COMPARISON OF THE BACTERIA WITHIN TICKS FROM ALLOPATRIC AND SYMPATRIC POPULATIONS OF *DERMACENTOR ANDERSONI* AND *DERMACENTOR VARIABILIS* NEAR THEIR NORTHERN DISTRIBUTIONAL LIMITS IN CANADA

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Shaun J. Dergousoff

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Abstract

Understanding the ecology and epidemiology of tick-borne diseases requires detailed knowledge of the complex interactions among the tick vector, the microorganisms they carry and the vertebrate hosts used by ticks, as well as the environmental conditions experienced by all three groups of organisms in this triad. In this thesis, I addressed questions relating to the biology and vector ecology of the Rocky Mountain wood tick (*Dermacentor andersoni*) and the American dog tick (*Dermacentor variabilis*). Comparisons were made of the distribution of both tick species, the vertebrate hosts used by immature ticks, and the types and prevalence of bacteria in individual ticks from multiple localities near the northern extent of their geographic ranges in western Canada. The results revealed that the distributions of both *D. andersoni* and *D. variabilis* have expanded since the 1960s, and there is now a broad zone of sympatry in southern Saskatchewan. In this zone of sympatry, *D. andersoni* and *D. variabilis* immatures were found to use the same species of small mammals as hosts and, in some cases, the same host individuals. This provides for the possibility of cross-transmission of bacteria from one tick species to the other. Bacteria of several genera (e.g. *Rickettsia*, *Francisella*, *Arsenophonus* and *Anaplasma*) were detected in *D. andersoni* and/or *D. variabilis*, some of which represented new tick-bacteria associations. However, most bacterial species were highly host (tick)-specific, except for three examples of apparent host switching from one tick species to the other at localities where the two tick species occurred in sympatry. The findings of this thesis provide a basis for understanding microbial transmission, the structure of tick-borne microbial communities, the risk of tick-borne disease in humans and animals, and the vector potential of *D. andersoni* and *D. variabilis* in geographical areas where they have not been studied previously.

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Chapter 1. Introduction

1.1. Vector-borne diseases

Microorganisms, including a number of different viruses, bacteria and protozoans, are the cause a variety of infectious diseases in humans, wildlife and domestic animals. A significant proportion of these infectious diseases are transmitted by haematophagous arthropod vectors that include insects (e.g. mosquitoes, black flies, tsetse flies, sand flies, triatome bugs, fleas and lice) and arachnids (e.g. ticks and mites) (Philip & Burgdorfer 1961, Balashov 1984, Spielman & James 1990, Azad & Beard 1998, DeFoliart *et al.* 2003, Mosbacher *et al.* 2010). Mosquitoes and ticks are considered the two most important groups of vectors in terms of the number and diversity of microorganisms they transmit to humans and animals (Parola & Raoult 2001). Many infectious diseases are considered emerging or re-emerging, as they are increasing in prevalence and/or changing in their geographic distribution (Gratz 1999, Parola & Raoult 2001, Petersen & Schriefer 2005, Eisen 2007, Telford & Goethert 2008). For example, Lyme borreliosis, which is caused by the bacterium *Borrelia burgdorferi* and is transmitted to humans by the bite of an infected tick, is now regarded an emerging disease in southern Canada because of the establishment of new populations of blacklegged ticks (*Ixodes scapularis*) in Ontario, Nova Scotia and Manitoba (Ogden *et al.* 2009).

Both soft ticks (Family: Argasidae) and hard ticks (Family: Ixodidae) are vectors of microorganisms that are human and animal pathogens (Jongejan & Uilenberg 2004). Hard and soft ticks have four life cycle stages; egg, larva, nymph and adult. Most ticks spend only a small proportion of their lifetime on a host while acquiring a blood meal in each stage prior to moulting (i.e. larvae and nymphs) or oviposition (i.e. adult females). There are also major

differences in the biology (e.g., life cycles) of soft and hard ticks that are important determinants of the epidemiology of tick-borne diseases. Soft ticks have multiple nymphal instars, each of which requires a host to feed upon and adult ticks, particularly the females that can feed multiple times. These nymphal instars and adults feed for a relatively short duration (minutes to hours) on hosts (Sonenshine 1991, Randolph 1998). Most soft ticks are nidicolous (nest dwelling), existing in protected habitats of nests, burrows, or rock or soil crevices. The restricted habitat and host usage limits dispersal of the tick and, therefore, the dispersal of any pathogenic agent(s) associated with those species of tick. In contrast, hard ticks, with the exception of some species of *Ixodes*, are non-nidicolous, and find their hosts by questing in an open environment. Hard ticks go through a single instar for each active life stage, each requiring a single large blood meal that is acquired over a period of days prior to moulting (larvae and nymphs) or prior to laying eggs (adult females). The number of hosts required to complete the life cycle of a hard tick varies from one to three, depending upon the species; however, many species require three separate hosts (Anderson & Magnarelli 2008). The feeding pattern of three-host ticks allows for the transmission of pathogens among vertebrate hosts because of the opportunity to acquire a pathogen from one animal and pass it to another when feeding in subsequent life stages. Ticks can also be important reservoir hosts for some pathogens (Azad & Beard 1998, Gyuranecz *et al.* 2011) because of their ability to survive relatively long time periods between blood meals (i.e. months to years) compared to many other arthropod vectors, such as mosquitoes.

Many of the biological and life cycle characteristics of ticks that are important to the epidemiology of vector-borne diseases differ from those of insect vectors (Randolph 1998). For example, mosquitoes are able to disperse on their own over much greater distances than can ticks, which are dependent on their vertebrate hosts for dispersal. Thus, patterns of pathogen

transmission by mosquitoes are vastly different than those by ticks. In addition, only female mosquitoes feed on vertebrate hosts, and, although they feed infrequently, they take a greater number of smaller meals than do hard ticks. Thus, mosquitoes can cause a much more rapid spread of a pathogen within and between vertebrate populations.

The epidemiology of vector-borne diseases are determined by the interactions among three groups of organisms, the arthropod vector, the microbial agent (bacteria, viruses and protozoans), and the vertebrate host used by vectors as a food source. The interrelationships of these organisms can be depicted in a triad of interactions (see Figure 1.1) (Nuttall *et al.* 2000). In addition, different biotic and abiotic factors can affect each of these organisms and influence the interactions among them, leading to altered patterns, both spatially and temporally, of tick-borne diseases (Wilson *et al.* 2002, Kurtenbach *et al.* 2006). An understanding of these complex relationships is necessary to estimate of the risk of exposure to humans and animals for vector-borne diseases, and for the implementation of effective management strategies essential for the diagnosis, treatment, prevention and control (and if possible, the elimination) of these diseases (Spielman & James 1990, Wobeser 2007).

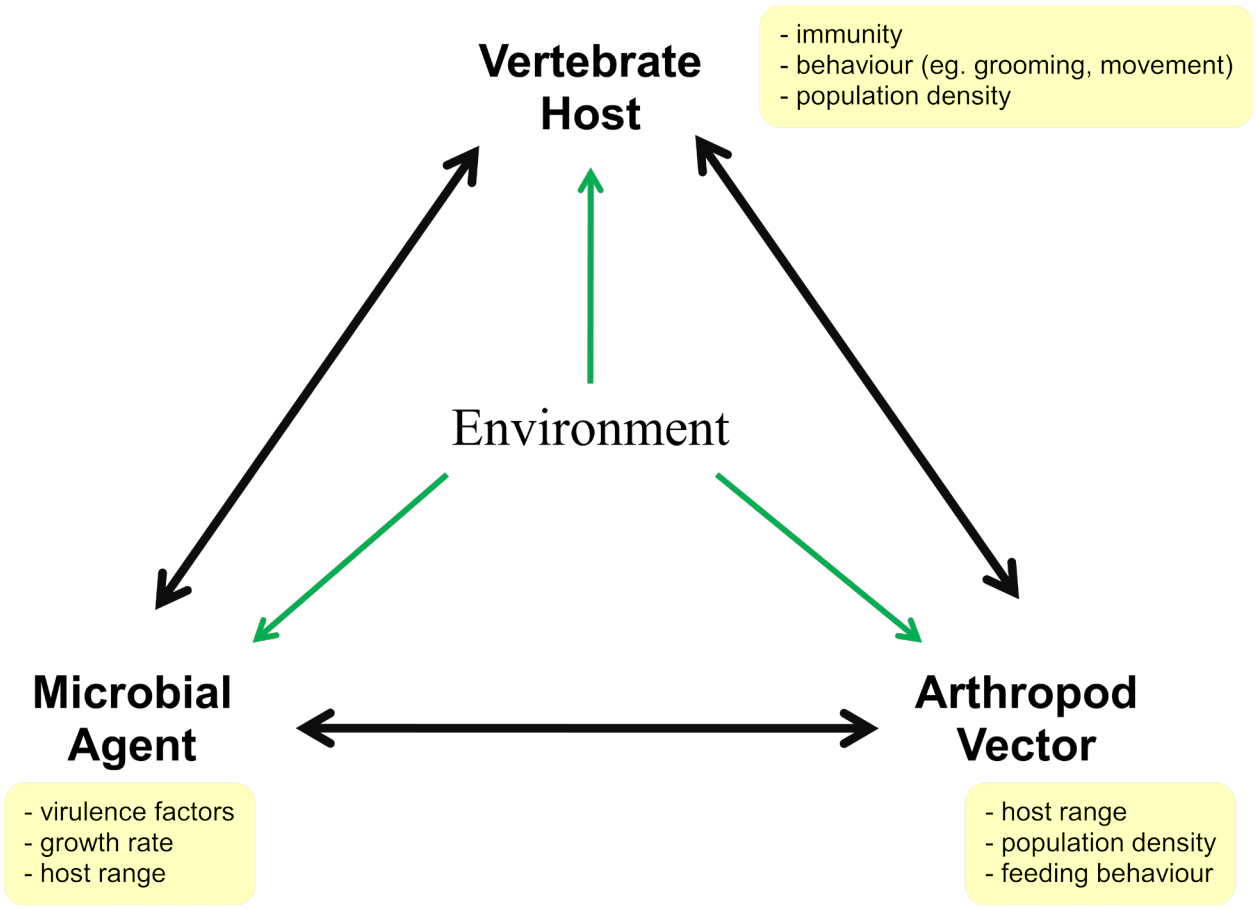

Fig. 1.1. Diagrammatic representation of the triad of biological interactions among arthropod vector(s), microbial agent, and the vertebrate host(s) that determine the epidemiology of vector-borne diseases. Environmental factors (abiotic and biotic) influence each of these interactions by affecting each of the organisms involved in this triangle of interactions in different ways, and therefore, altering the epidemiology of vector-borne disease. The diagram is a modification of the classical "epidemiologic triangle" (Comrie 2007) that is used to describe the occurrence of disease as a result of interactions among the disease agent, the diseased individual, and the environment. A number of factors relating to each organism (examples are indicated in the boxes next to each member) affect the interactions between the other organisms. Arrows represent the dynamics of the interactions between the organisms involved in the transmission cycle.

Vector-borne microorganisms are maintained and propagated in vertebrate and/or arthropod hosts through different modes of transmission. For a microorganism to be maintained in a transmission cycle involving an arthropod host that feeds only once during a life cycle stage, it must survive the moulting process of its host (i.e. transstadial transmission). The transmission cycle of many pathogenic agents involve horizontal transmission from an arthropod host to a vertebrate host, and then back to an arthropod host. This can occur when an infected blood-feeding arthropod takes a meal, passing the microorganism to the vertebrate host and a second arthropod acquires the microbe when it takes a blood meal on the systemically infected host at a later point in time. For example, the protozoan *Plasmodium falciparum*, the causative agent of malaria in humans, is transmitted in this way by mosquitoes of the genus *Anopheles* (Bousema & Drakeley 2011). Vector-borne microorganisms can also be transmitted from one arthropod host to another when an uninfected arthropod feeds on a vertebrate host without a systemic infection close to where an infected arthropod has recently fed (i.e. co-feeding transmission) (Jones *et al.* 1987, Randolph *et al.* 1996). This has been shown to occur for *Borrelia burgdorferi* in *Ixodes ricinus* feeding on sheep (Ogden *et al.* 1997). Some intracellular microorganisms (e.g. arboviruses,rickettsial organisms and protozoa) that infect an arthropod vector can be transmitted vertically from the female to its larval offspring through infected eggs (i.e. transovarial transmission) (Fine 1975, Randolph 1998, Howell 2007). This can be a very efficient method of maintaining a microorganism within a population, particularly in arthropods with a high fecundity. Some microorganisms use a combination of horizontal and vertical modes of transmission to ensure that they are passed on to a new vertebrate or arthropod host (Baldridge *et al.* 2009). For example, *Rickettsia rickettsii* is vertically transmitted from *Dermacentor*

andersoni females to their offspring and are maintained in an enzootic transmission cycle between ticks and a variety of mammals (Burgdorfer 1988, Azad & Beard 1998).

A central issue in the field of vector ecology is determining the relative effects of different biotic and abiotic factors on the transmission of vector-borne pathogens and on the epidemiology of vector-borne diseases. This requires information on the biology and ecology of the vectors, their vertebrate hosts and arthropod-borne microorganisms, for which there are still a number of questions remaining to be answered. For example, there have been changes in the epidemiology of some tick-borne diseases, including Rocky Mountain spotted fever and tularemia, over the past century in the USA (Eisen 2007, Telford & Goethert 2008), yet little is known about the underlying ecological and biological factors that caused these shifts in transmission and occurrence.

There are many different determinants of the ecology and epidemiology of vector-borne diseases. Some of these factors relate specifically to the pathogenic agent, the arthropod vector, and/or the vertebrate host. In addition, environmental factors have major effects on the interactions between members of the epidemiological triangle, which also influences the occurrence of vector borne diseases. With respect to the **vectors**, factors such as their distribution, population density, individual host range and susceptibility to infection by different species of pathogenic microorganisms are all determinants of the vectorial capacity and the epidemiology of vector-borne diseases. The occurrence of vector-borne diseases is necessarily linked to the distribution of the species that transmit the pathogen. Thus, an assessment of the risk of exposure for humans and animals to vector-borne pathogens is dependent on an accurate description of the current distribution of the different species of vector.

The epidemiology of a vector-borne disease is also influenced by the characteristics of the **vertebrate host(s)** used by vectors as a food source. These characteristics include population density of the vertebrate hosts, the defense mechanisms (e.g. immune and behavioural responses) of the host to resist feeding by the vector, and their susceptibility to infection by pathogens transmitted in the saliva of the blood-feeding vectors. Therefore, for tick-borne diseases, it is important to know which vertebrate species are used as hosts by ticks because it provides important clues as to potential transmission cycles of tick-borne microorganisms.

Attributes of **pathogenic agents** that are important determinants of vector-borne disease include their virulence factors, preference for arthropod and vertebrate hosts, mode(s) of transmission, environmental requirements, distribution, and prevalence. The risk of transmission to susceptible hosts will depend on the prevalence of microorganisms of veterinary or medical importance in a particular vector population. Given that the prevalence and abundance of vector-borne microorganisms show spatial variation, then it is important to determine the relative abundance of infected arthropod vectors at multiple localities in order to assess the potential risk of exposure to vector-borne pathogens for vertebrate hosts (i.e. humans and domestic animals).

Arthropod vectors can be infected simultaneously with a number of different species of microorganisms (Beard *et al.* 1993, Clay *et al.* 2006, Jones *et al.* 2009), creating the potential for a number of interactions among localized microbial populations. For example, the composition of the tick microbiome (i.e. all species of microorganisms present within an individual) can have important effects on the vector potential of ticks (Burgdorfer *et al.* 1981, Azad & Beard 1998, de la Fuente *et al.* 2003) however, relatively little has been done to study microbial communities of vectors and their epidemiological relevance.

Another key issue in the field of vector ecology is how the vector, pathogen and vertebrate host respond to different **environmental** conditions, such as those associated with changes in climate and landscape (Eisen & Eisen 2008, Vanwambeke *et al.* 2010), and how this influences the interactions among organisms and determines the transmission of pathogenic microorganisms. Studies have shown that temperature and humidity are important determinants of the distribution and relative abundance of arthropod vectors, and the prevalence of vector-borne diseases (McEnroe 1978, Sonenshine 1979, Wang *et al.* 2011). However, it is not always clear how different species of vector will respond to changes in these conditions. Climate-based models have been developed to estimate the risk of encountering vectors and predictions based on these models indicate that increases in the average daily temperature will result in spatial changes in vector abundance and expansion of the geographic range of some species (Eisen 2008, Ogden *et al.* 2008), and by implication, of the pathogens they carry. This is particularly important for vector populations that exist near their distributional limits, and likely at the limits of the conditions that are suitable for survival.

Therefore, some of the key questions that need to be addressed in order to understand the complex triad of interactions and the resulting epidemiology of vector-borne diseases are: (1) what species of arthropod are involved in the transmission of pathogenic microorganisms and what is the potential risk of exposure to these vectors for humans and domestic animals in different geographical areas?, (2) what species of vertebrate host are important in the life cycle of an arthropod vector, and also act as reservoir hosts for, and/or are involved in the transmission cycles of pathogenic microorganisms?, (3) what pathogenic microorganisms are present in arthropods and what is the risk of exposure to these pathogens for humans and domestic

animals?, and (4) are there any predictable patterns of infection (i.e. co-occurrences) among particularly types or species of pathogenic agents in specific types of vectors?

1.2. Research Objectives

In North America, there are over 80 species of tick (Merten & Durden 2000), some of which are known to be vectors of microorganisms that are pathogenic to humans and/or domestic animals (Jongejan & Uilenberg 2004). At least 32 species of hard tick, representing six genera, have been recorded in Canada (Smith *et al.* 1997). Two of the most common species in Canada are *Dermacentor andersoni* and *D. variabilis* (Gregson 1956, Lindsay *et al.* 1999, Copeland 2001), both of which are known to harbour pathogenic microorganisms in parts of their distributional ranges in North America (Azad & Beard 1998, Goethert *et al.* 2004, Scoles *et al.* 2005, Telford & Goethert 2008, Brackney *et al.* 2010). There are, however, a number of questions concerning the relative importance of their role as vectors for different pathogens (e.g. see Eisen 2007), the full extent of the diversity of the microorganisms within these ticks, and the ecological factors that influence their geographic distributions, particularly in areas where these two species coexist.

The overall aim of my PhD thesis research was to investigate fundamental questions with regard to the transmission cycles of tick-borne microorganisms (i.e. relationships comprising the epidemiological triangle) by comparing the ecology of two closely-related species of tick vector, *Dermacentor andersoni* and *D. variabilis*. This study compared the bacteria and vertebrates associated with these two tick species from a number of localities in Canada, particularly in Saskatchewan near the north-western distributional limit of *D. andersoni* and the north-eastern distributional limit of *D. variabilis*. An important aspect of this study was the comparison of the

bacteria present in individual ticks of different life cycle stages in both allopatric and sympatric populations of *D. andersoni* and *D. variabilis*.

1.3. *Dermacentor andersoni* and *Dermacentor variabilis* – their ecology and importance as vectors of pathogenic microorganisms, and the questions to be addressed in this thesis

The Rocky Mountain wood tick, *D. andersoni*, occurs throughout parts of western USA and southern parts of Canada west of central Saskatchewan, while the American dog tick, *D. variabilis*, occurs throughout much of the eastern USA, into Mexico, and southern parts of Canada east of central Saskatchewan (Bishopp & Trembley 1945, Gregson 1956, Wilkinson 1967, Sonenshine 1979, Merten & Durden 2000, James *et al.* 2006). Both tick species have largely allopatric distributions, except in some parts of Montana, Wyoming, Colorado, North Dakota and South Dakota, where they occur in sympatry (Bishopp & Trembley 1945, Wilkinson 1967, Sonenshine 1979, Merten & Durden 2000, James *et al.* 2006). *D. andersoni* and *D. variabilis* are three-host ticks, requiring three separate hosts on which to feed in order to complete their life cycle. The time taken to complete the life cycle by both tick species varies throughout their geographical ranges in response to different environmental conditions they experience. For example, *D. variabilis* in southern populations along the Atlantic coast have a one-year life cycle, while individuals in more northern populations (e.g. in Massachusetts and Nova Scotia) have a two-year life cycle (McEnroe 1974, Garvie *et al.* 1978, McEnroe 1978, Sonenshine 1979). For *D. andersoni*, it is known that the development times of engorged nymphs originating from Waterton National Park in Alberta (i.e. 'montane' population) differ from those individuals originating from Chin Lakes and Manyberries in Alberta (i.e. 'prairie' populations) in response to different photoperiodic regimes (Pound and George 1991).

In some parts of their geographical ranges, *D. andersoni* and *D. variabilis* also occur in sympatry with another species of *Dermacentor*, the winter tick, *D. albipictus* (Gregson 1956, Wilkinson 1967). Unlike *D. andersoni* and *D. variabilis*, *D. albipictus* is a one-host tick in that it can complete is life cycle using only a single host. Ungulates such as moose, caribou, elk, white-tailed deer, mule deer and cattle are used as hosts by *D. albipictus* (Gregson 1956, Kollars *et al.* 2000). Given that different species of vector may differ in their biology, ecology and vectorial capacity, it is essential that, in any study of the ecology and epidemiology of vector-borne disease, the different vectors can be unequivocally distinguished from one another, particularly for life cycle stages involved in the transmission of pathogenic microorganisms to vertebrate hosts. Adult male and female *D. albipictus* can be readily distinguished morphologically from *D. andersoni* and *D. variabilis* by their round spiracular plates and the relatively few large goblets within the spiracular plates (Gregson 1956). Although *D. andersoni* and *D. variabilis* are morphologically similar, they can also be distinguished from one another based on the shape and size of the goblets and their spiracular plates surrounding them. *D. andersoni* has spiracular plates with a sharp dorsal prolongation and medium number and size of goblets, while *D. variabilis* has spiracular plates with a blunt dorsal prolongation and many small goblets (Gregson 1956). In contrast, it is much more difficult to identify or distinguish among the larvae and nymphs of the three species of *Dermacentor* (Gregson 1956). **The inability to unequivocally identify *Dermacentor* immatures morphologically to the species level is a problem for studying the ecology of tick-borne pathogens, particularly in areas where two or more of these tick species occur in sympatry.** This is an important issue that needs to be resolved for any study conducted on the biology and ecology of *Dermacentor*, which includes an examination of their distributional limits in Saskatchewan. Therefore, the first objective was to establish

genetic markers that could be used in molecular assays to distinguish among the three species of *Dermacentor*, irrespective of life cycle stage **(Chapter 2).**

Given that *D. andersoni* and *D. variabilis* are known to be vectors of human and animal pathogens, such as *Francisella tularensis* and *Rickettsia rickettsii*, in certain parts of their distributional range (Walker 1998), an important question that needed to be addressed was: **what is the potential risk of exposure to these vectors in areas near their northern distributional limits in Saskatchewan?** The accepted distributions of *D. andersoni* and *D. variabilis* in this province are mainly based on records prior to the 1970's (Gregson 1956, Wilkinson 1967); however, anecdotal evidence suggests that the distributions of both species in Saskatchewan have expanded since then. The current distributional ranges of these species need to be determined. It also needs to be established if these two tick species in Saskatchewan occur in sympatry, as in geographical regions further to the south (i.e. Montana, Wyoming, Colorado, North Dakota and South Dakota) (Gregson 1956, Merten & Durden 2000). Therefore, an important objective of my research was to determine the distributional ranges of *D. andersoni* and *D. variabilis* in western Canada, mainly in Saskatchewan, and compare their current distributions with those based on the historical records for each species (**Chapter 3**).

Another important question that needed to be examined was, **what species of vertebrates are important hosts to the immature stages (larvae and nymphs) of *D. andersoni* and *D. variabilis*, and thus, may act as reservoir hosts for, and/or may be involved in the transmission cycles of pathogenic microorganisms?** It is known that the different life cycle stages of these two tick species prefer to parasitize different species of vertebrate host. Adults of both *D. andersoni* and *D. variabilis* utilize medium-sized to large mammals, including raccoons, skunks, horses, cattle, mule deer, dogs, cats and humans, as hosts throughout their geographic

ranges (Gregson 1956, Kollars 1996, Kollars *et al.* 2000, James *et al.* 2006). For example, *D. variabilis* adults have been collected from raccoons, skunks, humans and dogs in Saskatchewan and Manitoba (Dergousoff and Chilton, unpublished observations). The immature stages (i.e. larvae and nymphs) of both tick species use small mammals, such as voles, chipmunks, deer mice, jumping mice, white-footed mice, and ground squirrels, as hosts (Gregson 1956, Kollars 1996, Kollars *et al.* 2000). However, there is little published information as to which species of small mammals are used by *D. andersoni* and *D. variabilis* immatures in Saskatchewan. A key question that needed to be answered was, **do the immatures of these two tick species use the same host species (and host individuals) in areas where the two species occur in sympatry?** The answer to this question is important with respect to determining whether there is the potential for cross-transmission of pathogenic bacteria from one tick species to another. If so, this may represent one mechanism by which a pathogenic microorganism can expand its distributional range and, thus, lead to the spread of an infectious human and/or animal disease. Therefore, an important objective of my work was to determine which species of small mammals are used as hosts by the immature stages of *D. andersoni* and *D. variabilis*. This was examined at a locality where the tick species occur in sympatry, and at one locality where only *D. variabilis* was known to occur (**Chapter 3**). Determination of the different host associations of ticks also has important implications for the understanding how tick-borne microorganisms are maintained in nature.

Both *D. andersoni* and *D. variabilis* are important vectors for a variety of pathogens to humans and animals in some parts of their geographical ranges. For example, *D. andersoni* is the vector of the Colorado tick fever virus, *Rickettsia rickettsii*, *Francisella tularensis*, and *Anaplasma marginale* (Walker 1998, Scoles *et al.* 2006, Brackney *et al.* 2010), while *D.*

variabilis is known to be a vector of *R. rickettsii*, *F. tularensis*, and *A. marginale* (Walker 1998, James *et al.* 2006) . Some intracellular bacteria (i.e. *Ehrlichia chaffeensis*, *Ehrlichia canis* and *Ehrlichia ewingii*) have also been detected within *D. variabilis* (Everett *et al.* 1994, Murphy *et al.* 1998, Holden *et al.* 2003, Stich *et al.* 2008) and *D. andersoni* (*Coxiella burnetii*) (Sanders *et al.* 2008); however, it is not known if these ticks are vectors for these organisms. As a consequence, *D. andersoni* and *D. variabilis* infected with pathogens cause significant health problems to humans and domestic animals, particularly livestock. There are also substantial economic losses associated with livestock parasitized by *D. andersoni* or *D. variabilis* infected with pathogenic microorganisms. For example, it has been estimated that the financial losses in the USA due to cattle with bovine anaplasmosis (caused by *Anaplasma marginale*) was over $300 million per year (Kocan *et al.* 2003). In addition *D. andersoni* can have a direct effect on the health of domestic animals and wildlife by inducing paralysis (Lysyk 2010).

In addition to these pathogens, a number of endosymbiotic bacteria also reside within *D. andersoni* and *D. variabilis* (Noda *et al.* 1997, Scoles 2004, Clay *et al.* 2008). In this thesis, I will adopt the definition of endosymbionts as defined by Clay (2008), such that endosymbionts are microorganisms with no defined pathogenicity that form long-term associations with their hosts. These can be bacteria that are essential for the survival of the host (i.e., primary symbionts) or microorganisms that are not required by the host (i.e., secondary symbionts) (Vautrin & Vavre 2009). Depending on the nature of the relationships of symbionts with their tick host, different mechanisms or strategies are used to ensure their survival and successful maintenance in a host population. Primary symbionts are often vertically transmitted from one generation to the next, while secondary symbionts, because they are not necessary for survival of

the arthropod, can be transmitted to a new host through mechanisms of horizontal transmission (Clay *et al.* 2008), which may not be as efficient as vertical transmission.

Although *D. andersoni* and *D. variabilis* are hosts and vectors to a number of microorganisms, the types of pathogens and endosymbionts found in these ticks have not yet been fully characterized and the risk for transmission of some tick-borne microorganisms to animals and humans is not clear. This is partly due to limitations in the techniques used to detect and identify the microorganisms and partly due to the focus on only pathogenic species in most studies. Furthermore, most studies that have examined the bacterial species within *D. andersoni* and *D. variabilis* are based primarily on populations of these ticks in different regions of the USA (e.g. Gage *et al.* 1994, Smith *et al.* 2010, Stromdahl *et al.* 2011). In contrast, there has been limited number of surveys of tick-borne microorganisms in Canada (Humphreys & Campbell 1947, Teng *et al.* 2011). Therefore, two important questions that needed to be addressed were: **what species of pathogenic bacteria and endosymbiotic bacteria are found in *D. andersoni* and *D. variabilis* in different localities in Canada?, and what is the risk of exposure to potentially pathogenic tick-borne bacteria for humans and domestic animals in Saskatchewan?**

In western Canada, Colorado tick fever virus (Brown 1955), *R. rickettsii* (Humphreys 1947), and *F. tularensis* (Brown 1943, Gordon *et al.* 1983) have been detected in *D. andersoni*. However, the apparent incidence of tick-borne diseases has been relatively low in Canada, with only sporadic occurrences of certain diseases (Cimolai *et al.* 1988, Wobeser *et al.* 2009). It is important to determine if these organisms are endemic and if northern populations of tick play a role in their maintenance and transmission. An understanding of the current prevalence of tick-borne microorganisms is necessary to recognize any future changes for the risk of tick-borne

diseases. Thus, a major component of my research work was to determine which bacterial species are present in immature and adult *D. andersoni* and *D. variabilis*, and determine the prevalence of each bacterial species, in both allopatric and sympatric populations of these two tick species (**Chapters 5 to 8**).

The species composition of the microbial community within individual ticks can have important biological and epidemiological implications. For example, certain bacterial endosymbionts have been shown to affect the vectorial capacity of their tick hosts (Burgdorfer *et al.* 1981, Ginsberg 2008) . Given this, the following question needed to be examined: **are there predictable patterns of infection (i.e. co-occurrences) of different bacteria in *D. andersoni and D. variabilis* from different populations in Canada?** Thus, a comparison was conducted of the bacterial species composition of individual ticks from different life cycle stages and populations of both tick species (**Chapter 9**).

1.4. Anticipated significance of research

This research addresses some fundamental ecological questions of vector ecology that are important for assessing the risk of exposure for tick-borne pathogens to humans and animals and for understanding the transmission cycles that maintain these microorganisms in nature. One of the main goals of research in vector ecology is to determine how to control and prevent the transmission of tick-borne pathogens. An understanding of the relationships among the vector(s), pathogen and vertebrate host(s) are required to identify points in transmission cycles that can be exploited to disrupt the spread of tick-borne pathogens. This requires an analysis of certain fundamental questions of the biology and ecology of the vector(s), vertebrate host(s) and the pathogenic agent in epidemiological triangles. Despite the importance *of D. andersoni* and *D.*

variabilis as vectors for a number of pathogens, the vertebrate and microbial relationships of sympatric and allopatric populations for these two species have yet to be examined. The findings of this research are expected to provide information necessary to develop a detailed description of current distribution of *D. andersoni* and *D. variabilis* at the northern extent of their ranges. This will provide a basis for estimating the risk for exposure to potential vectors and provide a base-line for future studies of the (potentially changing) distribution of *D. andersoni* and *D. variabilis* and for studies aimed at determining the biotic and abiotic factors that limit tick distributions.

A comparison of the microorganisms in allopatric and sympatric populations of *D. andersoni* and *D. variabilis* will provide a basis for understanding microbial transmission, disease risk and the vector potential of *D. andersoni* and *D. variabilis* in localities where they have not been studied previously (i.e. at the northern-most extent of their distributional ranges) and where the ecological conditions and potential relationships likely differ from those in other areas where these ticks occur. This study may also provide clues to the potential transmission cycles and reservoir hosts for tick-borne microorganisms by identifying the species of mammals used as hosts by larvae and nymphs. Detailed information on the types and prevalence of the bacteria in ticks is necessary for future comparative analyses to determine how these associations may change over time.

The studies described in the following chapters represent necessary steps for understanding the vector ecology of two important tick species in North America. General principles examined in this study, such as those relating to the vertebrate host range and microbial community composition of ticks, would also be important to examine for other species of tick that are vectors for human and animal pathogens, particularly those with overlapping distributions. The

basic biological and ecological questions that still need to be answered to understand the epidemiology of tick-borne diseases will require a variety of approaches and an integration of disciplines, including those of ecology, physiology, and molecular biology, for a satisfactory understanding of the complex interactions that determine the epidemiology of tick-borne diseases.

1.5. References

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Chapter 2. Differentiation of three species of ixodid tick, *Dermacentor andersoni*, *D. variabilis* and *D. albipictus*, by PCR-based approaches using markers in ribosomal DNA[1]

2.1. Abstract

In this chapter, a practical PCR assay, based on the amplification of part of the second internal transcribed spacer ribosomal DNA (pITS-2 rDNA), was developed to distinguish *D. andersoni* from *D. variabilis*. In addition, single- strand conformation polymorphism (SSCP) analysis of the pITS-2 rDNA provided a reliable method of distinguishing specimens of the three species of ixodid tick. PCR and pITS-2 SSCP were also used to look for evidence of hybridization between *D. andersoni* and *D. variabilis* at two localities in Saskatchewan where they occur in sympatry. These molecular tools should be useful for the unequivocal identification of *D. andersoni* and *D. variabilis* at all life cycle stages, which is essential for studies on their ecology and on the transmission of tick-borne pathogens. Also, pITS-2 SSCP may be of potential use for discriminating among the other morphologically similar species within the genus *Dermacentor*.

2.2. Introduction

Three species of *Dermacentor* that occur in western Canada (*D. albipictus*, *D. andersoni* and *D. variabilis*) can be distinguished from one another by differences in their morphology. Adult *D. albipictus* lack a dorsal prolongation on their spiracular plates and have fewer but larger goblets within the spiracular plates than *D. andersoni* or *D. variabilis*. The spiracular plates of *D.*

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andersoni have a more pronounced dorsal prolongation and contain fewer but larger goblets compared with *D. variabilis* (Gregson 1956). However, there is variation in the features of the spiracular plate among specimens of *D. andersoni* and *D. variabilis*, which may make identification difficult, particularly for ticks collected at localities where the two species coexist. Hybrid adults, derived from laboratory experimental crosses between female *D. variabilis* and male *D. andersoni*, have also been shown to have spiracular plates that are intermediate in morphology between the two species (Oliver *et al.* 1972). Therefore, it is important that there are markers available that can be used to unequivocally distinguish specimens of *D. andersoni* from *D. variabilis*.

Molecular techniques have been used effectively to identify ticks to the genus and/or species level (Zahler *et al.* 1995, Norris *et al.* 1997, Norris *et al.* 1999, Poucher *et al.* 1999, Anderson *et al.* 2004, Shone *et al.* 2006), and to examine the phylogeny and/or taxonomic status of some species (Wesson *et al.* 1993, Zahler *et al.* 1995). The target regions used in these studies included the nuclear 18S ribosomal (r) RNA gene and second internal transcribed spacer (ITS-2), and the mitochondrial 12S and 16S ribosomal genes (Wesson *et al.* 1993, Zahler *et al.* 1995, Norris *et al.* 1997, Norris *et al.* 1999, Poucher *et al.* 1999, Anderson *et al.* 2004, Shone *et al.* 2006). For example, Zahler *et al.* (1995) examined the species status of *D. reticulatus* and *D. marginatus* using a comparison of the ITS-2 rDNA sequences of these two taxa, and those of *D. andersoni* and *D. variabilis*.

The aim of this chapter was to establish a practical and effective PCR-based assay to distinguish *D. variabilis* from *D. andersoni*, based on interspecific differences in the ITS-2 sequences (Zahler *et al.* 1995), and to determine if this marker provided any evidence of hybridization in areas where the two species occur in sympatry. Furthermore, the use of the

mutation scanning technique, single-strand conformation polymorphism (SSCP), was evaluated as a diagnostic tool to distinguish among specimens of *D. andersoni*, *D. variabilis* and *D. albipictus*.

2.3. Materials and methods

2.3.1. Collection of ticks

Adult ticks were collected by flagging grassy and shrubby vegetation along walking tracks in provincial parks in Saskatchewan (Blackstrap, Saskatchewan Landing and Buffalo Pound) and Alberta (Cypress Hills), Canada. Ticks were identified morphologically as either *Dermacentor andersoni*, *D. variabilis* or *D. albipictus* (Table 2.1), based on the shape of the spiracular plates, and on the relative size and number of the goblets within the spiracular plates (Wilkinson 1967). Adult *D. albipictus* were included in the study for comparative purposes. Each tick was frozen at -70°C until required for the molecular work. Also included for comparison were unfed *D. albipictus* larvae derived from the eggs of two engorged females collected from moose near Prince George (British Columbia) and Calgary (Alberta).

2.3.2. DNA purification and PCR

Genomic DNA (gDNA) was extracted and purified from either one or two legs, or the complete body of adult ticks using the DNeasy Tissue Kit™ (Qiagen). The use of only one or two legs permits the remainder of the specimen to be preserved for morphological examination or to be tested for the presence of pathogenic organisms. The leg(s) or complete body of an individual tick was/were placed into a 1.5 ml micropestle tube (Kontes) with 180 µl of ATL Buffer (Qiagen) and homogenized using a micropestle attached to a cordless drill. Proteinase K

Collection Site (Provincial Park)	**Coordinates** (decimal degrees)		**No. of adult individuals**		
	Lat. (N)	Long. (W)	*D. andersoni*	*D. variabilis*	*D. albipictus*
Blackstrap	51.79760	-106.45833	-	33	-
Saskatchewan Landing	50.64528	-107.96310	48	38	2
Buffalo Pound	50.57582	-105.31356	34	39	-
Cypress Hills	49.42682	-110.25441	20	-	-
Total			102	110	2

Table 2.1. Collection localities and number of adult *Dermacentor* used in this study.

(20 µl of 15 µg/µl) was then added to the micropestle tube and the sample incubated overnight at 55°C. Two hundred µl of AL Buffer (Qiagen) were added to the sample, vortexed and incubated for 10 min at 70°C. Then, 200 µl of 100% ethanol were added, and the solution was applied to a spin column. After rinsing the columns with the wash buffers AW1 and AW2 (Qiagen), gDNA was eluted with 100 µl AE buffer (Qiagen) and stored at -70°C. The gDNA from two whole individual *D. albipictus* larvae was extracted and purified using the same methodology.

Part of the ITS-2 (pITS-2) rDNA was amplified from gDNA using the forward primer DAVF (5'-TCA CAT ATC AAG AGA GCC TT-3') and reverse primer DAVR (5'-ACG TAC TTC GAA GGC AAA CA-3'), designed based on previously published sequences of *D. andersoni* and *D. variabilis* (GenBank accession nos. AY365355 to AY365363 and S83088; (Zahler *et al.* 1995, de la Fuente *et al.* 2005). The PCR was performed in 25 µl containing 200 µM of each dNTP (Promega), 3 mM $MgCl_2$, 50 pmol of each primer and 0.5 U of *Taq* polymerase (Promega) using a thermal cycler (Bio-Rad iCycler) with the following conditions: 95°C, 5 min (initial denaturation); 30 cycles of 95°C, 30 s (denaturation), 52°C, 30 s (annealing), and 74°C, 30 s (extension); followed by 74°C for 5 min (final extension). A negative (i.e. without gDNA) control was included in each PCR run. Individual amplicons were subjected to electrophoresis on SYBR® Safe (Molecular Probes) stained 2% agarose-TBE (EMD Biosciences; 89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) gels. A 100 bp TrackIt™ DNA ladder (Invitrogen) was used on gels as a size standard.

2.3.3. Single-strand conformation polymorphism (SSCP) analysis

Amplicons from representative samples of each species were also subjected to SSCP analyses. In thin-walled tubes, individual amplicons (1 µl) were mixed with 4 µl of DNase-free water and 5 µl of loading buffer (Gel Tracking Dye™, Promega), then denatured at 95˚C for 5 min prior to snap cooling in ice water for 5 min. Each sample (5 µl) was loaded into the wells of precast GMA™ S-50 gels (Elchrom Scientific) and subjected to electrophoresis for 18 h at 74 V and 7.4˚C (constant) in a horizontal SEA2000™ apparatus (Elchrom Scientific) connected to a temperature-controlled circulating water bath. Following electrophoresis, gels were stained for 30 min with SYBR® Gold (Molecular Probes), rinsed in distilled water and then photographed using a BioDoc-It™ (UVP) imaging system. Non-denatured amplicons were also included on SSCP gels to distinguish single-stranded from double-stranded DNA.

2.3.4. DNA sequencing and data analyses

The gDNA from two adult *D. andersoni*, two adult *D. variabilis* and the four *D. albipictus* specimens were amplified by PCR using the primers DermITS2-F (5'-GTG CGT CCG TCG ACT CGT T-3') and DermITS2-R (5'-TCG CCC AAC ACG GCG CTA CT-3') (Shone *et al.* 2006) and the conditions described above, except that an annealing temperature of 60ºC was used. Amplicons were column purified (MinElute PCR purification kit, Qiagen) and subjected to automated DNA sequencing (ABI Prism DNA Sequencer) using the same two primers in separate reactions. Sequences were aligned manually. Nucleotide sequence data have been deposited in the EMBL, GenBank™ and DDJB databases under the accession numbers AM498348 to AM498351. Pairwise comparisons of the number of fixed sequence differences (D) were determined using the formula D = 1-(M/L), where M is the number of alignment

positions at which the two sequences have a base in common, and L is the total number of alignment positions over which the two sequences are compared (Chilton *et al.* 1995).

