DIVERSITY AND ABUNDANCE OF FLEAS ON RICHARDSON'S GROUND SQUIRRELS AND IN THEIR BURROWS, AND THE BACTERIA WITHIN THE MICROBIOMES OF THESE FLEAS

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By

Jessica Thoroughgood-Smith

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ABSTRACT

The diversity and abundance of parasites among a host population varies across time and space. Parasites are restricted in an ecosystem by the range of hosts they infest (= the degree of host specificity), by the interactions with other parasites, environmental conditions, and the fitness of the host they parasitize. Some parasites are vectors of pathogens that affect wildlife, domestic animals and/or humans. For example, *Yersinia pestis*, the causative agent of plague, is transmitted from infected animals to susceptible animals by a variety of flea species. Black-tailed prairie dogs (*Cynomys ludovicianus*), a threatened species in North America, are highly susceptible to *Y. pestis* infection. They share multiple species of flea that are vectors of *Y. pestis* with Richardson's ground squirrels (*Urocitellus richardsonii*) and they co-exist in Grasslands National Park in southern Saskatchewan, Canada. Although work has been conducted previously on flea communities associated with black-tailed prairie dogs, little research has been conducted on fleas of *U. richardsonii*, particularly for populations located in their northern parts of the distributional range. Knowledge of the diversity and abundance of fleas parasitizing *U. richardsonii* and of the bacteria in flea microbiomes is imperative for understanding the risk and disease transmission to *U. richardsonii* and *C. ludovicianus*. In this thesis, I investigate the bacterial communities of the different flea species parasitizing Richardson's ground squirrels and assess if ecological concepts (e.g. host specificity, community structure) that apply to parasites on hosts can be applied to the bacterial of these fleas.

The first objective was to determine the species of fleas using genetic markers because of the difficulties distinguishing among species based on morphological characters. Also, this approach eliminated the need to chemical clear the internal structures and bloodmeal, permitting molecular-based studies of the fleas and their microbiomes. I assessed the suitability of five

molecular targets, the nuclear 18S ribosomal RNA (rRNA) and 28S rRNA genes, the nuclear internal transcribed spacer 2 (ITS2), and the mitochondrial cytochrome oxidase *c* subunit 1 and 2 genes (*cox*1 and *cox*2), to identify eleven flea species parasitizing Richardson's ground squirrels. The ITS2 and 28S rRNA gene were the best genetic markers for species identification, while *cox*2 was more useful for studying the population genetics of fleas.

The second objective was to investigate the community structure of fleas on *U. richardsonii* in Alberta and Saskatchewan. The dominant species on *U. richardsonii* near Lethbridge (Alberta) was *Oropsylla rupestris*, while *O. tuberculata* was the most abundant species on *U. richardsonii* near Moose Jaw (Saskatchewan). The sex and length of the host species as well as the month of collection were factors that significantly influenced the prevalence and abundance of fleas parasitizing Richardson's ground squirrels. Differences were also detected in the diversity and relative abundance of fleas in the burrows of *U. richardsonii* at a rural site (near Bradwell) and an urban site (Saskatoon). The most prominent species in the urban site was *O. rupestris*, while *O. bruneri* was the most prominent species at the rural site. Seasonal patterns of activity were observed with flea species peaking during different months.

I also investigated the prevalence and abundance of *Bartonella*, *Rickettsia*, and *Wolbachia* in the bacterial communities of nine species of fleas from the burrows of Richardson's ground squirrels in Saskatchewan. I found that the bacterial load of these samples and the prevalence of key bacterial species (e.g., *Bartonella*, *Rickettsia*, and *Wolbachia*) was very low based on qPCR analysis. A conventional PCR approach was taken to determine the diversity and abundance of *Bartonella*, *Rickettsia*, and *Wolbachia* in these samples. Distinct strains of *Wolbachia* were detected in different species of flea species and multiple strains of *Bartonella washoensis* were detected in *O. bruneri*. Three species of *Oropsylla* and *Neopsylla*

inopina contained a *Rickettsia*-like endosymbiont which have not been previously reported in fleas. Further investigation is required into the role and frequency of *Wolbachia* and *Rickettsia*-like endosymbiont in fleas. These findings provide insight into the diversity of fleas on Richardson's ground squirrels at their northern distribution and a framework for investigating the bacterial communities of these fleas.

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DEDICATION

To the girl that was told she couldn't,

I am proud of you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AB – Alberta

bp – base pair

cfu – colony-forming unit

CT – cycling threshold

DNA – deoxyribonucleic acid

cox1 – mitochondrial cytochrome oxidase subunit I gene

cox2 – mitochondrial cytochrome oxidase subunit II gene

gltA – citrate synthase gene

ITS – internal transcribe spacer

mt – mitochondrial

nj – neighbour-joining

PCR – polymerase chain reaction

qPCR – quantitative or real-time PCR

RGS – Richardson's ground squirrels

RLE – *Rickettsia*-like endosymbiont

SK – Saskatchewan

SSCP – single strand conformation polymorphism

ssrA – *Bartonella* transfer-messenger RNA

UPGMA - unweighted pair group methods using arithmetic means

wsp – *Wolbachia* surface protein

CHAPTER 1: GENERAL INTRODUCTION

1.1 PARASITES AND PARASITOLOGICAL CONCEPTS

Approximately 70% of extant animal species have a parasitic mode of existence for one or more phases of their life cycle (Clayton and Moore 1997, Bist et al. 2001). During the parasitic phase(s), they are dependent on one or more hosts for nutrients, shelter, reproduction and/or dispersal (Clayton and Moore 1997, Bist et al. 2001). Interactions among parasite species is one factor influencing species richness and abundance of parasite communities (Kamiya et al. 2014). Spatial and temporal factors have also been found to affect the community structure of parasites (Penczykowski et al. 2016). However, in general, diverse host taxa are expected to have a higher diversity of parasites (Poulin and Morand 2000, Kamiya et al. 2014). Parasite communities are also influenced by the characteristics of the host population (i.e., relative abundance and density), the diversity of parasites within/on an individual host, and the dispersal of parasites between individual hosts (Harbison and Clayton 2011, Cohen et al. 2015, Clark et al. 2018).

The range of host species that a parasite infests/infects is a measure of its host specificity. For those species that require two or more hosts to complete their life cycle, their relative host-specificity may differ significantly between life cycle stages. Some parasite species may be specific for a particular group of hosts (e.g., rodents or passerine birds). Those species that parasitize a wide range of host species are considered host generalists, while those restricted to one or a few host species are host specialists. Although some species can parasitize a range of host species, they may have preferred hosts where they achieve greater fitness (Krasnov et al. 2004). For example, ectoparasites feeding on birds or mammals (i.e., endotherms) may take less time to gain a blood meal compared to when they feed on reptiles (i.e., ectotherms) because of

the significant differences in body temperatures of these host groups (Pollock et al. 2015). The two major factors influencing host specificity are compatibility (e.g., physiological) with hosts and the chance of encountering a host species (i.e., influenced by distribution and abundance of host species) (Combes 2001). Furthermore, individuals of a parasite species are not randomly distributed throughout a host population but are aggregated on/in a relatively small number of hosts (Bist et al. 2001). Therefore, a large proportion of hosts lack that parasite species. This aggregation can be influenced by host factors such as home range, behaviour, sex, age, hormone level, and immune response (Soliman et al. 2001, Krasnov et al. 2005, Hillegass et al. 2008, Harrison et al. 2010, Krasnov et al. 2012, Kiffner et al. 2013, Waterman et al. 2014, Bohn et al. 2017, Warburton et al. 2017, Zduniak et al. 2023).

Host-parasite associations may be a consequence of a relationship between the ancestral species of both host and parasite species or by the colonization of a host species by a parasite species from another host species (i.e., host-switching) (Poulin et al. 2011). A strong association between host and parasite species can lead to evolutionary changes in both organisms at the microevolutionary level (e.g., population genetics) and macroevolutionary level (e.g., cospeciation) (Clayton and Moore 1997, Bist et al. 2001). Host-parasite coevolution is often inferred by comparing the evolutionary relationships (i.e., phylogenies) of both host and parasite species. Strict host-parasite co-speciation occurs where the phylogeny of the parasite group mirrors the phylogenies of the host group (= Fahrenholz's rule; Brooks 1985). The classic example of host-parasite cospeciation involves pocket gophers (Family Geomyidae) and their parasitic chewing lice (Family Trichodectidae). Hafner and Nadler (1988) used biochemical and molecular markers to demonstrate that lice (*Geomydoecus* and *Thomomydoecus* spp.) had coevolved with their pocket gopher hosts (*Thomomys*, *Geomys*, and *Orthogeomys* spp.).

However, there are also instances of host-switching events (Hafner and Nadler 1988). Host-switching is common in many parasite groups (e.g., Ricklefs and Fallon 2002, Hoberg and Brooks 2008, Millanes et al. 2014).

1.2 PARASITIC ARTHROPODS AS VECTORS OF HUMAN AND ANIMAL PATHOGENS

Some parasitic arthropods (i.e. ticks, fleas, lice, flies) are vectors of pathogens (i.e., bacteria, viruses and protozoa) that can have detrimental effects on the health of humans, wildlife, and/or domestic animals. Although mosquitoes are considered the most important vector for human pathogens (Schaffner et al. 2013), there are other important arthropod vectors, such as ticks, fleas, biting bugs and flies, that are responsible for transmitting pathogens to humans, wildlife and/or domestic animals (Lefèvre et al. 2022, Yanase et al. 2024). Vector-borne pathogens are expected to increase in frequency and distribution with global climate change as many vectors are expanding their distributional range in response to warmer temperatures enhancing vector reproduction, more rapid progression through the life cycle and increased survival (Ostfeld 2009, Semenza and Suk 2018, Rocklöv and Dubrow 2020). For example, the blacklegged tick or deer tick, *Ixodes scapularis*, the vector of *Borellia burgdorferi*, the bacterium responsible for Lyme borreliosis in humans and companion animals (i.e., horses & dogs) in the eastern United States and eastern Canada (Keirans et al. 1996, Eisen and Eisen 2018), continues to expand its range northwards (Khatchikian et al. 2015, Clow et al. 2017). Warmer climatic conditions in southern Canada have provided this tick species with a longer window of time during spring and summer to complete key phases of its life cycle, something that was not possible during the 1970s (Ogden et al. 2013, Ogden and Lindsay 2016). Given the increased risk of vector-borne diseases, such as Lyme disease, there has also been an increased investment

in investigating the bacterial community of vectors. An increased level of investigation is particularly important in species, such as black flies and mosquitoes, where the bacterial communities can suppress pathogen invasion and transmission (Jones et al. 2013a, Caragata et al. 2016, Woodford et al. 2018). For example, the presence of *Wolbachia* endosymbionts (i.e., mutualists) in female mosquitoes (*Anopheles* spp. and *Aedes aegypti*) inhibits reproduction and transmission of *Plasmodium*, the causative agent of malaria, to vertebrate hosts that includes humans (Moreira et al. 2009, Kambris et al. 2010, Hughes et al. 2011, 2014).

1.3 ORDER SIPHONAPTERA (FLEAS)

The order Siphonaptera (fleas) are bilaterally flattened wingless insects that are parasitic for part of their life cycle. There are over 2 200 species of siphonapterans that have been separated into four infraorder/super families and eighteen families (Krasnov 2008, Galloway 2019). The nomenclature of this insect group is continually changing with the use of molecular techniques to address fundamental evolutionary questions (Whiting et al. 1997, 2008, Whiting 2002). Siphonaptera represents a monophyletic group that are most closely related to two orders, Diptera (= true flies) and Mecoptera (= scorpion flies) (Durden and Traub 2002, Whiting 2002, Krasnov 2008). Within the Siphonaptera, species identification is only possible for adults as the other life stages do not have defining details for identification (Krasnov 2008). Adult fleas of different species can be identified based on minute differences in morphological characteristics that include shape and structure of the genitalia, as well as the distribution of setae and spines on the body (Holland 1985, Bitam et al. 2010). However, morphological identification of adult fleas first requires the chemical clearing of internal structures which prevents subsequent molecular-based studies of flea identification, population genetics, evolutionary relationships and the

community structure of bacteria (pathogens and endosymbionts) within individual fleas (Hastriter and Whiting 2003, Whiting et al. 2008, Belthoff et al. 2015). As many flea species play pivotal roles in the transmission of bacteria that are of medical and veterinary significance, it is imperative that genetic markers are developed and used for flea identification, to allow molecular studies to be conducted on their bacterial communities.

Fleas are holometabolous and are only parasitic during the adult stage; parasitism by larval fleas is indeed a rare phenomenon (Krasnov 2008). However, the larval stage of some species, such as *O. silantiewi* and *O. alaskensis*, feed on the skin and blood of their hibernating hosts (Krasnov 2008). Fleas parasitize a variety of animals including birds, bats, rodents, carnivores, and hominids (Whiting et al. 2008). Many flea species are host generalists but have a preferred host species which is termed their "true host" (Holland 1985). Some of the morphological structures of fleas, such as their sclerotized bristles, combs, ctenidia, spines and setae, are adapted for remaining attached to specific host species and these characteristics are related to the physical features of the hosts, particularly the type of skin, and/or fur (Krasnov 2008).

The duration of the life cycle varies among flea species, as does the number of offspring produced by female fleas (Krasnov 2008). For example, fleas that parasitize hosts that hibernate during winter have a life cycle averaging eight to nine months, whereas fleas parasitizing non-hibernating hosts take on average only two to three months to complete their life cycle (Krasnov 2008). In general, adult fleas lay eggs in their host's burrow or nest and will take 6-14 days to hatch (Krasnov 2008, Bitam et al. 2010). The number of eggs laid by female fleas and duration of the oviposition period differs among species, the type of host on which females had fed, and the temperature and relative humidity of the microhabitat in which eggs are laid (Krasnov 2008,

Bitam et al. 2010). Larvae that emerge from eggs feed on organic material in the burrow or nest of their host. During the larval phase, fleas are particularly sensitive to the composition of the soil on which they live because they require a microhabitat with a relative humidity above 40-50% (Krasnov 2008). The larvae go through three molts after which they form a silken cocoon for development through to the pupal stage (Krasnov 2008, Bitam et al. 2010). The duration of the pupal stage is dependent on environmental conditions of the microhabitat that include the relative humidity, temperature and CO_2 concentration, and/or vibration resulting from activity of hosts (Krasnov 2008). Adult fleas emerge from the cocoon. They will spend their time mainly in the burrow/nest of their host or on-host, the duration of which varies among species (Krasnov 2008). Reproduction does not occur until both adult males and females have fed on a suitable host (Krasnov 2008). After the initial feeding, fleas can live from several months to over a year depending on the species of fleas and the hosts available (Krasnov 2008).

1.4 FLEAS AS VECTORS OF HUMAN AND ANIMAL PATHOGENS

Fleas can acquire bacterial endosymbionts (non-pathogenic mutualists) by transovarial transmission, that is, passed from the adult female to her eggs, and then to larvae that hatch from the eggs and subsequent developmental stages. Also, these endosymbionts and pathogenetic bacteria can be acquired from the blood meal imbibed by adult fleas when feeding on an infected mammalian host. The most important flea-borne diseases include plague, murine typhus, and cat scratch disease.

Bubonic plague is one of the more notorious vector-borne diseases. It is caused by infection with the bacterium, *Yersinia pestis*. There are three types of *Y. pestis* transmission. Fleas consume a blood meal from an infected wild host and then transfer bacteria to a new

uninfected wild host (= sylvatic plague) or to a human host (= bubonic plague). There can also be transmission by respiratory droplets of *Y. pestis* from an infected person to a new human host (= pneumonic plague). This mode of transmission involves inhalation of respiratory droplets instead of infected flea bites. Susceptible hosts can also be infected by handling and/or consumption of deceased animals infected with the *Y. pestis.* There have been three major plague pandemics; the Justinian plague during the 6th century, "Black Death" during the 14th century, and the modern pandemic that has occurred since the 1850s (Eisen and Gage 2012). The most well-known of these, "Black Death", resulted in an estimated death toll of a quarter to a third of the population of Europe (Eisen and Gage 2012). The Oriental rat flea, *Xenopsylla cheopsis*, was the main vector of *Y. pestis* infections during the 14th century that transmitted the bacterium from infected rats to humans. The bacterium was later introduced to North America in the early 1900s via infected rats and fleas in the cargo of ships that landed in New Orleans, Louisiana, Galveston, Texas, and San Francisco, California (Eisen and Gage 2012). Although the bacterium was eliminated from Louisiana and Texas through increased sanitation, *Y. pestis* entered the sylvatic phase of local rodent hosts in California (Eisen and Gage 2012). This pathogen has spread throughout the western portion of North America northward into the prairie regions of Canada (Gibbons and Humphreys 1941, Antonation et al. 2014) and eastward from the west coast through the Rocky Mountains to the western portion of the Great Plains (Maher et al. 2010, Eisen and Gage 2012). In recent years, the average number of human cases of plague around the world is 2000 per year (Gage and Kosoy 2005, Bitam et al. 2010). Most (98%) of the incidences of human infection with *Y. pestis* have been reported in Madagascar (Nguyen et al. 2018) and the Democratic Republic of Congo (Bertherat 2019). For example, the plague epidemic in Madagascar in 2017 resulted in 2,417 cases of human infection and 209 deaths (Nguyen et al.

2018). The vectors of *Y. pestis* in Madagascar are *Xenopsylla cheopis* and *Synopsyllus fonquerniei* (Esquivel Gomez et al. 2023, Rasoamalala et al. 2024).

Some flea species are vectors of other pathogenic bacteria that includes species of *Rickettsia* and *Bartonella* (Eisen and Gage 2012). *Rickettsia* are obligate intracellular gram-negative bacteria that are separated into three groups: the typhus group, the transitional group, and the spotted fever group (Gillespie et al. 2008). Fleas are vectors of both the transitional and typhus groups of *Rickettsia*, specifically *R. typhi* and *R. felis* (Gillespie et al. 2008, Eisen and Gage 2012). *Bartonella* are intracellular facultative parasites of mammalian erythrocytes and endothelial cells (Kosoy 2010, Himsworth et al. 2020). Sciurid rodents, such as ground squirrels and prairie dogs, are mainly infected with *B. washoensis*, a causative agent of human myocarditis (Kosoy 2010).

1.5 FLEAS AS PARASITES OF SCIURID RODENTS AND VECTORS OF PATHOGENS IN NORTH AMERICA

Over 230 species of fleas are known to occur in North America (Hubbard 1947). At least 154 species, representing four super families and seven families, are known to occur in Canada (Galloway 2019). The most extensive research on siphonapterans in North America has focused on the flea communities of black-tailed prairie dogs (*Cynomys ludovicianus*), particularly in Colorado, New Mexico and South Dakota (e.g., Eads et al. 2015, Eads and Hoogland 2016, 2017, Eads and Biggins 2017). Black-tailed prairie dogs are particularly vulnerable to infection with *Y. pestis*, with deaths in prairie dog colonies ranging from 0-100% (Cully and Williams 2001, Pauli et al. 2006, Antonation et al. 2014). In Canada, black-tailed prairie dogs only occur in Grasslands National Park in southern Saskatchewan (COSEWIC 2011). Richardson's ground

squirrels (*Urocitellus richardsonii*) and black-tailed prairie dogs co-occur within the park and within the Northern Mixed Grassed Prairie of Montana and North Dakota (Augustine et al. 2023). This co-occurrence provides an opportunity for fleas, such as *Oropsylla rupestris, O. labis, O. tuberculata, Rhadinopsylla fraterna* and *Thrassis bacchi*, to transfer between the two rodent species (Holland 1985, Liccioli et al. 2020). Ground squirrels may play an important role in transferring *Y. pestis* infected fleas between reservoir host species and susceptible hosts (Lechleitner et al. 1968, Anderson and Williams 1997). In addition, ground squirrels and prairie dogs often share flea species and flea exchange between these hosts is particularly evident during plague outbreaks (Ecke and Johnson 1952, Anderson and Williams 1997, Cully and Williams 2001).

Plague has also been reported in Richardson's ground squirrels. For example, a severe outbreak occurred in *U. richardsonii* populations that covered an area of over 360 square kilometers of eastern Alberta during the late 1930s (Gibbons and Humphreys 1941). Two specimens of *O. labis* infected with *Y. pestis* were removed from a dead Richardson's ground squirrel (Gibbons and Humphreys 1941), suggesting that this flea species is an important vector of the bacterium. Five other flea species, *O. rupestris*, *O. tuberculata*, *Neopsylla inopina*, *R. fraterna* and *Hystrichopsylla dippiei*, were also collected from *U. richardsonii* in the same region (Gibbons and Humphreys 1941), two of which, *O. rupestris* and *O. tuberculata*, are known vectors of *Y. pestis* (Eskey and Hass 1939, Wilder et al. 2008, Eisen and Gage 2012). Another occurrence of plague in this region of eastern Alberta was reported in 1942 based on fleas (species not indicated) collected from *U. richardsonii* near Sunnynook, Youngstown and Hanna, and from the tissue of an infected Richardson's ground squirrel collected near Suffield (Ozburn 1944). According to Humphreys and Campbell (1947), *Y. pestis* was detected in ground squirrels

from eastern Alberta each year from 1939 to 1942 and in 1945. Two pools of fleas collected from 61 *U. richardsonii* in 1946 from an area of Saskatchewan adjacent to the infected area in Alberta also tested positive for *Y. pestis* (Humphreys and Campbell 1947). Similarly, *Y. pestis* was reported in fleas collected from *U. richardsonii* at two localities in North Dakota in 1941 (Prince 1943). Subsequently, populations of Richardson's ground squirrel in the Rocky Mountains and High Plains of Colorado and Wyoming were affected by an outbreak of plague in the 1970's (Virchow et al. 1992). In 2017, two dead Richardson's ground squirrels and one black-tailed prairie dog, collected in Grasslands National Park, located in southwestern Saskatchewan (Canada) were infected with *Y. pestis* (Liccioli et al. 2020). At least nine species of flea were removed from Richardson's ground squirrels and black-tailed prairie dogs, and from their burrows (i.e., using a swabbing technique) in Grasslands National Park, but of the 664 fleas tested for *Y. pestis*, only four *O. tuberculata* and one flea that could not be identified, were positive for the bacterium (Liccioli et al. 2020). Sylvatic plague in black-tailed prairie dogs in Grasslands National Park, the only population of this species in Canada, was first detected in 2010 (Antonation et al. 2014). Given that populations of black-tailed prairie dogs can suffer significant mortality (>90%) due to *Y. pestis* infection (Cully and Williams 2001), the Canadian population is at a significant risk of local extinction because of an increased threat of drought and sylvatic plague (Liccioli et al. 2020). Management of this population against the plague is complicated by coexistence of Richardson's ground squirrels (Liccioli et al. 2020) because the role of this species in the transmission and maintenance of *Y. pestis* in this area is poorly understood (Fitzgerald 1970, Anderson and Williams 1997, Cully and Williams 2001). Other mammals may also be acting as reservoir hosts for *Y. pestis* in this area. A serological survey conducted in 1995 on dogs and cats on farms close to Grasslands National Park revealed that

there had been contact between some animals and *Y. pestis* (i.e., 4% of dogs and 2% of cats), probably by hunting wild rodents (Leighton et al. 2001). This survey also detected dogs and cats that were serologically positive for *Y. pestis* on farms near Saskatchewan Landing Provincial Park (also in southwestern Saskatchewan), Dinosaur Provincial Park and Writing-On-Stone Provincial Park, both located in southeastern Alberta, all areas of which are located within the range of *U. richardsonii* (Cassola 2016).

Richardson's ground squirrels are commonly parasitized by two families of fleas, the Ceratophyllidae and the Ctenophthalmidae. The species of the Ceratophyllidae that parasitize *U. richardsonii* include *O. bruneri, O. labis, O. saundersi, O. tuberculata, O. rupestris, T. bacchi* and *T. pandorae*, while the two species of Ctenophthalmidae that occur on *U. richardsonii* are *N. inopina* and *R. fraterna* (Buckner 1964, Burgess 1955, Holland 1944, 1949,1985, Galloway and Christie 1990, Waterman et al. 2014, Hastriter 2023). Of these, *O. rupestris* and *O. tuberculata,* are known vectors of *Y. pestis* in North America (Eisen and Gage 2012). All of the flea species associated with Richardson's ground squirrels are considered host generalists because they parasitize a range of rodent species (Holland 1985), some of which are vulnerable to infection with *Y. pestis* or are suspected to be reservoir hosts (Eisen and Gage 2012). The rodents most susceptible to infection with *Y. pestis* in North America include Gunnison’s prairie dogs (*Cynomys gunnisoni*), white-tailed prairie dogs (*C. leucurus*), black-tailed prairie dogs (*C. ludovicianus*), Utah prairie dogs (*C. parvidens*), chipmunks (*Eutamias* spp.), wood rats (*Neotoma* spp.), California ground squirrels (*Otospermophilus beecheyi*), and rock squirrels (*S. variegatus*) (Gage and Kosoy 2005, Eisen and Gage 2012). Deer mice (*Peromyscus* spp.), rats (*Rattus rattus* and *R. norvegicus*), the eastern chipmunk (*Tamias striatus*), and voles (e.g., *Microtus*

californicus) are known to be reservoir species for *Y. pestis* (Gage and Kosoy 2005, Eisen and Gage 2009, Jahan et al. 2021).

Understanding the seasonal activity patterns of arthropod vectors is important for assessing the risks of infection with bacterial pathogens for the hosts parasitized by infected arthropods. Several studies have been conducted on the seasonal activity patterns and diversity of fleas that commonly parasitize Richardson's ground squirrels in the Aspen Parkland Ecoregion of southern Manitoba (Galloway and Christie 1990, Lindsay and Galloway 1997, 1998, Waterman et al. 2014). The diversity of fleas parasitizing Richardson's ground squirrels and black-tailed prairie dogs in Grasslands National Park in southern Saskatchewan has also been examined (Liccioli et al. 2020). However, there were differences in the species of fleas found on these sciurid rodents among these studies. *Oropsylla bruneri* was the only flea species detected on Richardson's ground squirrels in all four of the studies. While *O. rupestris* and *R. fraterna* were detected on Richardson's ground squirrels in Grasslands National Park (Liccioli et al. 2020) and Manitoba (Galloway and Christie 1990, Lindsay and Galloway 1997, 1998), these species were absent in the study by Waterman et al. (2014). The reason why they were not detected was probably a consequence of the restricted collection period for fleas (i.e., during the time when hosts emerged from hibernation).

Several studies have also examined if characteristics of the host (i.e., sex, immune response, body condition and mass) and/or environmental conditions influence the relative abundance of fleas on Richardson's ground squirrels in the Aspen Parkland Ecoregion of Manitoba (Waterman et al. 2014) and in the Mixed Grass Prairie Ecoregion of Saskatchewan (Jardine et al. 2006a, 2006b). The Waterman et al. (2014) study was restricted to the period when Richardson's ground squirrels first emerge from their burrows, whereas the studies by Jardine et

al. (2006a, 2006b) examined the seasonal activity patterns of *Bartonella* infections within Richardson's ground squirrels. Apart from the study of Lindsay and Galloway (1997, 1998) on the diversity and seasonal occurrence of fleas on Richardson's ground squirrels in Manitoba, there has been no detailed study of the seasonal occurrence of fleas associated with Richardson's ground squirrels in the other ecoregions in the Canadian prairies. Hence, we know very little about flea diversity and abundance on Richardson's ground squirrels from prairie regions of central and southern Saskatchewan or southeastern Alberta. These samples represent the north-western parts of the distributional range of *U. richardsonii* in Canada (Cassola 2016). This important knowledge gap needs to be addressed.

There is limited information available on the bacteria in the microbiomes of fleas parasitizing Richardson's ground squirrels. It is known that the bacterial communities of other arthropod vectors differ based on spatial, temporal, and host factors (Jones et al. 2010, 2012, 2013a, Colman et al. 2012, Hawlena et al. 2013). There have been a few studies conducted on the bacterial community structure in North American fleas (Jones et al. 2008, 2010, 2012, 2013b, Hawlena et al. 2013, Lawrence et al. 2015, Vasconcelos et al. 2018). For example, Hawlena et al. (2013) determined the relative abundance of bacteria within the microbiomes of *Orchopeas leucopus* and *Ctenophthalmus pseudagyrtes* from *Peromyscus leucopus* (the white-footed mouse) and *Microtus ochrogas*ter (the prairie vole) in southern Indiana using next-generation sequencing of the bacterial 16S rRNA gene. Similarly, Jones et al. (2008, 2010, 2012) used similar methods to examine bacterial abundance in *O. hirsuta*, *O. tuberculata cynomuris*, and *O. montana* parasitizing black-tailed prairie dogs in Colorado. The results of these four studies demonstrated that the microbiota of fleas have a low bacterial diversity and are dominated by *Rickettsia*, *Wolbachia* and/or *Bartonella* (Jones et al. 2008, 2010, 2012, Hawlena et al. 2013).

The question remains, are the bacteria in the microbiomes of the other flea species parasitizing *U. richardsonii* the same as those in *O. tuberculata cynomuris*? Studies on bacteria in the microbiomes of Richardson's ground squirrels are also limited. However, Jardine et al. (2005, 2006a, 2006b) did characterize the *Bartonella* species in the blood of Richardson's ground squirrels in Saskatoon, Saskatchewan. However, they did not investigate if the fleas parasitizing these animals also contained the same species/strains of *Bartonella*. The lack of information on the bacterial communities of fleas parasitizing Richardson's ground squirrels, except for *O. tuberculata*, is another important knowledge gap that needs to be addressed.

1.6 RESEARCH OBJECTIVE

The aim of this thesis is **to address the knowledge gaps concerning the diversity and abundance of fleas on Richardson's ground squirrels and the bacteria in their microbiome.** The key objectives to be examined are:

1) To establish genetic markers for the accurate species identification of *Oropsylla* (**Chapter 2**) and other fleas (**Chapter 3**) that are associated with Richardson's ground squirrels and black-tailed prairie dogs.
2) To determine the diversity of fleas, and their relative abundance on Richardson's ground squirrels at a locality in Alberta and a locality in southern Saskatchewan (**Chapter 4**). Given the sex-biased hypothesis (see review Krasnov et al. 2012) that male hosts have more ectoparasites than females, and that male *U. richardsonii* are generally larger in size than females (Michener and Koeppl 1985), I hypothesize that the sex of Richardson's ground squirrels will influence the prevalence and abundance of fleas that parasitize

them. I predict that only a small proportion of *U. richardsonii* will be parasitized by fleas and that males will have more fleas than females.

3) To determine the diversity of fleas within the burrows of Richardson's ground squirrels at a rural site and an urban site in Saskatchewan and examine if there are seasonal changes in the diversity and/or abundance of fleas (**Chapter 5**). Given that some flea species are known to have seasonal patterns in their activity (Krasnov 2008), I hypothesize that flea species parasitizing *U. richardsonii* will exhibit differences in their activity patterns over time. I predict that these seasonal patterns of activity will be more evident for those species that are "true parasites" of Richardson's ground squirrels.
4) To determine if the prevalence and abundance of bacteria of three genera, *Wolbachia*, *Bartonella* and *Rickettsia*, varies in the different flea species associated with Richardson's ground squirrels (**Chapter 6**). Since adult fleas acquire most of their bacteria from the blood-meal of their host, I hypothesize that the different flea species feeding on Richardson's ground squirrels will have similar bacterial communities. Given the low number of bacteria detected in *O. hirsuta* and *O. montana* parasitizing black-tailed prairie dogs (Jones et al. 2008), I predict that there will be low bacterial diversity and abundance in fleas parasitizing Richardson's ground squirrels, particularly species of *Oropsylla*.

I will use data obtained for the flea-Richardson's ground squirrel system to determine whether ecological concepts of parasite macro-communities (i.e., host specificity, aggregation, and community structure) apply to arthropod vectors (i.e., parasitic fleas) and bacteria in their microbiota.

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CHAPTER 2: MOLECULAR DIFFERENTIATION OF FOUR SPECIES OF *Oropsylla* (SIPHONAPTERA: CERATOPHYLLIDAE) USING PCR-BASED SINGLE STRAND CONFORMATION POLYMORPHISM ANALYSES AND DNA SEQUENCING[1]

2.1 ABSTRACT

It is often difficult to distinguish morphologically between closely related species of fleas (Siphonaptera). Morphological identification of fleas often requires microscopic examination of internal structures in specimens cleared using caustic solutions. This process degrades DNA and/or inhibits DNA extraction from specimens, which limits molecular-based studies on individual fleas and their microbiomes. Our objective was to distinguish between *Oropsylla rupestris* (Jordan), *Oropsylla tuberculata* (Baker), *Oropsylla bruneri* (Baker) and *Oropsylla labis* (Jordan and Rothschild) (Ceratophyllidae) using PCR-based single strand conformation polymorphism (SSCP) analyses and DNA sequencing. A 455 bp region of the nuclear 28S ribosomal RNA (rRNA) gene was used as the genetic marker. The results obtained for 36 reference specimens (i.e. fleas that were morphologically identified to species) revealed no intraspecific variation in DNA sequence, whereas the DNA sequences of the four species of *Oropsylla* differed from one another at two to six nucleotide positions. Each flea species also had a unique SSCP banding pattern. SSCP analyses were then used to identify another 84 fleas that

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had not been identified morphologically. DNA sequencing data confirmed the species identity of fleas subjected to SSCP. This demonstrates that PCR-SSCP combined with DNA sequencing of the 28S rRNA gene is a very effective approach for the delineation of four closely related species of flea.

2.2 INTRODUCTION

Fleas (Siphonaptera) are important vectors of human and animal pathogens (Eisen and Gage 2012). For example, the oriental rat flea, *Xenopsylla cheopis* (Rothschild) (Pulicidae), is the principal vector of *Yersinia pestis* (Lehmann and Neumsann), the causative agent of plague (Gage and Kosoy 2005). In North America, *Oropsylla* Wagner & Ioff spp. (Ceratophyllidae) are also vectors of *Y. pestis* (Lewis 2002, Gage and Kosoy 2005, Eisen et al. 2009); however, the ability to transmit this pathogenic bacterium successfully to susceptible hosts varies markedly among flea species (Gage and Kosoy 2005, Wilder et al. 2008, Eisen et al. 2009). For instance, *Oropsylla tuberculata cynomuris* (Jellison) is three times more efficient than *Oropsylla hirsuta* (Baker) in transmitting *Y. pestis* to laboratory mice (Wilder et al. 2008). Infection with *Y. pestis* poses a significant health risk to humans and rodents such as black-tailed prairie dogs, *Cynomys ludovicianus* (Ord) (Cully and Williams 2001, Gage and Kosoy 2005, Antonation et al. 2014, Salkeld et al. 2016). Outbreaks of *Y. pestis* can lead to 95-100% mortality within *C. ludovicianus* colonies (Pauli et al. 2006). In Canada, *C. ludovicianus* are restricted to a small area in and around Grasslands National Park (southern Saskatchewan), and are listed as threatened, in part due to the risk of sylvatic plague (COSEWIC 2011). Black-tailed prairie dogs infected with *Y. pestis* have been reported from within the Grasslands National Park (Antonation et al. 2014), and *Y. pestis* DNA has been detected in fleas collected from burrows in five of the 15 *C. ludovicianus*

colonies located within the park (Licciolli et al. 2020). Understanding the potential risk of *Y. pestis* infection to this and other populations of *C. ludovicianus* requires detailed knowledge of the vectors (i.e., flea species), and the ability to distinguish vectors from non-vectors of *Y. pestis*.

According to Lewis (2002), there are 12 species of *Oropsylla* in North America. These species have been separated into four subgenera, *Oropsylla* Wagner & Ioff, *Opisocrostis* Jordan, *Hubbardipsylla* Smit, and *Diamanus* Jordan (Lewis 2002). All species of *Oropsylla* can be distinguished from related taxa based on a combination of morphological characters, including the presence of a frontal tubercle, an ocular row of three setae, pigmented eyes that are normal in size, and an absence of postocular setae (Lewis 2002). They also possess a pronotal ctenidium and the teeth are greater than half the dorsal length of the pronotum (Lewis 2002). The characters used to distinguish among species of *Oropsylla* include the number of antesensial setae and acetabular setae, the presence/absence of small setae on the base abdominal sternite, the presence/absence of a pair of marginal spinelets on abdominal tergite 1, and the shape and structure of the male and female genitalia (Lewis 2002). Chemical maceration of specimens is generally required for the microscopic examination of the internal structures of fleas, and for individuals with a prominent blood meal that distorts internal structures (Belthoff et al. 2015). This can be a time-consuming process, and the compounds used for clearing specimens (e.g., lactophenol or potassium hydroxide) degrade DNA and/or inhibit DNA extraction, which limits molecular-based studies on the population genetics, phylogenetic relationships and microbiome composition of fleas.

Molecular approaches provide a valuable alternative or an adjunct to traditional (i.e. morphological) methods of species identification and to infer the evolutionary relationships of species. The genetic markers used to examine the phylogenetic relationships of taxa within the

order Siphonaptera have included the nuclear first and second internal transcribed spacers (ITS1 and ITS2) of the ribosomal DNA (rDNA), the small and large subunit rRNA genes (18S and 28S, respectively), the elongation factor 1-alpha gene, the mitochondrial (mt) cytochrome c oxidase genes I and II (COI and COII), and the mt large subunit rRNA gene (16S) (Whiting 2002, Vobis et al. 2004, Whiting et al. 2008, Marrugal et al. 2013, Zhu et al. 2015, Ghavami et al. 2018, Ying et al. 2018, Zurita et al. 2018). In contrast, few studies have defined genetic markers that can be used to distinguish among closely related species of fleas (Jones and Bitten 2010, Marrugal et al. 2013). However, Marrugal et al. (2013) showed that the DNA sequences of the ITS1 and ITS2 could be used to distinguish *Ctenocephalides felis* (Bouché) from *C. canis* (Curtis). Similarly, Jones et al. (2010) used sequences of COII to distinguish between *O. hirsuta* and *O. tuberculata* (Baker) collected from *C. ludovicianus* in Colorado.

Oropsylla (*Opisocrostis*) *tuberculata* and three other species, *O.* (*Opisocrostis*) *bruneri* (Baker), *O.* (*Opisocrostis*) *labis* (Jordan and Rothschild), and *O.* (*Oropsylla*) *rupestris* (Jordan), parasitize sciurid rodents in Saskatchewan (Holland 1985, Lewis 2002). Richardson's ground squirrels, *Urocitellus richardsonii* (Sabine), and *C. ludovicianus* are parasitized by *O. rupestris*, *O. tuberculata* and occasionally *O. labis*, while Franklin's ground squirrels, *Poliocitellus franklinii* (Sabine), and thirteen-lined ground squirrels, *Ictidomys tridecemlineatus* (Mitchell), are the principal hosts of *O. bruneri* (Holland 1985, Lewis 2002). Our objective was to differentiate these four species of *Oropsylla* using PCR-based single strand conformation polymorphism (PCR-SSCP) analyses and DNA sequencing of the nuclear 28S ribosomal RNA gene. SSCP is a mutation scanning technique that has been shown to be very effective for the species identification of parasitic arthropods (e.g., Dergousoff and Chilton 2007, Krakowetz et

al. 2010, Anstead et al. 2014).

2.3 MATERIALS AND METHODS

A total of 120 fleas (*Oropsylla* spp.) were collected from sciurid rodents, or their burrows, at three localities in southern and central Saskatchewan, Grasslands National Park (49.1784, -107.6829), Baildon (50.2510, -105.4432) and Beechy (50.8833, -107.3833). Fleas were fixed in 70% ethanol. A total of 36 *Oropsylla* were identified to species (Table 2.1) based on their morphological characters (Holland 1985, Lewis 2002). These represent the reference specimens for *O. rupestris, O. tuberculata, O. bruneri* and *O. labis*. Also included in this study were two specimens of *Foxella ignota* (Baker, 1895) collected from Richardson's ground squirrels near Beechy. Voucher specimens were deposited in the J.B. Wallis/R.E. Roughley Museum of Entomology (Department of Entomology, University of Manitoba, Winnipeg, Manitoba, Canada, R3T 2N2).

