rickettsia* (n=13; 37.1%). Three *D. andersoni* also tested positive for *Francisella* 16S rDNA. In addition, bacteria belonging to the classes Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Actinobacteria and Bacilli were widespread among the *D. andersoni* from Beechy (Table 7.5).

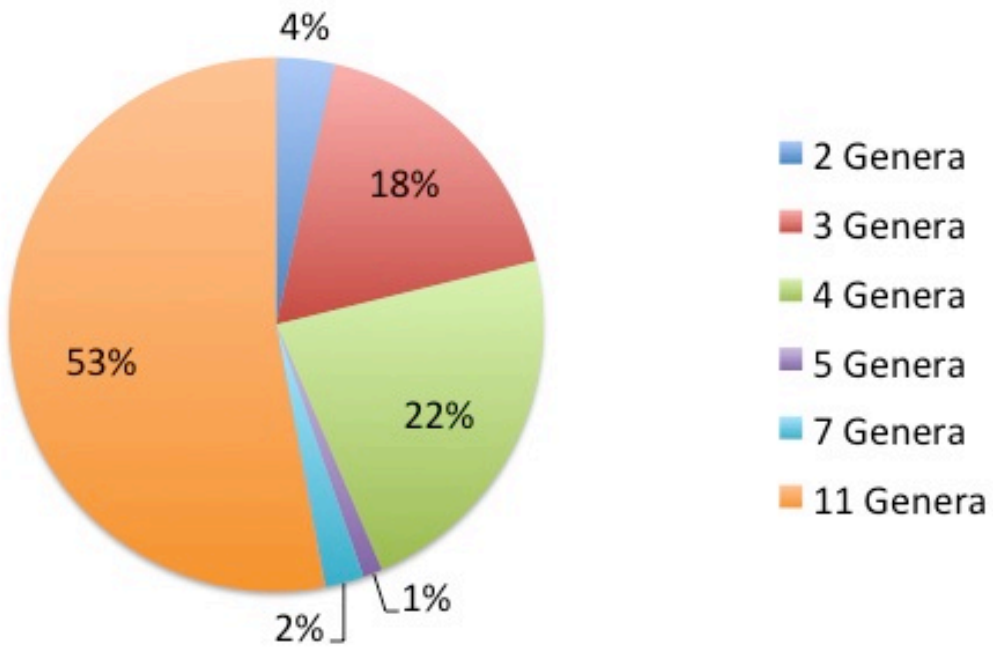


Fig. 7.8 Proportion of *I. kingi* from Clavet (n = 4A, 2N, 79L) infected with one – eleven different bacterial genera.

Locality (Coordinates) SSCP profile	Closest related genus	No. positive	Life cycle stage
Clavet, SK (51.9519°N, 106.4473°W)			
1	*Pseudomonas* *Ralstonia* *Stenotrophomonas*	4	2A, 2N
2	*Acidovorax* *Azoarcus* *Nocardia* *Streptomyces* *Thiomonas*	1	1L
3	*Rickettsia* *Staphylococcus*	1	1A
4	*Burkholderia* *Herbaspirillum* *Macrococcus* *Marinobacter* *Marinospirillum* *Pseudomonas* *Ralstonia* *Sphingomonas* *Stenotrophomonas* *Streptomyces* *Thiohalocapsa*	45	1A, 44L
5	*Pseudomonas* *Ralstonia* *Sphingomonas*	7	7L
6	*Acidovorax* *Devosia* *Ralstonia*	3	3L
7	*Ralstonia* *Rhadococcus* *Streptomyces*	1	1L

Table 7.4 Identification of bacteria in *Ixodes kingi* obtained by sequence analyses of re-amplified SSCP bands. Continued on next page.

Locality (Coordinates) SSCP profile	Closest related genus	No. positive	Life cycle stage
Clavet, SK (51.9519°N, 106.4473°W)			
8	*Acidovorax* *Brachymonas* *Comamonas* *Ralstonia*	17	17L
9	*Dichotomicrobium* *Dichotomicrobium* *Lysobacter* *Mesorhizobium* *Propionivibrio* *Pseudomonas* *Stenotrophomonas*	2	2L
10	*Ralstonia* *Rhodococcus*	1	1L
11	*Pseudomonas* *Stenotrophomonas*	1	1L
12	*Propionivibrio* *Pseudomonas* *Ralstonia* *Stenotrophomonas*	1	1L
13	*Ensifer* *Pseudomonas* *Ralstonia* *Stenotrophomonas*	1	1L
Beechy, SK (50°53'N, 107°23'W)			
1	*Rickettsia* *Rickettsiella*	1	1A
		Total: 86	

Table 7.4 Identification of bacteria in *Ixodes kingi* obtained by sequence analyses of re-amplified SSCP bands

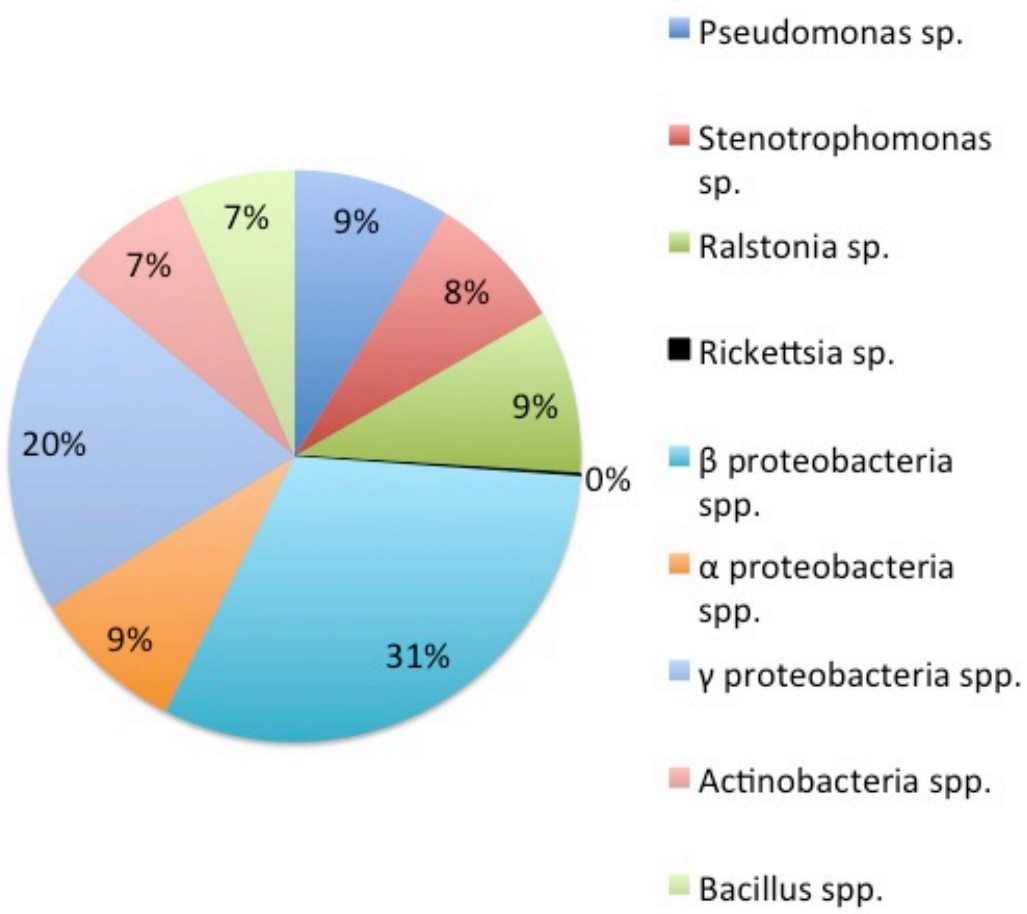


Fig. 7.9 Proportion of the different bacterial genera found in *I. kingi* (Clavet).

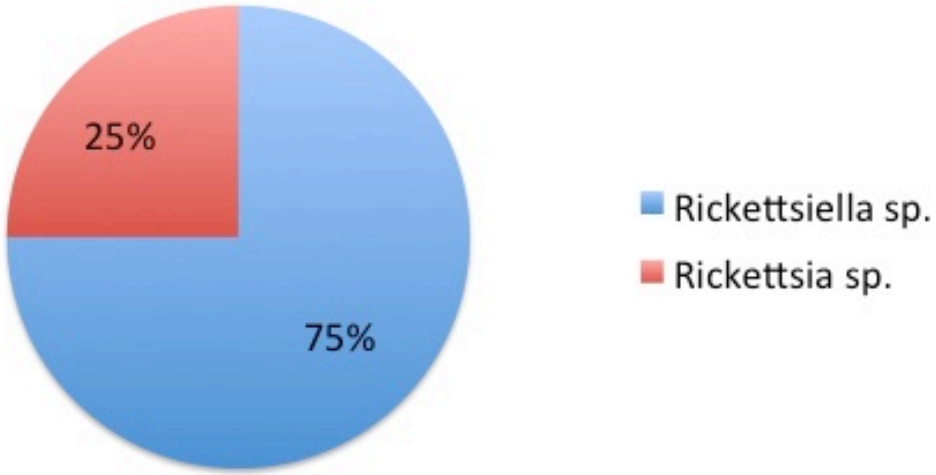


Fig. 7.10 Proportion of the different bacterial genera found in one *I. kingi* (Beechy).

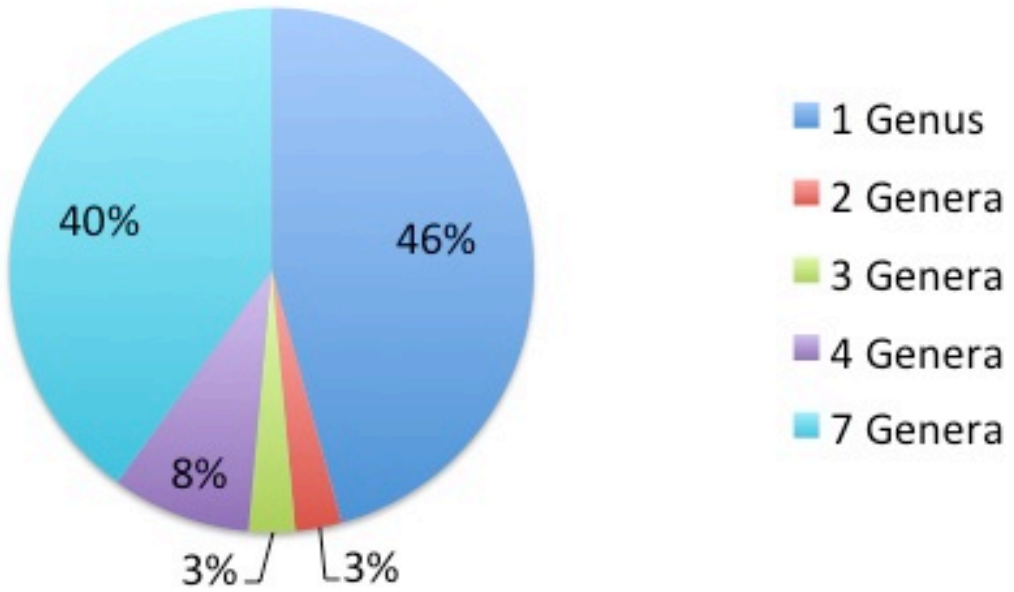


Fig. 7.11 Proportion of *D. andersoni* from Beechy (n = 19A, 16N) infected with one – seven different bacterial genera.

The three *D. andersoni* from Clavet tested positive for five genera of prokaryotic 16S rDNA (Fig. 7.13), with the majority of ticks (n=2; 66.7%) being infected with two genera of bacteria. Sequence analyses revealed that two of the three ticks were infected with *Pseudomonas* and *Stenotrophomonas* bacterial taxa, and there was also Betaproteobacteria and Gammaproteobacteria present (Fig. 7.14).

7.4.3 **Commonly occurring genera**

Several genera of bacteria were isolated from almost all of the tick species sampled (Table 7.6). For example, prokaryotic 16S rDNA of *Rickettsiella* was detected in high numbers in *I. angustus* (99%) and *I. sculptus* (76.7%), and was also detected in the single *I. kingi* from Beechy. The genus *Rickettsia* was found in three of the tick species screened and from multiple geographical locations. For instance, the 16S rDNA of *Rickettsia* was detected in *I. angustus* from Kootenay National Park, *I. kingi* from Clavet, and in the *D. andersoni* from both Beechy and Kootenay National Park. Bacteria of the genera *Ralstonia* and *Pseudomonas* were detected in similar proportions in the *I. kingi* (94.1% and 71.8%, respectively) from Clavet, as well as in *D. andersoni* from both Beechy (54.3% and 45.7%, respectively) and Clavet (66.7% and 33.3%, respectively).

Sequence alignments of short 16S rRNA gene fragments (~200 bp) belonging to several bacterial genera (i.e., *Rickettsiella, Pseudomonas, Sphingomonas* and *Staphylococcus*; Fig. 7.15-7.18) revealed differences in nucleotide composition, suggesting the possibility of different bacterial species or strains infecting different species of tick. For example, the *Rickettsiella* in *I. kingi* differed by 1 bp over an alignment length of 208 bp, when compared to the *Rickettsiella* in *I. sculptus* and *I. angustus* (Fig. 7.15). The *Rickettsiella* in *I. sculptus* differed by 2 bp over an

Locality (Coordinates) SSCP profile	Closest related genus	No. positive	Life cycle stage
Beechy, SK (50°53'N, 107°23'W)			
1	*Bacillus* *Rickettsia* *Staphylococcus*	1	1A
2	*Ralstonia*	5	3A, 2N
3	*Acidovorax* betaproteobacteria *Comamonas* *Pseudomonas* *Ralstonia* *Sphingobium* *Xanthomonadaceae*	14	11A, 3N
4	*Burkholderiales* *Comamonas* *Francisella* *Rickettsia*	3	2A, 1N
5	*Rickettsia*	9	1A, 8N
6	*Pseudomonas*	2	2N
7	*Sinomonas* *Thiomonas*	1	1A
Clavet, SK (51.9519°N, 106.4473°W)			
8	*Mesorhizobium* *Ralstonia* *Stenotrophomonas*	1	1L
9	*Pseudomonas* *Stenotrophomonas*	1	1L
10	*Pseudomonas* *Marinobacter*	1	1L
		Total: 38	

Table 7.5 Identification of bacteria in *Dermacentor andersoni* obtained by sequence analyses of re-amplified SSCP bands.

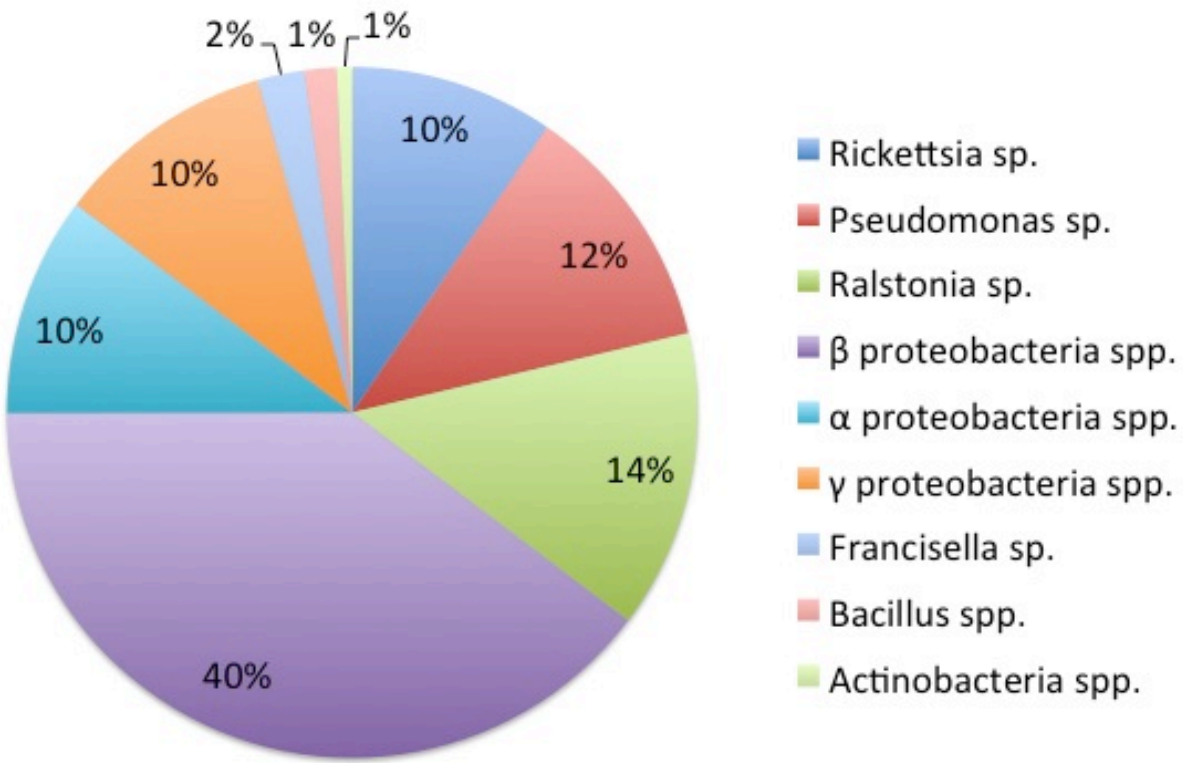


Fig. 7.12 Proportion of the different bacterial genera found in *D. andersoni* (Beechy).

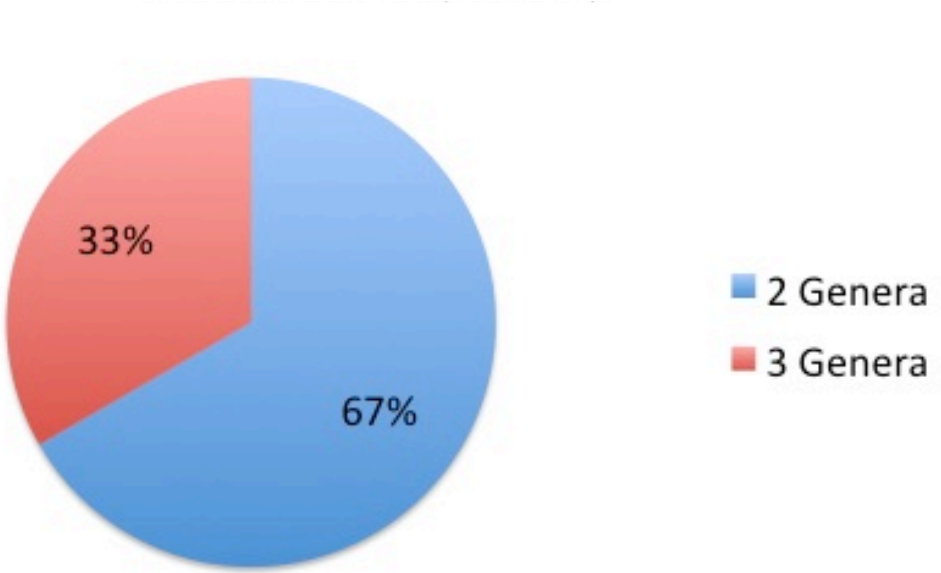


Fig. 7.13 Proportion of *D. andersoni* from Clavet (n = 3L) infected with two – three different bacterial genera.

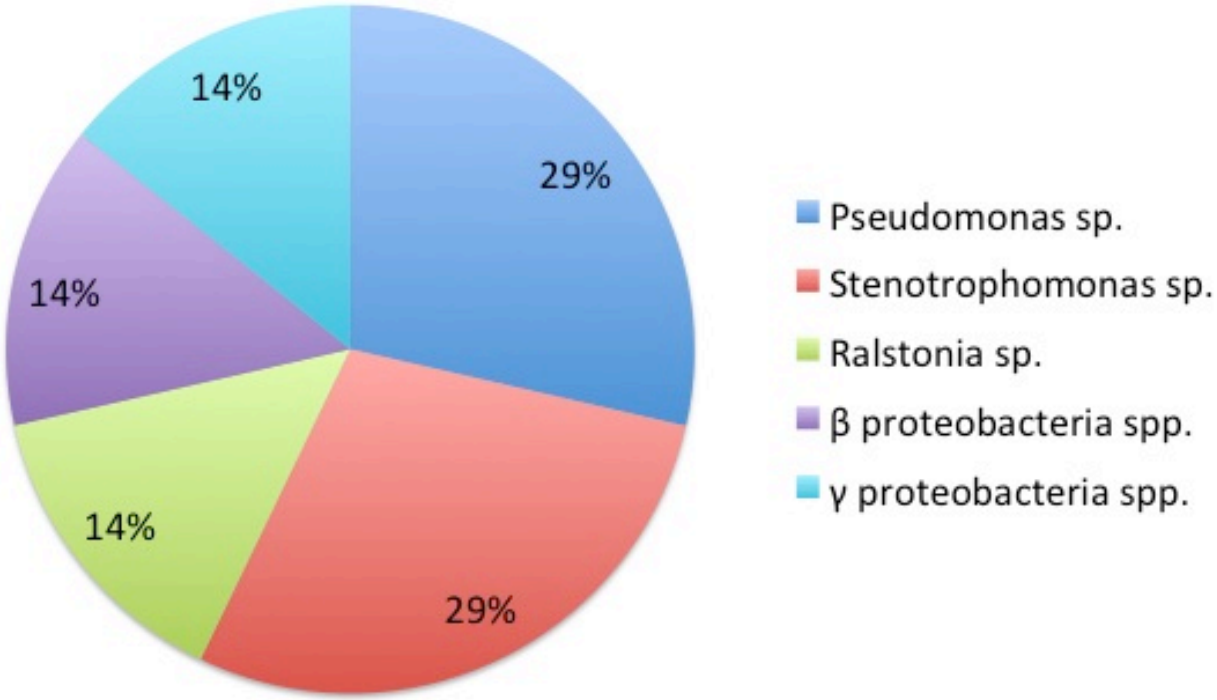


Fig. 7.14 Proportion of the different bacterial genera found in *D. andersoni* (Clavet).

Bacterial Class Bacterial Genus	*I. angustus* L*	 N*	 A*	*I. kingi* L	 N	 A	*I. sculptus* L	 N	 A	*D. andersoni* L	 N	 A
Alphaproteobacteria												
Devosia	-	-	-	+	-	-	-	-	-	-	-	-
Dichotomicrobium	-	-	-	+	-	-	-	-	-	-	-	-
Ensifer	-	-	-	+	-	-	-	-	-	-	-	-
Mesorhizobium	-	-	-	+	-	-	-	-	-	+	-	-
Rickettsia	+	+	-	-	-	+	-	-	-	-	+	+
Sphingobium	-	-	-	-	-	-	-	-	-	-	+	+
Sphingomonas	-	-	-	+	-	+	-	-	-	-	-	-
Betaproteobacteria												
Acidovorax	-	-	-	+	-	-	-	-	-	-	+	+
Azoarcus	-	-	-	+	-	-	-	-	-	-	-	-
Brachymonas	-	-	-	+	-	-	-	-	-	-	-	-
Burkholderia	-	-	-	+	-	+	-	-	-	-	-	-
Comamonas	-	-	-	+	-	-	+	-	-	-	+	+
Herbaspirillum	-	-	-	+	-	-	-	-	-	-	-	-
Propionvibrio	-	-	-	+	-	-	-	-	-	-	-	-
Ralstonia	-	-	-	+	+	+	-	-	-	+	+	+
Thiomonas	-	-	-	+	-	-	-	-	-	-	-	+
Order Burkholderiales	-	-	-	-	-	-	-	-	-	-	+	+
"Others"	-	+	-	-	-	-	-	-	-	-	+	+
Gammaproteobacteria												
E. of Curculio	+	+	-	-	-	-	-	-	-	-	-	-
Francisella	-	-	-	-	-	-	-	-	-	-	+	+
Klebsiella	-	-	+	-	-	-	-	-	-	-	-	-
Lysobacter	-	-	-	+	-	-	-	-	-	-	-	-
Marinobacter	-	-	-	+	-	+	-	-	-	+	-	-
Marinospirillum	-	-	-	+	-	+	-	-	-	-	-	-
Pasteurella	-	-	+	-	-	-	-	-	-	-	-	-
Pseudomonas	-	-	-	+	+	+	-	-	-	+	+	+
Rickettsiella	+	+	+	-	-	+	+	+	-	-	-	-
Stenotrophomonas	-	-	-	+	+	+	-	-	-	+	-	-
Thiohalocapsa	-	-	-	+	-	+	-	-	-	-	-	-
Order Xanthomonadaceae	-	-	-	-	-	-	-	-	-	-	+	+

* L = larva, N = nymph, A = adult, E = endosymbiont, + = bacteria present - = bacteria not detected

Table 7.6 Bacterial genera infecting the different life cycle stages (i.e., larvae, nymphs and adults) of *Ixodes angustus, I. kingi, I. sculptus* and *Dermacentor andersoni.* Continued on next page.

Bacterial Class Bacterial Genus	*I. angustus* L*	 N*	 A*	*I. kingi* L	 N	 A	*I. sculptus* L	 N	 A	*D. andersoni* L	 N	 A
Actinobacteria												
Nocardia	-	-	-	+	-	-	-	-	-	-	-	-
Rhodococcus	-	-	-	+	-	-	-	-	-	-	-	-
Sinomonas	-	-	-	-	-	-	-	-	-	-	-	+
Streptomyces	-	-	-	+	-	+	-	-	-	-	-	-
Williamsia	-	-	-	-	-	-	-	+	-	-	-	-
Bacilli												
Bacillus	-	-	-	-	-	-	-	+	-	-	-	+
Geobacillus	-	-	-	-	-	-	-	+	-	-	-	-
Lysinibacillus	-	-	-	-	-	-	-	+	-	-	-	-
Macrococcus	-	-	-	+	-	+	-	-	-	-	-	-
Staphylococcus	-	-	-	-	-	+	+	-	-	-	-	+
Clostridia												
Order Clostridiales	-	+	-	-	-	-	-	-	-	-	-	-

* L = larva, N = nymph, A = adult, E = endosymbiont, + = bacteria present - = bacteria not detected

Table 7.6 Bacterial genera infecting the different life cycle stages (i.e., larvae, nymphs and adults) of *Ixodes angustus, I. kingi, I. sculptus* and *Dermacentor andersoni.*

alignment length of 208 bp, when compared to the *Rickettsiella* in *I. angustus*. In addition, the *Rickettsiella* found in each of the three species of *Ixodes* differed by 1-3 bp when compared to the sequence of *Rickettsiella pyronotae* on GenBank (accession number HM017957).

The 16S rRNA sequences of two types of *Pseudomonas* were amplified from both *I. kingi* and *D. andersoni* individuals (Fig. 7.16). The sequence of *Pseudomonas* Type 1 from each tick species were identical over an alignment length of 209 bp, and BLAST searches revealed that they were identical to the 16S rRNA gene sequence of *Pseudomonas aeruginosa* (accession number JX843423). In comparison, the sequences of the *Pseudomonas* Type 2 from each tick species were also identical over an alignment length of 209 bp, and BLAST searches revealed that they were identical to the 16S rRNA gene sequence of *Pseudomonas fluorescens* (accession number JX960423). There were nine variable nucleotide positions in the aligned prokaryotic 16S rRNA gene sequences (210 bp) of the two types of *Pseudomonas*. A comparison of the 16S rRNA sequences of *Sphingomonas* revealed 10 bp differences between the *Sphingomonas* in *I. kingi* and the *Sphingomonas* in *D. andersoni* over an alignment length of 208 bp (Fig. 7.17). The *Sphingomonas* in *D. andersoni* differed by 3 bp in sequence to *Sphingomonas changhaiensis* (accession number JF459933) and the *Sphingomonas* in *I. kingi* had a 100% match on GenBank to an unknown *Sphingomonas* sp., *Sphingomonas azotifigens,* and *Caulobacter leidyia* (accession numbers JN697660, JN085438 and JF297626, respectively). Bacteria of the genus *Staphylococcus* were also found in *I. kingi, I. sculptus* and *D. andersoni*. When compared to the *Staphylococcus* in *I. kingi* and *I. sculptus,* the *Staphylococcus* in *D. andersoni* differed by 1 bp over an alignment length of 209 bp (Fig. 7.18). The *Staphylococcus* in *I. kingi* differed by 2 bp over an alignment length of 209 bp, when compared to the *Staphylococcus* in *I. sculptus*.