2.4. Results and discussion

The gDNA from 216 *Dermacentor* adults and larvae (Table 2.1) were subjected to the PCR. A single amplicon was resolved for each PCR reaction, but no bands were detected in the negative (i.e. no gDNA) controls. For all three species, there was no detectable intraspecific variation in the size of amplicons. However, the amplicon from *D. andersoni* (~430 bp) was significantly larger than that from *D. variabilis* (~360 bp) (Fig. 2.1). This was expected, based on a comparison of the published sequences of the complete ITS-2 rDNA for the two species (Zahler *et al.* 1995, de la Fuente *et al.* 2005). The primers DAVF and DAVR were specifically designed to amplify a part of the ITS-2 that included a 72 bp deletion in the sequence of *D. variabilis* relative to *D. andersoni*. Thus, the amplification of the pITS-2 and detection by agarose gel electrophoresis provided a simple, rapid and effective technique to distinguish between adult specimens of *D. variabilis* and *D. andersoni*. This PCR assay will also be particularly useful for distinguishing larvae and nymphs of these two species, particularly engorged individuals, which are more difficult to identify than adults. The PCR assay did not discriminate between *D. variabilis* and *D. albipictus* because their respective pITS-2 amplicons were of an equivalent size on an agarose gel (Fig. 2.1). Prior to the present study, no ITS-2 sequence data were available for *D. albipictus*; thus, the extent of the interspecific differences in the ITS-2 sequences between *D. albipictus* and *D. variabilis* was not known. Therefore, sequences of the pITS-2 rDNA were determined for four specimens of *D. albipictus* as well as two *D. andersoni* and two *D. variabilis* adults (Fig. 2.2). A comparison of the sequences revealed that the magnitude of sequence differences among *D. albipictus*, *D. andersoni* and

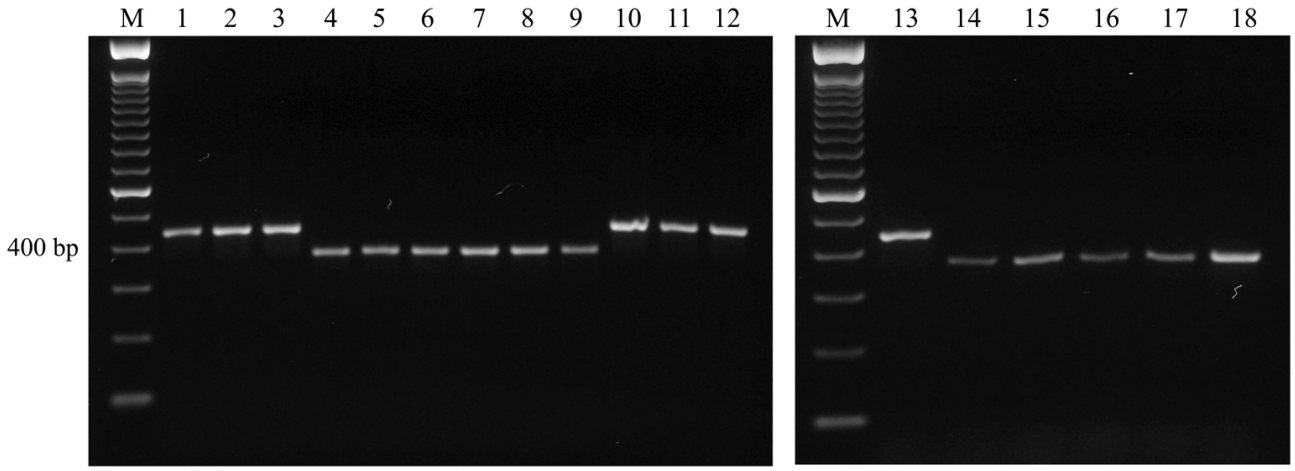

Fig. 2.1. Agarose gel depicting pITS-2 amplicons from representative individual adults of *D. andersoni* (lanes 1-3 and 10-13), *D. variabilis* (lanes 4-9 and 18) and *D. albipictus* (lanes 14 and 15), and larvae of *D. albipictus* (lanes 16 and 17). A 100 bp DNA ladder (M) was used as a size marker.

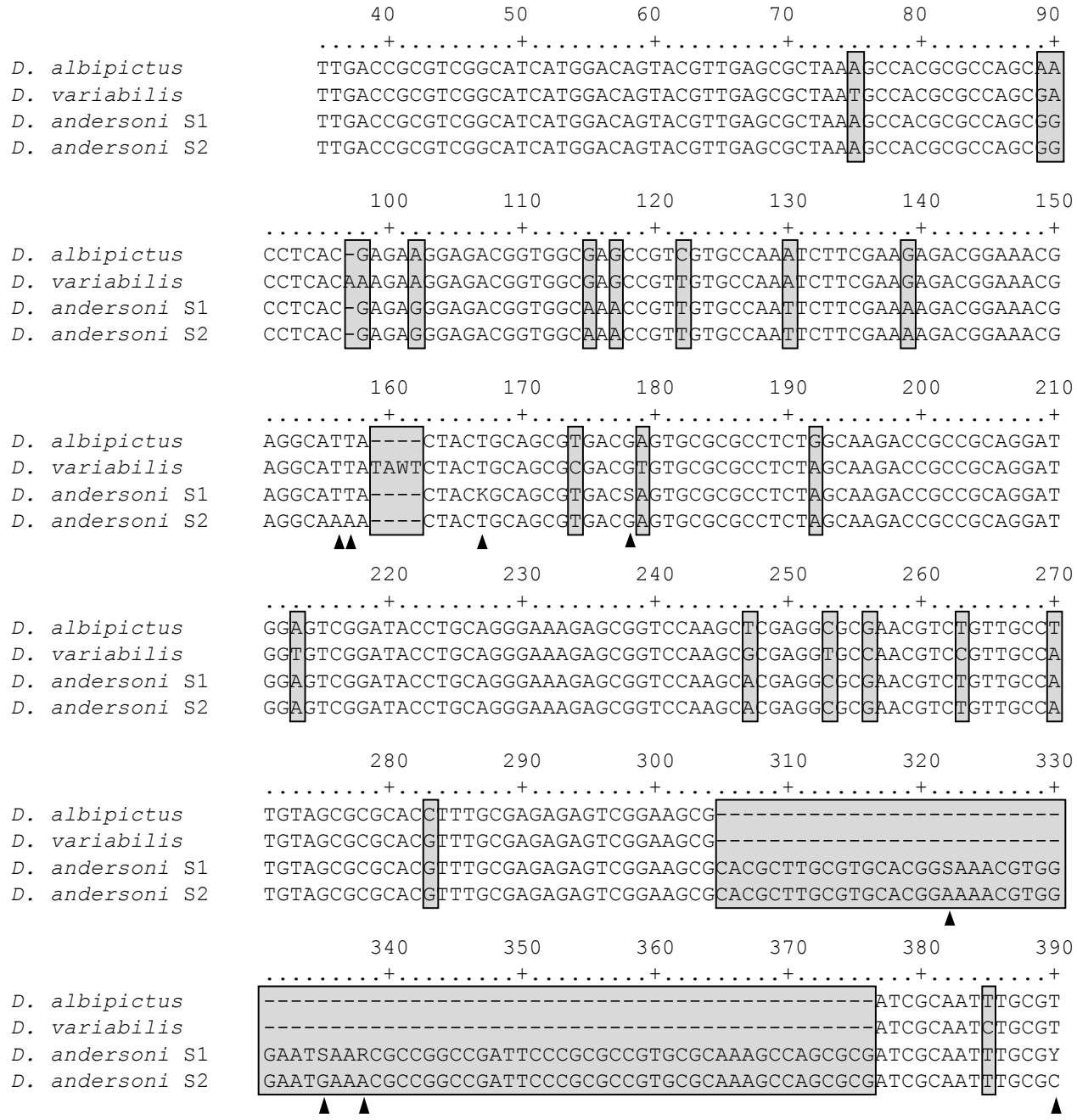

Fig. 2.2. Alignment of the pITS-2 rDNA sequences of *D. albipictus*, *D. variabilis* and *D. andersoni*. Shaded positions indicate interspecific sequence differences. Triangles indicate the positions of intraspecific variation between the two *D. andersoni* sequence types (S1 and S2). IUPAC codes (i.e. R = A & G; S = A & C, W = A & T and Y = C & T) are used at positions of intraindividual sequence polymorphism.

D. variabilis ranged from 7-27%, and that *D. variabilis* was genetically more similar to *D. albipictus* than to *D. andersoni*. Given these interspecific differences in the pITS-2 sequence, SSCP was investigated as a sequence-based diagnostic tool for distinguishing these three tick species. The pITS-2 amplicons from four specimens of *D. albipictus* (two larvae and two adults) each had the same banding pattern when subjected to SSCP analysis (Fig. 2.3). These four ticks also had identical pITS-2 sequences, even though some were collected from localities ~1100 km apart. Intraspecific variation in SSCP profiles was detected among samples of *D. andersoni* and *D. variabilis* (Fig. 2.3), which suggested that there was sequence variation within the ITS-2 rDNA. The two *D. andersoni* adults differed in their pITS-2 sequence at eight alignment positions (Fig. 2.2). The variation in SSCP profiles among *D. andersoni* individuals was consistent with the findings of de la Fuente *et al.* (2005), who detected nine different ITS-2 sequence types. No sequence differences were detected between the two specimens of *D. variabilis*, even though they had slight differences in their SSCP profile. However, no sequence data were available for the first 34 bp at the 5' end of the pITS-2 because DermITS2-F, rather than DAVF, was used as the forward primer in the sequencing reactions. Therefore, the variation in SSCP profiles in *D. variabilis* specimens may represent sequence variation at the 5' end of the pITS-2 and/or the formation of different conformational types of a single-stranded pITS-2 molecule. Despite the intraspecific variation in banding patterns, the SSCP profile of an amplicon could be used to infer species identity because *D. andersoni*, *D. variabilis* and *D. albipictus* each had a unique set of banding patterns (Fig. 2.3). In addition, there were significant differences among the three species in the relative migration rate of the double-stranded DNA fragments on SSCP gels (Fig. 2.3), providing another valuable marker for distinguishing among the three species of *Dermacentor* which occur in Canada.

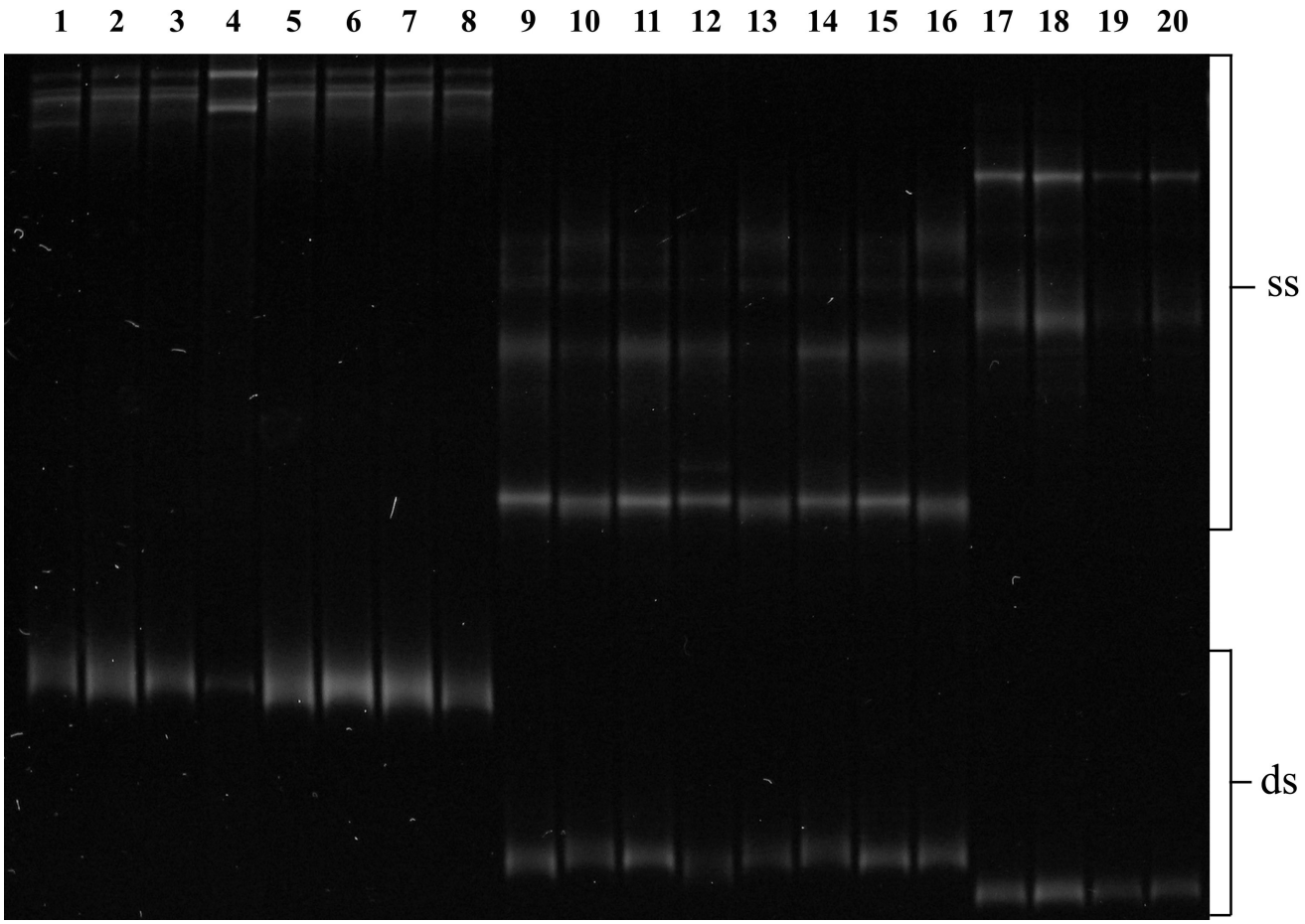

Fig. 2.3. SSCP analysis of the pITS-2 rDNA amplicons from representative individual adults of *D. andersoni* (lanes 1-8), *D. variabilis* (lanes 9-16) and *D. albipictus* (lanes 17 and 18), and individual *D. albipictus* larvae (lanes 19 and 20). A comparison of non-denatured and denatured amplicons (data not shown) were used to distinguish single-stranded DNA (ss) from double-stranded DNA (ds).

The establishment of genetic markers to distinguish unequivocally *D. andersoni* from *D. variabilis* provided the opportunity to determine whether there was any genetic evidence of hybridization between these two species in areas where they coexist. In Saskatchewan (Canada), there are localities where *D. andersoni* and *D. variabilis* occur in sympatry (Dergousoff and Chilton, unpublished data). This, together with overlapping periods of seasonal activity (Wilkinson 1967) and similarities in reproductive biology (Sonenshine 1985), provides the potential for hybridization between the two species. Furthermore, hybrids between *D. andersoni* and *D. variabilis* have been produced from experimental laboratory crosses (Oliver *et al.* 1972). In the latter study (Oliver *et al.* 1972), female *D. variabilis* crossed with male *D. andersoni* produced larvae, some of which were successfully raised through to the adult stage. The reciprocal crosses failed to produce viable offspring (Oliver *et al.* 1972). However, no hybrids were detected by morphological examination of several thousand specimens collected from sympatric populations of *D. andersoni* and *D. variabilis* in the Forsyth area of Montana (USA) (Oliver *et al.* 1972). Therefore, I examined whether there was any genetic evidence for hybridization between *D. andersoni* and *D. variabilis* in nature by conducting PCR and pITS-2 SSCP analyses on 159 adult ticks collected from Saskatchewan Landing and Buffalo Pound, two localities where *D. andersoni* and *D. variabilis* coexist (Dergousoff and Chilton, unpublished data). Following PCR, a single amplicon from each tick was detected by agarose gel electrophoresis. The size of each amplicon was either ~430 bp or ~360 bp, consistent with that for either *D. andersoni* or *D. variabilis* (respectively). Furthermore, all specimens had an SSCP profile consistent with that of either *D. andersoni* or *D. variabilis* (cf. Fig. 2.3) collected from allopatric populations (i.e. Cypress Hills and Blackstrap, respectively). Thus, employing the present molecular approach, there was no genetic evidence of hybridization between the two

species at Saskatchewan Landing or Buffalo Pound because no individual tick had a double-banded amplicon or a combination of *D. andersoni* and *D. variabilis* SSCP profiles (i.e. the expected patterns for a hybrid). This finding is consistent with the suggestion of Oliver *et al.* (Oliver *et al.* 1972) that hybridization, though possible under certain experimental conditions, is not likely to occur in nature possibly due to interspecific differences in seasonal activity.

In conclusion, a practical PCR assay was developed to discriminate between *D. andersoni* and *D. variabilis* based on a substantial size difference in sequences of the pITS-2 rDNA. Furthermore, PCR-SSCP of the pITS-2 will provide a useful diagnostic tool for the delineation of specimens of *D. andersoni*, *D. variabilis* and *D. albipictus*, irrespective of life cycle stage, which has important practical uses for studies of vector distribution, and of the ecology of *D. andersoni* and *D. variabilis* and the pathogens they transmit. PCR and PCR-SSCP was used to identify or confirm the identity of ticks collected by flagging for determining their distribution (Chapter 3) and to test for the presence of multiple bacterial species (Chapters 4-8).

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Chapter 3. Comparison of the geographic ranges of *D. andersoni* and *D. variabilis* in Saskatchewan and the vertebrate hosts used by larvae and nymphs.

3.1. Abstract

In this chapter, I determined the current distributional ranges of *D. andersoni* and *D. variabilis* in Saskatchewan, and compared these findings in light of historical collection records of both species in the province. The results of passive and active surveillance revealed that the two tick species have expanded their distributional limits further northwards, and eastwards (for *D. andersoni*) or westwards (for *D. variabilis*) since the 1960's (Gregson 1956, Wilkinson 1967). Furthermore, unlike in previous reports of the geographic ranges of *D. andersoni* and *D. variabilis* (Gregson 1956, Wilkinson 1967) where these two species had allopatric (i.e. separate) distributions in Saskatchewan, the results of the present study show that there is now a large zone of overlap (i.e. sympatry) in their distributions. This provided the opportunity to determine if immatures of the two tick species used the same species of small mammal as hosts particularly given that little is known of the hosts used by these ticks in Saskatchewan. Therefore, small mammals (voles, shrews and mice) were trapped in Saskatchewan Landing Provincial Park (a site where both tick species coexist) and Blackstrap Provincial Park (a site where only *D. variabilis* occurs) to determine the hosts used by immature ticks.

3.2. Introduction

Although *D. andersoni* and *D. variabilis* are the two most prevalent species of tick in Canada, and have been implicated as vectors of pathogenic agents, such as *Francisella tularensis* (Bow & Brown 1943, Gordon *et al.* 1983), *Rickettsia rickettsii* (Brown 1944), and Colorado tick

fever virus (Brown 1955), there are no recent published records of their distributional limits in western Canada. There is also little information of the hosts used by immature ticks of either species in Saskatchewan. Such information is vital to assess the potential risk of exposure to bacterial pathogens to humans and animals in western Canada, and to our understanding of the interactions between the three members of an epidemiological triangle, the vectors (ticks), the pathogens (e.g. bacteria) and the hosts (e.g. mammals) used by the vectors.

Examination of the literature reveals that many tick-borne pathogens occur focally (i.e. tularemia and Rocky Mountain spotted fever) (Azad & Beard 1998, Goethert & Telford 2009, Brown *et al.* 2011) and have occurrence patterns that overlap the distribution of particular species of vector (e.g Eisen 2007, Telford & Goethert 2008). Understanding the potential risks (both current and future) of pathogen transmission to humans and/or animals (domestic and wildlife) requires detailed ecological and biological knowledge, not only of the pathogen, but also of the vector(s). This includes information on the species of vector present, their geographic distributions, and the range of vertebrate hosts used by each species of vector. The development of predictive models for inferring the current distributional limits of important vector species (e.g. ticks) and the localities where they may become established in the future relies on detailed information on the biotic and abiotic factors influencing their survival, development and reproduction (e.g. Estrada-peña 1999, Hess *et al.* 2001, Brownstein *et al.* 2003, Ogden *et al.* 2005, Jackson *et al.* 2007).

The construction of these predictive models also requires data on the localities (including the environmental conditions, such as annual rainfall and temperature conditions, etc.) where the vector has been reported previously. The sources of such information include records from museum collections, and from both passive and active surveillance programs (e.g. Ogden *et al.*

2005, Jackson *et al.* 2007). Ticks can be acquired through active collection efforts, such as flagging or dragging at specific localities. These sampling techniques can be quantitative, providing an estimate of tick abundance through precisely measured sampling techniques, or can be qualitative, providing basic presence/absence data on the distribution of ticks and the location of established tick populations. Passive surveillance methods are also useful for providing qualitative information on the presence of different species of tick over large geographic areas, providing the recent travel history of the infected person/animal is known. For example, the presence of engorged *D. variabilis* females on a child visiting in Panama from Baltimore in the USA (Bermúdez *et al.* 2010), and on a woman returning to Australia from a visit to Madison in the USA (Halliday & Sutherst 1990), represent cases of ticks being dispersed (i.e. translocated) to different geographical regions of the world by tourists.

Some tick species, such as *Ixodes scapularis*, can also be dispersed over large distances by migratory passerines (e.g. Ogden *et al.* 2006, Ogden *et al.* 2008a, Ogden *et al.* 2008b), whereas those species that parasitize mammals, particularly rodents, will have limited dispersal capabilities. Such is the case for *D. andersoni* and *D. variabilis* because the larvae and nymphs are usually found on a variety of rodents and lagomorphs, while the adults occur on much larger mammals, including coyote (*Canis latrans*), mule deer (*Odocoileus hemionus*), and white-tailed deer (*Odocoileus virginianus*) (Bishopp & Trembley 1945, Gregson 1956, Dodds *et al.* 1969, Campbell & Mackay 1979, Burachynsky & Galloway 1985, Kollars *et al.* 2000). Although two *D. variabilis* nymphs have been reported on migratory passerines in Georgia (Durden *et al.* 2001) and Rhode Island (Hyland *et al.* 2000), these probably represent records of ticks on accidental hosts. Therefore, the ability for range expansion by a tick species will be directly related to the distances travelled by colonizing individuals while attached to their vertebrate

hosts. The longer the time that ticks spend feeding on hosts the greater the potential for dispersal over longer distances. Thus, it is important to know which vertebrate species are important for the maintenance of tick populations because host behaviour determines the dispersal distances of ticks and the potential for range expansion.

Abiotic factors are also important for determining the distributional limits of a tick species and the ability of a species to establish populations into new geographical areas. Wilkinson (1967) described the distributions of three species of *Dermacentor* (*D. andersoni*, *D. albipictus* and *D. variabilis*) in Canada with respect to climatic and environmental conditions experienced in different geographical areas. Temperature and relative humidity were two of the most important factors influencing the distribution and abundance of these three species of *Dermacentor* (Wilkinson 1967, McEnroe 1975, 1978, 1985) and ticks of other genera (e.g. Lindsay *et al.* 1995, Ogden *et al.* 2005). Temperature has an important effect on the tick life cycle because it influences the time required for metabolism of the blood meal, and the duration of key events, such molting, oviposition and the hatching of eggs. The survival times of individual ticks and eggs are also strongly influenced by temperature and relative humidity (Sonenshine 1991). Thus, range expansion of a particular tick species is dependent on their introduction to new localities through dispersal by vertebrate hosts into localities with permissive climatic conditions and suitable vertebrate hosts that allow the establishment and subsequent population growth of the tick population.

The current knowledge of the distributional ranges of *D. andersoni* and *D. variabilis* in western Canada are based on data collected prior to the 1970's. For example, there are a number of locality records in Alberta and Saskatchewan based on surveys conducted in the 1930's and 1940's to determine the threat of Rocky Mountain spotted fever and tularemia infection to

humans and livestock (Gibbons 1939, Brown 1944, Humphreys & Campbell 1947, Brown & Kohls 1950). The two publications that are commonly cited with regard to the distribution of *D. andersoni* and *D. variabilis* in Canada were published around 50 years ago (Gregson 1956, Wilkinson 1967). Therefore, decades have passed with very little research conducted on the distribution of these ticks.

The aim of this chapter was to use active and passive surveillance methods to determine the current distribution of *D. andersoni* and *D. variabilis*, particularly in Saskatchewan. This provides a basis to assess one component of the risk for acquiring tick-borne pathogens. In addition, a study was undertaken to identify some of the small mammal hosts of immature stages of *D. andersoni* and *D. variabilis*. This objective was important because it may provide clues as to which species of small mammal are important for maintaining tick populations and which species can be involved in the transmission cycles of tick-borne microorganisms.

3.3. Methods

3.3.1. Collection of adult ticks

Determination of the distributional ranges of *D. andersoni* and *D. variabilis* were based on the active collection of ticks at specific localities, and by the locality records of ticks submitted by passive surveillance. Unfed adult ticks were collected by flagging at five localities in Alberta and thirty in Saskatchewan during the spring and summer of 2005 to 2009. These collection localities were chosen based on prior information of tick activity and/or the presence of suitable "tick" habitat, based on descriptions by Gregson (1956), Wilkinson (1967) and Sonenshine (1991). Ticks were collected in regional and provincial parks, and in protected nature reserves, consisting of primarily mixed prairie grasslands and well vegetated areas near rivers and lakes.

Flagging was used to collect questing ticks on grasses and shrubs, situated along roadways, human and wild animal walking trails, and ecotones. The approximate coordinates of collection sites were recorded with a handheld GPS unit (Garmin eTrex Legend). Ticks from a single collection site were placed into 50 ml vials and stored at 4^{o}C until they were identified to the species level based on morphological characteristics (see Chapter 2). Individual ticks or groups of up to 10 ticks of the same sex and collected from the same locality were transferred into 1.5ml microcentrifuge tubes and stored at -70^{o}C until used in molecular studies to detect the presence of specific tick-borne bacteria (see Chapters 4 to 8).

Passive surveillance was also conducted to acquire records of tick activity from many more localities than was possible through flagging efforts. Ticks were received from veterinarians and the general public in Saskatchewan, Manitoba and Ontario throughout the spring and summer of five consecutive years (2005 to 2010). All ticks were identified to the species-level (see Chapter 2). Where possible, information was obtained on the locality from where ticks were collected, the date collected, and host type (i.e. human, cat, domestic livestock, etc.). Given that no GPS readings were available for ticks collected by passive surveillance, approximate coordinates were assigned to each collection record in order to plot the localities of the ticks onto a map. The distributions of *D. andersoni* and *D. variabilis* were compared by placing all collection sites recorded from the flagging and passive surveillance studies on maps (see Figure 3.1 and 3.2) using the geographic information services software ArcGIS 9.3 (Esri).

3.3.2. Collection of larval and nymphal ticks from small mammals

Small mammals were trapped in shrubs and grasses in areas with little human activity at Saskatchewan Landing Provincial Park (50° 39' 33" N, 108° 0' 4" W) during June and July of

2008 and April of 2009, and from Blackstrap Provincial Park (51° 47' 51" N, 106° 27' 29" W) during May to July of 2009. These two sites were selected because they either represented a locality where both tick species coexist (Saskatchewan Landing Provincial Park), or where only *D. variabilis* occurs (i.e. Blackstrap Provincial Park) and where a large proportion of adult ticks are infected with *Rickettsia* spp. (see Chapter 4). Small mammals were trapped with mouse snap-traps baited with peanut butter and oats. Traps were set in the morning and checked at least every four hours until sunset. All traps were reset, left overnight and checked just after sunrise the next morning. Trapping was carried out for two to three days in a row for each sampling effort. All small mammals collected were placed into individual bags and frozen at -20^{o}C within 18 hours of collection. Animals were thawed at a later date to examine for ticks. All ticks were removed from hosts and stored in 70% ethanol. All animal research was approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use. Permits to trap rodents were obtained from the Saskatchewan Ministry of Environment.

The species identity of each small mammal was determined using morphological descriptions in a field guide to North American mammals (Society 1996). Dr. Gary Wobeser (University of Saskatchewan) or Dr. Ray Poulin (University of Regina) confirmed the species identity of each individual collected. Immature ticks were examined using a dissecting microscope to confirm that they belonged to the Metastriata. The species identity of each larvae and nymph collected was determined using PCR-based assays. Genomic DNA was purified from whole individual immature ticks using the protocol described in Chapter 2. The species identity of each tick was confirmed using a PCR assay targeting a portion of the second internal transcribed spacer (ITS-2) of the nuclear ribosomal DNA. This target region can be used

discriminate between *D. andersoni* from *D. variabilis* / *D. albipictus* based on the amplicon size on an agarose gel (see Chapter 2). An RFLP analysis of the ITS-2 rDNA was then used to distinguish between amplicons of *D. variabilis* immatures from those of *D. albipictus* (see Chapter 6).

3.4. Results

3.4.1. Geographic distribution and seasonal activity of adult ticks

A total of 665 *D. andersoni* and 4072 *D. variabilis* adults were collected by flagging at over 30 localities (Figure 3.1) in Alberta, and Saskatchewan. The northern-most records for *D. andersoni* and *D. variabilis* were from Whiteshore Lake, SK (52° 7' 46" N, 108° 20' 1" W) and from Batoche National Historic Park (52° 45' 30" N, 106° 8' 5" W), respectively. *D. andersoni* was found as far east as Buffalo Pound Provincial Park (50° 34' 29" N, 105° 22' 13" W), while the most western collection site with *D. variabilis* was from Saskatchewan Landing Provincial Park (50° 39' 23" N, 107° 58' 11" W).

The results of the passive surveillance for ticks yielded a total of 87 *D. andersoni* and 4409 *D. variabilis* adults from 22 and approximately 190 localities, respectively, in Alberta, Saskatchewan, Manitoba and Ontario (see Figure 3.2 for distribution of ticks within Saskatchewan). The most northern records for *D. andersoni* and *D. variabilis* submitted from the passive surveillance were from LaLoche, SK (56° 28' 55" N, 109° 26' 7" W) and at Thompson Lake, SK (55° 59' 36" N, 105° 24' 32" W), respectively. *D. andersoni* was found as far east as Buffalo Pound Provincial Park (50° 34' 29" N, 105° 22' 13" W), and *D. variabilis* was found as far west as Three Hills, AB (51° 42' 24" N, 113° 15' 49" W). However, some caution must be used when attempting to determine the presence of different tick species with passive

surveillance records. It is not always possible to be certain of the accuracy of the locality where ticks were acquired because of travel and the period of time that can pass before the tick is detected. Thus, records of rare occurrences that are well outside the typical geographic range of a species should be considered suspect as to the locality where it was acquired and may represent an example of an introduction due to dispersal on a vertebrate host.

Based on the collection records of both the active and passive surveillance, adult ticks were active from late April to July of each year. *D. andersoni* adults were first detected on April 4th (2010) from Lake Minnewanka in Banff National Park, AB (51° 15' 39" N, 115° 15' 0" W), and the latest tick was collected on July 8 (2008) from Saskatchewan Landing Provincial Park. Active *D. variabilis* adults were first detected on April 19 (2009) at Saskatchewan Landing Provincial Park, and the latest dates ticks were collected was on October 10 (2009) at Blackstrap Provincial Park (51° 47' 31" N, 106° 25' 55" W).

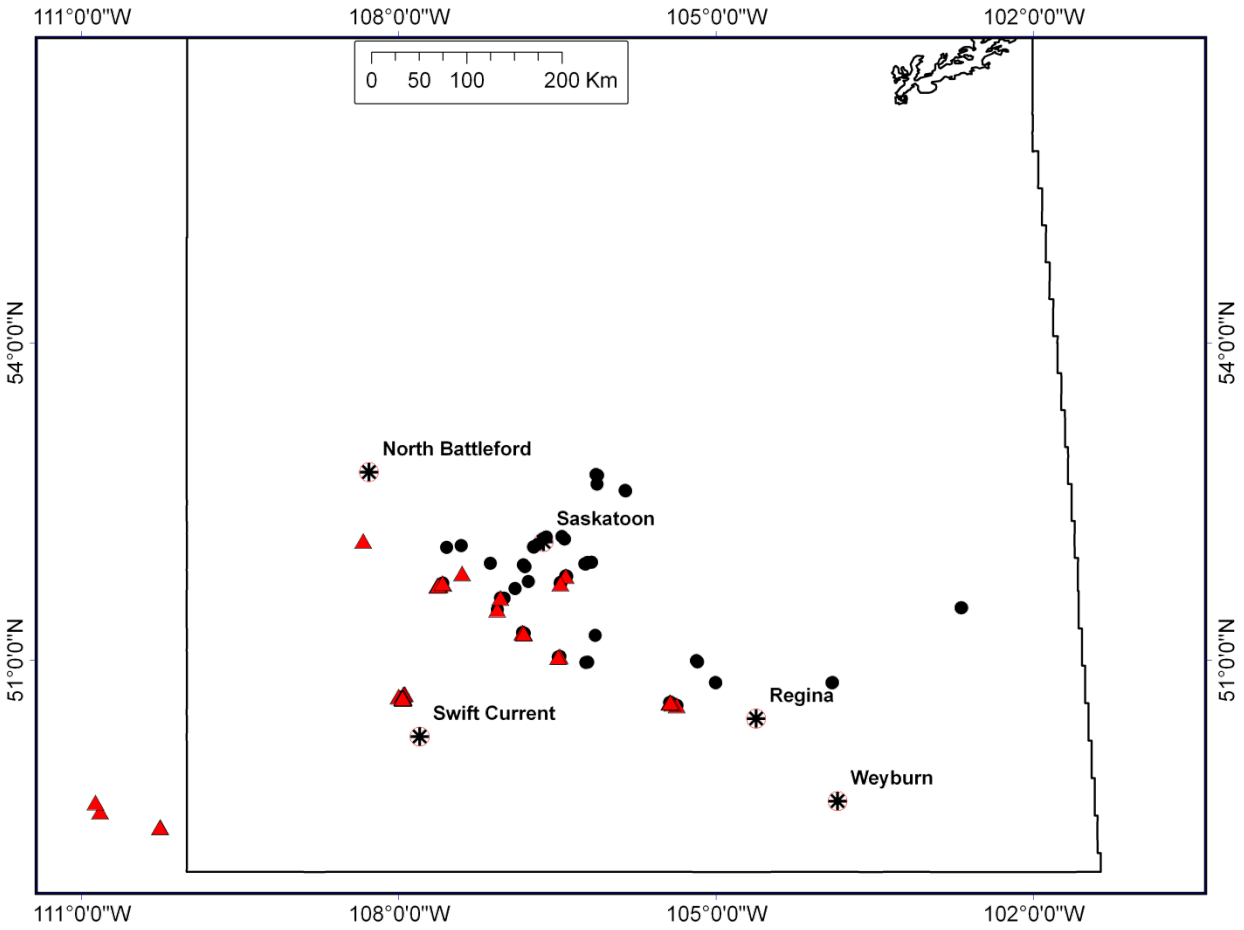

Fig. 3.1. Localities where *D. andersoni* (red triangles) and *D. variabilis* (black dots) adults were located, as determined by flagging.

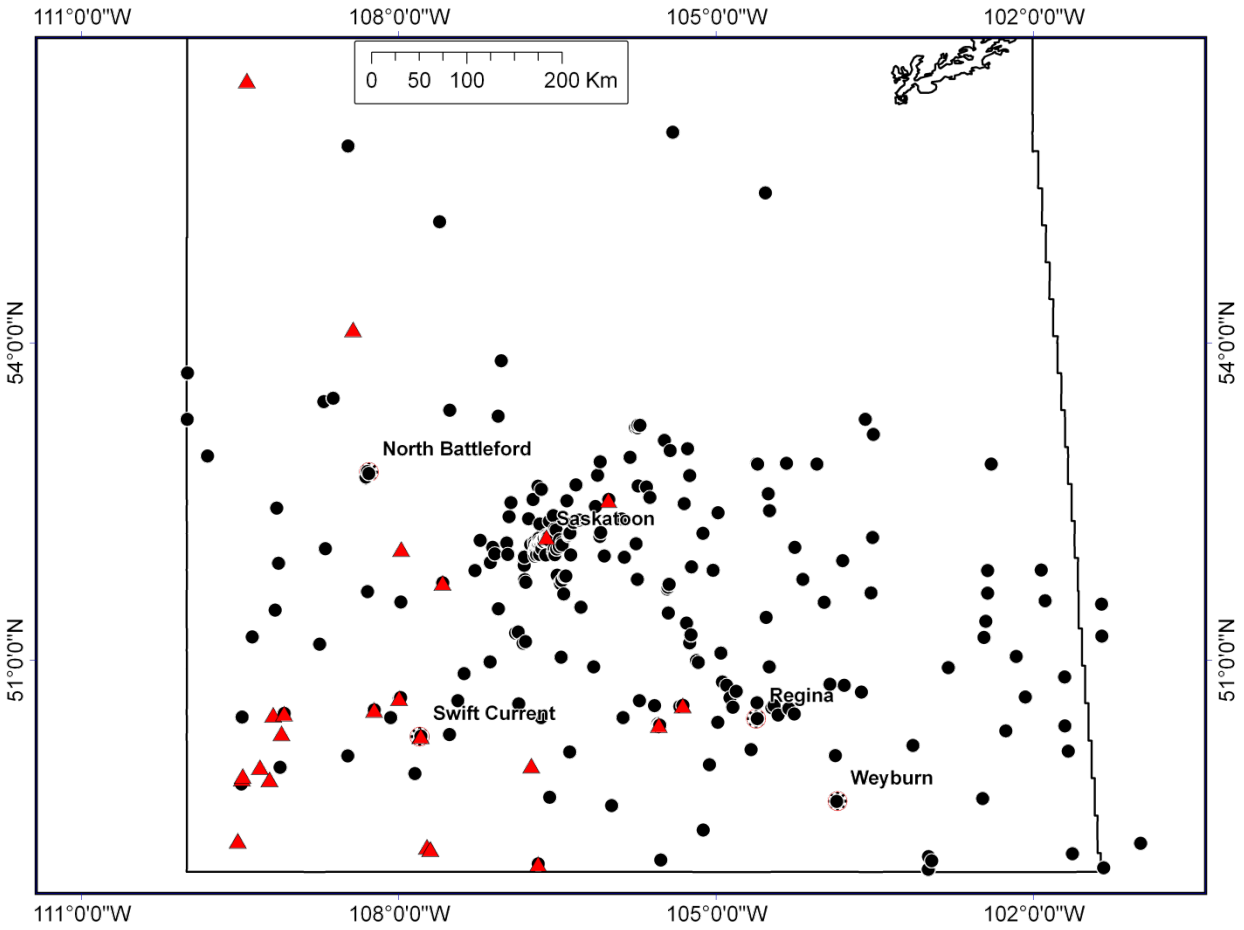

Fig. 3.2. Localities where *D. andersoni* (red triangles) and *D. variabilis* (black dots) adults were located, as determined through passive surveillance.

3.4.2. Host usage and seasonal activity of larval and nymphal ticks

Table 3.1 shows the number of immature ticks collected from small mammals at two sites, Saskatchewan Landing Provincial Park and Blackstrap Provincial Park. *D. andersoni* nymphs were collected from June 18 to July 10 (2008) and April 21 to April 31 (2009) at Saskatchewan Landing Provincial Park. *D. variabilis* larvae were also collected from May 27 to July 10 (2008) and April 21 to May 1 (2009) at Saskatchewan Landing Provincial Park and from May 29 to July 15 (2009) at Blackstrap Provincial Park. *D. variabilis* nymphs were active when sampling on June 17 and July 8 (2008) at Saskatchewan Landing Provincial Park and on May 29, July 16 and July 28 (2009) at Blackstrap Provincial Park.

Thirty four percent (27/79) of the small mammals trapped at Saskatchewan Landing Provincial Park were infested with ticks (Table 3.2). Nine *D. andersoni* nymphs were collected from four deer mice (*Peromyscus maniculatus*) and four meadow voles (*Microtus pennsylvanicus*). No *D. andersoni* larvae were found on any deer mice, shrews or voles at this locality. Seventy one *D. variabilis* larvae were collected off 10 deer mice, five meadow voles, and eight western jumping mice (*Zapus princeps*), and six nymphs were collected off one meadow vole and two western jumping mice trapped during June and July of 2008 and April of 2009 from Saskatchewan Landing Provincial Park (Table 3.1). A single shrew (*Sorex*. sp.) was also collected in 2008, but it was not infested with ticks.

Thirty percent (17/57) of the small mammals trapped at Blackstrap Provincial Park were parasitized by at least one tick (Table 3.2). One hundred and forty three *D. variabilis* larvae were collected off two deer mice, one western jumping mouse, five meadow voles and six southern red-backed voles (*Myodes gapperi*) at Blackstrap Provincial Park in 2009. In addition, seven *D. variabilis* nymphs were collected from four southern red-backed voles (Table 3.1). Eight shrews,

Species and life stage	Collection Locality	# ticks collected
D. andersoni Nymphs	Saskatchewan Landing Provincial Park	9
D. variabilis larvae	Saskatchewan Landing Provincial Park	71
D. variabilis nymphs	Saskatchewan Landing Provincial Park	6
D. variabilis larvae	Blackstrap Provincial Park	143
D. variabilis nymphs	Blackstrap Provincial Park	7

Table 3.1. Number of *D. andersoni* and *D. variabilis* larvae and nymphs off small mammals collected at Saskatchewan Landing Provincial Park and Blackstrap Provincial Park.

Collection locality	# Animals infested with ticks[2]							
	M. p.	*M. g.*	*P. m.*	*Z. p.*	*S. h.*	*S. c.*	*S. sp.*	*S. t.*
Saskatchewan Landing P. P.	6/15	N/C[3]	13/48	8/15	N/C	N/C	0/1	N/C
Blackstrap P. P.	5/12	9/17	2/13	1/4	0/4	0/1	0/3	0/3

Table 3.2. Small mammal species collected at Saskatchewan Landing Provincial Park and Blackstrap Provincial Park and proportion infested with at least one tick.

[2] *M. p. = Microtus pennsylvanicus* (meadow vole)
M. g. = Myodes gapperi (southern red-backed vole)
P. m. = Peromyscus maniculatus (deer mouse)
Z. p. = Zapus princeps (western jumping mouse)
S. h. = Sorex haydeni (prairie shrew)
S. c. = Sorex cinereus (masked shrew)
S. sp. = Sorex sp. (shrew – species undetermined)
S. t. = Spermophilus tridecemlineatus (13-lined ground squirrel)

[3] Not collected

representing at least three different species of *Sorex*, and three 13-lined ground squirrels (*Spermophilus tridecemlineatus*) were also collected, but none of these animals were parasitized by ticks.

3.5. Discussion

The distribution of tick-borne pathogens is largely dependent on the distribution of their vectors. Therefore, to understand the potential risk of humans and/or domestic animals acquiring a tick-borne pathogen, and to implement preventative measures to avoid these pathogens, it is important to know the distributional ranges of the vector(s). The aim of this chapter was to determine the distributional ranges of *D. andersoni* and *D. variabilis* in Saskatchewan and the small mammal hosts used by immature ticks.

Active (i.e. flagging) and passive sampling for ticks between March and August in four consecutive years showed that *D. andersoni* and *D. variabilis* were the most common species of tick collected in Saskatchewan. These results were consistent with those of a passive surveillance study conducted from 1998 to 2000, in which *D. variabilis* was, by far, the most often encountered species in Saskatchewan. Other species, such as *Ixodes scapularis*, *Haemaphysalis chordeilis*, *H. leporispalustris* and *Dermacentor albipictus* were rarely encountered in the present study. The distribution of *D. andersoni* and *D. variabilis* was quite patchy, particularly in central and southern parts of Saskatchewan because the environment has been modified for agricultural use. Thus, the more favourable habitat for ticks and their mammalian hosts (i.e. grasslands with shrubby vegetation (Wilkinson 1967)) was located sporadically throughout these regions of the province.