Total genomic DNA (gDNA) was extracted from the whole or partial body of each flea using either the DNeasy Blood & Tissue Kit or QIAmp DNA Mini Kit (Qiagen Inc.). Part (~490 bp) of the nuclear 28S rRNA gene (i.e., domains II and III) was amplified from gDNA by PCR using primers 28S-1 (5'-ATACGCCTTCGGCTTATGC-3') and 28S-2 (5'-AATAAGACGCCCCGGGATTG-3'). This target region was selected, and the two primers were designed, based on comparisons of the rDNA sequences of the 28S gene for three species of ceratophyllid flea: *F. ignota, Oropsylla montana* (Baker) and *Dactylopsylla bluei* (C. Fox) (GenBank accession numbers EU336137, EU336156 and EU336233, respectively). Although the 28S sequences of the three species are 99% similar, at least 58% of the sequence differences among them occur within domains II and III. The forward (28S-1) and reverse (28S-2) primers

Table 2.1: Fleas (n = 36) collected as reference specimens from black-tailed prairie dogs (*Cynomys ludovicianus*) and Richardson's ground squirrels (*Urocitellus richardsonii*) in southern Saskatchewan that were morphologically identified to species.

Flea species	No. of fleas	Host species	Locality
Oropsylla rupestris	2	*C. ludovicianus*	Grasslands National Park, SK
	13	*U. richardsonii*	Grasslands National Park, SK
	7	Uncertain*	Grasslands National Park, SK
O. tuberculata	5	*U. richardsonii*	Grasslands National Park, SK
	6	Uncertain*	Grasslands National Park, SK
O. bruneri	1	*U. richardsonii*	Baildon, SK
O. labis	1	*U. richardsonii*	Beechy, SK
	1	Uncertain*	Grasslands National Park, SK

* Samples collected from rodent burrows; as a result, the hosts for these fleas could not be confirmed.

correspond to positions 504-522 and 969-988 (respectively) in the 28S rDNA sequence of *F. ignota*. PCRs were performed on the gDNA of 120 specimens of *Oropsylla* and two of *F. ignota*, and negative (i.e. no gDNA template) control samples. PCR assays were conducted using 25 mL reaction mixtures containing 5X Phusion Green HF buffer (ThermoScientific), 1.7 mM $MgCl_2$, 200 mM of each dNTP, 0.75 mM of each primer, 1.25 U of Phusion HotStart II polymerase (ThermoScientific) and 1 mL of gDNA or water (i.e. for the negative control samples). The amplification conditions used were 96°C for 5 min, followed by 40 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, and then 72°C for 5 min. Amplicons were subjected to electrophoresis on SYBR-safe™ stained 1.5% agarose-TBE gels.

Shrimp alkaline phosphatase (0.014 U/µl) (New England BioLabs, Pickering, Canada) and exonuclease I (0.27 U/µl) (ThermoScientific) were added to the amplicons derived from the 36 reference specimens of *Oropsylla*. Samples were incubated at 37°C for 15 min, then at 80°C for 15 min. Purified amplicons were then subjected to automated DNA sequencing using primers 28S-1 and 28S-2 in separate reactions. The nucleotide sequences (446 bp) of the 28S rRNA gene for representative specimens of each of the four species of *Oropsylla* and *F. ignota* have been deposited on GenBank (accession numbers MT460405-MT460409). All DNA sequences were aligned manually, and pairwise comparisons were made of the number of fixed sequence differences between species using the methods described in Chilton et al. (1995).

SSCP analyses were conducted on all 122 fleas (i.e., including the 36 reference specimens). Each amplicon (4 mL) was mixed with 2 mL of ultrapure water (2 mL) and 2 mL of 6X Loading Dye (ThermoScientific). Samples were denatured at 95°C for 5 min after which they were snap cooled in ice water for 5 minutes. Each sample was loaded into a well of a precast GMA™ S-50 gel (AL-LABORTECHNIK e.U) placed within a horizontal SEA2000 apparatus

(Elchrom Scientific) that was connected to a temperature-controlled circulating water bath. The GMA gels were subjected to electrophoresis for 19 hours at 74 V and 7°C. Gels were then stained for 45 min with SYBR-Gold (Invitrogen), rinsed twice with distilled water, and photographed using a BioDoc^{TM} (UVP) imaging system. The species identities of fleas not identified morphologically were determined based on a comparison of their SSCP profiles with those of the reference specimens. Some samples (n = 61) were selected at random and sequenced to confirm species identification.

2.4 RESULTS AND DISCUSSION

Amplicons derived from each gDNA sample gave a single band (~500 bp) on agarose gels, whereas no amplicons were produced from the negative controls (not shown). Comparison of the DNA sequences of this target region for the 36 reference specimens revealed that each species of *Oropsylla* had a unique sequence (Table 2.2). The reference specimen of *O. bruneri* had two nucleotides (i.e. an A and a G) at position 424. In contrast, there was no intraspecific variation in DNA sequences among the multiple samples of *O. labis*, *O. tuberculata* and *O. rupestris*. There were 10 variable positions (excluding position 424) in the aligned sequences of the four species of *Oropsylla* (Table 2.2). These mutational changes in DNA sequence corresponded to 7 transitional (2 purine and 5 pyrimidine) and 4 transversional nucleotide changes. The 28S rDNA sequence of *O. labis* was most genetically similar to *O. bruneri*. They differed at 2 (0.5%) of the 446 positions in the aligned sequences, while both these species differed from *O. tuberculata* and *O. rupestris* at 6 (1.3%) positions. The same magnitude of sequence difference (i.e., 6 mutational changes) was detected between *O. tuberculata* and *O. rupestris*. SSCP analyses of the 28S rRNA gene amplicons revealed that all four species of

Oropsylla had a unique banding pattern (Figure 2.1). PCR-SSCP analyses conducted on another 84 *Oropsylla* that had not been identified morphologically to species revealed that each specimen had a SSCP profile consistent with that of one of the reference specimens of *O. tuberculata, O. rupestris*, *O. labis* or *O. bruneri* (Table 2.3). DNA sequencing data of 59 of the 84 *Oropsylla* specimens (selected at random) revealed that each flea had been accurately identified based on their SSCP profile. Most (85%) of the *Oropsylla* from *U. richardsonii* were identified as either *O. tuberculata* or *O. rupestris* (Tables 2.1 and 2.3). The two specimens of *F. ignota* also had unique SSCP profiles and DNA sequences when compared to the four species of *Oropsylla* (Figure 2.1).

Although SSCP has been used for species identification of other parasitic arthropods (e.g., Dergousoff and Chilton 2007, Krakowetz et al. 2010, Anstead et al. 2014), this mutation scanning technique, as far as we are aware, has not been applied to the identification of fleas. This molecular approach significantly reduces the time and costs associated with DNA sequencing of all individuals for species identification (Gasser et al. 2006). Only representative samples of each SSCP profile need to be sequenced to verify species identity. Also, a 446 bp region of the nuclear 28S rRNA gene was used in the present study as the genetic marker to distinguish among species of *Oropsylla*. This gene is generally used as a genetic marker to examine the phylogenetic relationships of fleas (Whiting 2002, Whiting et al. 2008, Zhu et al. 2015) rather than for species discrimination. The mitochondrial COI gene is the genetic marker most often used for the DNA barcoding of fleas (Šlapeta and Šlapeta 2016, Ying et al. 2018, McKee et al. 2019, Nziza et al. 2019, Wessels et al. 2018). Typically, “universal invertebrate” primers LCO1490 and HCO2198 (Folmer et al. 1994) are used to amplify 600-658 bp of the COI gene of fleas (e.g., Ying et al. 2018, McKee et al. 2019, Nziza et al. 2019). Unfortunately, these

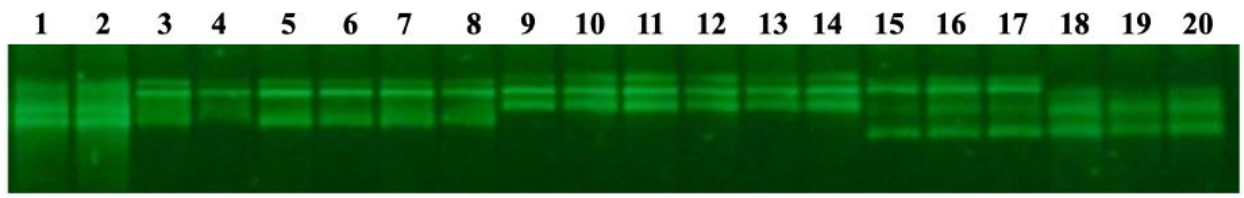
Table 2.2: Variable positions in the sequence alignment of the nuclear 28S rRNA gene of the reference specimens (listed in Table 2.1) of four species of *Oropsylla*. A dot indicates the same nucleotide as in the sequence of *O. rupestris*.

Flea species	Alignment Position:										
	6	84	116	165	166	251	366	408	424	437	442
O. rupestris	T	G	T	G	T	G	T	T	G	G	A
O. bruneri	C	T	.	.	.	.	C	C	R	A	T
O. labis	C	T	.	.	C	.	C	C	.	A	.
O. tuberculata	C	.	C	T	.	A	C	C	.	.	.

Table 2.3: Identification of fleas (not morphologically identified) based on the SSCP profile of their 28S rRNA gene amplicon. The species identification of 71% of specimens was confirmed by DNA sequencing.

Source	Locality	Number of individuals (no. sequenced)					
		O. rupestris	*O. tuberculata*	*O. bruneri*	*O. labis*	*F. ignota*	Total
U. richardsonii	Grasslands Nat. Park	4 (0)	1 (0)	-	-	-	5 (0)
	Beechy	11 (3)	4 (4)	-	2 (2)	2 (2)	19 (11)
	Baildon	16 (16)	25 (25)	8 (8)	-	-	49 (49)
C. ludovicianus	Grasslands Nat. Park	1 (0)	1 (1)	-	-	-	2 (1)
Unknown host	Grasslands Nat. Park	2 (0)	9 (0)	-	-	-	11 (0)
Total		34 (19)	40 (30)	8 (8)	2 (2)	2 (2)	86 (61)

Figure 2.1: Representative gel of single-stranded conformation polymorphism profiles of *Foxella ignota* (lanes 1 and 2), *Oropsylla tuberculata* (lanes 3-8), *O. rupestris* (lanes 9-14), *O. bruneri* (lanes 15-17) and *O. labis* (lanes 18-20).



COI amplicons cannot be subjected to SSCP analyses because this mutation scanning technique is only effective for amplicons that are 150-500 bp in size (Gasser et al. 2006). The variability in DNA sequences of COI among individuals of a species (i.e. different haplotypes) makes this gene a useful target for population genetic and phylogeographic studies of fleas (e.g., Šlapeta and Šlapeta 2016, Nziza et al. 2019). However, this intraspecific variation represents a limitation for the rapid identification of individual fleas from a diagnostic perspective, particularly when the extent of the genetic variation with COI has not been determined for the taxa of interest. The most useful genetic markers for species identification are those where there is no, or very little, intraspecific variation in DNA sequence (i.e., very few haplotypes or genotypes), and there are fixed genetic differences in sequence among species.

Our results demonstrate that SSCP combined with DNA sequencing of 485 bp amplicons of the 28S rRNA gene (= 446 bp plus 29 bp corresponding sequences of primers 28S-1 and 28S-2) provide a simple and effective molecular tool to distinguish among the four species of *Oropsylla* and *F. ignota*. Although several species of *Oropsylla* are vectors of *Y. pestis* in North America, they differ in their relative effectiveness to transmit this pathogen to vertebrate hosts (Jones and Britten 2010, Mize et al. 2017, Russell et al. 2018). Therefore, it is important that fleas are accurately identified to species in order to assess the potential risk of vector-borne disease for different populations of sciurid and geomyid rodents, particularly those that are at risk (e.g., the Canadian population of *C. ludovicianus*). It remains to be determined if the genetic marker used in the present study can also be used to distinguish other flea species that parasitize sciurid and geomyid rodents in North America (e.g., *U. richardsonii, C. ludovicianus, T. talpoides* and their relatives). However, comparison of the 28S sequence data obtained in the present study with the sequences of other flea species available on GenBank (e.g., *O. montana*,

O. hirsuta, *Neopsylla inopina* Rotschild and *Rhadinopsylla heiseri* (McCoy); accession numbers EU336156, KM891126, KM891055 and EU336138, respectively), suggests that it should be possible to use PCR-SSCP of domains II and III of the 28S rRNA gene to distinguish among species parasitizing these rodents.

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2.6 TRANSITION STATEMENT

It was demonstrated in Chapter 2 the utility of a 450 bp region of the nuclear 28S rRNA gene to differentiate among four species of *Oropsylla* that commonly parasitize Richardson's ground squirrels and black-tailed prairie dogs. In this next chapter, I examine if the sequence of a larger region (823 bp) of the nuclear 28S rRNA gene and the sequences of four other markers (two nuclear and two mitochondrial) can be used to distinguish among flea species that parasitize Richardson's ground squirrels.

CHAPTER 3: GENETIC DIFFERENTIATION OF FLEA SPECIES (Siphonaptera) ASSOCIATED WITH RICHARDSON'S GROUND SQUIRRELS (*Urocitellus richardsonii*)[2]

3.1 ABSTRACT

Fleas are vectors of a multitude of wildlife and human pathogens (e.g., bacteria, viruses and protozoa), but individual species of flea vary in their competency to transmit these pathogens. Therefore, to implement effective control and management strategies for vectors and vector-borne diseases, vectors need to be distinguished from non-vectors or those species that play little or no role in the transmission of pathogenic agents to vertebrate hosts. However, it is often difficult to distinguish among closely related species of flea because of their very similar morphologies. Molecular tools have been developed that allow for species differentiation in some arthropod groups. It is imperative that molecular tools be developed to distinguish among flea species parasitizing Richardson's ground squirrels (*Urocitellus richardsonii*) as these mammals are reservoir (i.e. maintenance) hosts for *Yersinia pestis*, the bacterium responsible for plague. Only some of the flea species parasitizing *U. richardsonii* are vectors of *Y. pestis*. They are also known to parasitize black-tailed prairie dogs (*Cynomys ludovicianus*), a mammal species that has a high mortality rate when infected with the plague. In this study, we investigated the effectiveness of three nuclear genes or spacers, namely the second internal transcribed spacer 2 (ITS2), and the 18S rRNA and 28S rRNA genes, and two mitochondrial genes, cytochrome oxidase *c* subunits 1 and 2 (*cox*1 and *cox*2), to differentiate among 11 flea taxa associated with *U. richardsonii* in the Canadian prairies. Results revealed that the ITS2 and *cox*2 were the best and most reliable genetic markers to distinguish among fleas of different species.

[2] This chapter is in collaboration with Kristen Daniels, Miranda Fellner, Breanne Bevelander, and Neil Chilton.

3.2 INTRODUCTION

It is often difficult to differentiate among individuals of two or more closely related species of parasitic arthropod because they are morphologically very similar. Consequently, molecular methods were developed, and are now commonly used, to determine the species identity of parasitic arthropods (Walton et al. 2007, Chan et al. 2014, Ondrejicka et al. 2017, Zhao et al. 2020, AL-Hosary et al. 2021). The DNA sequences of arthropods are also used to study their population genetics, phylogeography, and phylogenetic relationships (Giribet et al. 2001, Shao and Barker 2007, Goubert et al. 2016). However, the genetic loci or markers used to address these tasks often differ because of differences in the magnitude of the intraspecific and/or interspecific variation in DNA sequences. For example, the most useful species markers are those in which there is little or no intraspecific variation in DNA sequence, but consistent differences in DNA sequence among species. Such markers may also provide enough characters to infer phylogenetic relationships. In contrast, population genetic and phylogeographic studies require genetic markers that have substantial intraspecific variation in DNA sequence. Therefore, the DNA sequences of multiple genes need to be characterized for individuals of the arthropod group of interest to assess their utility as genetic markers for species identification, population genetics, phylogeography, and phylogenetic inference.

DNA sequences of the mitochondrial (mt) cytochrome oxidase *c* subunit 1 gene (*cox*I or *CO1*) are commonly used to differentiate and identify invertebrates, including a variety of arthropods (e.g., Chitimia et al. 2010, Arribas et al. 2016, Herbert et al. 2016, Klimov et al. 2019, Balzer et al. 2020) using “universal” primers to amplify the gene (described in Folmer et al. 1994). This gene is often referred to as the barcoding gene (Ratnasingham and Herbert 2007, Bianchi and Gonçalves 2021). However, some studies have questioned its usefulness for this task

(e.g., Deagle et al. 2014, Mioduchowska et al. 2018). Some studies have used other mitochondrial genes, such as the cytochrome oxidase c subunit 2 gene (*cox*2) for species identification (Maekawa et al. 1999, Austin et al. 2004, Rawson and Burton 2006). Other studies have used sequences of the first and second internal transcribed spacers (ITS1 and ITS2) of the nuclear ribosomal RNA for species delineation and identification of arthropods; for example, bark beetles (Gallego and Galián 2001), blowflies (Nelson et al. 2008), fleas (Vobis et al. 2004), and spider mites (Ben-David et al. 2007). The ITS1 occurs between the non-transcribed spacer and the 18S rRNA gene, while the ITS2 separates the 5.8S rRNA gene from the 28S rRNA gene.

Different species of flea (Order Siphonaptera) can be distinguished from one another based on microscopic examination of their internal structures (Holland 1985, Lewis 2002). However, specimens first need to be cleared for several hours or days using chemicals such as lactophenol or potassium hydroxide (Whiting et al. 2008, Hastriter and Whiting 2009, Belthoff et al. 2015). Such clearing agents often degrade DNA and/or inhibit DNA extraction, which limits molecular-based studies, that include determination of the bacterial species that make up the microbiota of fleas. The ability to investigate bacterial species of fleas in North America is particularly important as some are known vectors of *Yersinia pestis*, the causative agent of plague. For example, *Oropsylla tuberculata* and *O. montana* commonly transmit *Y. pestis* to black-tailed prairie dogs (*Cynomys ludovicianus*) (Gage and Kosoy 2005, Eisen et al. 2009). Black-tailed prairie dogs are exceptionally susceptible to *Y. pestis* infections and colony mortality rates can be up to 100% (Cully and Williams 2001, Pauli et al. 2006, Antonation et al. 2014).

Very little research has been conducted on the use of molecular tools for the identification of fleas in North America, which contrasts with the number of studies examining

the phylogenetic relationships of fleas (Whiting et al. 1997, Whiting 2002, Vobis et al. 2004, Whiting et al. 2008, Brinkerhoff et al. 2011, Jones and Britten 2010, Marrugal et al. 2013, Zhu et al. 2015, Zurita et al. 2016, Ghavami et al. 2018, Ying et al. 2018, Zhao et al. 2018, Zurita et al. 2018, 2019). However, Jones et al. (2010) used sequences of *cox*2 to distinguish between *O. hirsuta* and *O. tuberculata cynomuris* parasitizing *C. ludovicianus* in Colorado (USA). In the study by Zurita et al. (2016), *Ctenocephalides felis* could be distinguished from *C. canis* based on differences in nucleotide sequences of the ITS1, ITS2 and *cox*1. In contrast, the nuclear 18S rRNA gene did not discriminate between these two flea species (Zurita et al. 2016). The ITS1 has been used to distinguish cryptic species of *Pulex irritans* from Spain and Argentina (Zurita et al. 2019) as well as Iran (Ghavami et al. 2018). The nuclear 28S rRNA gene was used to distinguish between four species of *Oropsylla* parasitizing Richardson's ground squirrels (*Urocitellus richardsonii*) and/or *C. ludovicianus* in Saskatchewan, Canada (Chapter 2, Thoroughgood et al. 2021). Buhler et al. (2020) also used this same genetic marker to verify the species identity of the nest flea, *Ceratophyllus vagabundus vagabundus*, in the arctic.

Accurate identification of individual fleas to species is important irrespective of the type of investigation being conducted (e.g., ecological, evolutionary, phylogeography, population genetics, behavioural, and biochemical) because the interpretation of the data obtained may be misleading if an assumption is made that the study is focusing on a single species, when in fact, the specimens under investigation comprise two or more species (Andrews and Chilton 1999). In this study, we examine the potential of five genetic markers to discriminate between species of fleas that commonly parasitize *U. richardsonii* and *C. ludovicianus* in Saskatchewan.

3.3 MATERIALS AND METHODS

Fleas from two families, the Ceratophyllidae and Ctenophthalmidae, were collected in 2019 from cadaver Richardson's ground squirrels near Lethbridge, Alberta (49.6981, -112.7696) and the rural municipality of Baildon, Saskatchewan (Table 3.1). Total genomic DNA (gDNA) was extracted from each individual flea using the protocol described previously (Chapter 2: Thoroughgood et al. 2021). Species identifications of *O. bruneri, O. labis, O. tuberculata, Neopsylla inopina* and *Rhadinopsylla fraterna* were based on the morphological identification of specimens from Grasslands National Park by Dr. Terry Galloway. Each species was then subsequently characterized based on their DNA sequences for part (i.e., 446 bp) of the nuclear 28S ribosomal RNA (rRNA) gene (Chapter 2). DNA sequences of the 28S rRNA gene were also used to determine the identity of the other flea taxa to species or the genus level (i.e., *Aetheca wagneri, Catallagia* and *Megabothris*). This identity was determined based on a blast search where their sequences (446 bp) were compared to the 28S rRNA gene sequences of fleas on GenBank. One flea specimen could not be identified to the genus level but was placed in a clade containing only species of the family Ceratophyllidae based on phylogenetic analyses of its 446 bp sequence of the 28S rRNA gene (not shown). This flea is listed as an "unknown ceratophyllid species" in this chapter.

Five potential genetic markers were used to investigate their use for species differentiation of flea. This included three nuclear genes or spacers, the 18S rRNA gene, the second internal transcribed spacer (ITS2), and the 28S rRNA gene, and two mitochondrial (mt) genes, *cox*I and *cox*2 (Table 3.2). A larger fragment (834 bp) of the 28S rRNA gene was used in this study compared to that of Thoroughgood et al. (2021) to compare fleas. The primers for this new 28S rRNA gene PCR assay (Table 3.2) were designed based on the alignment of the 28S

Table 3.1: The number of fleas of each species characterized at five genetic markers.

Family	Number of specimens per genetic marker				
Species	ITS2 rDNA	18S rRNA gene	28S rRNA gene	*cox*2	*cox*1
Ceratophyllidae					
Oropsylla rupestris	5	1	11	11	11
Oropsylla tuberculata	10	4	6	9	10
Oropsylla bruneri	5	3	5	4	5
Oropsylla labis	5	1	2	4	1
Aetheca wagneri	6	3	4	5	0
Megabothris sp. 1	1	1	2	2	1
Megabothris sp. 2	1	1	1	1	1
unknown ceratophyllid	1	1	1	1	0
Ctenophthalmidae					
Neopsylla inopina	7	1	9	12	3
Rhadinopsylla fraterna	4	2	1	4	3
Catallagia sp.	5	3	3	2	0

Table 3.2: Primer sequences for each molecular target examined in this study.

Genetic marker	Primer	Sequence (5' --> 3')	Reference
ITS2			
	Sen-ITS2	GGGTCGATGAAGAACGCAGC	Vobis et al. (2004)
	ITSR	TTTAGGGGGTAGTCTCACCTG	Luchetti et al. (2005)
18S rRNA gene			
	1F	TACCTGGTTGATCCTGCCAGTAG	Wetzer et al. (2013)
	ai	CCTGAGAAACGGCTACCACATC	Whiting (2002)
	b0.5	ATGGTTGCAAAGCTGAAAC	Whiting (2002)
	9R	GATCCTTCCGCAGGTTCACCTAC	Whiting (2002)
28S rRNA gene			
	F1	TGAGAGTGCAGCTCTAAGTG	This study
	R1	AGTTCCGACGATCGATTTGC	This study
***cox*2**			
	A Leu	TCTAATATGGCAGATTAGTGC	Whiting (2002)
	B tLys	GTTTAAGAGACCAGTACTTG	Maekawa et al. (1999)
	2a	ATAGAKCWTCYCCHTTAATAGAACA	Whiting (2002)
	9b	GTACTTGCTTTCAGTCATCTWATG	Whiting (2002)
***cox*1**			
	LCO1490	GGTCAACAAATCATAAAGATATTGG	Folmer et al. (1994)
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. (1994)

rDNA sequences of several species of flea that included taxa within the Ceratophyllidae and Ctenophthalmidae (alignment data not shown). For each molecular target, PCRs were conducted using 25 µl reaction mixtures containing 5X Phusion Green HF buffer (ThermoScientific), 1.7 mM $MgCl_2$, 200 µM of each dNTP, 0.75 µM of each primer (Table 3.3), 0.5 U of Phusion HotStart II polymerase (ThermoScientific), and 1.5 µl of template gDNA or water, the latter representing the negative controls. The amplification conditions used are described in Table 3.3. Amplicons were subjected to 1.5% agarose-TBE gel electrophoresis. Size markers were included on each gel. Gels were stained with SYBR green and visualized under UV light. Amplicons were purified using the protocol described in Chapter 2 (Thoroughgood et al. 2021) and subjected to automated sequencing using one or both primers used for the amplification of the genetic marker.

The sequences obtained for each genetic marker were compared with the DNA sequences available on GenBank. The sequences for each genetic marker were aligned using the methodology described in Chapter 2 (Thoroughgood et al. 2021). Calculations were made of the number of sequence differences among each pair of flea taxa based on the aligned sequence data. 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). The D values of each target are presented in a matrix. UPGMA (unweighted pair group methods using arithmetic means; Sneath and Sokal 1973) analyses was conducted on the pairwise sequence data using the program PAUP (Swofford 2002). Phenograms or UPGMA trees depict the degree of genetic similarity or difference among taxa independent of the evolutionary history of the taxa being compared

Table 3.3: The conditions used to amplify each genetic marker from flea gDNA.

ITS2	94ºC for 5 mins, 35 cycles of 94ºC for 60 s, 55ºC for 60s, and 72ºC for 60 s, then 72ºC for 10 mins.
18S rRNA gene	Primer 1F & b0.5: 96ºC for 5 min, 35 cycles of 94ºC for 60 s, 60ºC for 60 s, and 72ºC for 60 s, then 72ºC for 5 mins. Primer ai & 9R: 95ºC for 5 mins; 35 cycles of 94ºC for 60 s, 65ºC for 60 s, and 72ºC for 60 s, then 72ºC for 5 mins.
28S rRNA gene	96ºC for 5 mins; 40 cycles of 94ºC for 60 s, 58ºC for 60 s, and 72ºC for 60s, then 72ºC for 5 mins.
***cox*1**	95ºC for 5 mins, 35 cycles of 95ºC for 60 s, 50ºC for 60s, and 72ºC for 60 s, then 72ºC for 5 mins.
***cox*2**	Primers F-leu & R-Lys: 95ºC for 5 mins; 35 cycles of 95ºC for 30 s, 56ºC for 30 s, and 72ºC for 2 mins, then 72ºC for 7 mins. Primers 2a & 9b: 95ºC for 5 mins; 35 cycles of 95ºC for 30 secs, 54ºC for 45 s, and 72ºC for 60 s, then 72ºC for 5 mins.

(Andrews and Chilton 1999). Phenograms therefore differ from cladograms which are used to infer phylogenetic relationships of taxa.

3.4 RESULTS

3.4.1 Molecular characterization of fleas using the internal transcribed spacer 2 (ITS2)

Single PCR amplicons (~ 500 bp) were detected on agarose gels for all flea gDNA samples examined, while no bands were detected on agarose gels for the negative control samples (images not shown). The ITS2+ (= 118 of the 5.8S rRNA gene, the complete ITS2 and 11 bp of the 28S rRNA gene) sequences ranged from 413 bp (in *O. bruneri*) to 434 bp (in *Megabothris* sp. 1) in size (Supplemental Fig. 3.1) and were aligned over a length of 457 positions. For the partial 5.8S rRNA gene, there were sequence differences detected at two positions (i.e., 110 and 114). The four species of *Oropsylla* had a guanine at position 110, whereas all other taxa had an adenine. At position 114, *R. fraterna* had an adenine in its DNA sequence, while all other taxa had a thymine. For the partial 28S rRNA gene, all species had identical sequences, except the three species of the Ctenophthalmidae had an adenine in their sequence at position 445, whereas all taxa in the Ceratophyllidae had a cytosine at this position.

The size of the ITS2 ranged from 283 bp (in *O. bruneri*) to 304 bp (in *Megabothris* sp. 1) (Supplemental Fig. 3.1). There were significantly more differences in ITS2 sequence among taxa when compared to that for the two partial genes. Sequence differences in the ITS2 among species occurred at 166 (51%) of the 326 alignment positions (Supplemental Fig. 3.1). As for the partial rRNA genes, there was intraspecific variation in DNA sequence of the ITS2 for all taxa for which multiple specimens were sequenced. There were sequence differences in the ITS for the

two taxa of the genus *Megabothris* (i.e., defined herein as sp. 1 and sp. 2). Each flea taxon had a unique ITS2 sequence.

The number of base pair differences in ITS2+ sequence among taxa ranged from 5 to 124 bp (Table 3.4). The number of differences among the four morphologically-well defined species of *Oropsylla* ranged from 13 to 33 bp. The phenogram produced from the UPGMA analyses of the pairwise comparisons of the differences in ITS2+ sequence demonstrates that the two families have differences in their sequence of 15% (Fig 3.1). The four species of *Oropsylla* formed a distinct cluster from the other taxa in the Family Ceratophyllidae. Also, all taxa within the Ceratophyllidae formed a distinct cluster to the exclusion of the three species from the Ctenophthalmidae. The magnitude of the sequence differences among *R. fraterna* and the other two species of the Ctenophthalmidae, *N. inopina* and *Catallagia* sp., was greater than that among taxa within the Ceratophyllidae (<7%).

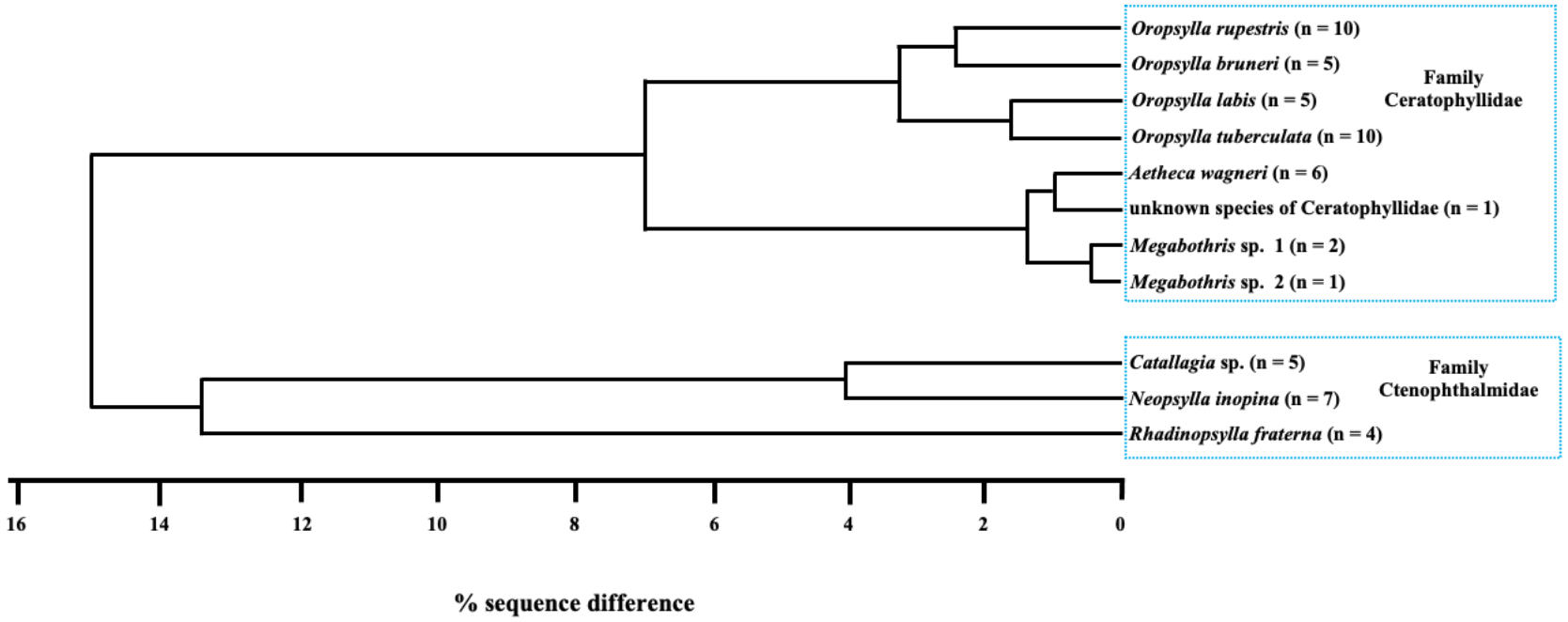
3.4.2 Molecular characterization of fleas using the nuclear 18S rRNA gene

A single band (~1,200 or 1,600 bp depending upon primer pair used) was detected on agarose gels for amplification of the 18S rRNA gene from the flea gDNA samples, whereas no bands were detected on agarose gels for the template lacking DNA (i.e., negative control) samples (images not shown). The size of the sequences for the 18S rRNA gene ranged from 1,823 bp (in *N. inopina* and *Catallagia* sp.) to 1,830 bp (in all other taxa except for *O. bruneri* where no sequence data was obtained for the first 403 bp). DNA sequences of the 18S rRNA gene for all taxa were aligned over 1,830 positions (Supplemental Fig. 3.2). There was no intraspecific variation in DNA sequences of the 18S rRNA gene for all taxa for which multiple specimens were sequenced. Differences in DNA sequence occurred at 79 (4.3%) of the 1,830

Table 3.4: Matrix showing the number of differences in the nucleotide sequences of the partial 5.8S rRNA gene (119 bp), the complete nuclear region of internal transcribed spacer 2 and partial 28S rRNA gene (11 bp) among pairwise comparisons of the eleven flea taxa.

	O. bruneri	*O. labis*	*O. tuberculata*	*A. wagneri*	*Megabothris* sp. 1	*Megabothris* sp. 2	Ceratophyllid sp.	*Catallagia* sp.	*N. inopina*	*R. fraterna*
O. rupestris	33	24	23	37	39	38	37	88	89	122
O. bruneri		31	32	46	49	49	46	91	90	124
O. labis			13	36	42	39	38	82	83	120
O. tuberculata				38	44	41	40	84	86	121
A. wagneri					12	7	7	75	78	113
Megabothris sp. 1						7	12	81	84	114
Megabothris sp. 2							5	74	77	111
Ceratophyllid sp.								73	76	110
Catallagia sp.									22	93
N. inopina										91

Figure 3.1: Phenogram depicting the genetic differences in nucleotide sequence of the partial 5.8S rRNA gene (119 bp), the complete nuclear region of internal transcribed spacer 2 and partial 28S rRNA gene (11 bp) among the eleven flea taxa.



alignment positions (Supplemental Fig. 3.2). These differences represented 38 transitional and 34 transversional mutational changes, and 7 indels. The number of sequence differences among species (Table 3.5) ranged from 0 to 58 (0-3.2%). There were no differences in 18S sequence between *O. rupestris* and *O. labis*, or among *A. wagneri* and the two species of *Megabothris* (Table 3.5). The phenogram produced from the UPGMA analyses of the pairwise comparisons of sequence differences in the 18S rRNA gene is shown in Figure 3.2. The four species of *Oropsylla* did not form a distinct cluster. However, all taxa within the Ceratophyllidae formed a distinct cluster to the exclusion of the three species from the Ctenophthalmidae, as did the three species of the Ctenophthalmidae (*R. fraterna*, *N. inopina* and *Catallagia* sp.). The magnitude of the sequence differences between *R. fraterna* and the other two species of the Ctenophthalmidae (~1.8%) approached the average sequence difference (~2.3%) separating taxa in the two families.

3.4.3 Molecular characterization of fleas using the nuclear 28S rRNA gene

The size of the sequences for the 28S rRNA gene ranged from 816 bp (in *O. rupestris* and *N. inopina*) to 819 bp (in *Catallagia* sp.). However, the first 16 bp of the 28S rRNA gene were not obtained for *O. tuberculata*. The DNA sequences of the 28S rRNA gene were aligned over 823 positions, 710 (86%) of which were invariant among the 11 flea taxa (Supplemental Fig. 3.3). There was no intraspecific variation in DNA sequences of the 28S rRNA gene for all taxa for which multiple specimens were sequenced. Table 3.6 shows the pairwise comparisons of the number of sequence differences in the 28S rRNA gene of the 11 taxa. All taxa had a unique sequence for the 28S rRNA gene. This included the two "species" of *Megabothris*, which differed at 5 (0.6%) of 818 positions in the sequence alignment. These five sequence differences represent four transitional and one transversional mutations. This difference in sequence among

Table 3.5: Matrix showing the number of differences within the nucleotide sequence of the 18S rRNA gene among the pairwise comparisons of the eleven flea taxa.

	O. bruneri	*O. labis*	*O. tuberculata*	*A. wagneri*	*Megabothris* sp. 1	*Megabothris* sp. 2	Ceratophyllid sp.	*Catallagia* sp.	*N. inopina*	*R. fraterna*
O. rupestris	1	0	4	11	11	11	3	55	51	40
O. bruneri		1	2	11	11	11	3	41	37	36
O. labis			4	11	11	11	3	55	51	30
O. tuberculata				15	15	15	7	58	54	43
A. wagneri					0	0	12	50	47	36
Megabothris sp. 1						0	12	50	47	36
Megabothris sp. 2							12	50	47	36
Ceratophyllid sp.								56	50	39
Catallagia sp.									34	44
N. inopina										15

Figure 3.2: Phenogram depicting the genetic differences in the nucleotide sequence of the nuclear 18S rRNA gene among the eleven flea taxa.