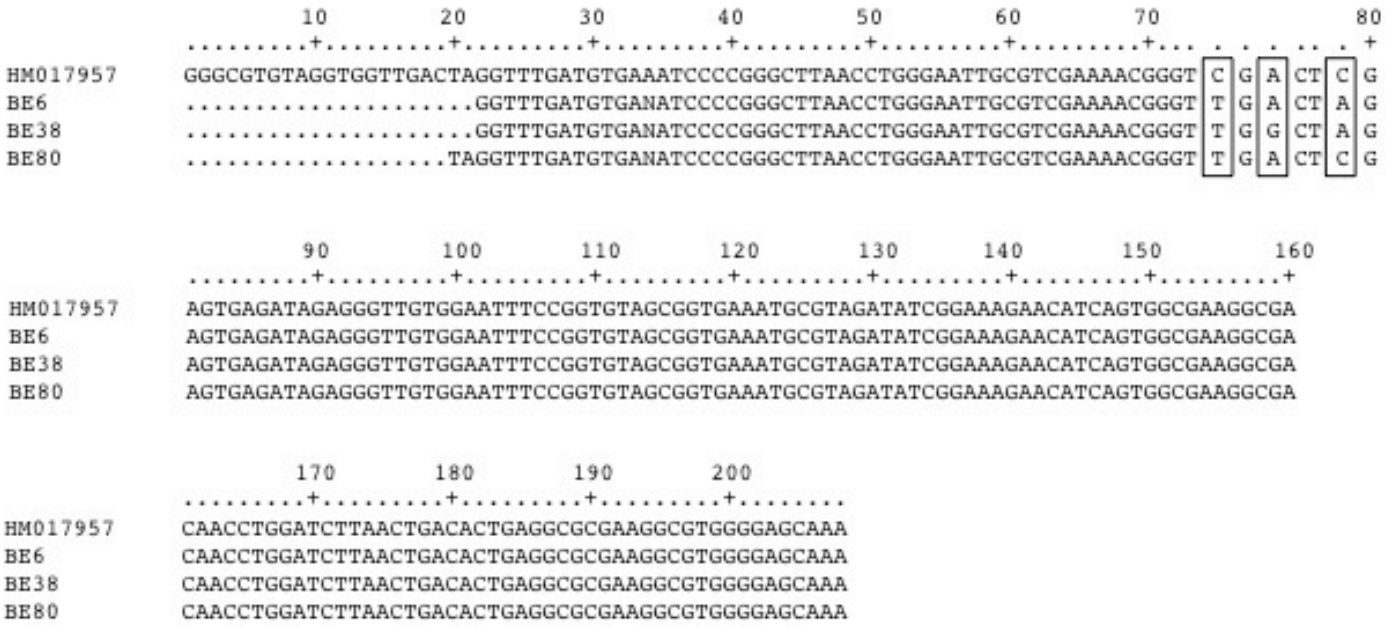


Fig. 7.15 Variable nucleotide positions in the aligned prokaryotic 16S rRNA gene sequences of *Rickettsiella* detected within *I. kingi* (BE6), *I. sculptus* (BE38) and *I. angustus* (BE80). Sequences are compared to that of *Rickettsiella pyronotae* (accession number HM017957).

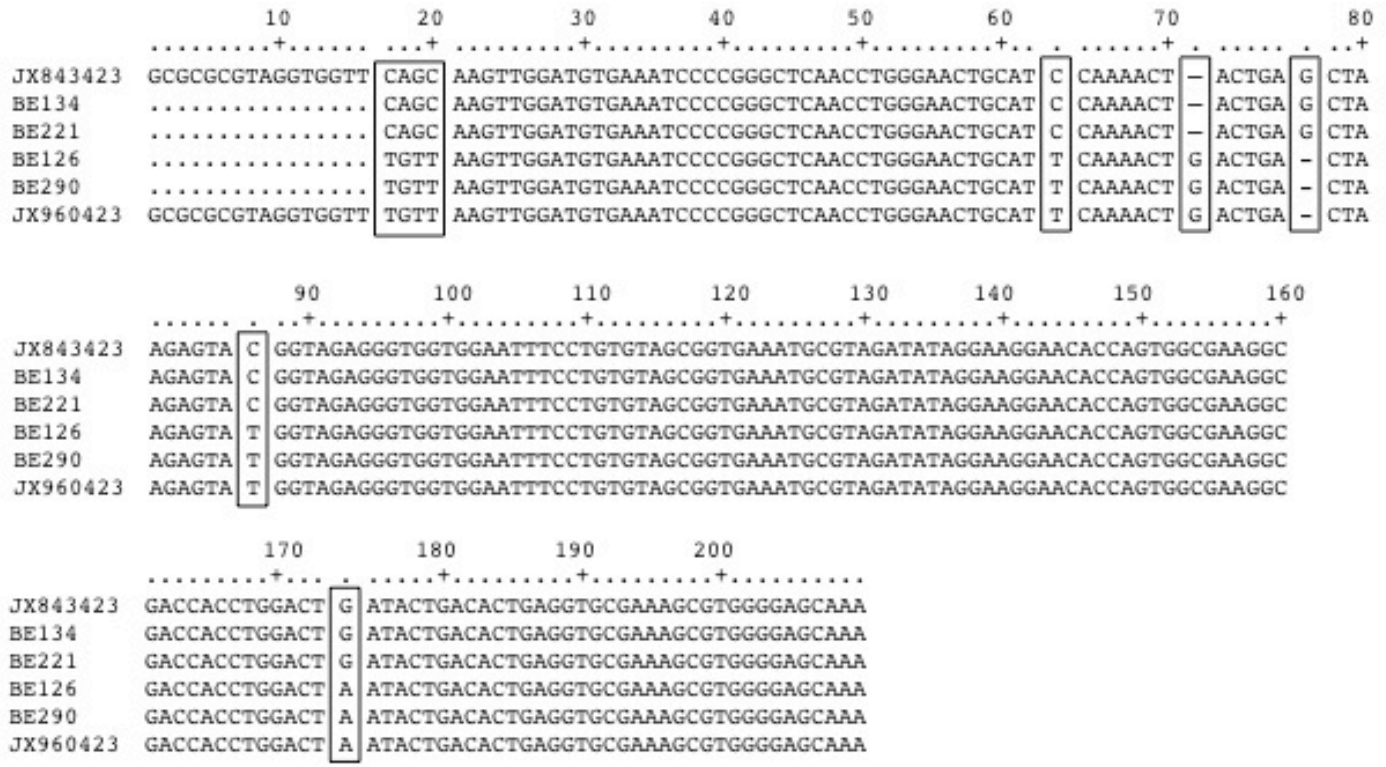


Fig. 7.16 Variable nucleotide positions in the aligned prokaryotic 16S rRNA gene sequences of two species of *Pseudomonas* both detected within *I. kingi* (BE221; BE290), and *D. andersoni* (BE134; BE126). Sequences are compared to those of *Pseudomonas aeruginosa* (accession number JX843423) and a *Pseudomonas fluorescens* (accession number JX960423).

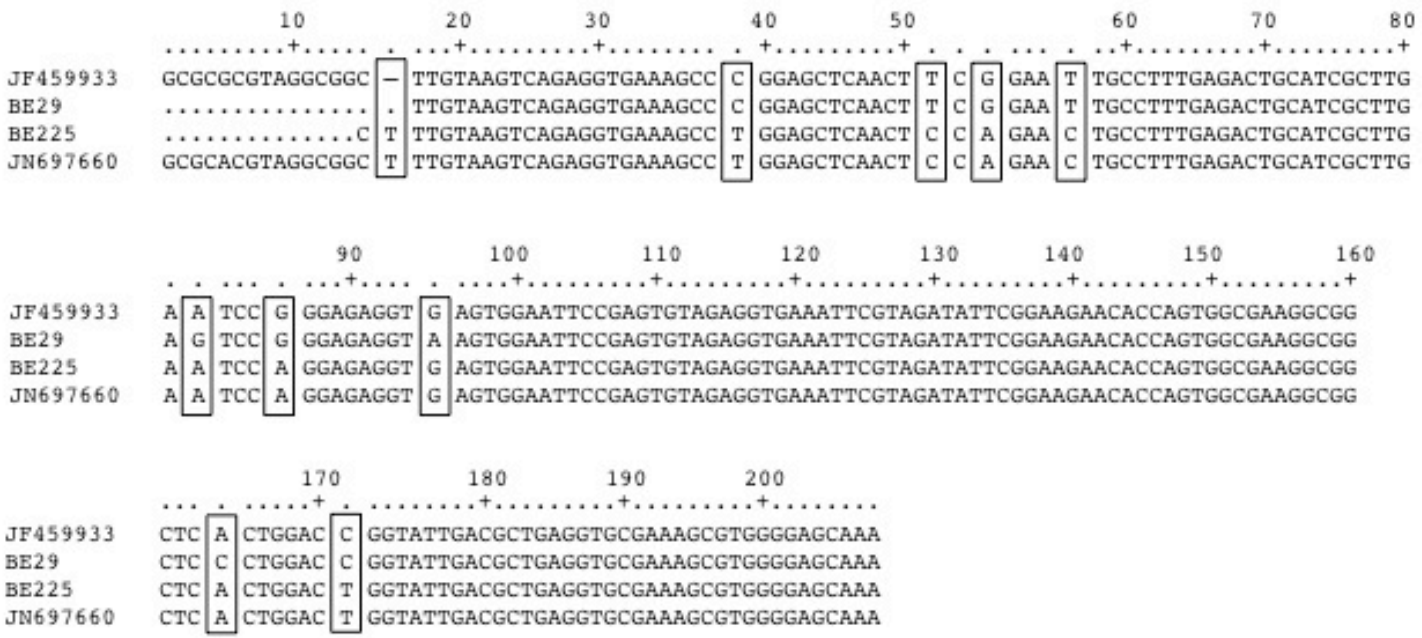


Fig. 7.17 Variable nucleotide positions in the aligned prokaryotic 16S rRNA gene sequences of *Sphingomonas* detected within *I. kingi* (BE225), and *D. andersoni* (BE29). Sequences are compared to those of *Sphingomonas changbaiensis* (accession number JF459933) and an unknown *Sphingomonas* species (accession number JN697660).

In addition, the *Staphylococcus* found in each of the three tick species differed by 1-2 bp when compared to the sequence of *Staphylococcus succinus* (accession number JX645230).

In contrast, sequence alignments of short 16S rRNA gene fragments belonging to several other bacterial genera (i.e., *Rickettsia, Ralstonia* and *Stenotrophomonas*; Figs 7.19- 7.21) did not reveal differences in nucleotide composition among amplicons of the same bacteria from different tick species. This suggests the possibility that some bacterial species are not tick species-specific, but occur within several species of tick. For example, the *Rickettsia* amplified from the gDNA of *I. kingi, I. angustus* and *D. andersoni* had identical sequences over an alignment length of 208 bp (Fig. 7.19). The 16S rRNA gene sequences of the *Rickettsia* in these three tick species were identical over this alignment length to *Rickettsia peacockii* (accession number NR_074488). The *Ralstonia* found in *I. kingi* and *D. andersoni* also had identical sequences over an alignment length of 207 bp (Fig. 7.20), and were identical in sequence to an unknown *Ralstonia* species (accession number EU475956). Bacteria of the genus *Stenotrophomonas* were also found in *I. kingi* and *D. andersoni,* and were identical to one another in 16S rRNA gene sequence over an alignment length of 208 bp (Fig. 7.21). A BLAST search revealed that these sequences were identical to the 16S rRNA gene sequence of *Stenotrophomonas maltophilia* (accession number JX426093).

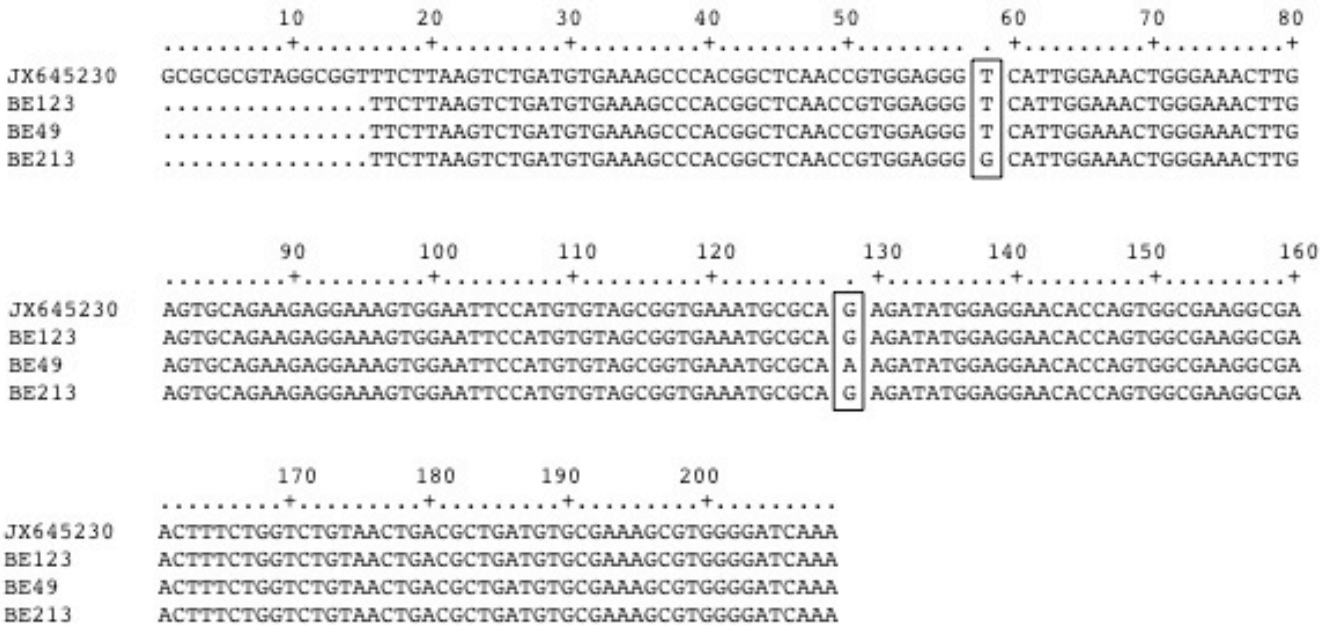


Fig. 7.18 Variable nucleotide positions in the aligned prokaryotic 16S rRNA gene sequences of *Staphylococcus* detected within *I. kingi* (BE213), *I. sculptus* (BE49) and *D. andersoni* (BE123). Sequences are compared to that of *Staphylococcus succinus* (accession number JX645230).

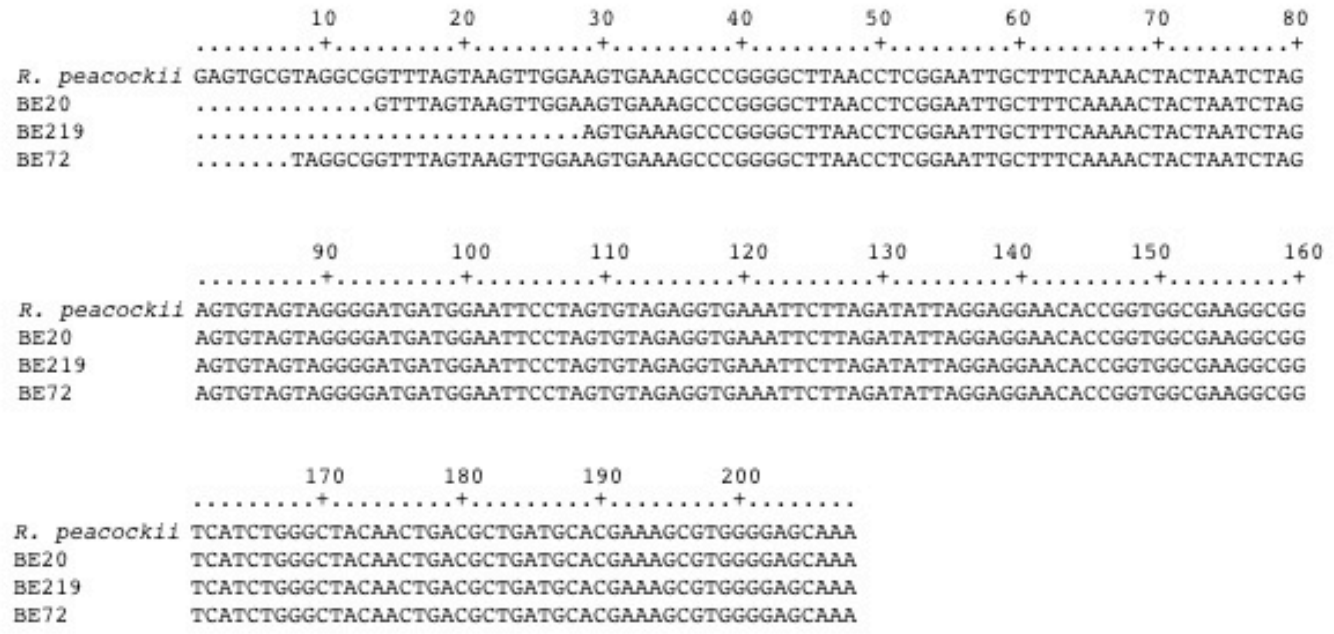


Fig. 7.19 Sequence alignment of the prokaryotic 16S rRNA gene sequences of *Rickettsia* detected within *I. kingi* (BE219), *I. angustus* (BE72) and *D. andersoni* (BE20). Sequences are compared to that of *Rickettsia peacockii* (accession number NR_074488).

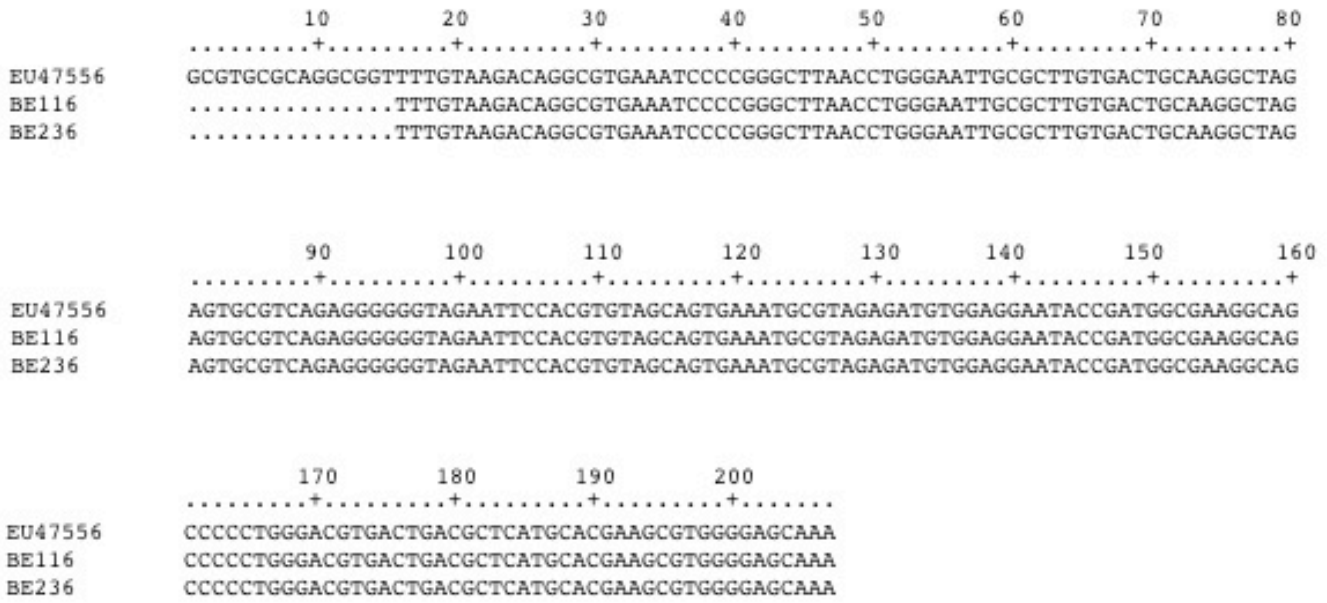


Fig. 7.20 Sequence alignment of the prokaryotic 16S rRNA gene sequences of *Ralstonia* detected within *I. kingi* (BE236), and *D. andersoni* (BE116). Sequences are compared to that of an unknown *Ralstonia* species (accession number EU475956).

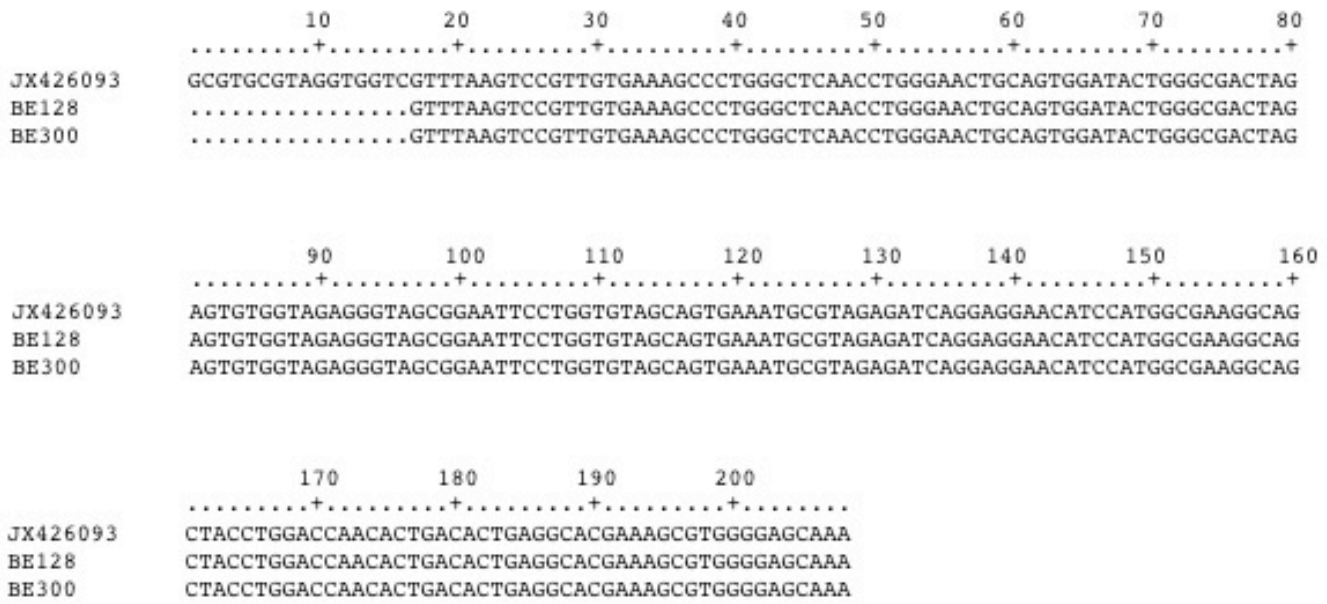


Fig. 7.21 Sequence alignment of the prokaryotic 16S rRNA gene sequences of *Stenotrophomonas* detected within *I. kingi* (BE300), and *D. andersoni* (BE128). Sequences are compared to that of *Stenotrophomonas maltophilia* (accession number JX426093).

7.5 **Discussion**

7.5.1 **Co-occurrence of microbes within individual ticks**

The numbers of co-occurring microbes within individual ticks differed among the four tick species. The majority of *I. angustus* (81.3%) and *I. sculptus* (83.4%) tested were infected with a single genus of bacteria, whereas the highest number of bacterial genera detected by PCR within individuals of these two tick species was three. Similar results were found in a study on the microbial communities of *Amblyomma americanum* where the majority of ticks were infected with only two or three microbial genera, with additional microbes being detected only sporadically (Clay *et al.,* 2008). Interestingly, 45.6% of the *D. andersoni* from Beechy were infected with only a single genus of bacteria, while 40% were infected with seven genera of bacteria. In comparison, just over half (53%) of the *I. kingi* from Clavet were infected with 11 bacterial genera, whereas 22.4% of individuals were infected with four genera. Previous studies on co-infections in ticks (Mixson *et al.,* 2006; Steiner *et al.,* 2008; Carmichael & Fuerst, 2010; Harrus *et al.,* 2010) have reported the presence of five or less bacterial species; however, these studies involved PCR-analyses using genus or species-specific primers that targeted specific bacterial taxa. Other studies employing a general 16S rRNA gene analysis (Clay *et al.,* 2008; Heise *et al.,* 2010) or a tag-encoding pyrosequencing approach (Andreotti *et al.,* 2011; Lalzar *et al.,* 2012; Hawlena *et al.,* 2013) have identified many more tick-infecting bacteria. Nine microbial taxa were identified in *I. scapularis* from northeastern U.S.A. (Moreno *et al.,* 2006), 15 bacterial genera were identified in *Rhipicephalus turanicus* from central Israel (Lalzar *et al.,* 2012), and 121 bacterial genera have recently been associated with *R. microplus* from southern Texas, U.S.A. (Andreotti *et al.,* 2011). Two studies on the bacterial diversity of *Amblyomma americanum* from the southeastern and midwestern U.S.A. detected 10 to 16 different microbial

taxa (Clay et al., 2008; Heise *et al*., 2010). These studies demonstrate a high frequency of co-infection, as well as an extremely variable relationship between the number of co-occurring microbes and the species of tick being infected. This raises the possibility of microbial interactions unique to a tick species or more specifically, individual ticks, which has been documented previously (Macaluso *et al*. 2002; de la Fuente *et al*. 2003).

7.5.2 **Bacterial diversity**

The results of the present study revealed differences in the number of different genera found within each species of tick. Seven different microbial genera were found in both *I. angustus* and *I. sculptus,* however *Rickettsiella* was the only bacterial genus these two tick species had in common. A total of 14 different bacterial genera were found in the *D. andersoni,* but the highest bacterial diversity was found in the *I. kingi,* which were PCR-positive for 16S rDNA of 26 different microbial genera. The majority of the bacteria infesting both the *I. kingi* and the *D. andersoni* belonged to the bacterial class Betaproteobacteria, whereas the majority of genera infesting the *I. sculptus* belonged to the bacterial class Bacilli.

Among the genera found in the three *Ixodes* species within this study, *Acidovorax, Bacillus, Burkholderia, Nocardia, Propionibacterium, Pseudomonas, Ralstonia, Rickettsia, Sphingobacterium, Staphylococcus, Stenotrophomonas,* and *Williamsia* are genera having been previously reported in a related species, the blacklegged tick, *I. scapularis* (Murrell *et al*., 2003; Moreno *et al*., 2006; Steiner *et al*., 2008; Yuan, 2010; Hawlena *et al*., 2013). Previous bacterial genera reported in *D. andersoni* that were also found in this study include *Bacillus, Francisella* and *Rickettsia* (Steinhaus, 1942; Dergousoff & Chilton, 2012; Dergousoff & Chilton, 2013). However, the identities of some bacteria in the present study need to be interpreted with caution

as this gene region was only used for genus-level identification, with some genera (e.g., *Ralstonia* and *Cupriavidus*) having identical sequences for this small part of the 16S rRNA gene. For the present study, any sample that had a sequence 100% identical to that of *Ralstonia* and *Cupriavidus* was considered as *Ralstonia*, since this bacterial genus has been previously reported in ticks (Moreno *et al*., 2006; Lalzar *et al*., 2012).

One unusual finding was the absence of *Coxiella*-like endosymbionts from all four species of tick tested in this study, as these bacteria have been detected in a variety of tick species (Noda *et al*. 1997; Mediannikov *et al*. 2003; Klyachko *et al*. 2007). In the study by Clay *et al*. (2008), a *Coxiella*-like symbiont was found in every *A. americanum* tick tested (n=900). A similar prevalence of infection was found in several other studies on *A. americanum* (Jasinskas *et al*., 2007; Klyachko *et al*. 2007; Heise et al., 2010). A *Coxiella*-like endosymbiont in *Haemaphysalis concinnae* also occurs at a high prevalence (Mediannikov *et al*. 2003), but another *Coxiella*-like endosymbiont occurs at a low prevalence in *Rhipicephalus sanguineus* (Bernasconi *et al*. 2002). However, examination of the literature on the bacterial communities of species within the genus *Ixodes* (Murrell *et al*., 2003; Moreno *et al*., 2006; Steiner *et al*., 2008; Yuan, 2010; Hawlena *et al*., 2013), revealed that *Coxiella*-like endosymbionts have only been detected in one species, *I. scapularis* (Yuan, 2010). This suggests that *Ixodes* may not be suitable hosts for *Coxiella*-like endosymbionts. These bacteria were also absent in all *D. andersoni* in this study; however, *Coxiella*-like symbionts have recently been detected within *D. silvarum* (Liu *et al*., 2013), indicating the presence of this microbe within ticks of the genus *Dermacentor*. Another microbe that was absent in all four species of tick was the *Arsenophonus*-type bacterium that has been found previously in Canadian populations of *D. andersoni* and *D. variabilis* (Dergousoff & Chilton, 2010). Additional studies on larger sample sizes of these four tick

species from different geographical areas are needed to determine the prevalence of these endosymbionts.

7.5.3 **Prevalence of specific microbes**

In the present study, *Rickettsiella, Rickettsia, Ralstonia* and *Pseudomonas* were frequently detected in the four tick species. In general, they were usually found in all the feeding life cycle stages (i.e., adult, nymph and larva) of ticks. For example, *Rickettsiella* were found in all three life cycle stages of *I. angustus,* larvae and nymphs of *I. sculptus,* and the single adult *I. kingi* from Beechy. There were no adult *I. sculptus,* nor larval or nymphal *I. kingi* from Beechy that were collected; therefore, the possibility that *Rickettsiella* bacteria may have been present in all life cycle stages of *I. sculptus* and *I. kingi* cannot be ruled out. The presence of unique *Rickettsiella* 16S rRNA gene sequences in each species of *Ixodes* raises the possibility of there being three different species of *Rickettsiella* infecting these ticks. This is examined further in Chapter 10. Given that there is geographical and occasional vertebrate host overlap between these tick species, a high degree of host specificity of the bacteria for each tick would have to exist if different *Rickettsiella* species were infecting each species of *Ixodes*. However, intraspecific variation has been documented in other bacterial genera (e.g., *Anaplasma*; Derdáková *et al*., 2011); therefore, a larger 16S rRNA gene fragment and/or additional genetic markers would need to be characterized in order to determine if multiple species of *Rickettsiella* are indeed present. Although *Rickettsiella* was not present within any *D. andersoni* in this study, additional ticks from different localities should be screened for the presence of this bacterium and included in future analyses as a possible negative control.