A comparison of the locality records for *D. andersoni* and *D. variabilis* acquired in this study to those previously published indicated that the distributional range of *D. andersoni* has changed very little, while the range of *D. variabilis* has expanded markedly. For, example, during the present study, *D. variabilis* consistently occurred at a relatively high abundance in and around the city of Saskatoon (52° 9' 50" N, 106° 36' 21"W), whereas approximately 40 years ago, there were no reliable records of this species north of 52^{o} longitude (Wilkinson 1967). In contrast, *D. andersoni* does not appear to occur at a noticeably higher latitude than previously reported (Wilkinson 1967). *D. andersoni* occurs in the western half of Saskatchewan, while *D. variabilis* is commonly found in the eastern half. Previous records indicated that the division between the ranges of these two species occurred around the middle of the province (~105^{o} longitude) and they did not overlap, being separated by at least 80 kilometers (Gregson 1956). Although eastward range expansion of *D. andersoni* appears to be limited, established populations of *D. variabilis* were identified at localities (107.4^{o} longitude) approximately 300 km further west than previously considered the limits of its distribution (Wilkinson 1967). This migration of ticks has created an area of overlap of their distributions (~200 km wide) in which they occur in sympatry at a number of localities between Buffalo Pound provincial Park and Saskatchewan Landing Provincial Park. The range expansion of *D. variabilis* may be due to transportation of adult ticks on pets or livestock as they travel to and from recreational areas and/or possibly due to gradual migration of ticks as they are transported on wild animals that use the relatively undisturbed natural corridor between Buffalo Pound Provincial Park and Douglas Provincial Park, and along the South Saskatchewan River, particularly around Diefenbaker Lake. These corridors connect many of the localities where both *D. andersoni* and *D. variabilis* occur together.

The geographic ranges of *D. variabilis* and *D. andersoni* were often considered to be due, in part to their requirements for different environmental conditions (Wilkinson 1967, McEnroe & Specht 1984, Yoder *et al.* 2007). However, both tick species have been detected together at sites that differed greatly in their plant composition, moisture levels and, possibly in the species of mammals (i.e. hosts) present. For example, *D. andersoni* and *D. variabilis* were collected at Buffalo Pound Provincial Park where there were trees, shrubs and dense grasses, and a relatively high humidity, while at Douglas Provincial Park, both tick species were collected along hiking trails in a sandy, dry region with sparse vegetation and relatively little grass. Thus, the range of conditions that limits the ability of these ticks to survive may have to be re-evaluated.

The timing of host-seeking activity by the different life stages (i.e. unfed larvae, nymphs and adults) of *D. andersoni* and *D. variabilis* is one important factor that influences the dynamics of pathogen transmission. In the present study, *D. andersoni* nymphs and *D. variabilis* larvae were both active (i.e. feeding) on small mammals immediately following snow melt in April and May (2009 and 2008, respectively) at Saskatchewan Landing Provincial Park. In addition, *D. andersoni* nymphs and *D. variabilis* larvae were also active on hosts in June and July, but some of these larvae may have been from a second generational cohort that recently emerged from eggs laid by females in the same summer. Activity of *D. variabilis* nymphs was first detected in late May, with most collected off small mammal hosts in June and July. Meanwhile, questing adults are commonly found as soon as the air temperature reaches approximately 5 to 8^{o}C after the snow melts in March or April until late June for *D. andersoni* and late July for *D. variabilis*. Therefore, the period of time that ticks in each of these life stages are actively seeking hosts overlaps, even though the peak host-seeking activity for each stage can differ.

Knowledge of the species of small mammals used as hosts by *D. andersoni* and *D. variabilis* is vital to determine the potential reservoir hosts of pathogenic bacteria and for understanding the potential transmission cycles of tick-borne microorganisms. There was, however, very limited information as to the types of hosts used by immatures of *D. andersoni* and *D. variabilis* in Saskatchewan. In other parts of their distributional range, these tick species use a variety of small mammals, such as deer mice, meadow voles, southern red-backed voles, and different species of squirrels (Burachynsky & Galloway 1985, Kollars *et al.* 2000). In the present study, small mammals were trapped at Saskatchewan Landing Provincial Park. This locality was specifically chosen because adults of both species were present in relative high abundance, suggesting both had established (i.e. reproducing populations). Given this, it provided the opportunity to compare the host usage and to obtain insight into seasonal activity of the two tick species at the same site. In addition, small mammals were trapped at Blackstrap Provincial Park because *D. variabilis* occurred at a high frequency, and it was one of the localities where this tick species was infected with *Rickettsia montanensis* (see Chapter 4).

The results of the study at Saskatchewan Landing Provincial Park revealed that *D. andersoni* nymphs were only collected on deer mice and meadow voles. No *D. andersoni* larvae were collected from any species of small mammal. This host usage by the *D. andersoni* nymphs was consistent with previous reports of this tick species in Canada (Gregson 1956). It has also been reported that *D. andersoni* immatures use western jumping mice, chipmunks (*Eutamias* sp.) and western bushy-tailed woodrats (*Neotoma cinerea occidentalis*) as hosts in other parts of its range (Gregson 1956). Although chipmunks and woodrats do not occur in Saskatchewan Landing Provincial Park, several western jumping mice were collected, but none were infected with *D. andersoni* immatures. Future studies should therefore be conducted based on a larger

sample size to determine the relative importance of other small mammal species present at the site, including western jumping mice, in maintaining *D. andersoni* populations.

In contrast to *D. andersoni*, western jumping mice at Saskatchewan Landing Provincial Park were found to be equally important as deer mice and meadow voles, as hosts for *D. variabilis* larvae and nymphs. This pattern of host usage was markedly different to that by *D. variabilis* immatures at Blackstrap Provincial Park, where western jumping mice were rarely infested by *D. variabilis* immatures. At this second site, meadow voles were most often used as a host for *D. variabilis* larvae. The southern red-backed vole (*Myodes gapperi*), which was not present at Saskatchewan Landing Provincial Park, was also an important host to *D. variabilis* larvae and nymphs. Shrews and 13-lined ground squirrels were not infested with *D. variabilis*. These results can be compared to those reported in the study by Burachynsky and Galloway (1985), which was conducted in an area of aspen parkland in Manitoba (i.e. Birds Hill Park) that was comprised of a similar environment to that of Blackstrap Provincial Park. In that study, 11 species of small mammal were trapped to determine the seasonal activity patterns of *D. variabilis* immatures. Nymphs were collected from six species; *Myodes gapperi*, *Peromyscus maniculatus*, *Microtus pennsylvanicus*, *Tamias striatus*, *Zapus hudsonius* and *Spermophilus tridecemlineatus*. However, most nymphs were collected from *M. gapperi*, *P. maniculatus* and *M. pennsylvanicus*. Burachynsky and Galloway (1985) collected *D. variabilis* larvae from seven of the 11 species of small mammal; *M. gapperi*, *M. pennsylvanicus*, *P. maniculatus*, *Z. hudsonius* and *Spermophilis franklinii.* Most larvae were collected from *M. gapperi* and *M. pennsylvanicus*. No *D. variabilis* immatures were collected from *Lepus americanus*, *Mus musculus*, *Sorex cinereus* or *Tamiascirus hudsonicus* (Burachynsky and Galloway, 1985). Thus, the species of mammals used as hosts by *D. variabilis* immatures most often were the same for Blackstrap

Provincial Park (SK) and Bird's Hill Park (MB). However, a greater diversity of mammals, including S. *tridecemlineatus,* was identified as hosts to larvae and nymphs at Bird's Hill Park.

The availability of specific species of small mammal hosts is unlikely to play a significant role in limiting the distribution of either *D. andersoni* or *D. variabilis*. Both tick species have a relatively broad host range (Bishopp & Trembley 1945, Gregson 1956, Kollars *et al.* 2000), and many species of mammals used as hosts have distributional ranges that exceed those of each tick species. Environmental factors, such as temperature may be more important in limiting the northward expansion of these ticks. The expansion of the distributional ranges of *D. andersoni* and *D. variabilis* in an eastward and westward direction is likely limited by the rate of dispersal by their vertebrate hosts, rather than environmental conditions. However, the role that environmental conditions other than temperature play in limiting the distribution of these ticks should be examined further.

The findings of the present study have shown that the geographic distributions of *D. andersoni* and *D. variabilis* have changed significantly over the last few decades, with the allopatric ranges of these two species in Saskatchewan expanding to a broad zone of sympatry in the central part of the province. This range expansion may increase the chance that pathogenic bacteria encounter potential vectors in localities that were once free from *D. andersoni* or *D. variabilis*. Similarities in the range of host species used by the different life stages of *D. andersoni* and *D. variabilis* may also have implications for the maintenance of their life cycles and for the transmission of tick-borne microorganisms, particularly in areas where the two tick species coexist. In addition, certain vertebrate species may be more suitable hosts to ticks than others (Kollars 1996, Kollars *et al.* 2000), allowing for successful completion of feeding and mating, due to differences in their physiology and grooming behaviour. Although deer mice,

meadow voles and southern red-backed voles are competent hosts for some tick-borne pathogens (Oliver *et al.* 2006, Childs & Paddock 2007, Wobeser *et al.* 2009, Walls *et al.* 1997), it is unknown if jumping mice are also suitable hosts. Thus, there may be differences in the potential for transmission of tick-borne microorganisms, based on the likelihood of different host species being infested by ticks.

Given the relatively broad zone of sympatry between *D. andersoni* and *D. variabilis*, together with similarities between these two tick species in host usage by immatures, and periods of host-seeking activity, may lead to cross-species transmission of microorganisms from one tick species to the other. This can occur through two different modes of horizontal transmission characterized by temporal differences in feeding. Microorganisms can be spread directly from one tick to another as they both feed close in space and time on a non-bacteremic host (i.e. co-feeding transmission) (Randolph *et al.* 1996), or indirectly by the transmission of the microbe to a host by an infected tick, followed by the feeding of another tick on the bacteremic host at some later point in time (Mather & Ginsberg 1994). The following five chapters of this thesis describe comparative studies that were conducted to determine if the two tick species have different bacterial communities, and if so, is there evidence of cross-transmission of bacteria from one tick species to the other.

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Chapter 4. Experimental approach to the identification of bacteria in ticks

4.1. Abstract

This chapter describes the experimental approach that was used to analyze the types and prevalence of bacteria present in *D. andersoni* and *D. variabilis* individuals. Initially, the genomic DNA from ticks was tested with a PCR designed to amplify a part of the bacterial16S rDNA that would allow the differentiation and identification of different sequences within individual ticks. The results of single strand conformation polymorphism and DNA sequence analyses indicated the presence a variety of bacteria from at least three different classes. Based on the results of these preliminary studies, a suitable approach for assessing the diversity of tick-borne bacteria was determined.

4.2. Introduction

A diverse range of pathogenic and nonpathogenic microorganisms (e.g. protozoa, viruses, and bacteria) are known to occur in many species of hard and soft ticks (Noda *et al.* 1997, Benson *et al.* 2004, Jongejan & Uilenberg 2004, Dennis & Piesman 2005). Traditionally, the detection and identification of these microbes relied on analyses of their physical, biochemical, and/or pathogenic properties and techniques have included bacterial culture, staining and microscopy, biochemical assays, and infection studies (Noguchi 1926, Humphreys & Campbell 1947, Bell *et al.* 1963, Busse *et al.* 1996). The development of serological techniques has also facilitated the species-level identification and typing of bacterial agents in ticks or animals (Philip *et al.* 1978, Philip *et al.* 1981). In addition, assays have been developed to detect serum antibodies in vertebrates, providing methods to assess the exposure of animals and humans to

tick-borne pathogens (Leighton *et al.* 2001, Yabsley *et al.* 2005, Apperson *et al.* 2008, Castellaw *et al.* 2010). Although many of these techniques were, and continue to be, important for studies of microorganisms, some of them have significant limitations in their usefulness for detecting and accurately identifying microbes because they can be time consuming or lack sensitivity and/or specificity (Busse *et al.* 1996). Molecular techniques have been shown to overcome these limitations by providing more rapid and reliable methods to study microorganisms (Engvall *et al.* 1996, Sjostedt *et al.* 1997, Harrus & Waner 2010).

DNA-based studies have led to the development of genetic markers that have been applied in assays used for diagnostic purposes (Wolfel *et al.* , Figueroa & Buening 1995, Sparagano *et al.* 1999, Ludwig 2007), for clarifying the host range and transmission cycle of tick-borne microorganisms (Chae *et al.* 2003, Castellaw *et al.* 2010, Robinson *et al.* 2010), and for elucidating the composition of tick microbiomes (Moreno *et al.* 2006, Clay *et al.* 2008, Andreotti *et al.* 2011). The use of molecular techniques has also facilitated the identification of many previously unrecognized species of bacteria, such as those that cannot be cultured or that are difficult to culture (Telford & Goethert 2004). This approach has led to the detection of many new species of *Rickettsia*, some of which are now associated with infection and disease in humans (Telford & Goethert 2008, Parola *et al.* 2009, Renvoisé *et al.* 2009).

The development of reliable molecular assays has also provided genetic markers to determine the phylogenetic (evolutionary) relationships of microorganisms (Weller *et al.* 1998, Inokuma *et al.* 2001, Scoles 2004, Novakova *et al.* 2009), and to assess the taxonomic status of tick-borne bacteria (Oh *et al.* 2009). Comparative analyses, based on DNA sequence data, have resulted in a change in the taxonomy of a number of bacterial species (Dumler *et al.* 2001). Studies on the genetic relationships of microorganisms are also useful for predicting their

physiological characteristics, virulence factors and host associations by comparing them to closely related species that are more thoroughly characterized (Strauss & Falkow 1997).

A number of genetic markers have been developed for the detection of specific genera, species, or subtypes of bacteria in ticks (see Sparagano *et al*. 1999 for a comprehensive review). The 16S rRNA gene has been a particularly useful target for the identification of a broad range of bacterial species and for examining their phylogenetic relationships because this gene is present in all prokaryotes and it differs in sequence among species, but there is relatively little intraspecific variation in DNA sequence (Weisburg *et al.* 1991, Sparagano *et al.* 1999, Maiwald 2004, Ludwig 2007). As a consequence, the prokaryotic 16S rDNA gene has been characterized for a large number of species and for many different bacterial isolates. These data are available in public databases (i.e. the DDBJ, EMBL and GenBank). Thus, the DNA sequences of unknown bacteria derived from the amplification of tick gDNA can be compared to the DNA sequences of previously identified bacteria (Drancourt *et al.* 2000, Telford & Goethert 2004).

Two important aims of my PhD research were to assess the diversity of the bacteria present in *D. andersoni* and *D. variabilis*, and to compare the microbial community composition of ticks from localities where these two tick species occur in sympatry and where their distributional ranges are allopatric with respect to one another. Given that tick-borne pathogens often occur in low frequency and at varying prevalences (Mixson *et al.* 2006, Goethert *et al.* 2009, Lane *et al.* 2010), it was important to obtain preliminary data as to which bacteria may be present in *D. andersoni* and *D. variabilis* populations in Canada. Thus, rather than screening large numbers of ticks by different PCR assays specific for particular groups of bacteria, I adopted a broad-range approach. Several studies have used relatively conserved primers to amplify part of the 16S rRNA gene of a wide variety of bacteria in combination with mutation scanning techniques, such

as temperature gradient gel electrophoresis (TGGE), denaturing gradient gel electrophoresis (DDGE), or single strand conformation polymorphism (SSCP) analysis to determine the composition of complex microbial communities (Schabereiter-Gurtner *et al.* 2003, Ammerman *et al.* 2004, Halos *et al.* 2006). These methods are useful for the differentiation of amplicons with DNA sequences that differ by one or more nucleotides, based on the electrophoretic mobility of denatured DNA (Gasser 1997). Mutation scanning techniques have great potential for the identification and characterization of multiple bacterial species concurrently; however, these methods have mainly been used in combination with DNA cloning to examine relatively few samples at a time.

In this chapter, PCR-based SSCP analysis and DNA sequencing were used to obtain preliminary information as to which genera of bacteria may be present in *D. andersoni* and *D. variabilis*. It has been used extensively to determine the genetic variation within and among tick populations (Hiss *et al.* 1994, Norris *et al.* 1996, Ketchum *et al.* 2009), however, it has rarely been used to simultaneously screen large numbers of ticks for species and subtypes of bacteria. Identification of the bacteria present in ticks by PCR-SSCP and DNA sequencing, as described in this chapter, provided the basis for subsequent larger-scale studies on the prevalence of specific bacteria in *D. andersoni* and *D. variabilis* from populations situated at the northern edge of their distributional range (Chapters 5-8).

4.3. Methods

The 20 *D. andersoni* and 23 *D. variabilis* adults used in the experiments were collected by flagging at Saskatchewan Landing Provincial Park and Blackstrap Provincial Park (Chapter 3). Total genomic DNA (gDNA) was purified from individual ticks using the methodology

described in Chapter 2. Two PCR experiments were conducted using 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 ~250 bp of the bacterial 16S rRNA gene. This target region was selected because it would allow for the detection of most bacterial species, while amplifying sequences of an appropriate size for SSCP analyses and still contain enough genetic variation for identification of different bacterial species present within individual ticks.

The first experiment was carried out to compare the relative effectiveness of three different DNA polymerases in the PCR amplification of bacteria from the gDNA of one *D. andersoni* and three *D. variabilis* adults. The enzymes tested were recombinant *Taq* DNA polymerases from Promega (Madison, WI, USA), Bio-Rad (iTaq; Hercules, CA, USA), and Fermentas (Burlington, ON, Canada). These particular polymerases were chosen because previous experiments have proven them to be useful for the amplification of a broad range of DNA targets in a variety of sample, but these enzymes can vary in their efficiency and specificity. For each enzyme, the PCR reactions were carried out using the dNTPs and PCR buffer supplied by the same manufacturer as the DNA polymerase. All PCR reactions were carried out in 25µl volumes containing 200 µM of each dNTP, 3 mM $MgCl_2$, 25 pmol (1 µM) of each primer, 0.5U of DNA polymerase, 2.5µl of 10x PCR buffer, and 2 µl of template gDNA. A negative control (i.e. without gDNA) was included in each set of reactions. PCRs were performed in a thermocycler (iCycler™; Bio-Rad) using the following conditions: 95°C for 5 min, followed by 30 cycles of 95°C for 30 s (denaturation), 58°C for 30 s (annealing), and 72°C (Fermentas and Bio-Rad DNA polymerases) or 74°C (Promega DNA polymerase) for 30 s (extension), and a final extension at 72°C or 74°C for 5 min. Amplicons were subjected to electrophoresis on SYBR® Safe-stained 2% 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. A 100 bp Ready-load ladder (Fermentas) was used on all agarose gels as a size standard.

In the second experiment, the gDNA of all 20 *D. andersoni* and 23 *D. variabilis* adults were subjected to PCR using primers 554f and 802r, the Fermentas recombinant *Taq* DNA polymerase, and the corresponding conditions used in the first experiment. Some of the positive amplicons produced from the adult ticks were column-purified (see Chapter 2) and subjected to DNA sequencing to verify the specificity of the PCR assay.

The PCR products of the second experiment were subjected to SSCP analyses using the protocol described in Chapter 2. SSCP gels were subjected to electrophoresis for 18 hours and the banding patterns of amplicons were examined using transillumination. A total of 19 bands with different mobility patterns from several amplicons were excised from the SSCP gels using Bandpick^{TM} (Elchrom Scientific, Cham, Switzerland), and then reamplified with primers 554f and 802r using the conditions described above. The amplicons obtained from the reamplification process were column-purified (see Chapter 2) and then sequenced using the forward primer (554f). A BLAST search (GenBank) was performed on the 16S rDNA sequence of each SSCP band (89-196 bp), excluding primer sites, to determine the possible identity (at the genus level) of the bacteria in the ticks.

4.4. Results

The results of the first experiment (i.e. comparison of the three different *Taq* DNA polymerases) revealed that a single amplicon of ~250 bp was produced from each of the four ticks with all three polymerases. However, the quantity of DNA produced was greater in the reactions with the reagents from Fermentas. No amplicon was detected for the negative control

(Fig. 4.1). As a consequence of these results, the Fermentas recombinant *Taq* DNA polymerase was used in the next experiment.

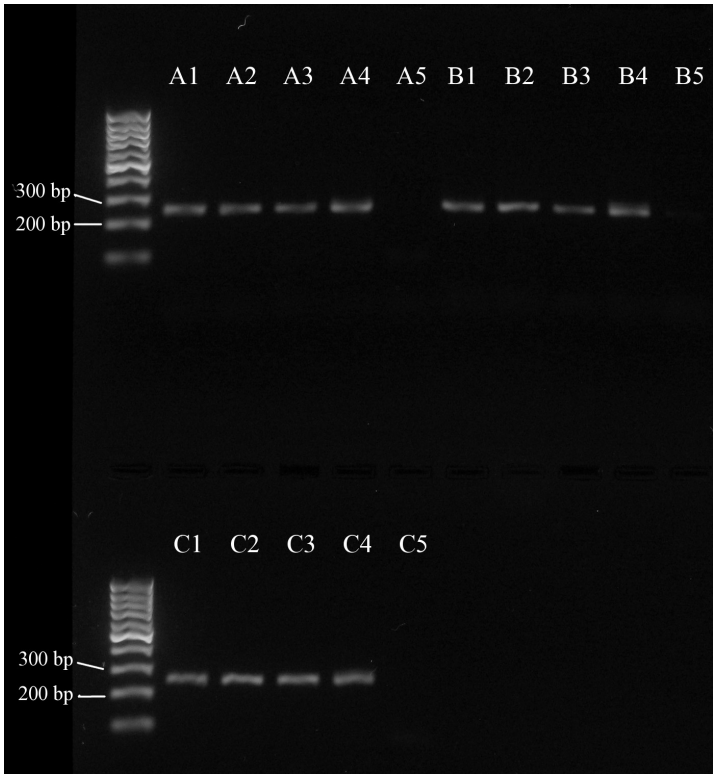

Figure 4.1. Agarose gel depicting the results of the PCR testing the relative effectiveness of three different *Taq* DNA polymerases. Samples no. 1-4 represent gDNA from individual ticks and no. 5 is the negative control (i.e. no gDNA template). Set A was performed with the DNA polymerases from Promega, B from Bio-Rad, and C from Fermentas.

In the second experiment, a single band of the expected size (~250 bp) was detected on agarose gels for amplicons derived from the gDNA of 19 (95%) *D. andersoni* (Fig. 4.2) and 18 (78%) *D. variabilis* adults (Fig. 4.3). No amplicons were detected in the negative (i.e. no gDNA) controls. Although several positive amplicons were sent for automated DNA sequencing, no readable sequence was obtained from any amplicon because each sample likely contained a mixture of bacterial types.

The number of SSCP bands comprising the banding pattern (i.e. SSCP profile) of the 37 amplicons varied from approximately five to eleven (Fig. 4.4). The 19 SSCP bands that were excised from the SSCP gel and subjected to DNA sequencing are indicated in Fig. 4.4. These 19 samples comprised eight SSCP bands derived from the gDNA of four *D. andersoni* adults and 11 bands from the gDNA of five *D. variabilis* adults. The quality of the DNA sequences (89-196 bp) obtained varied in terms of their signal strength and signal/noise ratio. However, BLAST comparisons of the sequences of 19 different SSCP bands revealed that they were genetically most similar to the 16S rDNA sequences of a variety of alpha-, beta-, and gamma-proteobacteria. The 16S rDNA sequences derived from the gDNA of *D. andersoni* adults were most similar to those of different species of four genera: *Pseudomonas*, *Arsenophonus*, *Francisella*, and *Rickettsia*. Some of the 16S rDNA sequences from the gDNA of *D. variabilis* adults were identical to those of "*Francisella*-like endosymbionts" (FLEs), while other sequences were most closely matched to those of an uncultured beta-proteobacterium or of *Rickettsia montanensis*. A comparison of the DNA sequence data with the SSCP profiles for the amplicons produced from the gDNA of *D. variabilis* revealed that at least 12 of the 18 PCR-positive ticks contained FLEs and four ticks contained bacteria most similar to *R. montanensis*.

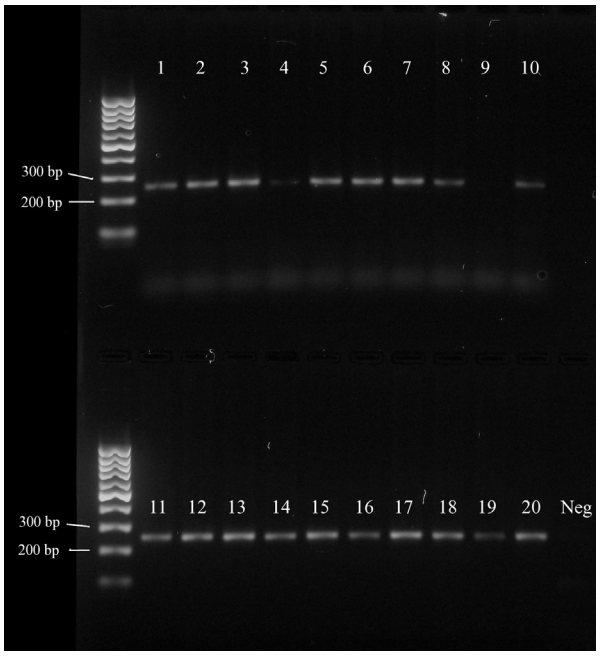

Figure 4.2. Agarose gel depicting the results of the PCR analyses targeting a 250 bp portion of the bacterial 16S rRNA gene, using primers 554f and 802r, with gDNA from 20 *D. andersoni* adults.

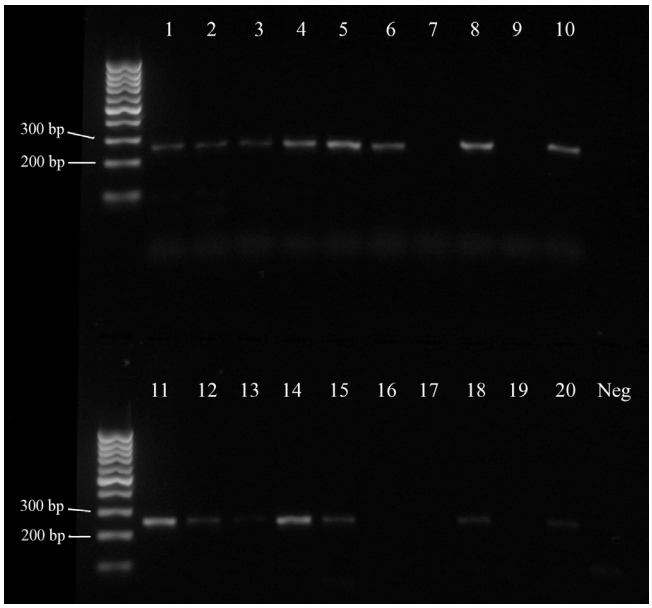

Figure 4.3. Agarose gel depicting the results of the PCR analyses targeting a 250 bp portion of the bacterial 16S rRNA gene, using primers 554f and 802r, with gDNA from 20 *D. variabilis* adults.

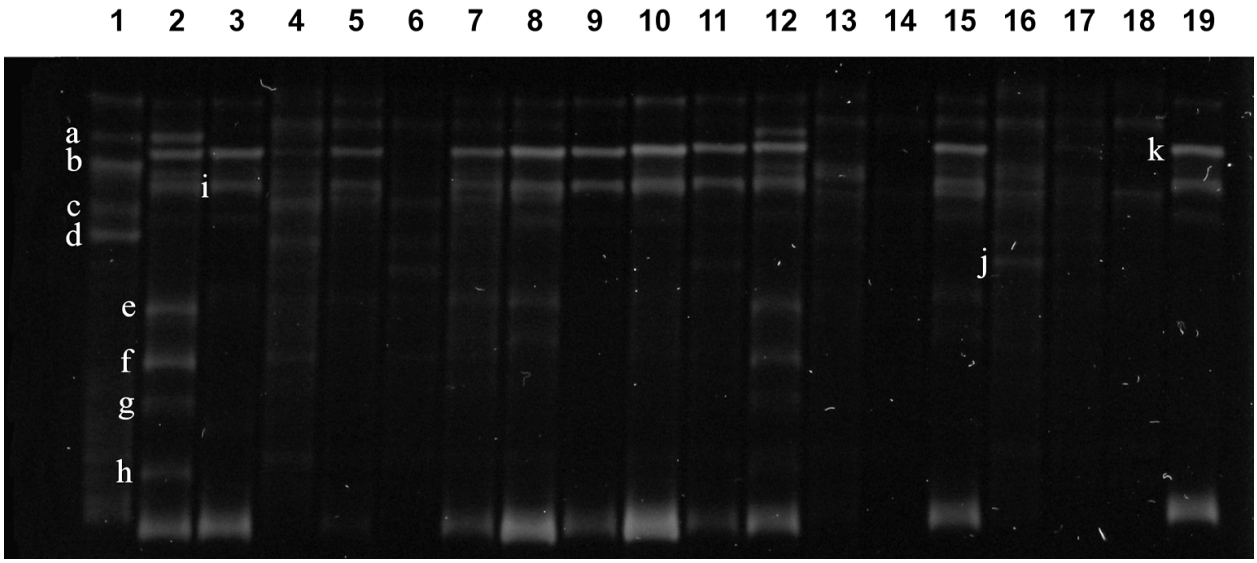

Figure 4.4. SSCP analysis of 16S rDNA amplicons from total gDNA of individual *D. andersoni* (lane 1) and *D. variabilis* adults (lanes 2-19). SSCP bands that were sequenced to determine the identity of the bacteria present are indicated by the letters a to k[4].

[4] Closest matches to sequences of SSCP bands (with corresponding GenBank accession numbers):

a – *Burkholderia* sp. (Accession no. AY839565) and *Pseudomonas aurantiaca* (Accession no. AY839234)

b – *Pseudomonas* sp. (Accession no. DQ472155)

c – *Pseudomonas* sp. (Accession no. AJ936936)

d – *Arsenophonus* sp. (Accession no. AY264674)

e – *Rickettsia montanensis* (Accession no. U11016)

f – *Francisella*-like endosymbiont of *D. variabilis* (Accession no. AY805307)

g – *R. montanensis* (Accession no. U11016)

h – *R. montanensis* (Accession no. U11016)

i – *Francisella*-like endosymbiont of *D. variabilis* (Accession no. AY805307)

j – Uncultured beta-proteobacterium (Accession no. AM182319)

k – *Francisella*-like endosymbiont of *D. variabilis* (Accession no. AY805307)

4.5. Discussion

The objective of this chapter used was to develop a "broad-range" assay to identify tick-borne bacteria using a PCR targeting part of the bacterial 16S rDNA, followed by SSCP and DNA sequencing analyses. Using this approach, I intended to describe the bacterial species present in individual ticks, without assumption of the types that may be present. This would facilitate the discovery of previously unrecognized associations between ticks and different bacteria, including previously unidentified species.

Although the amplicons produced a single band on agarose gels, multiple bands (~5-11) were detected for the same amplicons when subjected to SSCP analyses. These complex banding patterns on the SSCP gels were due to an abundance of different 16S rDNA sequences that were amplified by relatively conserved primers. Therefore, this broad-range PCR-SSCP approach does not provide a simple means to determine all of the bacterial species within many individual ticks. For example, in these preliminary studies, SSCP bands with nearly identical mobility were produced by amplicons from different bacterial species (data not shown). In addition, previous studies have also shown that there is the potential for amplification of eukaryotic DNA when using primers targeting the bacterial 16S rRNA gene (Huys *et al.* 2008). Due to this potential cross-reactivity between prokaryotic and eukaryotic rDNA and the potential difficulty in comparing and interpreting complex SSCP profiles to determine species composition of individual ticks, I chose to use a more targeted approach and developed PCR assays to detect specific genera of bacteria. The choice of which genera to examine was based on my preliminary results of the broad-range PCR used in this chapter, and on the potential veterinary and medical importance of certain groups of microorganisms. The following chapters describe the analyses of *Rickettsia* spp. (Chapter 5), *Francisella* spp. (Chapter 6), *Arsenophonus* spp. (Chapter 7), and

Anaplasma spp. (Chapter 8) in immature and adult *D. andersoni* and *D. variabilis* collected from multiple localities in western Canada.

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Chapter 5. Prevalence of *Rickettsia* in Canadian populations of *D. andersoni* & *D. variabilis*[5]

5.1. Abstract

In this chapter, the prevalence of rickettsiae in 15 Canadian populations of *D. andersoni* and *D. variabilis* was determined using PCR-based techniques targeting the rickettsial citrate synthase gene. The species identity of the rickettsiae was confirmed using sequence data of the 190 kDa (OmpA) surface protein gene. The results showed that *R. peacockii* was present in 76% of *D. andersoni* adults, while *R. montanensis* occurred at a much lower frequency (8%) in *D. variabilis* adults. This host specificity was maintained in localities where both tick species occurred in sympatry. *R. rickettsii* was not detected in any of the 1,326 adults tested. Two hundred and thirty six immature ticks from Saskatchewan Landing Provincial Park and Blackstrap Provincial Park were also examined for the presence of *Rickettsia*. *R. peacockii* was detected in all *D. andersoni* nymphs, but rickettsial DNA was not detected in *D. variabilis* larvae or nymphs. The findings of this study provide a better understanding of the prevalence of *Rickettsia* in Canadian populations of *D. andersoni* and *D. variabilis*.

5.2. Introduction

Dermacentor andersoni and *D. variabilis* are important vectors and reservoir hosts of *Rickettsia rickettsii* (Burgdorfer 1975), the etiological agent of Rocky Mountain spotted fever (RMSF) in humans. *R. rickettsii* occurs throughout the USA, but *D. andersoni* is the primary vector in the Rocky Mountain states, while *D. variabilis* is one of the vectors in the eastern USA (Treadwell *et al.* 2000, Chapman *et al.* 2006). Several species of non-pathogenic *Rickettsia* (e.g.

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R. bellii, *R. montanensis*, *R. peacockii*, and *R. rhipicephali*) have also been recorded in *D. andersoni* and/or *D. variabilis* in the USA (Bell *et al.* 1963, Feng *et al.* 1980, Gage *et al.* 1994, Roux & Raoult 1995).

RMSF has been a notifiable disease in the USA since the 1920's. Of the 3,649 reported cases of RMSF in the USA between 1997 and 2002, approximately 1.4% resulted in human deaths (Chapman *et al.* 2006). The number of RMSF cases fluctuates annually, but the highest annual incidence was recorded in 2002 with 3.8 cases per million individuals (Chapman *et al.* 2006). In contrast, RMSF is not a reportable disease in Canada. As a consequence, little is known about the frequency of RMSF cases, or of the distribution and prevalence of different rickettsial species in Canada, even though two vectors (*D. andersoni* and *D. variabilis*) are relatively common (Gregson 1956, Wilkinson 1967). The few published reports of RMSF in Canada indicate that most cases have occurred within the western province of Alberta. For instance, 27 cases of RMSF were reported in Alberta between 1923 and 1943, 12 of which occurred around Manyberries in 1923 (Duncan 1937, Gibbons 1939, Bow & Brown 1945).

The prevalence of *R. rickettsii* in ticks and wild mammals has been examined in the western Canadian provinces of British Columbia, Alberta and Saskatchewan (Gibbons 1939, Humphreys & Campbell 1947). These studies, conducted between 1938 and 1946, were based on the analyses of infection experiments using guinea pigs as hosts. The results showed that less than 0.01% of *D. andersoni* from British Columbia and Alberta were infected with *R. rickettsii* (Gibbons 1939, Humphreys & Campbell 1947). In addition, a serological survey of domestic dogs from rural Alberta and Saskatchewan conducted between 1994 and 1995 showed that approximately 3% of animals were positive for antibodies to *R. rickettsii* (Leighton *et al.* 2001). There was also serological evidence of *R. rickettsii* infection in snowshoe hares and a groundhog

near Ottawa, Ontario (Newhouse *et al.* 1964); however, there appears to be no reported cases of RMSF in eastern Canada (Humphreys 1947, Gregson 1956).

The detection and identification of *Rickettsia* in ticks have greatly improved in accuracy and sensitivity since the advent of PCR-based techniques. Several genes (e.g. 16S rRNA gene, *gltA*, *omp*A, *omp*B , gene D, *atpA*, *recA*, *virB4*, *dnaA*, *dnaK* and the *rrl-rrf* internal transcribed spacer) have been used effectively as targets to distinguish among species of *Rickettsia* and/or to infer their phylogenetic relationships (Roux & Raoult 1995, Niebylski *et al.* 1997, Roux *et al.* 1997, Fournier *et al.* 1998, Sekeyova *et al.* 2001, Fournier *et al.* 2003, Ammerman *et al.* 2004, Vitorino *et al.* 2007, Wikswo *et al.* 2008). The rickettsial citrate synthase (*gltA*) and the 190-kDa surface protein (*ompA*) genes have been used to distinguish among species of *Rickettsia* and to determine the prevalence of different *Rickettsia* in *D. andersoni* and *D. variabilis* adults (Bernasconi *et al.* 2002, Ammerman *et al.* 2004, Wikswo *et al.* 2008). Most studies that have determined the prevalence of *Rickettsia* in *D. andersoni* or *D. variabilis* within the USA are based on an examination of ticks from allopatric populations (Philip & Casper 1981, Anderson *et al.* 1986, Gage *et al.* 1994, Niebylski *et al.* 1997, Ammerman *et al.* 2004). Serological studies of the prevalence of *Rickettsia* in the USA have also been based on an examination of ticks from allopatric populations (Philip & Casper 1981, Anderson *et al.* 1986). Comparisons of the prevalence of rickettsiae in sympatric and allopatric populations of *D. andersoni* and *D. variabilis* would provide insight into the host specificity and transmission of *Rickettsia* species. There is no detailed information of the distribution and prevalence of rickettsial species in Canada, even though *D. andersoni* and *D. variabilis* are relatively common (Wilkinson 1967). The geographic ranges of these tick species in Canada are largely allopatric, except for a zone of sympatry in central Saskatchewan (Wilkinson 1967). The aim of this chapter was to determine

the species of *Rickettsia* present and their relative prevalence in adult ticks from allopatric and sympatric populations of *D. andersoni* and *D. variabilis* in Canada.

5.3. Methods

5.3.1. Collection of ticks

Adult ticks were collected by flagging grassy and shrubby vegetation at 15 different sites located across four provinces: Alberta (AB), Manitoba (MB), Saskatchewan (SK), and Ontario (ON) (Table 5.1). These collections were made in 2005 (May through July) and in 2007 (April through June). The geographic ranges of *D. andersoni* and *D. variabilis* in Canada are mostly allopatric, except for a zone of sympatry in central Saskatchewan (Wilkinson 1967). At two localities, only *D. andersoni* adults were collected, while at six sites only *D. variabilis* adults were collected (Table 5.1). Adults of both tick species were collected at the remaining seven sites. This sampling strategy provided the opportunity to compare the relative prevalence of *Rickettsia* in both allopatric and sympatric populations of *D. andersoni* and *D. variabilis*. All ticks were identified morphologically to the species level (Gregson 1956) prior to storage at -70^{o}C (i.e. until required for molecular analyses). In addition, immature ticks (nine *D. andersoni* nymphs, six *D. variabilis* nymphs and 71 *D. variabilis* larvae) were collected from 13 deer mice (*Peromyscus maniculatus*), seven meadow voles (*Microtus pennsylvanicus*) and eight western jumping mice (*Zapus princeps*) trapped in Saskatchewan Landing Provincial Park in June and July of 2008 and in April of 2009. Seven *D. variabilis* nymphs and 143 *D. variabilis* larvae were collected from one deer mouse, one western jumping mouse, five meadow voles and 10 southern red-backed voles (*Myodes gapperi*) from Blackstrap Provincial Park between May and July of 2009. Immature ticks were examined by microscopy to confirm they belonged to the Metastriata.

Their species identity was determined using the same PCR assay as for the adults, however amplicons were subjected to a RFLP analysis using *Alu*I (Fermentas), as per manufacturer's instructions. The RFLP analysis was used to confirm that the ITS-2 amplicons of *D. variabilis* individuals were not from *D. albipictus*, which has an ITS-2 amplicon of the same size. The ITS-2 sequence of *D. variabilis* lacks the restriction site for *Alu*I present in the ITS-2 sequence of *D. albipictus* (Chapter 2).

5.3.2. Isolation of genomic DNA and PCR of Rickettsia-specific genes from ticks

Total genomic DNA (gDNA) was extracted and purified from 1,326 adult ticks (508 *D. andersoni* and 818 *D. variabilis* (Table 5.1) and 236 immatures (Table 5.2) using a modification of the protocol of the DNeasy Tissue Kit^{TM} (Qiagen). Individual ticks were placed in 1.5ml micropestle tubes (Kontes), to which 180 µl of ATL buffer (Qiagen) was added. Ticks were homogenized by grinding with micropestles (Kontes) attached to a cordless drill. Proteinase K (20 µl @ 15 µg/µl) was added to the homogenate. Samples were incubated for 16 hours at 55^{o}C. The gDNA was purified according to the DNeasy tissue kit protocol, except that gDNA was eluted twice from the spin columns using 50 µl of AE buffer. The two elutions derived from the same tick were combined in a single tube and stored at -20^{o}C.

The presence of rickettsiae in adult and immature ticks was determined by amplification of a 381 bp fragment of *gltA* by PCR from the tick gDNA using the forward primer *Rp*CS877p (5'-GGG GAC CTG CTC ACG GCG G-3') and reverse primer *Rp*CS1258n (5'-ATT GCA AAA AGT ACA GTG AAC A-3') (Regnery *et al.* 1991, Eremeeva *et al.* 2003). This primer pair has been shown to amplify the *gltA* gene from all known species of *Rickettsia* (Regnery *et al.* 1991). PCR reactions were performed in 25 µl reaction volumes containing 200 µM of each dNTP, 2.5

mM $MgCl_2$, 50 pmol of each primer, 0.5U of iTaq DNA polymerase™ (Bio-Rad), PCR buffer, and 2 µl of template gDNA. Negative (i.e. no gDNA) and positive controls were included in each PCR run. The conditions used for PCR were: 95°C for 5 min, followed by 25 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, and a final extension at 74°C for 5 min. Individual amplicons were subjected to electrophoresis on SYBR® Safe (Molecular Probes) stained 2% agarose-TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) gels. A 100 bp TrackIt™ DNA ladder (Invitrogen) was used on gels as a size standard.

5.3.3. Screening for genetic variation and identification of rickettsiae in ticks

Genetic variation among *gltA* amplicons derived from *Rickettsia*-positive ticks was examined using single-strand conformation polymorphism (SSCP) analysis. In brief, 1-4 µl of each amplicon was mixed with 1-4 µl of DNase-free water and 5 µl of loading buffer (Gel Tracking Dye™, Promega), then denatured at 95˚C for 5 min prior to snap-cooling in ice water for 5 min. Individual samples (5 µl) were loaded into the wells of precast GMA™ S-50 gels (Elchrom Scientific) and subjected to electrophoresis for 18 h at 74 V and 7.4˚C (constant) in a horizontal SEA2000™ apparatus or an ORIGINS™ apparatus (Elchrom Scientific) connected to a temperature controlled circulating water bath. Following electrophoresis, gels were stained for 30 min with SYBR® Gold (Molecular Probes), rinsed in water and then photographed using a BioDoc-It™ (UVP) imaging system.