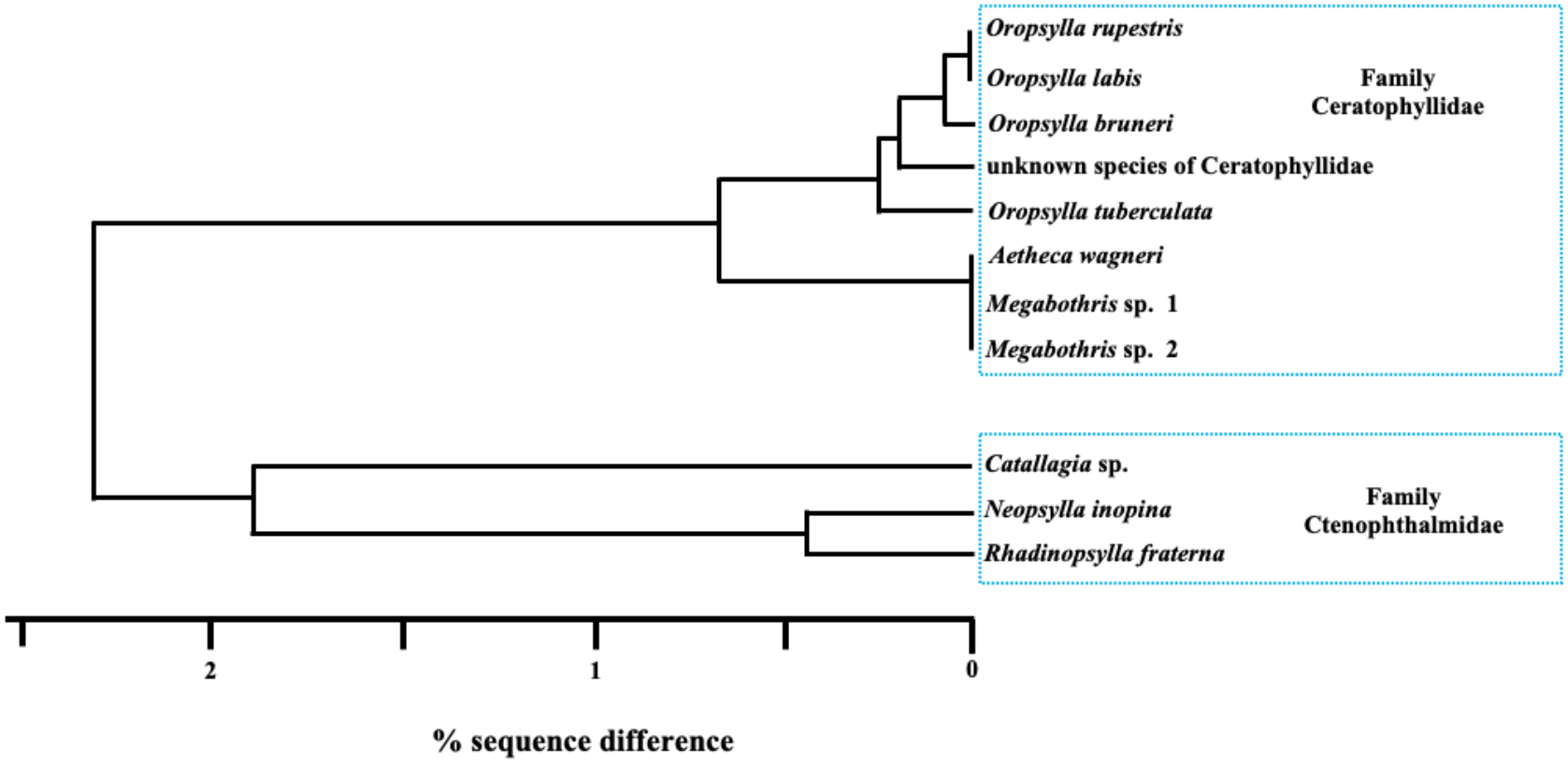


Table 3.6: Matrix showing the number of differences in the nucleotide sequence of the nuclear 28S rRNA gene among the pairwise comparisons of the eleven flea taxa.

	O. bruneri	*O. labis*	*O. tuberculata*	*A. wagneri*	*Megabothris* sp. 1	*Megabothris* sp. 2	Ceratophyllid sp.	*Catallagia sp.*	*N. inopina*	*R. fraterna*
O. rupestris	11	12	12	14	13	16	12	59	58	73
O. bruneri		5	11	20	21	22	18	63	64	72
O. labis			10	21	20	23	19	64	65	70
O. tuberculata				18	15	20	18	63	60	75
A. wagneri					3	6	8	61	57	76
Megabothris sp. 1						5	7	61	58	75
Megabothris sp. 2							10	64	61	78
Ceratophyllid sp.								60	58	74
Catallagia sp.									30	42
N. inopina										63

the two *Megabothris* taxa was of a similar magnitude to that between *O. bruneri* and *O. labis* (i.e., 0.7%; Table 3.6). The largest difference in 28S sequence (78 bp) occurred between *R. fraterna* and *Megabothris* sp. 2 (Table 3.6). The sequences of *A. wagneri* in the present study were identical to the sequences of the 28S rRNA gene for *Aetheca wagneri* on GenBank (accession number EU336147). The phenogram produced from the UPGMA analyses of the pairwise comparisons of sequence differences in the 28S rRNA gene is shown in Figure 3.3. The four species of *Oropsylla* formed a distinct cluster and differed from other taxa in the family by <3%. *Megabothris* sp. 1 was positioned with *A. wagneri* and not *Megabothris* sp. 2 (Fig 3.3). There was a distinct separation of the two families (i.e., average of ~9.7% difference in DNA sequence; Fig 3.3). Within the Ctenophthalmidae, *R. fraterna* differed in DNA sequence by ~8% from *Catallagia* sp. and *N. inopina*.

3.4.4 Molecular characterization of fleas using the mt cytochrome *c* oxidase 2 gene (*cox*2)

The nucleotide sequences for *cox2* were aligned over 756 bp positions (Supplemental Fig 3.4), of which 460 (61%) had nucleotides that were identical among all taxa. Different haplotypes (sequence variants) were of several species (e.g., *O. tuberculata* and *O. rupestris*). The magnitude of the intraspecific variation found in nucleotide sequence of the examined flea species was less than 3%. However, the magnitude of sequence difference among the three specimens of *Megabothris* exceeded this. The two specimens of *Megabothris* sp.1 differed in sequence from the single specimen of *Megabothris* sp. 2 at 47 or 50 (~6%) alignment positions (Table 3.7). The number of alignment positions that differed among species ranged from 47 to 189. The phenogram produced from the UPGMA analyses of the pairwise comparisons of sequence differences for *cox*2 is shown in Figure 3.4. The different sequence variants of each

Figure 3.3: Phenogram depicting the genetic differences in nucleotide sequence of the nuclear 28S rRNA gene among the eleven flea taxa.

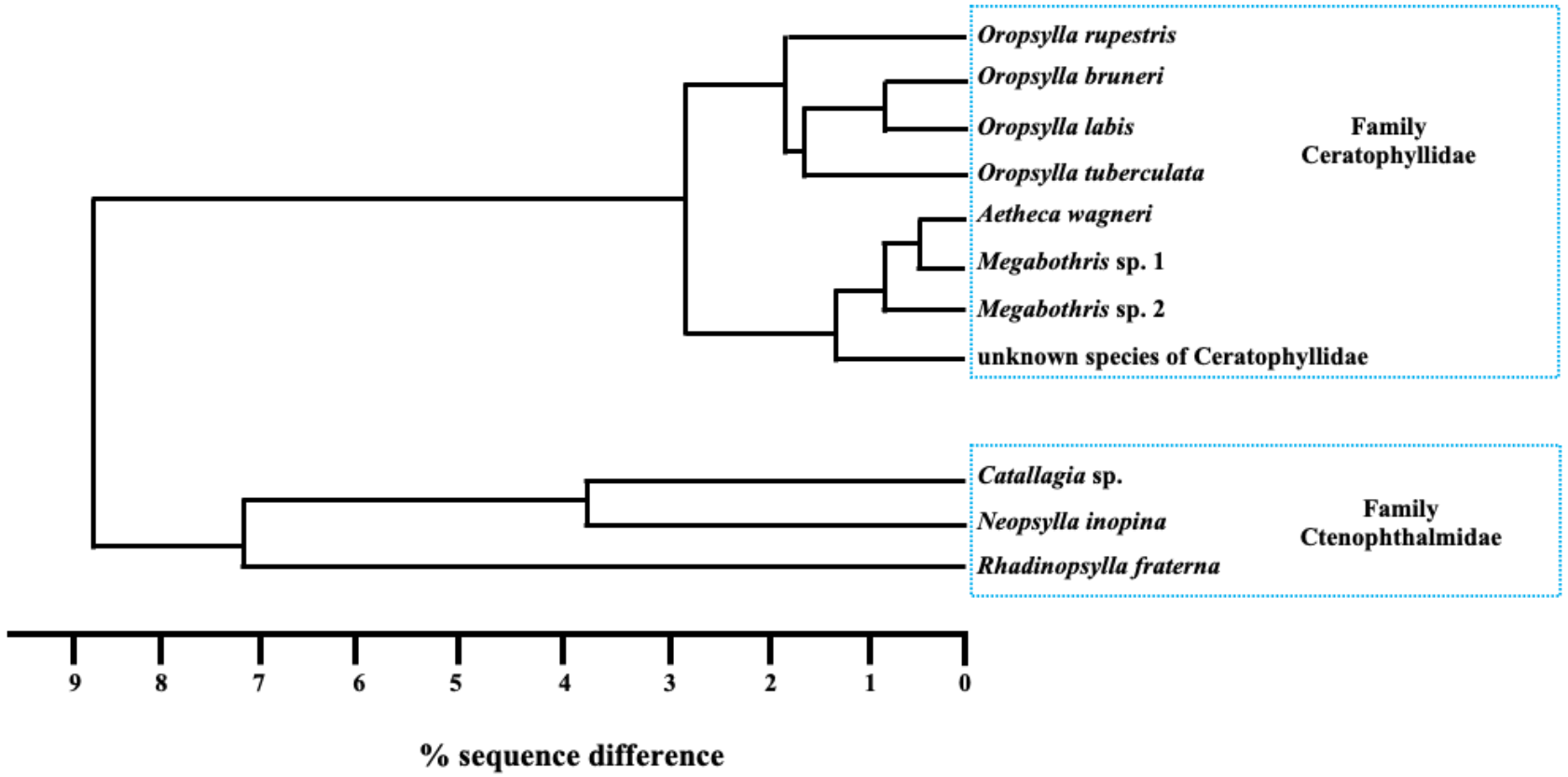


Table 3.7: Matrix showing the number of differences in the nucleotide sequences of *cox*2 among the eleven flea taxa.

	O. rupestris	***O. bruneri***	***O. labis***	***O. tuberculata***	***A. wagneri***	***Megabothris* sp. 1**
O. rupestris	0-5	71-74	77-83	66-73	78-89	96-116
O. bruneri		1-2	81-85	70-81	93-106	106-128
O. labis			2	74-77	111-113	141-145
O. tuberculata				1-19	97-100	100-122
A. wagneri					5	69-73
Megabothris sp. 1						6
Megabothris sp. 2						
Ceratophyllid sp.						
Catallagia sp.						
N. inopina						
R. fraterna						

	***Megabothris* sp. 2**	**Ceratophyllid sp.**	***Catallagia sp.***	***N. inopina***	***R. fraterna***
O. rupestris	99-121	87-95	122-143	131-154	154-167
O. bruneri	110-133	87-97	125-145	135-156	170-182
O. labis	142-143	101-103	134-137	149-160	174-182
O. tuberculata	112-130	101-103	138-140	149-156	169-174
A. wagneri	73-74	67-69	140-142	137-145	167-172
Megabothris sp. 1	47-50	92-93	152-153	141-155	157-161
Megabothris sp. 2	-	95	154-155	154-169	163-168
Ceratophyllid sp.		-	142-143	146-151	171-175
Catallagia sp.			1	129-131	184-189
N. inopina				1-14	166-174
R. fraterna					1-3

morphologically defined species were placed in separate clusters or groups. For *O. tuberculata*, this included the *cox2* sequences on GenBank for five *O. tuberculata cynomuris* collected from black-tailed prairie dogs in Boulder County, Colorado (Jones et al. 2010), which were positioned with the *O. tuberculata* from Richardson's ground squirrels. In addition, the four species of *Oropsylla* formed a distinct group in Ceratophyllidae and differed on average by 14% in DNA sequence when compared to the *cox2* sequences of other taxa in the family (Fig. 3.4). Furthermore, in the family Ctenophthalmidae, *Catallagia* sp. and *N. inopina* differed in *cox*2 sequence. *Rhadinopsylla fraterna* was placed on a branch external to the family to which it belongs, as well as from family Ceratophyllidae. It was the most divergent species in term of its *cox*2 sequence (i.e., differed on average by >22% from all other taxa).

The amino acid sequences were compared for all taxa based on the translation of the nucleotide sequence data for alignment positions 54 to 702. This was done to have an equivalent amino acid sequence length for all taxa. Thus, the amino acid sequences were aligned over a length of 216, of which 156 (72%) were identical among all taxa (Supplemental Fig 3.5). Table 3.8 shows the pairwise comparisons of the differences in amino acid sequence both within and among flea species. The magnitude of the intraspecific variation in sequence difference was relatively low (0 to 2 amino acids) while the level of interspecific differences was much larger ranging from 3 (i.e., 1.4% between *O. labis* and *O. tuberculata*) to 43 (i.e., 20% between *A. wagneri* and *R. fraterna*). The extent of the amino acid sequence difference among the four species of *Oropsylla* was 1.4% to 2.8% (Table 3.8). The amino acid sequences of the two specimens of *Megabothris* sp. 1 differed at 1 position (= 0.5% difference) whereas the specimen of *Megabothris* sp. 2 had 4-5 (1.9-2.3%) differences in amino acid sequence compared to the

Figure 3.4: Phenogram depicting the genetic differences in nucleotide sequence of the mitochondrial *cox*2 among eleven flea taxa. Dotted line represents threshold of the percentage of sequence differences of morphologically distinct species.

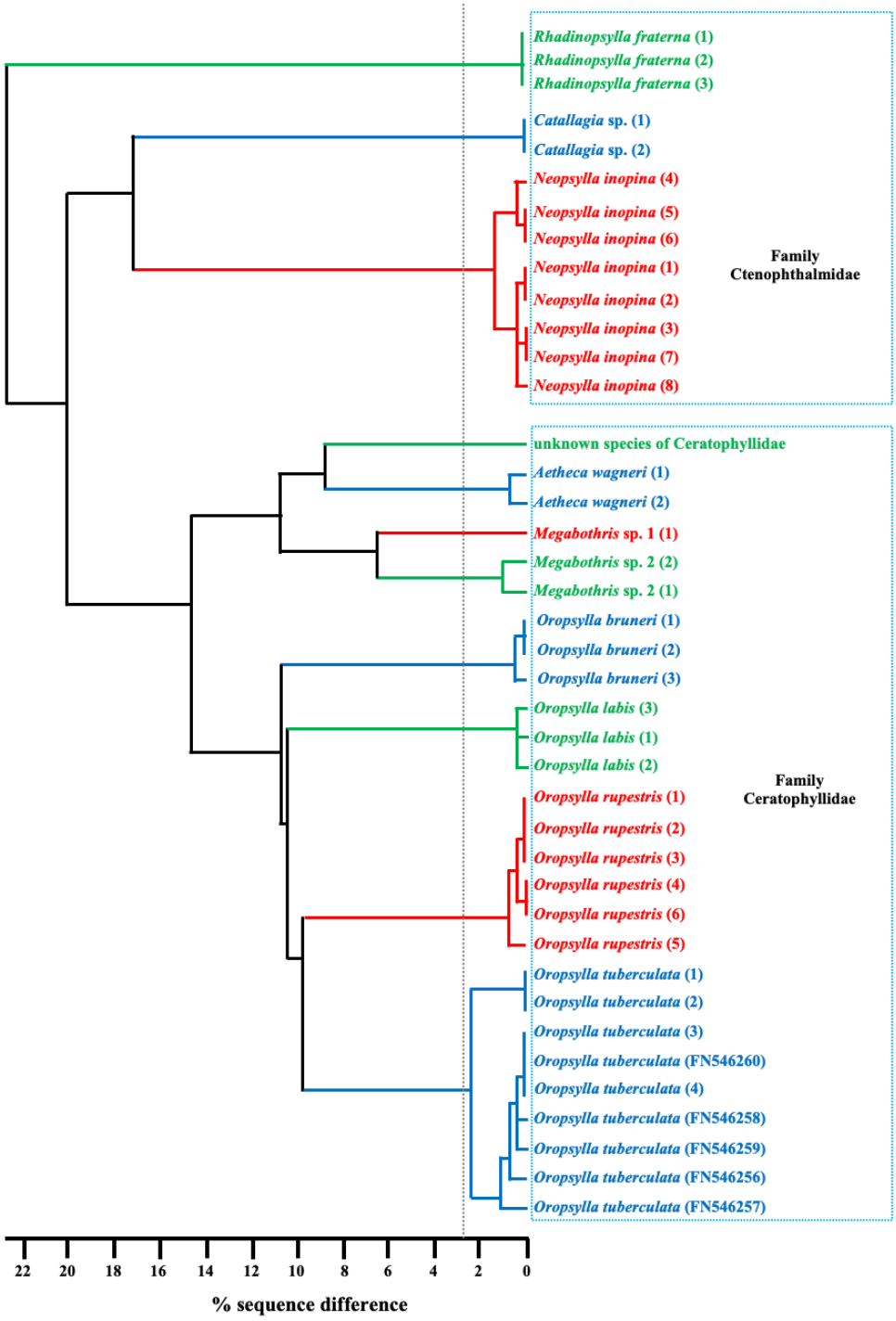


Table 3.8: Matrix showing the number of differences in the amino acid sequences of *cox*2 among the eleven flea taxa.

	O. rupestris	***O. bruneri***	***O. labis***	***O. tuberculata***	***A. wagneri***	***Megabothris* sp. 1**
O. rupestris	0-2	5-6	4-5	5-6	13-16	13-16
O. bruneri		0-1	5-6	4-6	12-16	11-14
O. labis			0	3	16-17	14-15
O. tuberculata				0	13-14	13-14
A. wagneri					1	6-8
Megabothris sp. 1						1
Megabothris sp. 2						
Ceratophyllid sp.						
Catallagia sp.						
N. inopina						
R. fraterna						

	***Megabothris* sp. 2**	**Ceratophyllid sp.**	***Catallagia* sp.**	***N. inopina***	***R. fraterna***
O. rupestris	15-17	11-13	23-31	26-33	33-36
O. bruneri	11-13	9-11	21-29	27-34	30-33
O. labis	16	12	29-30	32-34	35
O. tuberculata	15	11	28-29	32-34	34
A. wagneri	8-9	9-10	30-32	33-36	42-43
Megabothris sp. 1	4-5	6-7	31-33	33-36	38
Megabothris sp. 2	-	7	33-34	33-35	39
Ceratophyllid sp.		-	32-33	34-36	39
Catallagia sp.			1	20-23	30-31
N. inopina				0-2	32-33
R. fraterna					0

specimens of *Megabothris* sp. 1 (Table 3.8). The phenogram produced from the UPGMA analyses of the pairwise comparisons of amino acid sequence differences for *cox*2 are shown in Figure 3.5. The topology of the phenogram for the *cox2* amino acid sequence data was similar to that for the nucleotide sequence data (Fig. 3.4) except that *R. fraterna* was positioned with other members of the Family Ctenophthalmidae. As in the phenogram based on the nucleotide data, the four species of *Oropsylla* formed a group to the exclusion of other current members of the Family Ceratophyllidae and there was marked separation of fleas in the two families (Fig. 3.5).

3.4.5 Molecular characterization of fleas using the mt cytochrome *c* oxidase 1 gene (*cox*1)

Although amplicons of *cox*1 were amplified using the universal primers for all flea species except *N. inopina*, approximately 50% of the sequences produced from these amplicons were of too low of quality to provide reliable data. In addition, a blast search of each sequence (~596 bp) with data on GenBank, revealed that the sequences produced from one gDNA sample of *O. tuberculata* (specimen 18.020) and two gDNA samples of *R. fraterna* (specimens 18.105 and 18.111) were not flea *cox1* sequences but were in fact *cox*1 sequences identical to or genetically most similar to that of Richardson's ground squirrels (accession numbers JF457124 and KP698976). These sequences were still included in the *cox1* analyses to highlight this result.

Table 3.9 shows the pairwise comparisons of the differences in nucleotide sequence of *cox1* both within and among flea species for which reliable sequence data was obtained. The magnitude of the sequence differences among individuals of the same morphologically defined species ranged from 0-45 bp (= difference of 0-7.5%). Intraspecific variation on *cox*1 sequences was detected among specimens of *O. tuberculata* (Table 3.9). The range of sequence differences among the four species of *Oropsylla* (8.6-14.2%) was lower than that among genera of the

Figure 3.5: Phenogram depicting the genetic differences in amino acid sequence of mitochondrial *cox2* among 11 flea taxa.

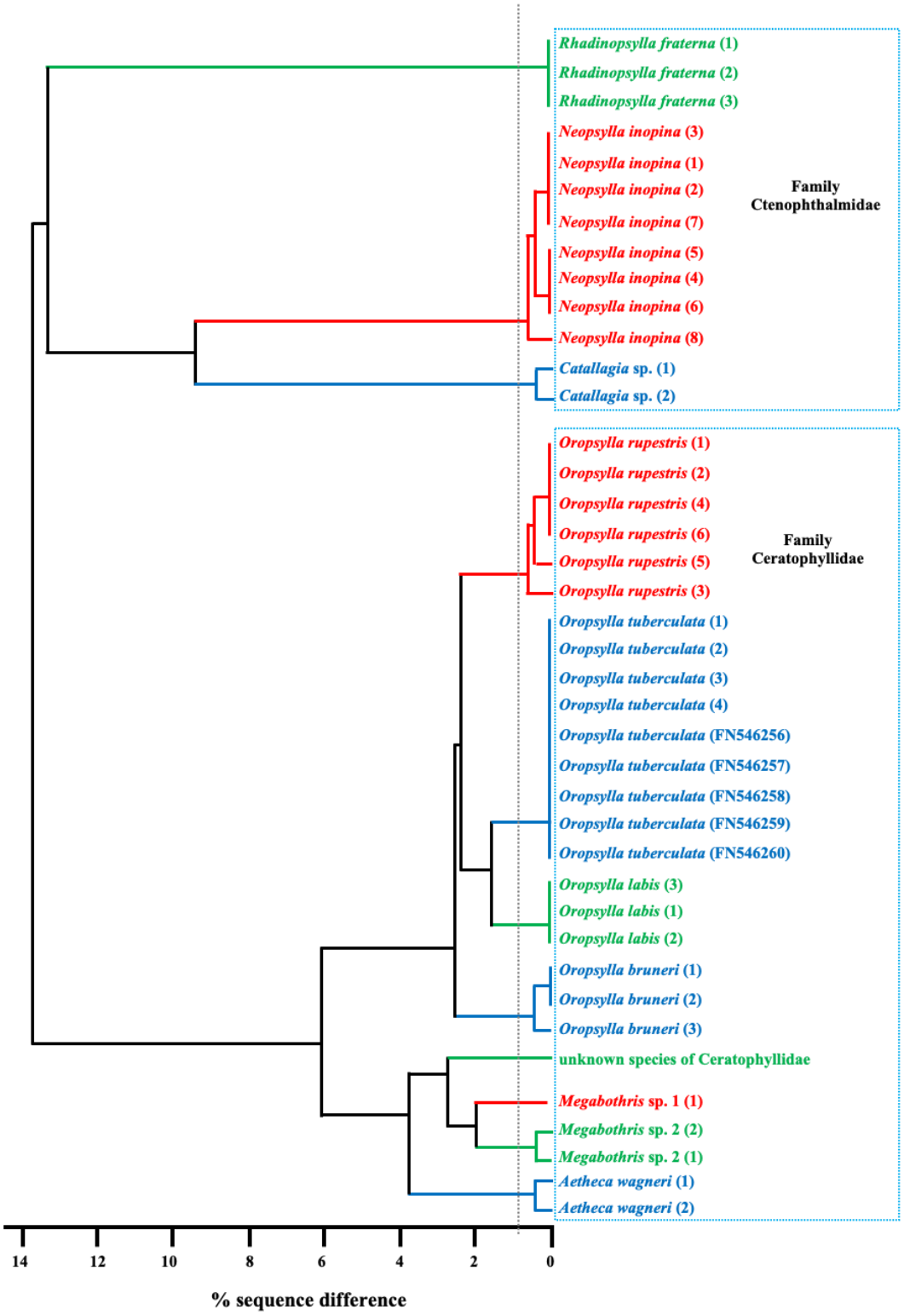


Table 3.9: Matrix showing the number of differences in the nucleotide sequences of mitochondrial *cox*1 among nine flea taxa.

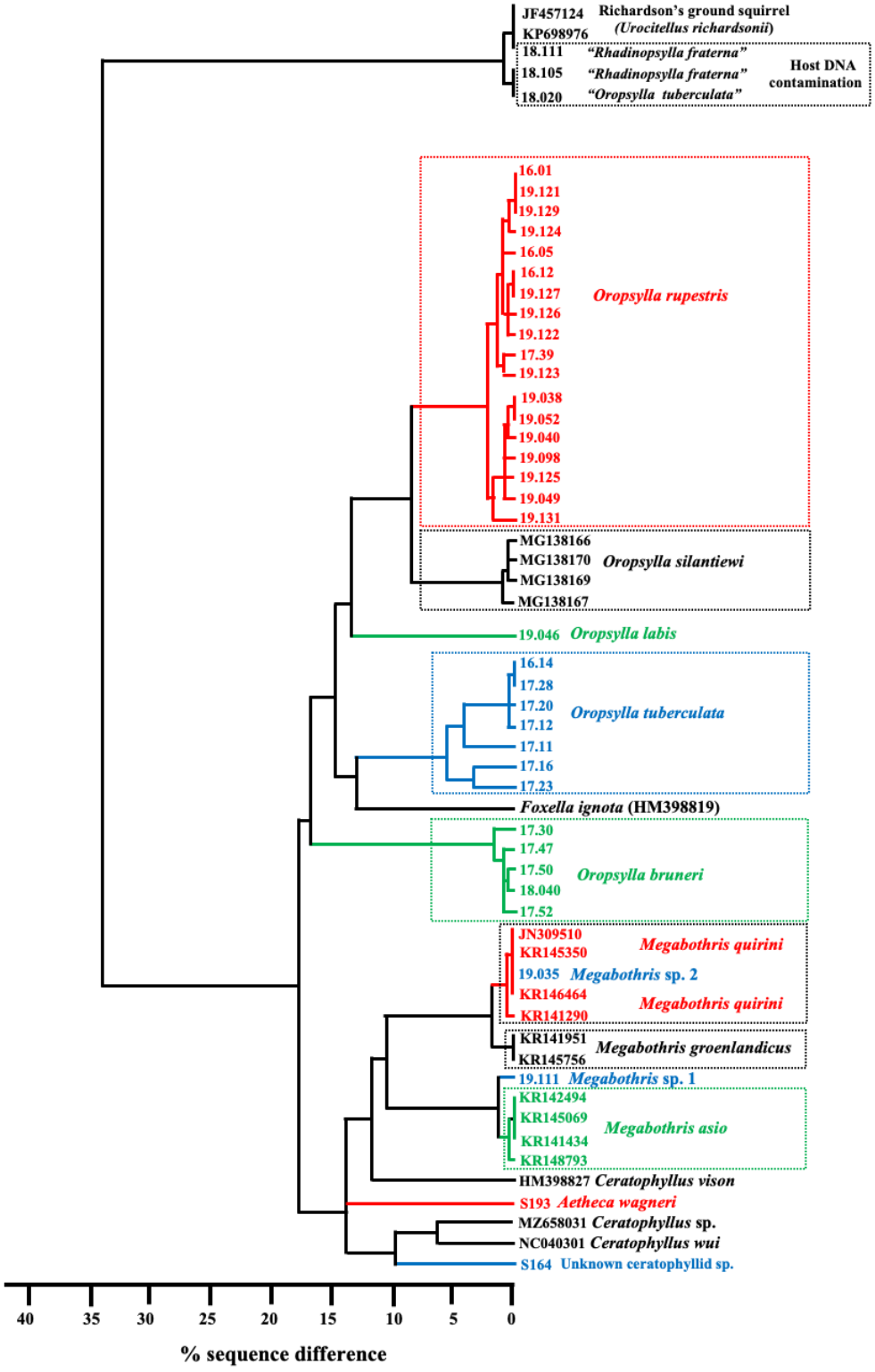
	O. rupestris	***O. bruneri***	***O. labis***	***O. tuberculata***	***A. wagneri***	***Megabothris* sp. 1**
O. rupestris	0-18	69-89	51-66	55-85	68-88	59-84
O. bruneri		2-10	69-73	62-84	84-88	84-89
O. labis			-	54-75	84	84
O. tuberculata				1-45	65-87	74-84
A. wagneri					-	66
Megabothris sp. 1						-
Megabothris sp. 2						
Ceratophyllid sp.						
R. fraterna						

	***Megabothris* sp. 2**	**Ceratophyllid sp.**	***R. fraterna***	***O. tuberculata* contaminated**	**RGS**
O. rupestris	71-91	70-94	134-173	158-173	158-172
O. bruneri	93-97	87-88	135-151	145-151	145-151
O. labis	90	92	144-163	162	165
O. tuberculata	73-91	72-100	144-173	146-173	146-171
A. wagneri	68	75	139-155	155	154
Megabothris sp. 1	51	72	140-162	162	159
Megabothris sp. 2	-	76	146-167	167	165
Ceratophyllid sp.		-	136-159	159	158
R. fraterna			0	0	0-6
O. tuberculata contaminated				-	6

Ceratophyllidae (9.9-16.8%). There were 51 (6%) differences in cox1 sequence between *Megabothris* sp. 1 and *Megabothris* sp. 2 (Table 3.9). The sequences of the amplicons of the flea gDNA contaminated with host DNA were identical to or differed by only 1% when compared to the *cox*1 sequences of *U. richardsonii* but differed by 20.6-29% when compared to the *cox1* sequences of taxa within the Siphonaptera.

The phenogram produced from the UPGMA analyses of the pairwise comparisons of sequence differences for *cox*1 is shown in Figure 3.6. The different sequence variants of each morphologically defined species were placed in separate clusters or groups (i.e., each species has a unique set of *cox*1 sequences). However, the four species of *Oropsylla* and the four sequences of *O. silantiewii* (GenBank accession numbers MG138166-MG13870) did not form a distinct group in Ceratophyllidae because *Foxella ignota* (accession number HM398829) was positioned between *O. bruneri* and the four other species of *Oropsylla* (Fig. 3.6). The phenogram also included sequences of 3 species of *Megabothris*, *M. quirini* (accession numbers JN309510, KR141290, KR145350, and KR146290), *M. asio* (accession numbers KR141434, KR142494, KR145069 and KR148793) and *M groenlandicus* (accession numbers KR141951 and KR145756), in addition to *Megabothris* sp. 1 and *Megabothris* sp. 2 from Richardson's ground squirrels. *Megabothris* sp. 2 was genetically very similar in *cox*1 sequence to two sequences of *M. quirini* and was positioned within the group containing all sequences of *M. quirini*, whereas *Megabothris* sp. 1 was genetically more similar to *M. asio* than to *Megabothris* sp. 2.

Figure 3.6: Phenogram depicting the genetic differences in nucleotide sequence of the mitochondrial *cox*1 among nine flea taxa. Also shown are the 3 flea sequences that represent host contamination.



3.5 DISCUSSION

Fleas are traditionally identified based on minute morphological characteristics that are difficult to assess without the chemical clearing of internal structures. This chemical clearing prevents subsequent investigations of flea genomics and their microbiota. The ability to accurately identify and distinguish among flea species that parasitize Richardson's ground squirrels and black tailed prairie dogs using one or more genetic markers is important given that five of the flea species examined in this study are vectors of *Y. pestis*, the causative agent of plague (Wilder et al. 2008, Eisen and Gage 2009, Hastriter 2023). This study investigated the relative usefulness of five potential genetic markers to differentiate 11 flea taxa associated with Richardson's ground squirrels in Saskatchewan and Alberta. These targets comprised three nuclear genes or regions (18S rRNA gene, the ITS2 and the 28S rRNA gene) and two mitochondrial genes (*cox*1 and *cox*2).

The findings of the present study have shown that the 18S rRNA gene is not a useful genetic marker for distinguishing among flea species. For example, there were no differences in DNA sequence among the two morphologically defined species, *O. rupestris* and *O. labis*, or among some species of different genera (i.e., *Aetheca* and *Megabothris* spp.). The relatively low number of nucleotide differences (0-15; 0-0.8%) among species within the Ceratophyllidae also suggests that there may little or no resolution of the phylogenetic differences of taxa within this family. In contrast, the differences in the sequences of the 18S rRNA gene among the three taxa of the Ctenophthalmidae (i.e., *Catallagia* sp., *N. inopina* and *R. fraterna*), which ranged from 15-44 (0.8-2.4%) mutational changes, suggests that this gene may have potential for species identification of taxa within this family, and provide enough characters to infer the evolutionary relationships of species within the Ctenophthalmidae. However, this family, unlike the

Ceratophyllidae, does not represent a monophyletic assemblage, but consists of at least seven paraphyletic lineages based on phylogenetic analyses of a combination of four genetic markers; the 18S rRNA gene, the 28S rRNA gene, *cox*2, and the elongation Factor 1-alpha gene (Whiting et al. 2008). Since the analyses conducted by Whiting et al. (2008) were on a combined data set, it is unclear if the 18S rRNA gene alone is useful for inferring the relationships of the major lineages (i.e., families) of the Siphonaptera. This gene did contribute 16% of the phylogenetic signal in the combined data set to infer evolutionary relationships of the taxa within the Siphonaptera (Whiting et al. 2008). The magnitude of sequence differences between taxa of the Ceratophyllidae and the Ctenophthalmidae obtained in the present study, that is 36-58 (2-3.2%) nucleotide differences, suggests that this gene has potential to infer phylogenetic relationships of ≥16 families of the Siphonaptera. More sequence data of the 18S rRNA gene from species in each of the recognized families is needed to determine if this gene can be used, independently of other genetic markers, to infer evolutionary relationships within Siphonaptera. Although phenograms are not phylogenetic trees, they can on occasion reflect the evolutionary relationships of trees based on cladistic analyses of genetic data (Andrews and Chilton 1999). The phenograms produced from analyses of the nucleotide sequence data of each genetic marker, and of the amino acid sequence data of *cox*2, show that the Ceratophyllidae forms a cluster to the exclusion of the Ctenophthalmidae which is congruent with the phylogenetic analysis conducted previously on molecular data (Whiting 2002, Whiting et al. 2008).

The ITS2 was the most effective genetic marker of the five target regions tested for species delineation of the flea species examined in this study because there was no intraspecific variation in DNA sequence among individuals of each morphologically distinct species, and each species had a unique DNA sequence. The sole exception to this was for three specimens of

Megabothris, where two individuals, one from Saskatchewan and one from Alberta, differed in ITS2 sequence at seven nucleotide positions when compared to the third specimen from Saskatchewan. Given this sequence difference and the lack of intraspecific variation in ITS2 sequence among the four morphologically distinct species of *Oropsylla*, it is most likely that the *Megabothris* specimens in the present study represent two species. This conclusion is not unreasonable given that at least two species of *Megabothris* have been reported on Richardson's ground squirrels (Holland 1985, Galloway and Christie 1990, Lindsay and Galloway 1997). *Megabothris asio*, which is normally a parasite of meadow voles, has been documented on Richardson's ground squirrels in Saskatchewan (Holland 1985), while *M. quirini*, a parasite of mice, has also been reported on Richardson's ground squirrels in Manitoba (Galloway and Christie 1990, Lindsay and Galloway 1997). Another species in the genus, *M. lucifer*, occurs in Saskatchewan on a variety of small rodents; however, to date, it has not been associated with Richardson's ground squirrels (Holland 1985). A fourth species in the genus that has been reported in southern Canada, *M. acerbus* is unlikely to be one of the species represented by the three *Megabothris* specimens as the distribution of its principal host, the eastern chipmunk (*Tamias striatus*), does not include Saskatchewan or Alberta. The existence of two species of *Megabothris* is also supported by sequence data of the 28S rRNA gene and *cox*2. For instance, for the 28S rRNA gene, the two *Megabothris* taxa had different sequences, and *Megabothris* sp. 1 was genetically more similar to *A. wagneri* than to *Megabothris* sp 2. Whereas for *cox*2, the magnitude of sequence differences among the two taxa (7%) is significantly greater than the level of intraspecific variation in DNA sequence (<2.6%) for *O. tuberculata*. In addition, the magnitude of sequence differences in the amino acid sequence of *cox*2 for the two *Megabothris* taxa (1.8%) exceeded the difference (1.3%) in amino acid sequences of the morphologically

distinct species, *O. tuberculata* and *O. labis*. The sequence data for *cox*1 also supports the hypothesis that the two *Megabothris* taxa represent different species based on differences in their DNA sequence. Unlike for the other genetic markers used in the present study, *cox*1 sequence data for three species of *Megabothris* (*M. quirini*, *M. asio* and *M. groenlandicus*) were available on GenBank. Inclusion of these *cox*1 sequences revealed that *Megabothris* sp. 2 falls within a cluster of sequences for *M. quirini* in the UPGMA analyses, indicating that *Megabothris* sp. 2 is *M. quirini*. In contrast, *Megabothris* sp. 1 was genetically most similar to *M. asio* but was positioned outside of the *M. asio* cluster. It is most likely that *Megabothris* sp. 1 is *M. asio*; however, this requires further investigation. Since the potential identities of the two *Megabothris* taxa examined in the present study are based on only a single genetic marker, they will be referred to in subsequent chapters of this dissertation as *Megabothris* sp. 1 and *Megabothris* sp. 2.

The utility of ITS1 and/or ITS2 for species delineation has been previously demonstrated for fleas (Zurita et al. 2016, Marrugal et al. 2013) and other insects (Nelson et al. 2008, Marinho et al. 2011, Park et al. 2018). Additionally, the ITS2 has significant potential as a diagnostic tool to distinguish among different species of fleas when combined with the mutation scanning technique, single-strand conformation polymorphism (SSCP). As shown in the previous chapter, four species of *Oropsylla* could be easily distinguished from one another based on PCR-SSCP of the 28S rRNA gene. The magnitude of the genetic differences in ITS2 sequences among flea species suggest that the ITS2 may represent a useful target for examining the evolutionary relationships of fleas. This DNA region has not been used previously to infer the phylogeny of fleas because of a lack of sequence data. Also, a secondary structure model of the ITS2 rDNA is needed to assist in the alignment of more divergent sequences (Letsch et al. 2009), thereby

increasing the chance that homologous characters (i.e., nucleotides) are compared for the phylogenetic analyses.

The 28S rRNA gene has been shown to be useful to infer the evolutionary relationships of fleas (Whiting et al. 2008) and other insects (Mardulyn and Whitfield 1999, Hovmöller et al. 2002, Gillespie et al. 2006). In the previous chapter, it was demonstrated that *O. rupestris*, *O. tuberculata*, *O. labis* and *O. bruneri* could be distinguished from one another based on differences in their sequences of ~500 bp of the 28S rRNA gene (Thoroughgood et al. 2021). In the present study, we designed primers to amplify a larger fragment (~830 bp) of the 28S rRNA gene. The results obtained in this study showed that all 11 flea species had a unique sequence for this gene, indicating the utility of this region for the differentiation of fleas on Richardson's ground squirrels. Given the relatively conserved nature of the 28S rRNA gene compared to the ITS2, additional sequence data of the 28S rRNA gene for other fleas is needed to determine if this genetic marker has broader application for the identification of fleas in different families.

Cytochrome oxidase *c* subunit 1 gene (*cox*1) is considered the universal barcode and is frequently used to identify insects (Chitimia et al. 2010, Arribas et al. 2016, Herbert et al. 2016, Klimov et al. 2019, Balzer et al. 2020). However, for some of the amplicons derived from flea gDNA samples representing specific taxa, we found frequent "contamination" of the *cox*1 of host DNA and/or produced sequences of very poor quality. These factors significantly reduced the effectiveness of this genetic marker for the identification of some flea species. Host contamination in *cox*1 amplicons may be a consequence of the blood meals imbibed by fleas, which can contribute to a significant increase in the volume of the midgut region of the flea body (Krasnov 2008). During a feeding period, fleas consume no more than 0.4 mg of blood which represents up to 1.6 mg of blood per mg of the flea body mass (Krasnov 2008). Recently, it has

been discovered that the so-called “universal invertebrate” PCR primers for *cox*1 can preferentially amplify the *cox*A gene of a specific group of bacteria (i.e., *Rickettsia*-like endosymbionts, RLEs) in a diverse range of insects (Pilgrim et al. 2020, 2021). Consequently, some of the so-called *cox*1 sequences of insects, are in fact, *cox*A sequences of RLEs (Pilgrim et al. 2017). There was no evidence in the present study that the *cox*1 primers are amplifying the *cox*A gene of RLEs in fleas parasitizing Richardson’s ground squirrels. Further research is needed to develop new PCR primers that amplify only *cox*1 from fleas and not their vertebrate hosts to allow for the assessment on the potential of this genetic marker for species delineation and identification of fleas, and to study their population genetics, phylogeographic patterns and evolutionary relationships.