Larval and nymphal *I. angustus,* as well as adult *I. kingi* from both Clavet and Beechy

tested positive for 16S rDNA of the genus *Rickettsia*. It is interesting that *Rickettsia* rDNA was not found in adult *I. angustus,* nor in larval and nymphal *I. kingi* from either locaity in Saskatchewan. Different species of rickettsiae have different transmission methods, some being transmitted transovarially (i.e., passed from parent to offspring) or transtadially (i.e., from one life stage to another). Additional samples, from all life cycle stages, need to be tested in order to gain a better understanding of the type of transmission demonstrated by the rickettsiae infesting *I. angustus* and *I. kingi* in this study. Rickettsial rDNA was also detected in *D. andersoni* nymphs and adults from Beechy, but was not detected in *D. andersoni* larvae from Clavet. However, once again the possibility cannot be ruled out that *Rickettsia* bacteria could be infesting all three life cycle stages of *D. andersoni* from both localities, as larvae were not collected from Beechy, and there were low numbers of larvae and no nymphs or adults collected from Clavet. Given that *D. andersoni* is not only a suitable host for the endosymbiont, *R. peacockii,* but also the human pathogen *R. rickettsii* (Burgdorfer *et al*. 1981; Niebylski *et al*. 1997; Macaluso *et al*. 2002; Carmichael & Fuerst, 2010), further studies are needed to identify the *Rickettsia* in *D. andersoni* from Beechy and Clavet, and determine if they are endosymbiotic or pathogenic. The presence of identical *Rickettsia* 16S rRNA gene sequences in *D. andersoni, I. kingi* and *I. sculptus* is indicative of all three species being infected with the same species of *Rickettsia*. However, the short 16S rRNA gene fragment amplified in this study is identical in many species of *Rickettsia* (e.g., *R. rickettsii* and *R. montanensis*). Additional genetic markers need to be characterized to determine the identity of the *Rickettsia* in these three species of tick. This is investigated further in Chapters 8 and 9 of this thesis.

Bacteria of the genera *Ralstonia, Pseudomonas* and *Stenotrophomonas* were found in all feeding life cycle stages of *I. kingi*. All three bacterial taxa were also found in adult and nymphal

D. andersoni from Beechy, and larval *D. andersoni* from Clavet; suggesting that all life cycle stages of *D. andersoni* are suitable hosts for these bacteria. However, all three of these bacterial genera were absent in the *I. sculptus* screened, even when the *I. sculptus* were found on the same host individuals as infected *I. kingi* and *D. andersoni*. Perhaps *I. sculptus* is not a suitable host for these three bacterial taxa, or there may exist bacterial competition within the arthropod host, resulting in a different microbial community when compared to the other two tick species feeding on the same mammal host.

7.5.4 **Bacterial communities of tick species feeding on the same host**

Ticks of different species feeding on the same host individual provides an opportunity to examine fundamental ecological and evolutionary questions relating to the structure and composition of bacteria in ticks. The influence of various factors (e.g., vertebrate host, environment, tick vector) on bacterial community composition of different tick species can be examined, and the specificity of a bacterium for its tick host can be assessed. In this study, four small mammal hosts (i.e., 2 ground squirrels and 2 pocket gophers) were infected with multiple species of tick. One Richardson's ground squirrel (*Spermophilus richardsonii*) from Beechy was parasitized by *I. kingi* (n=1), *I. sculptus* (n=11) and *D. andersoni* (n=33). Bacteria of the genus *Rickettsiella* were found in the *I. kingi* individual, and eight *I. sculptus*. However, *Rickettsiella* was absent in *D. andersoni*. Some other bacteria found within the *D. andersoni* individuals (e.g., *Francisella*) were not found in either of the *Ixodes* species. Another *S. richardsonii* from the same location was parasitized by two *I. sculptus* and two *D. andersoni*. Once again, there was no sharing of bacteria between these two species. Bacteria of the genus *Rickettsiella* was detected in the *I. sculptus,* and *Rickettsia, Staphylococcus* and *Bacillus* were found in the *D. andersoni*.

These results indicate that it may not be the vertebrate host, but the tick, that influences the bacterial community composition. Similar results have been found in a recent study on the bacterial community composition of fleas and ticks (Hawlena *et al*., 2013).

In contrast, two northern pocket gophers (*Thomomys talpoides*) from Clavet were parasitized by both *I. kingi* and *D. andersoni* that were infected with several of the same bacterial genera. Thirty-one *I. kingi* and one *D. andersoni* from one *T. talpoides* were all PCR-positive for 16S rDNA of *Pseudomonas* and *Stenotrophomonas*. In addition, four *I. kingi* and one *D. andersoni* from a second *T. talpoides* shared the bacterial genera *Ralstonia, Stenotrophomonas* and *Mesorhizobium*. Additional studies are needed to determine if these bacteria belong to the same species, or if different species of the same bacterial genera are specific to these small mammal ticks.

7.5.5 **Summary**

Ticks are important vectors of disease-causing agents; therefore, an understanding of the identity and prevalence of the bacterial species they harbor and can potentially transmit is of both medical and veterinary importance. It is also important in our understanding of the ecological and evolutionary factors that influence the structure and composition of microbial communities. In the present study, there were significant differences in the microbial communities of the *Ixodes* and *Dermacentor* species. Factors such as the tick species, tick life cycle stage and geographic location, and to a lesser extent, type of small mammal host, appear to have an important role in determining the bacterial community structures of the tick species examined. However, these results only represent a preliminary examination of the bacteria present within these four tick species because species-level characterization of bacterial taxa (e.g., *Rickettsia*

and *Rickettsiella*) are needed to determine if different tick species share the same species of bacteria. This would also provide insight into the potential interactions among microbes as well as the specificity of these microbes to their tick hosts. Additional studies are needed to determine the functional role of the microbes detected in this study for each tick species examined, as well as determine their effects on human and animal health.

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Chapter 8 Detection of a novel *Rickettsia* (Alphaproteobacteria: Rickettsiales) in rotund ticks (*Ixodes kingi*) from Saskatchewan, Canada[5]

8.1 Abstract

A novel *Rickettsia* was detected in the rotund tick, *Ixodes kingi* Bishopp, 1911, based on comparative DNA sequence analyses of four genes: the rickettsial-specific 17-kDa antigen gene, citrate synthase gene (*gltA*), the outer surface membrane protein A gene (*ompA*), and the 16S rRNA gene. The rickettsiae in *I. kingi* differed in nucleotide sequence from those of other *Rickettsia* species by 5.8-18.3% for the 17-kDa gene, 0.9-13.9% for *gltA,* 5.5-22.8% for *ompA*, and 0.9-1.6% for the 16S rRNA gene. Phylogenetic analyses of the sequence data revealed that this putative new species of *Rickettsia,* provisionally named *Candidatus* Rickettsia kingi, does not belong to the spotted fever group or typhus group of rickettsiae but represents a sister taxon to *R. canadensis* and *Candidatus* Rickettsia tarasevichiae. This novel *Rickettsia* was found in 60 of the 87 (69%) ticks examined, which included all feeding life cycle stages of *I. kingi*. Although adult *I. kingi* occasionally parasitize dogs and humans, it remains to be determined if this *Rickettsia* is pathogenic to these host species.

[5] Part of this chapter was reprinted from:
Anstead CA, Chilton NB. 2013. Detection of a novel *Rickettsia* (Alphaproteobacteria: Rickettsiales) in rotund ticks (*Ixodes kingi*) from Saskatchewan, Canada. Ticks Tick-borne Dis. **4**:202-206, with permission from Elsevier Journals.

8.2 **Introduction**

Rickettsia are obligate intracellular bacteria that are transmitted to vertebrates by arthropod vectors that include ticks, fleas, lice and mites (Fournier & Raoult, 2009; Merhej & Raoult, 2011). These alphaproteobacteria are the causative agents of disease (e.g., spotted fever and typhus) in many parts of the world (Fournier & Raoult, 2009; Merhej & Raoult, 2011). There are at least 30 recognized species of *Rickettsia*, 19 of which are considered human pathogens (Merhej & Raoult, 2011). A number of other putative species of *Rickettsia* have also been proposed based on sequence differences in two or more genes (e.g., Almeida *et al.,* 2011; Izzard *et al.,* 2009; Pacheco *et al.,* 2011; Phan *et al.,* 2011; Shpynov *et al.,* 2003). Historically, the genus *Rickettsia* has been divided into the spotted fever group (SFG), the typhus group (TG), *R. canadensis* and *R. bellii* (Fournier & Raoult, 2009). The SFG contains the majority of species within the genus, while *R. typhi* and *R. prowazekii* are the members of the TG (Fournier & Raoult, 2009). The TG is associated primarily with lice and fleas, whereas *R. canadensis*, *R. bellii* and the SFG (except for *R. akari* and *R. felis*) use ixodid ticks as vectors (Fournier & Raoult, 2009; Merhej & Raoult, 2011).

In North America, at least six species of *Ixodes* (i.e., *I. scapularis*, *I. pacificus*, *I. cookei*, *I. dentatus, I. brunneus* and *I. texanus*), all of which are known to parasitize rodents (Allan, 2001; Bishopp & Trembley, 1945; Kolonin, 2007), have been shown to contain SFG rickettsiae (Allan, 2001; Anderson *et al.,* 1986; Billings *et al.,* 1998; Clifford *et al.,* 1969; Magnarelli *et al.,* 1985; Phan *et al.,* 2011). The rotund tick, *Ixodes kingi*, is also a common parasite of rodents (i.e., murids, heteromyids, geomyids and sciurids), as well as other vertebrates, in western North America (Allan, 2001; Bishopp & Trembley, 1945; Gregson, 1971; Salkeld *et al.,* 2006). Although rotund ticks are known to be vectors of several pathogens, including *Coxiella burnetii*,

the causative agent of Q-fever, and *Francisella tularensis*, the causative agent of tularemia (Sidwell *et al.,* 1964; Thorpe *et al.,* 1965), there are no published reports of rickettsiae in *I. kingi*. In this paper, we report the discovery of a new species of *Rickettsia* in all feeding life cycle stages of *I. kingi* from a locality in central Saskatchewan, Canada.

8.3 **Materials and Methods**

For this study, a total of 87 *I. kingi* (i.e., 3 females, 1 male, 2 nymphs and 81 larvae) were collected from northern pocket gophers (*Thomomys talpoides*) trapped near Clavet, Saskatchewan (Anstead & Chilton, 2011). Total genomic (g) DNA was extracted from each tick as described by Dergousoff and Chilton (2007). The presence of rickettsiae in ticks was determined by nested (n)-PCR targeting a 434-base pair (bp) fragment of the rickettsial-specific 17-kDa antigen gene using primers 17k-5 and 17k-3 (first phase) and then primers 17KD1 and 17KD2 (second phase) (Heise *et al.,* 2010). All PCRs were conducted in 25 μl volumes with 2 μl of gDNA template used in phase 1, and 1 μl of purified amplicon (using the protocol of Dergousoff & Chilton, 2012) from phase 1 used as the template for phase 2. Negative (i.e., no gDNA) controls were included in each set of reactions. The PCR conditions used were: 95°C for 5 min, 35 cycles of 95°C for 60 sec, 58°C for 60 sec and 72°C for 60 sec, and a final cycle of 72ºC for 5 min (for phase 1), and 95°C for 5 min, 30 cycles of 95°C for 30 sec, 61°C for 30 sec and 72°C for 30 sec, and a final cycle of 72ºC for 5 min (for phase 2). Amplicons were subjected to electrophoresis on SYBR® Safe-stained 1.5% agarose-TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) gels and their banding patterns were visualized by UV transillumination. Single strand conformation polymorphism (SSCP) analysis (Gasser *et al.,* 2006) was then used as a pre-screen to examine for genetic variation before selecting representative amplicons for

DNA sequencing. Amplicons of the 17-kDa gene for *R. peacockii* and *R. montanensis* from *Dermacentor andersoni* and *D. variabilis* (respectively) were used on gels as mobility controls. Five amplicons of the rickettsiae in *I. kingi* were purified and subjected to automated DNA sequencing using primers 17KD1 and 17KD2 in separate reactions.

Four additional genetic markers were used to characterize the rickettsiae in *I. kingi*. Part (382-bp) of the citrate synthase gene (*gltA*) was amplified from the gDNA of 12 rickettsial-infected ticks using primers *Rp*CS.877p and *Rp*CS.1258n (Regnery *et al.,* 1991) and the conditions used by Dergousoff *et al.,* (2009). Then, 532-bp of the outer membrane protein A gene (*ompA*) was amplified from the gDNA of 3 rickettsial-infected ticks using primers *Rr*190.70p and *Rr*190.602n (Regnery *et al.,* 1991). The same PCR conditions were used as for *gltA*, except that the number of amplification cycles was increased to 30. Part (556-bp) of the 16S rRNA gene of 2 rickettsial-infected ticks was amplified using primers 16S-Rick-F1 (5'–TGGCTCAGAACGAACGCTATCGG-3') and 16S-Rick-R2 (5'-ACCTCTACACTAGAAATTCCATCA-3') and the following conditions: 95°C for 5 min, 30 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec, and then 72ºC for 5 min. Amplicons of *gltA*, *ompA* and the 16S rRNA gene derived from the gDNA of two or three rickettsial-infected ticks were purified and subjected to DNA sequencing as described above. An attempt was made to amplify ~800-bp of the outer membrane protein B gene (*ompB*) from the rickettisae in *I. kingi* using primers 120-2788 and 120-3599 and the PCR conditions of Roux and Raoult (2000), except that the number of cycles was increased to 35, and the annealing temperature raised from 50°C to 52°C. The gDNA of *R. peacockii*-infected *D. andersoni* were included as positive controls in the PCR analyses.

BLAST searches (GenBank) were performed on the DNA sequences of each gene to determine the genetic similarity of the rickettsiae in *I. kingi* to the different taxa within the genus *Rickettsia*. The DNA sequences of the rickettsiae in *I. kingi* were aligned manually with those of other *Rickettsia* species, and phylogenetic analyses were performed separately on the sequence data of each gene using the neighbor joining (NJ) and maximum parsimony (MP) methods in PAUP (Swofford, 2003). For the MP analyses of the 17-kDa gene, *gltA* and the 16S rRNA gene, characters were treated as unordered and were equally weighted; alignment gaps were treated as 'missing' characters and the sequences of *R. bellii, Ehrlichia ruminantium* and *Orientia tsutsugamushi* were used as the outgroups (respectively). Midpoint rooting was used in the MP analysis of the *ompA* sequence data. Exhaustive searches with TBR branch swapping were used to infer the shortest trees. The length, consistency index excluding uninformative characters, and the retention indices of each most parsimonious tree were recorded. Bootstrap analyses (1000 replicates for the NJ analyses and 100 replicates for MP analyses) were conducted to determine the relative support for clades in the consensus trees.

6.4 **Results**

A single band of the expected size (~450-bp) for the partial rickettsial 17-kDa gene was detected on agarose gels for amplicons derived from the gDNA of 60 of the 87 (69%) ticks. These 60 PCR-positive samples represented all feeding life cycle stages of *I. kingi* (i.e., 55 larvae, 1 nymph and 4 adults). No bands were detected on agarose gels for the negative control (i.e., no gDNA) samples. The banding patterns of all 60 PCR-positive samples on SSCP gels were identical to one another but differed to those of *R. peacockii* and *R. montanensis* (data not shown). There were no differences in the DNA sequences of five representative samples

(GenBank accession number HE647694), but each differed in sequence by 5.8-18.3% (i.e., 21-72 bp) when compared to those of the 17-kDa gene for taxa within the genus *Rickettsia*. A BLAST search of the 394 bp 17-kDa gene sequence of *Rickettsia* in *I. kingi* revealed that it was genetically most similar (94.9%) to the sequence of *R. canadensis* (Table 8.1). The NJ tree produced from the phylogenetic analysis of the aligned sequence data revealed strong statistical support (98% bootstrap value) for the *Rickettsia* in *I. kingi* representing a sister taxon to *R. canadensis* and *Candidatus* R. monteiroi to the exclusion of members of the SFG and TG (Fig. 8.1). An MP analysis of the sequence data set (i.e., 100 cladistically informative characters) produced 41 equally most-parsimonious trees (strict consensus tree not shown), with a length of 297, a CI of 0.58 and a RI of 0.72. As with the NJ tree, there was support (71% bootstrap value) for the inclusion of *Rickettsia* in *I. kingi* within a clade that included *R. canadensis* and *Candidatus* R. monteiroi (Fig. 8.1).

There were no differences in the DNA sequences of *gltA* (GenBank accession number HE647692) for amplicons derived from the gDNA of three rickettsial-infected *I. kingi*. However, they differed in sequence by 0.9% to 13.9% (i.e., 3-48 bp) when compared to the *gltA* sequences of species of *Rickettsia* available on GenBank. The closest match in sequence was to a sequence of *Candidatus* R. tarasevichiae (Table 8.1). The NJ tree produced from analyses of the *gltA* sequence data (342 alignment positions) showed that this putative new species of *Rickettsia* formed a clade, with strong bootstrap support (84%), with *R. canadensis, Candidatus* Rickettsia monteiroi, *Candidatus* R. tarasevichiae and an undescribed species of *Rickettsia* (sp. H820) from north-eastern China, to the exclusion of other members in the genus (Fig. 8.2).

Gene	*Rickettsia* (Genbank Accession no.)	% sequence similarity
17-kDa gene	*R. canadensis* (CP000409)	94.9 (374 of 394 bp)
	Candidatus R. monteiroi (FJ269036)	92.1 (363 of 394 bp)
	Candidatus R. hoogstraalii (EF629538)	87.8 (346 of 394 bp)
	R. monacensis (EF380355)	87.3 (344 of 394 bp)
gltA	*Candidatus* R. tarasevichiae (EF445981)	99.1 (338 of 341 bp)
	Rickettsia sp. H820 (JF714219)	98.8 (336 of 340 bp)
	R. canadensis (CP000409)	97.9 (334 of 341 bp)
	R. asiatica (AB297808)	96.8 (330 of 341 bp)
	Candidatus R. monteiroi (FJ269035)	96.5 (329 of 341 bp)
	R. helvetica (JQ669952)	96.5 (329 of 341 bp)
ompA	*Rickettsia* sp. H820 (JF714220)	94.5 (464 of 491 bp)
	R. canadensis (CP000409)	86.0 (425 of 494 bp)
	R. tamurae (DQ103259)	86.0 (426 of 496 bp)
	Candidatus R. cooleyi (AF031535)	85.5 (423 of 495 bp)
16S rRNA gene	rickettsial endosymbiont (AY961085)	99.3 (549 of 553 bp)
	R. massiliae (CP003319)	99.1 (548 of 553 bp)
	Most other SFG[a] *Rickettsia*	98.9 (547 of 553 bp)
	Candidatus R. tarasevichiae[b] (AM418457)	98.8 (398 of 403 bp)
	R. canadensis (CP000409)	98.4 (544 of 553 bp)

[a] SFG = spotted fever group, [b] only a partial sequence available for this taxon

Table 8.1 Closest relative sequences to the partial 17-kDa gene, *gltA*, *ompA* and 16S rRNA gene, sequences of the *Rickettsia* detected in the *I. kingi* from Saskatchewan, Canada

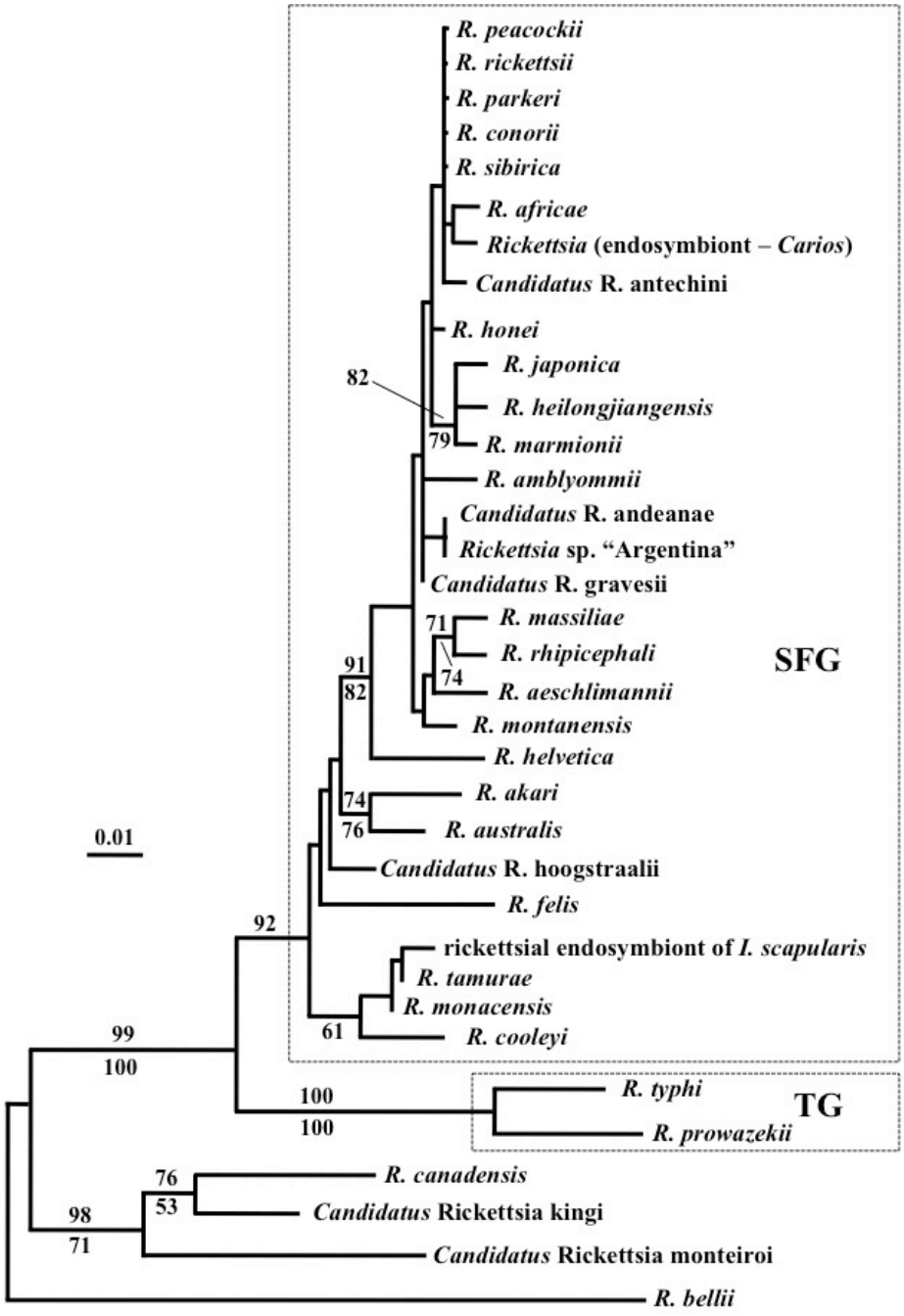

Fig. 8.1 Neighbor-joining tree depicting the relationships of the sequences for the rickettsial 17-kDa gene of *Candidatus* Rickettsia kingi and those of other *Rickettsia* species. SFG and TG refer to the spotted fever group and typhus group of *Rickettsia*, respectively. The scale bar represents the inferred substitutions per nucleotide site. The relative support for clades in the tree produced from the NJ and MP analyses are indicated above and below branches, respectively.

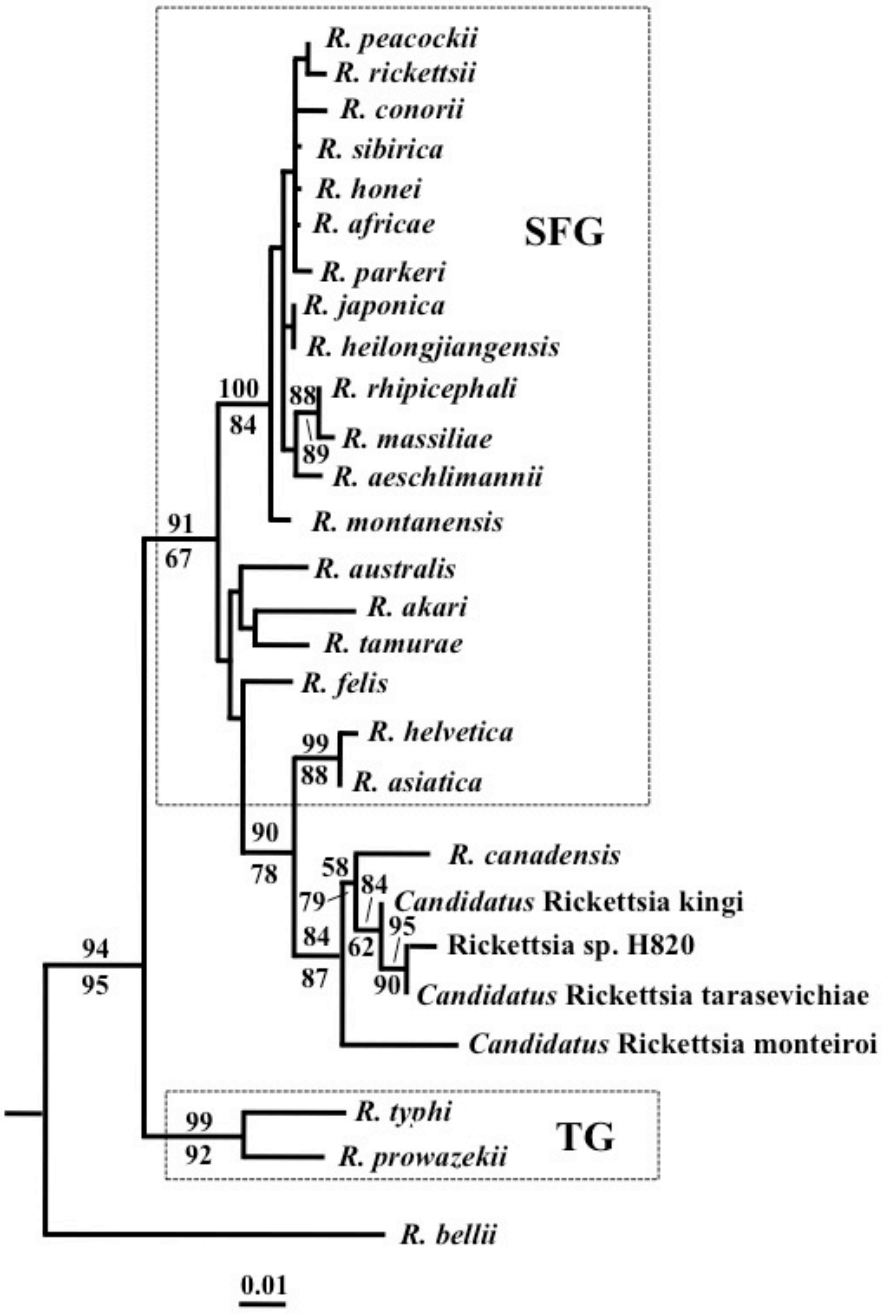

Fig. 8.2 Neighbor-joining tree depicting the relationships of the citrate synthase gene (*gltA*) sequences for *Candidatus* Rickettsia kingi and those of other *Rickettsia* species. SFG and TG refer to the spotted fever group and typhus group of *Rickettsia*, respectively. The scale bar represents the inferred substitutions per nucleotide site. The relative support for clades in the tree produced from the NJ and MP analyses are indicated above and below branches, respectively.

The same clade was also strongly supported (87%) by the bootstrap analyses of the six equally most-parsimonious trees (length of 237, a CI of 0.60 and an RI of 0.75) based on 59 cladistically informative characters (tree not shown).

For *ompA*, the DNA sequences of three rickettsiae-positive *I. kingi* (GenBank accession number HE647693) were identical to one another but differed by 5.5-22.8% (i.e., 3-112 bp) when compared to the sequences of species within the genus *Rickettsia*. The closest matches in sequence were those of *Rickettsia* sp. H820 and *R. canadensis* (Table 8.1). The NJ tree produced from the analyses of the aligned sequence data (491 alignment positions) produced a clade with total bootstrap support that included *R. canadensis*, *Rickettsia* sp. H820 and the *Rickettsia* from *I. kingi*, to the exclusion of all members of the SFG rickettsiae (Fig. 8.3). This clade also had 100% statistical support in the 30 equally most-parsimonious trees (length of 462, a CI of 0.58 and an RI of 0.74) based on 145 cladistically informative characters (tree not shown).