Multiple samples representing each SSCP profile were column-purified with the MinElute PCR purification kit™ (Qiagen) and subjected to automated DNA sequencing (Plant Biotechnology Institute, NRC, Saskatoon) using primers *Rp*CS877p and *Rp*CS1258n in separate reactions. The species identity of rickettsiae in these samples was determined by comparing their

gltA sequences to those of *Rickettsia* species deposited in GenBank using BLAST searches. A second genetic marker, a 532 bp fragment of *ompA* found in the spotted fever group (SFG) rickettsiae (Regnery *et al.* 1991), was utilized to provide further confirmation of the species identity of the rickettsiae present in *D. andersoni* and *D. variabilis* adults. In this case, the gDNA from a single individual of each tick species that contained rickettsiae was subjected to PCR using the forward primer *Rr*190.70p (5'-ATG GCG AAT ATT TCT CCA AAA-3') and the reverse primer *Rr*190.602n (5'-AGT GCA GCA TTC GCT CCC CCT-3') (Regnery *et al.* 1991). The same PCR conditions were used as for amplification of the *gltA* gene, except that 30 amplification cycles were used and 1.5mM $MgCl_2$ was used in the reaction mixture. Amplicons were column purified and sequenced using primers *Rr*190.70p and *Rr*190.602n in separate reactions. The nucleotide sequences of the *gltA* and *ompA* genes for representative samples in the present study have been deposited in the EMBL, GenBank™ and DDJB databases under the accession numbers FM883668 to FM883671.

The presence of rickettsiae in immature ticks was confirmed by screening all 236 individuals with a nested PCR designed to amplify a portion of the rickettsial 17kDa surface antigen gene. The first reaction amplified a 547 bp fragment using the forward primer 17k-5 (5'-GCT TTA CAA AAT TCT AAA AAC CAT ATA-3') and reverse primer 17K-3(5'-TGT CTA TCA ATT CAC AAC TTG CC-3') (Heise *et al.* 2010). PCR reactions were performed in 25 µl reaction volumes containing 200 µM of each dNTP, 2.5 mM $MgCl_2$, 25 pmol of each primer (1µM), 0.5U of iTaq DNA polymerase™ (Bio-Rad), PCR buffer, and 2 µl of template gDNA. Negative (i.e. no gDNA) and positive controls were included in each PCR run. The conditions used for PCR were: 95°C for 5 min, followed by 35 cycles of 95°C for 60 s, 58°C for 60 s, and 72°C for 60 s, and a final extension at 72°C for 5 min. PCR products were purified from dNTPs

and primers by mixing 10 µl of the reaction mixture with 0.15 µl (0.15 U) shrimp alkaline phosphatase (Fermentas), 0.15 µl (3 U) of exonuclease I (New England BioLabs) and 0.7 µl 10X Bio-Rad PCR buffer for 15 min. at 37^{o}C. The purification reaction was stopped by incubating the mixture at 80^{o}C for 15 min. The second PCR reaction amplified a 434 bp fragment using the primers 17kD1 (5'-GCT CTT GCA ACT TCT ATG TT-3') and 17kD2 (5'-CAT TGT TCG TCA GGT TGG CG-3') (Heise *et al.* 2010). Reaction conditions were the same as used for the external primers, except that 1 µl of purified PCR products were used as template and the amplification steps consisted of 30 cycles of 95^{o}C for 30 s, 61^{o}C for 30 s, and 72^{o}C for 30 s. To confirm the source of the bacterial DNA, All *gltA* amplicons were compared to those from adult ticks by SSCP. Amplicons from 4 *D. andersoni* nymphs and one *D. andersoni* adult were subjected to DNA sequencing using the primers 17k-5 and 17K-3.

5.3.4. Phylogenetic analyses

Sequences of the *ompA* gene for the two samples were compared to sequences of rickettsiae available in GenBank using BLAST searches. The gltA sequence of the *Rickettsia* from *D. variabilis* adults was not 100% identical to any sequence on GenBank; therefore, a phylogenetic analysis was conducted to determine the species identity of this *Rickettsia*. The *omp*A sequences of the rickettsiae from *D. variabilis* and *D. andersoni* were aligned with sequence data of *R. rickettsii* (accession numbers AY319293, DQ452933, DQ150693, DQ150687 and U43804), *R. peacockii* (AF129884, AH013412, AH013413, AY357765 and AY357766), *R. montanensis* (AY543681, AY543682, AY543683 and U43801) and *Rickettsia australis* (AF149108) (Roux *et al.* 1996, Ammerman *et al.* 2004, Baldridge *et al.* 2004, Wikswo *et al.* 2008). The phylogenetic analysis was carried out using the neighbor-joining (NJ) method

in PAUP v4.0b2 (Swofford 2003). *R. australis* was used to root the tree (Vitorino *et al.* 2007). The relative support for clades in the tree produced from the NJ analyses was determined using 1,000 bootstrap replicates.

5.4. Results

5.4.1. Determination of the prevalence of Rickettsia in adult ticks

A large proportion (76%) of the 508 adult *D. andersoni* gDNA samples tested by PCR were positive for (i.e. infected with) *Rickettsia*. The prevalence of rickettsial infection in *D. andersoni* adults varied among the different collection sites (36 to 96%), with the lowest prevalence recorded within Danielson Provincial Park (Table 5.1). Of the 161 adult ticks collected from two allopatric populations of *D. andersoni* (i.e. Lethbridge and Cypress Hills), 109 (68%) tested positive for *Rickettsia* by PCR. A greater proportion (80%) of *D. andersoni* were positive for *Rickettsia* by PCR at locations where this species was sympatric with *D. variabilis* (i.e. Saskatchewan Landing, Grasslands National Park, Buffalo Pound Provincial Park, Douglas Provincial Park, Danielson Provincial Park, Outlook, and Harris). The prevalence of *Rickettsia* in *D. andersoni* males (73%; n = 205) and females (79%; n = 303) was not statistically different ($\chi^2_1 = 2.32$, p = 0.305, $N = 508$) (data not shown). All nine *D. andersoni* nymphs collected from small mammals in Saskatchewan Landing Provincial Park were also PCR positive for *Rickettsia* (Table 5.2).

Locality	Coordinates (decimal degrees)		No. *D. andersoni* adults		No. *D. variabilis* adults	
	Lat. (N)	Long. (W)	No. Tested	No. Positive for *Rickettsia* (%)	No. Tested	No. Positive for *Rickettsia* (%)
Lethbridge, AB	49.73721	-112.84751	100	72 (72%)	-	-
Cypress Hills, AB	49.42682	-110.25441	61	37 (61%)	-	-
Saskatchewan Landing Prov. Park, SK	50.64528	-107.96310	101	97 (96%)	100	0 (0%)
Grasslands National Park, SK	49.21666	-107.70000	17	15 (88%)	1	0 (0%)
Buffalo Pound Prov. Park, SK	50.57582	-105.31356	35	30 (86%)	100	2 (2%)
Douglas Prov. Park, SK	51.02966	-106.46590	14	13 (93%)	40	0 (0%)
Danielson Prov. Park, SK	51.25933	-106.89580	61	22 (36%)	100	0 (0%)
Outlook, SK	51.48807	-107.05817	18	17 (94%)	12	0 (0%)
Harris, SK	51.73448	-107.58370	101	84 (83%)	12	0 (0%)
Saskatoon, SK	52.14731	-106.43278	-	-	38	0 (0%)
Blackstrap Prov. Park, SK	51.79760	-106.45833	-	-	141	46 (33%)
Bradwell, SK	51.91052	-106.23321	-	-	100	7 (7%)
Wakaw, SK	52.60297	-105.85426	-	-	44	0 (0%)
Minnedosa, MB	50.24715	-99.83870	-	-	100	8 (8%)
Kenora, ON	49.90153	-94.49324	-	-	30	2 (7%)

Table 5.1. Localities and coordinates of the collection sites of *D. andersoni* and *D. variabilis* within Canada and the number of ticks that were positive for infection with *Rickettsia* using PCR analyses of the *gltA* gene.

	Collection Locality	# ticks tested for *Rickettsia*	# PCR-positive
D. andersoni Nymphs	Saskatchewan Landing Provincial Park	9	9
D. variabilis larvae	Saskatchewan Landing Provincial Park	71	0
D. variabilis nymphs	Saskatchewan Landing Provincial Park	6	0
D. variabilis larvae	Blackstrap Provincial Park	143	0
D. variabilis nymphs	Blackstrap Provincial Park	7	0

Table 5.2. *D. andersoni* and *D. variabilis* immatures tested for the presence of rickettsial DNA and the number of ticks that tested positive in PCRs targeting the *gltA* and *ompA* genes.

The proportion of *D. variabilis* adults that tested positive for *Rickettsia* (8%) was significantly less than that of *D. andersoni* adults (Table 5.1). Of the 365 *D. variabilis* adults collected from the seven locations where this species was sympatric with *D. andersoni*, only two (< 1%) individuals were infected with *Rickettsia*. Similarly, none of the 77 *D. variabilis* immature (71 larvae and 6 nymphs) collected from small mammals in Saskatchewan Landing Provincial Park were PCR-positive for *Rickettsia* (Table 5.2). A greater proportion of *D. variabilis* (14%) from the six allopatric populations (i.e. Saskatoon, Blackstrap, Bradwell, Wakaw, Minnedosa and Kenora), contained *Rickettsia*. Nonetheless, the prevalence of *Rickettsia* in most allopatric populations of *D. variabilis* was relatively low (0 to 8%), except within Blackstrap Provincial Park, where 33% of the *D. variabilis* adults were *Rickettsia*-positive (Table 5.1). There was heterogeneity in the prevalence of *Rickettsia* within Blackstrap Provincial Park with a significantly greater ($p < 0.001$) proportion of *Rickettsia*-infected *D. variabilis* on the western side of Blackstrap Lake (39%; n = 115) than on the eastern side (4%; n = 26) (data not shown). At all 13 sites where *D. variabilis* adults were collected, there was no significant difference ($P = 0.420$) in the prevalence of *Rickettsia* in *D. variabilis* males (9%; n = 382) vs. females (7%; n = 436). None of the 150 *D. variabilis* immatures (143 larvae and 7 nymphs) collected from small mammals at Blackstrap Provincial Park were PCR-positive for *Rickettsia*; however, a majority (96%) of the immatures (i.e. 7 nymphs and 137 larvae) were collected from the eastern side of the lake, where the prevalence of *Rickettsia* was low.

5.4.2. Identification of Rickettsia in ticks

SSCP was used to compare the 461 *gltA* amplicons derived from *D. andersoni* and *D. variabilis*. Two different SSCP banding patterns (i.e. profiles) were detected among samples; one

profile (type I) was displayed by all *D. andersoni* adults and nymphs positive for *Rickettsia*, the second (type II) only by *D. variabilis* adults positive for *Rickettsia* (Fig. 5.1). The gDNA of samples representing type I and type II SSCP profiles (11 adult *D. andersoni* and eight adult *D. variabilis*, respectively) were subjected to automated DNA sequencing. The *gltA* sequences derived from 11 column-purified amplicons of type I were identical to each other and to the sequence for *R. peacockii* (GenBank accession number AF129885) (Simser *et al.* 2001). The eight type II *gltA* amplicons derived from *Rickettsia*-infected *D. variabilis* individuals were identical in nucleotide sequence to one another, but differed from those derived from *D. andersoni* at three nucleotide positions (100, 150 and 202) in the sequence alignment (data not shown). The sequences of *Rickettsia* from *D. variabilis* adults were identical to those of *R. montanensis* (accession number U74756) (Roux *et al.* 1997). The *gltA* amplicons from the nine PCR-positive *D. andersoni* nymphs had identical SSCP banding patterns to those of *R. peacockii* in *D. andersoni* adults.

The presence of *R. peacockii* in *D. andersoni* and *R. montanensis* in *D. variabilis* adults was confirmed by the amplification and sequencing of a 532 bp fragment of *ompA* (Regnery *et al.* 1991) from a single individual of each tick species that contained rickettsiae. The *ompA* amplicon from *D. andersoni* was identical in sequence to that reported previously for *R. peacockii* (accession number U55821) (Niebylski *et al.* 1997). The *ompA* amplicon from *D. variabilis* most closely matched the sequence for *R. montanensis* (accession number AY543682) (Ammerman *et al.* 2004), but it differed at a single nucleotide position. The results of a phylogenetic analysis showed that there was strong statistical support for the inclusion of the *Rickettsia* species from *D. variabilis* within the clade of *R. montanensis* (Fig. 5.2).

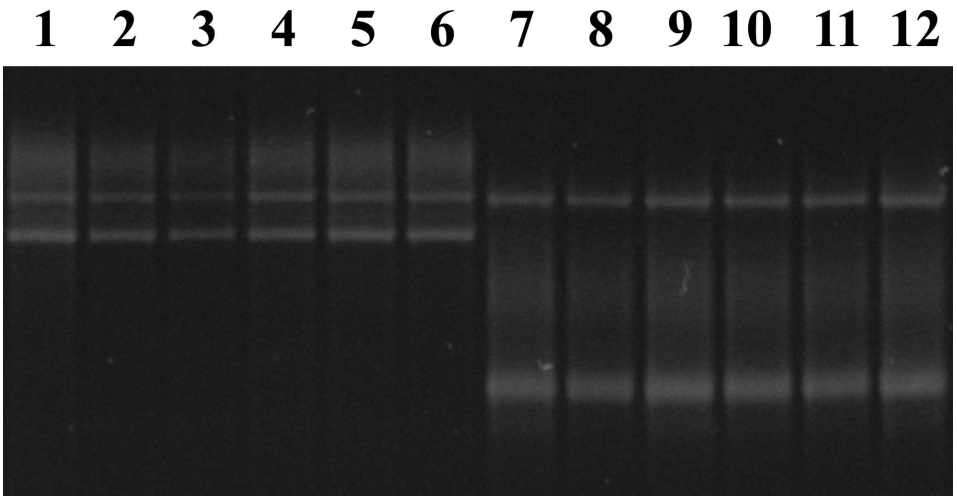

Fig. 5.1. SSCP analysis of *gltA* amplicons from total gDNA from *D. andersoni* (SSCP profile I) and *D. variabilis* (SSCP profile II). Lanes 1-6 and 7-12 contain *gltA* amplicons derived from single *D. andersoni* and *D. variabilis* individuals, respectively.

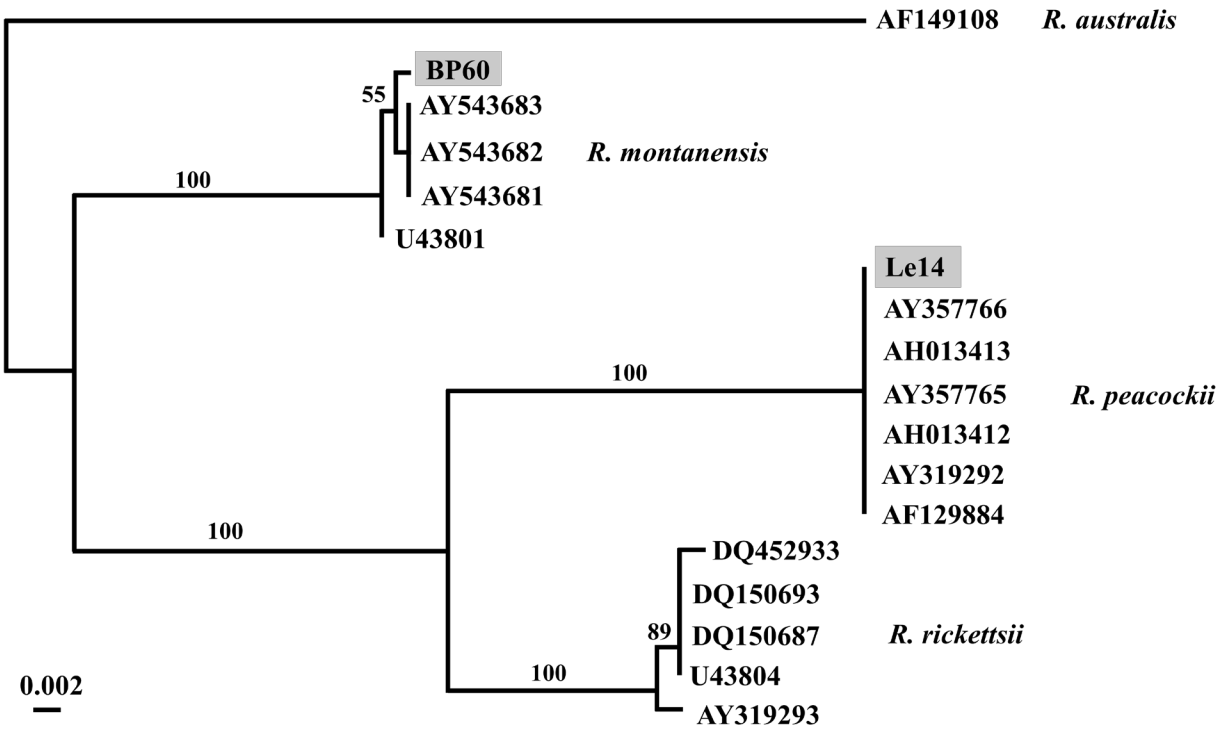

Fig. 5.2. A neighbor-joining tree depicting the relationships of the *ompA* sequences of *Rickettsia* from *D. andersoni* (Le14) and *D. variabilis* (BP60) obtained in the present study with those of *R. peacockii* (accession numbers AF129884, AH013412, AH013413, AY357765 and AY357766), *R. montanensis* (AY543681, AY543682, AY543683 and U43801), *R. australis* (AF149108), and *R. rickettsii* (AY319293, DQ452933, DQ150693, DQ150687 and U43804) derived from GenBank. The numbers above the branches in the tree indicate the statistical support following bootstrap analyses (1000 iterations) for each clade. *R. australis* was used to root the tree (Stenos & Walker 2000).

The presence of *Rickettsia* in all nine *D. andersoni* nymphs was confirmed by the amplification of a 434 bp fragment of the 17kDa surface protein gene. Only the ticks that were PCR positive in the *gltA* PCR were positive in the nested PCR. To confirm the identity of the *Rickettsia*, a 499 bp fragment of the 17kDa protein gene was sequenced from 4 nymphs and a single adult that tested positive by the *gltA* PCR. The sequence of all four amplicons derived from the nymphs were identical to each other and to the sequence of *R. peacockii* (GenBank accession number CP001227), but differed by a single nucleotide from the sequence derived from the adult.

5.5. Discussion

This chapter describes the prevalence and identity of rickettsiae in both sympatric and allopatric populations of *D. andersoni* and *D. variabilis* adults in Canada and immatures using PCR, SSCP and DNA sequencing analyses of the *gltA* gene. The identity of the rickettsiae in each tick species was further supported by comparative sequence data analyses of the *ompA* gene. Both the *gltA* and *ompA* genes have been shown to be useful genetic markers for distinguishing among species of *Rickettsia* (Regnery *et al.* 1991, Roux *et al.* 1996, Wikswo *et al.* 2008). In the present study, no *Rickettsia* were detected in the *D. variabilis* nymphs and larvae collected from Saskatchewan Landing Provincial Park or from Blackstrap Provincial Park. In contrast, the molecular analyses of 508 *D. andersoni* and 818 *D. variabilis* adults from 15 localities and nine *D. andersoni* nymphs revealed the presence of *R. peacockii* in *D. andersoni* and *R. montanensis* in *D. variabilis.* This host specificity was maintained at the seven localities where both tick species occurred in sympatry. Furthermore, the other rickettsial species recorded in *D. andersoni* and/or *D. variabilis* in the USA (i.e. the pathogenic *R. rickettsii* and the non-

pathogenic *R. bellii* and *R. rhipicephali* (Azad & Beard 1998)) or Canada (i.e. *R. rhipicephali* (Teng *et al.* 2011)) were not detected in any individuals of either tick species.

The prevalence of *R. peacockii* in *D. andersoni* adults in the present study was 76%. This finding is consistent with prevalences of 70-80% in *D. andersoni* from the eastern side of Bitterroot Valley in Montana (USA) (Burgdorfer *et al.* 1981, Niebylski *et al.* 1997). However, on the western side of Bitterroot Valley, only 8-16% of *D. andersoni* are infected with *R. peacockii* (Burgdorfer *et al.* 1981). *R. peacockii* is closely related to *R. rickettsii* (Niebylski *et al.* 1997), yet unlike *R. rickettsii,* which is pathogenic to *D. andersoni* (Niebylski *et al.* 1999), *R. peacockii* appears to be non-pathogenic and has no effect on the fecundity of infected *D. andersoni* females (Niebylski *et al.* 1999). Also, *R. peacockii* may not infect and cause disease in small mammals because of its inability to produce functional OmpA (Baldridge *et al.* 2004) and RickA (Simser *et al.* 2005) proteins, which are involved in cell adhesion and cell to cell transfer, respectively.

I detected equivalent prevalences of *R. peacockii* in *D. andersoni* males and females based on PCR analyses of total tick gDNA, whereas Niebylski *et al.* (1997) did not detect *R. peacockii* in *D. andersoni* males from Montana. However, Niebylski *et al*. (1997) conducted their PCR analyses on specific tissues (i.e. the hemolymph, salivary glands, reproductive tissues, malpighian tubules, midgut and hypodermal tissues), even though *R. peacockii* has been shown to occur in only the posterior diverticula of the midgut and small intestine of male ticks (Burgdorfer *et al.* 1981). Therefore, *D. andersoni* males probably represent dead-end hosts for *R. peacockii*. In *D. andersoni* females, *R. peacockii* occurs primarily in the ovarial tissues (resulting in transovarial transmission) (Burgdorfer *et al.* 1981), and to a lesser extent in the midgut diverticula and malpighian tubules (Burgdorfer *et al.* 1981), but not in the hemolymph, salivary glands, or hypodermal tissues (Niebylski *et al.* 1997). Although the salivary glands of some

larval *D. andersoni* have been shown to contain *R. peacockii* (Burgdorfer *et al.* 1981), and horizontal transmission of this rickettsial species is therefore theoretically possible, it is assumed that it does not occur (Burgdorfer *et al.* 1981, Niebylski *et al.* 1997). The absence of horizontal transmission of *R. peacockii* is further supported by transmission experiments and field surveys involving a small number of mammalian species, such as meadow voles (Burgdorfer *et al.* 1981, Niebylski *et al.* 1997), Swiss mice (Niebylski *et al.* 1997), ground squirrels (Norment & Burgdorfer 1985), chipmunks (Norment & Burgdorfer 1985), and guinea pigs (Burgdorfer *et al.* 1981). These studies have not determined a suitable mammalian host for *R. peacockii* even though meadow voles, ground squirrels and chipmunks are hosts for *D. andersoni* (Bishopp & Trembley 1945, Gregson 1956, James *et al.* 2006).

R. peacockii has only been reported in *D. andersoni* in this and previous studies (Burgdorfer *et al.* 1981, Niebylski *et al.* 1997), suggesting that it is specific for this tick species. This is likely due to its mode of transmission, which is thought to be exclusively transovarial (i.e. from female ticks to their offspring) (Burgdorfer *et al.* 1981), such that approximately 70% of females in the subsequent generation have been shown to be infected in laboratory studies (Niebylski *et al.* 1997). Thus, *R. peacockii* can be maintained in a large proportion of a tick population only though the mechanism of transovarial transmission.

In the present study, *R. montanensis* was detected only in *D. variabilis* adults, even in localities where both *D. variabilis* and *D. andersoni* coexist. Several studies have demonstrated the occurrence of *R. montanensis* from other parts of the distributional range of *D. variabilis* (Feng *et al.* 1980, Anderson *et al.* 1986, Pretzman *et al.* 1990, Ammerman *et al.* 2004). Philip and Casper (1981) reported *R. montanensis* in *D. andersoni* from the western side of Bitterroot Valley (Montana), based on serotyping of rickettsiae from ticks. However, this probably

represents a case of an incorrect identification of the rickettsiae. Philip and Casper demonstrated that there were four serotypes within 106 rickettsial isolates from *D. andersoni* and attributed these to be *R. rickettsii* (9%), *R. rhipicephali* (44%), *R. bellii* (i.e. 369-C; 39%) and *R. montanensis* (i.e. *R. montana*; 8%). In contrast, Burgdorfer et al. (1981) showed that *R. peacockii* occurs on the western side of Bitterroot Valley at a prevalence of 8 to 16%. It is, therefore, likely that the fourth rickettsial species detected by Philip and Casper (1981) was not *R. montanensis* but *R. peacockii*, especially if the antibodies used in their assay were cross-reactive with both species. If this were the case, then *R. montanensis* would also represent a rickettsial species that is host-specific for *D. variabilis.*

The average prevalence of *R. montanensis* in *D. variabilis* adults in the present study (i.e. 8%) was significantly lower than for *R. peacockii*, but was similar to that in other studies where *R. montanensis* was detected in relatively few *D. variabilis* individuals in populations from Ohio (< 0.1%) (Pretzman *et al.* 1990), Massachusetts (1%) (Feng *et al.* 1980), and Maryland (4%) (Ammerman *et al.* 2004). *R. montanensis* was found in an equivalent proportion of male and female *D. variabilis*, which is consistent with that in populations from Maryland (Ammerman *et al.* 2004). The prevalence of *R. montanensis* varied markedly among sampling localities, with the greatest prevalence occurring at Blackstrap Provincial Park (33%), but it was not detected in *D. variabilis* adults at eight other localities. Even within Blackstrap Provincial Park, there was significant heterogeneity in the prevalence of *R. montanensis* in ticks, with a significantly greater prevalence of infection on the western side of Blackstrap Lake than on the east side. The relatively low prevalence of *R. montanensis* in ticks compared to that for *R. peacockii* suggests that horizontal transmission is required for the maintenance of this species in populations of *D. variabilis*. *R. montanensis* has been detected in mice (*Peromyscus* spp.) and voles (*Microtus*

spp.) (Niebylski *et al.* 1999), hosts used by *D. variabilis* (Bishopp & Trembley 1945) (Gregson 1956), thus, small mammals may act as reservoirs for this *Rickettsia*. Furthermore, infection experiments have shown that *R. montanensis* also has some pathogenic effects in voles (Burgdorfer *et al.* 1981).

The prevalence of *R. rickettsii* in *D. andersoni* adults in the Bitterroot Valley of Montana varies from 1.5 to 5% (Burgdorfer 1975), while infections of *R. rickettsii* in *D. variabilis* range from 0.1% in Ohio (Pretzman *et al.* 1990) to 8.6% in Maryland (Schriefer & Azad 1994). The lack of detection of *R. rickettsii* in *D. andersoni* from the nine localities in Canada may be associated with the relatively high proportion of ticks infected with *R. peacockii*. Single-species rickettsial infections are typical in *Dermacentor* (Gage *et al.* 1994, Ammerman *et al.* 2004, Wikswo *et al.* 2008), except for the report of a single *D. variabilis* adult from Ohio infected with *R. bellii*, *R. montanensis*, and *R. rickettsii* (Carmichael & Fuerst 2006) and of a single *D. occidentalis* infected with *R. bellii* and *R. rhipicephali* (Wikswo *et al.* 2008).The greater incidence of RMSF on the western side of Bitterroot Valley compared to the eastern side of the valley has been shown to be associated with a significantly lower prevalence of *R. peacockii* (Burgdorfer *et al.* 1981, Niebylski *et al.* 1997). Only 8 to 16% of *D. andersoni* on the western side of the Bitterroot Valley are infected with *R. peacockii* (Burgdorfer *et al.* 1981), whereas the prevalence is 70-80% for ticks on the eastern side (Burgdorfer *et al.* 1981, Niebylski *et al.* 1997), which is equivalent to the average prevalence of *R. peacockii* in *D. andersoni* in the present study (76%). It has also been shown that establishment of *R. rickettsii* in the ovarial tissues of *D. andersoni* is prevented by an "interference phenomenon" when ticks are already infected with *R. peacockii* (Burgdorfer *et al.* 1981). *D. variabilis* adults infected with *R. montanensis* are also known to prevent the establishment of *R. rickettsii* (Burgdorfer 1988). Thus, *R. peacockii* and *R.*

montanensis have epidemiological significance with respect to *R. rickettsii* because of a negative effect on its enzootic maintenance. However, the relatively low prevalence of *D. variabilis* adults infected with *R. montanensis* in 13 of the Canadian localities we examined would not account for the apparent absence of *R. rickettsii*. Therefore, other factors must be responsible for this observation.

5.6. References

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Chapter 6. Prevalence of *Francisella* and *Francisella*-like endosymbionts (FLEs) in *Dermacentor andersoni* and *D. variabilis* from localities near their northern distributional limits

6.1. Abstract

This chapter describes a molecular analysis of the prevalence and diversity of *Francisella* and *Francisella*-like endosymbionts (FLEs) in 1,042 Adult and 236 immature *D. andersoni* and *D. variabilis* from 12 localities near their northern distributional limits. Ticks were tested for infection by these bacteria using PCR, PCR-SSCP and DNA sequencing, which targeted 373 bp of the 16S ribosomal RNA gene. The results showed no evidence for the presence of *Francisella tularensis* in any ticks. In contrast, FLEs were detected in 86% of the *D. andersoni* adults and 93% the nymphs. Similarly, FLEs were detected in 93% of the *D. variabilis* adults and 53% of the immatures. Ten types of FLE were identified; the three most prevalent types (1, 2 and 3) have been detected previously in other parts of the distributional ranges of *D. andersoni* or *D. variabilis*. The 16S sequences of the other seven FLE types have not been previously reported. Eight types of FLE were found exclusively in a single species of tick. FLE types 1 and 2, which were detected primarily in *D. andersoni* and *D. variabilis* respectively, also occurred in a few heterospecific ticks at locations where both tick species occurred in sympatry. The results of this study expand our knowledge of the diversity of *Francisella* and have implications for diagnostic tests and epidemiological studies of *F. tularensis* in tick populations near their northern distributional limits.

6.2. Introduction

There are four recognized species within the genus *Francisella* (Huber *et al*. 2010), three of which can cause disease in humans (Sjöstedt 2005). *Francisella tularensis*, the causative agent of tularemia in the Northern Hemisphere (Foley & Nieto 2010), varies considerably in its transmission patterns, virulence and disease presentation in different geographical areas (Staples *et al*. 2006, Keim *et al*. 2007, Eisen *et al*. 2009, Molins *et al*. 2010, Reese *et al*. 2010). In North America, there are two common subspecies of *F. tularensis*: *F. t. tularensis* and *F. t. holarctica* (Foley & Nieto 2010). Human infections with *F. t. holarctica* are mainly acquired through direct contact with infected beavers, muskrats, or lagomorphs, whereas in the USA, *F. t. tularensis* is often acquired by tick bites (Choi 2002, Eisen 2007). The American dog tick, *Dermacentor variabilis* (Say, 1821), and the Rocky Mountain wood tick, *Dermacentor andersoni* Stiles, 1908, are important for the transmission of *F. tularensis* in the eastern and western USA, respectively (Foley & Nieto 2010, Goethert & Telford III 2010). These two tick species, as well as *D. albipictus*, *D. occidentalis*, *D. hunteri*, and *D. nitens*, are also hosts of a number of bacteria that are closely related to *F. tularensis* (Niebylski *et al*. 1997, Scoles 2004, Goethert & Telford 2005). These so-called *Francisella*-like endosymbionts (FLEs) are generally of undetermined pathogenicity, but sometimes assumed to be nonpathogenic (Scoles 2004, Escudero *et al*. 2008). However, infection studies with the "*Dermacentor andersoni* symbiont" (DAS) showed it to be pathogenic for chicken embryos and guinea pigs (Burgdorfer *et al*. 1973).

Francisella tularensis is also endemic in Canada (Wobeser *et al*. 2009). However, compared to the USA, relatively few human cases of tularemia have been documented (Bow & Brown 1946, Isaac-Renton *et al*. 2010), some of which have occurred in Saskatchewan and Alberta in western Canada (Bow & Brown 1946, Harris 1956, Martin *et al*. 1982, CCWHC 1995,

Saskatoon Health Region 2007). Most human cases of tularemia in western Canada have been associated with contact with infected wildlife (McNabb 1930, Scott & Macbeth 1946, Black & Thomson 1958, Walker & Moore 1971, Jellison 1974) or livestock (Gwatkin *et al*. 1942, Bow & Brown 1943). Sporadic occurrences of tularemia have been reported in beavers (Langford 1954), muskrats (Langford 1954, Fyvie *et al*. 1959), jackrabbits (Bow & Brown 1943), snowshoe hares (Wobeser *et al*. 2009), ground squirrels (Bow & Brown 1943) and sheep (Gwatkin *et al*. 1942). The most recent outbreak occurred in deer mice (*Peromyscus maniculatus*) following a population explosion in 2005 near Madison, Saskatchewan. The causative agent was identified as *F. t. holarctica*, but the source of infection was not determined (Wobeser *et al*. 2007).

Although the transmission cycle of *F. tularensis* in Canada is not well defined, ticks (*Dermacentor* spp.) have been implicated as potential vectors in western Canada. For example, adult *D. andersoni* were important in some of the first recognized cases of human and animal tularemia in southern Alberta (Gwatkin *et al*. 1942, Bow & Brown 1943). *Francisella tularensis* has been recovered from *D. andersoni* in British Columbia, southern Alberta and southern Saskatchewan during surveys conducted between 1938 and 1946 (Gibbons 1939, Humphreys 1947, Humphreys & Campbell 1947). In 1982, *F. tularensis* was detected in adult *D. andersoni* from Saskatchewan Landing Provincial Park based on the results of transmission experiments in rabbits (Gordon *et al*. 1983). Despite these reports, the prevalence of *F. tularensis* and FLEs in ticks in western Canada is unknown. The aim of the present study was to use PCR-based methods to determine the prevalence of *Francisella* and FLEs in sympatric and allopatric populations of *D. andersoni* and *D. variabilis* from 12 localities near their northern distributional limits, which includes Saskatchewan Landing Provincial Park, a location where *F. tularensis* in adult ticks has been detected previously.

6.3. Methods

6.3.1. Collection of ticks.

A total of 1,042 adult male and female ticks (425 *D. andersoni* and 617 *D. variabilis*) were collected by flagging grass and shrubs or were removed from vertebrate hosts at 12 localities in southwestern Canada (Table 6.1). Questing ticks obtained by flagging were collected in May and June of 2005 and from April to June in 2006. Some adult ticks were also collected from humans, horses, dogs, skunks and raccoons between May and June in 2005 and 2007. Immature ticks (nine *D. andersoni* nymphs, six *D. variabilis* nymphs and 71 *D. variabilis* larvae) were collected from 13 deer mice (*Peromyscus maniculatus*) seven meadow voles (*Microtus pennsylvanicus*) and eight western jumping mice (*Zapus princeps*) trapped in Saskatchewan Landing Provincial Park in June and July of 2008, and in April of 2009. An additional seven *D. variabilis* nymphs and 143 *D. variabilis* larvae were collected from one deer mouse, one western jumping mouse, five meadow voles and ten southern red-backed voles (*Myodes gapperi*) from Blackstrap Provincial Park (Saskatchewan) May through July of 2009. All adult ticks were identified based on morphological examination. Adults of *D. andersoni* and *D. variabilis* are easily distinguished from one another, and from those of *D. albipictus* (a species that occurs in sympatry with the other two species (Wilkinson 1967)), based on differences in the morphology of their spiracular plates (Gregson 1956). The species identity of representative individuals was also verified using a PCR-based assay (Chapter 2). Immature ticks were examined by microscopy to confirm they belonged to the Metastriata. Their species identity was determined using the same PCR assay as for the adults, however amplicons were subjected to a RFLP analysis using *Alu*I (Fermentas), as per manufacturer's instructions. The RFLP analysis was used to confirm that the ITS-2 amplicons of *D. variabilis* individuals were not from *D. albipictus*, which has an ITS-2 amplicon

of the same size. The ITS-2 sequence of *D. variabilis* lacks the restriction site for *Alu*I present in the ITS-2 sequence of *D. albipictus* (Chapter 2).

6.3.2. DNA preparation.

Total genomic DNA (gDNA) was extracted and purified from each tick using a modification of the tissue protocol for the DNeasy Tissue Kit™ (Qiagen, Valencia, USA). Individual ticks were placed in 1.5ml micropestle tubes (Kontes), to which 180 µl of ATL buffer (Qiagen) was added. Ticks were homogenized by grinding with micropestles (Kontes) attached to a cordless drill. Proteinase K (20 µl @ 15 µg/µl) was added to the homogenate. Samples were incubated for 16 hours at 55°C. The gDNA was purified according to the DNeasy tissue kit protocol, except that gDNA was eluted twice from the spin columns using 50 µl of AE buffer. The two elutions derived from the same tick were combined in a single tube and stored at -20°C.

6.3.3. PCR and SSCP of 16S rRNA gene.

The presence of *Francisella* DNA in adult ticks was tested using a PCR targeting 373 bp of the bacterial 16S rRNA gene with the primers NC-Fran16S-F (5' - CAA CAT TCT GGA CCG AT – 3') and NC-Fran-16S-R (5' - TGC GGG ACT TAA CCC AAC AT – 3'), which were designed to be specific for *Francisella* spp. PCR reactions were carried out in 25µl volumes containing 200 µM of each dNTP (Fermentas, Burlington, Canada), 3 mM $MgCl_2$, 25 pmol (1 µM) of each primer, 0.5U of recombinant *Taq* DNA polymerase (Fermentas), 2.5µl 10x PCR buffer with $(NH_4)_2SO_4$ (Fermentas), and 2 µl of template gDNA. A negative control (i.e. without gDNA) and positive control was included in each set of reactions. PCRs were performed in a thermocycler (iCycler™; Bio-Rad, Hercules, CA, USA) using the following conditions: 95°C for

5 min, followed by 30 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 5 min. The presence of *Francisella* DNA in immature ticks was tested using a nested PCR (nPCR). The first phase of the nPCR targeted 1,141 bp of the bacterial 16S rRNA gene with primers (5'-TAC CAG TTG GAA ACG ACT GT-3') and F5 (5'-CCT TTT TGA GTT TCG CTC C-3') (Forsman *et al*. 1994). Each reaction contained 200 µM of each dNTP (Fermentas), 2.5 mM $MgCl_2$, 25 pmol (1 µM) of each primer, 0.5U of recombinant *Taq* DNA polymerase (iTaq; Bio-Rad), 2.5µl 10x PCR buffer (Bio-Rad), and 2 µl of template gDNA. The PCR conditions used were 95°C for 5 min, followed by 30 cycles of 95°C for 60 s, 65°C for 60 s, and 72°C for 60 s, and a final extension at 72°C for 5 min. PCR products, including those of the negative controls, were then purified by adding shrimp alkaline phosphatase (0.014 U/µl) (New England BioLabs, Pickering, Canada) and (0.27 U/µl) exonuclease I (Fermentas), and incubating the mixture at 37°C for 15 min., and then at 80°C for 15 min. The second phase of the nPCR was conducted with 2µl of purified PCR products (including the negative control samples) using primers NC-Fran16S-F and NC-Fran16S-R and the same PCR conditions used for the adult ticks. Additional negative control samples were also included. Amplicons were subjected to electrophoresis on SYBR® Safe-stained 1.5% agarose-TBE gels and their banding patterns were visualized by UV transillumination. Some of the initial amplicons produced from the adult ticks were subjected to DNA sequencing to verify the specificity of the PCR assay.

Single strand-conformation polymorphism (SSCP) analyses were performed on all samples that were PCR-positive using the methodology described in Chapter 2. This mutation scanning technique can be used to differentially display DNA sequences that differ by one or more nucleotides (Gasser *et al*., 2006). In the present study, SSCP was used to pre-screen all amplicons for genetic variation before selecting representative samples of each different SSCP

profile for DNA sequencing. Samples that were sequenced previously were used as mobility controls in SSCP gels. Where possible, multiple amplicons of each SSCP profile were prepared for DNA sequencing.

6.3.4. DNA sequence analyses and nucleotide sequence accession numbers.

Amplicons from 76 adult ticks and 21 immature ticks were column-purified (MinElute DNA purification kit; Qiagen) then sequenced using primers NC-Fran16S-F and NC-Fran16S-R in separate reactions. The 16S rDNA sequences, excluding primer sites, were manually aligned and a BLAST search was performed to determine sequence similarity with those of other bacterial 16S rDNA sequences deposited in GenBank. The sequences of representative samples obtained in the present study have been deposited in GenBank under accession numbers FR872824-FR872833. A minimum spanning network tree depicting the relationships of the different genetic types of FLEs was constructed using the TCS program (Fig. 6.1) (Clement *et al*. 2000).

6.4. Results

A total of 1,042 adult and 236 immature (214 larvae and 22 nymphs) ticks were tested individually for the presence of *Francisella* DNA by PCR. All samples that were positive by PCR produced a single band of the expected size (approximately 370 bp) on an agarose gel. The proportion of adult ticks at each locality that were PCR-positive for *Francisella* DNA ranged from 73% to 100%; however, significantly more *D. variabilis* (93%) were positive than *D. andersoni* adults (86%) (χ^2_1= 12.09, p<0.05, N = 1,042) (Table 6.1). There were also significant differences in the proportion of male and female *D. andersoni* (82% and 90%, respectively) (χ^2_1=

378.29, p<0.001, $N = 425$) and *D. variabilis* (88% and 97%, respectively) ($\chi^2_1 = 150.10$, p<0.001, $N = 617$) that were PCR-positive for *Francisella* DNA. For the immature ticks, 89% of the *D. andersoni* nymphs, 69% of the *D. variabilis* nymphs and 52% of the *D. variabilis* larvae were PCR-positive for *Francisella* DNA (Table 6.2). A significantly lower proportion of *D. variabilis* larvae from Saskatchewan Landing Provincial Park were PCR-positive for *Francisella* DNA than those from Blackstrap Provincial Park ($\chi^2_1 = 26.75$, p<0.001, $N = 214$).

At least 20 different SSCP banding patterns (i.e. profiles) were detected among the 1,068 PCR products. Many amplicons had SSCP patterns that were comprised of at least two different patterns, suggesting that some ticks contained more than one sequence type that differed from one another in sequence by one or more nucleotides. DNA sequencing analyses of representative amplicons of each SSCP banding pattern type revealed that the bacteria present in ticks were not *F. tularensis* but *Francisella*-like endosymbionts. A total of ten different sequence types of FLE were identified among the tick samples (Tables 6.3 and 6.4). Multiple FLE sequence types were detected in 24% of the adult ticks. The proportion of PCR-positive adult ticks that contained more than one sequence type was significantly different between *D. andersoni* (3%) and *D. variabilis* (38%) ($\chi^2_1 = 150.18$, p<0.001, $N = 940$) (Table 6.3). None of the 128 *Dermacentor* immatures contained multiple FLE types (Table 6.4).