In contrast to the results obtained for *cox*1, there were no “contamination” issues in the amplification and DNA sequencing of *cox*2 from fleas in the present study. Furthermore, each flea species examined had a unique set of DNA sequences for *cox*2. This genetic marker could be used for species identification; however, its usefulness as a diagnostic marker for species identification requires detailed information of the magnitude of intraspecific variation for each species. This is important if cryptic (i.e., genetically distinct but morphologically similar) species exist of siphonapterans. The 11 flea species examined in the present study also had a unique amino acid sequence(s) for *cox*2. Additional data needs to be collected from other flea species, including those of other families, to determine if the amino acid sequence can be used for species identification.

The results of the present study also suggest that *cox*2 would be useful in the study of the population genetics and phylogeography of flea species. This is supported by the study of de la Cruz and Whiting (2003), who used the sequence data from *cox*2 and the mt cytochrome *b* gene

to determine the population genetic structure and phylogeographic patterns of *Pulex simulans* from six populations in Peru. Similarly, Brinkerhoff et al. (2011) used *cox*2 as a genetic marker to examine the dispersion patterns of *Oropsylla hirsuta* following plague outbreaks in seven colonies of black-tailed prairie dogs in Colorado.

One interesting finding of the present study was that there was no clear separation of the *O. tuberculata* from Richardson's ground squirrels in Saskatchewan and *O. tuberculata* from black-tailed prairie dogs in Boulder County, Colorado (Jones et al. 2010) based on the analyses of *cox*2 sequence data. This outcome was surprising given that this flea species is considered by some to represent different subspecies based on the host species parasitized and the geographical regions in which they were collected. In the two reviews of Canadian fleas, Holland (1949, 1985) listed *Opisocrostis tuberculatus tuberculatus*, now *Oropsylla tuberculata tuberculata*, parasitizing Richardson's ground squirrels throughout its range in Alberta, Saskatchewan and Manitoba, and of the Columbian ground squirrel (*Urocitellus columbianus*) in southern Alberta and south-eastern British Columbia. This flea also parasitizes the northern Idaho ground squirrel (*Urocitellus brunneus*) in several areas of Idaho (Yensen et al. 1996). Holland (1949, 1985) also referred to another subspecies, *O. t. cynomuris*, that occurs on black-tailed prairie dogs in Montana, Wyoming and Colorado. Lewis (1975) also lists *O. t. cynomuris* as a parasite of *U. columbianus* and *C. gunnisoni* (Gunnison's prairie dog) from Montana, Wyoming, Colorado, Utah and Mexico. However, Holland (1949) also indicates that although black-tailed prairie dogs occur in a small area of south-western Saskatchewan, collections of these fleas of *O. tuberculata* from these animals were *O. t. tuberculata* and not *O. t. cynomuris.* Lewis (2002), in his review of North American species of *Oropsylla*, questioned the validity of the two subspecies. He refers to the description of *O. t. cynomuris* by Jellison (1947) who indicates that females are smaller in

size, have a smaller spermatheca and have a sharper lobe on the caudal margin of sternum VII, subtended by a deeper sinus. Lewis (2002) concludes that separating taxa within the Siphonaptera based solely on size is not valid taxonomic reasoning. Lewis further argues against the separation of the two taxa as separate subspecies is not warranted given there is considerable intraspecific variation in size among all species within *Oropsylla* (Lewis 2002). The results of the *cox2* data obtained in the present study supports the conclusion made by Lewis (2002).

In conclusion, PCR assays of *cox*1 (the barcoding gene) using the standard "universal primers" can result in the amplification of host DNA, and not flea DNA, as was the case for *O. tuberculata* and several other flea species. This severely reduces the potential of this gene for the delineation of flea species parasitizing Richardson's ground squirrels. In contrast, the ITS2 and 28S rRNA gene are viable molecular markers for the identification and differentiation of the different flea species parasitizing Richardson's ground squirrels and other rodents. The utility of these molecular markers is limited by the available sequence data of previously morphologically identified species. Further analysis to incorporate genetic sequences of important disease vectors into genetic datasets is required to increase the utility of these markers. It is possible that some flea species in other families may have identical sequences for the 28S rRNA gene given that this gene is relatively more conserved that the ITS2. Conversely, it has been demonstrated that the ITS2 is a very important genetic marker for the species-specific identification of other invertebrates that are difficult to identify morphologically (e.g., nematodes, see Gasser et al. 2024). Therefore, the ITS2 is likely to be more robust than the 28S rRNA gene for species identification of taxa within Siphonaptera. Although the 11 flea species examined in the present study did not share the same DNA sequences for *cox*2, the considerable intraspecific variation in sequence, like that of some other protein-coding genes in the mt genome (e.g., *cox*1), reduces the

practical utility of this gene as a genetic marker for species identification. Nonetheless, *cox*2 represents a valuable marker for population genetics and phylogeographic studies on species within the Siphonaptera.

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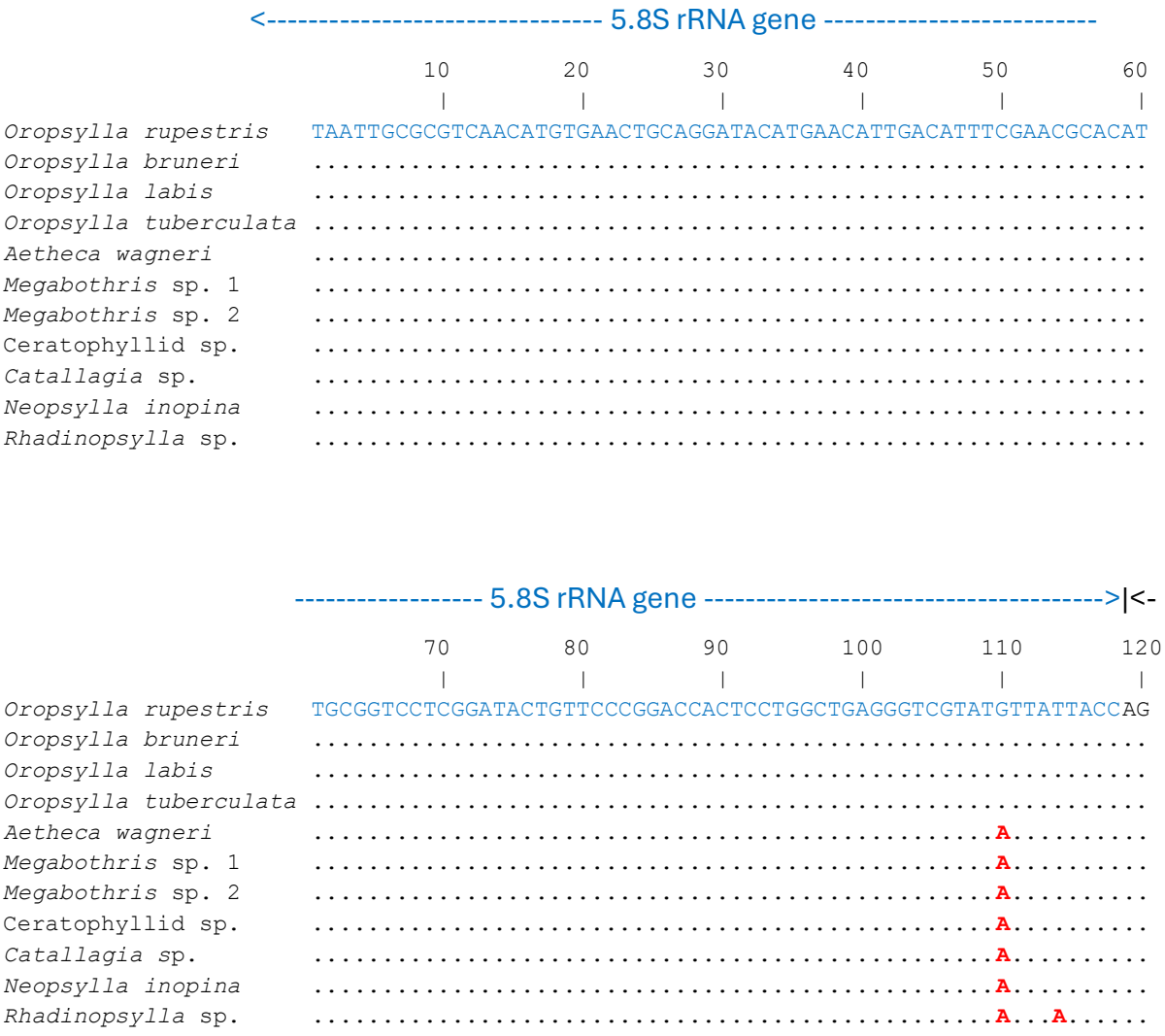
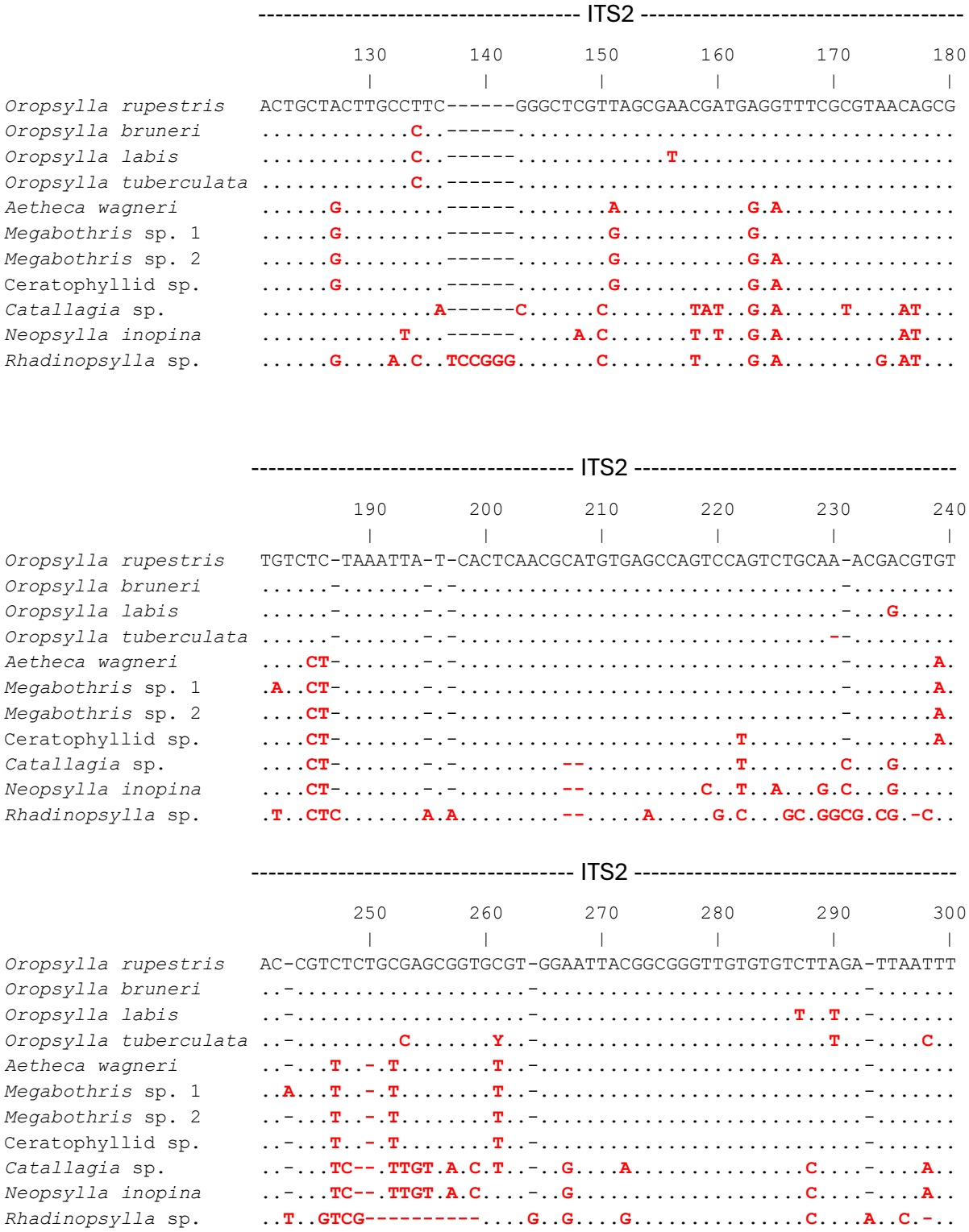
Zurita A, Callejón R, García-Sánchez ÁM, Urdapilleta M, Lareschi M. 2019. Origin, evolution, phylogeny and taxonomy of *Pulex irritans*. Med Vet Entomol. 33(2):296–311. https://doi.org/10.1111/mve.12365.

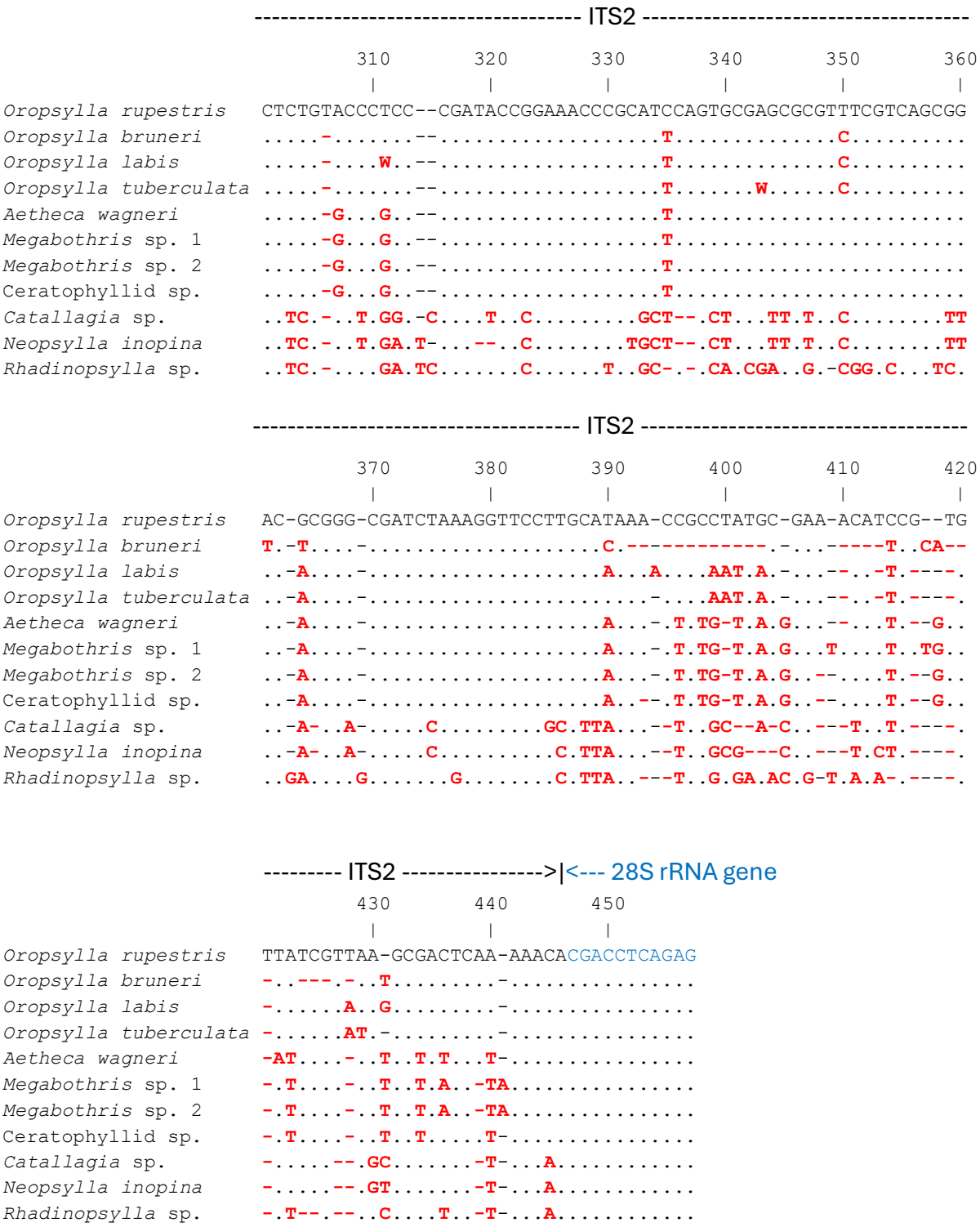
Supplemental Figure 3.1: Alignment of the nucleotide sequences of the partial 5.8S rRNA gene, the complete nuclear of internal transcribed spacer 2 and partial 28S rRNA gene of the eleven flea taxa. Dots represent the identical nucleotide as in the sequence of *Oropsylla rupestris*.



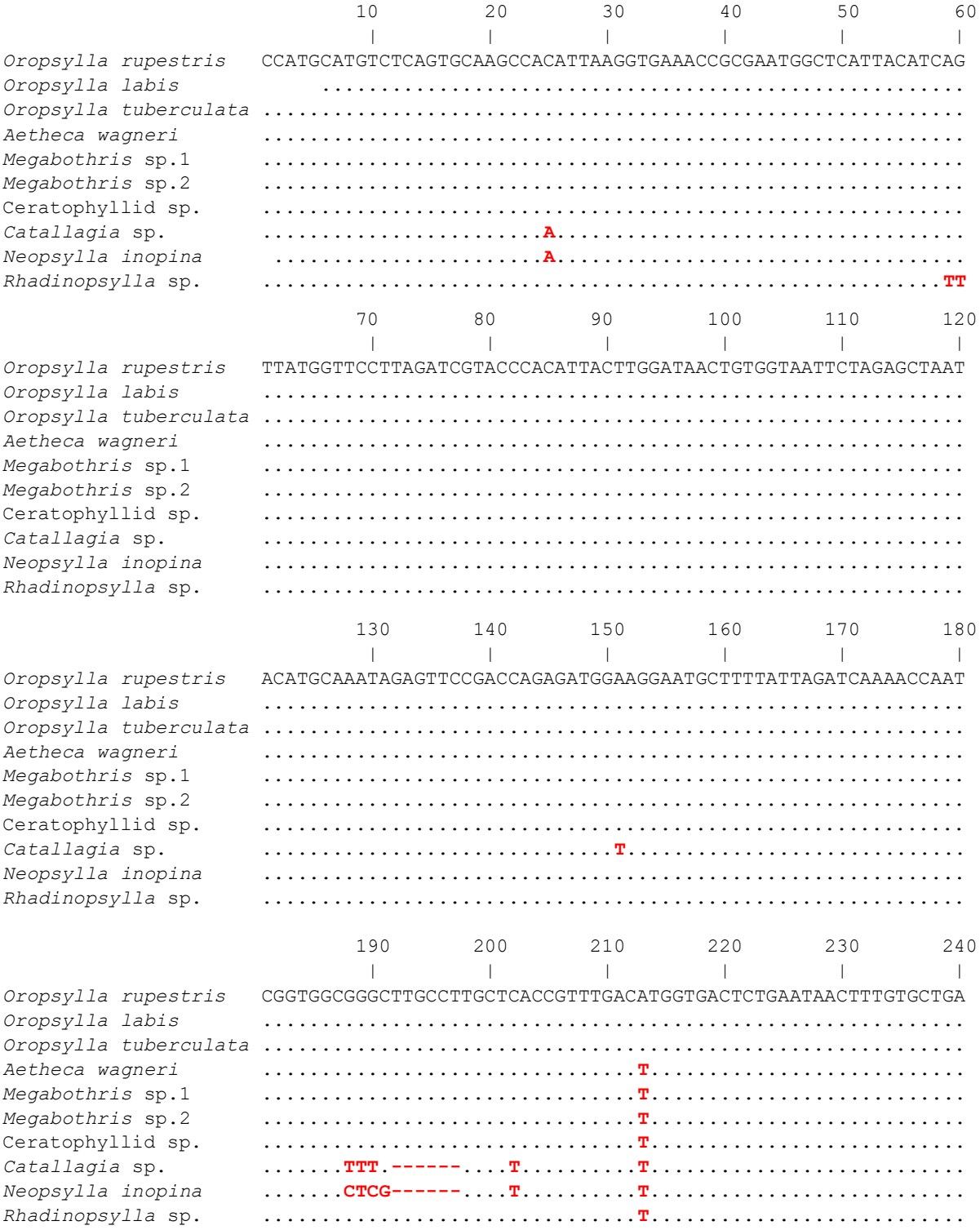
Supplemental Figure 3.1 (continued)



Supplemental Figure 3.1 (continued)



Supplemental Figure 3.2: Alignment of the nucleotide sequences of the nuclear 18S rRNA gene of the ten flea taxa. Dots represent the identical nucleotide as in the sequence of *Oropsylla rupestris*.



Supplemental Figure 3.2 (continued)

```
                              250       260       270       280       290       300
                               |         |         |         |         |         |
Oropsylla rupestris           TCGCACGGTCTCGTACCGGCGACGCATCTTTCAAATGTCTGCCTTATCAACTGTCGATGG
Oropsylla labis               ............................................................
Oropsylla tuberculata         ............................................................
Aetheca wagneri               ............................................................
Megabothris sp.1              ............................................................
Megabothris sp.2              ............................................................
Ceratophyllid sp.             ............................................................
Catallagia sp.                ............................................................
Neopsylla inopina             ............................................................
Rhadinopsylla sp.             ............................................................

                              310       320       330       340       350       360
                               |         |         |         |         |         |
Oropsylla rupestris           TAGTTTCTGCGACTACCATGGTTGTTACGGGTAACGGGGAATCAGGGTTCGATTCCGGAG
Oropsylla labis               ............................................................
Oropsylla tuberculata         ............................................................
Aetheca wagneri               ............................................................
Megabothris sp.1              ............................................................
Megabothris sp.2              ............................................................
Ceratophyllid sp.             ............................................................
Catallagia sp.                ............................................................
Neopsylla inopina             ............................................................
Rhadinopsylla sp.             ............................................................

                              370       380       390       400       410       420
                               |         |         |         |         |         |
Oropsylla rupestris           AGGGAGCCTGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAC
Oropsylla bruneri*                                                    ..............
Oropsylla labis               ............................................................
Oropsylla tuberculata         ............................................................
Aetheca wagneri               ............................................................
Megabothris sp.1              ............................................................
Megabothris sp.2              ............................................................
Ceratophyllid sp.             ............................................................
Catallagia sp.                ............................................................
Neopsylla inopina             ............................................................
Rhadinopsylla sp.             ............................................................

                              430       440       450       460       470       480
                               |         |         |         |         |         |
Oropsylla rupestris           TCCCGGCACGGGGAGGTAGTGACGAAAAATAACGATACGGGACTCATCCGAGGCCCCGTA
Oropsylla bruneri             ............................................................
Oropsylla labis               ............................................................
Oropsylla tuberculata         ............................................................
Aetheca wagneri               ............................................................
Megabothris sp.1              ............................................................
Megabothris sp.2              ............................................................
Ceratophyllid sp.             ............................................................
Catallagia sp.                ............................................................
Neopsylla inopina             ............................................................
Rhadinopsylla sp.             ............................................................
```

Supplemental Figure 3.2 (continued)

```
                                490       500       510       520       530       540
                                 |         |         |         |         |         |
Oropsylla rupestris             ATCGGAATGAGTACACTTTAAATCCTTTCACGATTAACAATTGGAGGGCAAGCCTGGTGC
Oropsylla bruneri               ...........................................................
Oropsylla labis                 ...........................................................
Oropsylla tuberculata           ...........................................................
Aetheca wagneri                 ...........................................................
Megabothris sp.1                ...........................................................
Megabothris sp.2                ...........................................................
Ceratophyllid sp.               ...........................................................
Catallagia sp.                  ..............................A..A.GG.T................T.......
Neopsylla inopina               ..............................A....GG.T.C..............T.......
Rhadinopsylla sp.               ..............................A....GG.T.C..............T.......

                                550       560       570       580       590       600
                                 |         |         |         |         |         |
Oropsylla rupestris             CAGCAGCCGCGGTAACTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCGGTTAAAAAGC
Oropsylla bruneri               ...........................................................
Oropsylla labis                 ...........................................................
Oropsylla tuberculata           ...........................................................
Aetheca wagneri                 ...........................................................
Megabothris sp.1                ...........................................................
Megabothris sp.2                ...........................................................
Ceratophyllid sp.               ...........................................................
Catallagia sp.                  ................T..........................................
Neopsylla inopina               ................T..........................................
Rhadinopsylla sp.               ................T..........................................

                                610       620       630       640       650       660
                                 |         |         |         |         |         |
Oropsylla rupestris             TCGTAGTTGAATCTGTGTCCCACACTGTCGGTTCACCGCTCGCGGTGTCCAACTGGCATG
Oropsylla bruneri               ...........................................................
Oropsylla labis                 ...........................................................
Oropsylla tuberculata           ...........................................................
Aetheca wagneri                 ...........................................................
Megabothris sp.1                ...........................................................
Megabothris sp.2                ...........................................................
Ceratophyllid sp.               ...........................................................
Catallagia sp.                  ............T......................................T..........
Neopsylla inopina               ............Y..........................................
Rhadinopsylla sp.               ...........................................................

                                670       680       690       700       710       720
                                 |         |         |         |         |         |
Oropsylla rupestris             TCTGTGGGACGTCCTGCCGGTGGGCGCAGCCCCTCAAAAGGCGGCCCAACTCAAAATCCT
Oropsylla bruneri               ...........................................................
Oropsylla labis                 ...........................................................
Oropsylla tuberculata           ...........................................................
Aetheca wagneri                 ...........................................................
Megabothris sp.1                ...........................................................
Megabothris sp.2                ...........................................................
Ceratophyllid sp.               ...........................................................
Catallagia sp.                  ................A.................G..........................
Neopsylla inopina               ................A.................G..........................
Rhadinopsylla sp.               ................A.................G..........................
```

Supplemental Figure 3.2 (continued)

```
                          730       740       750       760       770       780
                           |         |         |         |         |         |
Oropsylla rupestris       ACCACGGTGCTCTTCACCGAGTGTCGAGGTGGGCCGGTACGTTTACTTTGAACAAATTAG
Oropsylla bruneri         ...........................................................
Oropsylla labis           ...........................................................
Oropsylla tuberculata     ...........................................................
Aetheca wagneri           ...........................................................
Megabothris sp.1          ...........................................................
Megabothris sp.2          ...........................................................
Ceratophyllid sp.         ...........................................................
Catallagia sp.            ...........................................................
Neopsylla inopina         ...........................................................
Rhadinopsylla sp.         ...........................................................

                          790       800       810       820       830       840
                           |         |         |         |         |         |
Oropsylla rupestris       AGTGCTTAAAGCAGGCTCATTCGCCTGAATATTCTGTGCATGGAATAATGGAATAGGACC
Oropsylla bruneri         ...........................................................
Oropsylla labis           ...........................................................
Oropsylla tuberculata     ...........................................................
Aetheca wagneri           ...........................................................
Megabothris sp.1          ...........................................................
Megabothris sp.2          ...........................................................
Ceratophyllid sp.         ...........................................................
Catallagia sp.            ..........................................................A..........
Neopsylla inopina         ...........................................................
Rhadinopsylla sp.         ...........................................................

                          850       860       870       880       890       900
                           |         |         |         |         |         |
Oropsylla rupestris       TCGGTTCTATTTTGTTGGTTTTCGGAACTCCGAGGTAATGATTAATAGGGACAACTGGGG
Oropsylla bruneri         ...........................................................
Oropsylla labis           ...........................................................
Oropsylla tuberculata     ...........................................................
Aetheca wagneri           ...........................................................
Megabothris sp.1          ...........................................................
Megabothris sp.2          ...........................................................
Ceratophyllid sp.         ...........................................................
Catallagia sp.            ...........................................................
Neopsylla inopina         ...........................................................
Rhadinopsylla sp.         ...........................................................

                          910       920       930       940       950       960
                           |         |         |         |         |         |
Oropsylla rupestris       GCATTCGTATTGCGACGTTAGAGGTGAAATTCTTGGATCGTCGCAAGACGGACAGAAGCG
Oropsylla bruneri         ...........................................................
Oropsylla labis           ...........................................................
Oropsylla tuberculata     ...........................................................
Aetheca wagneri           ...........................................................
Megabothris sp.1          ...........................................................
Megabothris sp.2          ...........................................................
Ceratophyllid sp.         ...........................................................
Catallagia sp.            ..........................................................T......
Neopsylla inopina         ...........................................................
Rhadinopsylla sp.         ...........................................................
```

Supplemental Figure 3.2 (continued)

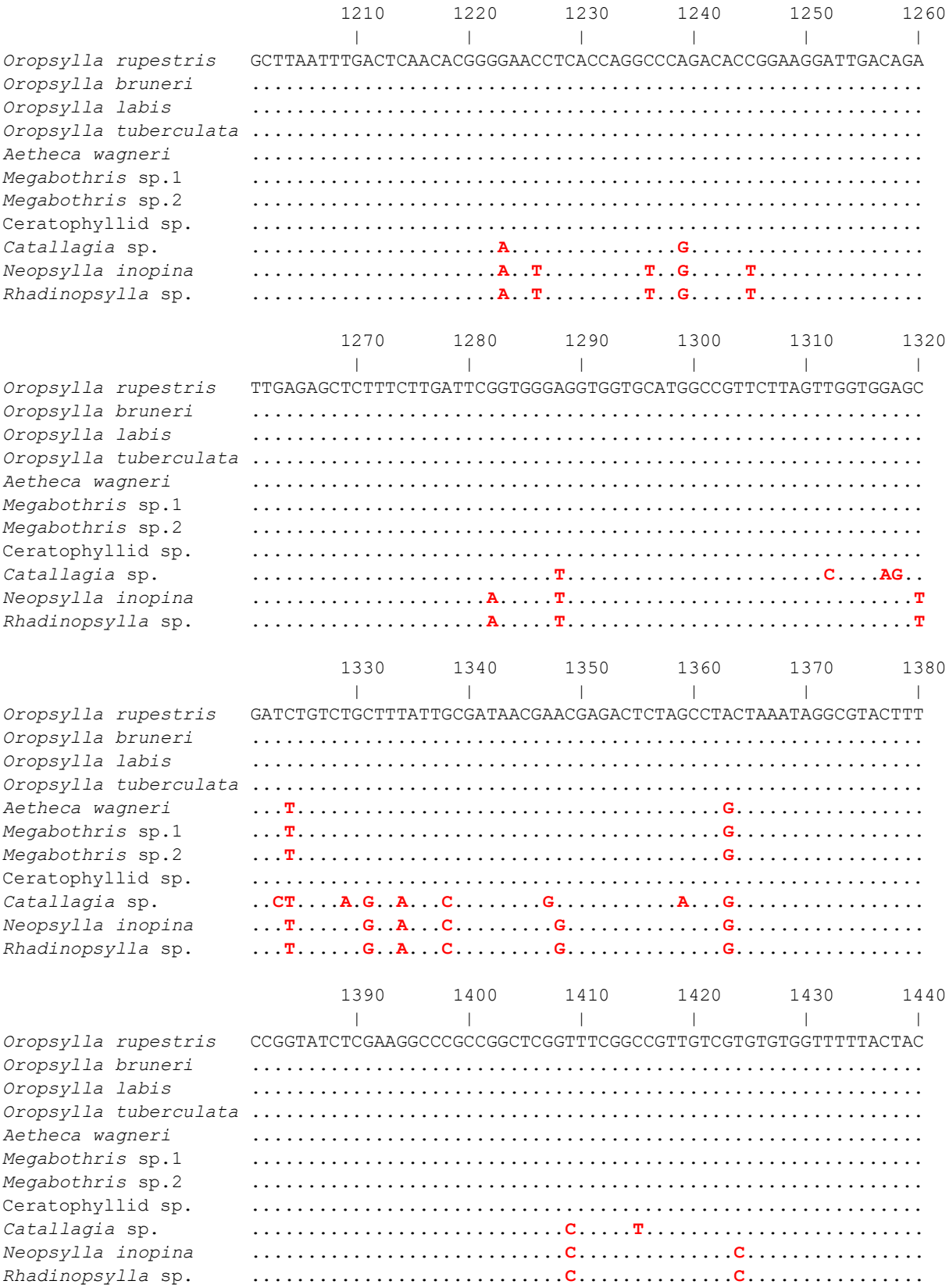
```
                              970       980       990       1000      1010      1020
                               |         |         |         |         |         |
Oropsylla rupestris           AAAGCATTTGCCAAATGTGTTTTCATCAATCAAGAACGAAAGTTAGAGGTTCGAAGGCGA
Oropsylla bruneri             ............................................................
Oropsylla labis               ............................................................
Oropsylla tuberculata         ............................................................
Aetheca wagneri               ............................................................
Megabothris sp.1              ............................................................
Megabothris sp.2              ............................................................
Ceratophyllid sp.             ............................................................
Catallagia sp.                ............................................................
Neopsylla inopina             ............................................................
Rhadinopsylla sp.             ............................................................

                              1030      1040      1050      1060      1070      1080
                               |         |         |         |         |         |
Oropsylla rupestris           TCAGATACCGCCCTAGTTCTAACCATAAACGATGCCAGCTAGCGATCCGCCGAAGTTCCT
Oropsylla bruneri             ............................................................
Oropsylla labis               ............................................................
Oropsylla tuberculata         ............................................................
Aetheca wagneri               ............................................................
Megabothris sp.1              ............................................................
Megabothris sp.2              ............................................................
Ceratophyllid sp.             ............................................................
Catallagia sp.                ............................................................
Neopsylla inopina             ............................................................
Rhadinopsylla sp.             ............................................................

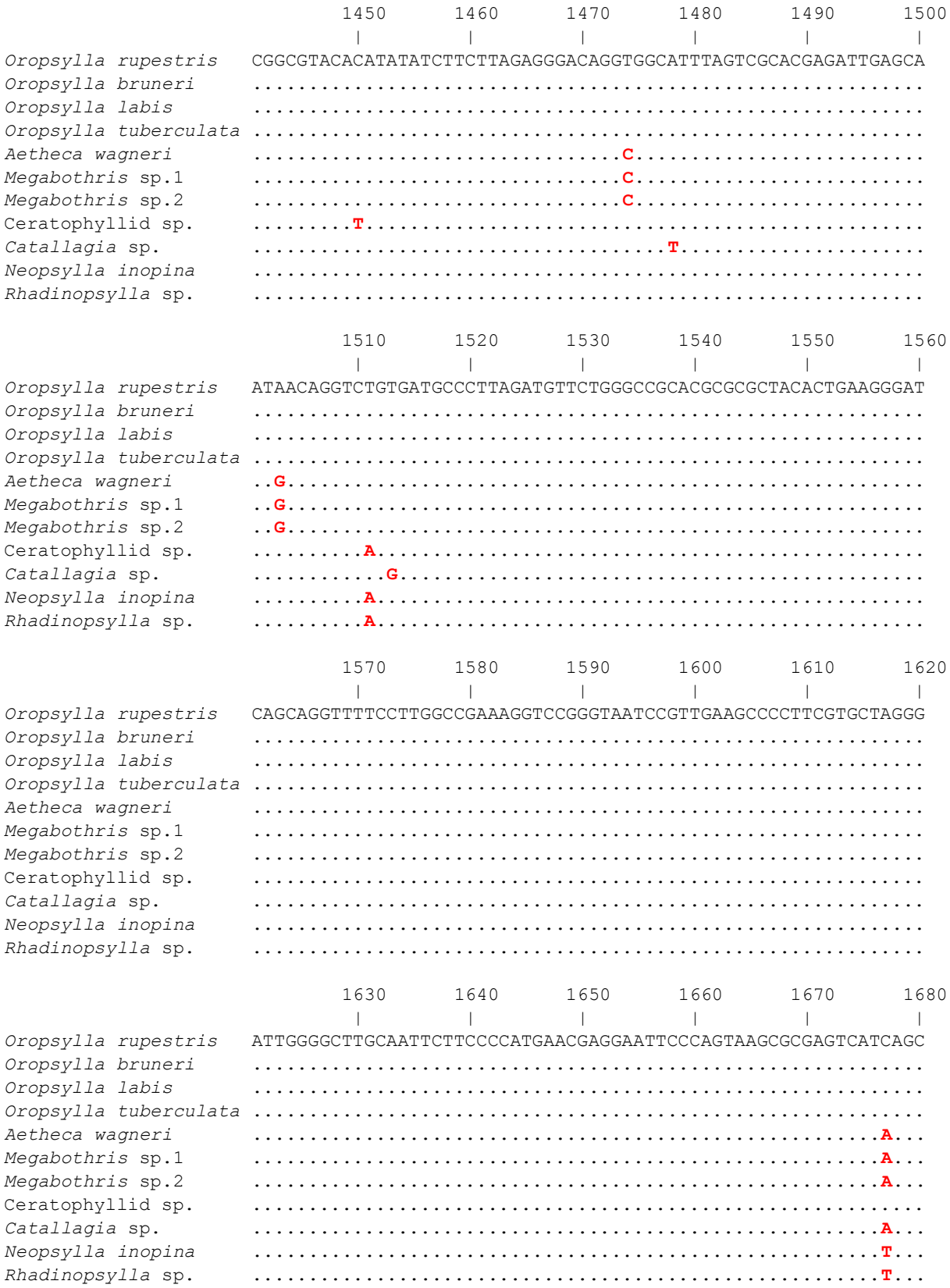
                              1090      1100      1110      1120      1130      1140
                               |         |         |         |         |         |
Oropsylla rupestris           CCGATGACTCGGCGGGCAGCTTCCGGGAAACCAAAGCTTTTGGGTTCCGGGGGAAGTATG
Oropsylla bruneri             ............................................................
Oropsylla labis               ............................................................
Oropsylla tuberculata         ............................................................
Aetheca wagneri               ............................................................
Megabothris sp.1              ............................................................
Megabothris sp.2              ............................................................
Ceratophyllid sp.             ............................................................
Catallagia sp.                ............................................................
Neopsylla inopina             ............................................................
Rhadinopsylla sp.             ............................................................

                              1150      1160      1170      1180      1190      1200
                               |         |         |         |         |         |
Oropsylla rupestris           GTTGCAAAGCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAGCCTGCG
Oropsylla bruneri             ............................................................
Oropsylla labis               ............................................................
Oropsylla tuberculata         ............................................................
Aetheca wagneri               ............................................................
Megabothris sp.1              ............................................................
Megabothris sp.2              ............................................................
Ceratophyllid sp.             ............................................................
Catallagia sp.                ............................................................
Neopsylla inopina             ..........................................A.................
Rhadinopsylla sp.             ..........................................A.................
```

Supplemental Figure 3.2 (continued)



Supplemental Figure 3.2 (continued)



Supplemental Figure 3.2 (continued)