The partial 16S rRNA gene sequences (556 bp) of two rickettsiae-positive *I. kingi* (GenBank accession no. HF548205) were identical to one another but differed by 0.7-1.6% (i.e., 4-9 bp) when compared to the sequences of species within the genus *Rickettsia* and other rickettsial endosymbionts. The closest match in sequence was to the 16S rRNA gene sequence of a rickettsial endosymbiont of the stone beetle, *Coccotrypes dactyliperda*, and to members of the SFG rickettsiae (Table 8.1). A phylogenetic analysis placed the sequence of the rickettsiae of *I. kingi* within a clade that contained the SFG rickettsiae, *R. canadensis* and *R. bellii* (i.e., to the exclusion of the TG rickettsiae); however, there was little resolution of the taxa within this clade (tree not shown). There was no amplification of *ompB* for the rickettsiae in *I. kingi*, whereas the amplicons were produced for the positive controls (i.e., *R. peacockii* derived from the total gDNA of *D. andersoni*). The partial sequences (770 bp) of *ompB* for two *R. peacockii* amplicons

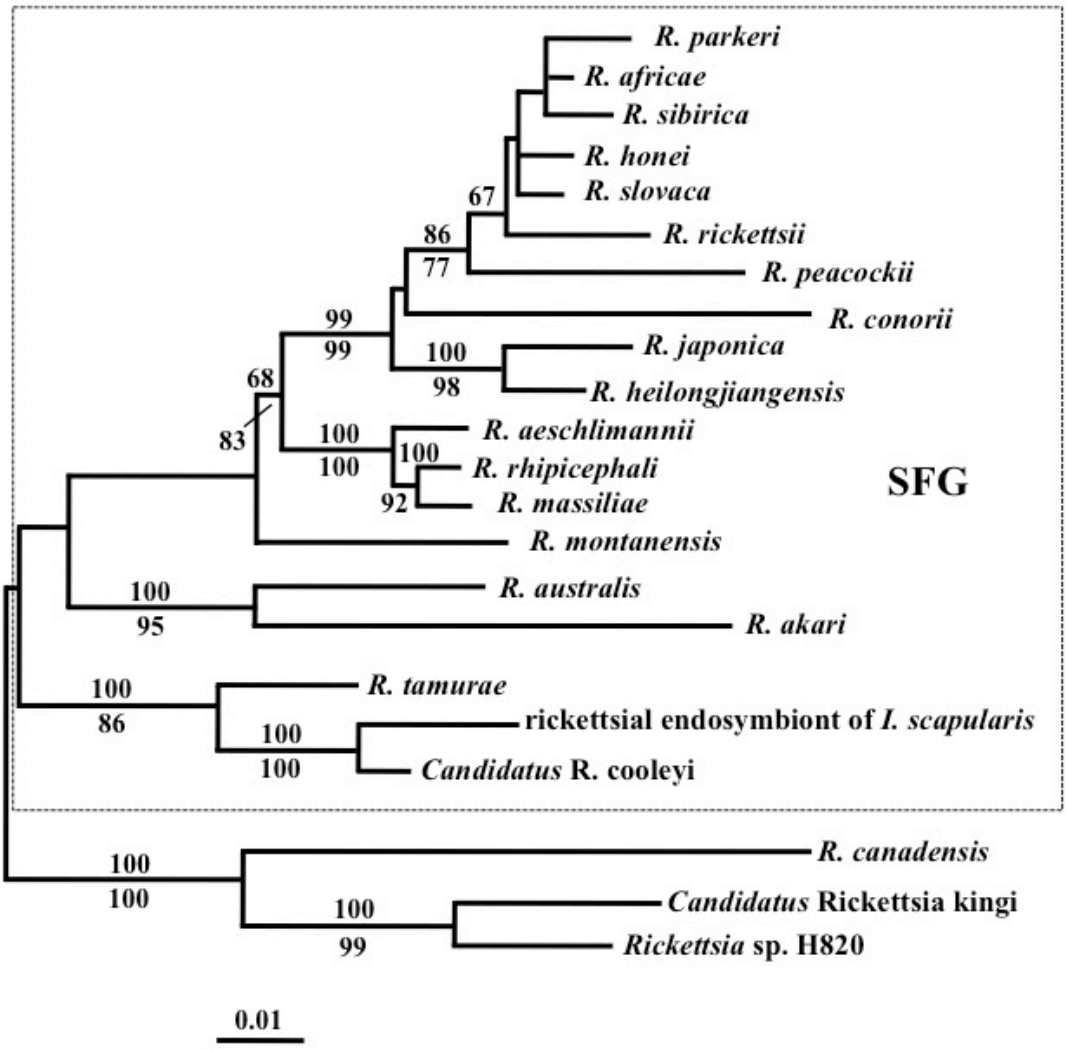

Fig. 8.3 Neighbor-joining tree depicting the relationships of the outer membrane protein A gene (*ompA*) sequences for *Candidatus* Rickettsia kingi and those of other *Rickettsia* species. SFG refers to the spotted fever group of *Rickettsia*. Representatives of the TG rickettsiae are not included because there are no *ompA* sequences for these taxa (Ngwamidiba et al., 2006). The scale bar represents the inferred substitutions per nucleotide site. The relative support for clades in the tree produced from the NJ and MP analyses are indicated above and below branches, respectively.

(accession number HF548206) were 100% identical to the *ompB* sequence for this species (accession number CP001227) on GenBank.

6.5 **Discussion**

This study, as far as we are aware, represents the first published record of the detection of *Rickettsia* within *I. kingi*. Furthermore, this bacterium, present within all feeding life cycle stages of *I. kingi*, represents a novel species of *Rickettsia* because it differs in DNA sequence at four gene loci (i.e., 17-kDa gene, *gltA, ompA* and 16S rRNA gene) when compared to the sequences of all recognized and putative species within the genus. The magnitude of sequence differences of this novel *Rickettsia* compared to other members of the genus for *gltA* (0.9-13.9%), *ompA* (5.5-22.8%) and the 16S rRNA gene (0.9-1.6%), exceeds the levels (0.1%, 1.3% and 0.2%, respectively) recommended by Fournier and Raoult (2009) to indicate the existence of potential new species. In addition, the magnitude of the difference (i.e., 7 bp) in DNA sequence of the 17-kDa gene for the *Rickettsia* in *I. kingi* and *R. canadensis* (i.e., the closest match; Table 1), is greater than that between two recognized species within the genus, *R. peacockii* and *R. montanensis* (i.e., 5 bp). Therefore, the putative new species of *Rickettsia* within *I. kingi* is provisionally named *Candidatus* Rickettsia kingi in accordance with the recommended nomenclature for new rickettsiae that have not been established in pure culture (Fournier & Raoult, 2009).

A fifth genetic marker (i.e., *ompB*) was also used to characterize *Candidatus* R. kingi. However, no amplicons for *ompB* could be obtained for *Candidatus* R. kingi, whereas this gene amplified for *R. peacockii* derived from the total gDNA of *D. andersoni*. This suggests that *ompB* may not be present in *Candidatus* R. kingi. Although *ompB* has been reported for most

species of *Rickettsia*, it is absent in *R. canadensis* (Roux & Raoult, 2000; Ngwamidiba *et al.*, 2006), the species most genetically similar to *Candidatus* R. kingi based on the sequence comparisons of the rickettsial 17-kDa gene.

The results of the phylogenetic analyses for three gene loci (i.e., 17-kDa gene, *gltA,* and *ompA*) revealed that *Candidatus* R. kingi does not belong to the SFG or TG rickettsiae, but represents a sister taxon to *R. canadensis,* a species first reported in the rabbit tick *Haemaphysalis leporispalustris* in Canada (McKiel, 1967). In addition, there is strong statistical support for the existence of a clade comprising *R. canadensis*, *Candidatus* R. kingi, and possibly several other putative species of *Rickettsia*: *Candidatus* R. tarasevichiae, *Candidatus* R. monteiroi and *Rickettsia* sp. H820. Although there is no published information on *Rickettsia* sp. H820 (other than sequence data on GenBank indicating this bacterium was detected in north-eastern China; the vector was not stated), the other potential members of this clade have also been reported in ixodid ticks: *Candidatus* R. tarasevichiae in *I. persulcatus* from Russia and Japan (Eremeeva *et al.,* 2007; Inokuma *et al.,* 2007) and *Candidatus* R. monteiroi in *Amblyomma incisum* from Brazil (Pacheco *et al.,* 2011). Of these rickettsiae, only *R. canadensis* is considered a potential human pathogen (Merhej & Raoult, 2011) based on serological evidence that it may have been the agent responsible for the Rocky Mountain spotted fever-like symptoms displayed by four human patients in North Carolina and Texas (Bozeman *et al.,* 1970).

Although *Candidatus* R. kingi represents a sister taxon to *R. canadensis*, it remains to be determined if this putative new species is of pathogenic significance with respect to human health. *Ixodes kingi* is predominantly a parasite of a range of different rodents, however, it has been reported to occur on a wide variety of hosts that includes wildlife (lagomorphs and carnivores), domestic animals (dogs and cats), and humans (Allan, 2001; Bishopp & Trembley,

1945; Gregson, 1971; Salkeld *et al*., 2006). The high prevalence of this agent (69%) among individuals in the study population of *I. kingi* suggests that this tick is a very suitable host for *Candidatus* R. kingi. We tested the gDNA of six *I. kingi* females collected from four dogs, one cat and one human in Saskatchewan during 2010 for the presence of rickettsiae using PCR; however, none were positive (unpublished data). The localities from where these ticks were collected (i.e., Vanguard, Unity, Swift Current, Bracken and Grasslands Provincial Park) are situated approximately 157 to 316 kms from the locality (i.e., near Clavet) where the rickettsial-infected *I. kingi* individuals were collected. However, there may be local foci for infection with *Candidatus* R. kingi, given that dispersal of *I. kingi* will be relatively restricted when compared to related species that are dispersed by birds. Additional studies are therefore needed to determine the relative prevalence of *Candidatus* R. kingi in tick populations from different parts of the distributional range of *I. kingi*, and to establish if the bacterium has any pathogenic effect on humans and/or domestic animals.

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Chapter 9 A novel *Rickettsia* detected in the vole tick, *Ixodes angustus,* from western Canada

9.1 Abstract

The gDNA of ixodid ticks from western Canada were tested by PCR for the presence of *Rickettsia*. No rickettsiae were detected in *Ixodes sculptus*, whereas 18% of *I. angustus* and 42% of *Dermacentor andersoni* examined were PCR-positive for *Rickettsia*. The rickettsiae from each tick species were characterized genetically using multiple genes. Rickettsiae within *D. andersoni* had sequences at four genes that matched those of *R. peacockii*. In contrast, the *Rickettsia* present within the larvae, nymphs and adults of *I. angustus* had novel DNA sequences, at four of the five genes characterized, when compared to the sequences available on GenBank for all recognized species of *Rickettsia* and all other putative species within the genus. This finding suggests that the rickettsiae in *I. angustus* represent a new species, provisionally named *Candidatus* Rickettsia angustus. Phylogenetic analyses of the sequence data revealed that *Candidatus* Rickettsia angustus does not belong to the spotted fever or typhus groups of rickettsiae but represents a member of a clade that contains *R. canadensis*, *Candidatus* R. tarasevichiae, *Candidatus* R. monteiroi and *Candidatus* R. kingi.

9.2 **Introduction**

The *Rickettsia* are obligate gram-negative intracellular bacteria of arthropods: ticks, fleas, lice and mites (Parola *et al*., 2005; Fournier & Raoult, 2009). Many species of *Rickettsia* are the causative agents of human disease, such as spotted fever and typhus (Fournier & Raoult, 2009; Merhej & Raoult, 2011). There are at least 30 recognized species within the genus (Merhej & Raoult, 2011); however, a number of other putative species have also been recently proposed (e.g., Izzard *et al*., 2009; Almeida *et al*., 2011; Pacheco *et al*., 2011; Phan *et al*., 2011; Anstead & Chilton, 2013; Doornbos *et al*., 2013). Most species of *Rickettsia* can be separated into two groups, the spotted fever group (SFG) and the typhus group (TG), based on their pathogenicity and phenotype (Fournier *et al*., 2003; Parola *et al*., 2005; Fournier & Raoult, 2009). Two species, *R. canadensis* and *R. bellii*, do not belong to the SFG or TG; the latter of which is considered the sister taxon to all other species within the genus (Parola *et al*., 2005; Fournier & Raoult, 2009). *R. canadensis*, which has morphological similarities to both the SFG and TG rickettsiae (Mediannikov *et al*., 2007), has been reported from several tick species (i.e., *Haemaphysalis leporispalustris, Dermacentor andersoni*, *D. variabilis* and *Ambylomma americanum*) in North America (McKiel *et al*., 1967; Brinton *et al*., 1971; Mediannikov *et al*., 2007).

Ticks are the most important vectors of SFG rickettsiae (Telford & Parola, 2007). In North America, there are at least 34 species of *Ixodes* (Allan, 2001); at least six of which, *I. scapularis*, *I. pacificus*, *I. cookei*, *I. dentatus, I. brunneus* and *I. texanus*, have been shown to contain SFG rickettsiae (Clifford *et al*. 1969; Magnarelli *et al*. 1985; Anderson *et al*., 1986; Billings *et al*., 1998; Allan, 2001; Phan *et al*., 2011). Other ticks in North America, such as *D. andersoni* and *D. variabilis*, are also vectors and reservoirs of SFG rickettsiae (i.e., *R. peacockii* and *R. montanensis*, respectively) (Niebylski *et al*., 1997; Ammerman *et al*., 2004; Dergousoff & Chilton, 2009). Both

D. andersoni and *D. variabilis* are also known vectors of *R. rickettsii*, the causative agent of Rocky Mountain spotted fever (Burgdorfer, 1975). All of these species of *Ixodes* and *Dermacentor* use rodents and/or insectivores as hosts for some part of their life cycle (Bishopp & Trembley, 1945; Allan, 2001; Kolonin, 2007; Dergousoff *et al*., 2013).

Recently, a novel *Rickettsia,* based on comparative DNA sequence analyses of four genes, was detected in a population of rotund ticks, *Ixodes kingi*, collected from northern pocket gophers (*Thomomys talpoides*) from Saskatchewan in Canada (Anstead & Chilton, 2013). Phylogenetic analyses of the sequence data revealed that this putative new species, provisionally named *Candidatus* Rickettsia kingi, did not belong to the SFG or TG of rickettsiae but represented a sister taxon to *R. canadensis* and *Candidatus* Rickettsia tarasevichiae (Anstead & Chilton, 2013), the latter of which occurs in *I. persulcatus* from eastern Russia (Shpynov *et al*., 2003) and Japan (Hiraoka *et al.,* 2005). The distributional range of *I. kingi* in western Canada (Gregson, 1971) overlaps that of several species of *Ixodes*, including the sculptured tick, *I. sculptus* (Hixson, 1932; Bishopp & Trembley, 1945; Gregson, 1956; Durden & Keirans, 1996; Allan, 2001; Salkeld *et al*., 2006), and the vole tick, *I. angustus* (Bishopp & Trembley, 1945; Gregson, 1956; Robbins & Keirans, 1992; Sorensen & Moses, 1998; Murrell *et al*., 2003); two tick species that parasitize some of the same rodents and insectivores as *I. kingi* (Bishopp & Trembley, 1945; Burgess, 1955; Gregson, 1956; Miller and Ward, 1960; Hilton & Mahrt, 1971; Robbins & Keirans, 1992; Durden & Keirans, 1996; Sorensen & Moses, 1998; Salkeld *et al*., 2006; Kolonin, 2007). The ranges of all three *Ixodes* species overlap those of *D. andersoni* and/or *D. variabilis*, known vectors of SFG rickettsiae (Burgdorfer, 1975; Niebylski *et al*., 1997; Ammerman *et al*., 2004; Dergousoff & Chilton, 2009). The geographical distribution of *I. angustus* is unusual compared to *I. kingi* and *I. sculptus*, in that it occurs in North America, Russia and Japan (Robbins and Keirans, 1992;

Shpynov *et al.,* 2003). Although *I. angustus* has been implicated as a vector of pathogenic bacteria, such as *Borrelia burgdorferi* (Damrow *et al.,* 1989), there are no reports of the presence of *Rickettsia* in this tick species or *I. sculptus*. Therefore, the aim of the present study was to determine if *I. angustus* and/or *I. sculptus* in western Canada contain rickettsiae, and if so, whether the bacteria belong to the SFG or the clade containing *R. canadensis*, *Candidatus* R. tarasevichiae and *Candidatus* R. kingi.

9.3 Materials and Methods

9.3.1 DNA extraction, PCR and single-strand conformation polymorphism (SSCP)

Total genomic DNA (gDNA) was extracted and purified from the complete bodies of 378 individual ticks (Table 9.1) using the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany), and the modifications described previously (Dergousoff & Chilton, 2007; Anstead & Chilton, 2013). These ticks represented 268 *I. angustus* and two *D. andersoni* collected from 46 red-backed voles (*Clethrionomys gapperi*), three long-tailed voles (*Microtus longicaudus*), two western heather voles (*Phenacomys intermedius*), one masked shrew (*Sorex cinereus*), two golden-mantled ground squirrels (*Callospermophilus lateralis*), and one deer mouse (*Peromyscus maniculatus*) that were live-trapped at three sites (i.e., Verdant Forest, Numa Forest and Marble Canyon) within the Kootenay National Park (50°68'N, 115°93'W), British Columbia. A total of 58 *I. sculptus*, six *I. kingi* and 40 *D. andersoni* collected from 17 Richardson's ground squirrels (*Spermophilus richardsonii*) near Beechy (50°53'N, 107°23'W), Saskatchewan, and four *I. sculptus* from a thirteen-lined ground squirrel (*Ictidomys tridecemlineatus*) collected 8 km southwest of Clavet (51.9519°N, 106.4473°W), Saskatchewan. All ticks were identified to species by morphological examination and confirmed using genetic markers (Anstead & Chilton, 2011, 2013).

Locality (Coordinates) Tick species	Life cycle stage	No. tested	No. *Rickettsia* positive
Kootenay N.P., BC (49°44'N 112°50'W)			
I. angustus	larvae	176	45 (26%)
	nymphs	68	3 (4%)
	adults	24	1 (4%)
D. andersoni	adults	2	1 (50%)
Beechy, SK (50°53'N, 107°23'W)			
I. sculptus	larvae	34	0
	nymphs	21	0
	adults	3	0
I. kingi	larvae	1	0
	nymphs	4	0
	adults	1	0
D. andersoni	nymphs	20	17 (85%)
	adults	20	17 (85%)
Clavet, SK (51.9519°N, 106.4473°W)			
I. sculptus	nymphs	4	0

Table 9.1 The number of *Ixodes angustus*, *I. sculptus*, *I. kingi* and *D. andersoni* tested that were positive for infection with *Rickettsia* using PCR analyses of the rickettsial 17-kDa antigen gene.

The presence/absence of *Rickettsia* DNA in each tick was tested by nested (n)-PCR targeting a 434-bp fragment of the rickettsial-specific 17-kDa antigen gene using primers 17K-5 (5'-GCTTTACAAAATTCTAAAAACCATATA-3') and 17K-3 (5'-TGTCTATCAATTCACAACTTGCC-3') for the first phase, and primers 17kD1 (5'-GCTCTTGCAACTTCTATGTT-3') and 17kD2 (5'-CATTGTTCGTCAGGTTGGCG-3') for the second phase (Heise *et al*., 2010), and the protocols and cycling conditions described previously (Dergousoff & Chilton, 2007; Anstead & Chilton 2013). All PCR positive samples were then subjected to single strand conformation polymorphism (SSCP) analyses (Gasser *et al*., 2006) to pre-screen for genetic variation. This mutation scanning technique can be used to differentially display genetic variation between DNA sequences that are 150-450-bp in size, and that differ by one or more nucleotides (Gasser *et al*., 2006). Representative amplicons (n=5) of each different SSCP profile type were purified (Dergousoff & Chilton, 2012) prior to automated DNA sequencing using primers 17kD1 and 17kD2. Amplicons from phase one of the n-PCR of three rickettsiae-infested *I. angustus* were also purified and subjected to automated DNA sequencing using primers 17K-5 and 17K-3.

To confirm the presence of rickettsial DNA in individual ticks, a second PCR assay, targeting 491-bp of the outer membrane protein A gene (*ompA*), was conducted on the total gDNA of a subset of ticks (n = 45) shown to be PCR-positive for the 17-kDa antigen gene. PCRs were carried out using primers *Rr*190.70p (5'-ATGGCGAATATTTCTCCAAAA-3') and *Rr*190.602n (5'-AGTGCAGCATTCGCTCCCCCT -3') (Regnery *et al*., 1991) and the following conditions: 95°C for 5 min, followed by 30 cycles of 95°C for 30 sec, 50°C for 30 sec, 72°C for 30 sec, and a final cycle of 72°C for 8 min. Purified amplicons from two PCR-positive samples from *I. angustus* were sequenced using primers *Rr*190.70p and *Rr*190.602n in separate reactions.

Four additional genetic markers were used to characterize the rickettsiae in *I. angustus* and *D. andersoni*. First, a 1,060-bp fragment of the citrate synthase gene (*gltA*) was amplified from the gDNA of two rickettsiae-infested *I. angustus* larvae and one rickettsiae-infested *D. andersoni* nymph using the primers CS2dF (5'-ATGACCAATGAAAATAATAAT-3') and *Rp*CS.1258n (5'-ATTGCAAAAAGTACAGTGAACA-3') (Regnery *et al*., 1991; Roux *et al*., 1997) and the following conditions: 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 50°C for 1 min, 72°C for 1 min, and a final cycle of 72°C for 8 min. The amplicons were purified and sequenced using primers CS2dF and *Rp*CS.1258n in separate reactions. Next, part (1,332-bp) of the prokaryotic 16S rRNA gene of six rickettsiae-infested ticks (i.e., four *I. angustus* & two *D. andersoni*) was amplified using primers Rick-16S-F3 (5'-ATCAGTACGGAATAACTTTTA-3') and Rick-16S-R4 (5'-TGCCTCTTGCGTTAGCTCAC-3') using the following conditions: 95°C for 5 min, 30 cycles of 95°C for 45 sec, 58°C for 45 sec, 72°C for 45 sec, and then 72ºC for 5 min. Primers Rick-16S-F3 and Rick-16S-R4 were designed specifically to amplify the 16S rDNA of *Rickettsia* because those most often used for this purpose in other studies (i.e., primers fd1 and rp2; Weisburg *et al*., 1991) also co-amplified the 16S rDNA of other bacteria present within the ticks. The purified 16S rDNA amplicons were subjected to automated DNA sequencing using primers Rick-16-F3 & Rick-16-F4 in separate reactions. In addition, part (488-bp) of the surface cell antigen 1 (*sca*1) gene of three rickettsial-infested ticks was amplified using primers SCA1-F2 (5'-GGTGATGAAGAAGAGTCTC-3') and SCA1-R2 (5'-CTCTTTAAAATTATGTTCTAC-3') and the following conditions: 95°C for 5 min, 35 cycles of 95°C for 30 sec, 50°C for 30 sec, 72°C for 30 sec, and then 72ºC for 5 min. Purified amplicons from three *I. angustus* larvae were subjected to automated DNA sequencing using primers SCA1-F2 & SCA1-R2. Amplification of *sca*1 was not achieved for any gDNA

samples from *D. andersoni* (n=5). Amplification of 812-bp of the outer membrane protein B gene (*ompB*) using the primers 120.3599 (5'-TACTTCCGGTTACAGCAAAGT-3') and 120.2788 (5'-AAACAATAATCAAGGTACTGT-3') was also attempted. PCR conditions of Roux and Raoult (2000) were used, except that the number of cycles was increased to 35, and the annealing temperature was raised from 50°C to 52°C.

Negative controls (i.e., no gDNA) were included in each PCR assay conducted. In addition, the gDNA of *Candidatus* R. kingi from *I. kingi* larvae, nymphs and adults (Anstead & Chilton, 2013), *R. peacockii*-infested *D. andersoni* adults, *R. montanensis*-infested *D. variabilis* adults (Dergousoff *et al.,* 2009) were included in each PCR assay and SSCP analysis as positive controls. The amplicons of these positive controls were also sequenced for each gene region to confirm the correct target genes had been successfully amplified.

9.3.2 **Sequence analyses**

BLAST searches (GenBank) were performed on the DNA sequences of each gene to determine the genetic similarity of the rickettsiae in *I. angustus* and *D. andersoni* to the different taxa within the genus *Rickettsia*. For each gene region, DNA sequences were aligned manually with those of *Rickettsia* species available on GenBank (Table 9.2). Phylogenetic analyses were performed separately on the sequence data of each gene using the neighbor joining (NJ) and maximum parsimony (MP) methods in PAUP (Swofford, 2003). For the MP analyses, characters were treated as unordered and were equally weighted, and alignment gaps were treated as 'missing' characters. Heuristic searches with TBR branch swapping were used to infer the shortest trees. The lengths, consistency indices (excluding uninformative characters), and the retention indices of the most parsimonious trees were recorded. The sequences of *Orientia*

Taxon	Gene				
	16S rRNA	17-kDa	*gltA*	*ompA*	*sca*1
R. aeschlimannii	U74757	DQ3799979	AY259084	U43800	DQ306900
R. africae	L36098	CP001612	U59733	U43790	DQ306901
R. akari	L36099	CP000847	U59717	CP000847	DQ306902
R. amblyommii	-	AY375162	-	-	-
R. australis	L36101	M74042	U59718	AF149108	DQ306903
R. bellii	L36103	AF445380	U59716	-	DQ306904
R. canadensis	L36104	CP000409	U59713	CP000409	DQ306905
R. conorii	L36105	AE006914	U59730	U43806	DQ306906
R. cooleyi	-	AF031534	-	-	-
R. felis	L28944	CP000053	AF210692	-	DQ306907
R. heilongjiangensis	-	AB473988	AY280709	AY280711	-
R. helvetica	L36212	AF181036	U59723	-	DQ306908
R. honei	L36220	AF027124	U59726	U43809	DQ306909
R. japonica	L36213	D16515	U59724	U43795	DQ306910
R. marmionii	-	AY737683	-	-	-
R. massiliae	L36214	CP000683	U59719	U43799	DQ306911
R. monacensis	-	EF380355	-	-	-
R. montanensis	L36215	U11017	U74756	U43801	DQ306912
R. parkeri	L36673	EF102237	U59732	U43802	DQ306913
R. peacocki	DQ062433	CP001227	DQ100162	AY357766	-
R. prowazekii	M21789	CP001584	M17149	-	DQ306914
R. rhipicephali	L36216	DQ865207	U59721	U43803	DQ306915
R. rickettsii	L36217	AY281069	U59729	U43804	DQ306916
R. sibirica	L36218	AF445384	U59734	U43807	DQ306918
R. slovaca	L36224	-	-	U43808	DQ306917
R. tamurae	-	AB114825	AY394896	AB114823	-
R. typhi	L36221	AE017197	U59714	-	DQ306919
R. endosymbiont of *I. scapularis*	D84558	EF689734	-	EF689735	-
R. endosymbiont of *C. kelleyi*	-	AY763102	-	-	-
Rickettsia sp. 'Argentina'	-	EU826507	-	-	-
Candidatus R. andreanae	-	GU395295	-	-	-
Candidatus R. antechini	-	DQ372953	-	-	-
Candidatus R. gravesii	-	DQ269436	-	-	-
Candidatus R. hoogstraalii	-	EF629538	-	-	-
Candidatus R. kingi	HF935068	HF935071	HF935074	HF935077	HF935080
Candidatus R. monteiroi	FJ269037	FJ269036	FJ269035	-	JF734727
Candidatus R. tarasevichiae	AF503168	-	AF503167		- -
Undescribed *Rickettsia* sp. 'H820'	JF714221	-	-	JF714220	-

Table 9.2 The GenBank accession numbers of the rickettsial DNA sequences used in phylogenetic analyses.

tsutsugamushi and *Midichloria mitochondrii* were used as outgroups in the MP analyses of the 16S rRNA gene and *gltA* (respectively), while the sequences of *R. bellii* were used as the outgroup in the MP analyses of the 17-kDa gene and *sca*1. Midpoint rooting was used in the MP analysis of the *ompA* sequence data. Bootstrap analyses (1000 replicates for the NJ analyses and 100 replicates for MP analyses) were conducted to determine the relative support for clades in the consensus trees.

9.4 **Results**

Three hundred and seventy eight ticks were each tested for the presence of *Rickettsia* DNA by n-PCR of the 17-kDa antigen gene, of which 84 were positive (Table 9.1). Each of these amplicons had a single band of the expected size (~450-bp) on 1.5% agarose-TBE gels. No bands were detected on agarose gels for the negative control samples. *Rickettsia* DNA was not detected in any of the *I. sculptus* or the *I. kingi* from Beechy (SK). In contrast, 49 (18%) of the 268 *I. angustus* individuals (1 female, 3 nymphs and 45 larvae) from three sites in Kootenay National Park in British Columbia (BC), and 35 (83%) of the 42 *D. andersoni* (18 adults and 17 nymphs) collected from two localities (Kootenay National Park, BC and Beechy, SK) were PCR-positive for *Rickettsia* DNA (Table 9.1). For *D. andersoni*, there was no significant difference ($\chi^2_1 = 0.8$, $P > 0.05$) in the proportion of nymphs and adults containing rickettsiae. Significantly more *D. andersoni* individuals were PCR-positive for *Rickettsia* DNA than *I. angustus* individuals ($\chi^2_1 = 77.78$, $P < 0.001$). None of the *I. angustus* from Numa Forest (i.e., 1 larva) or Marble Canyon (i.e., 2 adults, 1 nymph and 1 larva) within Kootenay National Park were PCR-positive for *Rickettsia*; however, most (98%) of the *I. angustus* were collected from Verdant Forest. At this site, a significantly ($\chi^2_2 = 17.32$, $P < 0.001$) greater proportion of *I. angustus*

larvae were PCR-positive for *Rickettsia* DNA than *I. angustus* nymphs or adults (Table 9.3). In addition, a significantly ($\chi^2_1 = 37.81$, $P < 0.001$) greater proportion of *I. angustus* larvae collected in 2007 were PCR-positive for *Rickettsia* DNA than larvae collected in 2005 (Table 9.3).

The results of a second PCR assay, targeting *ompA*, confirmed the presence of rickettsial DNA in 45 samples (selected at random) that were PCR-positive for the 17-kDa antigen gene. Each of the amplicons consisted of a single band of the expected size (~533-bp) on 1.5% agarose-TBE gels, while no bands were detected for the negative control samples. The SSCP banding patterns of the 17-kDa gene for amplicons of the positive control samples (i.e., *R. peacockii*, *R. montanensis* and *Candidatus* R. kingi) and representative samples of PCR-positive individuals of *I. angustus* and *D. andersoni* are shown in Figure 9.1. The SSCP banding patterns (i.e., profiles) of the PCR-positive samples derived from *D. andersoni* individuals from Kootenay National Park and Beechy were identical to that of the *R. peacockii* control samples. A BLAST search of the 17-kDa gene sequence of the *Rickettsia* in *D. andersoni* revealed that it was genetically identical to the sequence of *R. peacockii* (Accession no. CP001227). The SSCP profiles of the *I. angustus* PCR-positive samples were identical to one another but differed to those of *R. peacockii*, *R. montanensis* and *Candidatus* R. kingi controls (Fig. 9.1). The DNA sequences (464-bp) of three representative *I. angustus* amplicons used in the SSCP analyses revealed that they differed at 1 nucleotide position when compared to the sequence of *Candidatus* R. kingi (accession no. HE647694). When sequences of a slightly larger fragment (497-bp) of the 17-kDa gene were obtained (i.e., using 17K5/17K3 amplicons), the rickettsiae in *I. angustus* all had identical sequences to one another but differed in sequence to *Candidatus* R. kingi at 3 alignment positions (Table 9.4). A BLAST search revealed that the 17-kDa gene

Year	Adults		Nymphs		Larvae	
	No.	No. (%) PCR +ve	No.	No. (%) PCR +ve	No.	No. (%) PCR +ve
2005	13	0	31	2	67	1
2006	2	0	13	1	5	2
2007	7	1	22	0	103	42
Total	22	1 (4.5%)	66	3 (4.5%)	175	45 (25.7%)

Table 9.3 The number of larval, nymphal and adult *I. angustus* collected in different years at Verdant Forest within Kootenay National Park (British Columbia, Canada), and the number of ticks that were PCR-positive for rickettsiae using the 17-kDa antigen gene as a genetic marker.