Locality	Coordinates (decimal degrees)		*D. andersoni*		*D. variabilis*	
	Lat. (N)	Long. (W)	No. tested	No. *Francisella*-positive	No. tested	No. *Francisella*-positive
Lethbridge, AB	49.73721	-112.84751	100	73 (73%)	-	-
Saskatchewan Landing Prov. Park, SK	50.64528	-107.96310	82	79 (96%)	96	92 (96%)
Grasslands National Park, SK	49.21666	-107.70000	17	15 (88%)	1	1 (100%)
Buffalo Pound Prov. Park, SK	50.57582	-105.31356	33	28 (85%)	79	70 (89%)
Douglas Prov. Park, SK	51.02966	-106.46590	14	14 (100%)	40	40 (100%)
Danielson Prov. Park, SK	51.25933	-106.89580	61	59 (97%)	99	98 (99%)
Outlook, SK	51.48807	-107.05817	18	16 (89%)	12	11 (92%)
Harris, SK	51.73448	-107.58370	100	83 (83%)	12	9 (75%)
Blackstrap Prov. Park, SK	51.79760	-106.45833	-	-	105	95 (91%)
Wakaw, SK	52.60297	-105.85426	-	-	44	44 (100%)
Minnedosa, MB	50.24715	-99.83870	-	-	99	85 (86%)
Kenora, ON	49.90153	-94.49324	-	-	30	28 (93%)
Total			**425**	**367 (86%)**	**617**	**573 (93%)**

Table 6.1. Localities and coordinates of the collection sites of adult *D. andersoni* and *D. variabilis* and the number (and percentage) of ticks positive for the presence of *Francisella* 16S rDNA at each locality.

Locality	*D. andersoni* nymphs		*D. variabilis* larvae		*D. variabilis* nymphs	
	No. ticks tested	No. *Francisella*-positive	No. ticks tested	No. *Francisella*-positive	No. ticks tested	No. *Francisella*-positive
Blackstrap	-	-	143	92 (64%)	7	7 (100%)
Saskatchewan Landing	9	8 (89%)	71	19 (27%)	6	2 (33%)

Table 6.2. Localities of the collection sites of *D. andersoni* nymphs and *D. variabilis* larvae and nymphs and the number (and percentage) of ticks positive for the presence of *Francisella* 16S rDNA at both localities.

	FLE types in *D. andersoni*					FLE types in *D. variabilis*										
Locality	**1**	**5**	**1+2**	**1+9**	**1+10**	**1**	**2**	**3**	**4**	**8**	**2+3**	**2+3+4**	**2+7**	**3+4**	**3+7**	**3+8**
Lethbridge	73	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Saskatchewan Landing	76	-	2	1	-	-	55	2	-	-	35	-	-	-	-	-
Grasslands National Park	15	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-
Buffalo Pound Prov. Park	27	1	-	-	-	-	34	8	-	-	24	2	1	1	-	-
Douglas Prov. Park	13	-	-	-	1	-	24	-	-	-	16	-	-	-	-	-
Danielson Prov. Park	59	-	-	-	-	1	64	3	-	1	29	-	-	-	-	-
Outlook	16	-	-	-	-	-	5	-	-	-	6	-	-	-	-	-
Harris	76	-	3	4	-	-	7	-	-	-	2	-	-	-	-	-
Blackstrap Prov. Park	-	-	-	-	-	-	33	14	-	-	47	-	-	-	1	-
Wakaw	-	-	-	-	-	-	34	-	-	-	10	-	-	-	-	-
Minnedosa	-	-	-	-	-	-	44	4	1	-	34	-	-	-	1	1
Kenora	-	-	-	-	-	-	19	-	-	-	8	-	-	1	-	-
Total	**355**	**1**	**5**	**5**	**1**	**1**	**320**	**31**	**1**	**1**	**211**	**2**	**1**	**2**	**2**	**1**

Table 6.3. Genetic types of *Francisella*-like endosymbionts (FLEs) detected in adult *D. andersoni* and *D. variabilis* from each locality (coordinates given in Table 6.1).

	FLE types in *D. andersoni*		FLE types in *D. variabilis*			
Locality	**1**	**5**	**2**	**3**	**6**	**7**
Saskatchewan Landing	7	1	93	4	1	1
Blackstrap Prov. Park	-	-	20	-	-	1
Total						

Table 6.4. Genetic types of *Francisella*-like endosymbionts (FLEs) detected in *D. andersoni* nymphs, and *D. variabilis* larvae and nymphs from each locality.

There were 28 variable nucleotide positions in the alignment of the partial 16S rDNA sequences (321-336 bp) of the ten FLEs (Table 6.5). This included a 12 bp deletion (positions 172-183) in the sequence of FLE type 4, which was otherwise identical in sequence to FLE type 3. Pairwise comparisons of all ten sequences revealed differences at one to 21 nucleotide positions between each type of FLE. A comparison of the partial 16S rDNA sequences of the ten FLEs with sequence data available on GenBank revealed that FLE types 4 to 10 had unique sequences, whereas the sequences of FLE types 1 to 3 were reported previously. For instance, the 336 bp sequence of FLE type 1 was identical to the 16S rDNA sequence of FLEs detected in *D. andersoni* (GenBank accession nos. AY375397 and AY375398 (Scoles 2004)). FLE type 2 was identical in sequence to an FLE in *D. variabilis* (accession nos. AY795979 (Goethert & Telford 2005) and AY375406 (Scoles 2004)) and FLE type 3 was identical to that of the "DVF" FLE in *D. variabilis* (accession nos. AY795976 - AY795978 (Goethert & Telford 2005)). Most of the 10 FLE types were found in either *D. andersoni* or *D. variabilis*. However, two of the FLE sequence types occurred in adult ticks of both species. FLE type 1 was the most prevalent sequence variant in adult *D. andersoni*, found in 99.7% of the PCR-positive ticks; however, it also occurred in a single *D. variabilis* adult (<1%) (Table 6.3). This was also the most prevalent FLE type in the *D. andersoni* nymphs (Table 6.4). The FLE type 2 sequence variant was the most prevalent type in *D. variabilis*, occurring in 93-94% of PCR-positive adult and immature ticks (Tables 6.3 and 6.4). This type also occurred in five (1%) *D. andersoni* adults, each of which also contained the FLE type 1 variant (Table 6.3). In contrast, seven of the FLE types, which occurred at relatively low prevalences in the present study, were found exclusively in a single tick species. The DNA sequences of the least common FLE types in *D. andersoni* (types 5, 9 and 10) differed by a single nucleotide compared to the sequence of type 1, the most prevalent type in *D. andersoni*.

FLE type	Alignment position																												
	61	82	172	173	174	175	176	177	178	179	180	181	182	183	188	189	196	198	206	208	215	217	227	287	288	289	306	326
1	G	C	G	A	A	T	T	G	A	C	G	G	G	G	G	C	G	T	G	A	T	C	C	G	G	G	C	C
5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.
9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.
10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.
2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.
4	A	T	-	-	-	-	-	-	-	-	-	-	-	-	.	.	T	G	C	G	.	T	T	A	.	.	.	.
3	A	T	.	.	.	.	.	.	.	.	A	.	.	.	.	.	T	G	C	G	.	T	T	A	.	.	.	.
6	A	T	.	.	.	.	.	.	.	.	A	.	.	.	.	.	T	G	C	G	.	T	T	A	.	.	.	G
8	A	T	.	.	.	.	.	.	.	.	A	.	.	.	.	T	T	G	C	G	.	T	T	A	.	.	.	.
7	A	T	.	.	.	.	.	.	.	.	A	.	.	.	.	.	T	G	C	G	.	T	T	A	A	T	.	.

Table 6.5. Multiple sequence alignment of the 28 variable nucleotide positions of the 16S rDNA fragment (336 bp) obtained from the ten FLE types found in *D. andersoni* and *D. variabilis* adults from 12 localities in western Canada. A dot (.) represents the identical nucleotide and a dash (-) indicates a deletion with respect to the sequence of the type 1 FLE. The alignment position indicates the nucleotide position relative to the 3' end of the forward primer.

Similarly, the FLE types which were relatively rare in *D. variabilis* (i.e. 4, 6, 7 and 8) had 16S rDNA sequences most similar to that of FLE type 3 (Fig. 6.1), the second most common type of FLE in *D. variabilis* (Table 6.3). It is unlikely that single nucleotide substitutions in rare FLE types was due to polymerase error because of the low rate of nucleotide misincorporation during PCR (i.e. an accuracy rate of 4.5×10^4, according to the manufacturer; see http://www.fermentas.com/en/products/all/pcr-qpcr-rt-pcr/standard-pcr/ep040-taq-dna-recomb). FLE type 3 was detected in 44% of the *D. variabilis* adults positive by PCR, most (85%) of which occurred in *D. variabilis* adults that also contained FLEs of type 2 (Table 6.3). FLE type 6 was only detected in a single *D. variabilis* larva (Table 6.4).

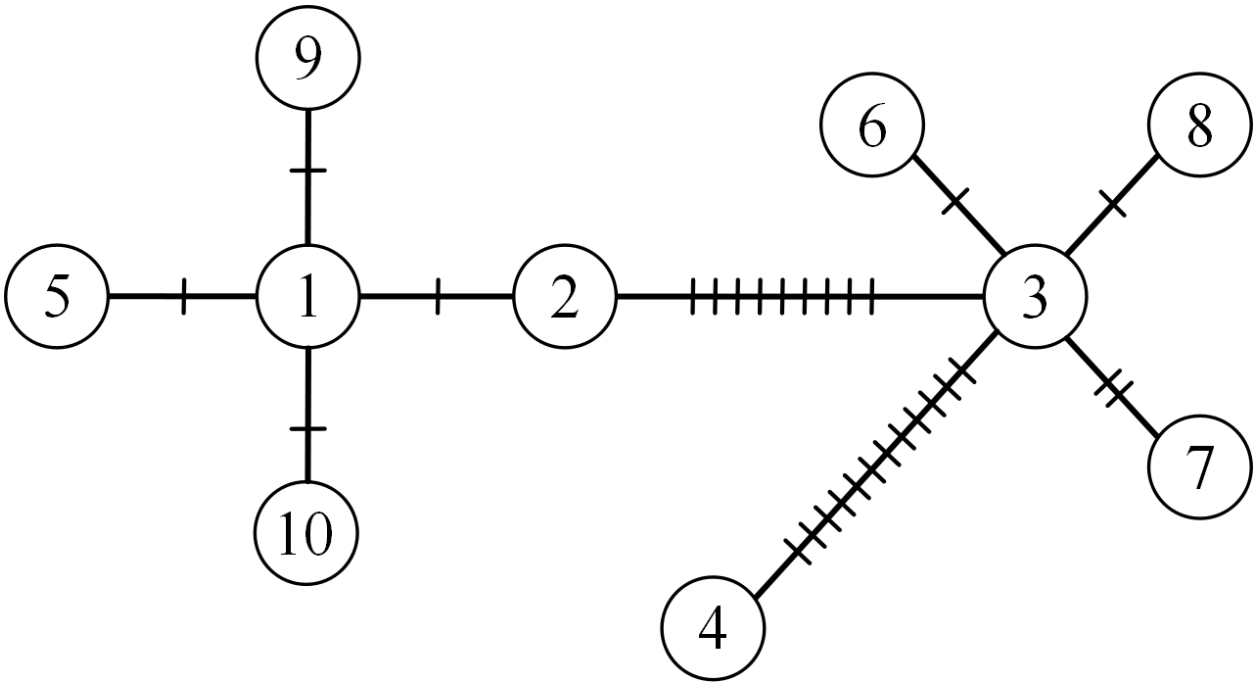

Fig. 6.1. Minimum spanning network tree depicting the relationships of the ten FLE sequence types detected in *D. andersoni* and *D. variabilis* from western Canada, based on comparisons of the partial sequence of the 16S rRNA gene. FLE types 5, 9, and 10 were found exclusively in *D. andersoni*, while types 3, 4, 6, 7, and 8 were found only in *D. variabilis*. Types 1 and 2 were detected in both *D. andersoni* and *D. variabilis*. Cross-hatches indicate the number of nucleotide differences between sequences of adjacent types**.**

6.5. Discussion

Francisella tularensis is endemic in western Canada (Martin *et al*. 1982, Wobeser *et al*. 2009). Despite this, little is known of the relative prevalence of this pathogen in different geographical areas, or of its natural transmission cycle, in western Canada. In the present study, we did not detect, using PCR, the presence of *F. tularensis* in any of the 425 *D. andersoni* and 617 *D. variabilis* adults, and nine *D. andersoni* and 225 *D. variabilis* immatures collected from 12 localities in Alberta, Saskatchewan and Manitoba. This included 82 *D. andersoni* or 96 *D. variabilis* adults collected by flagging from Saskatchewan Landing Provincial Park in 2005, a locality where, in 1982, questing *D. andersoni* adults were found to be infected with *F. tularensis* (Gordon *et al*. 1983). Similarly, none of the nine *D. andersoni* nymphs, six *D. variabilis* nymphs and 71 *D. variabilis* larvae collected from small rodents in Saskatchewan Landing Provincial Park in 2008 and 2009 were infected with *F. tularensis*. However, these findings are not unexpected since *F. tularensis* infections in ticks are often sporadic (Goethert & Telford 2009). This appears to be the case for ticks in Saskatchewan Landing Provincial Park because *F. tularensis* was also not detected in any *D. andersoni* collected in 1971, and between 1974 and 1981(Gordon *et al*. 1983). Given that *F. tularensis* is often maintained in endemic foci at a low prevalence (Goethert *et al*. 2009, Goethert & Telford 2009, Gyuranecz *et al*. 2011), estimations of the prevalences of *F. tularensis* in northern populations of *D. andersoni* and *D. variabilis* in western Canada will require surveys to be conducted involving a large number of ticks from numerous locations over multiple consecutive years.

Although *F. tularensis* was not detected in *D. andersoni* or *D. variabilis* in our study, we did detect the DNA of FLEs in a large proportion of adult ticks at each locality. This was not surprising given that FLEs have been reported from many genera of ixodid and argasid ticks (e.g.

Amblyomma, *Dermacentor*, *Haemaphysalis*, *Ixodes*, *Rhipicephalus*, *Argas* and *Ornithodoros*) (Suitor & Weiss 1961, Niebylski *et al*. 1997, Noda *et al*. 1997, Sun *et al*. 2000, Scoles 2004, Machado-Ferreira *et al*. 2009, de Carvalho *et al*. 2010). The combined results of our SSCP and DNA sequencing analyses revealed the existence of ten types of FLEs that differed in sequence from one another by 1-22 bp over an alignment length of 336 bp. The partial 16S rDNA sequences of seven FLEs (types 4-10) represented new sequence types based on comparisons with sequence data available on GenBank, whereas the three most prevalent types of FLEs (types 1, 2, and 3) were identical in sequence to FLEs reported in *D. andersoni* or *D. variabilis* from a number of locations in the USA and Canada (Scoles 2004, Goethert & Telford 2005, Kugeler *et al*. 2005). The partial 16S sequences of FLE types 1 and 2 were also identical in sequence to those previously found in other species of *Dermacentor* (i.e. *D. hunteri*, *D. nitens*, *D. occidentalis*, or *D. albipictus*)(Scoles 2004, Kugeler *et al*. 2005). Therefore, a larger fragment of the 16S gene, or a second genetic marker would be needed to distinguish FLE types 1-3 in *D. andersoni* and *D. variabilis* from those FLEs in the other species of *Dermacentor*. Nonetheless, we were able to identify different genotypes of FLEs in *D. andersoni* and *D. variabilis* based on a relatively small part of the 16S rDNA sequence.

A large proportion of *D. andersoni* and *D. variabilis* (86% and 93%, respectively) at localities near their northern distributional limits contained FLEs, which are similar to rates of infection (i.e. 55% to 97%) for FLEs in these two species in more southern parts of their geographical ranges (Niebylski *et al*. 1997, Goethert & Telford 2005). These bacteria occur in the malphigian tubules and/or the ovaries of female ticks (Burgdorfer *et al*. 1973, Niebylski *et al*. 1997, Noda *et al*. 1997) and transovarial transmission of FLEs has been shown to be very efficient (96-100%) in *D. andersoni* (Niebylski *et al*. 1997) and *D. variabilis* (Goethert &

Telford 2005). The infection rate of FLEs in *D. variabilis* larvae in the present study also suggests that FLEs are vertically transmitted. Transovarial transmission is an important mechanism by which FLEs are maintained in a large proportion of individuals within the tick populations. However, many *D. andersoni* and *D. variabilis* males in western Canada were also found to contain FLEs, but it is not known if these individuals would contribute to the maintenance of FLEs in tick populations. Few studies have examined *D. andersoni* or *D. variabilis* males for the presence of FLEs. However, the salivary glands and reproductive tissues of male *D. andersoni* from Bitterroot Mountains (Montana, USA) were found not to contain the "DAS" FLE found in *D. andersoni* females from the same locality (Niebylski *et al*. 1997). If vertical transmission is the only means by which FLEs are maintained in a tick population, then *Dermacentor* males would represent a dead end host for FLEs.

In the present study, 24% of the ticks containing FLEs were infected with multiple types. This is consistent with the results of previous studies where co-infections of multiple types of FLEs in *Dermacentor* adults were relatively common (Scoles 2004, Goethert & Telford 2005). However, we found a significant difference between the two tick species in the frequency of multiple infections. Very few *D. andersoni* (3%) were infected with multiple types of FLEs, whereas significantly more infected *D. variabilis* (38%) were co-infected with two or three types of FLE. There was also a notable difference between *D. andersoni* and *D. variabilis* in the types of FLEs they contained. Five types of FLEs (1, 2, 5, 9 and 10) were found in *D. andersoni*, while six types of FLEs (1, 2, 3, 4, 7 and 8) were detected in *D. variabilis*. Most of the FLE types were specific for a single tick species; however, the two most prevalent types were found in both species of tick but in different relative frequencies. Type 1 FLE, the most common FLE in *D. andersoni* was found in a single male *D. variabilis* from Danielson Provincial Park. Similarly,

type 2 FLE, the most common type in *D. variabilis* at all localities where this tick occurs, was detected in five *D. andersoni* females, three from Harris and two from Saskatchewan Landing Provincial Park. All five of these *D. andersoni* females also contained FLE type 2. Infections of FLE type 1 in *D. variabilis* and FLE type 2 in *D. andersoni* appeared to be rare and only occurred at localities where these tick species were sympatric. This infection pattern may be explained by horizontal transmission from one species of tick to the other through an vertebrate host, but horizontal transmission of FLEs has been shown not to occur in experimental infections (Burgdorfer *et al*. 1973, Niebylski *et al*. 1997, Baldridge *et al*. 2009). There is a recent report of the detection of FLEs (e.g. GenBank accession no. EU315913) in wood mice (*Apodemus sylvaticus*) that were also detected in ticks in Europe (Escudero *et al*. 2008), which suggests that horizontal transmission of some FLE types from ticks to small mammals can occur. Furthermore, Goethert *et al*. (2005) detected the "DVF" FLE in the hemolymph of *D. andersoni* adults and suggested that the salivary glands may also be infected. At localities where *D. andersoni* and *D. variabilis* occur in sympatry, immature stages of both species do parasitize the same small mammal hosts (e.g. mice and voles) (see Chapter 3), thus there is the potential for horizontal transmission of FLEs from one tick species to the other via a vertebrate host. However, there was no evidence of FLEs of type 1 in *D. variabilis* larvae and nymphs, or of types 2 or 3 in *D. andersoni* nymphs from Saskatchewan Landing Provincial Park.

It is not known if there is an epidemiologic significance of FLEs in ticks. Studies have not indicated that these organisms are pathogenic to ticks or affect their fecundity (Burgdorfer *et al*. 1973, Niebylski *et al*. 1997, Goethert & Telford 2005, Baldridge *et al*. 2009). Furthermore, multiple FLE types can be co-transmitted transovarially (Goethert & Telford 2005), or with *Rickettsia* sp. (Niebylski *et al*. 1997), or *Anaplasma phagocytophilum* (Baldridge *et al*. 2009).

Thus, the presence of FLEs does not appear to inhibit the vertical transmission of other FLE strains or distantly related organisms. Additional studies are needed to determine if there is a negative correlation between the presence of FLEs and the occurrence of *F. tularensis* in ticks and if they affect the vectorial capacity of their tick hosts (Goethert & Telford 2005).

In conclusion, multiple types of FLEs were found in northern populations of *D. andersoni* and *D. variabilis*. However, each FLE type was primarily found in a single tick species, and this host-specificity of FLEs was generally maintained at locations where both tick species occurred in sympatry because there were very few examples of potential transfer of FLEs from *D. andersoni* to *D. variabilis*, and *vice-versa*. Although the three most common FLE types have been found previously in *D. andersoni* or *D. variabilis* in other parts of their geographical range, seven of the FLE types detected in the present study represented new sequence types. This finding expands our knowledge on the genetic diversity of FLEs in ticks. The continual discovery of new FLEs in ticks (Machado-Ferreira *et al*. 2009, Sréter-Lancz *et al*. 2009) and species of *Francisella* in fish and mammals (Kamaishi *et al*. 2005, Nylund *et al*. 2006, Soto 2010) and humans (Escudero *et al*. 2010, Huber *et al*. 2010), shows that the family Francisellaceae is much more diverse than previously realized (Keim *et al*. 2007). Furthermore, the presence of multiple FLEs in *D. andersoni* and *D. variabilis* that are genetically similar to *F. tularensis* has important implications for diagnosis and epidemiological studies of tularemia in Canada. These require molecular techniques that can reliably distinguish among the different subspecies and subtypes of *Francisella* and the different types of FLEs (Goethert *et al*. 2004, Kugeler *et al*. 2005, Escudero *et al*. 2008). Our study has demonstrated that PCR-SSCP, combined with DNA sequencing, is an effective approach to examine a large number of ticks for the presence of different bacteria within the family Francisellaceae.

6.6. References

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Chapter 7. *Arsenophonus* sp. in Canadian populations of *Dermacentor andersoni* and *D. variabilis*[6]

7.1. Abstract

In this chapter, the presence of *Arsenophonus*-type bacteria was determined by PCR and DNA sequencing for 338 *D. andersoni* and 448 *D. variabilis* adults from western Canada. Fifty-one (15%) of the *D. andersoni* were found to be infected with *Arsenophonus*, whereas only a single *D. variabilis* was infected. The prevalence of *Arsenophonus* in *D. andersoni* varied among localities (0–27%). The 16S rDNA sequences of *Arsenophonus* in Canadian *D. andersoni* and *D. variabilis* were identical to one another, but the results of a phylogenetic analysis showed that they were genetically distinct from, and may represent a different species to, the *Arsenophonus* in *D. variabilis* and *Amblyomma americanum* in eastern USA.

7.2. Introduction

Preliminary results of the broad-range PCR assay for the detection of bacteria in ticks showed that a few ticks contained a bacterium belonging to the genus *Arsenophonus* (Chapter 4). The principal hosts for *Arsenophonus* species are insects (Balas *et al.* 1996, Hypša & Dale 1997, Dale *et al.* 2006, Trowbridge *et al.* 2006, Duron *et al.* 2008, Sorfová *et al.* 2008); however, this gammaproteobacteria has been reported in the American dog tick, *D. variabilis*, and the lone star tick, *Amblyomma americanum,* in some eastern states of the USA (Grindle *et al.* 2003, Clay *et al.* 2008). Although the American dog tick has a broad distribution in North America that includes the eastern two-thirds of the USA (Sonenshine 1979) and southern Canada

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(Saskatchewan, Manitoba, western Ontario and Nova Scotia) (Gregson 1956, Dodds *et al.* 1969), the prevalence of *Arsenophonus* in Canadian populations of *D. variabilis* is unknown. In contrast, there have been no previous reports of *Arsenophonus*-like organisms within *D. andersoni.* In this chapter, the prevalence of *Arsenophonus*-type bacteria in *D. andersoni* and *D. variabilis* was determined at several localities in western Canada, including those where the two tick species occur in sympatry.

7.3. Methods

7.3.1 Collection of ticks

Adult ticks were collected at eight localities in western Canada between 2005 and 2007 (Table 7.1). Larvae and nymphs were removed from mice and voles collected at Saskatchewan Landing Provincial Park and Blackstrap Provincial Park (Table 7.2). The specific details of these ticks are given in Chapters 5 and 6. All ticks were identified morphologically to the species level (Gregson 1956) and stored at -70^{o}C until needed for molecular analyses.

7.3.2. DNA purification and PCR

Total genomic DNA (gDNA) was purified from 822 individual ticks (133 male and 205 female *D. andersoni*, and 221 male and 263 female *D. variabilis*) using the DNeasy Blood and Tissue Kit (Qiagen) with a modified protocol (Chapter 5). In addition, a total of 236 immature ticks were also tested for the presence of *Arsenophonus*. A 710 bp fragment of the bacterial 16S ribosomal (r)RNA gene was amplified from the gDNA of all ticks using primers 554F (5' –TCG GAA TTA CTG GGC GTA AA - 3') and NC-Arsen16S-R (5' – GGC TCG CCT CTC TCT GTA TAC G - 3').

Locality[a]	Coordinates (decimal degrees)		*D. andersoni*		*D. variabilis*	
	Lat. (N)	Long. (W)	No. tested	No. PCR-positive	No. tested	No. PCR-positive
Lethbridge, AB	49.73721	-112.84751	100	27 (27%)	-	-
Saskatchewan Landing Prov. Park,	50.64528	-107.96310	85	21 (25%)	100	0 (0%)
Grasslands National Park, SK	49.21666	-107.70000	17	2 (12%)	1	0 (0%)
Buffalo Pound Prov. Park, SK	50.57582	-105.31356	35	0 (0%)	100	0 (0%)
Harris, SK	51.73448	-107.58370	101	1 (1%)	12	1 (8%)
Blackstrap Lake, SK	51.79760	-106.45833	-	-	141	0 (0%)
Minnedosa, MB	50.24715	-99.83870	-	-	100	0 (0%)
Kenora, ON	49.90153	-94.49324	-	-	30	0 (0%)

[a] AB = Alberta, SK = Saskatchewan, MB = Manitoba, and ON = Ontario

Table 7.1. Localities and geographic coordinates of the collection sites of *D. andersoni* and *D. variabilis* adults, and the number of ticks positive for *Arsenophonus* by PCR.

Locality	*D. andersoni* nymphs		*D. variabilis* larvae		*D. variabilis* nymphs	
	No. tested	**No. PCR-positive**	**No. tested**	**No. PCR-positive**	**No. tested**	**No. PCR-positive**
Saskatchewan Landing Prov. Park,	9	0	71	0	6	0
Blackstrap Lake SK	-	-	143	0	7	0

Table 7.2. Localities of the collection sites of *D. andersoni* nymphs and *D. variabilis* immatures, and the number of ticks positive for *Arsenophonus* by PCR.

The primers correspond to nucleotide positions 554 to 573 and 1242 to 1263, respectively, of the 16S rDNA sequence from *Escherichia coli* (GenBank accession number J01859). The reverse primer was designed to be specific for *Arsenophonus*, whereas the forward primer is useful for amplifying the 16S rDNA from all bacteria. PCRs were performed in a thermocycler using reaction volumes of 25 µl containing 200 µM of each dNTP, 2 mM $MgCl_2$, 25 pmol (1 µM) of each primer, 0.5U of recombinant *Taq* DNA polymerase (Fermentas), 2.5µl 10x PCR buffer with KCl (Fermentas), and 2 µl of gDNA. A negative control (i.e. without gDNA) was included in each PCR run. The PCR conditions used were: 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and a final extension at 74°C for 5 min. Individual 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.

The amplicons of eight *Arsenophonus*-positive *D. andersoni* gDNA samples and the single *Arsenophonus*-positive *D. variabilis* gDNA sample were column-purified using the MinElute PCR purification kit (Qiagen) and subjected to automated DNA sequencing with the forward and reverse primers in separate reactions. The nucleotide sequences reported in this chapter have been deposited in the EMBL, GenBank and DDJB databases under the accession numbers FN667675 and FN667676.

The 16S rDNA sequences (653 bp) of the nine representative *Arsenophonus*-positive ticks were manually aligned and a BLAST search was performed to determine sequence similarity to those of *Dermacentor*-associated *Arsenophonus* (GenBank accession numbers AY265341 to AY265348) (Grindle *et al.* 2003) and several insect-associated *Arsenophonus* (GenBank accession numbers AY264674, AY264669, DQ508172, U91786, DQ314778, AY264664, AY264672, AY264677)

Isolate	Nucleotide position[a]																								
	613	632	647	648	673	690	700	720	736	746	750	788	820	830	838	916	928	973	1031	1033	1048	1093	1098	1113
AY265341	-	-	G	A	A	-	-	G	A	T	C	-	-	-	-	A	G	T	G	T	G	G	T	G
AY265342	-	-	-	A	A	-	-	C	A	T	-	-	C	-	-	A	A	C	G	T	A	A	T	A
AY265343	A	C	-	G	A	G	C	G	G	T	-	-	C	-	-	G	A	T	G	T	A	A	C	A
AY265344	-	-	-	A	G	-	-	G	A	T	-	-	C	-	-	A	A	T	G	T	A	A	T	A
AY265345	-	-	-	A	A	-	-	G	A	T	-	G	-	-	-	A	A	T	G	T	A	A	T	A
AY265346	-	-	-	A	A	-	-	G	A	T	-	G	-	A	-	A	A	T	G	T	A	A	T	A
AY265347	-	-	-	A	A	-	-	G	A	T	-	-	C	-	-	A	A	T	G	T	A	A	T	A
AY265348	-	-	-	A	A	-	-	G	A	T	-	G	C	-	C	A	A	T	G	C	A	T	T	A
D. andersoni[b]	-	-	-	A	A	-	-	G	A	C	-	-	-	-	-	A	A	T	A	T	A	A	T	A
D. variabilis[b]	-	-	-	A	A	-	-	G	A	C	-	-	-	-	-	A	A	T	A	T	A	A	T	A

[a] Relative to *Escherichia coli* 16S sequence (Accession number J01859)

[b] From this study

A dash (-) represents a deletion in a sequence.

Table 7.3. Comparison of variable nucleotide positions in 16S rDNA sequences of *Arsenophonus* isolates from *D. variabilis* in Indiana, USA (accession numbers AY265341 to AY265348; Grindle *et al*. 2003), and from Canadian populations of *D. andersoni* and *D. variabilis*.

(Hypša & Dale 1997, Thao & Baumann 2004, Trowbridge *et al.* 2006, Sorfová *et al.* 2008). A neighbor-joining tree was constructed using PAUP version 4.0b10 (Swofford 2003) (Fig. 7.1), with the 16S rDNA sequence of *Providencia stuartii* (accession number AF008581) used as an outgroup, based on the phylogenetic analyses of Trowbridge *et al*. (2006).

7.4. Results

Fifty-two of 822 adult tick gDNA samples produced amplicons (Table 7.1) and were of the expected size (≈700 bp) on agarose gels. These PCR-positive samples were derived from only one (0.2%) *D. variabilis* (n = 484) and 51 (15%) *D. andersoni* (n = 338) adults. There was no significant difference in the proportion of males to females infected with *Arsenophonus* (χ^2_1 = 0.94, P > 0.05). There was no difference in the 16S rDNA sequence of the nine representative amplicons. No amplicons were produced from the larval *D. variabilis* or from the nymphs of *D. andersoni* and *D. variabilis*. A BLAST search of these sequences revealed 99.5% similarity to the sequence of an *Arsenophonus* endosymbiont of *D. variabilis* (isolate Dv17.10.1; GenBank accession number AY265345; Grindle *et al.* 2003).

7.5. Discussion

The 16S rDNA sequences of nine representative amplicons, including the one from *D. variabilis*, were identical to one another, but differed in sequence when compared to those of *Arsenophonus* from previous studies. The closest match (99.5% genetic similarity) was to the sequence of an *Arsenophonus* endosymbiont of *D. variabilis* (Grindle *et al.* 2003).

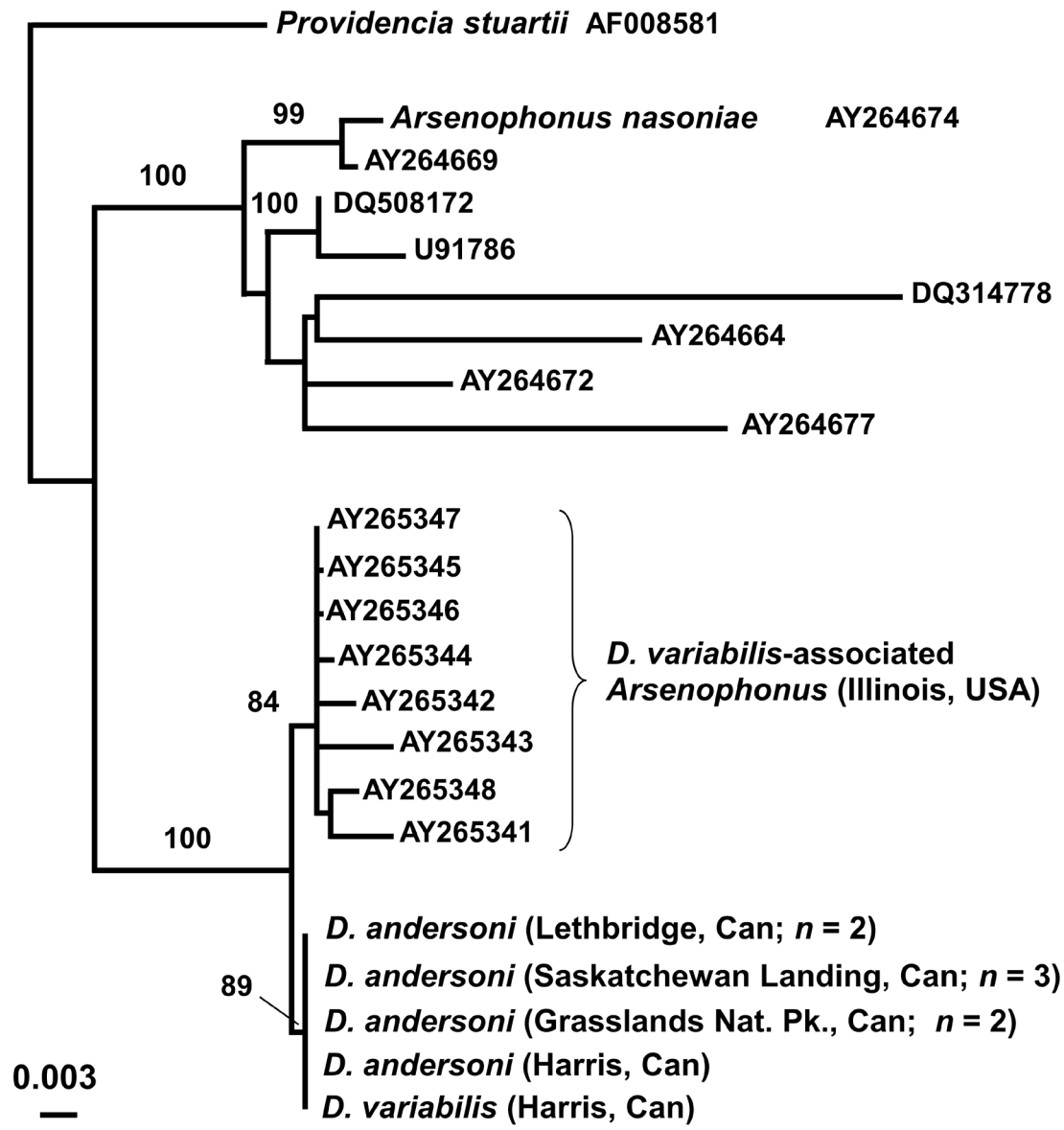

Fig 7.1. Neighbor-joining tree depicting the relationships of *Arsenophonus* from Canadian populations of *D. andersoni* and *D. variabilis*, *D. variabilis* from Indiana, USA, and several insect-associated species based on analyses of 16S rDNA sequence data. *Providencia stuartii* (AF008581; Mollet *et al.* 1997) was included used as an outgroup in the phylogenetic analyses.

Nonetheless, the *Arsenophonus* sequences obtained in the present study differed at three nucleotide positions when compared to the sequence of isolate Dv17.10.1 (Table 7.3). In addition, there were two positions in the sequence alignment (positions 746 and 1031; Table 7.3) where the *Arsenophonus*-type bacterium in *D. andersoni* and one *D. variabilis* differed from all other isolates of *Arsenophonus* reported from ticks.

The results of the phylogenetic analysis (Fig. 7.1) revealed that the *Arsenophonus* detected in ticks form a clade to the exclusion of those in insects, which is consistent with the findings of Trowbridge *et al*. (2006). In addition, *Arsenophonus* in *D. variabilis* from Indiana (USA) formed a clade with very strong statistical support (bootstrap value of 100%) to the exclusion of the *Arsenophonus* in *D. andersoni* from Alberta and Saskatchewan (Canada). In eastern and central USA, *D. variabilis* is sympatric with *A. americanum* (see Merten and Durden 2000). Clay *et al*. (2008) found that the *Arsenophonus* in *A. americanum* was identical in sequence to that of an isolate of *Arsenophonus* in *D. variabilis* from Indiana (isolate DvL3.2; Grindle *et al*. 2003), indicating that this *Arsenophonus* species is not specific for either tick species. Although the prevalence of *Arsenophonus* in *D. variabilis* from eastern USA was not indicated by Grindle *et al*. (2003), these bacteria in *A. americanum* occur at a prevalence of 0-90% (Clay *et al.* 2008). Surprisingly, we did not detect *Arsenophonus* in *D. variabilis* from several localities in Saskatchewan, Manitoba or Ontario. The single exception was one *D. variabilis* adult from Harris (SK) that was infected with *Arsenophonus*-type bacteria identical in sequence to those in one *D. andersoni* adult from Harris and another seven in *D. andersoni* adults from three localities in southern Saskatchewan and Alberta (Table 7.1). Also, no sequence variation was detected among the *Arsenophonus* in *D. andersoni* collected from Lethbridge (AB) and Harris (SK), localities that are 430 km apart. These results suggest that the *Arsenophonus* in *D.*

andersoni in south-western Canada may represent a different species to the *Arsenophonus*-type bacteria in *D. variabilis* and *A. americanum* in eastern USA, and that this species can also occur in *D. variabilis* in localities where it is sympatric with *D. andersoni*.

The presence of the same *Arsenophonus* species in *D. andersoni* and *D. variabilis* from a locality where the tick species are sympatric suggests horizontal transmission of the bacterium. There is evidence of horizontal transmission in some species of insect-associated *Arsenophonus* (Thao & Baumann 2004, Dale *et al.* 2006). It is known that *A. nasoniae* can be transmitted both vertically and horizontally from one wasp host to another (Skinner 1985), while other insect-associated *Arsenophonus* can be transmitted transovarially (Hypša & Dale 1997). Vertical transmission is known to occur in *D. variabilis* and *A. americanum* because *Arsenophonus*-like bacteria have been detected in larvae reared from engorged female ticks (Grindle *et al.* 2003, Clay *et al.* 2008). Thus, *Dermacentor*-associated *Arsenophonus* may also be transmitted from one host to another by two different modes of transmission.

Most *Arsenophonus* species are considered to be secondary symbionts (i.e. not essential for survival of the host) (Hypša & Dale 1997, Thao & Baumann 2004, Dale *et al.* 2006, Trowbridge *et al.* 2006). However, it is well established that *A. nasoniae* infection in females of the parasitoid wasp *Nasonia vitripennis* increases the mortality of males during the egg stage, resulting in the production of predominantly female offspring (Skinner 1985). This "son-killer trait" leads to a sex-ratio distortion in *N. vitripennis*. In contrast, there is evidence that this sex-ratio distortion does not occur in *A. americanum* as a consequence of *Arsenophonus* infection (Clay *et al.* 2008). Rather, infection may affect the microbial community structure within individual ticks and populations of ticks. For example, Clay *et al*. (2008) found that there was a negative correlation of the infection rates of *Arsenophonus* and spotted fever group *Rickettsia* in

populations of *A. americanum*, indicating that infection with one endosymbiotic species may prevent or reduce infection by other bacteria. The *Arsenophonus* found in *D. andersoni* is also likely a secondary symbiont, since relatively few ticks were infected at each locality, and there was no difference between sexes in the proportion of infected ticks. Further investigation is required to determine if there is any correlation of infection rates of *Arsenophonus* relative other bacterial species within *D. andersoni*.

In conclusion, this chapter represents the first report of *Arsenophonus*-type bacteria in *D. andersoni.* The results suggest that this species of *Arsenophonus* can be transmitted to *D. variabilis* where it is sympatric with *D. andersoni*. Phylogenetic analyses show that *Arsenophonus* found in *D. variabilis* and *A. americanum* in eastern USA are genetically distinct from, and may represent a different species to, the *Arsenophonus*-type bacteria found in *D. andersoni* and *D. variabilis* in western Canada.

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Chapter 8. Prevalence of *Anaplasma bovis* in *Dermacentor andersoni* from Saskatchewan Landing Provincial Park and in *D. variabilis* from Blackstrap Provincial Park and Saskatchewan Landing Provincial Park[7]

8.1. Abstract

The aim of this chapter was to use molecular techniques to determine if *Anaplasma* were present in *D. andersoni* and *D. variabilis* at localities near their northern distributional limits in Saskatchewan. Nested PCR analyses of the bacterial 16S rRNA gene were conducted on the total genomic DNA of 109 *D. andersoni* and 327 *D. variabilis* individuals. Single strand conformation polymorphism analysis and DNA sequencing of the 11 PCR-positive amplicons from *D. andersoni* individuals revealed the presence of three species of bacteria, none of which have been previously reported in *D. andersoni*. Although *A. marginale* was not identified in *D. andersoni* adults or nymphs, a novel genotype of *A. bovis* was detected in eight individuals. This discovery represents the first report of *A. bovis* in Canada. The potential implications of this finding with respect to animal health and anaplasmosis surveillance in Canada are discussed. The other two bacterial species detected were genetically similar to "*Candidatus* Midichloria mitochondrii" and *Ignatzschineria larvae*, the latter of which has been associated with human disease in Europe. *Anaplasma* sp.,*Wolbachia* sp. and an unidentified organism of the order Rickettsiales, were also detected in a single *D. variabilis* adult each. Seventy-five *D. variabilis* immatures were also found to be positive by PCR; however, SSCP analyses and DNA sequencing revealed that none of the amplicons were derived from species of *Anaplasma*. No *D. variabilis* individuals were infected with *A. bovis*.