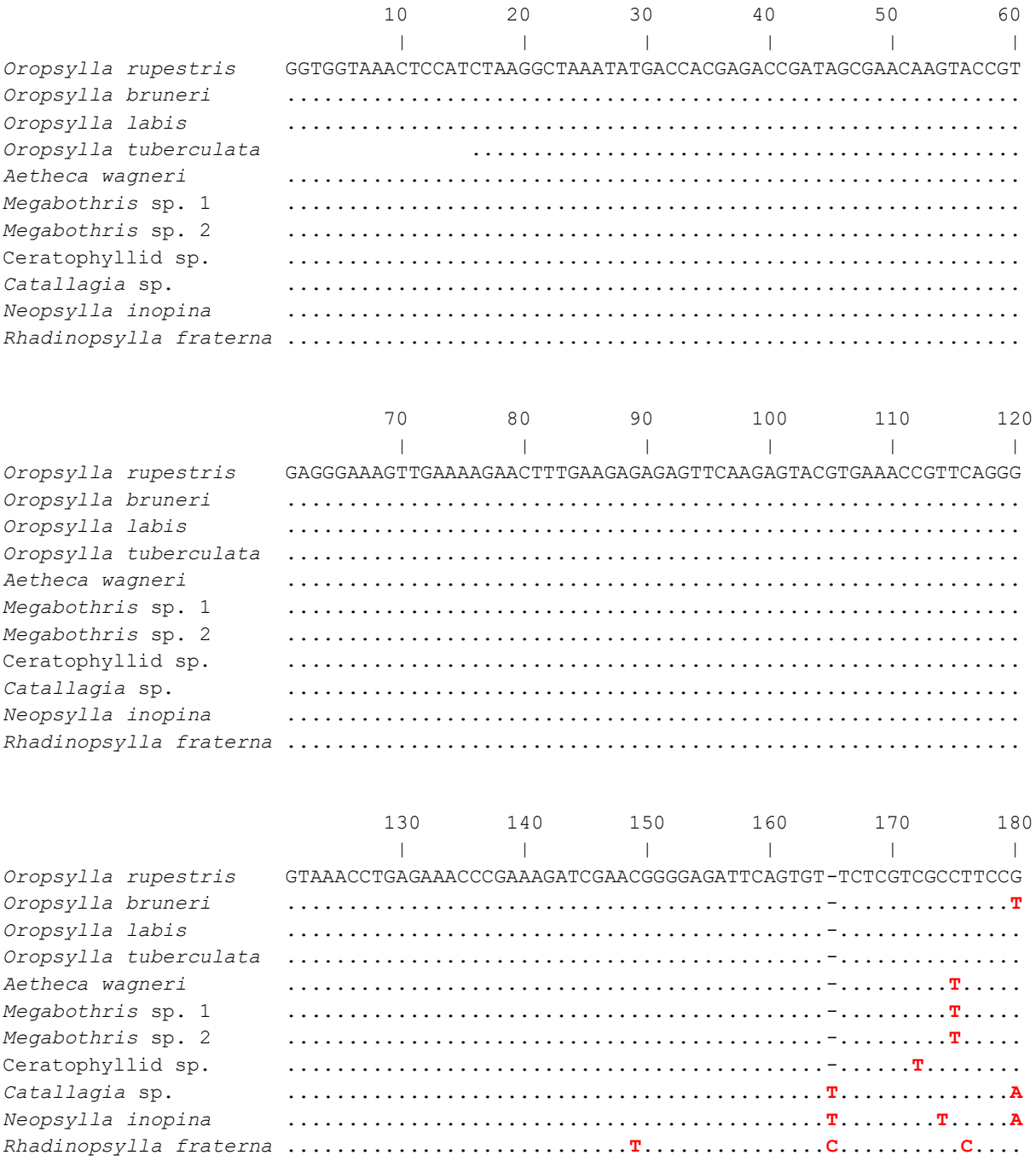
```
                                1690      1700      1710      1720      1730      1740
                                  |         |         |         |         |         |
Oropsylla rupestris             TTGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTACTACCGATTGAGTG
Oropsylla bruneri               ............................................................
Oropsylla labis                 ............................................................
Oropsylla tuberculata           ............................................................
Aetheca wagneri                 .C..........................................................
Megabothris sp.1                .C..........................................................
Megabothris sp.2                .C..........................................................
Ceratophyllid sp.               ............................................................
Catallagia sp.                  ............................................................A.T
Neopsylla inopina               ....................T.......................................A..
Rhadinopsylla sp.               ....................T.......................................A..

                                1750      1760      1770      1780      1790      1800
                                  |         |         |         |         |         |
Oropsylla rupestris             ATTTATTGAGGTCTTCGGACCGGTGCGCGGGGGCGTTTCGACGTTGCCGTTGTTGCTGGG
Oropsylla bruneri               ...........................................A................
Oropsylla labis                 ............................................................
Oropsylla tuberculata           ..........................................A.................
Aetheca wagneri                 ..............................CA..........A......A...........
Megabothris sp.1                ..............................CA..........A......A...........
Megabothris sp.2                ..............................CA..........A......A...........
Ceratophyllid sp.               ............................................................
Catallagia sp.                  .....A........................CA..........A......A...........
Neopsylla inopina               .....G........................CA..........A......A...........
Rhadinopsylla sp.               .....G........................CA..........A......A...........

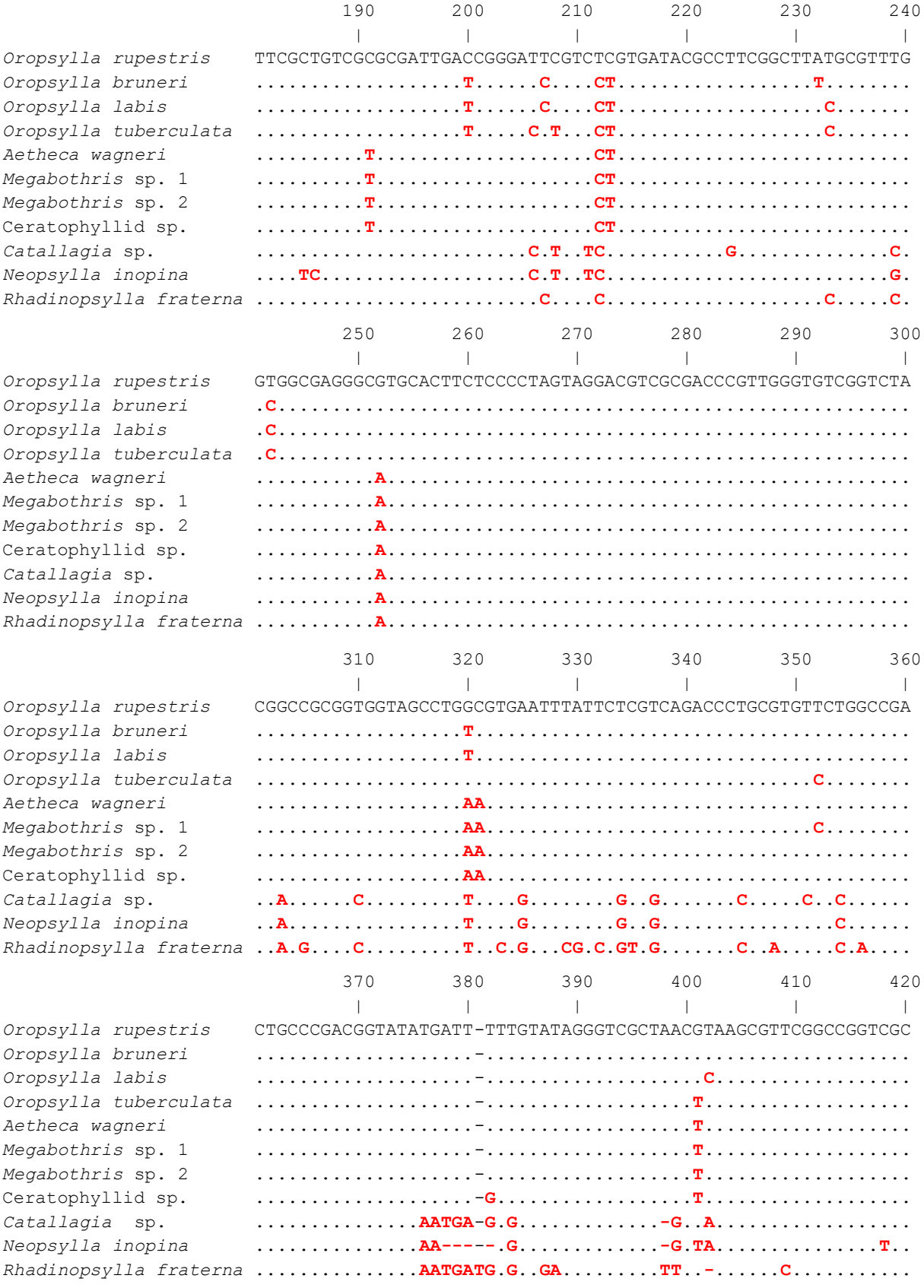
                                1810      1820      1830
                                  |         |         |
Oropsylla rupestris             AAGATGACCAAAATTGATTACTTAGAGGAA
Oropsylla bruneri               ........
Oropsylla labis                 ............................
Oropsylla tuberculata           ........A........AA.
Aetheca wagneri                 ............................
Megabothris sp.1                ............................
Megabothris sp.2                ............................
Ceratophyllid sp.               ............................
Catallagia sp.                  ............T.......-.........
Neopsylla inopina               ............C.......-.........
Rhadinopsylla sp.               ............C.......-.........
```

* No sequence obtained for the first 403 bp of *O. bruneri*.

Supplemental Figure 3.3: Alignment of the nucleotide sequences of the nuclear 28S rRNA gene of the ten flea taxa. Dots represent the identical nucleotide as in the sequence of *Oropsylla rupestris*.



Supplemental Figure 3.3 (continued)



Supplemental Figure 3.3 (continued)

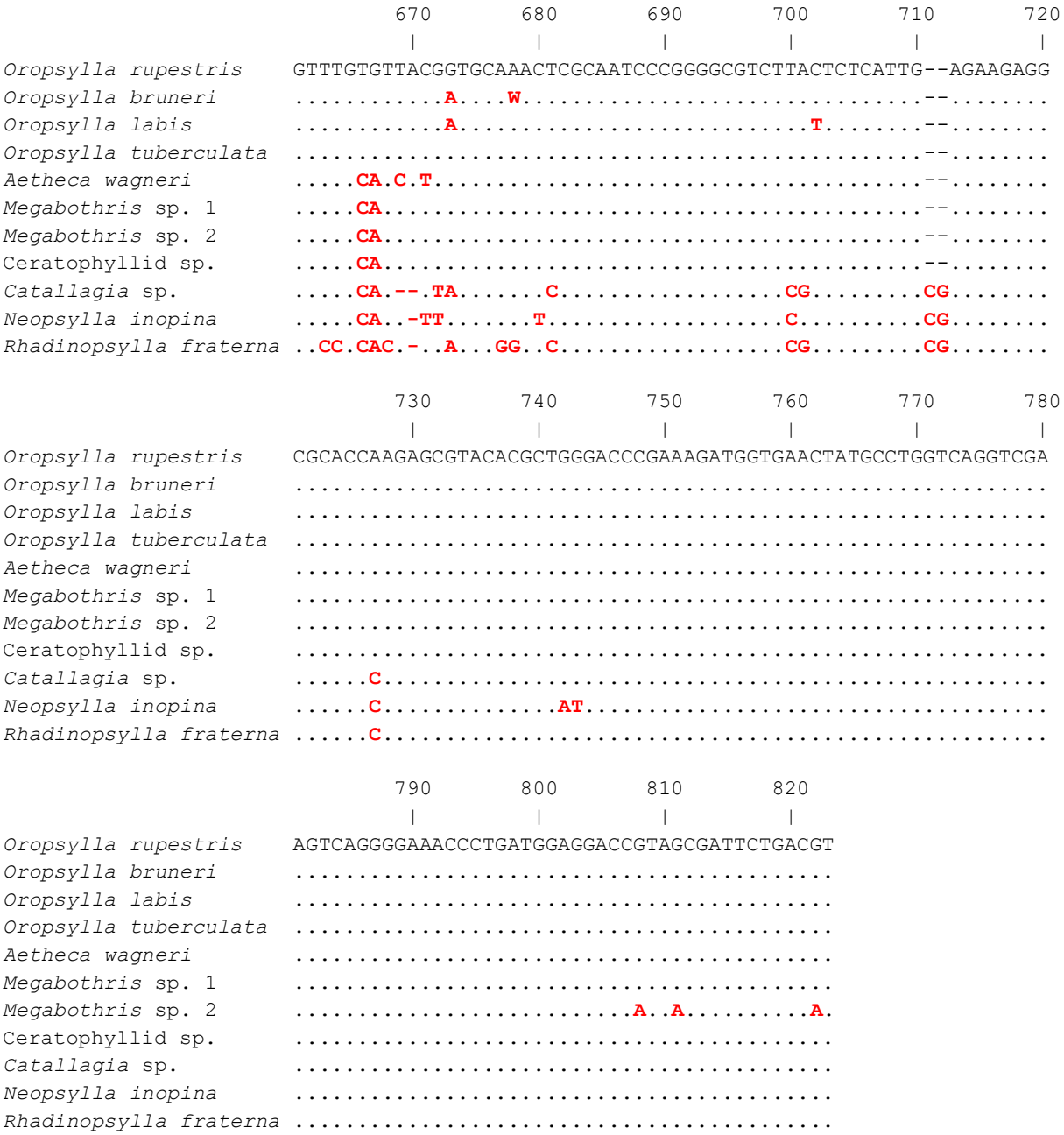
```
                                430       440       450       460       470       480
                                 |         |         |         |         |         |
Oropsylla rupestris             AAGCGTGTGCGGTTCTTTTGGCTGTCGGACCTGGTCCGGCACCGATCC-CGCTCGTGCTG
Oropsylla bruneri               ...........................................-..........
Oropsylla labis                 ...........................................-..........
Oropsylla tuberculata           ...........................................-..........
Aetheca wagneri                 ...........................................-..........
Megabothris sp. 1               ...........................................-..........
Megabothris sp. 2               ...........................................-..........
Ceratophyllid sp.               ...........................................-..........
Catallagia sp.                  .....C.GA.......C.C...C.....................A..CGC..T......
Neopsylla inopina               .....C.GA.......C.C...C.....................A..CGC.........
Rhadinopsylla fraterna          .....C.GA.......C.C...C.....................CGC.........

                                490       500       510       520       530       540
                                 |         |         |         |         |         |
Oropsylla rupestris             TTGGTGGCCGTGTCCTCGGACAGACTCATACAGGTCAGCGATGCTATTGCTTTGGGTACT
Oropsylla bruneri               ............................................................
Oropsylla labis                 ............................................................
Oropsylla tuberculata           ......A.....................................................
Aetheca wagneri                 ......A.....................................................
Megabothris sp. 1               ......A.....................................................
Megabothris sp. 2               ......A.....................................................
Ceratophyllid sp.               ............................................................
Catallagia sp.                  ....C.A...........A.........T...C............................
Neopsylla inopina               ......AT..........A.........C...CA..........................
Rhadinopsylla fraterna          ....C.A.............G.....TC..C..............................

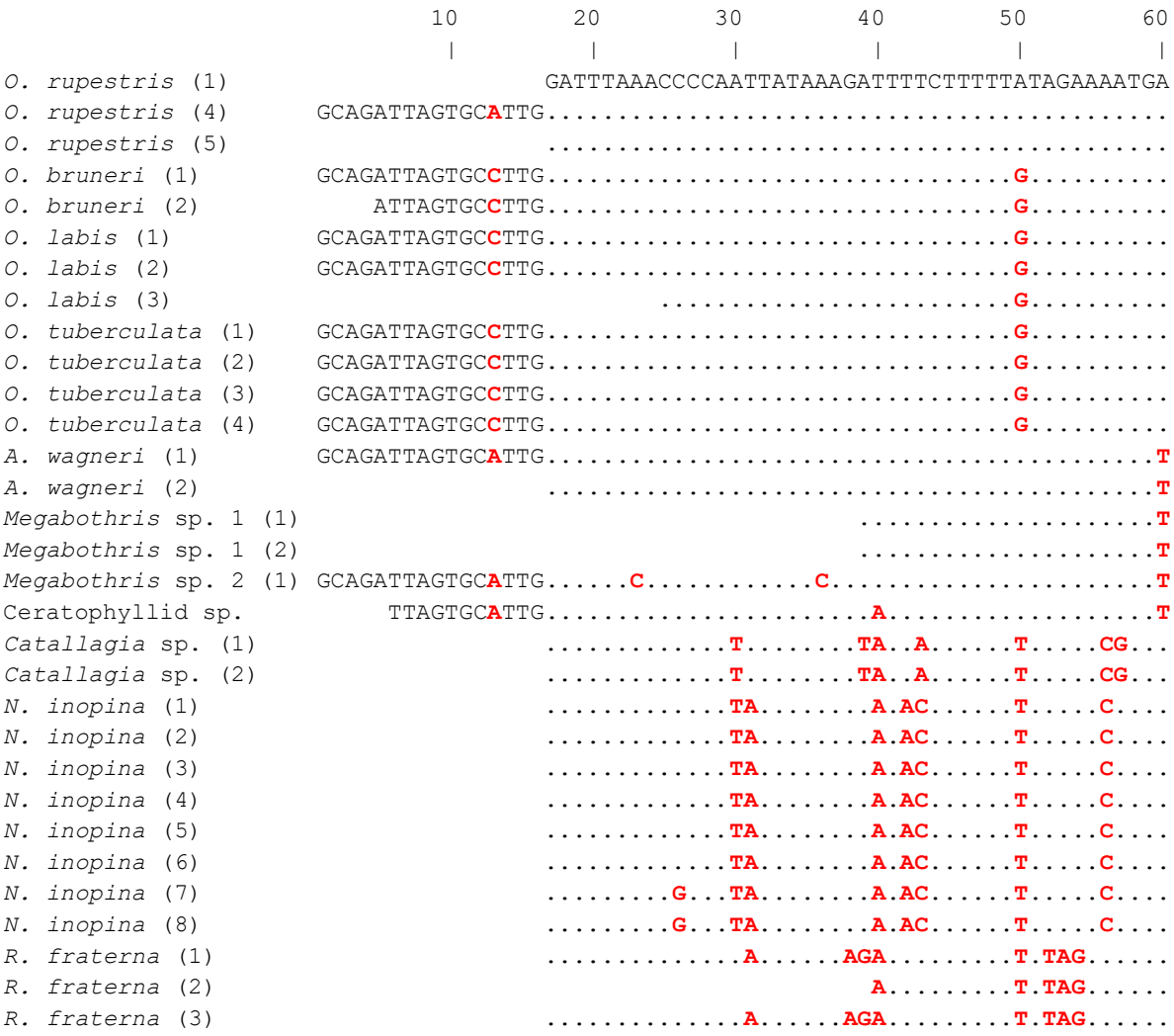
                                550       560       570       580       590       600
                                 |         |         |         |         |         |
Oropsylla rupestris             TTCAGGACCCGTCTTGAAACACGGACCAAGGAGTCTAGCATGTGCGCAAGTCATTGGGAA
Oropsylla bruneri               ............................................................
Oropsylla labis                 ............................................................
Oropsylla tuberculata           ............................................................
Aetheca wagneri                 ............................................................
Megabothris sp. 1               ............................................................
Megabothris sp. 2               ............................................................
Ceratophyllid sp.               ............................................................
Catallagia sp.                  ............................................................
Neopsylla inopina               ............................................................
Rhadinopsylla fraterna          ............................................................

                                610       620       630       640       650       660
                                 |         |         |         |         |         |
Oropsylla rupestris             TTACAAAACCTAAAGGCGTAATGAAAGTGAAGGTTCGCTTATGTGGACTGAGGGATGATG
Oropsylla bruneri               .C.........................................C.............R
Oropsylla labis                 .C.........................................C.............
Oropsylla tuberculata           .C.........................................C.............
Aetheca wagneri                 ...........................................A.............
Megabothris sp. 1               ...........................................A.............
Megabothris sp. 2               ...........................................A...T.........
Ceratophyllid sp.               ..............................A.............................
Catallagia  sp.                 A................C.........A.................................
Neopsylla inopina               C............................................................
Rhadinopsylla fraterna          .C................C.........A...........T..C.................
```

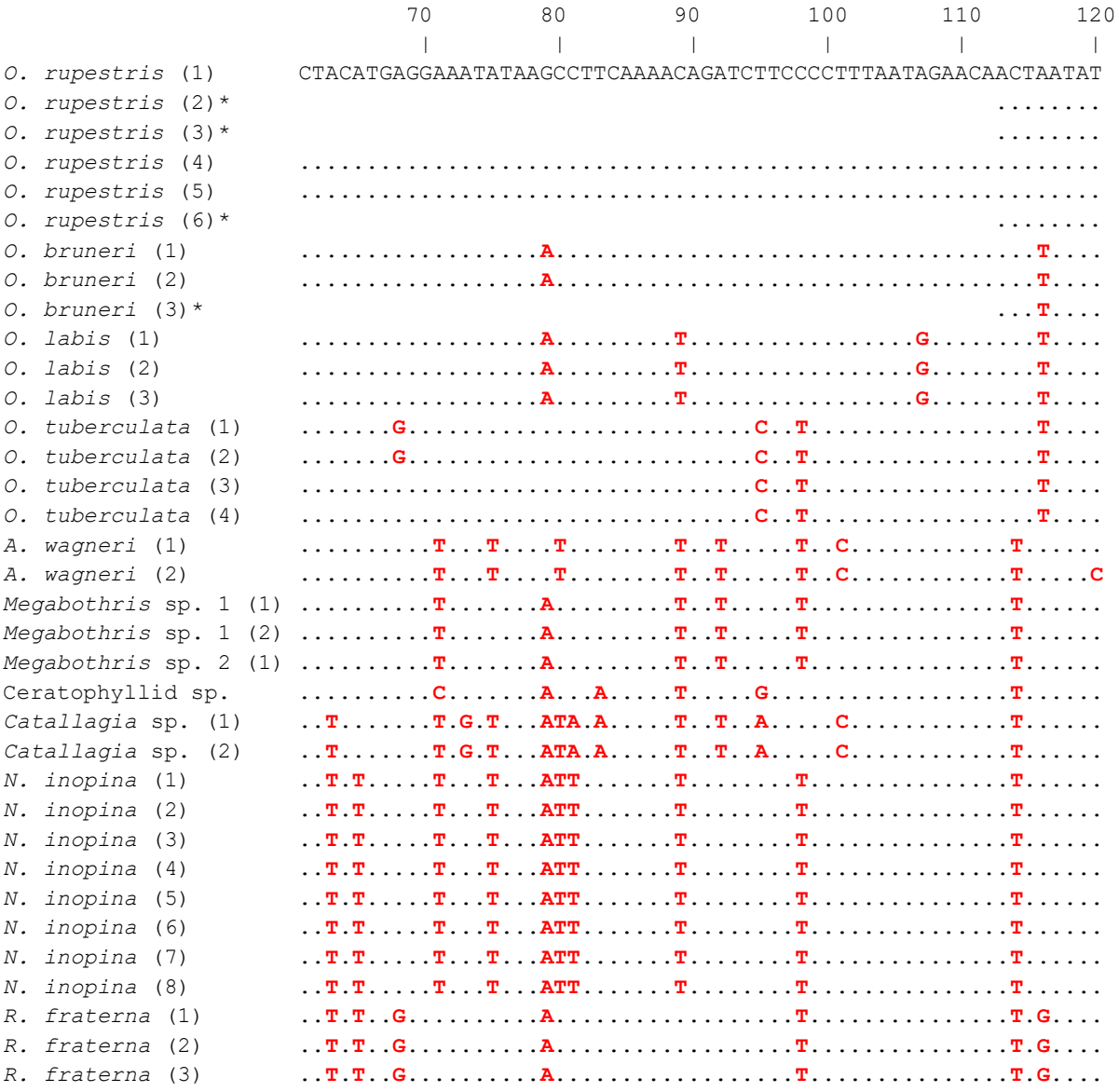
Supplemental Figure 3.3 (continued)



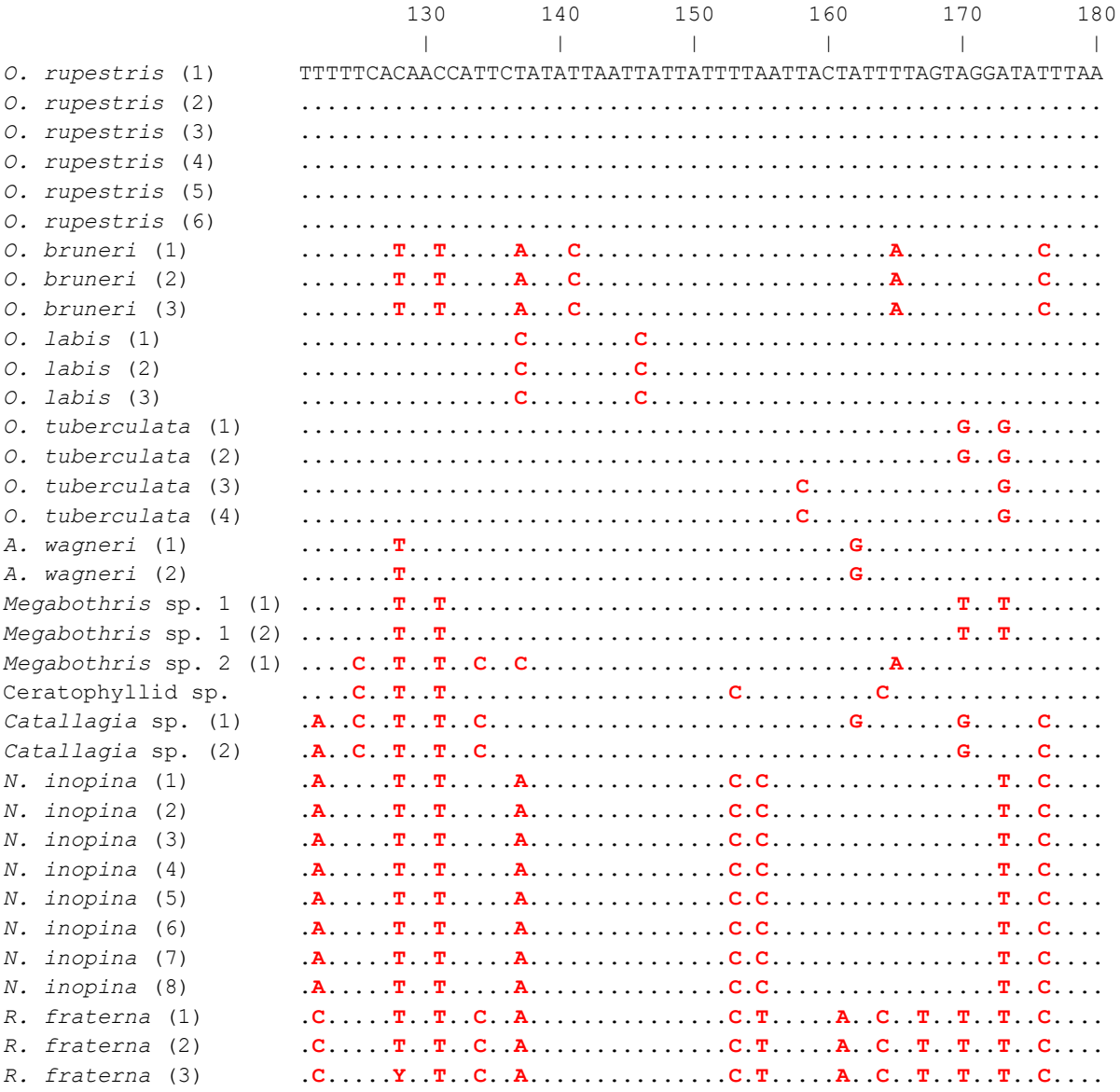
Supplemental Figure 3.4: Alignment of nucleotide sequences of *cox*2 for different haplotypes of the eleven flea taxa. Dots represent the identical nucleotide as in the sequence of *Oropsylla rupestris* haplotype (1).



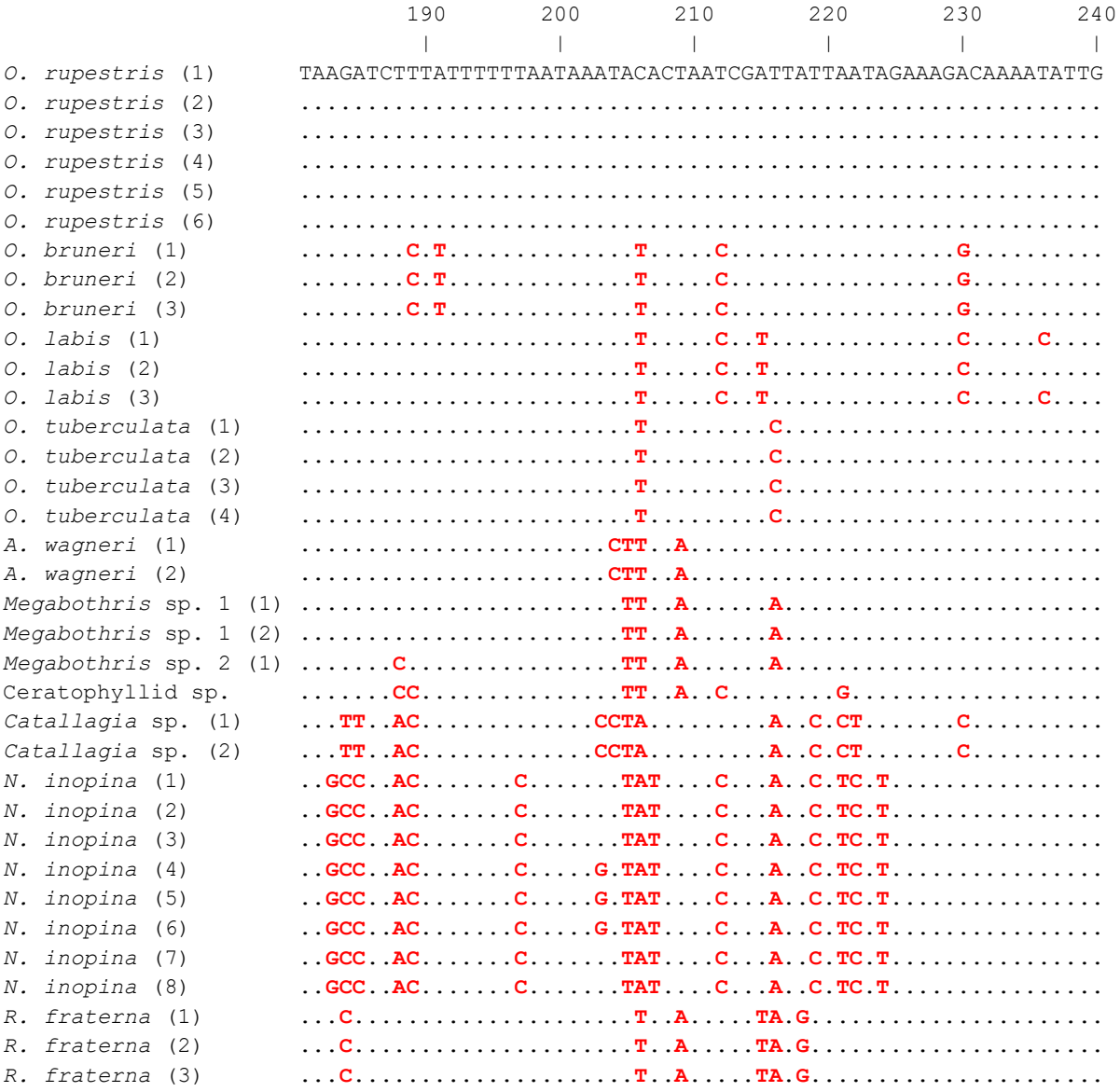
Supplemental Figure 3.4: (continued)



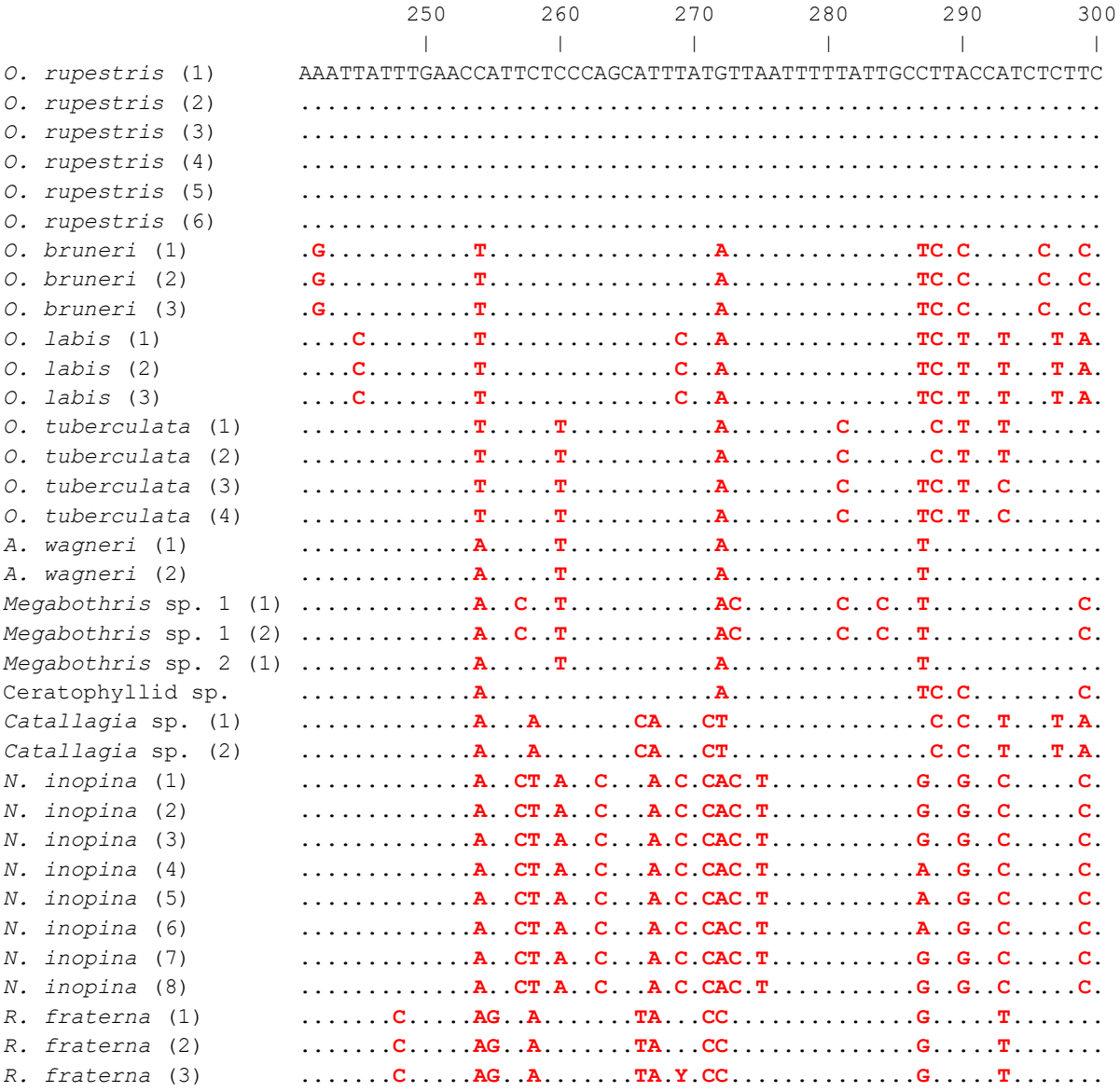
Supplemental Figure 3.4: (continued)



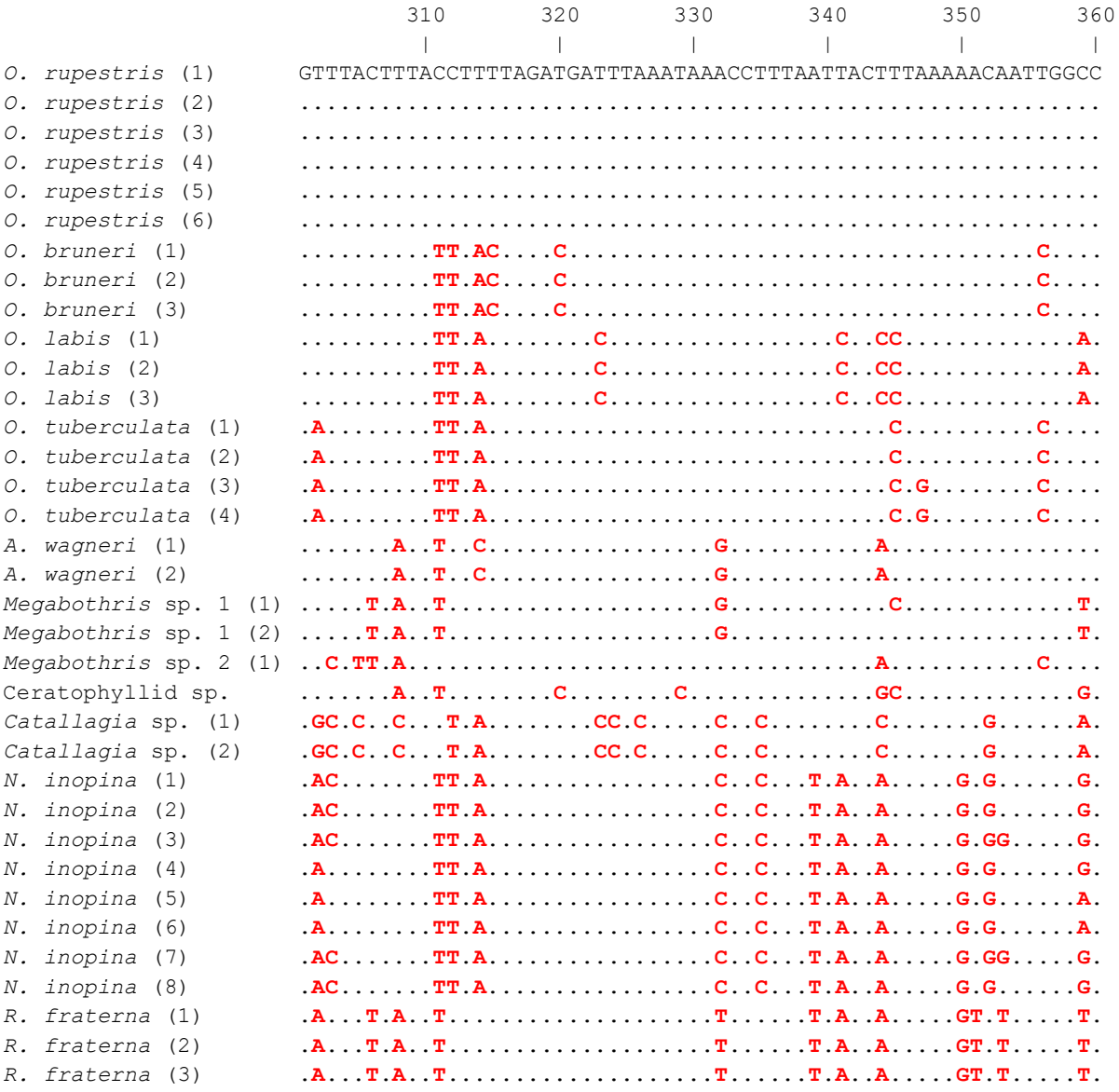
Supplemental Figure 3.4: (continued)