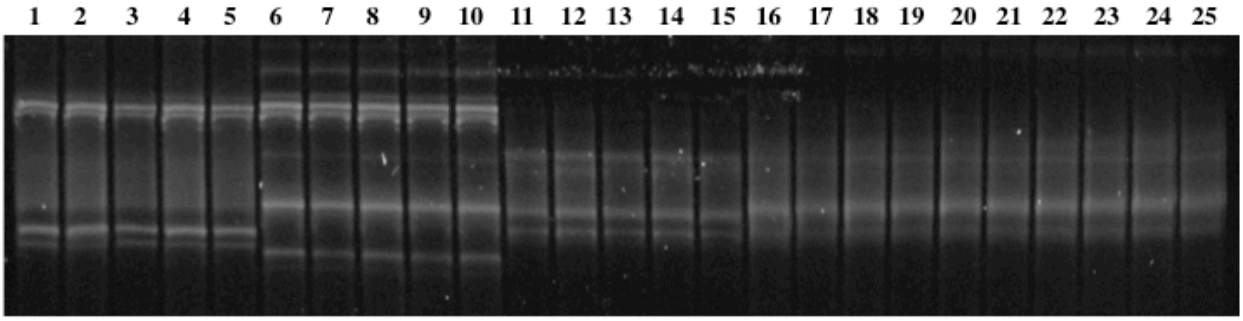

Fig. 9.1 SSCP profiles of amplicons of the *Rickettsia* 17-kDa gene for representative specimens of *R. peacockii* (lanes 1-5) *R. montanensis* (lanes 6-10), *Candidatus* R. kingi (lanes 11-15) and the rickettsiae in *Ixodes angustus* (lanes 16-25).

Gene	*Rickettsia* (Genbank accession no.)	% sequence similarity
16S rRNA gene	*Candidatus* R. kingi (HF935068)[a]	99.7 (1,328 of 1,332 bp)
	Rickettsia of *Curculio hilgendorfi* (AB604688)	99.4 (1,325 of 1,333 bp)
	R. bellii (CP000087)	99.3 (1,323 of 1,332 bp)
	Candidatus R. tarasevichiae (AF503168) [b]	99.3 (1,281 of 1,290 bp)
	R. felis (CP000053)	99.3 (1,324 of 1,333 bp)
17-kDa gene	*Candidatus* R. kingi (HF935071) [a]	99.4 (494 of 497 bp)
	R. canadensis (CP003304)	95.3 (466 of 489 bp)
	R. canadensis (CP000409)	94.7 (463 of 489 bp)
	Candidatus R. monteiroi (FJ269036) [b]	92.4 (404 of 437 bp)
	R. rhipicephali (CP003342)	89.4 (446 of 499 bp)
gltA	*Candidatus* R. kingi (HF935074) [a]	99.9 (1,059 of 1,060 bp)
	Candidatus R. tarasevichiae (AF503167)	98.7 (1,046 of 1,060 bp)
	R. canadensis (AB297809)	97.8 (1,037 of 1,060 bp)
	R. canadensis (CP000409)	97.5 (1,034 of 1,060 bp)
	Candidatus R. monteiroi (FJ269035)	96.5 (1,008 of 1,045 bp)
ompA	*Candidatus* R. kingi (HE647693)	99.4 (488 of 491 bp)
	Rickettsia sp. H820 (JF714220)	94.9 (466 of 491 bp)
	R. tamurae (DQ103259)	87.2 (429 of 492 bp)
	Candidatus R. cooleyi (AF031535)	87.1 (426 of 489 bp)
	R. canadensis (CP000409)	86.7 (430 of 495 bp)
*sca*1	*Candidatus* R. kingi (HF935080)[a]	100 (488 of 488 bp)
	R. canadensis (CP003304)	96.3 (470 of 488 bp)
	Candidatus R. monteiroi (JF734727) [b]	95.5 (426 of 446 bp)
	R. helvetica (DQ306908)	94.7 (462 of 488 bp)
	R. felis (DQ306907)	94.3 (460 of 488 bp)

[a] Sequence data determined in this study, [b] only a partial sequence available for this taxa

Table 9.4 Closest relative sequences to the partial 16S rRNA gene, 17-kDa gene, *gltA*, *ompA*, and *sca1* sequences of the *Rickettsia* detected in *I. angustus* from Kootenay National Park (British Columbia, Canada).

sequence of the rickettsiae in *I. angustus* differed at 3 to 55 (0.6-12%) nucleotide positions when compared to the 17-kDa sequences of taxa within the genus *Rickettsia*.

The DNA sequences of the rickettisae detected in *D. andersoni* from Kootenay National Park and Beechy were identical in sequence to those of *R. peacockii* for *gltA* (Accession number DQ100162) and *ompB* (Accession number CP001227), and 99.9% similar (i.e., at 1,214 of 1,215 bp) to the 16S rRNA gene sequence of *R. peacockii* (Accession number DQ06243). However, no *sca*1 amplicons were obtained for the rickettisae in *D. andersoni* or for the *R. peacockii* controls.

Amplicons were obtained for the rickettsiae detected in *I. angustus* for four of the five additional target regions: *gltA*, *ompA*, 16S rRNA gene, and *sca*1. No amplicons were obtained for *ompB*. The *gltA* sequences (1,060-bp) of two representative samples of rickettsiae from *I. angustus* were identical to one another but differed in sequence at 1 to 150 bp (0.1-14%) when compared to the *gltA* sequences of species within the genus *Rickettsia*. The closest match in sequence was to a sequence of *Candidatus* Rickettsia kingi (Table 9.4).

The rickettsiae in *I. angustus* also had a unique sequence for *ompA* (491-bp) and the 16S rRNA gene (1,332-bp); differing in sequence by 0.6% to 17% (3-85 bp) and 0.3% to 2% (4 -25 bp), respectively, when compared to the sequences of these genes for *Rickettsia* species on GenBank. For each gene region, the sequences of the rickettsiae in *I. angustus* had the closest match to the sequences of *Candidatus* Rickettsia kingi (Table 9.4). The DNA sequences of *sca*1 amplicons derived from the gDNA of three rickettsial-infested *I. angustus* were identical to those of *Candidatus* Rickettsia kingi (Table 9.4), but differed in sequence from those of other taxa in the genus *Rickettsia* by 4% to 17% (18-85 bp).

The NJ tree produced from the phylogenetic analysis of the 17-kDa sequence data revealed total statistical support (100% bootstrap value) for the *Rickettsia* in *I. angustus*

representing a sister taxon to a clade that contained *Candidatus* Rickettsia kingi and *R. canadensis*, to the exclusion of members of the SFG and TG (Fig. 9.2). The MP analysis of the same data set (i.e., 117 cladistically informative characters) produced over 1000 equally most-parsimonious trees (strict consensus tree not shown), with a length of 365, a CI of 0.69 and a RI of 0.73. As with the NJ tree, there was strong support (bootstrap value of 94%) for the inclusion of the rickettsiae in *I. angustus* within a clade that included *Candidatus* Rickettsia kingi, *R. canadensis* and *Candidatus* R. monteiroi (Fig. 9.2).

The NJ analyses of the *ompA* aligned sequence data produced a tree with a clade, with total bootstrap support, that included the *Rickettsia* from *I. angustus*, *R. canadensis*, *Rickettsia* sp. H820 and *Candidatus* Rickettsia kingi, to the exclusion of all members of the SFG rickettsiae (Fig. 9.3). This clade also had 100% statistical support in the 30 equally most-parsimonious trees (length of 462, a CI of 0.67 and a RI of 0.77) based on 149 cladistically informative characters (tree not shown).

The NJ tree produced from analyses of the *gltA* sequence data also showed that the *Rickettsia* in *I. angustus* formed a clade, with total bootstrap support (100%), with *R. canadensis, Candidatus* Rickettsia kingi, *Candidatus* Rickettsia monteiroi and *Candidatus* Rickettsia tarasevichiae, to the exclusion of other members in the genus (Fig. 9.4). The same clade was also strongly supported (98%) by the bootstrap analyses of the 84 equally most-parsimonious trees (length of 829, a CI of 0.71 and a RI of 0.73) based on 219 cladistically informative characters (tree not shown). In contrast, there was limited resolution of taxa in the NJ and MP analyses of the 16S rRNA gene sequence data (Fig. 9.5). However, the rickettsiae in *I. angustus* represented the sister taxon to *Candidatus* Rickettsia kingi with strong statistical support (bootstrap values of 96% and 85% for NJ and MP analyses, respectively).

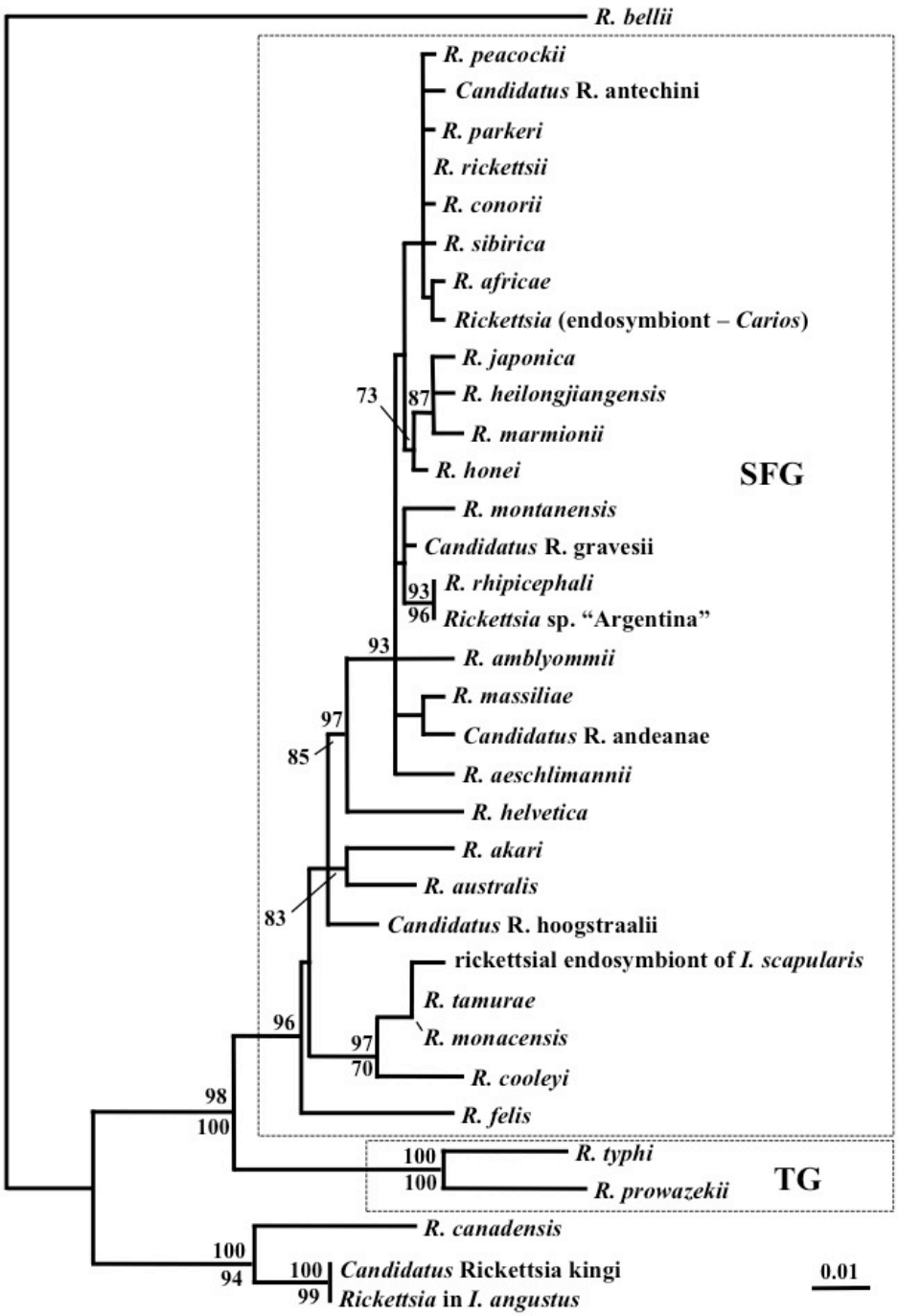

Fig. 9.2 Neighbor-joining tree depicting the relationships of the sequences for the rickettsial 17-kDa gene of the rickettsiae in *Ixodes angustus* and those of other *Rickettsia* species. SFG and TG refer to the spotted fever group and typhus group of *Rickettsia*, respectively. The scale bar represents the inferred substitutions per nucleotide site. The relative support for clades in the tree produced from the NJ and MP analyses are indicated above and below branches, respectively.

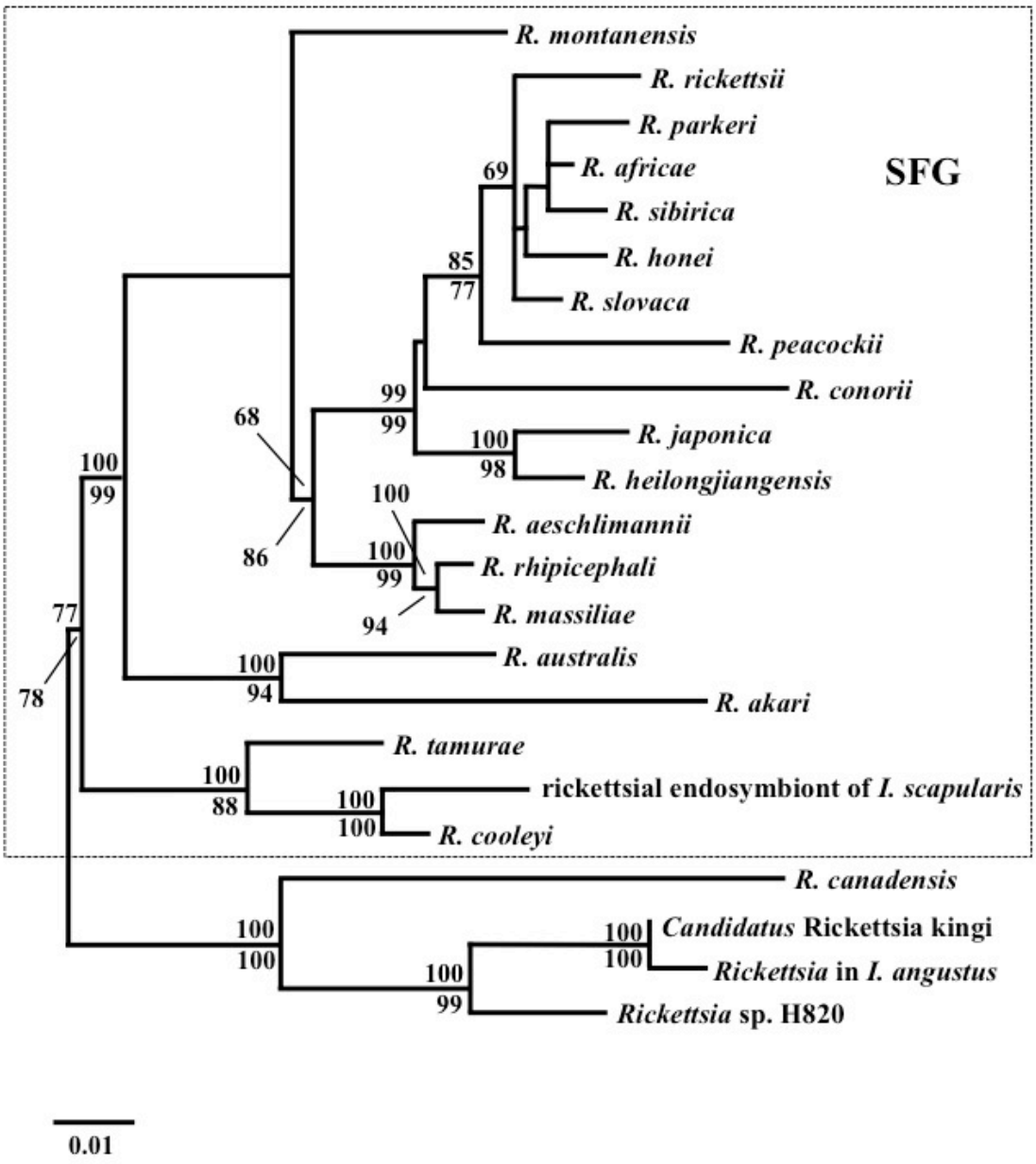

Fig. 9.3 Neighbor-joining tree depicting the relationships of the outer membrane protein A gene (*ompA*) sequences for the rickettsiae in *Ixodes angustus* and those of other *Rickettsia* species. SFG refers to the spotted fever group of *Rickettsia*. Representatives of the TG rickettsiae are not included because there are no *ompA* sequences for these taxa (Ngwamidiba *et al*., 2006). The scale bar represents the inferred substitutions per nucleotide site. The relative support for clades in the tree produced from the NJ and MP analyses are indicated above and below branches, respectively.

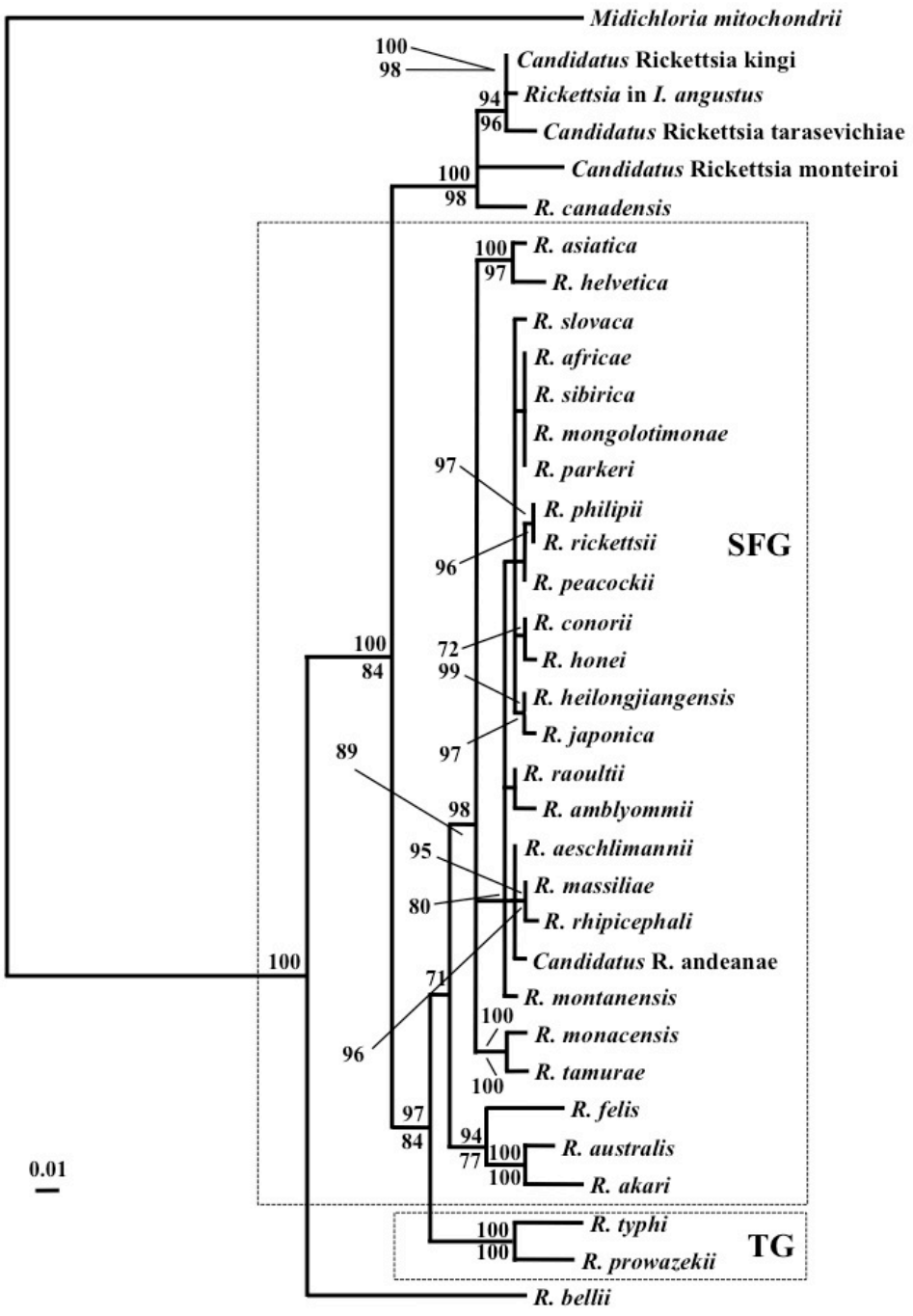

Fig. 9.4 Neighbor-joining tree depicting the relationships of the citrate synthase gene (*gltA*) sequences for the rickettsiae in *Ixodes angustus* and those of other *Rickettsia* species. SFG and TG refer to the spotted fever group and typhus group of *Rickettsia*, respectively. The scale bar represents the inferred substitutions per nucleotide site. The relative support for clades in the tree produced from the NJ and MP analyses are indicated above and below branches, respectively.

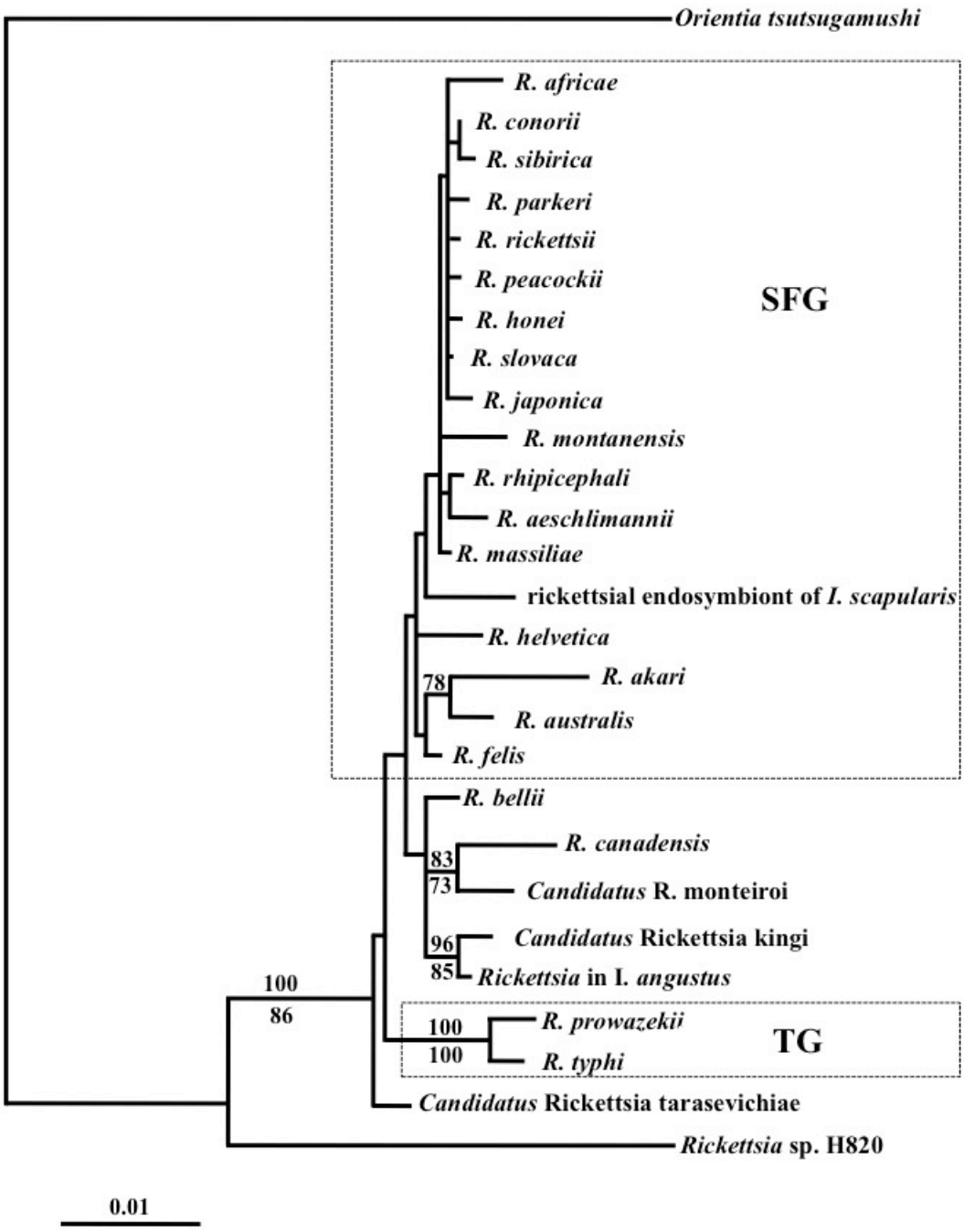

Fig. 9.5 Neighbor-joining tree depicting the relationships of the sequences for the 16S-rRNA gene of the rickettsiae in *Ixodes angustus* and those of other *Rickettsia* species. SFG and TG refer to the spotted fever group and typhus group of *Rickettsia*, respectively. The scale bar represents the inferred substitutions per nucleotide site. The relative support for clades in the tree produced from the NJ and MP analyses are indicated above and below branches, respectively.

The NJ analyses of the *sca*1 sequence data produced a tree containing a clade, with strong bootstrap support (85%), that comprised the *Rickettsia* in *I. angustus*, *R. canadensis*, *Candidatus* Rickettsia monteiroi and *Candidatus* Rickettsia kingi, to the exclusion of other members in the genus (Fig. 9.6). The same clade was not supported by the bootstrap analyses of the 60 equally most-parsimonious trees (length of 262, a CI of 0.63 and a RI of 0.59) based on 85 cladistically informative characters (data not shown).

9.5 **Discussion**

Many species of *Rickettsia* use ticks as hosts (Parola *et al*., 2005; Telford & Parola, 2007; Fournier & Raoult, 2009); however, in the present study, rickettsial DNA was not detected in any of the 58 *I. sculptus* larvae, nymphs or adults feeding on Richardson's ground squirrels at Beechy in Saskatchewan (SK), or the four *I. sculptus* nymphs feeding on a thirteen-lined ground squirrel collected near Clavet (SK). This was markedly different to the presence of rickettsiae in 34 (85%) of 40 *D. andersoni* nymphs and adults feeding on Richardson's ground squirrels at Beechy. It is possible that *I. sculptus* may not represent a suitable host for *Rickettsia*, however, individuals from different localities throughout the large geographical range of this tick species in North America need to be tested because it is known that prevalence of rickettsiae can vary significantly among tick populations (e.g., Dergousoff & Chilton, 2009). For example, although the sample size was small, none of the *I. kingi* individuals feeding on Richardson's ground squirrels at Beechy were PCR-positive for rickettsiae (Table 1), whereas 69% of the 87 *I. kingi* feeding on northern pocket gophers (*Thomomys talpoides*) at Clavet were found to contain *Candidatus* R. kingi (Anstead & Chilton, 2013).

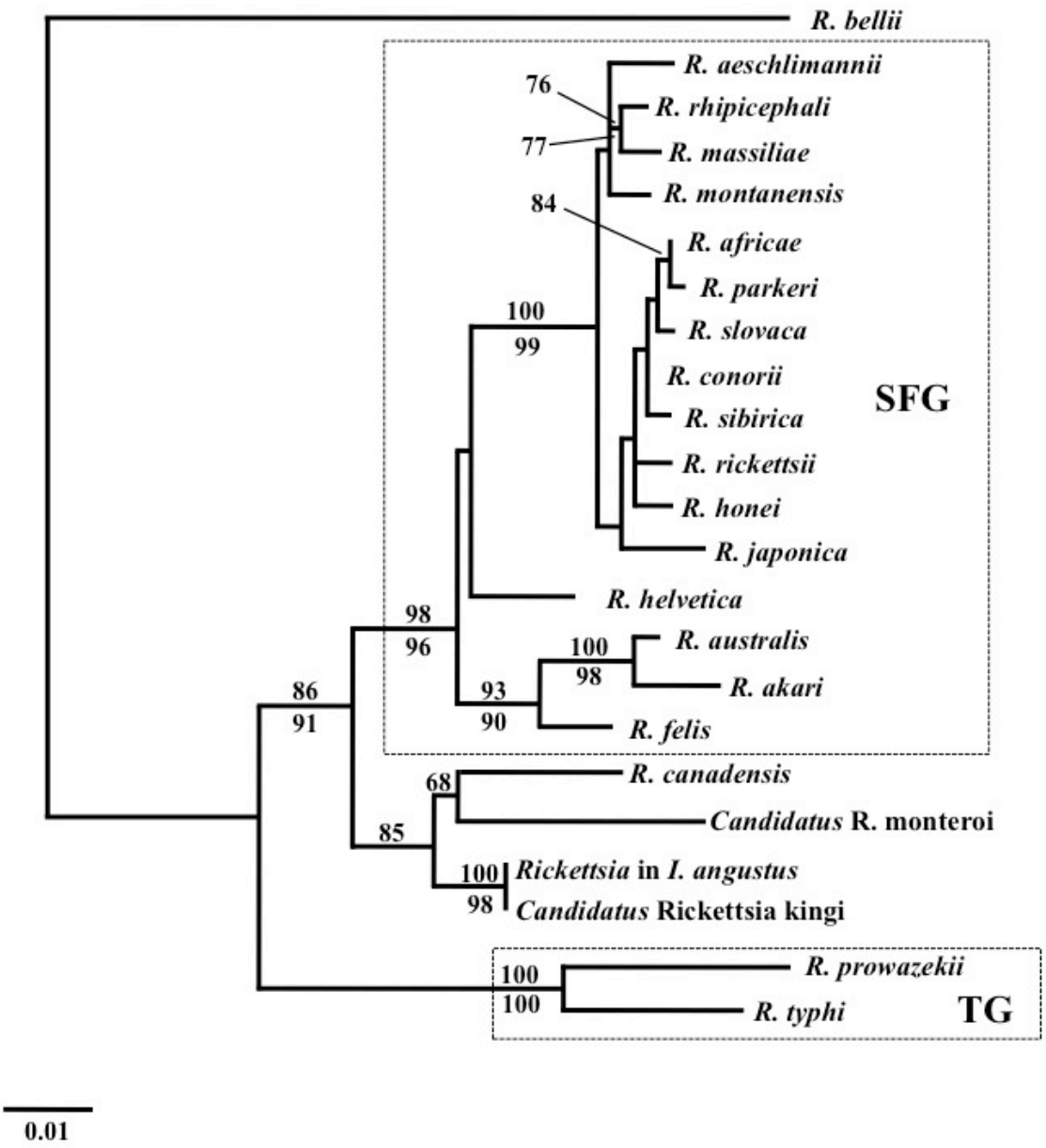

Fig. 9.6 Neighbor-joining tree depicting the relationships of the surface cell antigen 1 gene (*sca*1) sequences for the rickettsiae in *Ixodes angustus* and those of other *Rickettsia* species. SFG and TG refer to the spotted fever group and typhus group of *Rickettsia*, respectively. The scale bar represents the inferred substitutions per nucleotide site. The relative support for clades in the tree produced from the NJ and MP analyses are indicated above and below branches, respectively.