[7] Part of this chapter was published in the following paper:
Dergousoff, S. J. and N. B. Chilton. 2011. Novel genotypes of *Anaplasma bovis*, "*Candidatus* Midichloria" sp. and *Ignatzschineria* sp. in the Rocky Mountain wood tick, *Dermacentor andersoni*. With permission from Elsevier.

8.2. Introduction

Anaplasma (order Rickettsiales, family Anaplasmataceae) are obligate intracellular Alphaproteobacteria that are transmitted to mammals mainly by ticks (Rymaszewska & Grenda 2008). Four species, *A. marginale*, *A. centrale*, *A. ovis* and *A. bovis*, are the causative agents of anaplasmosis, a disease that occurs worldwide in tropical and subtropical areas and has a significant impact on livestock production (Kocan *et al.* 2003, Rymaszewska & Grenda 2008, Kocan *et al.* 2010).

Bovine anaplasmosis is enzootic in many parts of the USA, including some states bordering western Canada (Kocan *et al.* 2003, Kocan *et al.* 2010). The Rocky Mountain wood tick, *Dermacentor andersoni*, is the major vector of *A. marginale*, the causative agent of bovine anaplasmosis in northwestern USA (Kocan *et al.* 2003, Kocan *et al.* 2010). Although adult *D. andersoni* from western Canada have been shown to be competent vectors for the transmission of *A. marginale* to cattle (Scoles *et al.* 2006, Lankester *et al.* 2007), there have been only a few isolated cases of bovine anaplasmosis in Canada prior to 2008, and these were subsequently eradicated (Whiting 2005, Lankester *et al.* 2007). Thus, Canadian cattle herds have been considered free of the disease (Whiting 2005). However, from 2008 to 2011, a number of cattle were positive for infection with *A. marginale*, as determined by the standard testing methods of the MSP5 cELISA and PCR (Howden 2010, CFIA 2011). Outbreaks of bovine anaplasmosis in Canada, including two occurrences in Saskatchewan, have usually been associated with the importation of infected animals from the USA (Whiting 2005, CFIA 2006). However, the distribution of bovine anaplasmosis may change as a consequence of range expansion by the vectors, such as *D. andersoni*, a tick species that appears to be expanding its distribution eastwards and northwards in Saskatchewan (Chapter 3). Therefore, the aim of the present study

was to use molecular techniques to test for the presence of *Anaplasma* in *D. andersoni* and *D. variabilis* adults and immatures from a locality near the northeastern distributional limit of this tick species in Saskatchewan.

8.3. Methods

8.3.1. Collection of ticks.

A total of 109 *D. andersoni*, 100 adults (22 males and 78 females) and nine nymphs (Table 8.1), were collected from Saskatchewan Landing Provincial Park (50.6592N, 108.0012W) in southern Saskatchewan. Adult ticks were collected in May of 2005 by flagging grass and shrubs from coulees and ridges on both sides of the lake. Nymphs were collected from deer mice (*Peromyscus maniculatus*) and meadow voles (*Microtus pennsylvanicus*) captured in snap-traps during June and July of 2008 and April of 2009. One hundred *D. variabilis* adults were collected by flagging at Saskatchewan Landing Provincial Park in May of 2005. In addition, 214 larvae and 13 nymphs were collected off deer mice, meadow voles, and western jumping mice (*Zapus princeps*) trapped during June and July of 2008 and April of 2009 from Saskatchewan Landing Provincial Park. Immature *D. variabilis* were also collected off deer mice, meadow voles, southern red-backed voles (*Myodes gapperi*), and western jumping mice from Blackstrap Provincial Park (51.79760N, 106.45833W) in May to July of 2009 (Table 8.1). The morphological identity of each tick was confirmed using PCR-based assays described in previous chapters (Chapters 2 and 6).

Locality	*D. andersoni*			*D. variabilis*				
	Adults (2005)	Nymphs (2008)	Nymphs (2009)	Adults (2005)	Nymphs (2008)	Larvae (2008)	Larvae (2009)	Nymphs (2009)
Saskatchewan Landing	100	5	4	100	6	67	4	0
Blackstrap	0	0	0	n/t[8]	n/t	n/t	143	7

Table 8.1. Number of ticks screened for *Anaplasma* and *Ehrlichia* from Saskatchewan Landing Provincial Park and Blackstrap Provincial Park.

[8] Not tested

8.3.2. DNA preparation.

Total genomic DNA (gDNA) was extracted and purified from each tick using a modification of the protocol of the DNeasy Tissue Kit™ (Qiagen, Valencia, USA; see Chapter 2). Individual ticks were placed in 1.5ml micropestle tubes (Kontes), to which 180 µl of ATL buffer (Qiagen) was added. Ticks were homogenized by grinding with micropestles (Kontes) attached to a cordless drill. Proteinase K (20 µl @ 15 µg/µl) was added to the homogenate. Samples were incubated for 16 hours at 55°C. The gDNA was purified according to the DNeasy tissue kit protocol, except that gDNA was eluted twice from the spin columns using 50 µl of AE buffer. The two elutions derived from the same tick were combined in a single tube and stored at -20°C.

8.3.3. PCR of 16S rRNA gene.

The presence of *Anaplasma* and *Ehrlichia* in *D. andersoni* was tested using a nested PCR (nPCR). The gDNA of all ticks collected prior to 2009 were subjected to nPCR using the following protocol: The first phase of the nPCR targeted 1,462 bp of the 16S rRNA gene of *Anaplasma* and *Ehrlichia* using primers EC9 (5' – TAC CTT GTT ACG ACT T – 3') and EC12A (5' – TGA TCC TGG CTC AGA ACG AAC G – 3') (Kawahara *et al.* 2006). PCR reactions were carried out in 25µl volumes containing 200 µM of each dNTP, 3 mM $MgCl_2$, 25 pmol (1 µM) of each primer, 0.5U of recombinant *Taq* DNA polymerase (Fermentas, Burlington, Canada), 2.5µl 10x PCR buffer with $(NH_4)_2SO_4$ (Fermentas), and 2 µl of gDNA. A negative control (i.e. without gDNA) was included in each set of reactions. PCRs were performed in a thermocycler (iCycler™, Bio-Rad; Hercules, USA) using the following conditions: 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 30 s, and a final

extension at 72°C for 5 min. Two internal primers, PER1 (5' – TTT ATC GCT ATT AGA TGA GCC TAT G – 3') and PER2 (5' – CTC TAC ACT AGG AAT TCC GCT AT – 3') were used in phase two of the nPCR to amplify 451 bp of the 16S rRNA gene of all species of *Anaplasma* and *Ehrlichia* (Munderloh *et al.* 1996). PCRs were conducted as in the first phase, except that 1 µl of the purified products from phase one, including those of the negative controls, were used as the DNA templates and the annealing temperature was raised to 56°C. Additional negative controls were included in each set of PCRs. For those immature ticks collected in 2009 (four *D. andersoni* nymphs, four *D. variabilis* larvae from Saskatchewan Landing Provincial Park, and seven *D. variabilis* nymphs and 143 *D. variabilis* larvae from Blackstrap Provincial Park). This was done to attempt to increase the sensitivity of the PCR assay. In the first phase of the nPCR, reactions were carried out as described above, except that iTaq (Bio-Rad, Hercules, CA, USA) was used and the $MgCl_2$ concentration was lowered to 2.5mM. In addition, only 1µl of gDNA was used as template in the PCR reaction. The number of amplification cycles was reduced to 30, but the annealing temperature was increased to 59°C. The second phase of the nPCR also used 1µl of the purified products as the template and the PCR conditions used were 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 5 min. Amplicons were subjected to electrophoresis on SYBR® Safe-stained 1.5% agarose-TBE gels and their banding patterns were visualized by UV transillumination.

8.3.4. Single-strand conformation polymorphism (SSCP) analyses.

This mutation scanning technique was used on all positive amplicons (n=93) derived from the second phase of the nPCR to differentially display DNA sequences that differ by one or more

nucleotides (Gasser *et al.*, 2006). Each amplicon (1-5 µl) was mixed with DNase-free water (1-4 µl) and 5 µl of loading buffer (Gel Tracking Dye™; Promega, Madison, USA). Samples were denatured at 95˚C for 5 min then snap cooled in ice water for 5 min prior to loading into individual wells of precast GMA™ S-50 gels (Elchrom Scientific, Cham, Switzerland) that had been placed a horizontal SEA2000™ apparatus (Elchrom Scientific) containing 1x TAE buffer. A temperature controlled circulating water bath connected to the electrophoretic apparatus maintained a constant temperature of 7.4°C for 18 h, while the samples were subjected to electrophoresis at 74 V. Gels were stained for 30 min with SYBR® Gold[f] (Invitrogen, Carlsbad, USA) then rinsed in distilled water and photographed.

8.3.5. DNA sequence analyses and nucleotide sequence accession numbers.

All PCR-positive samples were column-purified (MinElute DNA purification kit; Qiagen) and subjected to automated DNA sequencing using primers PER1 and PER2 in separate reactions. A BLAST search was performed to determine sequence similarity of each sequence with those of other bacteria deposited in GenBank. Sequences of representative samples obtained in the present study have been deposited in GenBank under accession numbers FN665374, FN665375 and FR667203. The phylogenetic relationships of the three bacterial species in *D. andersoni* were determined using neighbor-joining analyses of the 16S rRNA sequences of representative taxa from different bacteria groups.

8.4. Results

8.4.1. PCR detection of bacterial 16S rDNA

No bands were detected on agarose gels for any of the negative control samples from either the first or second phase of the nPCR. Amplicons were produced from the second phase of the nPCR for 11 (10%) of the 105 *D. andersoni* samples collected in 2005 and 2008 (i.e. from one nymph, four males and six females) nPCR. The amplicon from one tick was approximately 475 bp, while the amplicons from nine other samples were approximately 450 bp in size. In addition, the amplicon of another *D. andersoni* individual contained two bands (450 and 475 bp), suggesting the presence of at least two types of bacteria. However, only a single bacterial species could be detected in this amplicon when subjected to DNA sequencing. The results of the SSCP analyses (Fig. 8.1) showed that there were three distinct banding patterns (i.e. profiles) among the 10 samples that produced a single band on agarose gels. DNA sequencing of these amplicons revealed that each comprised the 16S rDNA sequence of a single bacterial species; however, the sequences of each amplicon were not identical. Amplicons with the same SSCP profile had an identical sequence, whereas those with different banding patterns had different DNA sequences. Two of the four *D. andersoni* nymphs collected in 2009 produced an amplicon of approximately 475bp when subjected to nPCR with the modified protocol. SSCP analysis of the two amplicons produced two different banding patterns, neither of which matched SSCP profiles derived from *D. andersoni* nymphs or adults collected in previous years.

The original nPCR protocol produced amplicons on an agarose gel, one of which was estimated to be ~500bp and the other two ~450 bp, from three out of the 100 *D. variabilis* adults collected in 2005. Each of these amplicons produced different SSCP banding patterns, none of which matched any SSCP banding patterns produced by amplicons from *D. andersoni*.

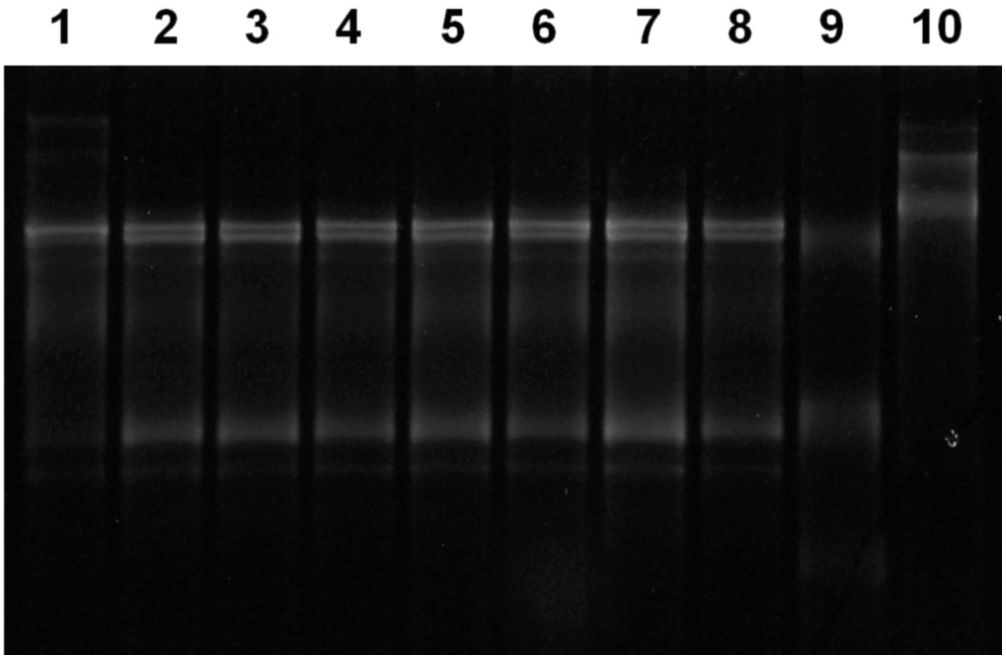

Fig. 8.1. SSCP analysis of the bacterial 16S rDNA amplicons from nPCR of gDNA from *D. andersoni*. Banding patterns in Lanes 1-8 represent rDNA from *A. bovis*, lane 9 from "*Candidatus* Midichloria mitochondrii", and lane 10 from *Ignatzschineria* sp.

This suggested the presence of three unique types of bacteria. No amplicon was detected from gDNA of the 67 *D. variabilis* larvae or six nymphs from Saskatchewan Landing Provincial Park.

Three of the seven *D. variabilis* nymphs and 71 of the 143 larvae collected from Blackstrap Provincial Park in 2009 were positive by the modified nPCR. All amplicons were approximately 475 bp in size, except for a single amplicon that was approximately 450bp. However, none of the four *D. variabilis* larvae collected from Saskatchewan Landing Provincial Park in 2009 were positive. SSCP analysis of each amplicon resulted in many different SSCP profiles, none of which were identical to those from *D. andersoni* or *D. variabilis* from Saskatchewan Landing Provincial Park.

8.4.2. Phylogenetic analyses

The 16S rDNA sequences of all bacteria detected in *D. andersoni* were not identical to any sequence deposited in GenBank. The sequences (404 bp) of eight amplicons were most similar (97-99%) to the 16S sequences of species within the genus *Anaplasma*. Results of a phylogenetic analysis (Fig. 8.2) revealed that the *Anaplasma* in *D. andersoni* was placed within a clade consisting of only *A. bovis* genotypes. However, the 16S rDNA sequence of the *A. bovis*-like organism in *D. andersoni* was unique because it differed by five to seven nucleotides when compared to the sequences of all other *A. bovis* genotypes. The rDNA sequence (404 bp) of another bacterium from a single *D. andersoni* male was 98.3% similar (i.e. 7 bp differences) to the 16S rDNA sequence of an uncultured bacterium of the order Rickettsiales (accession no. AF497583) derived from a tick (*Haemaphysalis wellingtoni*) in Thailand (Parola *et al.* 2003). This bacterial species in *D. andersoni* falls within a clade (Fig. 8.2) that includes "*Candidatus* Midichloria mitochondrii" and other unnamed Rickettsiales. The 16S rDNA sequences of the

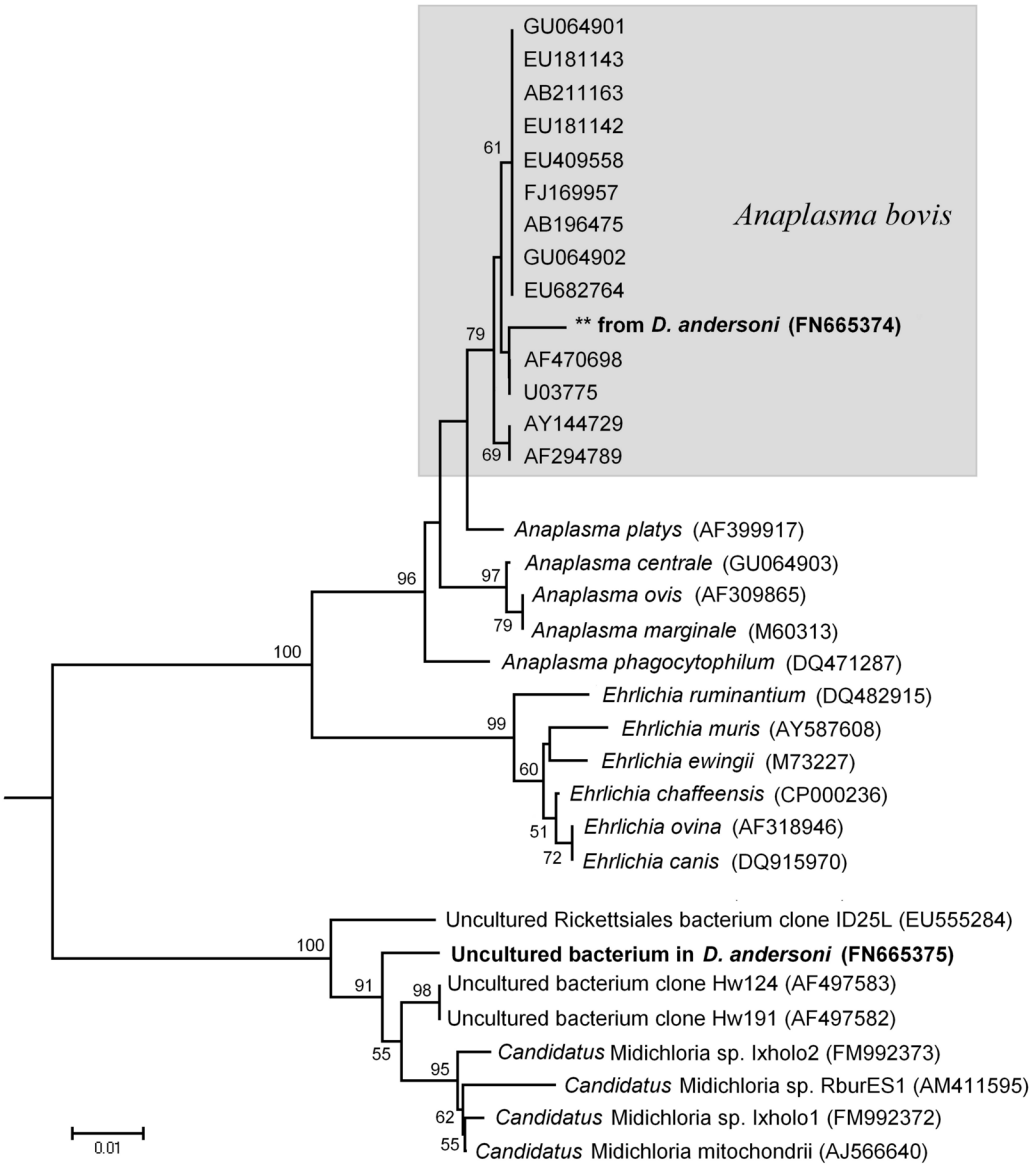

Fig. 8.2. Phylogenetic tree depicting the position of the two different Alphaproteobacteria detected in *D. andersoni* relative to representative taxa within the order Rickettsiales based on a neighbor-joining analysis of the 16S rRNA gene. The GenBank accession number for each sequence is indicated (in brackets). Numbers above branches are bootstrap values. *Rickettsia rickettsii* (accession number CP000766) was used as the outgroup (Epis *et al.* 2008). The bar represents 0.01 inferred substitutions per nucleotide site.

third bacterial species from one male and one female tick were identical to one another and 95.8% (413 of 431 bp) similar to the 16S sequence of *Ignatzschineria larvae*. It was genetically most similar to the unpublished sequence of an *Ignatzschineria* sp. found in a swine effluent holding pit (accession no. DQ337535). This bacterium falls within a clade (Fig. 8.3) comprised of genotypes of *Ignatzschineria* and other unnamed Gammaproteobacteria.

Two of the amplicons produced from gDNA of *D. andersoni* nymphs that were collected in 2009 were subjected to DNA sequencing, which revealed the presence of two different gammaproteobacteria. The sequence of one amplicon was 98% similar (355/362) to *Lysobacter* sp. (GenBank accession no. EF072637), and the sequence of the other amplicon was 97% (348/358 bp) similar to *Luteimonas* sp. (GenBank accession no. FJ50462).

The three amplicons produced from *D. variabilis* adults from Saskatchewan Landing Provincial Park were subjected to DNA sequencing and determined to be derived from different species of Alphaproteobacteria. The 16S rDNA sequence of one amplicon was most similar to an uncultured bacterium (GenBank accession no. AF497583), which was also the closest match to a sequence derived from a single *D. andersoni* male that fell within a clade that includes "*Candidatus* Midichloria mitochondrii". However, there was only a 94% (372/397 bp) match between the sequence from *D. variabilis* and AF497583, whereas the sequence derived from *D. andersoni* was 98% similar to that of AF497583. The second 16S rDNA sequence (404 bp) was an exact match to the sequence of a *Wolbachia* symbiont of *Koreoculio minutissimus* (GenBank accession no. AB604664). Based on a 452 bp, the 16S rDNA sequence of an amplicon derived from a third *D. variabilis* adult was most similar to a species of *Anaplasma*. This sequence was the same as those of *A. marginale*, *A. centrale* and *A. phagocytophilum*, excluding four ambiguous sites.

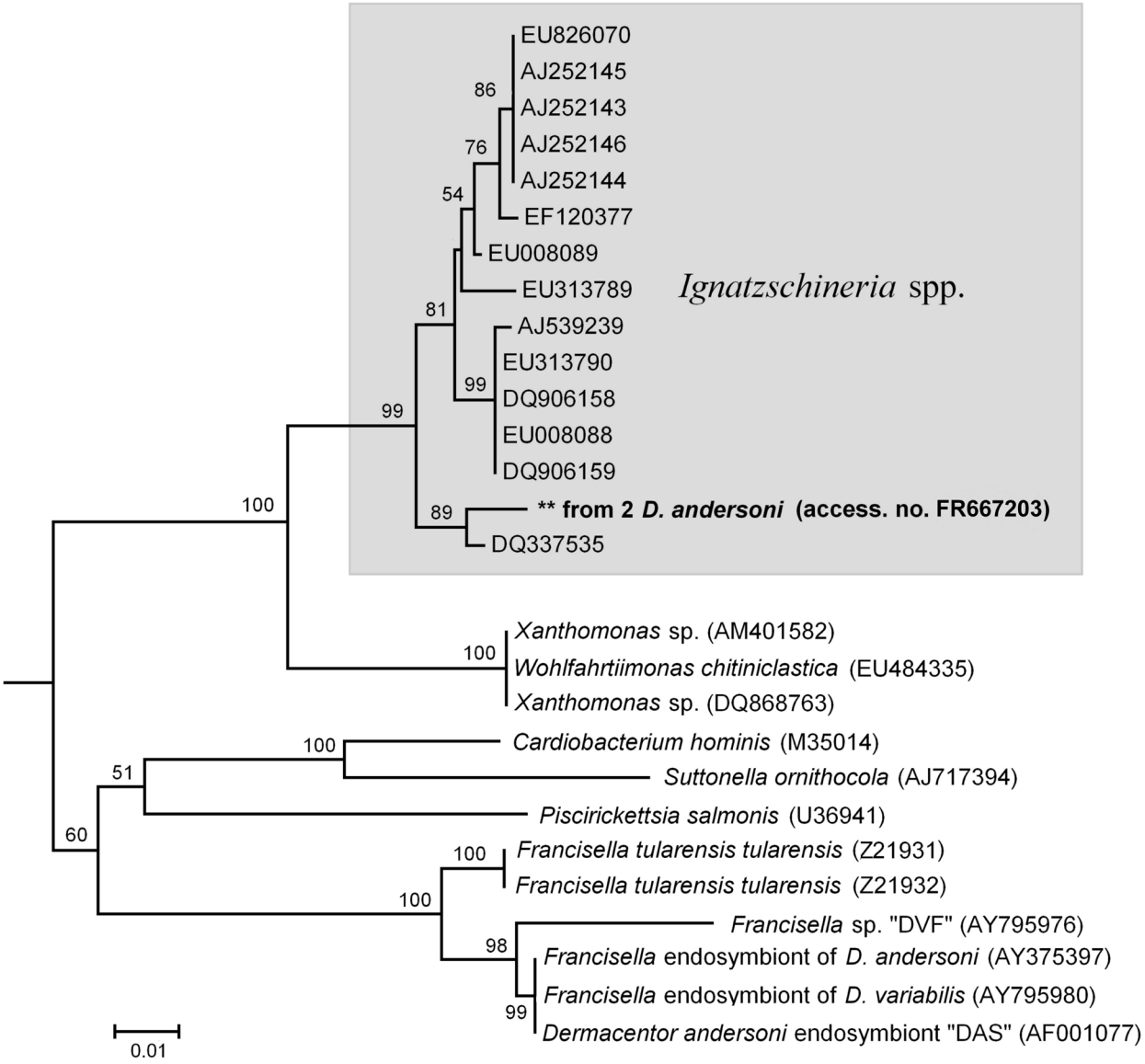

Fig. 8.3. Phylogenetic tree depicting the position of the gammaproteobacterium detected in two adult *D. andersoni* relative to representative taxa of Gammaproteobacteria based on a neighbor-joining analysis of the 16S rRNA gene. The GenBank accession number for each sequence is indicated (in brackets). Numbers above branches are bootstrap values. *Legionella pneumophila* (accession number AF129523) was used as the outgroup. The bar represents 0.01 inferred substitutions per nucleotide site.

The sequence was determined for 27 representative amplicons of the different SSCP profiles produced from *D. variabilis* larvae and nymphs collected at Blackstrap Provincial Park. Sequences of each amplicon (354 to 432 nucleotides) were compared to those in GenBank. BLAST searches of the ~475 bp amplicons revealed varying degrees of sequence identity to 16S rDNA sequences of at least five different genera of Gammaproteobacteria, including *Pseudomonas*, *Stenotrophomonas*, *Xanthomonas*, *Pseudoxanthomonas*, and *Luteimonas* and one of Deltaproteobacteria, *Geobacter*. The 16S rDNA sequence of the amplicon that was ~450 bp was 94% similar to an uncultured alphaproteobacterium.

8.5. Discussion

There was no evidence of *A. marginale* in any of the nine *D. andersoni* nymphs collected from small rodents, or the 100 *D. andersoni* adults collected by flagging. However, *A. bovis* DNA was detected in eight *D. andersoni* (one nymph, two males and five females) using nPCR, SSCP and DNA sequencing of the 16S rRNA gene. Furthermore, the rDNA sequence of the *A. bovis* detected in the nymphal tick was identical to those of the eight adult ticks collected three years earlier, indicating that this organism may be endemic at a low prevalence in the tick population and, presumably in one or more suitable vertebrate hosts. As far as I am aware, this represents the first published report of *A. bovis* in Canada and in ticks of the genus *Dermacentor*.

Anaplasma bovis has been detected previously in the genomic DNA of *Haemaphysalis longicornis* in Korea (Oh *et al.* 2009, Lee & Chae 2010), Japan (Kawahara *et al.* 2006) and China (Sun *et al.* 2008), *H. concinna* in Russia (Shpynov *et al.* 2006), *H. lagrangei* in Thailand (Parola *et al.* 2003), *H. megaspinosa* in Japan (Yoshimoto *et al.* 2010), *Rhipicephalus evertsi* in South Africa (Tonetti *et al.* 2009) and *R. turanicus* in Israel (Harrus *et al.* 2010). *Amblyomma*

variegatum and *R. appendiculatus* have also been implicated as vectors of *A. bovis* in Africa (Scott 1994) and *Hyalomma* sp. in Iran (Donatien & Lestoquard. 1936). *A. bovis* infections of mammals are most commonly reported in ungulates, such as cattle and buffalo from Africa, the Middle East, South America, and Japan (Uilenberg 1993, Ooshiro *et al.* 2008), and in deer from Japan (Kawahara *et al.* 2006) and South Korea (Lee *et al.* 2009). The DNA of *A. bovis* has also been detected in cottontail rabbits (Goethert & Telford 2003) and raccoons (Sashika *et al.* 2011). In cattle, *A. bovis* infects monocytes (Uilenberg 1993, Scott 1994). Infection is often asymptomatic (Stewart 1992), but *A. bovis* can cause a variety of clinical symptoms, including fever and reduced body weight and possibly death of naïve or stressed cattle (Uilenberg 1993, Scott 1994). However, the 16S rDNA sequences of the *A. bovis* in *D. andersoni* were genetically unique (differences of 5 to 7 nucleotides) when compared to the sequences of *A. bovis* detected in other studies, including the strain found in cottontail rabbits in the USA (Goethert & Telford 2003). It will be important to determine if this novel genotype of *A. bovis* in *D. andersoni* is transmissible to cattle, if it has an impact on the health of livestock, and its prevalence in different geographical areas.

The discovery of *A. bovis* DNA in host-seeking *D. andersoni* adults may have important implications for anaplasmosis surveillance in Canada, the aim of which is to determine whether cattle and bison within Canada are infected with *A. marginale*. There is a significant economic cost (i.e. reduced animal production and compensation costs for quarantine and destruction of infected livestock) associated with diagnosed cases of bovine anaplasmosis in Canada (Whiting 2005). Given this, it is essential that there are diagnostic tests that unequivocally distinguish between cattle infected with *A. marginale* from those infected with *A. bovis*, or other species within the family Anaplasmataceae. The diagnostic tests used to screen blood from cattle to

detect the presence of and/or exposure to *A. marginale* include the MSP5 competitive-inhibition enzyme-linked immunosorbent assay (cELISA) and a nested PCR of the *msp5* gene (Torioni de Echaide *et al.* 1998, Van Donkersgoed *et al.* 2004, Howden *et al*. 2010). The MSP5 protein is highly conserved in *A. marginale*, *A. centrale*, *A. ovis* and *A. phagocytophilum* (de la Fuente *et al.* 2005), but it has not been characterized in *A. bovis*. Studies have shown that some of the diagnostic tests used to detect animals infected with *A. marginale* cross-react with other *Anaplasma* species (Molloy *et al.* 1999, Dreher *et al.* 2005, Scoles *et al.* 2008). However, it is unknown if the tests used to identify *A. marginale* infected cattle and bison will also detect animals exposed to *A. bovis*. Therefore, more work is needed to determine if the presence of *A. bovis* in *D. andersoni* represents a complicating factor for the bovine anaplasmosis surveillance program in Canada.

The DNA of a bacterium within the genus *Anaplasma* was detected in a *D. variabilis* female from Saskatchewan Landing Provincial Park. The 452 bp sequence of this bacterium was identical to 16S rDNA sequences (in GenBank) of *A. marginale*, *A. centrale*, *A. phagocytophilum*, and *A. ovis*. The identity of this organism needs to determined using a larger portion of the 16S rRNA gene where there are sequence differences among species of *Anaplasma* (Inokuma *et al.* 2001, Oh *et al.* 2009). The DNA of two different Alphaproteobacteria was also detected in adult *D. variabilis* from Saskatchewan Landing Provincial Park. Both of these organisms likely represented endosymbionts; one belonged to the genus *Wolbachia* and the other was genetically similar to the bacterium from *D. andersoni* and belonged to a clade that included *Candidatus* Midichloria mitochondrii and other Rickettsiales.

Although the primers used in the nPCR were designed (Munderloh *et al.* 1996, Kawahara *et al.* 2006) to amplify the 16S rDNA sequences of *Anaplasma* and *Ehrlichia* species, the results

showed that they were able to amplify16S rDNA of two other bacterial species from the gDNA of *D. andersoni* adults and several other bacteria in *D. andersoni* and *D. variabilis* larvae and nymphs, depending on the DNA polymerase used in the PCR. The 16S rDNA sequence of one species found in a single male tick most closely resembled (98% similar) the 16S sequence of uncultured bacteria (strains Hw124 and Hw191) from two *Haemaphysalis wellingtoni* nymphs collected from red jungle fowl (*Gallus gallus*) in Thailand (Parola *et al.* 2003). Phylogenetic analyses of the 16S sequences of representative taxa within the order Rickettsiales revealed that this bacterial species falls within a clade that includes the bacterial strains Hw124 and Hw191 and the different strains of "*Candidatus* Midichloria mitochondrii" reported in several species of tick (Epis *et al.* 2008). This clade represents a sister group to the family Anaplasmataceae and appears to represent a novel family of Alphaproteobacteria (Epis *et al.* 2008), but the veterinary significance of the group remains to be determined. The bacterium detected in *D. andersoni* may also be a member of this genus given the 97% similarity in 16S rDNA sequence (i.e. 394 of 404 bp) to the "*Candidatus* Midichloria mitochondrii" strain in *Ixodes ricinus* (GenBank accession no. AJ566640). This would, therefore, represent the first report of such an organism in a North American species of *Dermacentor*.

The other bacterial species amplified by nPCR from the gDNA of one female and one male *D. andersoni* was a gammaproteobacterium. The16S rDNA sequence of this bacterium was 96% similar to the 16S sequence of *Ignatzschineria* (syn. *Schineria*) *larvae* and belonged within a clade comprising isolates of *Ignatzschineria*, to the exclusion of other Gammaproteobacteria. *Ignatzschineria larv*ae was first isolated from larvae of the parasitic fly *Wohlfahrtia magnifica* (Tóth *et al.* 2001). Therefore, our study probably represents the first report of the detection of *Ignatzschineria* in ticks. Given that *Ignatzschineria* has also been shown to be associated with

human infections in France (Roudiere *et al.* 2007), it will be important to determine if the *Ignatzschineria* sp. in *D. andersoni* is of animal and/or human health significance.

Many *D. andersoni* nymphs and *D. variabilis* larvae and nymphs were positive by the modified nPCR; however, most of the sequenced amplicons were derived from different gammaproteobacteria. A bacterium belonging to the class Alphaproteobacteria was detected in only a single *D. variabilis* larva when using the modified PCR protocol. The specificity of the nPCR was compromised following replacement of the DNA polymerase with a different type, even though the conditions were changed to be more stringent (i.e. increased $MgCl_2$ concentration, increased primer annealing temperatures, and reduced number of amplification cycles). This shows that changes to an assay that is meant to detect specific organisms must be made with caution.

In conclusion, novel genotypes of *A. bovis*, "*Candidatus* Midichloria" sp. (Alphaproteobacteria) and *Ignatzschineria* sp. (Gammaproteobacteria) were all amplified by nPCR from the gDNA of *D. andersoni*. In addition, a single *D. variabilis* adult was found to be infected with a species of *Anaplasma*, potentially one of *A. marginale*, *A. centrale*, *A. phagocytophilum*, or *A. ovis*. The four bacterial species could be readily distinguished from one another using SSCP analyses of the 16S rRNA gene. More work is needed to genetically characterize this novel genotype of *A. bovis* and to determine its prevalence, reservoir hosts, pathogenicity, and potential importance to the Canadian surveillance program for bovine anaplasmosis.

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Chapter 9. Comparison of the bacterial communities in *D. andersoni* and *D. variabilis*.

9.1. Abstract

Two important aims of my PhD research were to assess the diversity of the bacteria present in *D. andersoni* and *D. variabilis*, and to compare the microbial community composition of ticks from different localities near their northern distributional limits. In this chapter, I compared the diversity of bacterial species within adult *D. andersoni* and *D. variabilis*, as determined by molecular analyses conducted in previous chapters. I also determined if tick-borne microbial communities were structured or comprised of a random assemblage of species.

9.2. Introduction

In the previous four chapters of this thesis, molecular studies were conducted to determine the proportion of ticks from different populations that contained specific species of bacteria. However, each bacterial species does not exist in nature by itself, but occurs in a mixture of different organisms within an individual tick or tick species, that is, they are members of a bacterial community. Although ecologists have defined communities of free-living or parasitic organisms in many different ways, they are usually considered to represent an assemblage of different species of organisms 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. For example, Whittaker (1975) defined a community as "an assemblage of populations of plants, animals, bacteria and fungi that live in an environment and interact with one another, forming together a distinctive living system with its own composition, structure, environmental relations, development, and function." The structure of parasite communities has

often been examined at different hierarchical levels, which includes the infracommunity (Bush & Holmes 1986, Sousa 1994) (i.e. all parasites within a single host) and component community (Holmes & Price 1986) (i.e. all parasites within a collection of a single host species). For the purposes of this thesis, the different species of pathogenic and endosymbiotic bacteria present within an individual tick, and the bacteria present in a single tick species (i.e. in *D. andersoni* or in *D. variabilis*) will be considered as bacterial infracommunities and component communities, respectively.

Fundamental questions in community ecology include: do species within communities interact with one another?, and how do they respond to each other and to their environment? Interactions among species within a community can limit coexistence (species richness) within an assemblage and influence community structure (Ricklefs 2008). Comparative analyses of the composition of microbial communities in different tick populations can provide insights to the factors or mechanisms that structure communities. Consistent patterns among tick populations indicate common ecological determinants of community structure, whether they may be abiotic (i.e. environmental) or biotic (i.e. interactions among other microorganisms and/or the tick host). Analyses of component communities are useful to study interspecific competition, the regulation of biodiversity, the requirements for invasion and colonization of a host by different species, and the response of organisms to different environmental conditions.

Community structure and the interactions between members of a community have been investigated for many eukaryotic parasites (Poulin 1997), but this has received relatively little attention with respect to arthropod-borne microorganisms. Understanding the ecology microbial communities, particularly those in arthropod vectors, would provide insights into the interactions that affect colonization (i.e. the ability of a microbe to successfully establish in a vector) and,

therefore, affect the vectorial capacity of arthropod hosts. Thus, studies on parasite communities can be a useful way to look at the role of hosts as reservoirs or vectors in the transmission cycle of different microbial species.

It is evident that most communities, whether they involve free-living or parasitic organisms, are complex in structure (e.g. Zoetendal *et al.* 2004, Wu *et al.* 2005, Clay *et al.* 2006). It is not uncommon for different species of tick and their vertebrate hosts to be co-infected with multiple species of pathogenic and non-pathogenic microorganisms (Little *et al.* 1998, Varela *et al.* 2004, Steiner *et al.* 2008, Václav *et al.* 2010, Andreotti *et al.* 2011). There are several ways in which communities can be described and compared. Most studies on parasite communities (Holmes & Price 1986, Molloy *et al.* 1995, Muñoz & Cortés 2009), including those of arthropod-borne bacteria (Schabereiter-Gurtner *et al.* 2003, Clay *et al.* 2006, Clay *et al.* 2008, Jones *et al.* 2009, Heise *et al.* 2010), are primarily descriptive. In these studies, the criteria used to describe the composition of a community include species richness, species diversity, and species composition. Species richness, which is the total number of species present within the community, is usually evaluated in studies for groups of organisms that are taxonomically well known and that can be readily sampled. Species diversity, which takes into account the relative abundance of each species, can be calculated using a number of different indices (Krebs 1999, Maurer & McGill 2011). In contrast, the species composition of a community includes both the identity and relative abundance of particular organisms (Jost *et al.* 2011). Another important component is the interactions among community members, which for free-living organisms is often based on their trophic level (Menge & Sutherland 1976, Cousins 1991). All of the above criteria can be used to compare different parasite communities.

The structure of communities, including those of bacteria in arthropods (Guernaoui *et al.* 2011), have also been examined using more of a mechanistic approach (as defined by Schoener (Schoener 1986)). In such cases, the focus is on how infracommunities and component communities are organized. The co-occurrence of different species with overlapping niches can lead to competition for space and nutrients (Whittaker 1975, Hibbing *et al.* 2010). If these resources are limiting for the growth of co-occurring species that have similar growth requirements, this can lead to competitive exclusion and the loss of one of the species from that environment. Likewise, the composition of bacterial communities within individual ticks determines the potential interactions among the bacteria (Hibbing *et al.* 2010), and the interactions with their tick hosts. Thus, the species composition and order of colonization can affect community structure at a particular point in time. This is particularly important when considering the interactions of intracellular bacteria that occupy the same host cells where there is restricted space and a limited nutrient supply.

It is known that the presence of one bacterial species in an arthropod can influence the presence or absence of one or more bacterial species in that vector and/or host (Burgdorfer *et al.* 1981, Macaluso *et al.* 2002, de la Fuente *et al.* 2003). This, therefore, can have an impact on the community structure. Although ticks can be simultaneously infected with multiple species of *Rickettsia* (Carmichael & Fuerst 2010), a pre-existing infection in *D. variabilis* with an endosymbiotic *Rickettsia*, such as *R. montanensis*, has been shown to have an inhibitory effect on the transovarial transmission of a second rickettsial species (e.g. *R. rhipicephali*) (Macaluso *et al.* 2002). A similar mechanism has been suggested to account for the difference in prevalence of *Rickettsia rickettsii* on opposite sides of the Bitterroot Valley, where a low prevalence of *R. rickettsii* in *D. andersoni* is associated with a high prevalence of *R. peacockii* (Burgdorfer *et al.*

1981). There is also limited evidence that the composition of a microbial community within ticks can affect the acquisition and transmission of other pathogens in a positive manner (Clay & Fuqua 2010). For example, *Ixodes ricinus* immature were more likely to be infected with *Anaplasma* if they were co-infected with either *Borrelia lusitaniae* and/or *Rickettsia* (Václav *et al.* 2010).

The presence of a variety of species within a microbial community inside a host provides opportunities for interactions of the bacteria with the host and other bacteria. Although these interactions can have biological and epidemiological implications, the nature of these relationships is generally not well understood. Describing the diversity and composition of microbial communities within individual ticks, among ticks from different populations, and between tick species that live in the same geographical region is an important step in detecting potential interactions among microbial species and elucidating the factors or mechanisms that determine the structure of bacterial communities. Interactions among tick-borne bacteria that influence the prevalence of one or more bacterial species can be inferred through null model analyses of co-occurrence, which test for non-random patterns of species occurrence within a community (Clay *et al.* 2008).

The aim of this chapter was to describe and compare the bacterial communities within *D. andersoni* and *D. variabilis* at localities where these two tick species occur in sympatry and where their distributional ranges are allopatric with respect to one another. The combined data reported in chapters 5 to 8 were also used to determine if certain bacterial species occurred together more often than expected. In other words, are bacterial communities in the two tick species structured (i.e. occur in a predictable pattern), or represent a random assemblage of organisms. If the tick-borne microbial communities are structured, then this may be due to

competitive or facilitative interactions among microbes (Clay *et al.* 2006). Therefore, given that the presence of only specific bacteria in ticks (i.e. "observed" bacterial taxa) were examined from certain localities in this study, the data obtained in Chapters 5 to 8 were used to test the following five null hypotheses:

1) There is no difference in the mean species richness of the observed bacterial taxa within *D. andersoni* and *D. variabilis* adults, including those at localities where the two species occurred in sympatry.

2) There are no differences in the mean species richness of the bacterial communities in populations of *D. andersoni* adults from different localities, including those where *D. andersoni* is sympatric and allopatric with respect to *D. variabilis*.

3) There are no differences in the mean species richness of the bacterial communities in populations of *D. variabilis* adults from different localities, including those where *D. variabilis* is sympatric and allopatric with respect to *D. andersoni*.