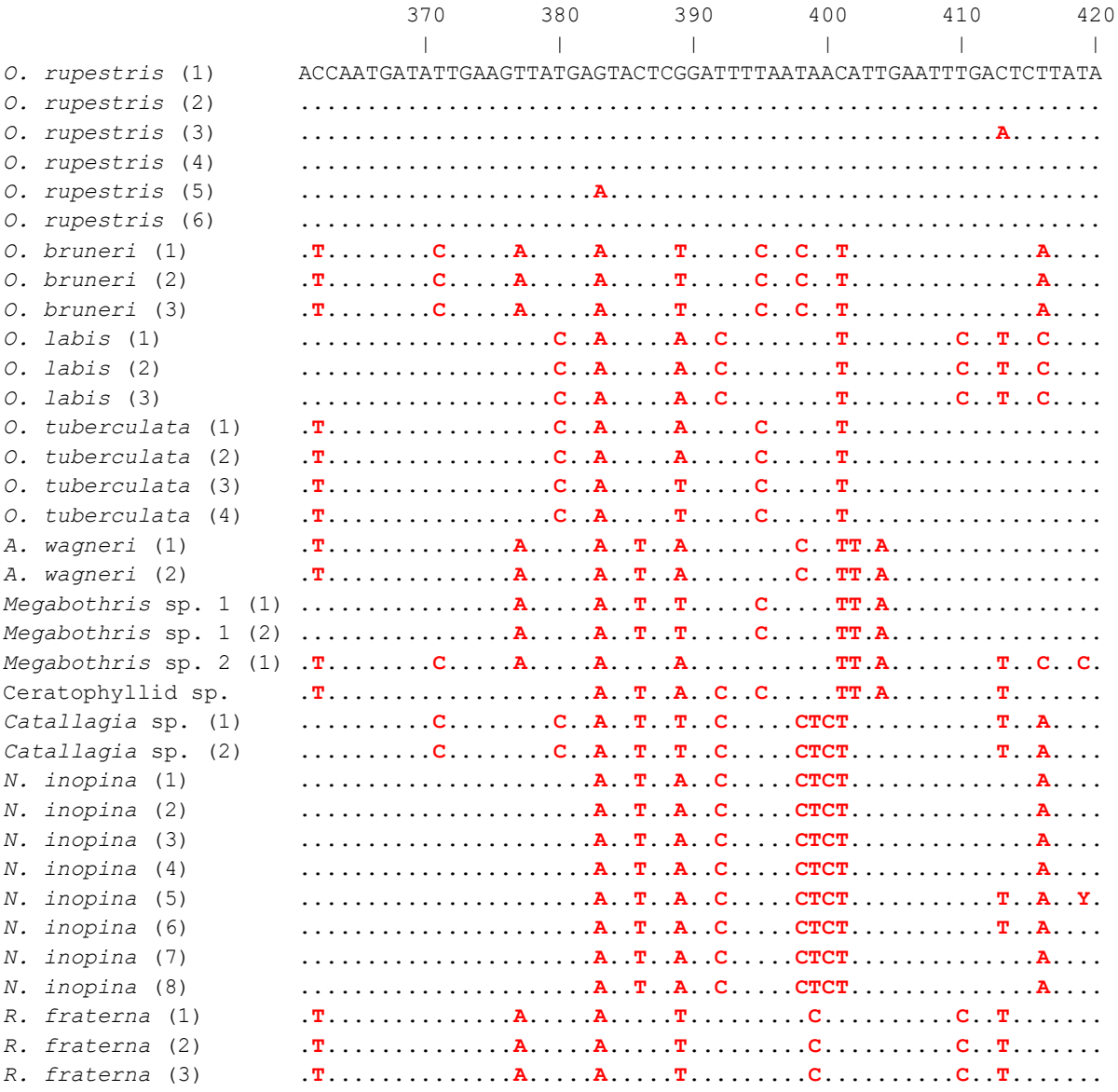
Supplemental Figure 3.4: (continued)



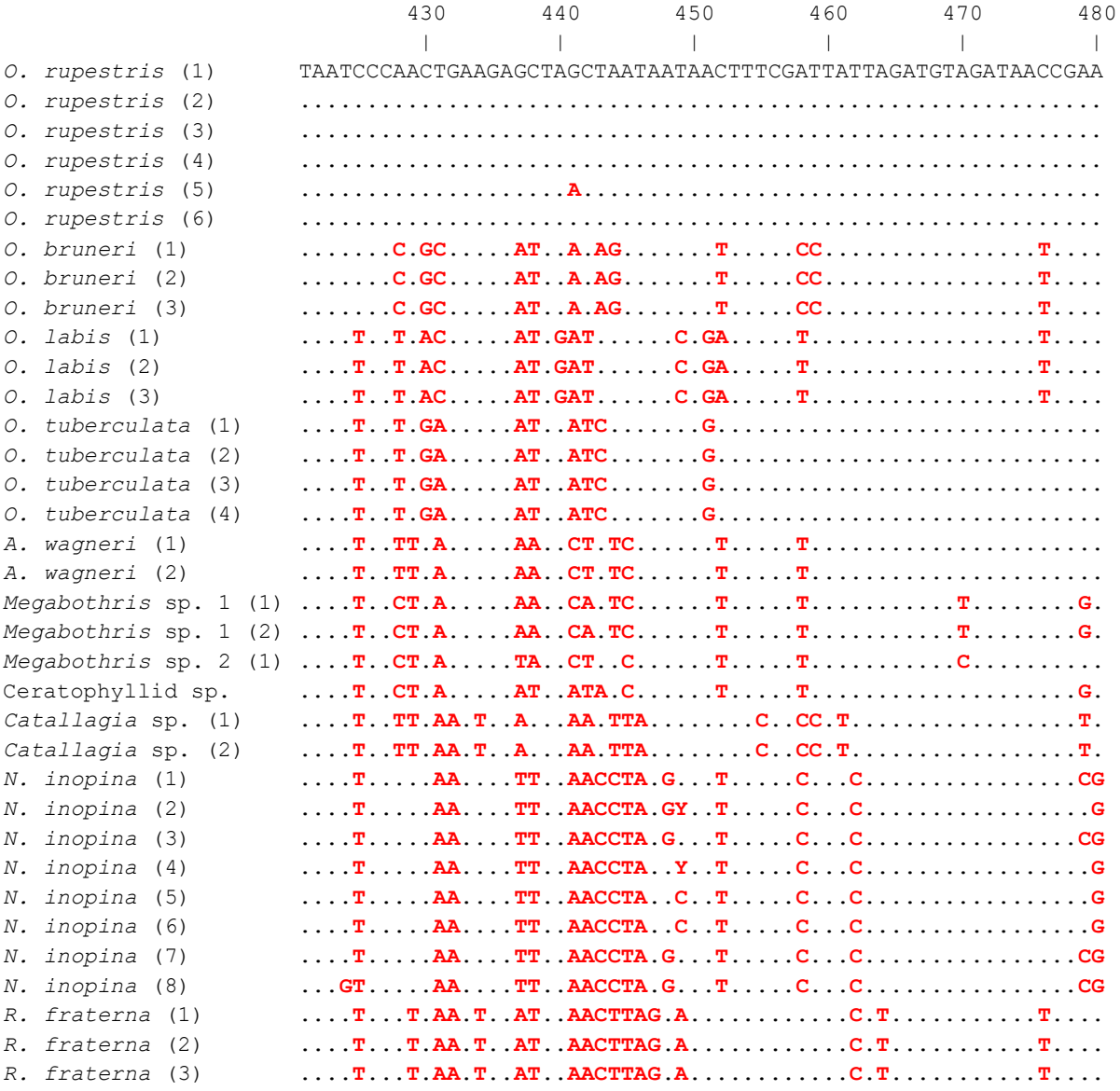
Supplemental Figure 3.4: (continued)



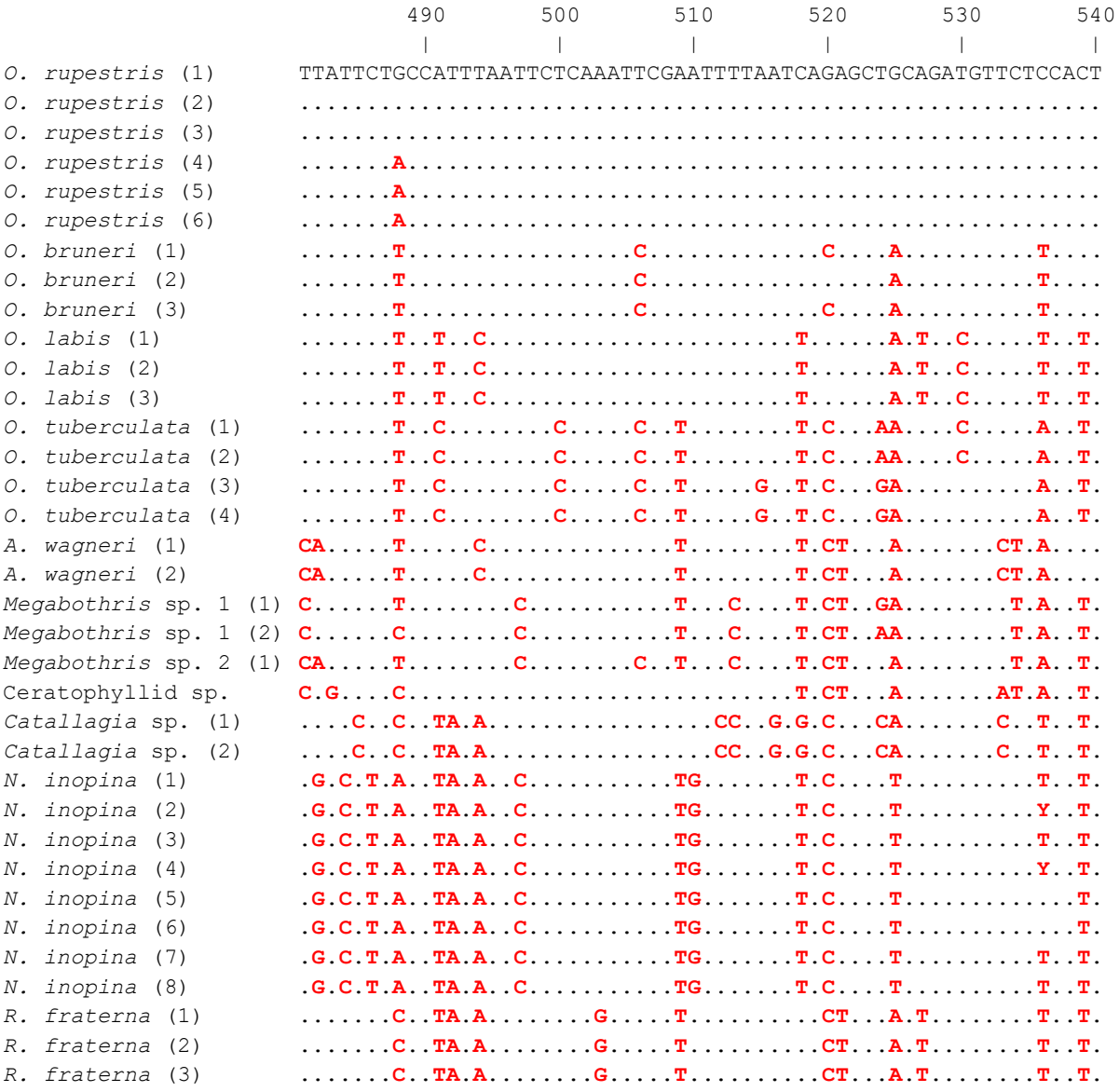
Supplemental Figure 3.4 (continued)



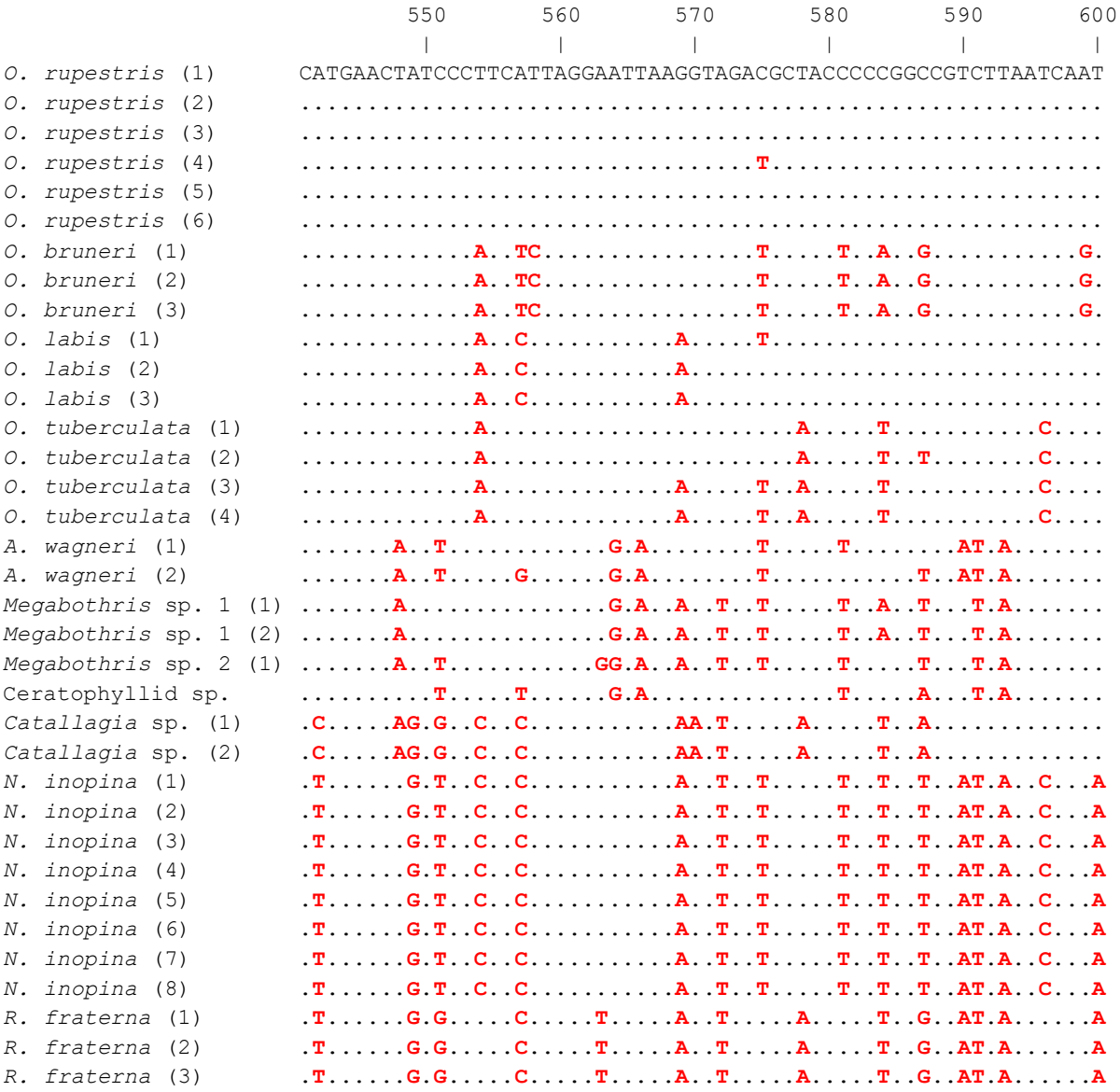
Supplemental Figure 3.4 (continued)



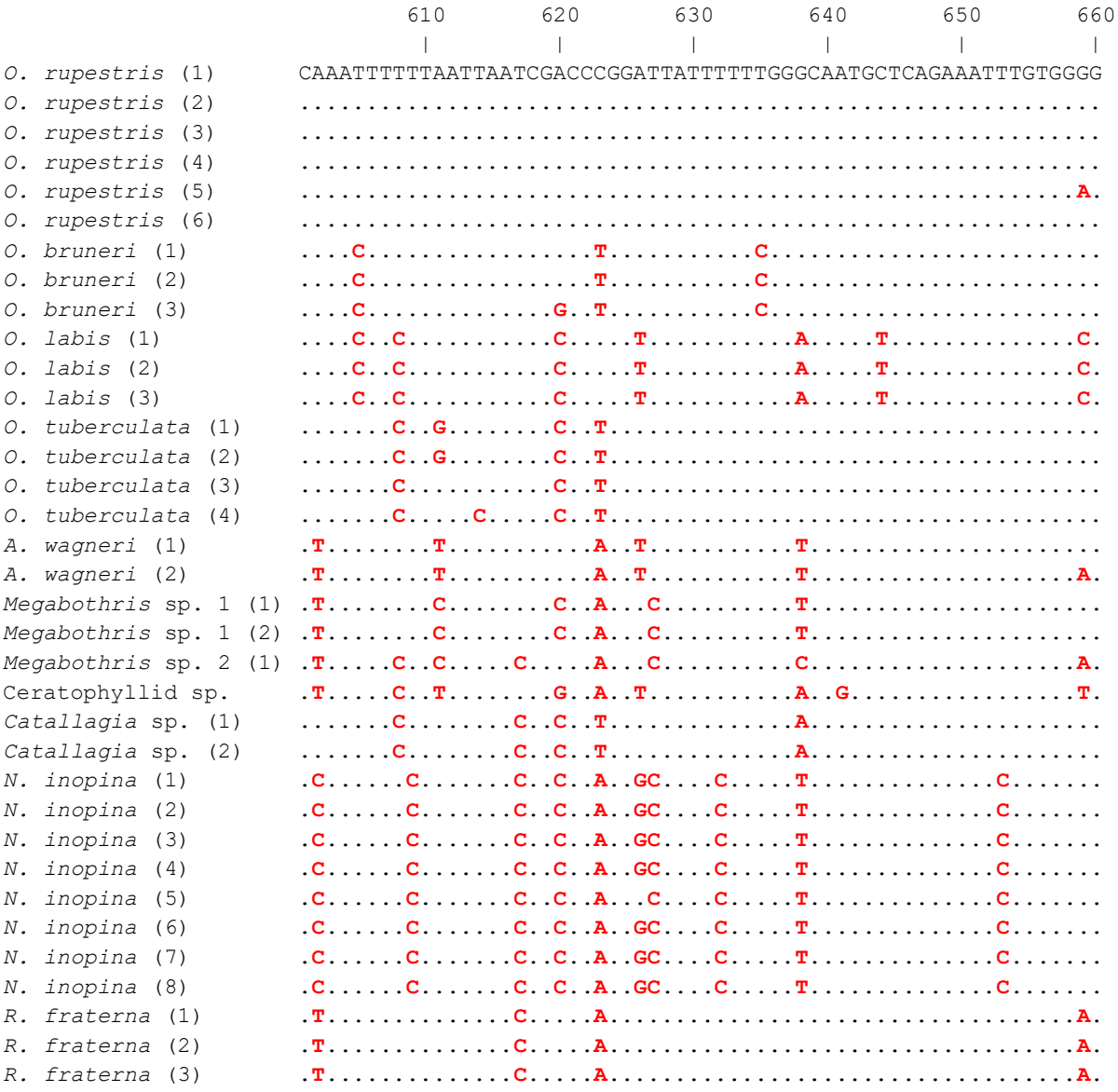
Supplemental Figure 3.4 (continued)



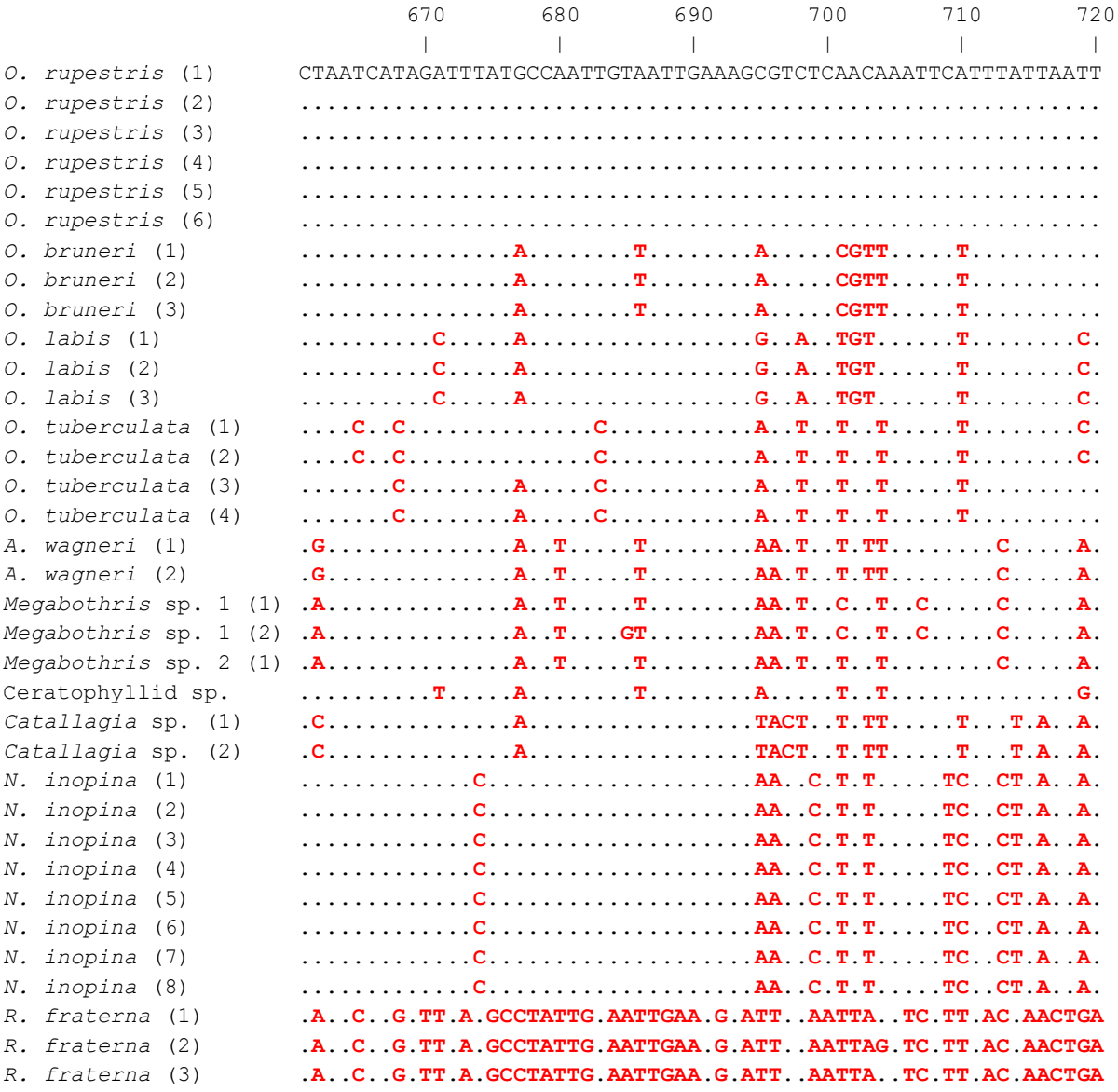
Supplemental Figure 3.4 (continued)



Supplemental Figure 3.4 (continued)



Supplemental Figure 3.4 (continued)



Supplemental Figure 3.4 (continued)

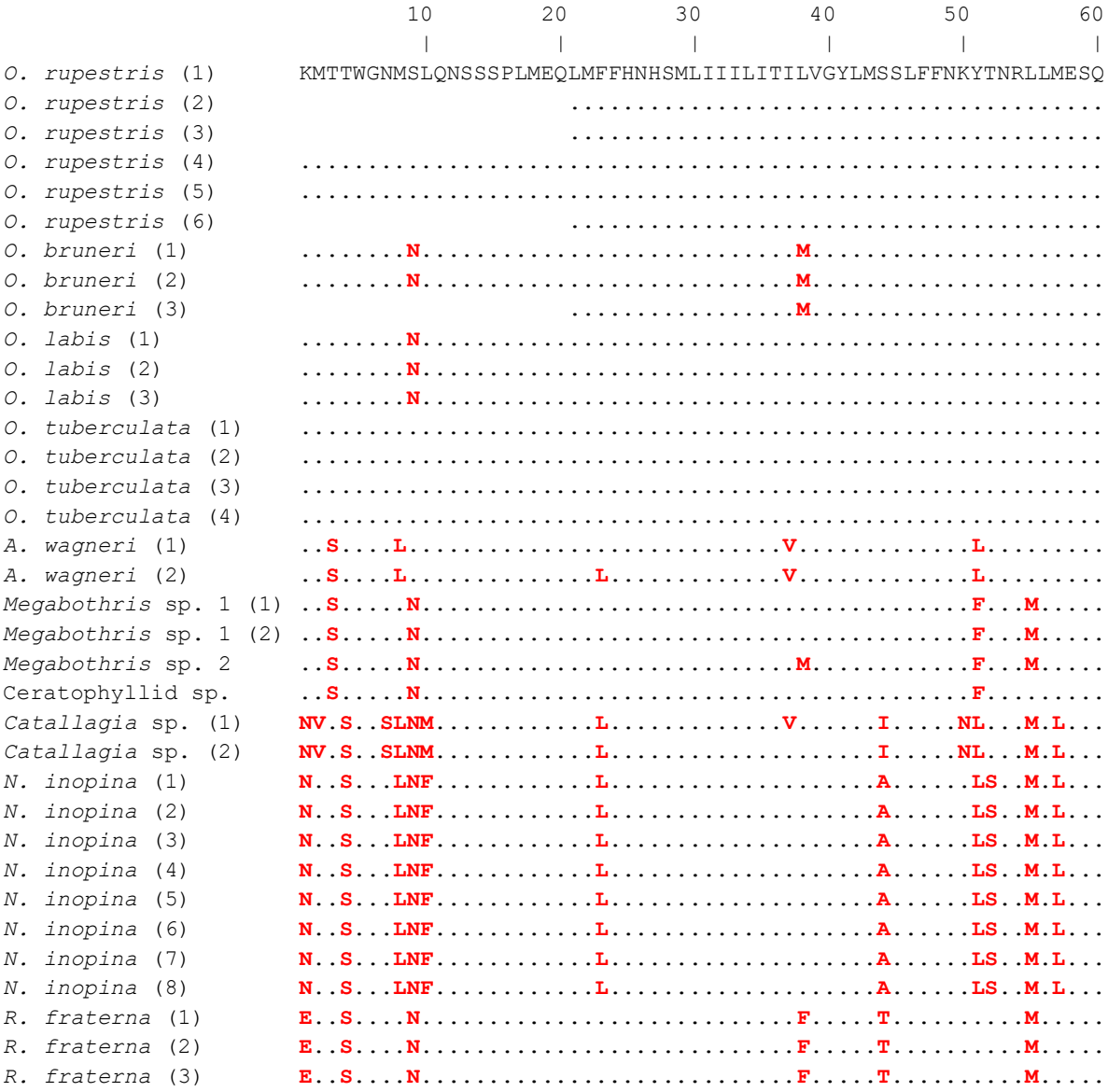
```
                                730       740       750
                                |         |         |
O. rupestris (1)          GAATTTACTCTAACTAATTCATAAGATGACTGAAG
O. rupestris (2)          ...................
O. rupestris (3)          ...................
O. rupestris (4)          ...................................
O. rupestris (5)          ................
O. rupestris (6)          ...................
O. bruneri (1)            .............T......................
O. bruneri (2)            .............T......................
O. bruneri (3)            .............T.....
O. labis (1)              .............T......................
O. labis (2)              .............T......................
O. labis (3)              .............T........G............
O. tuberculata (1)        .....C.T.....T......................
O. tuberculata (2)        .....C.T.....T......................
O. tuberculata (3)        .....C.......T......................
O. tuberculata (4)        .....C.......T
A. wagneri (1)            ......TT.....T......................
A. wagneri (2)            ......TT.....T......................
Megabothris sp. 1 (1)     ......TT.TCT.A.T.A.TCAT.AGATGACTG.AG
Megabothris sp. 1 (2)     ......TT.TCT.A.T.AGGCAT.AGATGACTG
Megabothris sp. 2 (1)     ..G...TT.TCT.A.T.A.TCAT.AGATGACTG.AG
Ceratophyllid sp.         ......TT.....T......................
Catallagia sp. (1)        .....C.......T.....
Catallagia sp. (2)        .....C.......T.....
N. inopina (1)            .......T.....T...........G........A
N. inopina (2)            .......T.....T...........G........A
N. inopina (3)            .......T.....T...........G.......GA
N. inopina (4)            .......T.....T.....
N. inopina (5)            .......T.....T...........G........A
N. inopina (6)            .......T.....T.....
N. inopina (7)            .......T.....T...........G.......GA
N. inopina (8)            .......T.....T...........G.......GA
R. fraterna (1)           ATTAA.T..T.TTAATTC
R. fraterna (2)           ATTAA.T..T.TTAATTC
R. fraterna (3)           ATTAA.T..T.T
```

* Sequence was unavailable for the first 112 bp for *O. rupestris* 2, 3 and 6 as well as *O. bruneri* 3.

For each haplotype, sample size = 1, except for the following:

O. rupestris (1) (n = 2)
O. rupestris (6) (n = 5)
O. bruneri (1) (n = 2)
O. labis (1) (n = 2)
O. tuberculata (1) (n = 3)
O. tuberculata (3) (n = 2)
O. tuberculata (4) (n = 3)
A. wagneri (1) (n = 4)
N. inopina (1) (n = 4)
N. inopina (3) (n = 2)
R. fraterna (1) (n = 2)

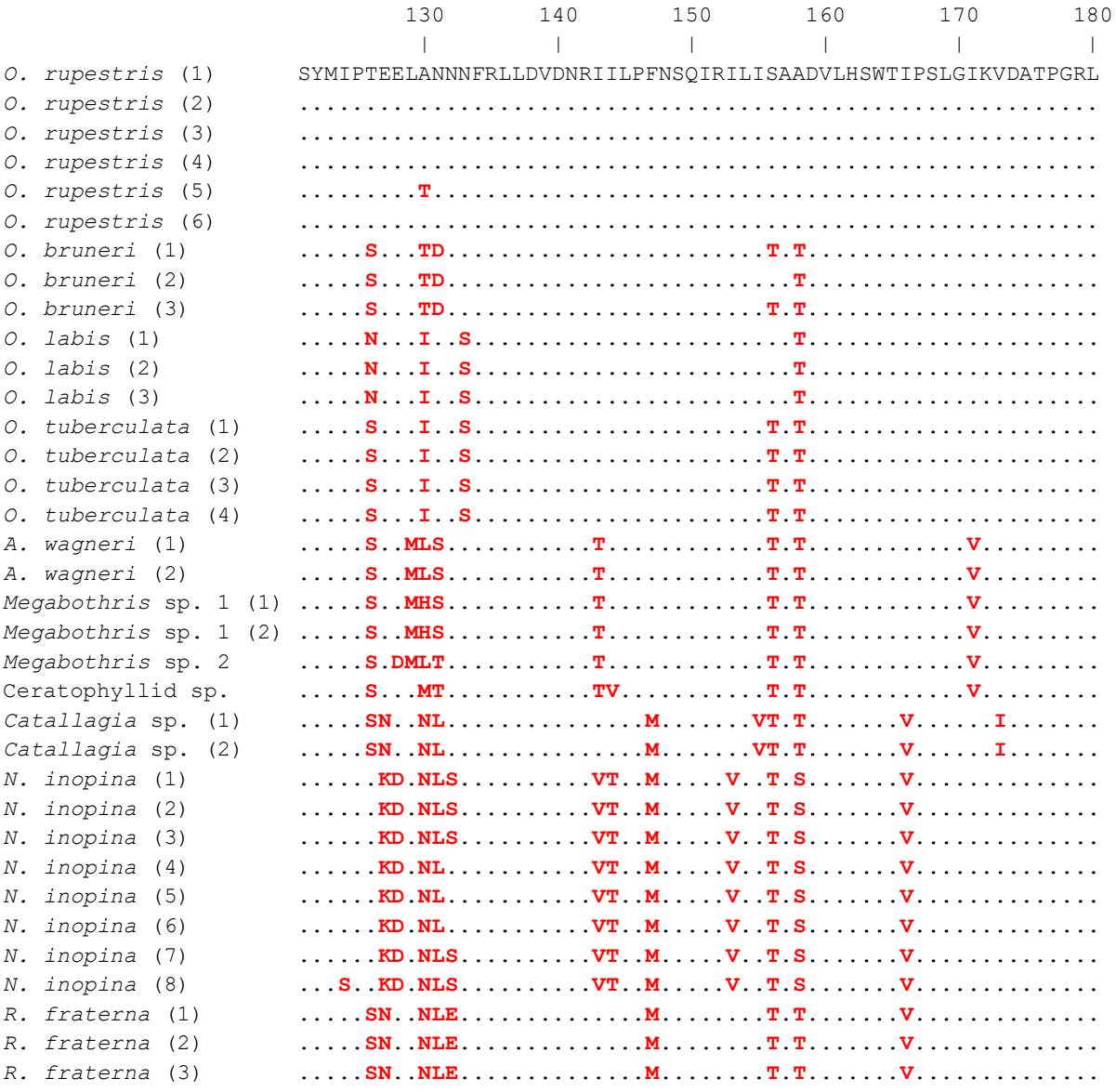
Supplemental Figure 3.5: Amino acid alignment of *cox*2 sequence of all species of fleas.



Supplemental Figure 3.5 (continued)

```
                                  70         80         90        100        110        120
                                   |          |          |          |          |          |
O. rupestris (1)          NIEIIWTILPAFMLIFIALPSLRLLYLLDDLNKPLITLKTIGHQWYWSYEYSDFNNIEFD
O. rupestris (2)           ...........................................................
O. rupestris (3)           ...........................................................E
O. rupestris (4)           ...........................................................
O. rupestris (5)           ...........................................................
O. rupestris (6)           ...........................................................
O. bruneri (1)             ...........................................................
O. bruneri (2)             ...........................................................
O. bruneri (3)             ...........................................................
O. labis (1)               ...........................................................
O. labis (2)               ...........................................................
O. labis (3)               ...........................................................
O. tuberculata (1)         ...........................................................
O. tuberculata (2)         ...........................................................
O. tuberculata (3)         ...........................................................
O. tuberculata (4)         ...........................................................
A. wagneri (1)             ...........................................................L...
A. wagneri (2)             ...........................................................L...
Megabothris sp. 1 (1)      ...........................................................L...
Megabothris sp. 1 (2)      ...........................................................L...
Megabothris sp. 2          ...........................................................L...
Ceratophyllid sp.          ...........................................................L...
Catallagia sp. (1)         ........I..IT.....................N......S..............S....
Catallagia sp. (2)         ........I..IT.....................N......S..............S....
N. inopina (1)             ...........IT.....................N..L...S..............S....
N. inopina (2)             ...........IT.....................N..L...S..............S....
N. inopina (3)             ...........IT.....................N..L...S..............S....
N. inopina (4)             ...........IT.....................N..L...S..............S....
N. inopina (5)             ...........IT.....................N..L...S..............S....
N. inopina (6)             ...........IT.....................N..L...S..............S....
N. inopina (7)             ...........IT.....................N..L...S..............S....
N. inopina (8)             ...........IT.....................N..L...S..............S....
R. fraterna (1)            .......VI..IT.....................N..L...S..............H....
R. fraterna (2)            .......VI..IT.....................N..L...S..............H....
R. fraterna (3)            .......VI..IT.....................N..L...S..............H....
```

Supplemental Figure 3.5 (continued)



Supplemental Figure 3.5 (continued)

```
                                 190       200       210
                                  |         |         |
O. rupestris (1)          NQSNFLINRPGLFFGQCSEICGANHSFMPIVIESVS
O. rupestris (2)          ....................................
O. rupestris (3)          ....................................
O. rupestris (4)          ....................................
O. rupestris (5)          ....................................
O. rupestris (6)          ....................................
O. bruneri (1)            ....................................
O. bruneri (2)            ....................................
O. bruneri (3)            ....................................
O. labis (1)              ....................................
O. labis (2)              ....................................
O. labis (3)              ....................................
O. tuberculata (1)        ....................................
O. tuberculata (2)        ....................................
O. tuberculata (3)        ....................................
O. tuberculata (4)        ....................................
A. wagneri (1)            .....F............................I.
A. wagneri (2)            .....F............................I.
Megabothris sp. 1 (1)     .....F............................I.
Megabothris sp. 1 (2)     .....F.........................G...I.
Megabothris sp. 2         .....F............................I.
Ceratophyllid sp.         .....F.............................
Catallagia sp. (1)        ....................................T.
Catallagia sp. (2)        ....................................T.
N. inopina (1)            ..T................................IP
N. inopina (2)            ..T................................IP
N. inopina (3)            ..T................................IP
N. inopina (4)            ..T................................IP
N. inopina (5)            ..T................................IP
N. inopina (6)            ..T................................IP
N. inopina (7)            ..T................................IP
N. inopina (8)            ..T................................IP
R. fraterna (1)           ..T.....................QIYAYCNWKNFN
R. fraterna (2)           ..T.....................QIYAYCNWKNFN
R. fraterna (3)           ..T.....................QIYAYCNWKNFN
```

For each haplotype, sample size = 1, except for the following:
O. rupestris (1) (n = 2)
O. rupestris (6) (n = 5)
O. bruneri (1) (n = 2)
O. labis (1) (n = 2)
O. tuberculata (1) (n = 3)
O. tuberculata (3) (n = 2)
O. tuberculata (4) (n = 3)
A. wagneri (1) (n = 4)
N. inopina (1) (n = 4)
N. inopina (3) (n = 2)
R. fraterna (1) (n = 2)

3.7 TRANSITION STATEMENT

In Chapter 3, I demonstrated that eleven species of flea that parasitize Richardson's ground squirrels could be distinguished from one another based on the DNA sequences of three markers, the ITS2, the 28S rRNA gene and *cox2*. Another gene, *cox*1, also has potential for species delineation but there are issues with host contamination when using the universal primers for the amplification of this gene. Therefore, fleas in studies reported in the next three chapters will first be identified using sequences of their 28S rRNA gene and/or the ITS2. The objective of the next chapter (Chapter 4) is to determine the diversity and relative abundance of fleas parasitizing Richardson's ground squirrels collected in Alberta and Saskatchewan from 2017 to 2019.

CHAPTER 4: HOST CHARACTERISTICS INFLUENCE FLEA ABUNDANCE ON RICHARDSON'S GROUND SQUIRRELS, *Urocitellus richardsonii*[3]

4.1 ABSTRACT

Rodents are important hosts of a variety of ectoparasites. The number of ectoparasites on a host may be influenced by the sex, age, body weight and/or condition of that host individual. In the present study, we investigated if the relative abundance of fleas (Siphonaptera) on Richardson's ground squirrels (*Urocitellus richardsonii*) was influenced by physical characteristics of host individuals. Ground squirrels were collected from two localities in the Canadian prairies, one in the province of Alberta and the other in Saskatchewan. A total of 579 fleas comprising nine species were collected from 142 (54%) of 264 ground squirrels. The number of fleas parasitizing hosts ranged from 1 to 3. The most abundant fleas were *Oropsylla rupestris* (73%), *O. tuberculata* (15%), and *Neopsylla inopina* (6%). The dominant flea species on *U. richardsonii* in Saskatchewan was *O. tuberculata*, while *O. rupestris* was the most abundant species in Alberta. The results of a negative binomial multivariate model showed that flea abundance was influenced by host sex, with males having a higher mean flea abundance than females. The length of the host's body, year and province in which hosts were collected also influenced flea abundance and diversity. We observed monthly variation in the flea abundance on male and female hosts and the diversity of fleas present.

[3] This chapter is in collaboration with D. Baloun, J. Lane, M.J. Voordouw and N.B. Chilton and is expected to be submitted for publication in Journal of Vector Ecology.