The rickettsiae detected in the *D. andersoni* individuals on Richardson's ground squirrels from Beechy (SK) and the one *D. andersoni* adult on a golden-mantled ground squirrel from Kootenay National Park (BC) were genetically characterized as *R. peacockii* at four loci (i.e., 17-kDa gene, *gltA, ompB* and the 16S rRNA gene). This is consistent with previous findings of this species of *Rickettsia* in *D. andersoni* from other localities in western North America (Burgdorfer *et al.,* 1981; Niebylski *et al*., 1997; Dergousoff & Chilton, 2009, Dergousoff, 2011). Attempts were made to characterize the *R. peacockii* in *D. andersoni* and the *R. peacockii* control samples at the *sca*1 locus because this gene has been recommended as a target for species delineation and used for inferring phylogenetic relationships of taxa within the genus *Rickettsia* (Ngwamidiba *et al*., 2006). Sequences of the variable (488-bp) region of *sca*1 have been determined for all recognized members of the SFG rickettsiae, (except for *R. peacockii),* and for *R. felis* and *R. prowazekii* (i.e., TG rickettsiae), *R. canadensis*, and *R. bellii* (Ngwamidiba *et al*., 2006). In the present study, no *sca*1 amplicons were obtained for *R. peacockii*, whereas amplicons (488-bp) were obtained and sequenced for several representatives of *Candidatus* R. kingi, and the rickettsiae within the different life cycle stages of *I. angustus*. This suggests that *sca*1 may be absent in *R. peacockii*, and may therefore reduce the effectiveness of this gene for species delineation for all taxa within the genus *Rickettsia*. BLAST searches comparing the sequences for conserved regions of the *sca*1 gene in several species of *Rickettsia* with the sequence of the complete genome for *R. peacockii* available on GenBank (Accession number CP001227) also indicate that *sca*1 is absent in *R. peacockii*.

Although no rickettsiae were detected in *I. sculptus*, 18% of the 268 *I. angustus* feeding on voles, and a small number of shrews, ground squirrels and mice within the Kootenay National Park (BC) were found to be PCR-positive for *Rickettsia*. The proportion of *I. angustus* individuals

containing rickettsiae differed among life cycle stages and year, with the greatest rickettsial presence detected in larvae collected from hosts in 2007. The results of the SSCP analyses of the 17-kDa amplicons of the rickettsiae present within all PCR-positive *I. angustus* samples revealed that they had an identical banding pattern (i.e., profile) to one another but differed markedly to the SSCP profiles of the control samples: *R. peacockii*, *R. montanensis* and *Candidatus* R. kingi (i.e., rickettsiae present in the total gDNA of *D. andersoni*, *D. variabilis* and *I. kingi*, respectively). Subsequent DNA sequencing of the 17-kDa gene amplicons of representative samples of the rickettsiae from *I. angustus* revealed that they had identical sequences to each other but differed in sequence by 3-56 bp when compared to the rickettsiae from *D. andersoni*, *D. variabilis* and *I. kingi*. PCR-SSCP is therefore an effective prescreening method to determine if there is genetic variation among rickettsial DNA derived from the total gDNA of individual ticks. A BLAST search of the sequence data further revealed that the rickettsiae in *I. angustus* had a novel 17-kDa gene sequence when compared to the sequences of this gene for all recognized and putative species of *Rickettsia*. Given this, the rickettsiae in *I. angustus* were genetically characterized at four additional gene loci (i.e., *gltA, ompA, sca*1 and the 16S rRNA gene). These rickettsiae had an identical sequence for *sca*1 to that of *Candidatus* R. kingi, but differed in sequence by 3.7-17.4% to all other taxa within the genus *Rickettsia*. The magnitude of sequence differences of the rickettsiae in *I. angustus* at four of five loci compared to other taxa within the genus (i.e., 0.6-11.7% for the 17-kDa gene, 0.1-14.2% for *gltA*, 1.6-17.3% for *ompA*, and 0.3-1.89% for the 16S rRNA gene) exceed the levels of sequence differences (i.e., 0.7%, 0.1%, 1.2% and 0.2%, respectively) that distinguish different species of *Rickettsia* (Fournier & Raoult, 2009; Fournier *et al.,* 2003). Therefore, the putative new species of *Rickettsia* within *I. angustus* is provisionally

named *Candidatus* Rickettsia angustus in accordance with the recommended nomenclature for new rickettsiae that have not been established in pure culture (Fournier & Raoult, 2009).

Phylogenetic analyses conducted on the sequence data of five genes (i.e., 17-kDa gene, *gltA, ompA,* 16S rRNA gene and *sca*1) for *Candidatus* R. angustus revealed that this taxon does not belong to the SFG or TG rickettsiae, but belongs to a clade that contains *R. canadensis*, and three other putative species of *Rickettsia*: *Candidatus* R. kingi, *Candidatus* R. tarasevichiae, and *Candidatus* R. monteiroi. Attempts were made to characterize *Candidatus* R. angustus using a sixth gene region, a ~800-bp fragment of *ompB*. However, no amplicons could be obtained for *Candidatus* R. angustus, whereas this gene was amplified for *R. peacockii* present within the total gDNA of *D. andersoni*. This suggests that *Candidatus* R. angustus lacks the ompB gene. This finding provides additional support for a relationship between *Candidatus* R. angustus and members of the *R. canadensis* clade because the *ompB* gene is also absent in *R. canadensis* (Roux & Raoult, 2000; Ngwamidiba *et al*., 2006) and could not be amplified in *Candidatus* R. kingi (Anstead & Chilton, 2013), whereas this gene is present within species of the SFG rickettsiae (Walker *et al*., 2007).

Although *I. angustus* is known to bite humans (Damrow *et al*., 1989; Estrada-Peña & Jongejan, 1999), and *Candidatus* R. angustus represents a sister taxon to *R. canadensis*, a potential human pathogen (Bozeman *et al*., 1970; Merhej & Raoult, 2011), it remains to be determined if this putative new species of *Rickettsia* is of pathogenic significance with respect to human health. Furthermore, it would be of importance, from an evolutionary standpoint, to determine if *Candidatus* R. angustus is present in other *I. angustus* populations, particularly those in eastern Russia (Robbins & Keirans, 1992; Shpynov *et al*., 2003) and Japan (Robbins & Keirans, 1992; Hiraoka *et al*., 2005), and whether other ixodid ticks are hosts for *Rickettsia*

belonging to the *R. canadensis* clade. Currently, the members of this clade would include *R. canadensis* present in *Haemaphysalis leporispalustris* and several other tick species in North America (McKiel *et al*., 1967; Brinton *et al*., 1971; Mediannikov *et al*., 2007), *Candidatus* R. tarasevichiae in *I. persulcatus* from Siberia and Japan (Eremeeva *et al*., 2007; Inokuma *et al*., 2007), *Candidatus* R. monteiroi in *Amblyomma incisum* from Brazil (Pacheco *et al*., 2011), *Candidatus* R. kingi in *I. kingi* from Saskatchewan (Canada) (Anstead & Chilton 2013), *Candidatus* R. angustus in *I. angustus* from British Columbia (Canada) (present study), and possibly the undescribed "*Rickettsia* sp. H820" from an unknown vector in north-eastern China (GenBank accession numbers JF714220 and JF714221). Interestingly, rickettsiae with a partial (i.e., 322-bp) *gltA* sequence identical to that of *R. canadensis* have also been detected in a single female *Haemaphysalis* sp. feeding on a dog from Fukuoka, Japan (Hiraoka *et al*., 2005). One possible explanation for the current distribution and host usage of the members of the *R. canadensis* clade is that the ancestral species originated in the Palearctic (i.e., eastern Russia, Japan or north-eastern China), and some of its descendants established in the Nearctic following dispersal by small mammals and their rickettsial-infected ticks across the Bering Strait land bridge (Beringia). Subsequently, rickettsiae colonized other tick species in North America and spread further southward into South America. It has been proposed previously that speciation in the group to which *I. angustus* belongs (i.e., the subgenus *Ixodiopsis*) is associated with the dispersal of boreal arvicoloid rodents during the Pleistocene from Siberia across the Bering Strait land bridge to North America, followed by several speciation events (Robbins & Keirans, 1992). The evolutionary history of rickettsiae within the *R. canadensis* clade may therefore be linked to the evolutionary history of tick species in which they occur; however, this hypothesis requires further investigation.

Nucleotide sequence accession numbers. The nucleotide sequences of the different genes for representative samples of the rickettsiae in *I. angustus* and *D. andersoni*, and for *Candidatus* I. kingi obtained in the present study have been deposited in GenBank under accession numbers HF935068-HF935081, respectively.

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Chapter 10 Molecular detection of novel *Rickettsiella* 16S rDNA sequences in *Ixodes angustus, I. kingi* and *I. sculptus* (Acari: Ixodidae)

10.1 Abstract

The genomic DNA from seven species of ixodid tick in western Canada was tested for the presence of *Rickettsiella* by PCR-analyses targeting the 16S rRNA gene. *Rickettsiella* were not detected in any *Dermacentor andersoni, D. variabilis, D. albipictus* or *Ixodes scapularis*, whereas 88% of *I. angustus* (n=270), 43% of *I. sculptus* (n=61), and 4% of *I. kingi* (n=93) examined were PCR-positive for *Rickettsiella*. A comparison of the SSCP profiles of the 16S rDNA amplicons of the *Rickettsiella*-positive individuals revealed that there were three different banding patterns, each corresponding to a different sequence type. Furthermore, each sequence type was associated with a different tick species. Phylogenetic analyses of the sequence data revealed that all three sequence-types were placed in a clade that contained species and pathotypes of the genus *Rickettsiella*. The bacterium in *I. kingi* represented the sister taxon to the *Rickettsiella* in *I. sculptus,* and both formed a clade with *R. grylli,* from crickets (*Gryllus bimaculuatus*), and '*R. ixodidis*' from *I. woodi*. In contrast, the *Rickettsiella* in *I. angustus* was placed external to a clade that contained nine pathotypes of *R. popilliae*. The magnitude of the genetic differences in 16S rRNA gene sequences and the phylogenetic relationships of the bacteria within the three tick species, suggest that each taxon represents a new species within the genus *Rickettsiella*.

10.2 **Introduction**

Terrestrial and marine invertebrates harbor a diverse range of bacterial species (e.g., Jeyaprakash *et al*., 2003; Campbell *et al*., 2004; Hongoh *et al*., 2005; Weinert *et al*., 2007; Goffredi, 2010), some of which are facultative or obligate mutualists, assisting their symbionts in metabolic processes or their ability to resist infection by pathogens (e.g., Burgdorfer *et al*., 1981; Graf *et al*., 2006), whereas others exploit their invertebrate hosts. For instance, some bacteria have pathogenic effects on vertebrates as a consequence of their transmission to these hosts by haematophagous arthropods (e.g., mosquitoes and ticks) (e.g., Tilly *et al*., 2008; Olszewski *et al*., 2009). Other exploitative bacterial species manipulate their invertebrate host to increase transmission to another host (Hurd, 2003; Thomas *et al*., 2005), or reduce the fitness of their invertebrate host as a consequence of their pathogenic effects (McGraw *et al*., 2002; Turley *et al*., 2009). For example, the genus *Rickettsiella* includes species known to be intracellular pathogens of arthropods (Cordaux *et al*., 1997; Roux *et al*., 1997). In many instances, infection with *Rickettsiella* results in death of the arthropod host (Dutky & Goodens, 1952; Federici, 1980; Federici, 1984; Adamo, 1998). However, it has also been shown that some *Rickettsiella* may be of benefit to their arthropod hosts. For example, the presence of *Rickettsiella* in pea aphids (*Acyrthosiphon pisum*) results in a change of host body color from red to green, which may decrease their risk of predation by lady bird beetles (Tsuchida *et al*., 2010).

Rickettsiella were first described in 1952 as "small *Rickettsia*" (Philip, 1956) and assigned to the order Rickettsiales (Alphaproteobacteria) (Weiss *et al*., 1984). However, phylogenetic analyses of 16S rDNA sequence data subsequently revealed that *Rickettsiella grylli* represented a sister taxon to two genera of Gammaproteobacteria: *Coxiella* and *Legionella* (Roux *et al*., 1997). As a consequence, the genus *Rickettsiella* was transferred from the Rickettsiales to the family

Coxiellaceae within the order Legionellales (Fournier & Raoult, 2005). The close association of these arthropod-borne bacteria with members of the Legionellales has been demonstrated repeatedly in many molecular-based studies (Cordaux *et al.,* 2007; Leclerque, 2008; Leclerque & Kleespies, 2008a, 2008b; Mediannikov *et al.,* 2010; Kleespies *et al.,* 2011; Leclerque *et al.,* 2011; Leclerque & Kleespies, 2012; Leclerque *et al.,* 2012; Shuster *et al.,* 2012).

A diverse range of arthropods, including insects (e.g., beetles, flies, crickets, locusts, cockroaches, wasps, midges, moths and aphids), collembolans, crustaceans (e.g., isopods and crabs) and arachnids (e.g., spiders, scorpions, ticks and mites) have been reported as hosts for *Rickettsiella* (Fournier & Raoult, 2005; Bouchon *et al*., 2012). Currently, there are only four recognized species of *Rickettsiella*: *R. popilliae*, *R. grylli, R. chironomi* and *R. stethorae* (Leclerque & Kleespies 2012). However, several pathotypes have been described based on the host species they infect, their pathogenic effects on hosts, and on genetic comparisons with other *Rickettsiella* (Bouchon *et al*., 2012; Leclerque *et al.,* 2012). Some pathotypes have been shown, using genetic comparisons, to have identical 16S rDNA sequences to one another (e.g., *'R. costelytrae*' and *'R. pyronotae*'; Leclerque *et al*., 2012); some are called a synonym of one of the four recognized species (Leclerque & Kleespies, 2008a; Bouchon *et al*., 2012; Leclerque *et al.,* 2012; Shuster *et al*., 2012), while others are considered as unassigned species (Bouchon *et al*., 2012).

Ticks have been shown to be suitable hosts for *Rickettsiella*. For example, a *Rickettsiella*, genetically similar to *R. grylli*, has been isolated in the ovarian tissues and malpighian tubules of unfed female *Ixodes woodi* (Kurtti *et al.,* 2002), while female *Dermacentor reticulatus* have been experimentally-infected with '*R. phytoseiuli'* isolated from the mite *Phytoseiulus persimilis* (Šuťáková & Řeháček, 1990). *Rickettsiella* DNA has also been detected by PCR in *I. woodi*, *I.*

tasmani and *I. ricinus* (Kurtti *et al.,* 2002; Vilcins *et al.,* 2009; Carpi *et al*., 2011; Tveten & Sjåstad, 2011). We have also recently detected *Rickettsiella* DNA in a few individuals of *I. sculptus* during a molecular-based study of the bacterial diversity in this tick species (Chapter 7). Therefore, the aim of the present study was to develop a PCR-based assay to screen for the presence of *Rickettsiella* in several species of *Ixodes* and *Dermacentor* in Canada that use small mammals as hosts, and to compare the 16S rRNA gene sequences of these bacteria with those available for different species and pathotypes of *Rickettsiella*.

10.3 **Materials and Methods**

Total genomic (g) DNA was extracted and purified from 270 *Ixodes angustus*, 93 *I. kingi*, 61 *I. sculptus*, six *I. scapularis*, 45 *Dermacentor andersoni*, two *D. variabilis*, and one *D. albipictus* (Table 10.1) using the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany), and the modifications described previously (Dergousoff & Chilton, 2007; Anstead & Chilton, 2013). PCR analyses were conducted on the total gDNA of each tick to test for the presence of *Rickettsiella* DNA. Initially, PCRs were conducted using the primers (RCL16S-211F and RCL16S-470R) and conditions of Tsuchida *et al*. (2010); however, no amplicons were produced for any sample. PCRs were then conducted using RCL16S-211F and a 16S rDNA universal bacterial primer (i.e., primer 802r: 5'-ACTACCAGGGTATCTAATCCTG-3'; Dergousoff, 2011), and the conditions of Tsuchida *et al*. (2010), but with modifications to the number of cycles (n=30) and the annealing temperature (58°C). This PCR assay produced amplicons from the ticks tested; however, subsequent sequencing of representative PCR products revealed the presence of multiple bacterial species within each amplicon. The same problem was encountered

Locality (Coordinates) Tick species	Life cycle stage	No. tested	No. (%) PCR-positive
Kootenay N.P., BC (49°44'N 112°50'W)			
Ixodes angustus	larvae	178	163 (92%)
	nymphs	68	55 (81%)
	adults	24	20 (83%)
Dermacenor andersoni	adults	2	0 (0%)
Beechy, SK (50°53'N, 107°23'W)			
I. sculptus	larvae	33	16 (49%)
	nymphs	21	8 (38%)
	adults	3	0 (0%)
I. kingi	larvae	1	0 (0%)
	nymphs	4	1 (25%)
	adults	1	1 (100%)
D. andersoni	nymphs	20	0 (0%)
	adults	20	0 (0%)
Clavet, SK (51.9519°N, 106.4473°W)			
I. sculptus	nymphs	4	2 (50%)
I. kingi	larvae	82	0 (0%)
	nymphs	2	0 (0%)
	adults	3	2 (66%)
I. scapularis	larvae	6	0 (0%)
D. andersoni	larvae	3	0 (0%)
D. variabilis	larvae	1	0 (0%)
	nymphs	1	0 (0%)
D. albipictus	nymphs	1	0 (0%)

Table 10.1 The number of larvae, nymphs and adults of different tick species collected at different localities in western Canada that were PCR-positive for *Rickettsiella*

when PCRs were conducted using the universal eubacterial primers (i.e., fD1 and rP2; Weisburg *et al*., 1991) that have been used to amplify the 16S rRNA gene of *Rickettsiella* in other studies (e.g., Kurtti *et al*., 2002; Leclerque & Kleespies, 2008a; Vilcins *et al*., 2009). As a consequence, two new primers, Rickella-F (5'-GTAGGAATCTGTCCTGGAG-3') and Rickella-R2 (5'-TGCTTATTCTGTGGGTACCG-3'), were designed specifically, based on a sequence comparison of all available nucleotide sequences available on GenBank, to amplify part (~380bp) of the 16S rRNA gene of *Rickettsiella*. PCR's were performed in 25μl volumes containing 2.5μl 10X i*Taq* PCR buffer (Bio-Rad), 3 mM $MgCl_2$, 200μM of each deoxynucleoside triphosphate (dNTP), 25pmol (1μM) of each primer, 0.5U/μl i*Taq* DNA polymerase (Bio-Rad), and 1.5μl of gDNA template. A negative control (i.e., without gDNA) sample was included in each set of PCR assays. PCR's were performed in a thermocycler (iCycler; Bio-Rad, Hercules, CA) using the following conditions: 95°C for 5 min, followed by 30 cycles of 95°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec, and a final extension step at 72°C for 5 min. Amplicons were subjected to electrophoresis on SYBR® Safe-stained 1.5% agarose-TBE (89mM Tris, 89mM boric acid, 2mM EDTA, pH 8.3) gels and their banding patterns were visualized by UV transillumination. All PCR-positive samples were pre-screened for genetic variation using single strand conformation polymorphism (SSCP) analyses (Gasser *et al*., 2006) following the same protocol as described previously (Dergousoff & Chilton, 2007). Amplicons from eight PCR-positive ticks (i.e., two *I. angustus,* four *I. kingi,* and two *I. sculptus*), representing the three different SSCP banding patterns (i.e., profiles), were purified (Dergousoff & Chilton, 2009) and subjected to automated DNA sequencing using primers Rickella-F and Rickella-R2 in separate reactions to confirm the identity of these bacteria within the three species of tick.

The *Rickettsiella* in the gDNA of two *I. angustus,* two *I. kingi,* and two *I. sculptus* were further characterized by amplifying a larger (~1,270 bp) fragment of the 16S rRNA gene using primers Rickella-F and 1387R-mod (5'-GGGCGGTGTGTACAAGGC-3') (Marchesi *et al.,* 1998). The same temperature conditions were used for the PCR as described above except that the duration of each phase was increased to 60 seconds. In addition, the $MgCl_2$ concentration was reduced to 2.5mM and the volume of gDNA template increased to 2 μl. All amplicons were purified prior to DNA sequencing with primers Rickella-F and 1387R-mod in separate reactions. BLAST searches (GenBank) were performed on the DNA sequence data. The DNA sequences of the *Rickettsiella* in each species of tick were aligned manually with the sequences of *Rickettsiella* available on GenBank. Phylogenetic analyses were performed using the neighbor joining (NJ) and maximum parsimony (MP) methods in PAUP (Swofford, 2003). For the MP analyses, characters were treated as unordered and were equally weighted, while alignment gaps were treated as 'missing' characters. The 16S rDNA sequence of *Coxiella burnetii* was used as the outgroup for the MP analyses. Heuristic searches with TBR branch swapping were used to infer the shortest trees. The length, consistency index excluding uninformative characters, and the retention indices of each most parsimonious tree were recorded. Bootstrap analyses (1000 replicates for the NJ analyses and 100 replicates for MP analyses) were conducted to determine the relative support for clades in the consensus trees.

10.4 **Results**

A total of 478 ticks were tested individually for the presence of *Rickettsiella* DNA by PCR (Table 10.1). No amplicons were obtained for any of the 48 *Dermacentor* individuals (i.e., 45 *D. andersoni*, two *D. variabilis*, and one *D. albipictus*), six *I. scapularis* larvae, or the negative control samples. Of the remaining 424 *Ixodes* individuals tested, 268 (63%) were PCR-positive (Table 10.1). All PCR-positive samples produced a single band on TBE-agarose gels of the expected size (~380 bp) for the partial fragment of the 16S rRNA gene amplified using primers Rickella-F and Rickella-R2 (data not shown). There was a significant difference ($\chi^2_2 = 224.9$, $P < 0.001$) in the proportions of *I. angustus, I. sculptus* and *I. kingi* that were PCR-positive for *Rickettsiella* (i.e., 88%, 43% and 4%, respectively). For *I. angustus,* there was no significant difference ($\chi^2_2 = 5.94$, $P > 0.05$) among life cycle stages in the proportions of individuals that were PCR-positive for *Rickettsiella* (Table 10.1). Although none of the three *I. sculptus* adults were infected with *Rickettsiella*, there was no significant difference ($\chi^2_2 = 0.37$, $P > 0.05$) in the proportions of *I. sculptus* larvae and nymphs that were PCR-positive for *Rickettsiella* (Table 10.1). Of the 95 *I. kingi* individuals screened for *Rickettsiella* DNA, some of the adult and nymphal ticks were PCR-positive, whereas none of the 81 larvae were PCR-positive. Furthermore, *I. kingi* and *I. sculptus* collected from Clavet and Beechy (SK), situated approximately 200km apart, were PCR-positive for *Rickettsiella*.

A comparison of the SSCP profiles of the 16S rDNA amplicons of the 268 *Rickettsiella*-positive individuals revealed that there were three different banding patterns (Fig. 10.1). The banding patterns of the four *I. kingi* samples (two from Beechy and two from Clavet) were identical to one another but differed to the banding patterns of all 26 amplicons derived from *I. sculptus* (24 from Beechy and two from Clavet). Similarly, there was no variation in SSCP

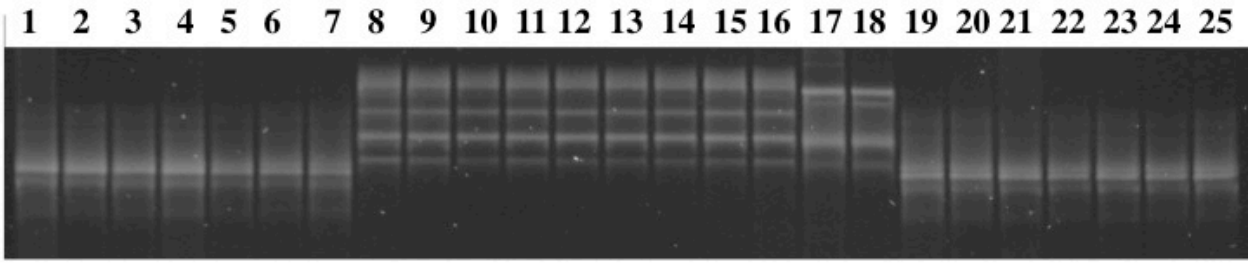

Fig. 10.1 SSCP profiles of representative 16S rRNA amplicons of *Rickettsiella* from the total gDNA of *Ixodes angustus* (lanes 1-7 and 19-25), *I. kingi* (lanes 17 & 18) and *I. sculptus* (lanes 8-16).

profiles of the 238 PCR-positive samples from *I. angustus*; however, the banding pattern of each sample differed from those derived from *I. sculptus* and *I. kingi*. A comparison of the DNA sequences (340 bp) of representative samples of each SSCP profile type revealed that samples with identical banding patterns had identical 16S rDNA sequences, whereas those that differed in banding pattern differed by 3-22 bp in sequence. BLAST searches of all three sequence types revealed that they were genetically most similar, but not identical, to the 16S rRNA gene sequences of species within the genus *Rickettsiella*.

Given the novel sequences of the *Rickettsiella* from *I. angustus*, *I. sculptus* and *I. kingi*, comparisons were made for the sequence of a larger fragment (1,272 bp) of the 16S rRNA gene for six *Rickettsiella*-infested ticks (i.e., two *I. angustus,* two *I. kingi,* and two *I. sculptus*). There were 55 variable positions in the sequence alignment of the three taxa, representing 36 transitional (23 purine and 13 pyrimidine) changes, 15 transversional changes, two multiple mutational changes and two indels (Table 10.2). The DNA sequences of the *Rickettsiella* in the two *I. angustus* were identical to one another but differed by 3.8% (i.e., 49 bp) from the *Rickettsiella* in the two *I. kingi*, and by 3.8% (i.e., 49 bp) from the *Rickettsiella* in the two *I. sculptus*. The DNA sequences of the *Rickettsiella* in *I. kingi* differed by 1.1% (i.e., 14 bp) from the *Rickettsiella* in the *I. sculptus* (Table 10.2). The DNA sequences of the *Rickettsiella* in *I. angustus*, *I. kingi* and *I. sculptus* differed by 2.0-6.6% (i.e., 25-82 bp), 2.6-6.2% (i.e., 33-78 bp) and 2.5-6.0% (i.e., 32-75 bp) respectively, when compared to the sequences of taxa within the genus *Rickettsiella* (Table 10.3).

Species	Nucleotide position																											
	1	3	35	52	64	68	82	84	87	116	124	165	223	240	281	320	322	328	330	331	332	335	337	350	405	452	509	512
'Rickettsiella kingi'	A	C	G	T	A	C	C	A	T	G	G	G	G	A	C	A	T	A	T	G	A	A	T	A	G	T	A	A
'Rickettsiella sculptus'	.	T	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	G	.	.	A	C	G	.
'Rickettsiella angustus'	C	T	A	C	–	A	A	G	G	T	A	A	A	C	A	G	C	T	C	T	G	G	C	G	A	C	.	C

Species	Nucleotide position																										
	696	706	715	719	725	726	871	875	876	877	878	888	889	894	908	909	985	997	1007	1009	1051	1056	1060	1115	1117	1135	1156
'Rickettsiella kingi'	A	A	T	G	–	C	G	G	A	A	A	C	T	G	T	G	T	G	A	A	A	G	C	A	G	T	A
'Rickettsiella sculptus'	.	C	.	A	–	.	A	.	.	G	.	.	.	A	.	.	.	A	.	.	.	A	.	G	.	.	.
'Rickettsiella angustus'	G	T	C	.	A	A	.	T	G	.	G	T	C	T	C	A	C	.	T	T	G	.	T	G	A	C	T

Table 10.2 Variable nucleotide positions in the aligned 16S rDNA sequences of '*Rickettsiella kingi*', '*R. sculptus*' and '*R. angustus*' detected within three species of *Ixodes* in western Canada. A dot indicates the same nucleotide as in the sequence of the '*R. kingi*'.