4) There is no difference in the mean species richness of the observed bacterial taxa within *D. variabilis* larvae and adults from Saskatchewan Landing Provincial Park.

5) The observed patterns of species occurrence in individual ticks do not differ from the patterns of occurrence expected in a random assemblage.

9.3. Methods

The presence/absence data for *Rickettsia peacockii*, *R. montanensis* (chapter 5), the 10 types of *Francisella*-like endosymbionts (FLEs) (chapter 6), *Arsenophonus* sp. (chapter 7) and other bacteria (Chapter 8) in individual ticks from eight localities were used in this chapter to compare bacterial species diversity between *D. andersoni* and *D. variabilis*. In this chapter, each

FLE type was treated as a different species. Infection data for all bacteria in *D. andersoni* adults (n = 333) and *D. variabilis* adults (n = 423) from eight localities (for details see chapters 5 to 8) were combined in presence/absence matrices (not shown). In addition, *D. variabilis* larvae from Blackstrap Provincial Park (n = 143) and Saskatchewan Landing Provincial Park (n = 71) were also used to compare the microbial species richness of infracommunities between localities and between different tick life stages. The infection data for *D. andersoni* and *D. variabilis* nymphs were not included in these analyses because of small sample sizes, and no *D. andersoni* larvae were collected during this study. Furthermore, analyses to examine species occurrence patterns could not be performed on the data for *D. variabilis* larvae because there were no instances of multiple infections (i.e. of different bacterial species) in these ticks.

Calculations were made of the mean (± S.D.) number of bacterial types in each tick species. The non-parametric Kruskal-Wallis one way analysis of variance test was used to compare the mean number of bacteria in *D. andersoni* adults from five localities in Alberta and Saskatchewan (Lethbridge, Saskatchewan Landing Provincial Park, Grasslands National Park, Buffalo Pound Provincial Park and Harris) and to compare the mean number of bacteria in *D. variabilis* adults from seven localities in Saskatchewan, Manitoba and Ontario (Saskatchewan Landing Provincial Park, Grasslands National Park, Buffalo Pound Provincial Park, Harris, Minnedosa and Kenora). In addition, a Mann-Whitney *U* test was used to determine if the mean number bacterial species in *D. andersoni* adults from Saskatchewan Landing Provincial Park was the same as that of *D. variabilis* adults from the same locality. These tests were also performed to compare the bacterial diversity in *D. andersoni* and *D. variabilis* at three other sites (Saskatchewan Landing Provincial Park, Buffalo Pound Provincial Park, and Harris) where both tick species occurred in sympatry.

The infection patterns of the different bacterial species within individual ticks were analyzed to determine if there is any evidence for competitive or facilitative interactions that influence the structure the microbial communities. The co-occurrence analysis tests for non-random species associations (Gotelli 2000), and has been used to examine communities for competitive interactions between species within a particular site (Gotelli 2000). These analyses are suitable for investigating interactions among members of tick-borne microbial communities, where individual ticks represent different "sites".

Co-occurrence analyses were conducted to determine if the composition of microbial infracommunities resulted from random associations, or if they have predictable patterns of species occurrence (Gotelli 2000). This was assessed by quantifying the patterns of infection in the ticks, using two indices of co-occurrence, the C-score and the number of checkerboard species pairs, and by comparing observed patterns with those produced for null models. The indices were calculated from presence/absence data from adult ticks using the null modeling software EcoSim Ver. 7.72 (Gotelli & Entsminge 2011). The average index score for the null model was calculated from 5000 simulations, producing randomized matrices. Row and column constraints were set to fixed rows (maintain same species occurrence frequencies as in original data set) and fixed columns (maintain same number of observed species per site (i.e. species richness total for each tick) as in original data set). Degenerate matrices were retained because all 5000 randomizations resulted in row or column totals of zero. These simulation conditions were used because they tend not to result in false positives (Type I errors) (Gotelli 2000).

The C-score index quantifies the average amount of co-occurrence among all unique pairs of species in a component community (i.e, from multiple populations of ticks) and measures the tendency of species to not occur together. If the C-score of the observed occurrence pattern

differs significantly from one expected by chance (as estimated by the C-score for a null model), the microbial community is considered to be structured (i.e. a non-random assemblage of species) (Gotelli 2000). A C-score that is significantly greater than expected indicates that a species pair occurs less often than expected by chance and is usually interpreted to indicate the presence of competition between at least two different species. A C-score that is significantly less than expected indicates that there are species pairs that occur more often than expected by chance, and may indicate a facilitative relationship.

The number of checkerboard species pairs, was also calculated using EcoSim to measure the number of species pairs that never co-occur in any tick (Gotelli 2000). An index score for the observed species pattern that is significantly greater than the value of the index for the null model indicates that there are more checkerboard species pairs than expected by chance, and can be interpreted to mean that the community is structured by competitive interactions. Test criteria for these analyses were the same as used to calculate the C-score.

9.4. Results

Figure 9.1 shows a comparison of the bacterial species richness present in adult ticks of the two species from all sites combined. At least twice as many *D. variabilis* adults (9 %) did not contain any bacteria (i.e. of the genera/species tested for) compared to the *D. andersoni* adults (4 %). With respect to first the null hypothesis (see p. 176), there was a significant difference (U = 52.94, $P < 0.001$) in the mean species richness of bacteria in *D. andersoni* adults (1.9 ± 0.8) and *D. variabilis* adults (1.5 ± 0.8). The mean bacterial species richness in *D. andersoni* and *D. variabilis* adults from all Grasslands National Park were not compared because only a single *D. variabilis* was tested for all the targeted bacteria (Table 9.1).

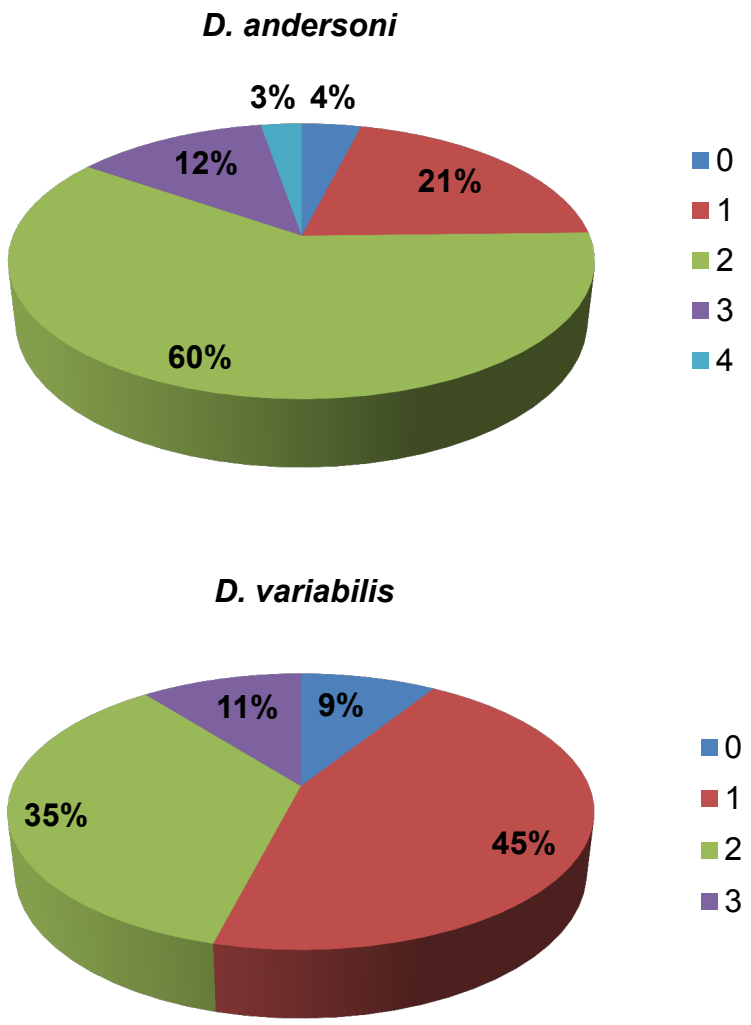


Fig. 9.1. The relative frequencies of single and mixed bacterial species infections in adult *D. andersoni* (n = 353) and *D. variabilis* (n = 448). Also shown is the proportion of ticks in which no bacteria were detected. The data for *D. andersoni* are based on the combined records for five localities (Lethbridge in Alberta, and Saskatchewan Landing Provincial Park, Grasslands National Park, Buffalo Pound Provincial Park, and Harris in Saskatchewan), while the data for *D. variabilis* are based on the combined records from seven localities (Saskatchewan Landing Provincial Park, Grasslands National Park, Buffalo Pound Provincial Park, Harris and Blackstrap Provincial Park in Saskatchewan, Minnedosa in Manitoba, and Kenora in Ontario).

	Locality								
Species	Lethbridge	Saskatchewan Landing P. P.	Grasslands N. P.	Buffalo Pound P. P.	Harris	Blackstrap P. P.	Minnedosa	Kenora	Statistical comparison
D. andersoni	1.7 +/- 0.8 n = 100	2.3 +/- 0.8 n = 83	1.9 +/- 0.6 n = 17	1.7 +/- 0.6 n = 33	1.8 +/- 0.6 n = 100	-[9]	-	-	$H = 33.981$ $P < 0.001$
D. variabilis	-	1.4 +/- 0.5 n = 96	1.0 n = 1	1.3 +/- 0.7 n = 79	1.0 +/- 0.9 n = 12	1.8 +/- 0.9 n = 105	1.6 +/- 0.9 n = 100	1.3 +/- 0.7 n = 30	$H = 26.117$ $P < 0.001$
Statistical comparison	-	$U = 68.16$ $P < 0.001$	-	$U = 10.213$ $P = 0.001$	$U = 12.68$ $P < 0.001$	-	-	-	

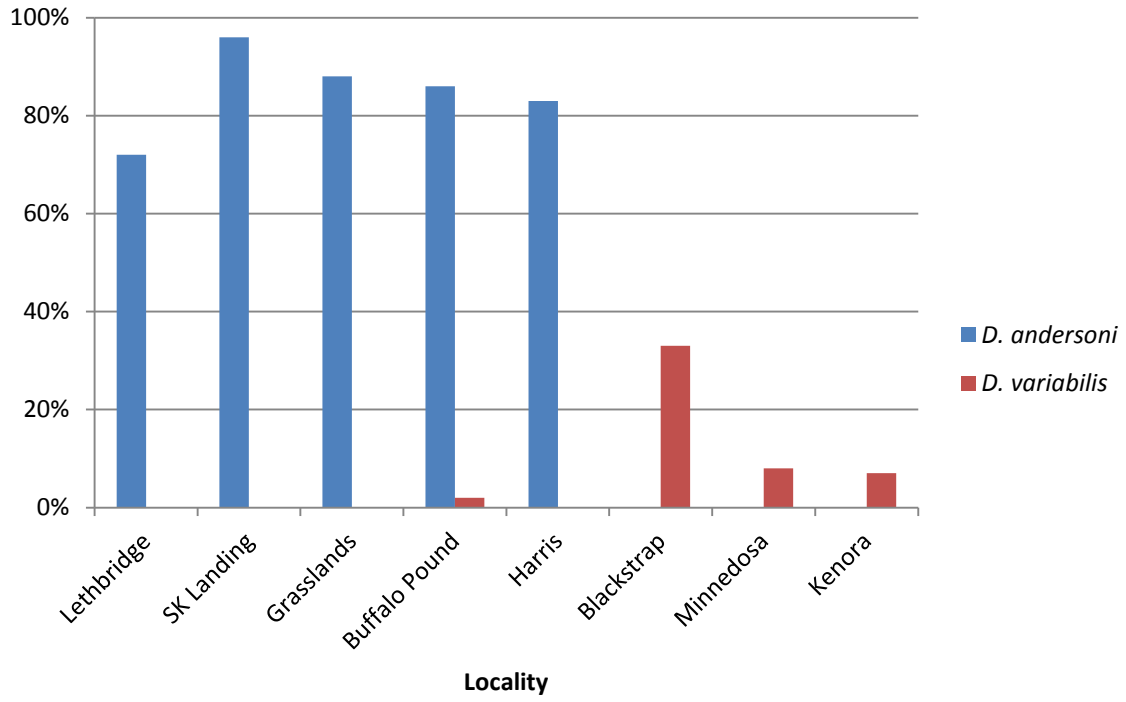
Table 9.1. The mean (+/- SD) bacterial species richness in adult ticks from different localities. Mean species richness of bacterial communities within *D. andersoni* and *D. variabilis* adults at localities where they are sympatric were compared with the Mann-Whitney non-parametric t-test (test statistic = *U*). Mean species richness of bacterial communities within *D. andersoni* or *D. variabilis* adults from multiple localities were compared with the Kruskal-Wallis non-parametric one-way ANOVA (test statistic = *H*).

[9] - = not applicable

However, at the other three localities where the two tick species were sympatric (Saskatchewan Landing Provincial Park, Buffalo Pound Provincial Park, and Harris), the mean species richness of bacteria in *D. andersoni* adults was significantly higher than in *D. variabilis* adults (Table 9.1). The difference in bacterial species richness between *D. andersoni* and *D. variabilis* was related to interspecific differences in bacterial species diversity and composition (Fig. 9.2). This is particularly evident for ticks collected from Saskatchewan Landing Provincial Park. Of the 11 bacterial species detected in ticks from this locality, nine were found in *D. andersoni* adults but only four in *D. variabilis* adults, two of which were not found in *D. andersoni* (Table 9.2).

Table 9.3 shows the bacterial species composition (including *Rickettsia*, FLEs and *Arsenophonus*) in *D. andersoni* adults from five localities, four of which (Saskatchewan Landing Provincial Park, Grasslands National Park, Buffalo Pound Provincial Park and Harris) represent sympatric populations, and the fifth (Lethbridge) an allopatric population with respect to the distribution of *D. variabilis*. Two of the seven bacterial species, *Rickettsia peacockii* and FLE type 1, were detected in *D. andersoni* at all five localities and the *Arsenophonus sp.* was detected at four localities; whereas, FLE types 2 and 9 were only detected at two localities and FLE type 5 and *Anaplasma* sp. were detected in *D. andersoni* at a single locality. The results of a non-parametric Kruskal-Wallis one-way analysis of variance to test the second null hypothesis (p. 176) revealed that there was a significant difference in the mean bacterial species richness of *D. andersoni* from the five different localities (Table 9.1); the highest of which was detected in adults from Saskatchewan Landing Provincial Park.

(A)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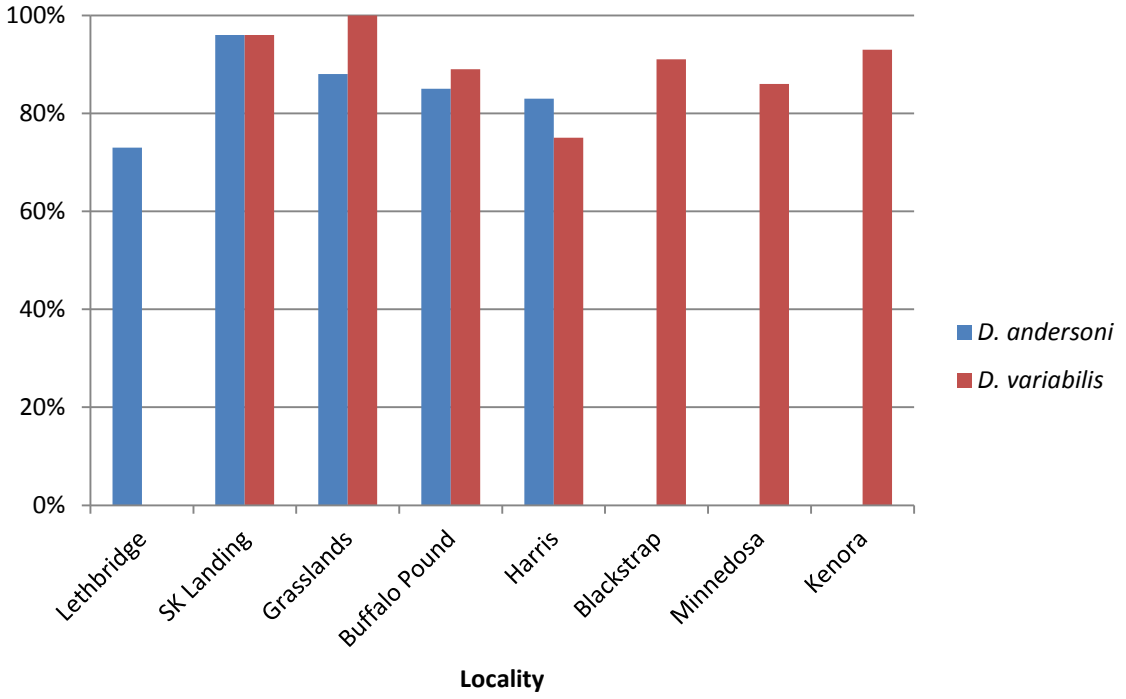
Lethbridge
SK Landing
Grasslands
Buffalo Pound
Harris
Blackstrap
Minnedosa
Kenora
Locality
D. andersoni
D. variabilis

(B)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Locality
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D. variabilis

(C)

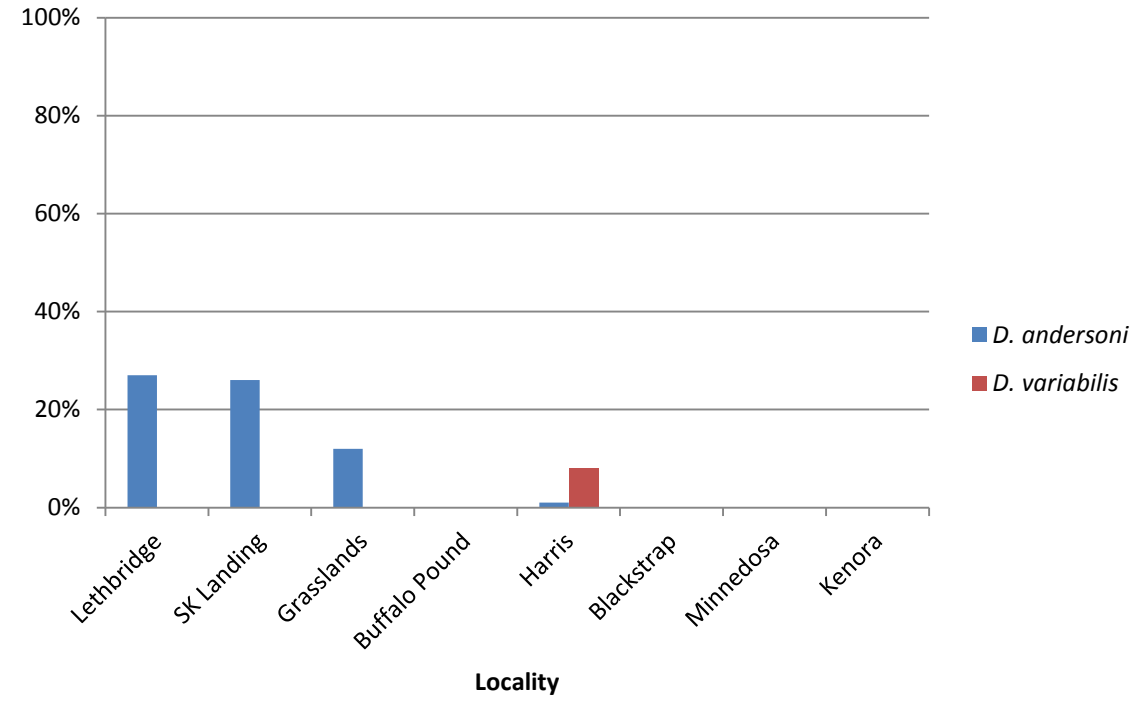

Fig. 9.2. Comparison of the prevalence of (A) *Rickettsia* spp., (B) FLEs and (C) *Arsenophonus* in *D. andersoni* and *D. variabilis* adults from different localities.

	D. andersoni		***D. variabilis***	
Bacterial taxon	No. PCR-positive	% positive	No. PCR-positive	% positive
Rickettsia montanensis	0/100	0	0/100	0
Rickettsia peacockii	96/100	96	0/100	0
FLE Type1	80/83	96	0/96	0
FLE Type2	2/83	2	91/96	94
FLE Type3	0/83	0	37/96	39
FLE Type4	0/83	0	0/96	0
FLE Type5	0/83	0	0/96	0
FLE Type6	0/83	0	0/96	0
FLE Type7	0/83	0	0/96	0
FLE Type8	0/83	0	0/96	0
FLE Type9	1/83	1	0/96	0
FLE Type10	0/83	0	0/96	0
Arsenophonus sp.	21/85	25	0/100	0
Serratia sp.	4/85	47	0/100	0
Anaplasma bovis	7/100	7	0/99	0
Candidatus Midichloria sp.	1/100	1	1/99	1
Ignatzschineria sp.	2/100	2	0/99	0
Wolbachia sp.	0/100	0	1/99	1

Table 9.2. Proportion of *D. andersoni* and *D. variabilis* adults from Saskatchewan Landing infected with different bacteria.

Bacterial taxon	Locality				
	Lethbridge	Saskatchewan Landing	Grasslands N.P.	Buffalo Pound	Harris
Rickettsia montanensis	0	0	0	0	0
Rickettsia peacockii	72	96	88	86	83
FLE_Type1	73	96	88	82	83
FLE_Type2	0	2	0	0	3
FLE_Type3	0	0	0	0	0
FLE_Type4	0	0	0	0	0
FLE_Type5	0	0	0	3	0
FLE_Type6	0	0	0	0	0
FLE_Type7	0	0	0	0	0
FLE_Type8	0	0	0	0	0
FLE_Type9	0	1	0	0	4
FLE_Type10	0	0	0	0	0
Arsenophonus sp.	27	26	12	0	1
Anaplasma bovis	N/T[10]	7	N/T	N/T	N/T

Table 9.3. Proportion of *D. andersoni* and *D. variabilis* adults from Saskatchewan Landing infected with different bacteria.

[10] Not tested

Table 9.4 shows the bacterial species composition in *D. variabilis* adults from seven localities, four of which (Saskatchewan Landing Provincial Park, Grasslands National Park, Buffalo Pound Provincial Park and Harris) represent sympatric populations and the other three (Blackstrap Provincial Park, Minnedosa and Kenora) representing allopatric populations with respect to the distribution of *D. andersoni*. One of the seven bacterial species, FLE type 2, was detected in *D. variabilis* at all seven localities and FLE type 3 was detected at six localities; whereas, the other five species were only detected at one to four localities. The results of a non-parametric Kruskal-Wallis one-way analysis of variance to test the third null hypothesis (p. 176) revealed that there was a significant difference in the mean bacterial species richness of *D. variabilis* among difference localities (Table 9.1). Adult *D. variabilis* from Blackstrap Provincial Park had the highest mean species richness of the bacterial infracommunities.

The mean bacterial species richness in larval and adult *D. variabilis* at Saskatchewan Landing Provincial Park and Blackstrap Provincial Park is shown in Table 9.5. The results of a Mann Whitney *U* test to examine the fourth null hypothesis (p. 176) revealed that, at both localities, larval ticks had a significantly lower bacterial species richness than adults. In addition, although there was no statistical difference in the mean bacterial species richness of the adult ticks at the two localities, larvae from Blackstrap Provincial Park had a significantly higher mean bacterial species richness than did larvae from Saskatchewan Provincial Park.

Co-occurrence analyses were conducted to test null hypothesis 5 (p. 176). The observed C-scores for both *D. andersoni* and *D. variabilis* were no different than expected for a random assemblage of species (Table 9.6). This conclusion was supported by the calculation of the observed number of checkerboard species pairs of bacteria in *D. andersoni* and *D. variabilis* adults, which were not significantly different than expected (Table 9.7).

Bacterial taxon	Locality						
	Saskatchewan Landing	Grasslands N.P.	Buffalo Pound	Harris	Blackstrap	Minnedosa	Kenora
Rickettsia montanensis	0	0	2	0	39	8	7
Rickettsia peacockii	0	0	0	0	0	0	0
FLE_Type1	0	0	0	0	0	0	0
FLE_Type2	94	100	77	75	76	79	90
FLE_Type3	39	0	44	17	59	40	30
FLE_Type4	0	0	4	0	0	1	3
FLE_Type5	0	0	0	0	0	0	0
FLE_Type6	0	0	0	0	0	0	0
FLE_Type7	0	0	1	0	1	1	0
FLE_Type8	0	0	0	0	0	1	0
FLE_Type9	0	0	0	0	0	0	0
FLE_Type10	0	0	0	0	0	0	0
Arsenophonus	0	0	0	8	0	0	0
Anaplasma bovis	0	N/T[11]	N/T	N/T	0	N/T	N/T

Table 9.4. Proportion (%) of *D. variabilis* adults from different localities infected with different bacterial species.

[11] Not Tested

Life cycle stage	Locality		
	Saskatchewan Landing Provincial Pk.	Blackstrap Provincial Pk.	Statistical comparison
adults	1.4 +/- 0.5 n = 96	1.8 +/- 0.9 n = 105	*U* = 0.023 P = 0.88
larvae	0.3 +/- 0.4 n = 71	0.6 +/- 0.5 n = 143	*U* = 25.71 P < 0.001
Statistical comparison	*U* = 91.25 P < 0.001	*U* = 96.44 P < 0.001	

Table 9.5. The mean (+/- SD) species richness of bacterial infracommunities in adult and larval *D. variabilis* from two localities.

	C-score (observed)	C-score (expected)	Tail probability P (obs > = exp)	Tail probability P (obs < = exp)	reject H_0?	Non-random occurrence pattern?
D. andersoni adults	251.30560	235.47820	0.05620	0.94380	Fail to reject	No
D. variabilis adults	375.27270	361.08940	0.05780	0.94240	Fail to reject	No

Table 9.6. Results of co-occurrence analyses (C-score) of adult ticks from eight different localities (Lethbridge, Saskatchewan Landing Provincial Park, Grasslands National Park, Harris, Blackstrap Provincial Park, Buffalo Pound Provincial Park, Minnedosa and Kenora).

	Index (observed)	Index (expected)	Tail probability P (obs > = exp)	Tail probability P (obs < = exp)	reject H_0?	Non-random occurrence pattern?
D. andersoni adults	16.00000	16.17760	0.79020	0.59960	Fail to reject	No
D. variabilis adults	39.00000	36.44280	0.10160	0.97980	Fail to reject	No

Table 9.7. Results of co-occurrence analyses (number checkerboard species pairs) of adult ticks from eight different localities (Lethbridge, Saskatchewan Landing Provincial Park, Grasslands National Park, Harris, Blackstrap Provincial Park, Buffalo Pound Provincial Park, Minnedosa and Kenora).

9.5. Discussion

In the present chapter, comparisons were conducted on bacterial communities in *D. andersoni* and *D. variabilis*. However, caution is advised in the interpretation of the results obtained because only a portion of the bacterial diversity of *D. andersoni* and *D. variabilis* was described in the studies reported in the previous chapters. This occurred as a consequence of the experimental approach used (i.e. the use of genus-specific PCR-based methods) because it did not allow for the identification of all potential bacterial species that occur within individual ticks. Nonetheless, sufficient data were collected to conduct a comparative analysis of the bacterial communities within *D. andersoni* and *D. variabilis* at localities where these two tick species occur in sympatry, and where their distributional ranges are allopatric with respect to one another. These analyses were conducted on the presence/absence data of two different species of *Rickettsia*, 10 genetic types of *Francisella*-like organisms, a single species of *Arsenophonus*, and other bacterial species in low prevalence, such as *Anaplasma bovis*, in *D. andersoni* and *D. variabilis* individuals from eight of the collection localities.

Many adult ticks were co-infected by various combinations of bacteria. The species composition and the relative frequencies of these bacteria in the different component communities (i.e. tick populations) were, therefore, analyzed to determine if the species richness differed between tick species, life stage (i.e. larvae *vs.* adults) and collection locality (i.e. tick population). In general, the mean species richness of bacteria in *D. andersoni* was greater than that for *D. variabilis*. The results of the statistical analyses showed that this was the case for *D. andersoni* and *D. variabilis* adults from Saskatchewan Landing Provincial Park. The same pattern was observed at other sites where the two tick species occurred in sympatry (i.e. Buffalo Pound Provincial Park and Harris), where there was a greater diversity of species detected in *D.*

andersoni than in *D. variabilis*. Likewise, the diversity of bacterial species differed significantly among different populations of adults of each tick species. The infection patterns of different bacterial species may relate to the type of symbiotic relationship between the bacteria and their invertebrate hosts. The prevalence of secondary symbionts can vary greatly among arthropod populations (Hansen *et al.* 2007, Skaljac *et al.* 2010). Primary symbionts, as per their definition of being essential to the host, should occur at a high prevalence, as detected for *Rickettsia peacockii* (Chapter 5) and the FLEs (Chapter 6). The prevalences of different bacterial species within ticks can also be related to their transmission cycles (Ewald 1987). Horizontally transmitted microbes would be more likely to occur at a lower prevalence than those species that are essential and transmitted vertically within tick populations. This is particularly true for a bacterium that enhances the fitness of its host and has an efficient mechanism of transmission, which would lead to a rapid increase in prevalence following introduction to a new host population (Himler *et al.* 2011).

A lower number of bacterial species were detected in the immature stages of ticks than in the adults. Also, multiple infections were only detected in adult ticks and *D. andersoni* nymphs. Of the 62% of 143 *D. variabilis* larvae infected with bacteria, but none contained multiple infections of the specific bacterial species tested for. Similarly, no mixed bacterial infections were detected in the 13 *D. variabilis* nymphs examined. However, this does not exclude the possibility that other bacterial species may be present in these immature ticks. Nonetheless, analyses of the presence/absence data for immature stages of *D. variabilis* indicated that the bacterial species richness in larvae was significantly less than that of the adults from Saskatchewan Landing Provincial Park. Such a difference in prevalence of bacteria in different life stages of tick has also been demonstrated in *Amblyomma americanum* (Heise *et al.* 2010),

and may be related to the different modes of transmission of the different bacterial species. For instance, the proportion of vertically transmitted symbionts (*Rickettsia* spp. and *Coxiella* symbionts) in female ticks has been demonstrated to increase following a blood meal (Heise *et al.* 2010). Such a strategy will increase the success rate of transovarial transmission for these organisms. In addition, the overall diversity of species has been shown to increase following a blood meal (Heise *et al.* 2010). Adult ticks may have acquired a greater variety of horizontally transmitted bacteria during a blood meal. Furthermore, there is a greater potential for population growth of existing microbial species within ticks because of the long period of time between blood meals. Therefore, the ability to detect particular bacterial species may be affected by the life cycle stage and the timing of sample collection because of changes in their relative abundance.

Most of the bacteria detected in *D. andersoni* and *D. variabilis* (i.e. *Rickettsia*, *Francisella*, and *Arsenophonus*) are mainly transmitted vertically and need to infect the ovaries and eggs of the females to be passed on to the next generation of tick. Due to these apparently overlapping niches, competitive interactions might be expected and result in competitive exclusion. However, there is no evidence for this from the analyses of ticks infected by multiple bacterial species. Rather, the results of my thesis suggest that distantly related bacteria (i.e. different genera of bacteria) can co-exist and potentially be co-transmitted from one generation to the next. In contrast, previous studies have demonstrated that other bacterial species or genotypes can inhibit the transovarial transmission of a closely related organism (Macaluso *et al.* 2002, de la Fuente *et al.* 2003).

Estimates of infection patterns can be used as evidence of a non-random assembly of microbial communities; however, the results of the co-occurrence analyses did not indicate that

some bacterial species tended to be associated together in *D. andersoni* and in *D. variabilis* adults more often than expected. Random patterns of species occurrence are often interpreted to indicate a lack of competitive (or facilitative) interactions among community members. These results suggest that there is no predictable pattern to the distribution of bacterial species within ticks; however, an examination of the presence-absence matrix used to calculate the co-occurrence indices suggests that some bacterial species do tend to be positively associated. For instance, *R. peacockii* and FLE type 1 tended to occur together more often within *D. andersoni* adults than they were with other types. In *D. variabilis* adults, FLE type 3 tended to occur more often with FLE type 2 than by itself (i.e. in comparisons with other FLEs) or with other species of bacteria. For instance, of the 44% of the *D. variabilis* infected with FLE type 3, most (86%) also contained FLE type 2. Conversely, only 44% of the *D. variabilis* adults infected with FLE type 2 contained FLE type 3. Patterns of co-occurrence of the most prevalent bacterial species may be linked to their mode of transmission. It is known that both *R. peacockii* and the FLEs are maintained in a tick population through transovarial transmission (Niebylski *et al.* 1997, Baldridge *et al.* 2009). Hence, co-infection patterns of these types of bacteria would be maintained and propagated from one generation to the next, given that this mode of transmission is very efficient (Niebylski *et al.* 1997, Baldridge *et al.* 2009).

The structures of microbial communities may also be a determined by other physiological, environmental, or historical factors. For example, the types and relative proportions of bacteria within ticks could be influenced by the availability of vertebrate hosts, the microhabitat, temperature, evolutionary and geographic history (i.e. phylogeography) of the bacteria and their tick hosts, and by anthropogenic changes (e.g. on the environment/animals).

The power of the co-occurrence analyses to detect non-random patterns of species occurrence may be diminished as a consequence of marked difference in prevalences of specific bacteria (Clay *et al.* 2008). In the present study, some species, such as *R. peacockii* and FLEs 1-3, always occurred at consistently high prevalences, whereas *R. montanensis*, FLE types 4-10 and *Arsenophonus* sp. always occurred at low prevalences. The inclusion of a greater number of bacterial species in these analyses, particularly those with more intermediate prevalences, may increase the likelihood of providing a more reliable estimate of deviation from a random assemblage. The low prevalence of *R. montanensis*, some FLE types, and the single *Arsenophonus* species resulted in degenerate matrices (ones with empty rows or columns i.e. absence of a particular species or sites with no species) for all 5000 iterations of the randomly generated null models. These degenerate matrices can increase the likelihood that the null hypothesis will be rejected (i.e. increase the likelihood of a Type I error) (Gotelli 2000), although this did not occur in these analyses.

In light of these limitations, conclusions based on the results of these analyses must again be made with caution. However, some general inferences can be made from the observed infection patterns. The occurrence of one species does not seem to result in the exclusion of another species because an examination of the presence/absence matrices does not provide evidence that two species cannot co-occur. Clay *et al*. (2008) found a negative correlation between the infection frequencies of *Arsenophonus* and *R. amblyommii* at all seven of their collection localities they examined. In contrast, the results of this thesis show that nearly all ticks infected with *Arsenophonus* sp. also contained rickettsial organisms (data not shown).

An analysis of the spatial and temporal variations in the composition of microbial communities (Jones *et al.* 2009, Heise *et al.* 2010) would provide a better understanding of the

mechanisms that determine community structure within ticks. Different microbial community compositions have been demonstrated in ticks, depending on the life stage and the degree of engorgement (Moreno *et al.* 2006, Heise *et al.* 2010), and can, therefore, be two important factors determining the diversity of the tick microbiome. This needs to be investigated further for *D. andersoni* and *D. variabilis*. Comparisons of microbial community composition conducted over an extended time course for different tick populations that also take into account differences in the ecology of different tick populations would be useful to determine if influences other than interactions within the microbial infracommunity determine its structure. As a result of the dynamic nature of the microbial community composition, the conditions imposed on potential new members of the community changes over time and the ability to successfully colonize a host may be dependent on the timing of infection.

The influence of interactions among tick-borne bacteria on the presence or transmission of other bacterial species may not be dependent on just the presence or absence of a particular bacterial species, but rather may be dependent on the relative infection intensity (relative abundance) of each (Burgdorfer *et al.* 1981). Thus, there may be no detectable effect of interactions among bacterial species by analyses of co-occurrence unless the relative proportions of certain bacteria reach a threshold in a large proportion of ticks. A measurement and comparison of the relative abundance of different constituents of the microbial community may provide further insight into the regulation of community structure.

Most studies on the bacterial infracommunities and component communities of arthropods are descriptive, comparing the presence/absence of species in one community to that of another community (Moreno *et al.* 2006, Van Overbeek *et al.* 2008, Heise *et al.* 2010). However, many of these studies were limited in their analysis of tick-borne microbial communities because of

small sample sizes and/or technical limitations that affect the ability to determine the identity of all members of the tick microbiome. The latter is often a problem in studies on bacterial communities, including the present study. The complexity of the source DNA pool can mask the presence of target DNA from species occurring in low abundance. However, new high-throughput DNA sequencing and analysis technologies have been recently developed that overcome these issues and will facilitate the identification of the entire tick microbiome (Clay & Fuqua 2010, Andreotti *et al.* 2011).

In conclusion, the present study described and compared some of the microbial diversity within *D. andersoni* and *D. variabilis*. Future studies will need to be conducted to delineate the complete composition of microbial communities and to provide a more comprehensive estimate of the diversity of tick-borne microbes in *D. andersoni* and *D. variabilis*. This will undoubtedly lead to the identification of new bacterial species and novel associations between ticks and microbial agents.

9.6. References

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Chapter10. General Discussion

10.1. Principal questions addressed in this thesis

The occurrence and prevalence of tick-borne diseases are determined by the intimate interrelationships of three different groups of organisms; the pathogenic agent, the tick vector and the vertebrate host used by the vector. The complex interactions between the members of this triad, or epidemiological triangle, are influenced by many different factors, especially environmental parameters, which together, have a strong impact on the prevalence and severity of disease in the different geographical areas in which the vector occurs. A number of questions relating to the biology and ecology of vectors, their vertebrate host(s), and the pathogen(s) they carry need to be answered in order to understand the transmission pattern of tick-borne pathogens, and the epidemiology of tick-borne diseases. Some of these questions include: what tick species transmit pathogenic microorganisms to their vertebrate hosts?, what is the diversity of pathogens (and endosymbionts) within individual ticks and the prevalence of each pathogen in different tick populations?, what vertebrate hosts are important in the life cycle of an arthropod vector and are they a reservoir hosts for pathogens?, are the vertebrate hosts of ticks involved in the transmission cycles of pathogenic microorganisms?, are there negative interactions (e.g. competitive exclusion) or positive associations (e.g. co-occurrence) between microorganisms in individual ticks from different populations?, and what is the potential risk of exposure to pathogens for humans and domestic animals in different geographical areas where the vectors occur? Once this information has been obtained, effective management strategies can be employed for the control of tick-borne pathogens and to predict the risk of future disease incidence.

My PhD thesis examined some of these questions in relation to the ixodid ticks, *Dermacentor andersoni* and *D. variabilis* (see Fig. 10.1). In particular, I compared the distributional ranges of *D. andersoni* and *D. variabilis* in Saskatchewan, the hosts used by immature ticks, and determined some of the species/types of bacteria (potential pathogens and endosymbionts) in allopatric and sympatric populations of each tick species situated near their northern distributional limits.

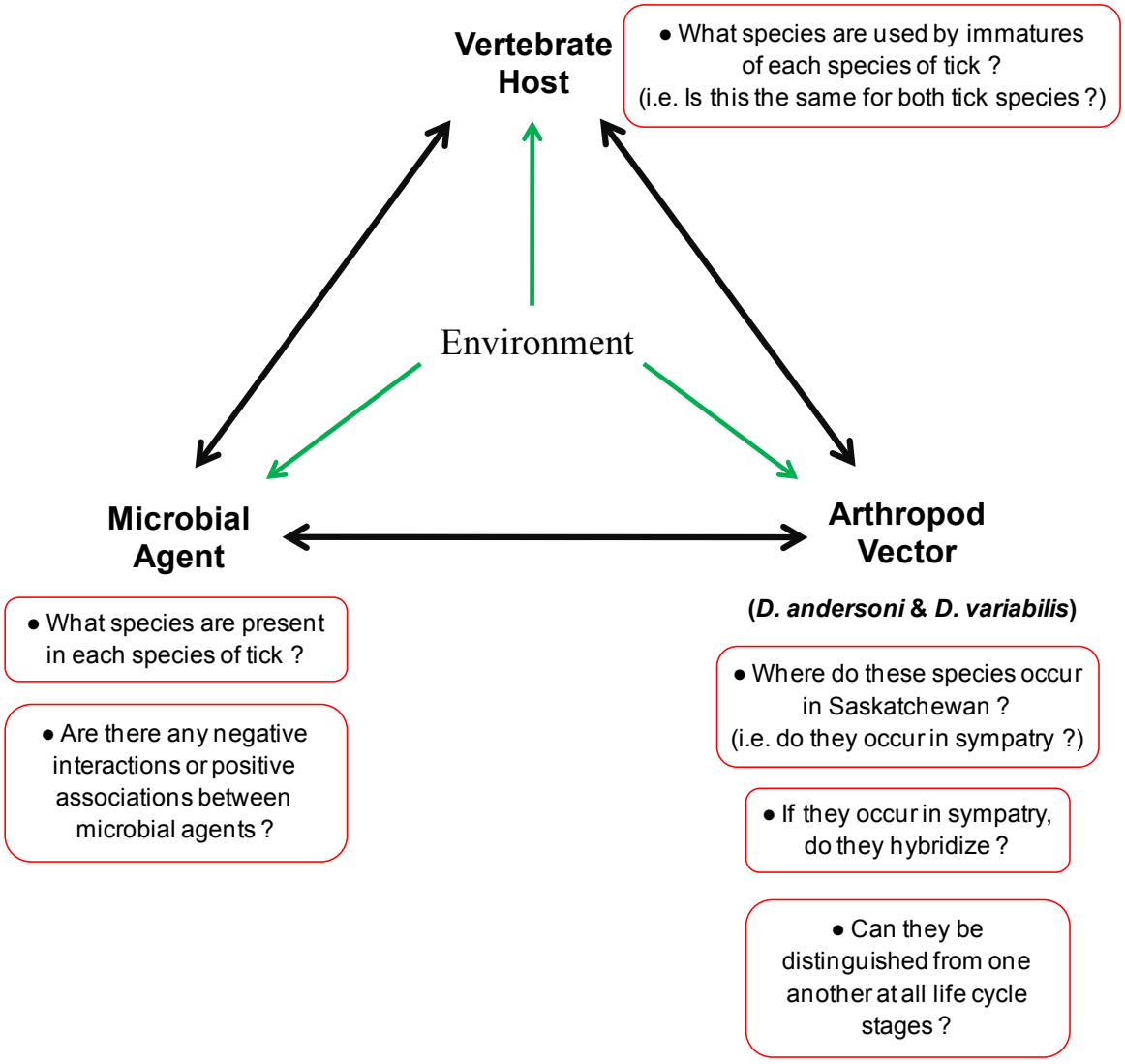

Fig. 10.1. The principal questions addressed in this thesis with respect to *D. andersoni* and *D. variabilis*, and their relationships with the other members of the triad. This is a modification of the classic epidemiological triangle of tick-borne diseases.