4.2 INTRODUCTION

Rodents are hosts to a variety of internal and external parasites that include helminths, fleas, ticks, and lice (Sackett 2018). Historical and ecological factors influence the diversity of parasites that exploit hosts within a population (Poulin 2004). For rodents, parasite abundance depends on well-known host factors such as the sex and age of host (e.g. Soliman et al. 2001, Hillegass et al. 2008, Harrison et al. 2010, Kiffner et al. 2013, Waterman et al. 2014, Bohn et al. 2017). However, other host factors, such as home range size, behaviour, hormone levels, and immune responses, can also influence parasite prevalence and abundance (Krasnov et al. 2012, Waterman et al. 2014, Warburton et al. 2017, Zduniak et al. 2023). These host factors can also contribute to the diversity and abundance of bacteria in the microbiomes of rodents (Weinstein et al. 2021, Bensch et al. 2023). Rodents are also important reservoir hosts for multiple bacterial pathogens that cause diseases in humans, domestic animals, and wildlife (Jardine et al. 2005, Eisen and Gage 2012). For example, the deer mouse (*Peromyscus maniculatus*) is considered to be a reservoir host of *Yersinia pestis* (Salkeld and Stapp 2008, Eisen et al. 2008), the causative agent of plague (Gage and Kosoy 2005, Eisen and Gage 2009). The risk of transmission of *Y. pestis* is dependent on the vector competence of the flea species and their abundance on hosts (Eisen et al. 2008, 2009, Eisen and Gage 2012). For example, the transmission efficiency of *Y. pestis* by the flea *Oropsylla tuberculata* feeding on laboratory mice is three times greater compared to *O. hirsuta* (Wilder et al. 2008). Transmission efficiency, as defined by Wilder et al. (2008), is the probability an infected flea will transmit the bacterium to a naïve host following a single feeding.

Richardson's ground squirrels (*Urocitellus richardsonii*) are semi-social rodents that occur across the northern Great Plains in Canada (i.e., Alberta, Saskatchewan and Manitoba) and

the United States (i.e., Montana, North Dakota, South Dakota, and Minnesota) (Michener and Koeppl 1985, Augustine et al. 2023). This species exhibits sexual dimorphism where adult males have a heavier body weight (300-450 g) upon emergence from hibernation than adult females (250 g) (Michener and Koeppl 1985). Following emergence, female ground squirrels gain weight by feeding on the vegetation of their environment prior to mating (Michener and Koeppl 1985). Juvenile males are also 25% heavier than juvenile females prior to the onset of hibernation (Bintz and Strand 1983, Dolman and Michener 1983, Michener and Koeppl 1985). Richardson's ground squirrels are semi-fossorial and hibernate in burrows that are located 4 to 15 m from the burrow entrance (Howell 1938, Quanstrom 1971, Michener and Koeppl 1985). These hibernation sites are situated approximately 1 to 2 m below the ground surface (Howell 1938, Quanstrom 1971, Michener and Koeppl 1985). In southern Alberta and Saskatchewan, Richardson's ground squirrels are active for 7 months of the year, with half of that time spent above ground (Michener and Koeppl 1985). There are differences between the sexes and age groups in hibernation and activity periods. Adult males begin their hibernation in June or early July, whereas adult females commence hibernation in July or early August (Michener and Koeppl 1985). Hibernation for juvenile females begins in late August or early September, while juvenile males commence their hibernation in September or early October (Michener 1984, Lindsay and Galloway 1997, Jardine et al. 2006a). Emergence from hibernation follows a similar pattern with adult males emerging first, followed by adult females, and then juveniles (Michener and Koeppl 1985, Jardine et al. 2006a).

Richardson's ground squirrels are hosts to a variety of ectoparasites that includes fleas (e.g., *O. rupestris*, *O. tuberculata*, and *Neopsylla inopina*), ticks (e.g., *Dermacentor andersoni* and *D. variabilis*), lice (e.g., *Linognathoides laeviusculus*) and mites (e.g. *Pneumonyssus* spp.,

and *Hirstionyssus occidentalis*) (Hilton and Mahrt 1971, Michener and Koeppl 1985, Waterman et al. 2014, Thoroughgood and Chilton 2024). The intensity of a/the parasite infection is defined as the number of individuals belonging to the same parasite species in an infested host (i.e., hosts with zero parasites are excluded). The mean intensity of fleas on Richardson's ground squirrels in Manitoba depends on the time of year and the flea species present (Lindsay and Galloway 1997). For example, Lindsay and Galloway (1997) found that *O. rupestris*, a flea species commonly found on Richardson's ground squirrels (Holland 1985), had a mean intensity ranging from 6 to 34 fleas per infested host depending on the time of year, while the flea species *Rhadinopsylla fraterna* had a mean intensity ranging from 1-8 fleas per infested host (Lindsay and Galloway 1997). In Manitoba, *O. rupestris* had a mean intensity of fleas at host emergence of 4.6 fleas per host (range = 2.4 to 12.6 fleas per host) (Waterman et al. 2014). In Alberta, *O. rupestris* had a mean intensity of 1.9 fleas per host (range = 1 to 6), while *R. fraterna* had a mean intensity of 1 (Hilton and Mahrt 1971). Several studies have investigated whether host characteristics and/or environmental factors influence the prevalence and abundance of fleas parasitizing Richardson's ground squirrels (Waterman et al. 2014, Jardine et al. 2006a, 2006b). For example, in Manitoba in the spring (March and April 2010), the flea prevalence was greater for female ground squirrels than males immediately following their emergence from hibernation (Waterman et al. 2014). In contrast, a study in Saskatchewan between April and September in 2004 found that male ground squirrels were more likely to be parasitized by fleas than females when month of sampling was not included in the analysis (Jardine et al. 2006b). This study found no effect of host age on flea prevalence (Jardine et al. 2006b). However, another study in Saskatchewan found that adult males were more likely to be infested by fleas when the analysis was adjusted for month (Jardine et al. 2006a). That study also found that flea prevalence on

Richardson's ground squirrels differed among months with prevalence decreasing from April to June followed by an increase until the end of collection in September (Jardine et al. 2006a). Seasonal variation in flea prevalence can occur because of the differences in the activity periods of different flea species (Lindsay and Galloway 1997, Thoroughgood and Chilton 2024). For example, Lindsay and Galloway (1997) found that *O. bruneri*, *O. rupestris*, *N. inopina*, and *R. fraterna* had unique seasonal activity peaks during the time when Richardson's ground squirrels were active above ground. In addition, *O. rupestris* and *N. inopina* did not have overlapping peaks in their mean intensity or prevalence with other flea species (Lindsay and Galloway 1997).

Different host and environmental factors affect the abundance of parasites within a host population. Although previous work has investigated factors associated with flea parasitism on Richardson's ground squirrels, we wanted to further study the composition of the flea community. Previous work has either focused on specific time periods or not identified the species of fleas found. The objective of the present study was to determine if host characteristics (sex and weight), and the month and/or year of sampling influence the abundance of different flea species on Richardson's ground squirrels.

4.3 MATERIALS AND METHODS

4.3.1 SAMPLE COLLECTION

Richardson's ground squirrels were collected by farmers on four properties; one located six kilometers east of Lethbridge (49.695453, -112.844938) in southern Alberta and the others located 25-28 kilometers from Moose Jaw (50.391535, -105.534766) in southern Saskatchewan. Ground squirrels were collected between mid-March and mid-August for both provinces in 2018 and 2019 and only for Saskatchewan in 2017. The collection area on each property consisted of an area with a maximum radius of two kilometers. Data from the three properties near Moose

Jaw were combined for the statistical analyses because the distance separating the properties was 500 meters to 10 kilometers and the seasonal environmental conditions on each property were very similar to one another. Farmers were asked to immediately place dead hosts individually into Ziploc bags and to record the date and location of capture. Hosts were placed in -20°C freezers prior to transportation to the University of Saskatchewan.

Ectoparasites were removed from the body and fur of thawed hosts, and from the Ziploc bags, using fine forceps. Each ectoparasite was placed into an Eppendorf tube containing 70% ethanol. Ticks (n = 13) and mites (n = 7) were collected from hosts, but these ectoparasites were excluded from this study due to their small sample sizes. Fleas were identified based on the DNA sequence of the second internal transcribed spacer (ITS2) of the nuclear ribosomal DNA (Chapter 3) and/or part of the nuclear 28S rRNA gene (Thoroughgood et al. 2021). The sex, weight (g), and length (cm) of each ground squirrel was recorded. Host weight was measured to the nearest gram using an electronic balance. The spinal length of each ground squirrel was measured from the occipital condyles to the caudal vertebrae to the nearest 0.5 cm (Waterman et al. 2014).

4.3.2 STATISTICAL METHODS

The purpose of the statistical analysis was to determine which factors influence the abundance of fleas (i.e., the total number of fleas, irrespective of flea species) on individual Richardson's ground squirrels. The counts of ectoparasites on infested hosts can follow a Poisson distribution but more often a negative binomial distribution when ectoparasites are highly aggregated on some hosts. In the present study, the negative binomial distribution was a better fit

than the Poisson distribution to the flea counts on the Richardson's ground squirrels (Log-likelihood ratio test: $p < 0.0001$).

A generalized linear model (GLM) with a negative binomial distribution (glm; MASS package; R) and a log-link function was used to analyze the flea counts on the Richardson's ground squirrels (Bolker et al. 2009, Zuur et al. 2009). Explanatory factors included host sex, and province of collection (2 levels: Saskatchewan, Alberta), year (3 levels: 2017, 2018, and 2019) and month of capture (6 levels: March, April, May, June, July, August), whereas host weight (grams) was included as a continuous covariate. We defined year and month as categorical factors rather than continuous variables as the temporal relationships are unlikely to be linear. Since there are differences in the seasonal activity patterns between male and female Richardson's ground squirrels (Michener 1977, 1983), an interaction between host sex and month was included in the flea abundance models. As Richardson's ground squirrels display sexual size dimorphism (Michener and Koeppl 1985), we also included models with interactions between host sex and body weight or body length. The relative performance of the candidate models was assessed using an Akaike Information Criterion (AIC)-based approach and the ratio between the residual deviance and degrees of freedom. Models were ranked based on their AIC scores and models with the lowest AIC scores are considered the best models based on the principle of parsimony (i.e., they explain most of the variation in the response variable with the fewest number of parameters). Model-averaged parameter estimates were calculated to determine how the explanatory variables influenced the flea counts on the ground squirrels.

The statistical analyses were done using R (version 4.2.1, R Core Development Team 2022). The glm() function in the MASS package was used to create the GLMs. The mod.sel() and mod.avg() functions in the MuMIn package were used for AIC-based model selection and

for calculating the model-averaged parameter estimates.

4.4 RESULTS

A total of 264 *U. richardsonii* (110 females and 154 males) were collected during this study, of which 142 (54%) were parasitized by fleas (Table 4.1). Of the 125 ground squirrels from Alberta (Fig 4.1), 79% of individuals collected in 2018 (April to July) and 45% of individuals collected in 2019 (April to July) were infested with fleas (Table 4.1 and Fig 4.2). Of the 139 ground squirrels collected in Saskatchewan, 39% of individuals collected in 2017 (March to July with one sample in August), 59% of individuals collected in 2018 (April, June and July), and 45% of individuals collected in 2019 (April to July) were infested with fleas (Table 4.1). The total number of fleas collected was 579, with a mean abundance of 2.2 fleas per host, and a mean intensity of 4.1 fleas per infested host (Table 4.1). The greatest number of fleas were collected from Alberta in 2018. The mean abundance of fleas from this sample was 5.1 fleas per host, with a mean intensity of 6.4 fleas per infested host (Table 4.1). Comparison of the flea abundance over the three-year sampling period in Saskatchewan showed that the *U. richardsonii* collected in 2018 had the highest mean abundance of fleas (2.5 fleas per host). The mean intensity of fleas in 2018 was 4.3 fleas per infested host (Table 4.1).

Nine species of fleas were identified in this study (Table 4.2). We were unable to determine the species identity of 12 fleas from Alberta. *Oropsylla labis* was only found on ground squirrels in Alberta, while *Aetheca wagneri*, *O. bruneri*, and *R. fraterna* were only found on ground squirrels in Saskatchewan. The flea species with the highest total prevalence were *O. rupestris* (73%), *O. tuberculata* (15%), and *N. inopina* (5.5%) (Table 4.2). In Alberta, *O. rupestris* was the dominant species (93%), while *O. tuberculata* (41%) and *O. rupestris* (37%)

were the dominant species in Saskatchewan (Fig 4.3). The relative abundance of the different flea species varied between provinces, except for a species of *Megabothris* where only one individual was collected on a host in each province. Although *N. inopina* and *O. tuberculata* were found in both provinces, only one *N. inopina* and six *O. tuberculata* were found on ground squirrels in Alberta. The prevalence of the two dominant flea species in Saskatchewan varied between years, with *O. rupestris* being more prevalent in 2019, while *O. tuberculata* was the more prevalent species in 2017 and 2018 (Fig 4.4). The highest prevalence of fleas was observed in April, June, and July (Table 4.3).

Prevalence, abundance and intensity of fleas on hosts varied between province of capture and sex of host (Table 4.4). There was no significant difference ($t = -0.051$, $df = 239.88$, $p = 0.96$) in mean body weights (± S.D.) of female (294.9 ± 84.4 g) and male (295.4 ± 87.5 g) ground squirrels. Additionally, there was no significant difference ($t = 0.353$, $df = 234.9$, $p = 0.72$) in mean body lengths (± S.D.) of female (17.8 ± 2.6 cm) and male (17.7 ± 2.6 cm) ground squirrels. Body weight and length are colinear ($r^2 = 0.438$, $F = 204.5$, $p < 2.2e\text{-}16$; Fig 4.5) and therefore are not used in the same model. The negative binomial distribution was a better fit to the flea count data compared to the Poisson distribution (Likelihood ratio test: $c^2 = 679.85$, $p < 2.2\ e\text{-}16$). The ratio of the residual deviance to the residual degrees of freedom was close to 1.0 further indicating that the negative binomial distribution was a good fit to the model. The best model included province, year, month, length, host sex, and the interaction between month and sex (Table 4.5). Only models that included length had support, with the top model having 73% of the support with an interaction between month and sex. Although Richardson's ground squirrels show sexual size dimorphism, the inclusion of an interaction between sex and length was not preferred over the model without interactions (AIC: 923.3 > 923.1; Table 4.5) or in comparison

to the sex:month interaction (AIC: 923.3 > 919.8; Table 4.5). The same result was found after the inclusion of the sex:body weight interaction to the sex:month interaction (AIC: 937.8 > 930.6; Table 4.5) and a model without interactions (AIC: 937.8 > 936.0; Table 4.5) when body weight was included instead of body length.

In addition, log-likelihood ratio tests were used to determine the statistical significance of the explanatory factors for the best model. Host sex ($p = 0.0015$), body length ($p = 0.0003$), sampling year ($p = 0.0001$), province ($p = 0.0402$), and the host sex:month interaction ($p = 0.0164$) had significant effects on flea abundance (Table 4.6). Only month did not significantly affect the abundance of fleas on Richardson's ground squirrels ($p = 0.3600$). Parameter estimates based on the weighted models found that the flea abundance was significantly higher in Alberta and in the year 2018 (Table 4.7). Additionally, longer individuals had a significantly higher abundance of fleas ($p = 0.0039$, Table 4.7), as illustrated in Figure 4.7 ($r^2 = 0.0277$, $F = 7.465$, $p = 0.007$). Based on the estimated marginal means, flea abundance was 1.6 times higher in Alberta compared to Saskatchewan and 1.3 times higher in 2018 than 2017 and 2019 combined (Table 4.8). Males had a flea abundance (0.008 fleas per male) that was 1.6x higher than females (0.005 fleas per female) (Table 4.8). The abundance of fleas on males (2.9 ± 8.6 fleas) was higher that on females (1.15 ± 1.91 fleas) (Fig 4.6). When looking at specific months, females had a flea abundance 2.1 times greater than males in May, while males had a higher flea abundance in the other months (Table 4.8).

Figure 4.1: The number of female (black) and male (grey) Richardson's ground squirrels (*U. richardsonii*) collected from 2017 to 2019 in Alberta and Saskatchewan.

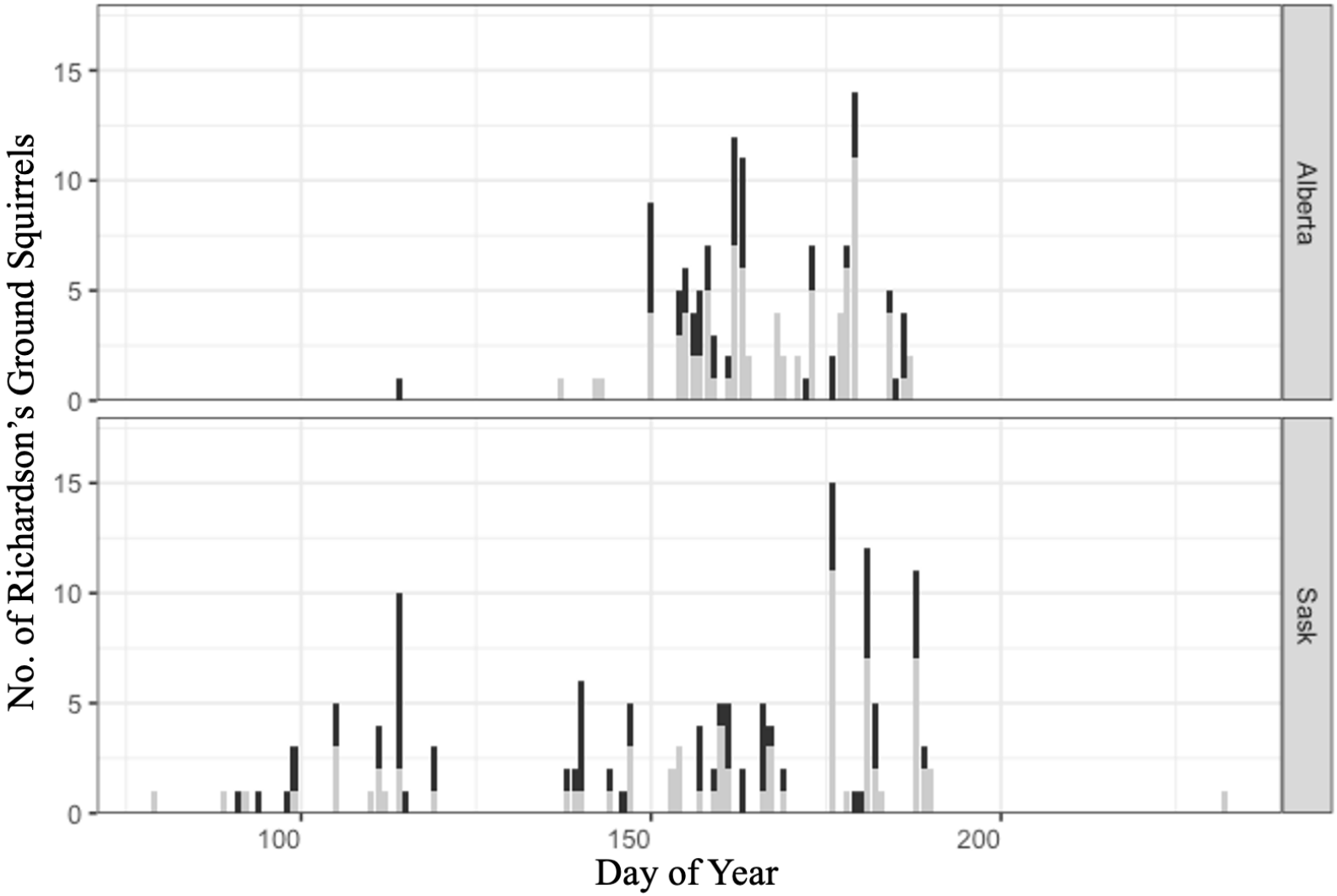


Table 4.1: The number of Richardson's ground squirrels (*U. richardsonii*) collected from Alberta and Saskatchewan (2017–2019) and the proportion infested with fleas, and the mean abundance and mean intensity of fleas.

Location	Year	No. of hosts			No. of infested hosts			Total no. of fleas	Mean abundance of fleas	Mean intensity of fleas
		♀	♂	Total	♀	♂	Total(%)			
Alberta	2018	17	41	58	13	33	46 (79)	296	5.1	6.4
	2019	27	40	67	8	22	30 (45)	79	1.2	2.6
Saskatchewan	2017	21	15	36	7	7	14 (39)	37	1.0	2.6
	2018	20	19	39	11	12	23 (59)	99	2.5	4.3
	2019	25	39	64	10	19	29 (45)	68	1.1	2.3
Total		110	154	264	49	93	142 (54)	579	2.2	4.1

Figure 4.2: Number of female (black) and male (grey) Richardson's ground squirrels (*U. richardsonii*) infested with fleas in different years in Alberta and Saskatchewan.

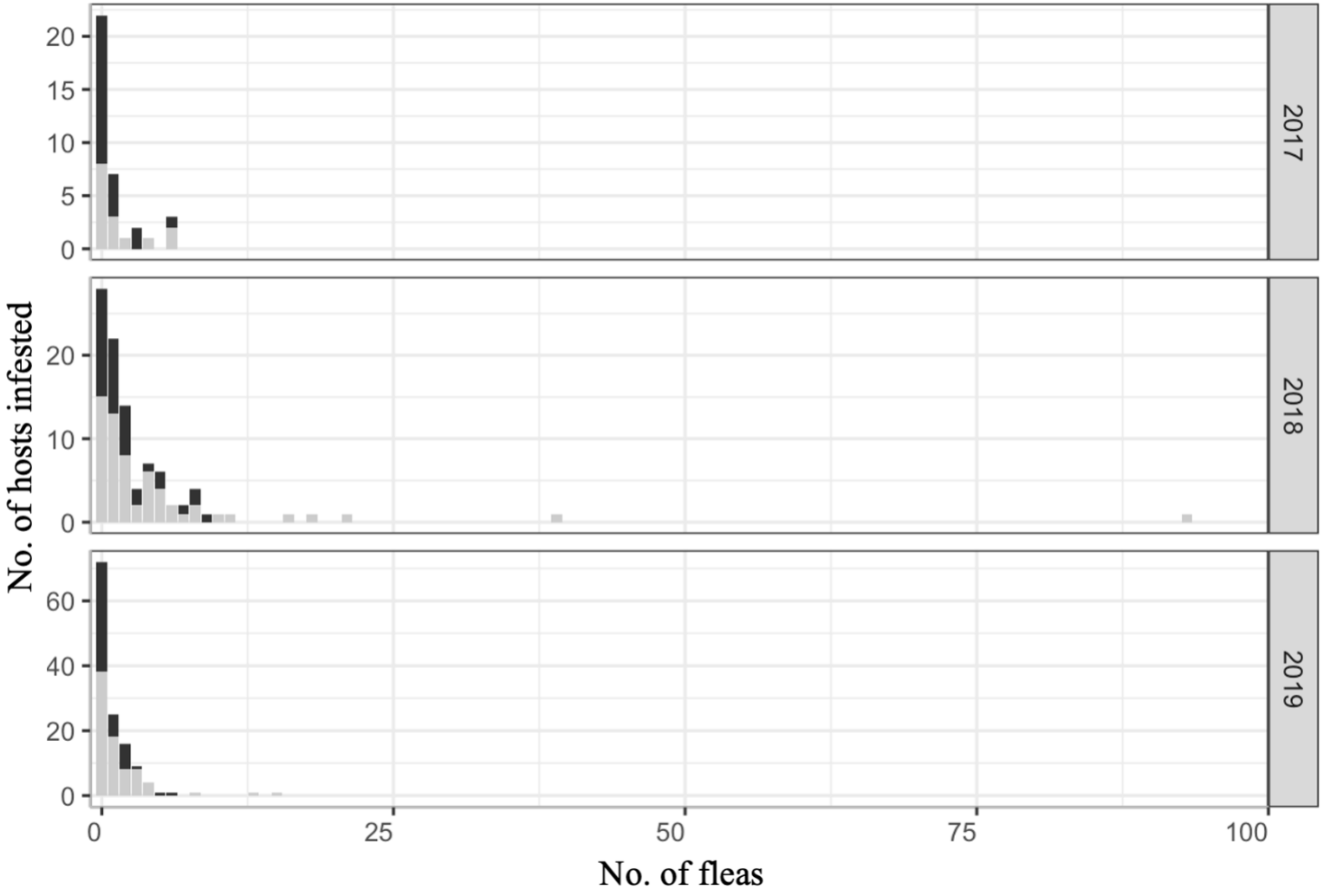


Table 4.2: Number of fleas of each species on Richardson's ground squirrels (*U. richardsonii*) collected in Alberta and Saskatchewan in different years.

Flea species	Alberta		Saskatchewan			
	2018	2019	2017	2018	2019	Total
Oropsylla rupestris	280	69	7	28	40	424
O. bruneri	0	0	1	7	3	11
O. labis	2	4	0	0	0	6
O. tuberculata	2	4	18	52	13	89
Aetheca wagneri	0	0	0	1	0	1
Megabothris sp. 1	0	1	0	0	0	1
Megabothris sp. 2	0	0	0	0	1	1
Neopsylla inopina	1	0	11	10	10	32
Rhadinopsylla fraterna	0	0	0	1	1	2
Unidentified	11	1	0	0	0	12
Total	296	79	37	99	68	579

Figure 4.3: Comparison of the flea community composition on Richardson's ground squirrels (*U. richardsonii*) collected in Alberta and Saskatchewan.

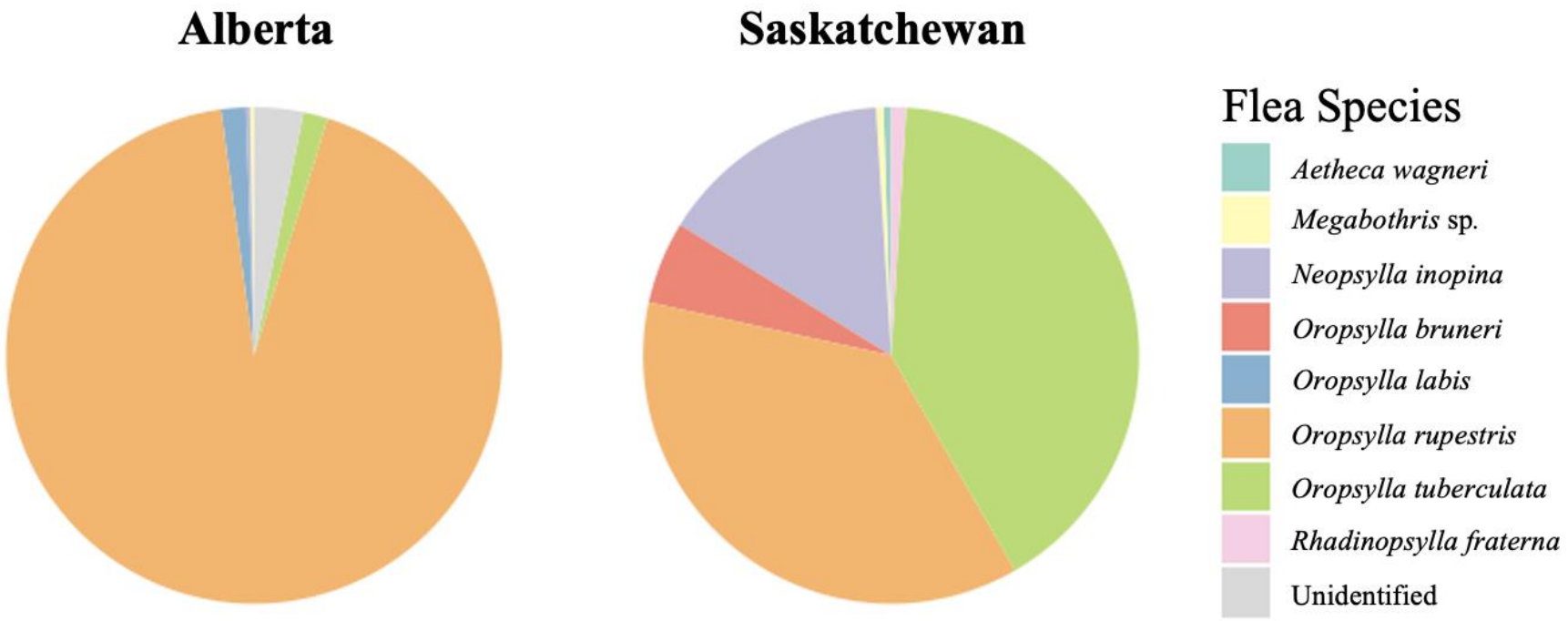


Figure 4.4: Comparison of the flea community composition on Richardson's ground squirrels (*U. richardsonii*) collected from Alberta and Saskatchewan in 2017 to 2019. No Richardson's ground squirrels were collected from Alberta in 2017. Note the values of n represent the number of fleas taken on ground squirrels for each province and year.

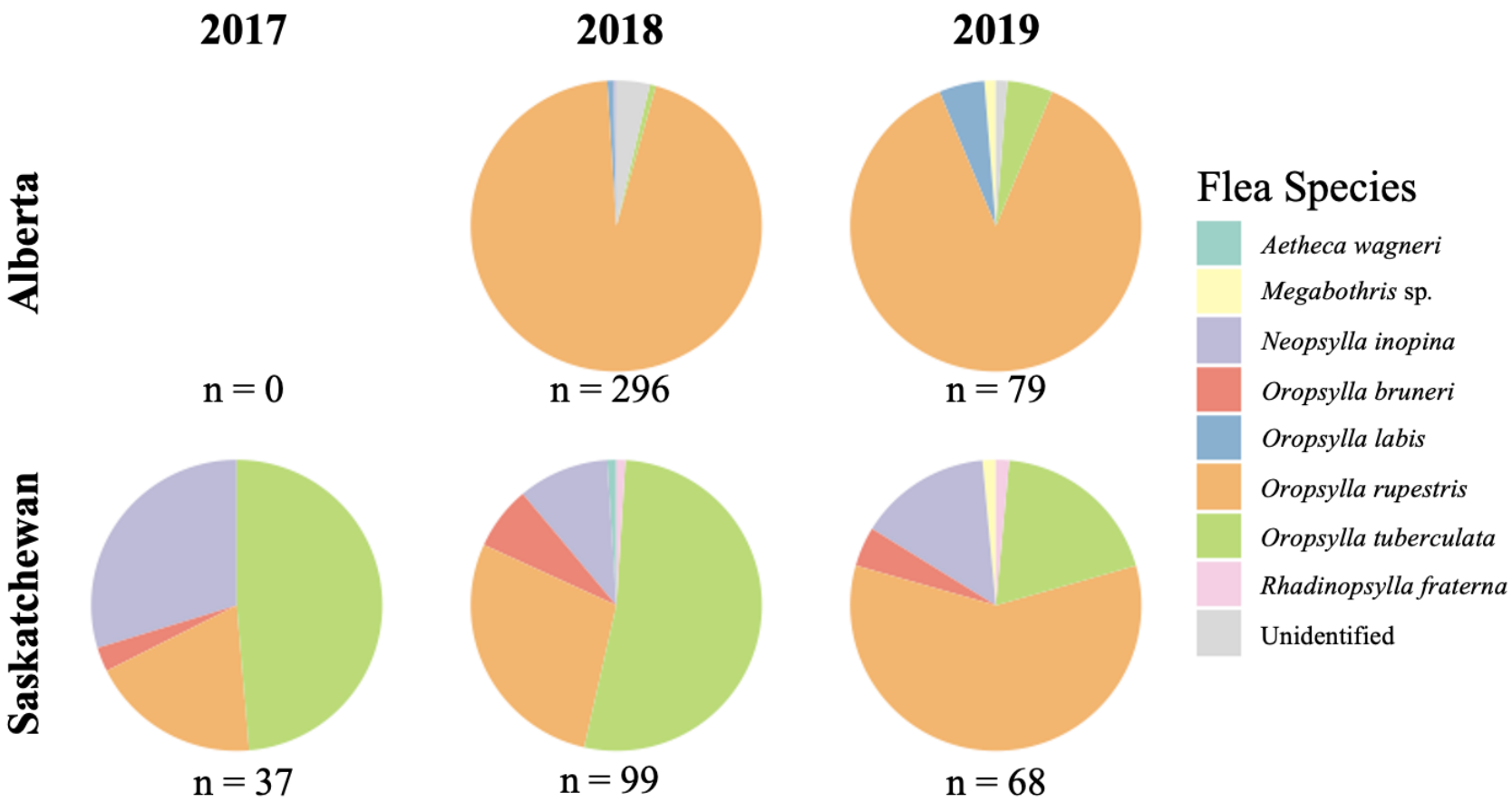


Table 4.3: The number of fleas of each species on Richardson's ground squirrels (*U. richardsonii*) collected during different months in Alberta (AB) and Saskatchewan (SK).

Flea Species	Province	March	April	May	June	July	Total
Oropsylla rupestris	AB	0	0	5	316	28	349
	SK	0	5	4	27	39	75
O. bruneri	SK	0	3	1	5	2	11
O. labis	AB	0	0	0	5	1	6
O. tuberculata	AB	0	0	1	5	0	6
	SK	1	75	4	2	1	83
Aetheca wagneri	SK	0	0	0	1	0	1
Megabothris sp. 1	AB	0	0	0	1	0	1
Megabothris sp. 2	SK	0	0	0	1	0	1
Neopsylla inopina	AB	0	0	1	0	0	1
	SK	0	18	3	9	1	31
Rhadinopsylla fraterna	SK	0	1	0	1	0	2
Unidentified fleas	AB	0	0	0	11	1	12
Total		1	102	19	384	73	579

Table 4.4: Prevalence, abundance and intensity of fleas on Richardson's ground squirrels (*U. richardsonii*) in Alberta (AB) and Saskatchewan (SK). Prevalence is the percentage of potential hosts in the population that were infested by fleas, abundance is the mean number of fleas per potential host found in a population, and intensity of infection is the mean number of fleas per infested hosts in a population.

Province	Sex	March	April	May	June	July	August
Number of hosts:							
AB	Female	0	1	5	33	5	0
	Male	0	0	7	67	7	0
SK	Female	0	20	11	27	8	0
	Male	2	12	7	37	14	1
Prevalence:							
AB	Female	-	0%	60%	42%	80%	-
	Male	-	-	14%	75%	57%	-
	Total	-	0%	33%	64%	67%	-
SK	Female	-	55%	36%	37%	38%	-
	Male	50%	83%	29%	43%	64%	0%
	Total	50%	66%	33%	41%	55%	0%
Abundance:							
AB	Female	-	0.00	1.20	0.85	4.60	-
	Male	-	-	0.14	4.63	1.00	-
	Total	-	0.00	0.58	3.38	2.50	-
SK	Female	-	1.55	0.82	0.78	1.00	-
	Male	0.50	5.92	0.43	0.68	2.50	0.00
	Total	0.50	3.19	0.67	0.72	1.95	0.00
Intensity:							
AB	Female	-	-	2.00	2.00	5.75	-
	Male	-	-	1.00	6.20	1.75	-
	Total	-	-	1.75	5.28	3.75	-
SK	Female	-	2.82	2.25	2.10	2.67	-
	Male	1.00	7.10	1.50	1.56	3.89	-
	Total	1.00	4.86	2.00	1.77	3.58	-

Figure 4.5: Comparison of the body length and weight of individual Richardson's ground squirrels collected from Alberta and Saskatchewan displayed with a linear model line.

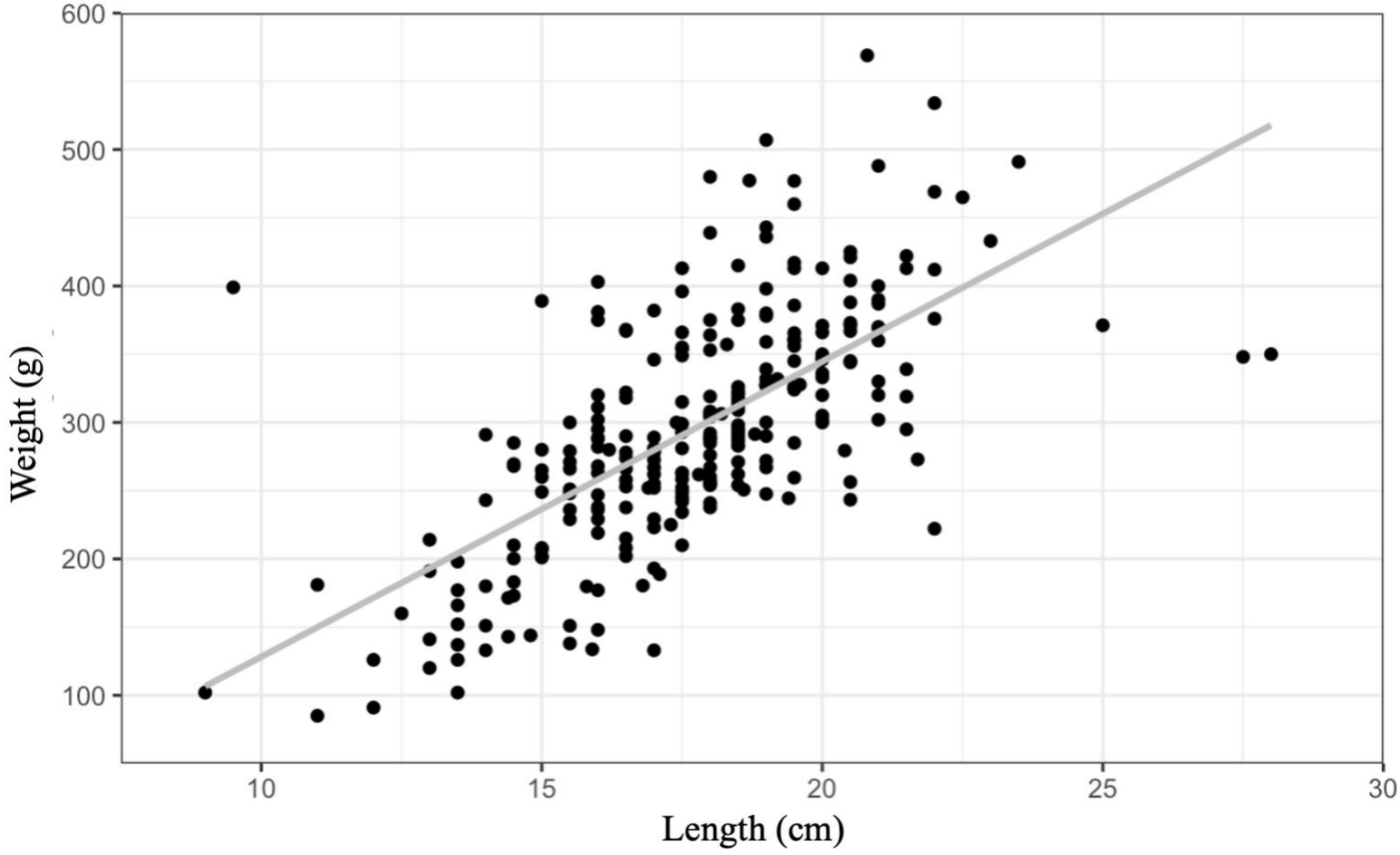


Table 4.5: Model selection table of generalized linear models with negative binomial errors of flea abundance on Richardson's ground squirrels (*U. richardsonii*). Models were ranked according to their Akaike information criterion (AIC). For each model, the degrees of freedom (df), log-likelihood (logLik), AIC score, and difference in AIC scores between each model and the top model (ΔAIC) are shown. The model weights are based on the DAIC values. The r ratio is the ratio of the residual deviance to the residual degrees of freedom and indicates whether the negative binomial distribution is a good fit to the model.