	Taxon	Accession no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	*R. costelytrae*[1]	JF490030	-	99.8	99.9	99.8	99.8	99.5	99.7	99.4	99.4	97.6	96.5	97.9	97.4	97.5	94.4	95.2	95.1	97.1	94.5	97.1
2	*R. tipulae*[1]	EU180598	3	-	99.8	99.7	99.7	99.4	99.6	99.1	99.2	97.5	96.4	97.9	97.2	97.3	94.2	95.0	95.0	97.1	94.5	97.1
3	*R. melolonthae*[1]	EF408231	1	2	-	99.8	99.8	99.6	99.8	99.2	99.3	97.5	96.5	98.0	97.3	97.5	94.2	95.1	95.1	97.1	94.5	97.1
4	*R. agriotidis*[1]	HQ640943	3	4	2	-	99.7	99.4	99.6	99.1	99.1	97.4	96.3	97.9	97.3	97.4	94.1	94.9	95.2	97.1	94.4	97.1
5	ex *Myrmeleon bore*[1]	AB291637	3	4	2	4	-	99.6	99.8	98.9	99.1	97.5	96.3	98.0	97.2	97.3	94.2	94.9	94.9	97.0	94.4	97.0
6	ex *Poecilus chalcities*[1]	EF608533	6	7	5	7	5	-	99.5	98.9	98.9	97.0	96.0	97.6	96.9	97.0	94.0	94.8	94.8	96.7	94.2	98.6
7	ex *Harpalus pennsylvanicus*[3]	GU815103	4	5	3	5	2	5	-	99.0	99.0	97.2	96.1	97.6	97.1	97.2	94.1	94.9	94.7	96.8	94.2	98.6
8	ex *Eisenia fetidia*[1]	FJ542982	8	11	9	14	11	14	12	-	99.7	97.3	96.5	97.6	97.3	97.3	94.1	95.2	95.1	97.1	94.2	97.0
9	ex *Folsomia candida*[4]	AF327558	8	10	9	11	11	14	12	4	-	97.6	96.4	97.6	97.3	97.3	94.3	95.1	94.9	97.1	94.1	96.9
10	*R. grylli ex Gryllus*[1]	U97545	30	32	32	33	32	37	35	33	30	-	97.2	96.3	96.4	97.3	94.1	94.8	95.1	96.2	93.8	96.0
11	*R. ixodidis*[2]	AF383621	44	46	45	47	47	51	49	44	36	35	-	95.1	96.2	96.4	93.5	94.2	94.3	95.2	93.3	95.5
12	***R. angustus***[1]		26	27	25	27	25	30	30	30	30	46	62	-	96.2	96.2	93.9	94.1	93.4	96.3	93.5	96.3
13	***R. kingi***[1]		33	35	34	34	35	39	37	34	34	39	47	49	-	98.9	93.8	94.1	94.4	94.9	96.1	96.7
14	***R. scultpus***[1]		32	34	32	33	34	38	35	33	33	33	46	49	14	-	94.0	94.3	95.3	95.2	96.3	96.4
15	ex *Ceciotrioza sozanica*[6]	AF286124	71	72	72	74	72	75	75	74	72	74	83	77	78	75	-	95.8	94.4	94.0	93.0	93.4
16	ex Oribatid mites[5]	GU906700	61	62	63	62	64	64	65	65	61	62	65	74	75	72	53	-	95.0	94.9	94.2	94.6
17	ex *Acyrthosiphon pisum*[3]	AB522704	62	63	62	62	64	66	66	62	65	62	73	76	72	62	71	63	-	95.4	93.6	94.4
18	ex *Asellus aquaticus*[7]	AY447040	36	37	37	36	38	42	40	36	37	47	61	47	64	61	74	64	57	-	93.9	96.0
19	*Diplorickettsia massiliensis*[5]	GQ857549	69	70	70	72	72	73	73	74	75	73	85	82	49	47	88	73	81	77	-	94.2
20	*R. grylli ex Armadillidium*[1]	AAQJ02000001	36	37	37	37	38	40	40	37	39	50	57	47	41	45	83	68	71	50	74	-

[1]1255 bp, [2]1269 bp, [3]1256 bp, [4]1253 bp, [5]1260 bp, [6]1259 bp and [7]1257 bp

Table 10.3 Pairwise comparison of the number of nucleotide differences (lower diagonal) and percent sequence similarity (upper diagonal) between different members of the genus *Rickettsiella*.

The NJ analysis of the 16S rDNA sequence data revealed that the *Rickettsiella* in *I. kingi* represented the sister taxon to the *Rickettsiella* in *I. sculptus* with 100% statistical support (Fig. 10.2). Both taxa were also members of a clade, with some statistical support (i.e., bootstrap value of 78%) that also contained '*R. ixodidis*' and *R. grylli* (ex *Gryllus bimaculatus*). In contrast, the *Rickettsiella* in *I. angustus* was placed external, with strong statistical support (80% bootstrap value), to a group comprising two clades, the first containing seven pathotypes of *R. popilliae*, and the second containing *Rickettsiella* ex *Eisenia fetida* and *Rickettsiella* ex *Folsomia candida* (Fig. 10.2). The single most-parsimonious tree (not shown) produced by the MP analysis of the sequence data (i.e., 158 cladistically-informative characters) had a length of 607, a CI of 0.57 and a RI of 0.59. As with the NJ analysis, there was very strong statistical support (i.e., bootstrap value of 99%) for a sister taxa relationship between the *Rickettsiella* in *I. kingi* and *I. sculptus* in the MP tree, but no support for these taxa forming a clade with *R. grylli* (ex *Gryllus bimaculatus*) and '*R. ixodidis*'. There was also no statistical support in the MP tree for the inclusion of the *Rickettsiella* in *I. angustus* in a clade with other taxa in the genus.

10.5 **Discussion**

A new PCR assay was developed to test for the presence of *Rickettsiella* 16S rDNA in the total gDNA of 478 individual ticks representing seven species collected from three different localities in Canada, Kootenay National Park (BC), Beechy and Clavet (SK). A total of 268 ticks (i.e., 23 adults, 66 nymphs & 179 larvae), all *Ixodes* sp., were PCR-positive for *Rickettsiella* using our PCR assay. None of the 45 *D. andersoni*, two *D. variabilis*, and one *D. albipictus* tested were infected with *Rickettsiella*. It is possible that these three tick species may not

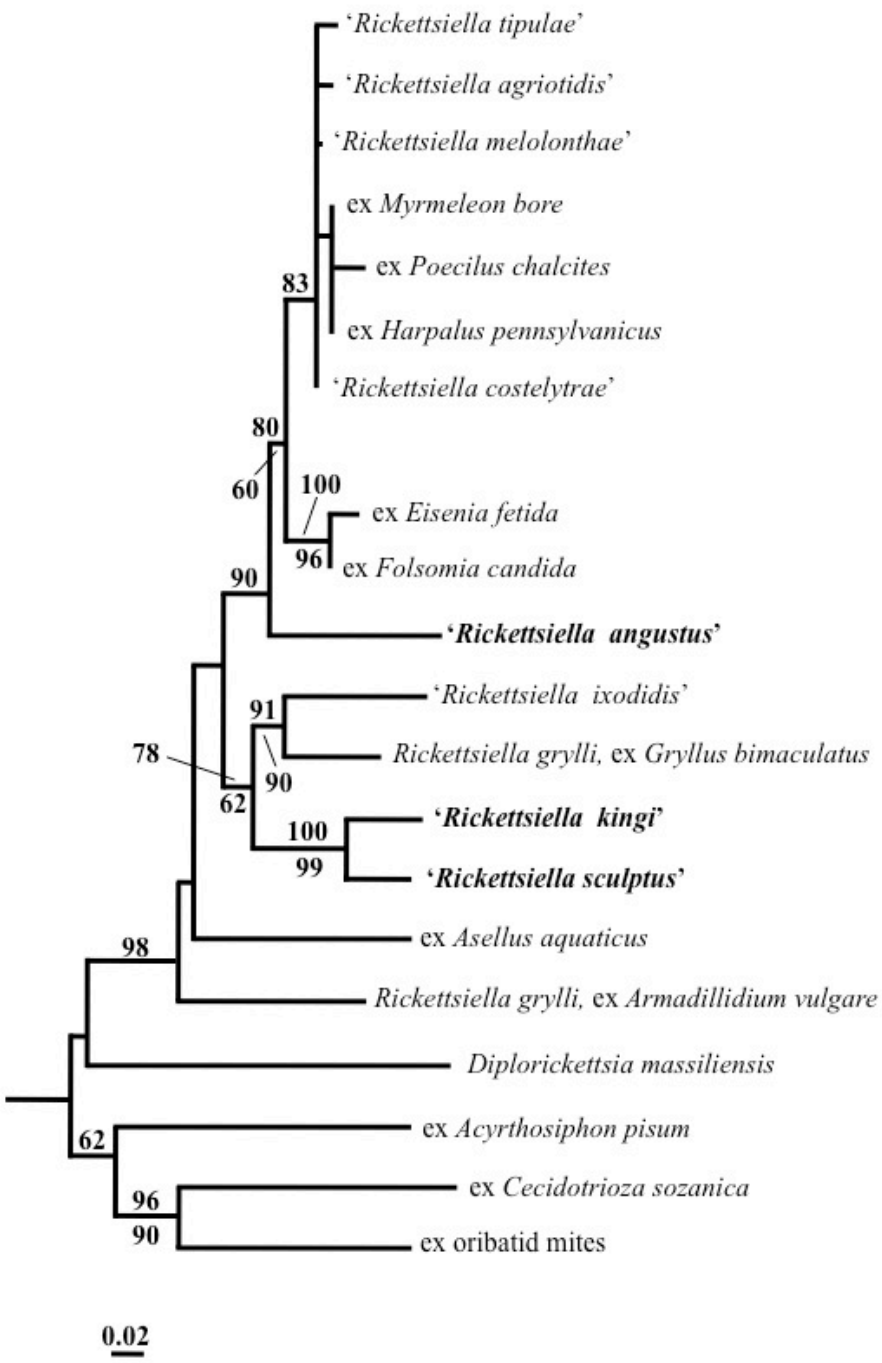

Fig. 10.2 Neighbor-joining tree depicting the relationships of the 16S rRNA gene sequences of *'Rickettsiella angustus', 'R. kingi', 'R. sculptus'* and other species and pathotypes of the genus *Rickettsiella*. The scale bar represents the inferred substitutions per nucleotide site. The relative support for clades in the tree produced from the NJ and MP analyses are indicated above and below branches, respectively.

represent suitable hosts for this genus of gammoproteobacteria, however more individuals from a number of localities need to be screened to test this hypothesis. Nonetheless, many of the *D. andersoni* individuals tested were found parasitizing the same host individuals as *I. scultpus* (at Beechy, SK) or *I. angustus* (at Kootenay NP, BC) that were found to contain *Rickettsiella*. This suggests that there was no transfer of *Rickettsiella* to *Dermacentor* individuals feeding with *Rickettsiella*-infected ticks on small mammals. There are no reports, as far as we are aware, of species within the genus *Dermacentor* as hosts for *Rickettsiella*, even though *D. reticulatus* females have been shown experimentally to be suitable hosts for '*R. phytoseiuli*' (Šuťáková & Řeháček, 1990). Interestingly, *Rickettsiella* has been detected in ticks, but thus far, only in members of the genus *Ixodes*: *I. ricinus* in the Palearctic (Carpi *et al*., 2011; Tveten & Sjåstad, 2011), *I. woodi* in the Nearctic (Kurtti *et al*., 2002) and *I. tasmani* in Australia (Vilcins *et al*., 2009). The results of the present study also detected *Rickettsiella* DNA in three of four species of *Ixodes*: *I. angustus*, *I. sculptus* and *I. kingi*. In addition, all feeding life cycle stages (i.e., larvae, nymphs and adults) of *I. angustus* were infected with *Rickettsiella*, whereas for *I. kingi*, all but the larval stage contained the bacterium, and for *I. sculptus*, only the larvae and nymphs contained *Rickettsiella*. There were also significant differences among the three species of *Ixodes* in the proportions of individuals infected with *Rickettsiella*. The majority (88%) of the 270 *I. angustus* individuals were infected with *Rickettsiella,* compared to only 43% of the 61 *I. sculptus* individuals tested. In contrast, only four (4%) of the 95 *I. kingi* individuals were infected with *Rickettsiella*. The PCR-positive ticks included *I. sculptus* and *I. kingi* individuals collected from both Beechy and Clavet.

The 16S rDNA amplicons (~380 bp) of the 268 PCR-positive samples were compared using SSCP. This mutation scanning technique has been used effectively to differentially display

genetic variation between DNA sequences that are 150-450-bp in size, and that differ by one or more nucleotides (e.g., Dergousoff & Chilton, 2007; Dergousoff *et al*., 2009; Dergousoff & Chilton, 2012; Anstead & Chilton, 2013). Three different SSCP banding patterns (i.e., profiles) were detected among the 268 *Rickettsiella* 16S rDNA amplicons, each profile type associated with a different tick species. DNA sequencing of representative samples confirmed that those with the same SSCP profile had identical 16S rDNA sequences, whereas those with different SSCP profiles differed in sequence. The partial (340 bp) 16S rDNA sequences of the *Rickettsiella* in *I. kingi* differed from the *Rickettsiella* in the *I. sculptus* by 3 bp, while both taxa differed from the *Rickettsiella* in *I. angustus* by 22 bp. A larger number of nucleotide differences (i.e., 14-49 bp; 1.1-3.8%) were detected among the *Rickettsiella* from the three species of *Ixodes* when a much larger fragment (1,272 bp) of the 16S rRNA gene was analyzed. BLAST searches of the sequence data showed that each taxon was closest in sequence to a member of the genus *Rickettsiella*; however, the *Rickettsiella* in *I. angustus*, *I. sculptus* and *I. kingi* each had novel 16S rDNA sequences when compared to those of all recognized species and pathotypes of *Rickettsiella*. Given this, we propose to provisionally name the *Rickettsiella* in *I. angustus*, *I. sculptus* and *I. kingi* as '*Rickettsiella angustus*', '*Rickettsiella sculptus*' and '*Rickettsiella kingi*' (respectively) in accordance with the nomenclature used in other studies (e.g., Leclerque *et al*., 2011; Leclerque & Kleespies, 2012).

Phylogenetic analyses of the 16S rDNA sequence data revealed that '*R. angustus*' was placed external to a clade, comprising two groups; the first containing seven pathotypes of *R. popilliae* (i.e., *'R. tipulae', 'R. agriotidis', 'R. melolonthae', 'R. costelytrae*', *Rickettsiella* in *Myrmeleon bore, Rickettsiella* in *Poecilus chalcites* and the *Rickettsiella* in *Harpalus pennsylvanicus*), and the second containing the *Rickettsiella* in the earthworm, *Eisenia fetida*,

and the *Rickettsiella* in the springtail, *Folsomia candida*. The magnitude of sequence differences between '*R. angustus*' and other members of the genus, including '*R. sculptus*' and '*R. kingi*', ranged from 25-82 bp (2.0-6.6%), which is greater than the differences (i.e., 1-7 bp; 0.1-0.6%) among seven pathotypes of *R. popilliae*. This suggests that *'R. angustus*' represents a new species of *Rickettsiella* based on the results of the phylogenetic analyses and the magnitude of differences in 16S rDNA sequences when compared to other members of the genus.

Similarly, the 16S rDNA sequences of '*R. sculptus*' and '*R. kingi*' differed from those of other *Rickettsiella* by 32-78 bp (2.5-6.2%), which is similar to or exceeds the levels of sequence difference (i.e., ~3%) among closely related species of bacteria (Stackebrandt & Goebel, 1994). This suggests that '*R. sculptus*' and '*R. kingi*' represent distinct species to other members of the genus. However, species delineation within the genus *Rickettsiella* is controversial (Bouchon *et al.,* 2012). The results of the phylogenetic analyses revealed that *'Rickettsiella kingi'* and *'R. sculptus'* formed a clade in the NJ tree, with strong bootstrap support, with *R. grylli* (a pathogen of the cricket, *Gryllus bimaculatus*) and '*R. ixodidis'* in the tick, *I. woodi*, whereas there was no support in the MP analyses for this sister taxa relationship. This suggests that '*R. sculptus*' and '*R. kingi*' are not pathotypes of any recognized species of *Rickettsiella*. Although there was strong support for a sister taxa relationship in both the NJ and MP analyses between '*R. sculptus*' and '*R. kingi*', these two *Rickettsiella* differed in 16S rDNA sequence by 1.1% (i.e., 14 bp), which is greater than differences among pathotypes of *R. popilliae*. In addition, '*R. sculptus*' and '*R. kingi*' were host-specific because they were found in different tick species that were feeding on the same small mammal hosts at two localities separated by approximately 200km. Therefore, there was no evidence of cross-transmission of the *Rickettsiella* in *I. sculptus* to *I. kingi*, or *vice-versa*. There were also major differences in the proportions of *I. sculptus* and *I. kingi* individuals

infected with *Rickettsiella*. These combined results suggest that '*R. sculptus*' and '*R. kingi*' each represent a new species of *Rickettsiella*.

In conclusion, three novel *Rickettsiella* were detected in the total gDNA of three species of *Ixodes* in North America that use small mammals as hosts. More work is needed to determine whether these putative new species of *Rickettsiella* have pathogenic or beneficial effects on their tick hosts, as has been shown for other members of the genus (e.g., Dutky & Goodens, 1952; Tsuchida *et al.,* 2010), and if other species of *Ixodes* in North America are hosts for *Rickettsiella*.

Nucleotide sequence accession numbers. The nucleotide sequences of the 16S rRNA gene for representative samples of the *Rickettsiella* in *I. angustus*, *I. kingi* and *I. scutptus* have been deposited in GenBank under accession numbers HF912419, HF912420, HF912421.

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Chapter 11. General Discussion

11.1 Principal questions addressed in this thesis

Arthropods are important hosts and vectors for a diverse community of microorganisms (e.g., pathogenic and endosymbiotic bacteria; Hill *et al*., 2005; Clay *et al*., 2006; Jones *et al*., 2009). The distributional ranges of some arthropods have or are predicted to change because of altered environmental conditions associated with global warming (Lindsay *et al*., 1998; Githeco *et al*., 2000; Ogden *et al*., 2008). As a consequence, the incidences of arthropod-borne diseases in some geographical regions are also changing. However, for many arthropod species, there is limited information on the composition and diversity of their bacterial communities.

Ixodid ticks of small mammals are important vectors of human and animal pathogens (Parola & Raoult, 2001; Jongejan & Uilenberg, 2004; Dantas-Torres *et al*., 2012). Understanding the ecology of tick-borne diseases requires knowledge of the interactions between tick vectors, their hosts (e.g., small mammals), and the microorganisms they harbor. Some of the questions that can be examined include: what tick species parasitize different small mammal hosts?, what is the composition, prevalence and diversity of bacteria within individual ticks?, do bacteria exhibit specificity for tick vectors? and what is the potential risk of exposure for humans and animals to tick-borne pathogens? The answers to these questions provide important information that can then be incorporated into the surveillance programs aimed at the control of ticks and tick-borne pathogens. The data gathered can also be used to examine fundamental questions relating to the interactions between parasites, their hosts and their bacterial endosymbionts.

The focus of my PhD research was an examination of some of the questions stated previously in relation to four species of ixodid tick that parasitize small mammals in western Canada (Fig. 11.1). Specifically, I identified ticks (to the species-level) parasitizing small mammals at three localities in western Canada using a combined morphological and molecular approach (**Chapters 2-4**), and examined the genetic variation and phylogenetic relationships of these tick species (**Chapters 5 & 6**). In addition, I compared the composition and diversity of bacteria within four tick species (**Chapter 7**), and further characterized the bacteria of two genera to determine if they represented different species within each species of tick (**Chapters 8-10**).

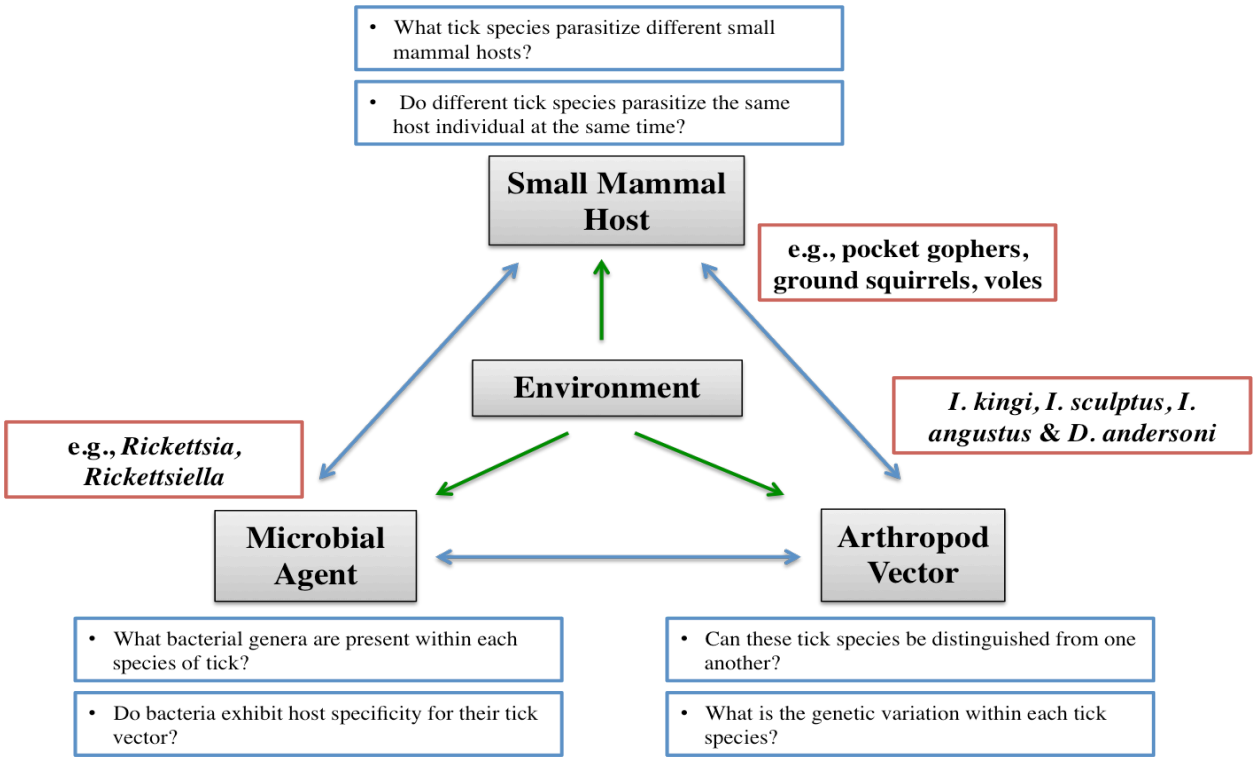

Fig. 11.1 The principal questions addressed in this thesis in respect to *I. kingi*, *I. sculptus*, *I. angustus* and *D. andersoni* and their relationships to other members in the triangle (i.e., small mammal host, microbial agents, and the environment).

11.2 **Identification of ticks**

An important objective of this thesis was to determine which tick species were parasitizing small mammals at three localities in western Canada. It is necessary to accurately identify ticks to the species level, irrespective of life cycle stage, in order to address two key questions: (1) what tick species parasitize different small mammal hosts? and (2) do different tick species parasitize the same host individual at the same time?

Although it is not difficult to use morphological characters to identify adult ticks of the different species within the genus *Dermacentor* in North America, it is much more difficult to distinguish among adults of species within the genus *Ixodes*. In this thesis, light microscopy and scanning electron microscopy (SEM) were used to identify adult and immature (i.e., larvae and nymphs) ticks to the species level using morphological characters. Species-level identification was based on the size and shape of the hypostome, coxal spurs, spiracular plate and goblet cells (Brinton *et al*., 1965; Keirans & Litwak, 1989). SEM was used to confirm the identification of some adult ticks, and as a means to identify immature ticks to the species-level. Although SEM is an effective and high-resolution tool for species-level identification, specimens prepared for SEM cannot be subsequently included in molecular-based studies that examine population genetics or the bacterial communities of ticks. Given this and the difficulties of identifying some immature ticks by morphological examination, a molecular approach (i.e., PCR-single strand conformation polymorphism (SSCP) analysis combined with DNA sequencing of three targets) was therefore employed to accurately identify the ticks feeding on small mammal hosts from Clavet and Beechy in Saskatchewan, and Kootenay National Park in British Columbia.

Initially, I targeted the second internal transcribed spacer (ITS2) of the ribosomal DNA as a potential genetic marker to identify ticks on small mammals. This DNA region was selected

because it has been used for distinguishing among closely related species of ticks (Wesson *et al*., 1993; Zahler *et al*., 1995; Poucher *et al*., 1999; Dergousoff & Chilton, 2007). In particular, Poucher *et al*. (1999) used PCR-RFLP of the ITS-2 to distinguish among 17 species of *Ixodes* in North America. However, the ITS-2 rDNA sequences of these species were not published or deposited on GenBank. My preliminary molecular analyses showed that *I. scapularis*, *I. kingi, I. sculptus* and *I. angustus* could be distinguished from one another based on sequence differences in the ITS-2 rDNA (not reported herein); however, several difficulties were encountered in further examination of this potential genetic marker. For instance, I could not replicate the RFLP patterns for *I. kingi, I. sculptus* and *I. angustus* as shown by Poucher *et al*. (1999). In addition, minisatellites (i.e., small repeated nucleotides; [e.g., $AT_{(n)}$]) were detected in the ITS-2 rDNA sequences of some species of *Ixodes,* making techniques such as PCR-RFLP and PCR-SSCP more difficult to interpret. Therefore, this potential genetic marker was not pursued further in this thesis, but it may be of use in future studies on these ticks following method modification.

The usefulness of the D3 domain and flanking core regions (=$D3^{+}$) of the nuclear 28S rRNA gene was also examined as a potential genetic marker for species-level identification of ticks (Chapter 5) because this DNA target had been used for species identification in some other arthropods (e.g., Wheeler & Hayashi, 1998; Maraun *et al*., 2003; Maraun *et al*., 2004). One important outcome of this study was the development of a new primer pair and PCR-protocol designed to amplify the $D3^{+}$ of ticks (and other arthropods), because other primers previously used to amplify this gene in ticks (McLain *et al*., 2001) co-amplified the $D3^{+}$ of fungi from some specimens of *Ixodes* (Chapter 5). Amplification of the 28S rRNA gene of fungal contaminants from invertebrate gDNA samples is often a problem, given the relatively high genetic similarity in the sequences and secondary structure for many regions of the 28S gene among distantly

related organisms, particularly in the core regions (Wuyts *et al*., 2001). Therefore, development of a new primer pair effectively eliminated the accidental amplification of fungal contaminants from the gDNA of ticks. Despite this method modification, the results obtained in this thesis showed that although some species of *Ixodes* had different sequences of the $D3^+$, *I. angustus* and *I. sculptus* had identical $D3^+$ sequences. Hence, this demonstrated that the $D3^+$ could not be used to unequivocally distinguish among all species of *Ixodes* that have been reported in western Canada. Furthermore, there were no interspecific differences in the $D3^+$ sequences of four species of *Dermacentor* that occur in North America. In contrast, the $D3^+$ sequences of the six species of *Ixodes* considered in the present thesis were distinct from the $D3^+$ sequences of *D. albipictus, D. andersoni, D. occidentalis* and *D. variabilis*. Although the $D3^+$ region of the 28S rRNA gene is useful to distinguish among ticks at the genus level, it is not suitable as a species marker for ixodid ticks.

The mt 16S rRNA gene has been used frequently in population genetic studies of several species of *Ixodes* (e.g., Caporale *et al*., 1995; Norris *et al*., 1999; Qiu *et al*., 2002; de la Fuente, 2005), and to examine the species status of taxa within the genus *Ixodes* (Norris *et al*., 1997). Therefore, the 3' end of the 16S gene was examined as a genetic marker to identify and distinguish among different tick species from different species of small mammal. The results obtained showed that each tick species within the two genera examined (*Dermacentor* and *Ixodes*) had different sequences of the 16S gene that could be distinguished from one another by PCR-SSCP, hence providing a reliable method to identify and distinguish among tick species.

11.3 **Tick species on small mammals in western Canada**

The mt 16S rRNA gene was used to confirm the species identity of the two species of *Ixodes* and two species of *Dermacentor* collected from northern pocket gophers (*Thomomys talpoides*) near Clavet, Saskatchewan. The majority of ticks parasitizing these hosts were identified as adults, nymphs or larvae of *I. kingi*; however, an unusual finding of the study was the detection of a small number of *I. scapularis* larvae (Chapter 2). This tick species is the most important vector of *Borrelia burgdorferi* (i.e., causative agent of Lyme Disease) in eastern North America (Thompson *et al.,* 2001; Bacon *et al.,* 2008). It is also an important vector of the bacterium *Anaplasma phagocytophilum*, the causative agent of human granulocytic anaplasmosis (Thompson *et al.,* 2001). The presence of *I. scapularis* larvae on *T. talpoides* near Clavet was unexpected because the nearest known established population of *I. scapularis* is situated more than 700 km to the southeast in southern Manitoba (Ogden *et al.,* 2008). Flagging for questing ticks and further examination of the ticks feeding on *T. talpoides* in two subsequent years failed to detect the presence of additional *I. scapularis* near Clavet, suggesting that there is no established population of this species at this site. It has been predicted that by the 2020's, environmental conditions in southern Saskatchewan may become suitable to support the establishment of populations of *I. scapularis* (Ogden *et al.,* 2006). Therefore, given the medical importance of this tick species and its pathogenic bacteria, it is important that there are surveillance programs aimed at the early detection, notification, and control of this tick vector and the disease-causing agents it carries. The results of the present study demonstrate the utility of PCR-SSCP and DNA sequencing of the mt 16S rRNA gene for the identification of ticks of socio-economic importance.