10.2. Determination of the distributional ranges of *D. andersoni* and *D. variabilis*

One important objective of my research was to determine the current distributional limits of *D. andersoni* and *D. variabilis* in Saskatchewan, and establish whether there overlap in their geographic ranges within the province. Knowledge of the distributions of these two vectors is essential for estimating the risk of exposure for humans and domestic animals to potential pathogenic microorganisms carried by these tick species. This objective was achieved using both passive and active collection methods. The results obtained suggest that the geographic range of *D. andersoni* in Saskatchewan may have expanded to the north and to the east compared to the distributional records of the 1950's and 1960's (Gregson 1956, Wilkinson 1967). However, there were a limited number of historical records, so this was difficult to determine with certainty. In contrast, the incidence records for *D. variabilis* showed that it occurs further north than previously reported (Gregson 1956, Wilkinson 1967). The validity of some of these records, which were acquired through passive surveillance, need to be treated with some caution with respect to their accuracy (e.g. locality names). This is because reports of ticks, at or near these localities, are quite rare, and they are well outside the range of the localities where this species is known to consistently occur. Furthermore, the locality information for these records of ticks provided through the passive surveillance were based on the location from where the tick was recovered from the host. This may not be the locality from where it was actually acquired, particularly if the host (i.e. person or dog) had travelled within a few days prior to removing the tick(s). This uncertainty in the accuracy of some of the reported collection locations is a drawback of the passive surveillance methods. Nonetheless, the strength of this collection method lies in the large number of samples that can be acquired over a much broader geographic range than is possible through more accurate and direct sampling methods, such as flagging.

A major finding of the present study was that the geographic range for *D. variabilis* has extended much further west of locations based on comparisons with the historical records for this species prior to 1967 (Gregson 1956, Wilkinson 1967). There is currently an area at least 200 km wide in southern Saskatchewan where there is overlap in the ranges of *D. andersoni* and *D. variabilis*. Range expansion of *D. variabilis* may have occurred as a result of multiple introductions into new areas following the westward translocation of ticks attached to wild animals (e.g. deer and coyotes) or domestic animals (e.g. dogs and livestock). It is known, for example, that the establishment *D. variabilis* populations in Nova Scotia arose following the importation of tick-infected dogs from the USA around 1900 (McEnroe 1985), with subsequent range expansions in that province during the 1940's (Dodds *et al.* 1969). Therefore, it is likely that there has been a gradual expansion of the distributional range of *D. variabilis* in Saskatchewan over several decades as deer and other wild animal hosts have gradually moved through relatively undisturbed natural corridors along protected areas and waterways. One possible route taken by *D. variabilis*-infected hosts is westwards along the South Saskatchewan River to localities occupied by *D. andersoni*. However, it is also important to note that major changes in environmental conditions, such as those associated with global warming, can also lead to changes in the abundance and geographic range of ticks and their hosts (Nelson & Mech 1984, Ogden *et al.* 2006). This is because the ability of ticks to successfully complete the different phases of the life cycle (e.g. feeding on-host, reproduction and off-host survival) is strongly influenced by abiotic factors, such as temperature and humidity (McEnroe 1974, 1978, Chilton & Bull 1994).

Changes in the distributional ranges of these ticks have both ecological and epidemiological implications. Both *D. andersoni* and *D. variabilis* were found in the same

collection sites at several localities in Saskatchewan. The zone of sympatry between *D. andersoni* and *D. variabilis* provides a unique opportunity to examine questions relating to their potential roles as vectors of pathogenic microorganisms. The coexistence of the two species, particularly if the immature ticks parasitize the same host individuals, may be an important mechanism for the range expansion of tick-borne pathogens, and for the spread of these microorganisms to different species of vector and vertebrate host. As individuals of a species establish into new areas, they are potentially exposed to new ecological niches in which they can encounter different of vertebrate and microbial species. These new relationships may result in transmission cycles that differ from those in other parts of the geographic range of the tick. In addition, overlapping ecological niches can create the potential for the introduction of tick-borne microorganisms from one species of tick to the other.

It will be important to continue to monitor the distribution of *D. andersoni* and *D. variabilis* to detect changes in the risk to vertebrate hosts for infection by tick-borne pathogens as a result of increased encounters with these two vector species. In addition, continued surveillance efforts for these tick species in Saskatchewan will increase the likelihood of detecting the spread of other species of tick that are of medical and veterinary importance that currently do not have established populations within the province. For example, this would help determine if (and where) populations of *Ixodes scapularis*, a vector of human and animal pathogens (e.g., *Borrelia burgdorferi*, *Anaplasma phagocytophilum* and *Babesia microti*), become established within Saskatchewan. It has been predicted that by the 2020's, environmental conditions in southern Saskatchewan may become suitable to support the establishment of *I. scapularis* populations (Ogden *et al.* 2006).

The ability to determine the distributional range of any species relies on the ability to accurately distinguish individuals of that species from individuals of related species, irrespective of their life cycle stage. Although it is not difficult to distinguish among adults of the different species of *Dermacentor* in North America, it is difficult to identify the larvae ad nymphs to the species-level (Gregson 1956). Therefore, a simple PCR-based method, using the second internal transcribed spacer (ITS-2) of the nuclear ribosomal DNA as the genetic marker, was developed to distinguish between *D. andersoni* and *D. variabilis* (Chapter 2). Two additional PCR-based methods (i.e. RFLP and SSCP) were also used to distinguish among *D. andersoni, D. variabilis* and a third species, the winter tick, *D. albipictus* (Chapter 2). The winter tick also occurs in sympatry with *D. andersoni* and *D. variabilis*. These molecular techniques also have the potential to distinguish among a wider range of *Dermacentor* species and other genera of ticks, given that each species has unique set of ITS-2 rDNA sequences (Zahler *et al.* 1995, Murrell *et al.* 2001, Shone *et al.* 2006). The development of genetic markers to accurately distinguish among the three species of *Dermacentor* in Canada also provided the opportunity to determine the hosts used by immature ticks (Chapter 3) and to examine fundamental questions relating to their reproductive ecology (i.e. do these species hybridize in the zone of overlap?).

It has been demonstrated in laboratory experiments that *D. andersoni* and *D. variabilis* adults from allopatric populations of each species have the potential to interbreed and produce viable hybrids, depending upon the type of cross-mating (Oliver *et al.* 1972). For instance, no viable progeny were produced when *D. andersoni* females were crossed with *D. variabilis* males, whereas viable offspring were produced from crosses involving *D. andersoni* males with *D. variabilis* females (Oliver *et al.* 1972). However, only a few offspring were produced from these interspecific matings and they had greatly reduced survival. Although hybridization can occur in

laboratory crosses between *D. andersoni* and *D. variabilis*, it is assumed to be rare in nature (Oliver *et al.* 1972). Given the potential for interbreeding, and that both species coexist in some localities in Saskatchewan (Chapter 3), there was the question as to whether *D. andersoni* and *D. variabilis* hybridize in the zone of sympatry. Using the genetic markers developed in Chapter 2, 82 *D. andersoni* and 77 *D. variabilis* adults from Saskatchewan Landing Provincial Park and Buffalo Pound Provincial Park were tested for evidence of hybridization. Offspring resulting from a cross-mating between *D. andersoni* and *D. variabilis* adults would be heterozygous for a marker based on the nuclear ribosomal DNA (i.e. ITS-2) because nuclear DNA is inherited both maternally and paternally, rather than maternally inherited as for mitochondrial DNA (Ballard & Whitlock 2004). The expected pattern of the ITS-2 amplicon from an hybrid (F1) individual would be two bands on an agarose gel, since the single bands of the amplicons of *D. andersoni* and *D. variabilis* individuals are of a different size (~430 and ~360 bp respectively; Chapter 2). Similarly, as the SSCP profiles of the ITS-2 for *D. andersoni* and *D. variabilis* differ markedly, then hybrid (F1) individuals would have the combined SSCP profiles of the two parents (i.e. of both species). However, results of the molecular analyses indicated there was no evidence for F1 hybrids as a result of a cross-mating between *D. andersoni* and *D. variabilis* adults at two localities in the zone of sympatry between these two tick species. If hybridization does occur, a larger number of ticks would need to be tested to detect hybrid F1 individuals because very few offspring may survive to the adult stage (Oliver *et al*. 1972). The detection of any F2 offspring produced by a back-cross of F1 hybrids with and adult of either parental species can be problematic using a single genetic marker. Therefore, identification of hybrid offspring would require multiple nuclear markers (Boecklen & Howard 1997) that could be displayed using techniques, such as AFLP (Tranah *et al*. 2003).

Although no hybrid individuals were detected during this study, a morphologically abnormal *D. andersoni* male was collected from Alexander Wilderness Park (Lethbridge, Alberta) (Dergousoff & Chilton 2007). This abnormal tick was missing the fourth leg and coax IV on the right side of the body (see Fig. 1, Dergousoff & Chilton 2007). Despite the morphological anomalies, this abnormal male tick was kept alive in the laboratory at 4°C for at least 130 days. However, it was not determined whether this individual had reduced reproductive fitness because of the abnormalities. The only other naturally occurring morphological anomaly previously reported in *D. andersoni*, for which I am aware, was a gynandromorph, an individual containing a combination of some male and female characteristics (Homsher & Yunker 1981). Structural malformations in *D. andersoni* are, therefore, rare as only a single abnormal individual was detected in a collection of 103 adults from the Alexander Wilderness Park and hundreds of other adults collected from vegetation at other locations across Canada (Chapter 3).

The broad geographic distribution of *D. andersoni* and *D. variabilis*, and their limited mobility compared some other species of tick that can be dispersed by birds, can result in the (reproductive) isolation among individuals from different localities. This can lead to the genetic differentiation among populations and differences in morphological and behavioural characteristics, due to different selective pressures placed on tick populations by their environment and their vertebrate hosts, and the composition of the tick microbiome. Studies have examined the genetic diversity of *D. andersoni* (de la Fuente *et al.* 2005a, Lysyk & Scoles 2008, Patterson *et al.* 2009) and *D. variabilis* (Krakowetz *et al.* 2010) using mitochondrial genes as genetic markers. Genetic differences have been detected between "montane" and "prairie" populations of *D. andersoni* (Lysyk & Scoles 2008, Patterson *et al.* 2009). In addition, differences in feeding behaviour (Wilkinson 1972, Scott & Brown 1986), vectorial capacity

(Scoles *et al.* 2005), paralyzing ability (Wilkinson 1985), and development (Pound & George 1991) have also been reported for *D. andersoni* populations from different geographic localities. Some of these differences may be related to the different environmental (which includes climatic) conditions experienced by these populations.

Some of the greatest changes in climatic variables, such as temperature, are predicted to occur in areas near the northern distributional limits of *D. andersoni* and *D. variabilis* (Wilkinson 1967, Ogden *et al.* 2006). Thus, the effect of these changes may be greater at the distributional limits of these tick species compared to those in more southern parts of their ranges. Little is known of the relative influence of different climatic variables on the distribution of ticks as they respond to changes in these important ecological conditions (Mills *et al.* 2010). It was important, therefore, to determine the distribution of these ticks and identify relationships with their vertebrate hosts and microorganisms that infect them to be able to determine if these relationships change in the future. Knowledge of the vertebrate hosts used by different species of tick is also important for understanding their role in maintaining the tick population and the transmission cycles of tick-borne microorganisms.

10.3. Determination of the vertebrate hosts used by immature ticks

An important objective of this thesis was to determine which species of small mammals were important for supporting the tick populations (Chapter 3). This was undertaken because there is little information as to the hosts used by the immature ticks of either species in Saskatchewan. The result obtained showed that, at Saskatchewan Landing Provincial Park, western jumping mice and meadow voles were parasitized by *D. variabilis* nymphs, while *D. variabilis* larvae were also found in these small mammals and on deer mice. At Blackstrap

Provincial Park, southern red-backed voles were also hosts to a significant proportion of *D. variabilis* larvae. All *D. variabilis* nymphs at Blackstrap Provincial Park were collected on southern red-back voles. For *D. andersoni*, deer mice and meadow voles were parasitized by nymphs at Saskatchewan Landing Provincial Park. Thus, *D. andersoni* nymphs and *D. variabilis* larvae were detected on the same host species, and sometimes on the same host individuals, at a locality where they occurred in sympatry. A much larger number of nymphs need to be examined to determine if other species, including deer mice, also act as hosts for immature ticks.

The mammalian hosts of *D. andersoni* and *D. variabilis* immatures, are potentially involved in the transmission cycles of horizontally transmitted tick-borne microorganisms and may be important reservoir hosts for these microorganisms. In addition, the overlapping host range of these two tick species may provide the opportunity for horizontal transmission of tick-borne microorganisms from one tick species to the other. This can occur, either from a systemically infected host, or by feeding close in space to an infected tick on a host that is not systemically infected (i.e. co-feeding transmission). Thus, the occurrence of two species of tick vector that occur in sympatry may have important epidemiological implications.

A number of questions regarding the use of different vertebrate species as hosts by *D. andersoni* and *D. variabilis* remain to be answered. For example, more work is needed at Saskatchewan Landing Provincial Park to determine which species of rodent are used as hosts by *D. andersoni* larvae. Studies also need to be conducted to determine the full range of suitable hosts and quantify the relative importance of the different host species for maintaining the tick life cycle. This can be determined through more extensive trapping efforts at a greater number of localities over a longer time period than for the study conducted in this thesis. Molecular tools can also be used to determine the range of hosts used by different tick life stages through blood

meal analysis (e.g., Kent & Norris 2005, Rodrigues & Maruniak 2006). However, identification of the host species used by ticks using such methods is much more difficult and has been less successful than in studies on other vectors, such as mosquitoes (e.g., Kirstein & Gray 1999, Pichon *et al.* 2005, Allan *et al.* 2010). This is due to significant degradation of host DNA during the long period of time since the blood meal was acquired by the tick in its previous life stage; whereas, mosquitoes host DNA is analyzed relatively recently following a blood meal. Clarification of the relative importance of different mammal species for supporting tick populations would require comparative feeding experiments to measure the relative fecundity of ticks.

Another important question that needs to be addressed in the future is, what is the relative importance of different vertebrate hosts for the transmission and maintenance of tick-borne microorganisms? A stronger inference about the role that the different mammal species (particularly those species used by immature ticks) play in the transmission cycle of tick-borne microorganisms could be made by determining which bacterial species naturally infect these mammals. This could be determined by testing the blood or other organs (e.g., spleen) of individual mammals for the presence of specific bacteria that were detected in the ticks that parasitized those host individuals. Although not reported previously in this thesis, I attempted to detect bacterial DNA in the spleens of all small mammals trapped from Saskatchewan Landing Provincial Park and Blackstrap Provincial Park using a nested PCR and broad-range primers targeting the bacterial 16S rDNA. The PCR analyses produced amplicons from all spleen genomic DNA samples. However, readable DNA sequences for bacteria could not be obtained from any of these amplicons. The non-specific and sensitive nature of this assay also resulted in problems with contamination, as evidenced by the production of amplicons in the negative

controls. This is a common issue when using primers that are able to amplify DNA from a broad range of bacteria species, particularly in a nested PCR (Galkiewicz & Kellogg 2008, Huys *et al.* 2008, King *et al.* 2008). I also performed PCR assays with genus-specific primers to detect the DNA of several bacterial genera (i.e. *Rickettsia*, *Francisella*, and *Anaplasma*) in the spleen of all small mammals, some of which were infested by immature ticks containing these microorganisms. Unfortunately, none of the host samples were positive by PCR for *Rickettsia* or *Francisella* and technical difficulties were experienced when testing for the presence of *Anaplasma*. Due to these technical issues, and time limitations, further analyses of the bacterial species in small mammals were abandoned. Renewed efforts to determine the presence of tick-borne bacteria in the spleen and/or blood of the mammalian hosts to immature ticks need to be made to ascertain their role as potential amplification hosts and sources of infection to ticks.

10.4. Determination of the bacterial species in *D. andersoni* and *D. variabilis*

A novel and important aspect of my research was the comparison of the diversity and prevalence of microorganisms in *D. andersoni* and *D. variabilis* in areas where these tick species are found alone (i.e. in allopatric populations), as well as in areas where the two species coexist (i.e. in sympatric populations) (Chapters 4-9). In addition, these studies also included a comparison of the bacteria in immature and adult ticks collected from the same locality. This was of significance because most studies that have examined the microorganisms of either of these tick species have done so only for only adult individuals, or the progeny of adult female ticks reared in the laboratory (e.g., Roland *et al.* 1998, Goddard *et al.* 2003, Ammerman *et al.* 2004). Results from these studies therefore provided insight into the host (i.e. vector) specificity, transmission cycles and risk of exposure of tick-borne bacteria.

Although these molecular-based studies focused on specific genera of bacteria, there was a marked difference in the bacterial species present in *D. andersoni* compared to those in *D. variabilis* for populations located near their northern distributional limits. Bacteria of the genus *Rickettsia* were found in both tick species; however *R. peacockii* was present in only *D. andersoni*, while *R. montanensis* was present in only *D. variabilis* (Chapter 5). This finding is consistent with other studies conducted on these ticks for other part of their geographical distributions (Niebylski *et al.* 1997, Ammerman *et al.* 2004, Moncayo *et al.* 2010, Stromdahl *et al.* 2011, Teng *et al.* 2011). Both these rickettsial species are considered to be non-pathogenic endosymbionts (Azad & Beard 1998). There was no evidence of *R. rickettsii*, the causative agent of Rocky Mountain spotted fever in humans, present in either tick species for populations that were examined in Canada. Similarly, there was no evidence for the presence of *Francisella tularensis*, the causative agent of tularaemia in humans and animals, in any of the populations of *D. andersoni* and *D. variabilis* examined in this thesis (Chapter 6). However, there were significant differences between the two tick species in the types of *Francisella*-like endosymbionts (FLEs) they carried. The detection of FLEs in *D. andersoni* and *D. variabilis* was not unexpected as studies conducted in other geographical areas have detected different FLEs in the two species (Sun *et al.* 2000, Scoles 2004). One of the interesting findings of the present study was the discovery of seven new types of FLES, three in *D. andersoni* and four in *D. variabilis*.

Another important discovery was the detection of a new species within the genus *Arsenophonus* in *D. andersoni* adults (Chapter 7). Hence, this represents a new microbe-tick association. It was also expected that *Arsenophonus* would be detected in *D. variabilis* adults, based on studies conducted in Indiana (Grindle *et al.* 2003). However, only a single *D. variabilis*

adult was infected with *Arsenophonus.* This tick was collected at a locality where it was sympatric with *D. andersoni*. Interestingly, this bacterial species is different to the *Arsenophonus* species reported in *D. variabilis* from Indiana (Grindle *et al.* 2003), but was identical to the species of *Arsenophonus* in *D. andersoni* based on sequence analyses of the bacterial 16S gene.

Ticks from two localities, Saskatchewan Landing Provincial Park and Blackstrap Provincial Park, were also examined for the presence of *Anaplasma* and *Ehrlichia* because species within these two genera are known have pathogenic effects on humans and/or animals (Doudier *et al.* 2010) and have been reported in Canada (Howden *et al.* 2010). For example, *Anaplasma marginale*, which is transmitted by *D. andersoni* and *D. variabilis* in the USA, has a major impact on the health of cattle (Kocan *et al.* 2010). However, it was expected that *A. marginale* would not be detected by PCR in either *D. andersoni* or *D. variabilis* because it is not endemic in Canada (Howden *et al.* 2010). The results of PCR analyses using the 16S rRNA gene detected one *D. variabilis* female infected with *Anaplasma*, but the species identity of this bacterium could not be verified. Based on the available sequence information, it could be *A. marginale*, *A. centrale*, *A. ovis* or *A. phagocytophilum*. Further analyses are needed to identify and characterize of this species of *Anaplasma*. As a consequence of these PCR analyses, one important finding of my work was the discovery of a bacterium genetically similar, based on 16S sequence data, to *A. bovis* in adult and nymphal *D. andersoni* at Saskatchewan Landing Provincial Park. There are no previously published reports of *A. bovis* in Canada. This discovery may have important implications for the health of cattle in the same regions because *A. bovis* is known to have a pathogenic effect on livestock in other countries. The potential pathogenic effect of this *A. bovis* strain on different species of mammals, particularly for bovids, needs to be determined. It is also important to determine if the presence of this bacterium is a health risk for

cattle located on farms located adjacent to collection site and for cattle that are free to roam within the park. Therefore, a number of questions remain unanswered regarding this bacterium. Although the 16S rRNA gene is a useful marker for distinguishing among bacterial species, it is important to further characterize this strain of *A. bovis* using several genes that have been used to examine genetic variation in other species within the genus (e.g. de la Fuente *et al.* 2005b, Zhou *et al.* 2010).

In addition, a number of other bacteria were accidentally detected in *D. andersoni* or *D. variabilis* because the "genus–specific" primers occasionally amplified the 16S gene of other bacterial genera (Chapter 8). For example, bacteria genetically-most similar (i.e. identical at 839 of 841 bp = 99.8 %) to *Serratia proteamaculans* and *Serratia proteamaculans quinovora* were discovered in *D. variabilis* adults from a single locality. As far as I am aware, this represents the first report of this type of bacterium in ticks in North America. It is also of interest to note that this bacterium was found only in some of the ticks feeding on skunks and raccoons, whereas ticks collected from the same locality, but obtained while feeding on dogs or while questing on vegetation (i.e. unfed adult ticks), were not infected with this bacterium. This suggests some association between this bacterium and medium-sized mammals. This discovery is of interest because *S. proteamaculans* is a pathogen of New Zealand grubs (Grkovic *et al.* 1995) and *S. p. quinovora* has been associated with a fatal case of pneumonia in a human (Bollet *et al.* 1993). Further studies are necessary to determine the mode of transmission of these microorganisms, and if they have pathogenic potential for human and/or animal hosts for ticks.

PCR analyses conducted in the present study also resulted in the detection DNA from an organism most similar (95.8% identity) to *Ignatzschineria larvae*, a species that has been isolated from the larvae of a parasitic fly (Tóth *et al.* 2001). This discovery is of interest because it is

likely the first report of this type of bacterium in ticks. In addition, *I. larvae* has been isolated from humans (Maurin *et al.* 2007, Roudiere *et al.* 2007); however, it is still has to be determined if this bacterium has any significance with regard to human or animal health. While attempting to detect *Anaplasma* in ticks by PCR, DNA sequences of another two endosymbiotic alphaproteobacteria (*Midichloria mitochondrii* and *Wolbachia* sp.) were detected. *Midichloria mitochondriii*, a bacterium most closely related to bacteria in the order Rickettsiales, was found in a single *D. andersoni* adult. The 16S sequence of the *Wolbachia* sp. was identical to that of a bacterium from an insect. The non-specific detection of these organisms highlights the need to be cautious when interpreting results of PCR-based assays, even when using primers that are meant to be specific for particular species or genera. It is also gives an indication of the diversity of organisms residing in *Dermacentor* ticks.

Another important finding of my work was the discovery of the high degree of host (i.e. vector) specificity of many of these bacterial species, and that this host specificity was maintained in areas where the two tick species coexisted. There were only three exceptions to this; one of which included the single *D. variabilis* adult in the zone of sympatry infected with *Arsenophonus*, which was found primarily in *D. andersoni* (Chapter 7). Also, two of the more frequent types of FLEs, type 1 in *D. andersoni* and type 2 in *D. variabilis*, were also found in very low frequency in *D. variabilis* and *D. andersoni* (respectively) collected from sympatric populations of these ticks (Chapter 6). These results provided valuable information as to the potential transmission cycles of these tick-borne microorganisms.

10.5. Inferences on the modes of bacterial transmission

A comparison of the vertebrate hosts and microbial species associated with *D. andersoni* and *D. variabilis* provides clues as to the potential transmission cycles and important hosts for the tick-borne microorganisms (see Fig. 10.2). Those bacteria that are specific for a single species of tick and occur at a relative high prevalence, such as *R. peacockii* and FLEs, are most likely to be vertically transmitted from one generation to the next.

Horizontal transmission between ticks through an intermediate vertebrate host may not be necessary for these bacteria to be maintained in tick populations. Indeed, previous studies have shown that *R. peacockii* and some FLEs are passed transovarially and there is little evidence to suggest that *R. peacockii* and FLEs can be transmitted horizontally (Niebylski *et al.* 1997, Baldridge *et al.* 2009). Amplification in a vertebrate host may not be necessary if bacteria, particularly for those species residing within the ovaries, are able to reproduce within ticks and take advantage of the high fecundity of their host (i.e. female ticks) by infecting a large proportion of eggs. Some bacterial endosymbionts have developed successful methods of spreading and maintaining themselves in a host population through highly efficient vertical transmission while also conferring a fitness advantage to those that are infected (Himler *et al.* 2011). The exclusive use of vertical transmission would also make infection of male ticks unnecessary for the propagation of the bacteria, even though there were no differences in the proportion of male and female ticks infected with *R. peacockii* and FLEs.

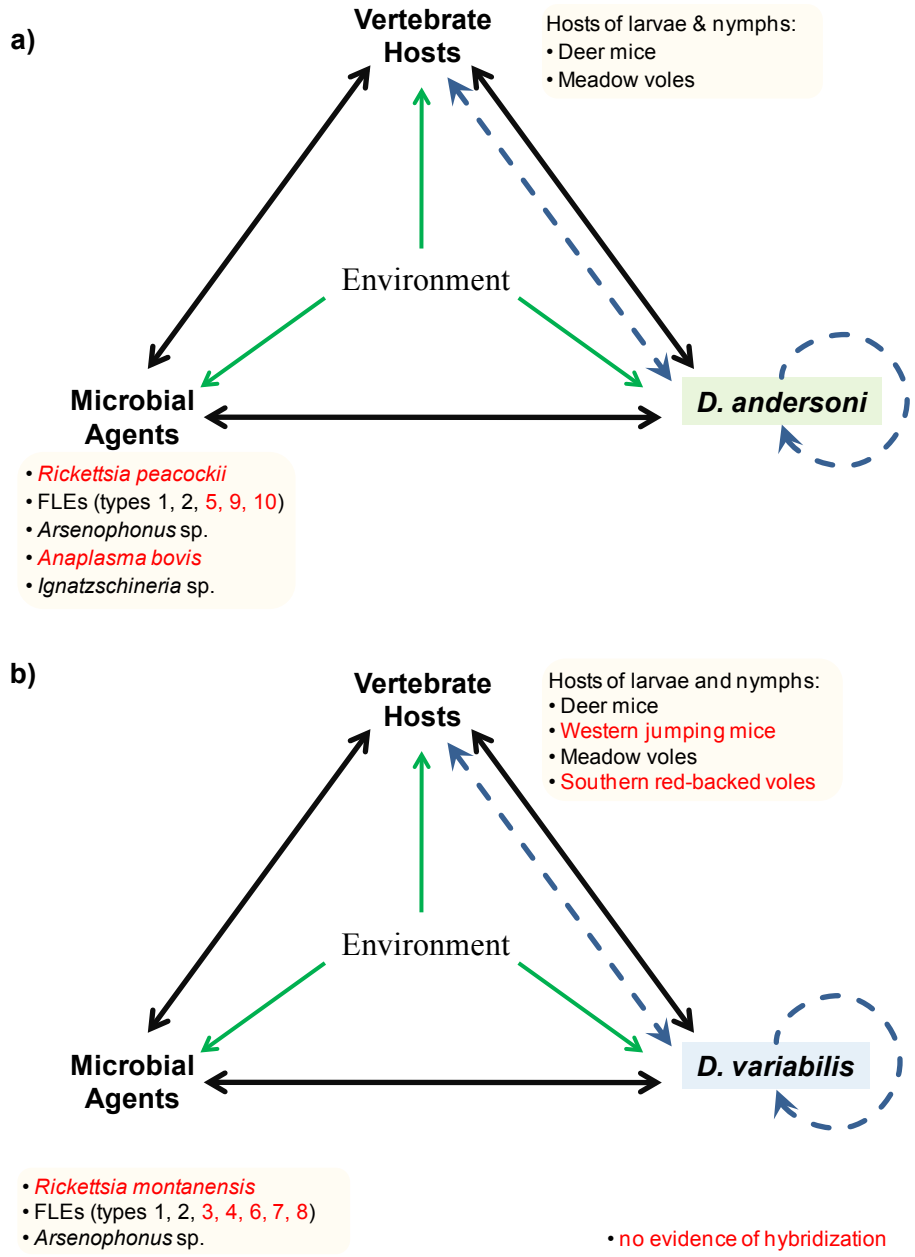

Fig. 10.2. Diagrammatic representation of the epidemiological triangle or triad of complex interactions as it relates to the two vectors examine in this thesis; (a) *D. andersoni* and (b) *D. variabilis*. Solid arrows represent the interactions between the organisms, while the broken arrows represent the direction of the modes of transmission (i.e. vertical and horizontal) for the microbial agent. Also shown are the small mammal hosts used by immature *D. andersoni* and *D. variabilis* and some of the microbes detected in individual ticks from localities near their northern distributional limits in Canada. Names of the hosts and bacteria in red indicate differences with respect to *D. andersoni* and *D. variabilis*.

In contrast, there is indirect evidence, based on the prevalence data of bacteria, of horizontal transmission of some species. This is particularly the case for bacteria that were detected in both tick species in the zone of sympatry but that differed in their relative prevalence, and that were absent in allopatric populations of one of the tick species. For example, the most prevalent FLE types (1 and 2) were found primarily in a single species of tick (*D. andersoni* and *D. variabilis*, respectively); however, they were also found in a small number of individuals from the other species of tick from localities where they were sympatric. This pattern of infection could be explained by the bacteria being transmitted mainly vertically by its primary tick host, together with horizontal transmission resulting in cross-infection into the other tick species. For this mechanism to be feasible, these bacteria must be transmissible and infective to at least one species of vertebrate host used by both *D. andersoni* and *D. variabilis*. Although many studies indicate that FLEs are not horizontally transmitted, there has been at least one report (Escudero *et al.* 2008) of an FLE detected in a wild small mammal that was genetically identical to those found in co-occurring ticks.

Horizontal transmission is also a possible mechanism by which *Arsenophonus* is transmitted among individuals of *D. andersoni*, and may also explain the rare occurrence of *Arsenophonus* in *D. variabilis*, which may be an example of cross-species transmission. Horizontal transmission, rather than transovarial transmission, is invoked as the possible mechanism because of the relatively low prevalence of *Arsenophonus* in *D. andersoni* adults (Chapter 7). In addition, the prevalence of *Arsenophonus* in *D. andersoni* varied greatly among tick populations. This pattern of infection is similar to that of a closely related species of *Arsenophonus* in different populations of the lone star tick, *Amblyomma americanum* in the USA (Clay *et al.* 2008). These bacteria are likely to be secondary symbionts, as has been suggested for

other species of *Arsenophonus* that occur in whiteflies (Thao & Baumann 2004) and the louse fly (Dale *et al.* 2006). Other species of *Arsenophonus* have been shown to be transmitted vertically in other arthropods (Duron *et al.* 2008); however, horizontal transmission of secondary symbionts within and between species of arthropod has been suggested for other *Arsenophonus* species (Russell *et al.* 2003, Thao & Baumann 2004, Taylor *et al.* 2011). The other symbionts that were detected at a low frequency (e.g., *Anaplasma bovis*, *Serratia proteamaculans*, and *Rickettsia montanensis*) in *D. andersoni* and *D. variabilis* are also likely to be maintained through horizontal transmission (exclusively, or in combination with vertical transmission), involving a vertebrate host in which the bacterium can survive and reproduce. An amplification host is necessary for those species that occur in a small percentage of a tick population and/or are vertically transmitted inefficiently (Fine 1975). In areas where different arthropods occur in sympatry, horizontal transmission is an important source of new symbiotic relationships in different species of arthropod (Duron *et al.* 2010).

The presence of *Anaplasma bovis* in a small number of *D. andersoni* adults and immatures suggests that it is probably maintained in a transmission cycle involving ticks and small mammals in Saskatchewan Landing Provincial Park. Vertical transmission probably does not contribute significantly to the maintenance of *Anaplasma* species; therefore, the transmission cycle of *A. bovis* likely involves a vertebrate host (s) that is used by *D. andersoni* immatures and is able to act as a reservoir host for this bacterium. However, the actual transmission cycle needs to be clarified. Further work is needed to determine if cross-species transmission to *D. variabilis* can occur because *D. variabilis* is also found at the same locality, uses the same small mammal hosts for the immature stages as *D. andersoni*, and has periods of host-seeking activity that overlap with *D. andersoni* (Chapter 3). Transfer of *A. bovis* to *D. variabilis* could have

significant epidemiological consequences through an increased potential for transmission to vertebrates due to a greater abundance of *A. bovis*-infected ticks at this locality. In addition, the potential period of transmission to large animals by the bite of an adult tick would be longer than if only *D. andersoni* was a vector for this bacterium.

10.6. Examination of the bacterial community structure in ticks

Arthropods of medical, veterinary or economic importance, like all living organisms, are colonized by a variety of symbionts (Zindel *et al.* 2011). The species composition of microbial infracommunities within ticks will be influenced by the interactions among the different species of microorganisms present. The results of my research did not reveal any negative associations between bacterial species, or any positive associations among bacteria; however, this may be a consequence of the sampling methods used to determine the bacteria within individual ticks (see Chapter 9, section 9.4). Nonetheless, one bacterial species, *Rickettsia peacockii*, which was detected in *D. andersoni* (Chapter 4), is known to prevent the transmission of a related tick-borne pathogen, *Rickettsia rickettsii*, from one generation of tick to the next (Burgdorfer *et al.* 1981). Hence, the presence of *R. peacockii* alters the vectorial capacity of *D. andersoni* individuals for *R. rickettsii*. Furthermore, *R. rickettsii* can also be lethal to its tick host (Niebylski *et al.* 1999). Thus, as a consequence of inhibiting the transovarial transmission of this pathogen, the endosymbiotic *Rickettsia* not only benefits the host, but also enhances its own reproductive potential (Lively *et al.* 2005). Similarly, other endosymbionts are believed to confer a protective effect to their arthropod hosts (Brownlie & Johnson 2009). The high prevalence of *R. peacockii* in all populations of *D. andersoni* examined (Chapter 4) combined with its ability to prevent the transmission of *R. rickettsii*, may be one explanation as to why *R. rickettsii* was not detected in

any *D. andersoni* examined in the present study. A similar explanation could be used to explain the lack of detection of *Francisella tularensis* in *D. andersoni* or in *D. variabilis* because a majority of ticks contained the non-pathogenic FLEs (Chapter 5). However, further studies are required to determine if FLEs and/or other species of non-pathogenic bacteria identified in *D. andersoni* and *D. variabilis* also reduce the infectivity and transmission of other bacterial pathogens. The degree to which pathogenic bacteria are excluded from a tick may not be based solely of the presence of an endosymbiotic bacterium, but may also be influenced by its relative intensity of infection (Burgdorfer *et al.* 1981). Therefore, changes in the relative abundance of different species within a microbial community may have important epidemiological implications.

The species composition of microbial infracommunities, and the interactions among the members of these communities and between the microorganisms and the arthropod host, can have significant effects on the physiology, reproduction, evolution and vectorial capacity of the host (Clay *et al.* 2006, Feldhaar & Gross 2009, Hibbing *et al.* 2010, José Gosalbes *et al.* 2010, Zindel *et al.* 2011). For example, some bacterial endosymbionts have been shown to alter reproduction of their insect hosts through the mechanisms of cytoplasmic incompatibility or distortions of the sex-ratio of individuals within a population (McGraw & O'Neill 1999, Ferree *et al.* 2008, Himler *et al.* 2011). Manipulating the sex ratio of their hosts, so that a large proportion of the offspring are females, enhances the vertical transmission of endosymbionts to the next generation. This is particularly important when they are propagated exclusively by transovarial transmission. Although several bacteria closely related to those that use these strategies (i.e. species of *Arsenophonus* and *Wolbachia*) were detected in *D. andersoni* or *D. variabilis*, there is

no evidence that a similar type of reproductive manipulation by these bacteria occurs in ticks (Clay *et al.* 2008); however, this warrants further investigation.

The effects of infection with bacterial symbionts in different arthropod species are often not readily apparent. Likewise, the functional roles of the different types of microorganisms detected in *D. andersoni* and *D. variabilis* remain largely unknown. However, previous studies have shown that certain bacterial symbionts can have significant effects on their hosts physiology, and may even be vital for the host's survival and reproduction. For example, removal of bacteria from within the tick *Amblyomma americanum* through antibiotic treatment can result in its reduced fitness (Zhong *et al.* 2007). Although some bacteria, such as *Rickettsia peacockii* and the FLEs, are highly prevalent in tick populations (Chapters 4 and 5) and are predominately transmitted vertically to ticks, it is not known if they are beneficial for the survival of individual hosts. It is possible that the relationship between these endosymbionts and their tick hosts may be in the process of evolving into a mutualistic relationship in which both organisms are highly dependent on one another for their survival. It has been speculated that non-pathogenic endosymbionts from the genus *Rickettsia* and *Francisella* evolved from an ancestral species similar to the pathogens within each genus, which have a broad host range, but have become adapted to conditions within the tick host (Azad & Beard 1998, Scoles 2004, Weinert *et al.* 2009).

The relationships between ticks and their endosymbiotic bacteria are likely the result of long-term associations, being shaped by selective pressures produced by features of the tick, the bacteria and their vertebrate hosts (Wernegreen 2002). Symbiotic bacteria are important drivers of arthropod evolution (Duron 2010) and the environment within the tick hosts provides the conditions that tick-borne microorganisms need to adapt to, resulting in co-evolution of the two

groups of organisms. Such evolutionary changes can lead to a strict association of bacteria with specific species of arthropod host (Azad & Beard 1998). The close associations between arthropods and the bacteria they harbour can allow for horizontal gene transfer between bacteria, and between bacteria and the eukaryotic host (Davison 1999, Hotopp *et al.* 2007, Baldridge *et al.* 2010). These exchanges of genetic material lead to rapid genomic changes in the bacteria and/or the host and, therefore, potentially to changes in important physiological characteristics.

The co-evolution of bacteria and host is a continuous process, as illustrated by the wide variety of rickettsial species that range from important human and animal pathogens that are transmitted among arthropod vectors and a variety of vertebrate hosts to those that likely have a relationship with a single species of arthropod and are only vertically transmitted (Azad & Beard 1998). Through adaptation to surviving and propagating in specific hosts, many rickettsial species have experienced a reductive genome evolution, sometimes resulting in the loss of virulence factors (Felsheim *et al.* 2009, Merhej & Raoult 2010). Bacterial species that are considered primary symbionts in other species of arthropod have also experienced a reduced genome, losing the ability to produce essential nutrients, while at the same time, their arthropod host also experienced the same type of function loss for other genes involved in nutrient processing (Wernegreen 2002). This has created interdependence between the bacterial endosymbionts and the arthropod host based metabolic needs (i.e. metabolic coupling) and a streamlining of the genome for greater efficiency (José Gosalbes *et al.* 2010).

10.7. Future work

This study has identified a variety of bacterial species in *D. andersoni* and *D. variabilis*, including some bacteria that have not been reported previously in these tick species. However, it

is evident that this represents only a fraction of the bacterial diversity within these ticks based on comparisons with studies conducted on other species of ixodid tick, such as *Amblyomma americanum, Ixodes Scapularis* and *Ixodes ricinus* (Clay *et al.* 2006, Clay *et al.* 2008, Van Overbeek *et al.* 2008, Heise *et al.* 2010). Further studies should employ new molecular tools to identify and characterize all the microbial species within the microbiome of all life stage of *D. andersoni* and *D. variabilis* from different populations throughout their extensive geographical ranges. In addition, the types and prevalence of bacteria in these ticks should also be examined for temporal variation, sampling from the same localities throughout their active feeding period (i.e. April to July) over multiple years to determine if the microbial community composition changes over time and in different life stages. Such studies would provide a greater understanding of the vector potential of these ticks, the epidemiological significance of different microbial relationships, and the interactions among bacteria within tick-borne microbial communities.

Although this study focused on the specific groups of bacteria in *D. andersoni* and *D. variabilis*, these ticks are also potential vectors of viruses that are pathogens to humans and/or animals. For example, *D. andersoni* is the vector for Colorado tick fever virus, a pathogen that was shown to exist in these ticks in Alberta and Saskatchewan (Cimolai *et al.* 1988). However, little is known about the current prevalence or transmission of this specific virus. Thus, it would be of medical relevance to conduct studies to determine if this viral pathogen currently occurs within different populations of *D. andersoni* in Canada.

The results obtained during this thesis have raised a number of other questions regarding the transmission cycles of tick-borne microorganisms and their relationships with the vertebrate and/or tick host. Some of these include: Why are some species of bacteria found only in one of

the two species of tick, even in areas where the ticks occur in sympatry? Why are there significant differences in the prevalence of some bacteria in different tick populations and species? Are particular strains of tick or different genotypes of tick more susceptible to infection and more suitable as vectors of particular bacteria? Finding answers to these questions is necessary to further define which biological factors influence the vectorial capacity of ticks.

10.8. Conclusions

The results of my study suggest that, although there is definite risk of exposure to potential vector species in many areas of western Canada, there appears to be little or no risk of acquiring tick-borne pathogens that are commonly associated with *D. andersoni* and *D. variabilis* (i.e. *Francisella tularensis*, *Rickettsia rickettsii* and *Anaplasma marginale*). Some of the tick-borne bacteria identified in my research are generally considered to be non-pathogenic symbionts. However, this does not preclude any epidemiological relevance to their presence. Other bacteria detected in *D. andersoni* and *D. variabilis* have been associated with human infection, but the presence of some potentially pathogenic bacteria in does not necessarily imply a vector-pathogen relationship. Transmission studies, along with field observations are required to implicate a tick as a vector and bacteria as cause of disease. However, the identification of tick-borne microorganisms provides a basis for further investigations into the role of ticks as vectors and understanding tick-microbe associations that are biologically, evolutionary and epidemiologically important. Analyses of the microbial community composition of arthropod vectors, such as mosquitoes, fleas and, especially ticks, can help identify important associations between ticks and microorganisms, potentially leading to the recognition of their role in infectious diseases, and of interactions that can affect the vectorial capacity of the vector.

The biotic and abiotic factors that determine the epidemiology of many arthropod-borne diseases are still poorly understood. In general, more work is needed to determine the host range (alternate vectors and mammalian hosts), geographic range, and pathogenicity (medical/veterinary relevance) of a number of known and newly recognized arthropod-borne microorganisms. In addition, the ecological factors that determine the distributional range, host associations and vectorial capacity of arthropod vectors need to be further investigated. Such studies are necessary to further understand the transmission cycles of arthropod-borne microorganisms and the epidemiology of vector-borne disease. It is particularly important to understand the ecological factors that affect the epidemiology of vector-borne disease as changes in human activity and environmental conditions will result in shifting patterns in the distributions of vectors and their associated pathogens, and in frequency of tick-borne diseases.

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