Another important discovery resulting from the examination of ticks on *T. talpoides* near Clavet was the detection of three *D. andersoni* larvae. Each tick was collected from a different host and each differed in the sequence of the 16S rRNA gene. Given the maternal inheritance of mt DNA, the detection of three different 16S haplotypes among the *D. andersoni* larvae indicates that they are the progeny of three adult females. The occurrence of *D. andersoni* near Clavet was surprising because this species has not been recorded previously this far east in Saskatchewan (see Wilkinson, 1967, Dergousoff *et al.,* 2013). Although the distributional range of *D. andersoni* has expanded eastwards in Saskatchewan since the 1970's, which may have implications for transmission of tick-borne pathogens to livestock and humans, the closest locality to Clavet where large numbers of questing *D. andersoni* adults have been collected is the township of Outlook (Dergousoff *et al.,* 2013), situated ~ 97 km to the southwest. The presence of two *D. variabilis* immatures (one nymph and one larva) on *T. talpoides* near Clavet was not unusual given that questing adults were also collected at this locality. However, immatures of *D. variabilis* are more commonly found feeding on other smaller rodents (e.g., *Clethrionomys gapperi, Microtus pennsylvanicus* and *Peromyscus maniculatus*) in this region (Dergousoff, 2011). This work highlights the value of PCR-based techniques (e.g., SSCP in combination with DNA sequencing) to distinguish among tick species, particularly for engorged larvae, where it is often more difficult to determine species identity based on morphological examination alone.

These molecular tools were also used to identify ticks collected from Richardson's ground squirrels (*Spermophilus richardsonii*) collected from Beechy, Saskatchewan (Chapter 3). These hosts were parasitized by three species of ixodid tick: *I. kingi, I. sculptus* and *D. andersoni*. The species found in the highest frequency was *I. sculptus*, followed by *D. andersoni*. A small number of *I. kingi*, representing all life cycle stages, were also identified feeding on *S.*

richardsonii. Detection of three tick species on the same host, all of which are known vectors of pathogenic agents, provides an interesting opportunity to examine fundamental ecological and evolutionary questions relating to the structure and composition of bacteria in ticks. This was examined in Chapter 7 of this thesis.

Interestingly, *D. variabilis* were collected from northern pocket gophers at Clavet, whereas no life cycle stages of this tick species were found on Richardson's ground squirrels at Beechy. All life cycle stages of *D. variabilis* have been recorded at Saskatchewan Landing Provincial Park (i.e. ~ 37 km to the southwest), but larvae and nymphs were found feeding on voles, shrews and mice at that location (Dergousoff *et al*., 2013). The absence of *D. variabilis* on Richardson's ground squirrels at Beechy may not be unexpected because, as far as we are aware, this tick species has never been reported feeding on Richardson's ground squirrels. Perhaps Richardson's ground squirrels are not suitable hosts for *D. variabilis*; however, this needs to be investigated further.

The ticks parasitizing small mammals within Kootenay National Park, British Columbia were identified as *I. angustus* and *D. andersoni* by morphological methods and molecular tools (Chapter 4). A total of 189 *I. angustus* were collected from six species of small mammal, whereas only two adult female *D. andersoni* were found on these small mammals. Both tick species are of medical and veterinary importance; *I. angustus* has been implicated in the spread of Lyme disease in the Pacific Northwest (Damrow *et al*., 1989; Banerjee *et al*., 1994; Eisen *et al*., 2006), while *D. andersoni* is a vector of other human and animal pathogens (Burgdorfer, 1975; Gordon *et al*., 1983; Foley & Nieto, 2010; Kocan *et al*., 2010). The results of this thesis highlights the need for accurate identification of ticks to the species-level, so that potential vectors of disease can be monitored. Furthermore, the results of my research have shown that

different tick species parasitize the same host species, and in some cases, the same host individual at all three localities: Clavet, Beechy and Kootenay National Park (Chapters 2-4). This has important implications in the spread of vector borne diseases, as the overlapping host ranges of these tick species may provide the opportunity for horizontal transmission of tick-borne microorganisms from one tick species to the other.

A number of questions regarding the use of different small mammal hosts by *I. kingi, I. sculptus, I. angustus* and *D. andersoni* remain unanswered. These questions include: Does each of these tick species have a preference for certain species of small mammal host? and does this host preference differ among geographical localities (i.e., where there are different species of small mammal)? Additional studies at different geographical localities are needed to clarify if host preference does exist for any of these tick species. However, the data obtained for *I. angustus* can be used to examine if this tick species prefers to parasitize certain species of small mammal. The results revealed that all life cycle stages of *I. angustus* had a preference for southern red-backed voles (*Clethrionomys gapperi*) in Kootenay National Park (Chapter 4). This finding is similar to that reported for *I. angustus* in a mature aspen forest near Lac La Biche in north-central Alberta (Sorensen & Moses, 1998). In contrast, *I. angustus* in western Oregon have been shown to prefer deer mice (*Peromyscus maniculatus*) and shrews (*Sorex vagrans*, *S. trowbridgii*, and *S pacificus*) as hosts (Easton & Goulding, 1974). It is possible that this difference in host preference is an effect of changing species composition; when voles are less available, *I. angustus* may parasitize *P. maniculatus* or other small mammal hosts in higher frequencies. Therefore, additional studies are needed at locations where shrews, voles and mice coexist or are in close proximity to northern pocket gophers and Richardson's ground squirrels to

determine if there are specific host preferences for the different species of *Ixodes* and *Dermacentor* species that occur in western Canada.

11.4 **Prevalence of ticks on small mammals in western Canada**

The distribution of parasites among host individuals is usually not random, as parasites typically aggregate on their hosts in nature (Shaw & Dobson, 1995). Most parasite individuals occur in/on a few host individuals, while most host individuals have only a few, if any, parasites (Anderson & May, 1978; Shaw & Dobson, 1995). Such aggregations can influence the population dynamics of the host, the parasite, and the microorganisms that the parasite contains (Anderson & Gordon, 1982; Jaenike, 1996). Parasite transmission is usually density dependent, and given that many small mammals are social, or group living, they are expected to have higher parasitic loads and a higher prevalence of disease (Côté & Poulin, 1995; Arneberg *et al*., 1998; Altizer *et al*., 2003; Hillegass *et al*., 2008). Strong aggregations of ticks with a potentially higher propensity to carry disease-causing agents have important implications in the spread of vector-borne disease. The data collected in Chapters 2-4 can therefore be used to examine the question, what is the prevalence of ticks on small mammal hosts?

Approximately 30% of the northern pocket gophers collected at Clavet were parasitized by ticks (Chapter 2). Five *T. talpoides* were parasitized by less than 12 ticks, whereas three hosts were parasitized by 21-50 ticks. This aggregation of ticks on northern pocket gophers is similar to that of other species of ticks that parasitize small mammals, such as *Ixodes scapularis* and *I. ricinus*, where the distribution pattern of ticks among individuals of a host population has important implications for the spread of vector-borne diseases (Brunner & Ostfeld,

2008; Harrison & Bennett, 2012). A similar pattern of prevalence was observed for ticks on Richardson's ground squirrels at Beechy where 44% of these small mammals were parasitized by ticks (Chapter 3). However, the majority of these hosts were parasitized by one or two ticks, while a single *S. richardsonii* was parasitized by 67 ticks. The prevalence of *I. kingi* found on northern pocket gophers at Clavet was greater than that of *I. kingi* on Richardson's ground squirrels at Beechy. The significance of this finding needs to be explored further. Additional studies on northern pocket gophers from Beechy, Richardson's ground squirrels from Clavet, and additional localities where both small mammal species occur in sympatry are needed in order to test if *I. kingi* prefers northern pocket gophers to Richardson's ground squirrels as hosts.

At Kootenay National Park, 55% of red-backed voles were parasitized by *I. angustus,* with a mean intensity of 6.4 (see Chapter 4). The majority of these hosts were parasitized by one, two or three ticks; however, two red-backed voles were parasitized by 30 and 54 ticks, respectively. The prevalence of *I. angustus* on red-backed voles fits the expected distribution of parasites on hosts, and more specifically, ticks on small mammals that live in close proximity to one another in burrow systems and have small home ranges. Studies have found that tick burdens on small mammal hosts were highly variable and that strong aggregations of ticks would only be found on small numbers of hosts (Anderson & May, 1978; Shaw & Dobson, 1995). This raises the question: does the bacterial community structure of a tick species change in composition if it is aggregated with tick individuals belonging to different species?

When multiple tick species parasitize one host individual, horizontal transmission of microorganisms from tick to tick can occur via 'co-feeding transmission', where ticks become infected via feeding alongside infected ticks (Harrison & Bennett, 2012). Given that ticks are not randomly distributed among a group of small mammals, the aggregation of ticks on small

mammal hosts leads to an increase in the number of ticks that could potentially become infected, increasing the probability of transmission potential via this 'co-feeding' route (Harrison & Bennett, 2012). Horizontal transmission of tick-borne microorganisms from one tick species to another can also occur from both tick species feeding on a systematically infected host; therefore, several tick species feeding on one host individual may have important epidemiological implications. Further investigation into the bacterial community structure of different tick species feeding on the same host species, or host individual (Chapters 7-10), are needed to understand the ecology of vector-borne diseases.

11.5 Genetic variation and phylogenetic relationships among tick species on small mammals in western Canada

Understanding the evolutionary ecology of vector-borne diseases requires a detailed knowledge of the population genetics of the vector (Kurtenbach *et al.,* 2006). Differences in genetically based traits among populations of organisms across the natural geographic range of a species of focus can be used to study the phylogeographic history of a species, as well as the rate of evolutionary change within that species. The results obtained from the genetic identification of ticks using the mt 16S rRNA gene were also used to explore the magnitude of genetic variation within each tick species, as well as their phylogenetic relationships (Chapter 6).

Six 16S haplotypes were detected among the *D. andersoni* individuals from small mammals at the three collection localities, four of which were the same as those reported previously by Patterson *et al*. (2009). Two new haplotypes were also found, one from Clavet, and the other from Kootenay National Park. In contrast, there was no variation in 16S sequence among individuals of *I. sculptus*. This lack of intraspecific sequence variation could be a consequence of sampling ticks from a host population over a relatively small area. Similarly, no

sequence variation was detected among all life cycle stages of *I. kingi* feeding on *T. talpoides* at Clavet (Chapter 2). However, three additional 16S haplotypes of *I. kingi* were detected among individuals collected from other localities in Saskatchewan (e.g., Beechy and Vanguard) (Chapters 3 & 6). Therefore, 16S sequence data are needed for individuals from many other localities in Saskatchewan to assess the magnitude of genetic variation in *I. sculptus* and the other species examined in this study. Interestingly, the *I. sculptus* from Saskatchewan differed significantly in 16S sequence from that of an *I. sculptus* individual from Colorado. The results of a phylogenetic analysis showed that the *I. sculptus* from the two different geographical regions did not form a monophyletic clade, suggesting that *I. sculptus* may represent a cryptic (i.e., genetically distinct but morphologically similar) species. Further studies are needed to explore this hypothesis.

Three 16S haplotypes were found within the *I. angustus* collected from Kootenay National Park, but a majority (78.4%) of individuals were of one haplotype (Chapter 4). The *I. angustus* from Kootenay National Park differ markedly in sequence compared to *I. angustus* from Durham (New Hampshire) and Vinalhaven (Maine) in the eastern United States, suggesting possible genetic divergence between tick populations at the western and eastern limits of the species distribution in North America. Little information exists on genetic variation in *I. angustus* despite its broad geographical range that includes North America, Russia and Japan (Robbins & Keirans, 1992; Shpynov *et al*., 2003). Consequently, additional population genetic studies are needed to determine the magnitude of genetic variation among *I. angustus* populations in different parts of the species distributional range. This knowledge will have implications for our understanding of the transmission of vector-borne pathogens.

Although *I. angustus* and *I. kingi* display more intraspecific variation than *I. sculptus,* they exhibit much less genetic variation than other *Ixodes* species. For example, genetic studies of *I. scapularis* (e.g., Norris *et al.,* 1996; Qiu *et al.,* 2002; Krakowetz *et al.,* 2011) in North America using the mt 16S rRNA gene reported the presence of large numbers of haplotypes, which could be the product of a greater dispersal and/or a high mutation rate. Migratory passerines are known to carry *I. scapularis* larvae and nymphs large distances from the United States into Canada each spring (Ogden *et al.,* 2008). In comparison, small mammals have a much more restricted home range, limiting the dispersal of their tick parasites. Additional genetic markers, in combination with the mt 16S DNA, may provide important insights into the genetic divergence and phylogeographic relationships of *D. andersoni, I. kingi, I. sculptus* and *I. angustus* populations throughout their distributional ranges. This will have important implications for studies on the different pathogens transmitted by these four tick species to humans, domestic animals, and wildlife. It is important to note however, a lack of sequence variation in the $D3^{+}$ was found among *I. scapularis* adults (Chapter 5), indicating that this gene region is not useful for examining the population genetics of ticks.

The phylogenetic relationships of *I. angustus, I. kingi* and *I. sculptus* with respect to other species within the genus were inferred using sequence data of the 3' region of the mitochondrial 16S rRNA gene that was aligned based on the secondary structure of the gene (Chapter 6). The results showed that these three tick species formed a clade that contained members of two subgenera, the *Pholeoixodes* and *Ixodiopsis*. In addition, the results supported the current view that *I. kingi* and *I. sculptus* are more closely related to one another (i.e., both members of the *Pholeoixodes*) than either species is to *I. angustus* (a member of the *Ixodiopsis*). This is consistent with their placement in different subgenera. However, the results of the phylogenetic

analyses led to the placement of *I. woodi* (a member of the subgenus *Ixodiopsis*) within a clade that contained members of the subgenus *Pholeoixodes* (i.e., *I. banksi, I. cookei, I. sculptus, I. kingi* and *I. dampfi*). There was some statistical support for a sister taxa relationship between *I. angustus* and *I. hexagonus*, the latter of which belongs to *Pholeoixodes*. The phylogenetic analyses of the 16S sequence data therefore suggest that each subgenus was not a monophyletic group, the taxonomic significance of which requires further investigation.

In summary, the 16S rRNA gene is an effective marker for species-level identification, population genetics studies and phylogenetic studies of ixodid ticks. Given that *I. kingi* and *I. sculptus* are more related to one another than either are to *I. angustus*, and that they all feed on the same hosts as *D. andersoni*, it would interesting to determine if similarities in the composition of the bacterial communities within these ticks are based on their phylogenetic relationships and/or the hosts they parasitize?

11.6 **Composition and diversity of bacteria within ticks in western Canada**

A major component of this thesis was the examination of the composition and diversity of the bacterial communities within individual ticks representing different life cycle stages of tick and different species of tick feeding on a number of small mammal hosts. Several questions that were examined include: are the bacterial communities of different tick species similar? and if so, is this determined based on their phylogenetic relatedness and/or the host species on which they feed?

Communities are usually considered to comprise an assemblage of different species living within a defined area or habitat (Bush *et al.,* 1997; Poulin, 2007; Ricklefs, 2008). Some definitions of community also take into account the interactions among species (Whitakker,

1975). For parasites, their host represents a type of interactive habitat, and several terms have been used to describe parasite assemblages at different hierarchical levels. For example, an infracommunity represents all parasites within a single host (Bush & Holmes, 1986; Sousa, 1994; Poulin, 2001), whereas a component community represents all parasites within a collection of a single host species (Holmes & Price, 1986). In terms of my thesis work, the different species of pathogenic and endosymbiotic bacteria present within an individual tick (e.g., a single *D. andersoni*) will be considered an infracommunity, whereas all the bacteria present in a tick species (e.g., all *D. andersoni* examined) will be considered a component community.

Studies of parasite component communities have been numerous in recent years (Esch *et al.,* 1990; Poulin, 1997). The findings of these studies have shown that the distribution of parasite species among infracommunities range from completely random to highly structured (Poulin, 2007). Several studies have shown that hosts with a widespread geographical distribution (e.g., Holarctic waterfowl) tend to harbor more parasite species than those hosts with a more restricted geographical distribution (e.g., pocket gophers) (Gregory, 1990). Although the structure and the interactions between members of a community have been investigated for many eukaryotic parasites (Poulin, 2007), very little has been examined with respect to microbial diversity within ticks, despite their important medical and veterinary importance.

To date, most studies that have been conducted have focused on a single tick species, or test for the prevalence of one species of bacteria within ticks (e.g., Bernasconi *et al.,* 2002; Moreno *et al.,* 2006). In addition, many studies have only examined the adults of a tick species, or the progeny of adult female ticks reared in a laboratory setting (e.g., Roland *et al.,* 1998; Goddard *et al.,* 2003; Ammerman *et al.,* 2004). Therefore, a novel and important aspect of my PhD research was the identification and comparison of the prevalence and diversity of

microorganisms in *I. angustus, I. kingi, I. sculptus* and *D. andersoni* (Chapters 7-10). In addition, my research work also included a comparison of the bacteria in all three life cycle stages for all four species of tick, and examined the bacterial community structure of multiple tick species parasitizing the same host species, or host individual. My results (Chapters 7-10) provided insight into the bacterial community structure of ticks of all life cycle stages feeding alone, as well as with other tick species. The host (i.e., tick vector) specificity of some tick-borne bacteria was also explored.

In the present study, PCR-SSCP analyses were used to determine the bacterial genera present within individual ticks (i.e., infracommunity level) of *I. angustus, I. kingi, I. sculptus* and *D. andersoni* (i.e., component community level). The composition of microbial infracommunities was found to be very diverse, with some tick individuals being infected with only one genus of bacteria while others were infected with 11 genera of bacteria. From a component community perspective, tick species such as *I. angustus* and *I. sculptus* were infected with approximately the same number (i.e., 1-3) of bacterial genera, while others (e.g., *I. kingi*) had a much higher infection rate (i.e., 2-11 different genera).

At least 40 genera of bacteria were detected in the ticks examined in this thesis. The numbers of co-occurring microbes within individual ticks differed among *I. angustus, I. kingi, I. sculptus* and *D. andersoni*. The majority of *I. angustus* (81%) and *I. sculptus* (83%) tested were infected with a single genus of bacteria, whereas the highest number of bacterial genera detected by PCR within individuals of these two tick species was three. Approximately 45% of the *D. andersoni* from Beechy were infected with only a single genus of bacteria, while 40% were infected with seven genera of bacteria. In comparison, just over half (53%) of the *I. kingi* from Clavet were infected with 11 bacterial genera, whereas 22% of individuals were infected with

four genera. These studies demonstrate a high frequency of co-infection, as well as an extremely variable relationship between the number of co-occurring microbes and the species of tick being infected. This raises the possibility of microbial interactions unique to a tick species, or more specifically, individual ticks.

Several genera of bacteria were isolated from almost all of the tick species tested. For example, prokaryotic 16S rDNA of *Rickettsiella* was detected in high numbers in *I. angustus* (99%) and *I. sculptus* (77%), and was also detected in a single *I. kingi* from Beechy. The genus *Rickettsia* was found in three species of tick (i.e., *I. kingi, I. angustus* and *D. andersoni*) and from multiple geographical locations. For instance, the 16S rDNA of *Rickettsia* was detected in the *I. angustus* (18%) from Kootenay National Park, the *I. kingi* from Clavet (69%), and in the *D. andersoni* (83%) from both Beechy and Kootenay National Park. Bacteria of the genera *Ralstonia* and *Pseudomonas* were detected in similar proportions in the *I. kingi* (94% and 72%, respectively) from Clavet, as well as in *D. andersoni* from both Beechy (54% and 46%, respectively) and Clavet (67% and 33%, respectively). In contrast, some bacterial genera were specific to one species of tick. For example, *Francisella* was only found in *D. andersoni* and *Pasteurella* was specific to *I. angustus*. Although several bacterial genera were found in several species of tick, the question remains: are different tick species infected with the same species of bacteria, or are different species of bacteria specific to their tick host?

In order to answer this question, two of the bacterial genera that were found in several species of tick (i.e., *Rickettsia* and *Rickettsiella*) and from multiple collection locations were characterized further (Chapter 8-10). First, the DNA sequences of *Rickettsia* found in the *I. kingi, I. angustus* and *D. andersoni* were determined for multiple genes. As a consequence, two novel species of *Rickettsia* were discovered, one infecting *I. kingi* (i.e., *Candidatus* R. kingi) and the

other infecting *I. angustus* (i.e., *Candidatus* R. angustus) (see Chapters 8 & 9, respectively). Phylogenetic analyses conducted on the sequence data of five rickettsial-specific genes for *Candidatus* R. kingi and *Candidatus* R. angustus revealed that these taxa did not belong to the spotted-fever group or typhus group rickettsiae, but belonged in a clade that contained *R. canadensis*, and two other putative species of *Rickettsia*: *Candidatus* R. tarasevichiae, and *Candidatus* R. monteiroi. Although *I. kingi* and *I. angustus* predominantly use small mammals as hosts, they are known to parasitize humans (Bishopp & Trembley, 1945; Gregson, 1971; Robbins & Keirans, 1992; Peavey *et al.,* 2000; Allan, 2001; Salkeld *et al.,* 2006; Kolonin, 2007). The other potential members of this rickettsial clade (i.e., *R. canadensis*, *Candidatus* R. tarasevichiae, *Candidatus* R. monteiroi) have all been reported in ixodid ticks that also bite humans (McKiel *et al*., 1967; Salkeld *et al*., 2006; Eremeeva *et al*., 2007; Inokuma *et al*., 2007; Pacheco *et al*., 2011). Of these rickettsiae, only *R. canadensis* is considered a potential human pathogen (Merhej & Raoult, 2011) based on serological evidence that it may have been the agent responsible for the Rocky Mountain spotted fever-like symptoms displayed by four human patients in North Carolina and Texas (Bozeman *et al*., 1970). However, any newly described *Rickettsia* from an invertebrate host, especially ticks, should be viewed as a potential pathogen. Although *Candidatus* R. kingi and *Candidatus* R. angustus represent a sister taxon to *R. canadensis*, it remains to be determined if these putative new species are of pathogenic significance with respect to human health. Additional studies are needed to establish whether these novel rickettsiae are found in other populations of *I. kingi* and *I. angustus* throughout North America, and to determine if these bacteria have any potentially pathogenic effects on their invertebrate and/or vertebrate hosts.

Second, the DNA sequences of the *Rickettsiella* found in *I. kingi, I. sculptus* and *I. angustus* were determined by targeting ~380bp of the prokaryotic 16S rRNA gene (Chapter 10). As a consequence, three putative species of *Rickettsiella* were detected, each of which was associated with a different tick species. Phylogenetic analyses of the sequence data revealed that the *Rickettsiella* in *I. kingi* represented the sister taxon to the *Rickettsiella* in *I. sculptus,* and both formed a clade with *R. grylli,* from crickets (*Gryllus bimaculuatus*), and '*R. ixodidis*' from *I. woodi*. In contrast, the *Rickettsiella* in *I. angustus* was placed external to a clade that contained nine pathotypes of *R. popilliae*. The magnitude of the genetic differences in the 16S rRNA gene sequences and the phylogenetic relationships of the bacteria within the three tick species, suggest that each taxon represents a new species of *Rickettsiella*. It is interesting to note that the phylogenetic relationships between the different species of *Rickettsiella* in the different species of *Ixodes* (Fig. 10.2; Chapter 10) mirror the phylogenetic relationships of *I. kingi, I. sculptus, I. angustus* and *I. woodi* (Fig. 6.3; Chapter 6). Although *Rickettsiella* was not detected in any *D. andersoni,* additional studies are needed at different geographical localities where *D. andersoni* parasitizes the same small mammals as *I. kingi, I. sculptus* and *I. angustus* to test for *Rickettsiella*. It would also be interesting to determine if other species of *Ixodes* in North America are hosts for *Rickettsiella*.

Sequence alignments of short 16S rRNA gene fragments belonging to several other bacterial genera (e.g., *Pseudomonas, Sphingomonas* and *Staphylococccus*) revealed differences in nucleotide composition among bacteria from different tick species; suggesting the possibility of different bacterial species within different species of tick. In contrast, some genera (e.g., *Ralstonia* and *Stenotrophomonas*) displayed identical short 16S rRNA sequences despite being found in several species of tick. These data raise questions regarding the tick-specificity of the

bacteria identified in this study. In the case of the *Rickettsia* in *I. kingi, I. angustus* and *D. andersoni,* identical sequences from the short 16S rRNA gene fragments did not imply that identical species of *Rickettsia* were infecting all three tick species. Therefore, how many more species of bacteria are tick specific and are some bacterial species shared between multiple species of tick? Species-level characterizations of the bacteria are needed to determine if different tick species share the same species of bacteria. This would also provide insight into the potential interactions among microbes as well as the specificity of these microbes for their tick hosts.

Ticks of different species feeding on the same host individual provides an opportunity to examine fundamental ecological and evolutionary questions relating to the structure and composition of bacteria in ticks. The influence of various factors (e.g., vertebrate host, environment and tick vector) on bacterial community composition of different tick species can be examined, and the specificity of a bacterium for its tick host can be assessed. Data can then be used to answer the question, is the specificity of relationships between tick and bacteria, and among bacterial communities within ticks, dependent on the vertebrate host or the tick species? Interestingly, there was no sharing of bacterial genera between three species of tick (i.e., *I. kingi, I. sculptus* and *D. andersoni*) that were parasitizing the same Richardson's ground squirrel hosts (Chapter 7). These results indicate that it may not be the vertebrate host, but the tick species, that influences the bacterial community composition. Similar results have been found in a recent study on the bacterial community composition of fleas and ticks (Hawlena *et al.,* 2013). However, *I. kingi and D. andersoni* parasitizing the same northern pocket gophers from Clavet were infected with several of the same bacterial genera. Additional studies are needed to address the question of whether the specificity of relationships between tick and bacteria, and among

bacterial communities within ticks, is dependent on the vertebrate host or the tick species. Further research is also needed to determine the host specificity of bacterial genera infecting ticks on small mammals and to investigate the questions: (1) do ticks share some bacterial genera, but not other genera? and (2) are some species of bacteria acquired from the host, whereas others are passed transovarially from female tick to her progeny? Another important question that needs to be addressed in the future is, do different small mammal hosts play different roles in the transmission and maintenance of tick-borne microorganisms? This question could be answered by testing the small mammals directly (i.e., using the blood or other organs as sample material) for the presence of naturally occurring microorganisms. This information could then be compared to the specific bacterial flora detected within the ticks that parasitize these host individuals. Overall, the results of Chapters 7-10 demonstrate that ticks harbor a diverse community of microorganisms, potentially allowing for ecological interactions among the microorganisms within ticks. Such interactions could affect pathogen prevalence and transmission within tick populations.

11.7 **Synthesis**

My PhD research provides a comprehensive analysis of several epidemiological triangles from three different geographical localities in western Canada. Although focus is placed on the interactions between members of specific epidemiological triangles (i.e., *I. angustus,* red-backed voles, *Rickettsiella*), the scientific approach can be applied to the study of numerous parasite-host associations. My research has many original components, making this research exciting in its novelty, and important in that it addresses questions that have not been previously examined concerning vector-host interactions in these particular geographic regions. The majority of

studies place focus on one species of tick, or one species of bacteria found within a tick species; however, my PhD research examined the bacterial community structure within multiple tick species parasitizing a single small mammal host species, and in some cases, the same small mammal host individual, which has important implications in the ecology of tick-borne bacteria.

The results of my research suggest that each tick species has a relatively unique bacterial community structure. Factors such as the tick species, tick life cycle stage, geographic location, and to a lesser extent, type of small mammal host, appear to have an important role in determining the bacterial community structures of the tick species examined. Future studies are needed on other tick species and ectoparasitic arthropods (e.g., fleas, lice and mites) that parasitize small mammals in western Canada to determine if they too have unique bacterial communities.

A similarity in the phylogenetic relationships of *Rickettsiella* and *Ixodes* suggests possible coevolution (i.e., cospeciation) of bacteria and their tick hosts. Coevolution is the reciprocal evolutionary change between interacting species (Álvarez-Castañeda, 2010). The close relationship between ticks and their microorganisms are likely the result of long-term associations, which are shaped by selective pressures produced by the tick, other bacteria, and the vertebrate host (Wernegreen, 2002). Tick-borne microorganisms need to adapt to conditions in their environment (i.e., within the tick host), resulting in coevolution of both groups of interacting organisms. These evolutionary changes can lead to the close associations of bacteria with specific-species of arthropod host (Azad & Beard, 1998).

Analyses of the bacterial community structure of other arthropod vectors, such as mosquitoes, fleas, and other species of ticks, can help identify important association between microorganisms and their arthropod hosts. This information can help to identify the role of

different microbes in infectious disease, and of the interactions that could affect the vector capacity of their host. These studies would also provide insight into whether other species of ectoparasite display coevolution with their bacterial endosymbionts. Certain ectoparasites have demonstrated a strict cospeciation with their vertebrate hosts (e.g., lice of pocket gophers; Reed & Hafner, 1997). It would be interesting to determine if the bacterial species within the lice have coevolved as well. Additional studies are needed to explore this exciting concept further.

In general, the complex interrelationships between many arthropod, microorganism and vertebrate host epidemiological triangles are poorly understood. However, my PhD research provides insight into the relationships of several tick species, their hosts and microbial communities, and makes available a framework for future studies on the microbial composition and diversity in other tick species. My research has contributed important information to our general knowledge of the distributional ranges, host usage and preferences, and the bacterial community structures of *I. kingi, I. sculptus, I. angustus and D. andersoni*. In addition, this work advances our understanding of the evolution and ecology of ticks and tick-borne bacteria.

11.8 **References Cited**

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