Rank	**Model Structure**	**df**	**logLik**	**AIC**	**ΔAIC**	**weight**	**r ratio**
1	fleas ~ sex * month + length + province + year	15	-443.9	919.8	0.0	0.73	0.99
2	fleas ~ sex + month + length + province + year	12	-448.9	923.1	3.3	0.14	0.98
3	fleas ~ sex * length + month + province + year	13	-447.9	923.3	3.5	0.13	0.98
4	fleas ~ sex * month + province + year	14	-450.2	930.1	10.4	0.00	1
5	fleas ~ sex * month + weight + province + year	15	-449.3	930.6	10.8	0.00	1
6	fleas ~ sex + weight + province + year	7	-459.8	934.0	14.2	0.00	0.97
7	fleas ~ sex * weight + province + year	8	-459.3	935.2	15.5	0.00	0.97
8	fleas ~ sex + month + weight + province + year	12	-455.4	936.0	16.2	0.00	0.99
9	fleas ~ sex + month + province + year	11	-457.1	937.2	17.5	0.00	0.99
10	fleas ~ sex + province + year	6	-462.5	937.4	17.6	0.00	0.97
11	fleas ~ sex * weight + month + province + year	13	-455.2	937.8	18.1	0.00	0.99
12	fleas ~ year	4	-470.2	948.5	28.7	0.00	0.96
13	fleas ~ length	3	-475.2	956.5	36.7	0.00	0.94
14	fleas ~ sex * month	11	-467.8	958.7	39.0	0.00	0.97
15	fleas ~ sex	3	-480.6	967.3	47.5	0.00	0.95
16	fleas ~ sex * weight	5	-479.8	969.9	50.2	0.00	0.95
17	fleas ~ province	3	-483.7	973.4	53.6	0.00	0.95
18	fleas ~ month	7	-482.3	979.0	59.3	0.00	0.96
19	fleas ~ weight	3	-488.5	983.0	63.3	0.00	0.95

Table 4.6: Log likelihood ratio tests of the explanatory variables for the top model in Table 4.5. For each explanatory factor, the chi-square statistic and the p-value are shown.

Variable	X^2	**p**
Host sex	10.13	0.0015
Month of collection	5.48	0.3600
Body length	12.85	0.0003
Year of collection	17.66	0.0001
Province	4.21	0.0402
Host sex:Month	10.28	0.0164

Table 4.7: Model-averaged parameter estimates from the model selection table in Table 4.5. The parameter estimates for each of the 19 models in Table 4.5 were averaged according to their weights. For each parameter, the estimate, standard error, adjusted standard error, z value, and p-value are shown.

	Estimate	Std. Error	Adjusted SE	z value	p
(Intercept)	-3.72	1.78	1.79	2.08	0.0377
Male-Female	-0.02	0.92	0.92	0.02	0.9859
April - March	0.86	1.54	1.55	0.56	0.5767
May - March	0.81	1.51	1.52	0.54	0.5924
June - March	0.60	1.51	1.52	0.39	0.6938
July - March	1.09	1.42	1.43	0.76	0.4446
August - March	-29.92	3.36×10^{6}	3.38×10^{6}	0.00	1.0000
Length (cm)	0.16	0.05	0.05	2.89	0.0039
2018-2017	1.01	0.35	0.35	2.90	0.0037
2019-2017	0.19	0.36	0.36	0.52	0.6011
SK-AB	-0.53	0.23	0.23	2.33	0.0200
April Male - March Female	0.94	0.83	0.83	1.12	0.2609
May Male - March Female	-1.01	0.99	1.00	1.01	0.3117
June Male - March Female	0.54	0.59	0.59	0.92	0.3604
July Male - March Female	0.00	0.00	0.00	NaN	NaN
August Male - March Female	0.00	0.00	0.00	NaN	NaN
Male length - Female length	0.02	0.05	0.05	0.31	0.7533
Weight (g)	1.02×10^{-5}	1.84×10^{-4}	1.84×10^{-4}	0.06	0.9559
Male weight - Female weight	9.45×10^{-7}	6.85×10^{-5}	6.87×10^{-5}	0.01	0.9890

Table 4.8: Estimated marginal means for each factor including the standard error (std. error), degrees of freedom (df), lower confidence (lower CL) and upper confidence (upper CL) levels.

Variable	Response	Std. error	df	Lower CL	Upper CL
Sex:					
Female	0.005	3059.9	250	0	Inf
Male	0.008	4302.7	250	0	Inf
Month:					
March	0.52	0.73	250	0.006	42
April	1.97	0.60	250	0.758	5
May	0.71	0.29	250	0.195	3
June	1.24	0.19	250	0.766	2
July	1.55	0.43	250	0.656	4
August	0	0	250	0	Inf
Year:					
2017	0.004	2430.5	250	0	Inf
2018	0.012	6695.9	250	0	Inf
2019	0.005	2935.4	250	0	Inf
Length:	0.006	3628.5	250	0	Inf
Province:					
AB	0.008	4740.6	250	0	Inf
SK	0.005	2777.3	250	0	Inf
Sex * Month					
Female:March	0.46	0.67	250	0.005	46
Female:April	1.08	0.42	250	0.324	4
Female:May	1.03	0.50	250	0.221	5
Female:June	0.83	0.19	250	0.400	2
Female:July	1.36	0.55	250	0.381	5
Female:August	0	0	250	0	Inf
Male:March	0.59	0.81	250	0.008	44
Male:April	3.60	1.58	250	0.907	14
Male:May	0.49	0.40	250	0.038	6
Male:June	1.85	0.31	250	1.093	3
Male:July	1.77	0.60	250	0.615	5
Male:August	0	0	250	0	Inf

Figure 4.6: A comparison of the number of fleas on female and male Richardson's ground squirrels (*U. richardsonii*).

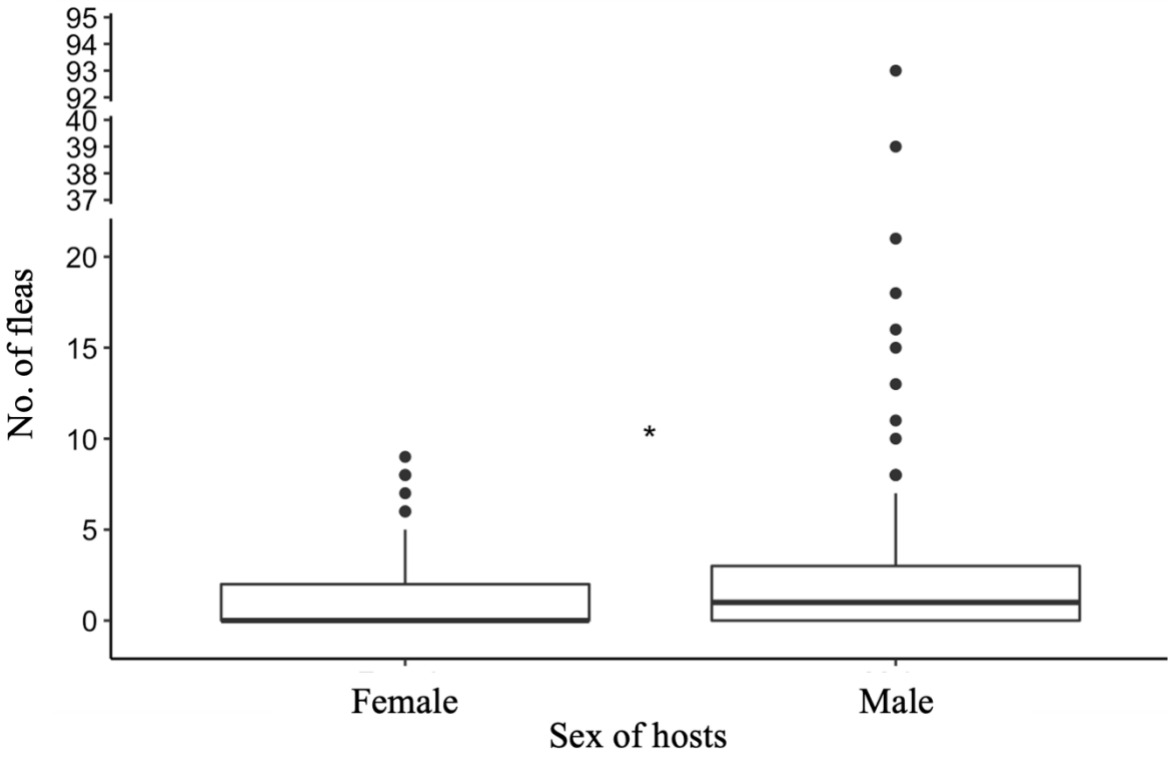
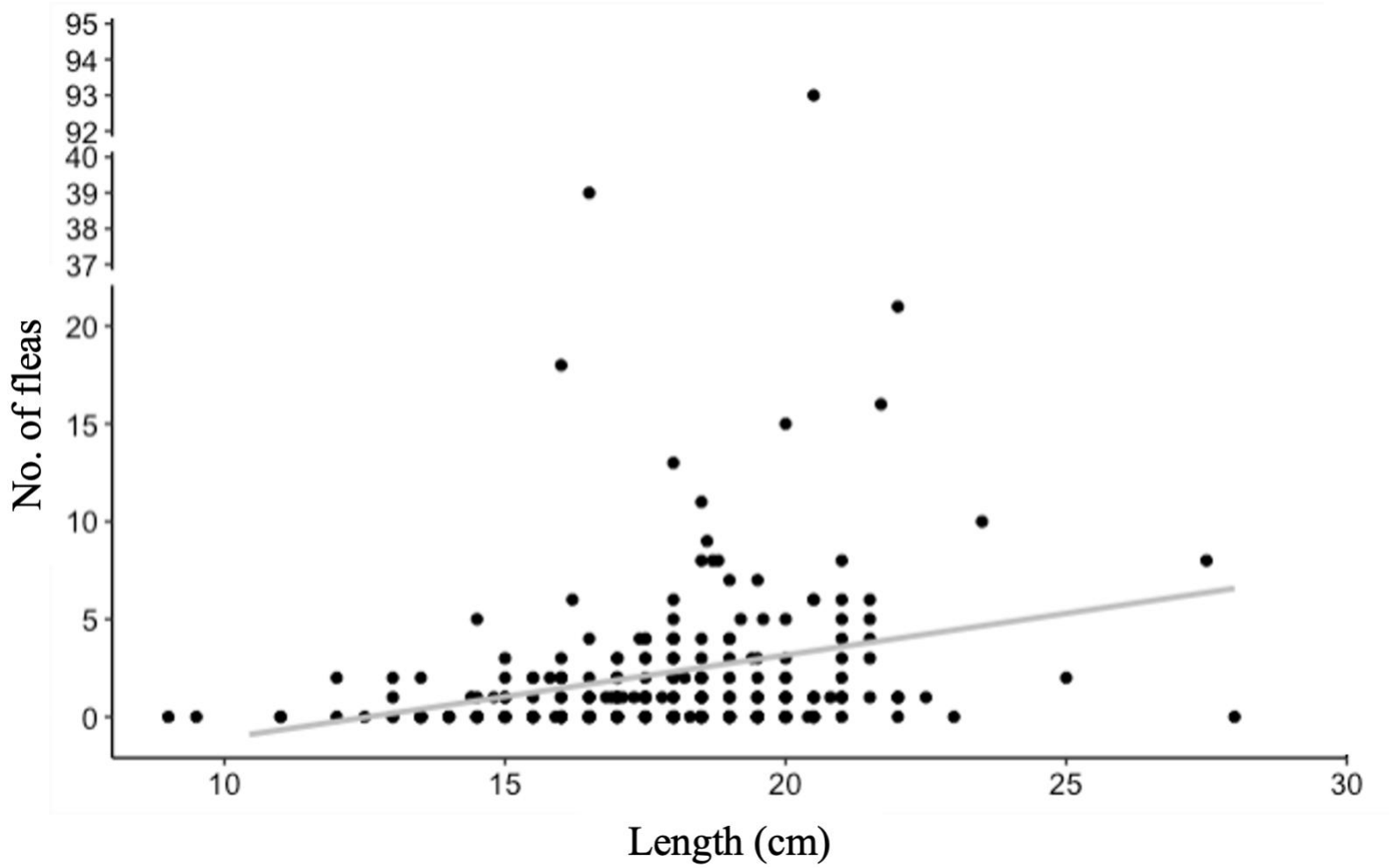


Figure 4.7: Comparison of number of fleas per the length of the individual Richardson's ground squirrels.



4.5 DISCUSSION

The life history traits and intimate historical relationship of ectoparasites and rodent hosts determine the relative abundance and prevalence of parasites within a population. Richardson's ground squirrels are regularly parasitized by a diversity of ectoparasites (i.e., fleas, lice, ticks, and mites) (Waterman et al. 2014). Several flea species that parasitize ground squirrels and prairie dogs are vectors of *Y. pestis*, the bacterium that causes plague. These include *A. wagneri, Epitedia wenmanni, O. rupestris, O. tuberculata, O. labis, O. bruneri,* and *Thrassis bacchi* (Holland 1985, Eisen and Gage 2012). Investigating the factors that influence the abundance of parasite vectors is important as rodents are common reservoirs for a variety of pathogens. In this study, we detected a high prevalence of two vector species (i.e., *O. rupestris* and *O. tuberculata*) of *Y. pestis*, and three additionally reported vector species (*A. wagneri, O. bruneri* and *O. labis*) but at relatively low abundance.

Parasites are not ubiquitous across a population but are found aggregated on a select number of individuals (Wilson et al. 2002, Sackett 2018). In the present study, 46% of hosts were not infested by fleas. The frequency of infestation by fleas decreased as more fleas are present (i.e. one flea 20.5%, two fleas 11.7%, 3 fleas 5.7%, etc.). The patterns of host usage by fleas on *U. richardsonii* are the same as those of many ectoparasites on vertebrate hosts (Brinkerhoff et al. 2006, Poulin 2007, Patterson et al. 2015, Eads and Hoogland 2016, Sackett 2018, Lehikoinen et al. 2021, Caron-Lévesque and Careau 2023). Defense mechanisms of the hosts, such as grooming and immune responses against infesting ectoparasites, can influence the abundance of fleas on the rodent host (Krasnov 2008). The highest abundance of fleas was found on *U. richardsonii* collected from Alberta in 2018. The prevalence of fleas on *U. richardsonii* in the present study are lower than those reported by Lindsay and Galloway (1997). Previous

studies investigating the prevalence of fleas on Richardson's ground squirrels found prevalences of 82% (Jardine et al. 2006b), 66% in Manitoba (Waterman et al. 2014), and 35% in Alberta (Hilton and Mahrt 1971) from live-trapped ground squirrels. Difference in flea prevalence among studies could be attributed to a number of factors including collection location, the season in which hosts were sampled, and sampling technique. As our samples were collected postmortem, some fleas may have vacated the host before collection. The majority (80%) of infested *U. richardsonii* in the present study were parasitized by only one flea; therefore competition (interspecific and intraspecific) for resources is unlikely to have occurred.

It is generally assumed that "true parasites", those specialized for a specific host taxon, are expected to achieve higher abundance on their preferred host than other species (Krasnov et al. 2004). *Oropsylla* species are considered to be specialized for North American species of ground squirrel, with *O. rupestris* considered to be the most common occurring on *U. richardsonii* (Jellison 1945, Holland 1985, Lewis 2002, Jones and Britten 2010). In the present study, the most prevalent flea species present on Richardson's ground squirrels was *O. rupestris* (73%). The next two most prevalent species were *O. tuberculata* (15%) and *N. inopina* (5.5%). These three species, as well as *O. bruneri*, *O. labis*, and *R. fraterna*, are considered true parasites of Richardson's ground squirrels (Burgess 1955, Hilton and Mahrt 1971, Holland 1985, Galloway and Christie 1990, Lindsay and Galloway 1997, 1998, Hastriter 2023). Another two species collected on *U. richardsonii* in the present study, *A. wagneri* and a species of the genus *Megabothris*, are considered incidental parasites (Holland 1985, Galloway and Christie 1990). These incidental parasites are primarily associated with a variety of small rodents but can attach to Richardson's ground squirrels in areas where *U. richardsonii* coexist with small rodents (Holland 1985, Galloway and Christie 1990, Lindsay and Galloway 1997, Hastriter 2023). Given

the variety of flea species found in this study, a general linear mixed effects models was considered for the abundance of each flea species; however, this was not pursued because of the limited sampling size for most flea species. We chose not to pursue abundance modeling for specific species at a high abundance as the seasonal activity patterns of the species would influentially bias the model towards month. Instead, we conducted modeling analyses of the total abundance of fleas on *U. richardsonii* throughout the above ground activity of these hosts.

Previous studies have used modeling to explain the total flea abundance and prevalence on Richardson's ground squirrels (Jardine et al. 2006a, 2006b, Waterman et al. 2014). Waterman et al. (2014) analyzed flea abundance and species diversity in Winnipeg, Manitoba during the months of March and April, whereas Jardine et al. (2006a, 2006b) analyzed flea prevalence and abundance on Richardson's ground squirrels near Saskatoon, Saskatchewan throughout their above ground activity period. Waterman et al. (2014) found that females had a higher prevalence of fleas than males, but no difference in flea abundance among female and male *U. richardsonii*. Additionally, Waterman et al. (2014) did not find flea abundance to be correlated with host sex, body mass or body condition. The only significant relationship Waterman et al. (2014) found for flea abundance was the hematocrit level (i.e., the proportion of red blood cells in the blood). In contrast, in the univariate model of Jardine et al. (2006b), flea prevalence and abundance were associated with the sex of the host. However, this association was no longer significant when incorporated into a multivariate model that adjusted for month of sampling and other factors (e.g. deltamethrin powder insecticide treatment group) (Jardine et al. 2006b).

Depending on the system, host factors such as age, size, sex, behaviour, geographical range, longevity, immune system, diet, and genetic diversity have been found to influence the variability of parasite load within a population (Poulin 2004, Sackett 2018). Population factors

such as host density and social behaviour can also influence parasite diversity and abundance (Poulin 2004). No single factor can predict the abundance and prevalence of parasitic infestation in a host population (Poulin 2004). Our negative binomial models for examining flea abundance on Richardson's ground squirrels based on a variety of host factors had a range of AIC values from 919.8 to 983.0. The parameter estimates were averaged based on the weighted value of the model through AIC selection. The top models with the highest weight (0.73) incorporated an interaction between month and sex which is biologically relevant for Richardson's ground squirrels. The above ground activity of *U. richardsonii* is based on age and sex cohorts that fluctuates depending on the activity period (Michener and Koeppl 1985). In comparison, models that included an interaction between weight and sex or length and sex did not have as much explanatory power. This is probably a consequence of the lack of significant difference in body size between the sexes of the Richardson's ground squirrels in our samples. Sex, length, sampling year, province of collection and the interaction between sex and month were found to significantly affect the flea abundance on hosts in the highest weighted model.

Given the results of previous studies conducted on rodents (Krasnov et al. 2005, Jardine et al. 2006b, Hillegass et al. 2008, Kiffner et al. 2013, Waterman et al. 2014), we expected that the host sex would be the most significant factor in predicting the flea abundance on *U. richardsonii*. In addition, we predicted that males would be more heavily infested than females based on the sex-biased parasitism hypothesis that has been applied to a variety of rodent-ectoparasite relationships; for example fleas on northern flying squirrels (Perez-Orella and Schulte-Hostedde 2005), ectoparasites on Cape ground squirrels in Africa (Hillegass et al. 2008) and the tick, *Ixodes ricinus* on wood mice (Harrison et al. 2010). Flea prevalence on *U. richardsonii* males was greater (Alberta 68% and Saskatchewan 52%) than for females (Alberta

48% and Saskatchewan 42%), with males having an overall flea abundance 1.6 times higher than females. These results are in contrast to what has been previously reported in Richardson's ground squirrels (Waterman et al. 2014), but is consistent with the findings of other rodent-flea systems (Jardine et al. 2006b, Krasnov et al. 2005). When comparing specific sampling months, males consistently had a higher abundance of fleas in every month, except in May, where females had a flea abundance that was two times higher. Waterman et al. (2014) investigated flea abundance during mating season of *U. richardsonii* (i.e., late-March to early-April). They found the females had an overall higher abundance of fleas than males. The higher abundance of fleas on females may be due to their increased occurrence in burrows during reproduction (Michener 1983, Michener and Koeppl 1985). Patterson et al. (2015) found that male red squirrels (*Tamiasciurus hudsonicus*) had higher flea infestations during the mating season, while females had higher flea infestation during lactation. Fleas are likely to occur in higher abundance within the burrows as flea reproduction occurs within the nest and burrows of their host (Krasnov 2008). Male rodents are more likely to be parasitized by ectoparasites due to a suppressed immune system from testosterone, increased body size, and increased home range (Krasnov et al. 2004, Waterman et al. 2014). Size variation between sexes has been proposed as a contributing factor in sex-biased parasitism of ectoparasites on rodents (Krasnov et al. 2004, Kiffner et al. 2013). Others argued that the male bias for parasitism is due to males being larger than females (Krasnov et al. 2004, Kiffner et al. 2013). In the present study, no significant difference was found in body size of male and female Richardson's ground squirrels. There was also no significant effect of host body weight on flea abundance on *U. richardsonii* however; body length significantly affected the abundance of fleas on hosts. Similar results have been found in fleas and lice infesting Richardson's ground squirrels (Waterman et al. 2014) and fleas on other

species of rodent (Krasnov et al. 2004, 2005). It is expected that larger hosts represent increased opportunity for nutrients and attachment sites for ectoparasites (Kiffner et al. 2013). In addition, heavier animals may represent healthier hosts that are able to support a higher load of parasites (Zduniak et al. 2023). The effect of weight on flea abundance may be compounded by other factors (i.e. health, age, energy, place in population) that were not investigated in this study. For example, adult Richardson's ground squirrels are heavier than yearlings and juveniles (Michener 1984, Michener and Koeppl 1985) and as in previous studies (Soliman et al. 2001, Hawlena et al. 2006, Jardine et al. 2006a, Sackett 2018), age has been determined to be a contributing factor to flea abundance/presence. Richardson's ground squirrels exhibit sexual dimorphism in size; however, size can additionally fluctuate with age, location, and year (Michener and Koeppl 1985). As we did not distinguish between *U. richardsonii* juveniles and adults, we are unable to determine if flea load is influenced by age.

Sampling year and province were included in the linear model to indicate if characteristics of the environment (i.e. soil compensation, precipitation, and vegetation) have an effect on flea abundance. These two variables were highly significant in the model, with Alberta having a flea abundance 1.6 times higher than Saskatchewan and 2018 having a 1.3 times higher abundance than other sampling years. Differences in flea abundance on hosts between years and between sampling locations, may be a consequence of climatic differences (i.e., temperature, precipitation, or both), but this requires further examination. Our data may be biased towards Alberta and 2018 as we collected substantially more fleas in Alberta in 2018 than in Saskatchewan and other years. Future studies should include climatic variables in models to predict the abundance of fleas and other ectoparasites on hosts. Yearly variation in flea abundance may reflect fluctuations in host grooming behaviour, food resource, host dispersal and

microclimate conditions of burrows (Eads et al. 2016, Hayes et al. 2017, Krasnov et al. 2022). As fleas are ectothermic and prone to desiccation (Krasnov 2008), the relative humidity and temperature in burrows can affect the survival of different life cycle stages which influences the prevalence and abundance of fleas on hosts (Krasnov et al. 1997, 2006, Bossard 2006, Hubbart et al. 2011). Prairie dogs are also parasitized by some flea species parasitizing ground squirrels (Holland 1985). In dry years, vegetation is reduced, prairie dogs have decrease energy resources to allocate to producing an immune response to flea infestation allowing flea populations to increase (Krasnov 2008, Eads and Hoogland 2016, Eads et al. 2016). This suggestion is based on the findings of Eads et al. (2016) who studied flea load on black-tailed prairie dogs in New Mexico. They found that flea densities on black-tailed prairie dogs were the greatest during dry years when hosts were spending less time grooming (Eads et al. 2016). During years with a wet spring and a cold summer, fleas are more abundant on hosts; however, this result was likely an artifact of the limited number of black-tailed prairie dog hosts due to high mortality rates from plague (Tripp et al. 2009, Brinkerhoff et al. 2010, Eads et al. 2016).

The flea species present and their relative abundance on *U. richardsonii* varied between provinces and collection periods. The most dominant species of flea collected from hosts in Lethbridge in southern Alberta was *O. rupestris*, while in those collected near Moose Jaw in southern Saskatchewan two species (*O. tuberculata* and *O. rupestris*) dominated. Variation in the flea communities between sites could be influenced by other host species present, yearly precipitation, and/or soil or vegetation composition (Krasnov et al. 2004, Eads and Hoogland 2016, Goldberg et al. 2020). Krasnov et al. (2010) found that the effect on environment in the dissimilarities between flea communities is likely related to the microclimate preferences of the fleas during their off-host life stages. Caution is advised in the interpretation of the results of

year and province effects on the model as the sampling methods and sample sizes varied and date of sampling was sporadic between years. Also, we only sampled one location within each province, and therefore our results should not be generalized to the entire province level. Observed differences in flea prevalence and diversity between the two provinces could also be due to differences in the period of collection between the two provinces. For example, we did not find any fleas on *U. richardsonii* in March or April in Alberta when *O. tuberculata* is present in Saskatchewan.

Seasonal variation in host and flea activity patterns can influence the abundance of fleas (Patterson et al. 2015), ratio of sex bias parasitism (Krasnov et al. 2004), and host sex ratio. Given that Richardson's ground squirrels have seasonal variation in the sex and age-class activity throughout a year (Michener and Koeppl 1985), we included an interaction between sex and month in our model. The abundance and presence of different flea species varied throughout the collection period. Prevalence was highest in Saskatchewan in April while *O. tuberculata* was in peak activity. While in Alberta was highest prevalence was in June and July during the active period of *O. rupestris*. Seasonal activity patterns for fleas have been observed previously on *U. richardsonii* (Lindsay and Galloway 1997), in their burrows (Thoroughgood and Chilton 2024), and other rodent species (Salkeld and Stapp 2008, Friggens et al. 2010). The seasonal activity of fleas is likely to be an important factor in pathogen transmission from rodents to susceptible hosts (Wilder et al. 2008, Tripp et al. 2016).

In conclusion, the present study showed that the sex of the host, location, and year of collection influenced the abundance of fleas found on *U. richardsonii*. The month of collection also influenced flea abundance which is probably a function of seasonal differences in the activity patterns of different flea species. *Oropsylla rupestris* and *O. tuberculata* had the highest

abundance and had unique peaks in intensity and activity periods. The overall prevalence and intensity of fleas was much lower in this study in comparison to previous studies which may be a consequence of our samples being opportunistically collected post-mortem. We suggest that more research needs to be conducted that explores the seasonal activity patterns of fleas parasitizing *U. richardsonii* and how microclimate in host burrows influences flea abundance.

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4.7 TRANSITION STATEMENT

In the last chapter, Richardson's ground squirrels were opportunistically collected by farmers in Alberta and Saskatchewan. I determined that the structure of the flea communities varied between collection sites and between years. Some seasonal activity patterns in fleas were observed; however, the opportunistic method of host collection prevented consistent sampling which would allow for detailed investigation into the seasonal activity pattern of the fleas. In the next chapter (Chapter 5), fleas were regularly collected from the burrows of Richardson's ground squirrel at a rural site and an urban site in Saskatchewan to further investigate flea diversity and abundance, and to determine if there are differences in seasonal activity patterns of fleas at different sites.

CHAPTER 5: COMPARISON OF FLEA DIVERSITY IN THE BURROWS OF RICHARDSON'S GROUND SQUIRRELS (*Urocitellus richardsonii)* IN AN URBAN AND RURAL SITE IN CENTRAL SASKATCHEWAN, CANADA[4]

5.1 ABSTRACT

Spatial and temporal differences in the relative abundance of arthropod vectors are an important factor influencing the risk of disease for mammalian hosts. Seasonal changes in the diversity and abundance of fleas (Siphonaptera) in Richardson's ground squirrel (*Urocitellus richardsonii*) burrows were studied at two sites in central Saskatchewan. A total of 225 fleas (151 at an urban site and 74 at a rural site) were collected. Flea prevalence differed among seasons at the urban site, but not at the rural site. Of the nine flea species detected (8 at the urban site and 6 at the rural site), *Oropsylla rupestris*, *O. bruneri*, *O. labis*, *O. tuberculata* and *Aetheca wagneri* are vectors of *Yersinia pestis*, the causative agent of plague. The presence and abundance of some fleas differed between sites and seasons. *Neopsylla inopina* and *O. rupestris* were the most abundant species at the urban site during the spring and summer (respectively), while *O. bruneri* was the most abundant species at the rural site. Our findings may have implications for the management of the black-tailed prairie dogs (*Cynomys ludovicianus*) in south-western Saskatchewan because they coexist with *U. richardsonii*, are hosts for *Oropsylla*, and are at great risk of plague exposure/infection.

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5.2 INTRODUCTION

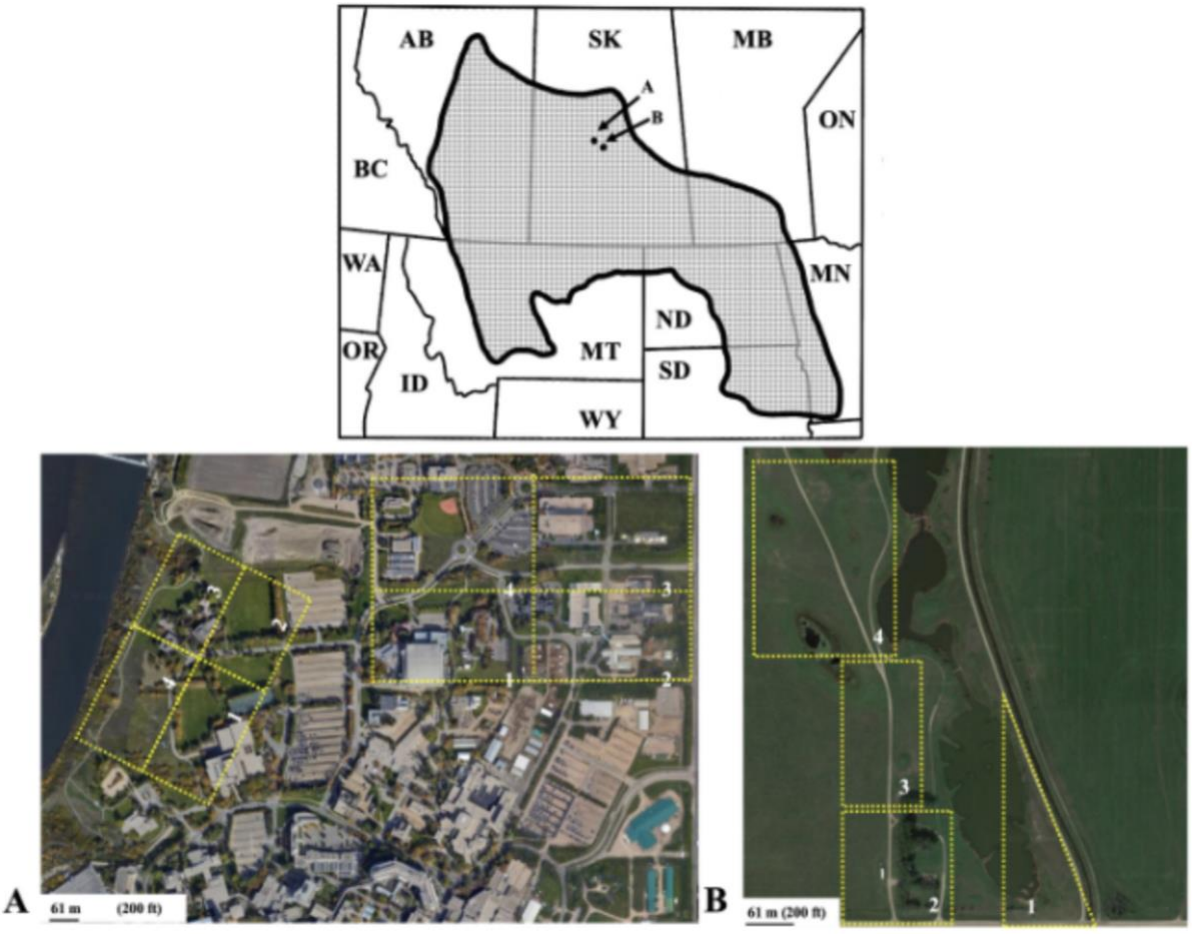
Fleas are hematophagous insects, some species of which are vectors of *Yersinia pestis, Rickettsia felis, R. typhi,* and *Bartonella henselae*, the causative agents of plague, flea-borne spotted fever, murine typhus, and cat scratch disease, respectively (Eisen and Gage 2009, 2012). Some of the key factors influencing the risk of infection for susceptible mammalian hosts to these bacterial pathogens include the intensity of fleas infesting the host, the proportion of fleas carrying a pathogen, the relative competency of different flea species to transmit a pathogen to the vertebrate host, and the body condition of the host (Brinkerhoff et al. 2006, Eisen and Gage 2012, Eads et al. 2013, 2016, Eads and Hoogland 2016, 2017).

Several flea species are involved in the transmission of *Y. pestis* in North America. These include *Oropsylla hirsuta*, *O. montana*, *O. tuberculata*, *Orchopeas sexdentatus*, and *Hoplopsyllus anomalous* (Eisen and Gage 2012), all of which use rodents as hosts (Holland 1985, Galloway and Christie 1990, Eisen and Gage 2012). There are also 21 other flea species that parasitize rodents in North America that have been shown experimentally to be vectors of *Y. pestis* (see review by Eisen and Gage 2012). This group includes *O. rupestris* and *O. labis*, the latter of which was associated with a dead Richardson's ground squirrel (*Urocitellus richardsonii*) in an area in eastern Alberta (Canada) where there was a serious outbreak of plague (Gibbons and Humphreys 1941).

The distributional range of *U. richardsonii* on the northern great plains includes the areas of southern and central eastern Alberta, southern and central Saskatchewan, southwestern Manitoba in Canada, and northern and western Montana, North Dakota (except for the southwestern region), northeastern South Dakota and western Minnesota in the United States (Cassola 2016) (Figure 5.1). The most extensive study of fleas on Richardson's ground squirrels

was conducted by Lindsay and Galloway (1997) in livestock pastures in the Aspen Parkland Ecoregion near Winnipeg in Manitoba. Ten flea species were detected on *U. richardsonii*; the four most common species were *O. bruneri, O. rupestris, Neopsylla inopina,* and *Rhadinopsylla fraterna* (Lindsay and Galloway 1997). They also found that infestations on hosts by each of these flea species peaked at different times of the year (Lindsay and Galloway 1997). In the study by Liccioli et al. (2020), conducted in Grasslands National Park, located in the Mixed Grassland Ecoregion of southwestern Saskatchewan, most fleas collected from Richardson's ground squirrels and black-tailed prairie dogs (*Cynomys ludovicianus*), and the burrows of *C. ludovicianus*, were either *O. rupestris* or *O. tuberculata*. They also found seasonal differences in the total number of fleas found in burrows of black-tailed prairie dogs but did not indicate which flea species varied over time. Despite the broad geographical range of *U. richardsonii*, few studies have examined the species diversity and seasonal activity of fleas that parasitize this ground squirrel. Therefore, we conducted the present study to compare the diversity and seasonal abundance of fleas in the burrows of Richardson's ground squirrels at a rural site and an urban site located in the northern part of the distribution of *U. richardsonii* within the Moist Mixed Grassland Ecoregion of central Saskatchewan. Also, given that the level of human disturbance can influence the diversity and relative abundance of fleas on other species of mammalian host (Friggens and Beier 2010), we examined if the diversity, prevalence and/or relative abundance of fleas in *U. richardsonii* burrows at a rural site differed from those at an urban site where there is greater anthropogenic disturbance.

Figure 5.1: Location and aerial photographs of the urban site (A) in Saskatoon and the rural site (B) near Bradwell in central Saskatchewan (SK). Also shown on the map is the distributional range of *Urocitellus richardsonii* based on Cassola (2016). The density of Richardson's ground squirrel burrows in quadrants 1-4 at each site are given in Table 5.1.



5.3 MATERIALS AND METHODS

The diversity and seasonal patterns of fleas in active burrows of *U. richardsonii* were investigated at two sites (one urban and one rural site) in central Saskatchewan, located ~345 km northeast of Grasslands National Park and ~160 km south of the northern distributional limit of *U. richardsonii* in Saskatchewan (Fig 5.1). Flea sampling was conducted from April 30 to October 2 in 2019; thus sampling covered three seasons: spring (April to June), summer (July and August) and fall (September and October). Active burrows were defined as those where a Richardson's ground squirrel was observed in the burrow, and/or there was visible evidence of disturbed earth or *U. richardsonii* feces at the burrow entrance (Eads 2017). The urban site covered an area of 4.143 ha on the University of Saskatchewan campus in Saskatoon (52°08'12.2"N 106°37'59.9"W), whereas the rural site (51°52'54.8"N 106°16'20.5"W), located near Bradwell, was an area of 4.9 ha consisting of mixed-grass prairie. Although the rural site was located far from any developed area, it was a managed area (frequently mowed) used for retriever competitions (i.e., an area of frequent dog activity). The urban site, located 37 km to the northwest of the rural site, was an area of manicured cool grass lawn. Both sites were divided into quadrats, the size of which was based on the density of active burrows (Table 5.1). The urban site was divided into two areas with four unequal sections with each area containing at least 20 *U. richardsonii* burrows in each section, whereas the rural site was divided into four unequal sections each containing a minimum of 20 burrows (Fig 5.1).

Table 5.1: Summary of collection of fleas from burrows at each site as well as the size of the sample site and density of Richardson's ground squirrel burrow entrances per hectare.

Quadrant	Urban Site			Rural Site		
	Area (ha)	Burrow density*	No. of fleas	Area (ha)	Burrow density*	No. of fleas
1	1.344	273	41	0.797	69	11
2	0.669	262	21	0.968	65	24
3	0.430	521	26	0.811	51	9
4	1.700	87	63	2.320	21	30
Total	4.143	105	151	4.896	42	74

* = “burrow entrances”/ha

Fleas were collected from active *U. richardsonii* burrows using a swabbing technique (Gage 1999, Eads 2017). The white swab consisted of a 12" by 12" piece of cotton attached to a plumber's drain snake with an elastic band. This device was inserted into a burrow to a depth of 30 cm to 1 m, and then shaken for 30 seconds to simulate the presence/movement of a Richardson's ground squirrel in the burrow to increase the chance that fleas attach to the cotton swab (Gage 1999, Eads 2017, Eads et al. 2021). The cotton swab was removed from the drain snake and immediately placed into a Ziploc bag. A clean cotton swab was used for the sampling of each *U. richardsonii* burrow. Sampling for fleas was conducted during the mornings (i.e., between 8 am and 12 pm) two days per week at both the urban site and rural site. On day 1 of each week, 10 active burrows in one area of the urban site were sampled for fleas and this was followed by the sampling of another 10 active burrows from a second area. This process was repeated at the urban site at day 3 of each week, but sampling was conducted from different quadrats. For the rural site, 20 burrows in one area were sampled for fleas on day 2 of each week and this process was repeated for another area at this site on day 4 of each week. To reduce the chances of eliminating the local flea population, we rotated sampling through different areas of a study site, so that one area was only swabbed once every two weeks. Ziploc bags containing the cotton swabs and fleas were transported to the lab, then placed in a -20°C freezer for four hours (i.e. to reduce flea movement). The contents of the bag were sorted, and each flea was placed into a separate sterile Eppendorf 1.5 ml tube containing 70% ethanol and stored at -20°C until required for molecular analysis. Chi-square (χ^2) tests or contingency tests were used to compare flea prevalence (= proportion of swabs that had fleas) between sites and among seasons, while Mann Whitney U tests or Kruskal Wallis tests were used to compare mean flea intensity (=

average number of fleas in burrows that contained one or more fleas) between sites and among seasons.

Given that fleas collected in the present study were to be used in a future microbiome study, it was not possible to morphologically identify fleas to species because the clearing agents required to examine their internal structures would degrade DNA and/or prevent DNA extraction. Therefore, the identification of fleas was determined based on their DNA sequences of the part of the nuclear 28S rRNA gene and/or the nuclear second internal transcribed spacer (ITS-2) ribosomal DNA. Total genomic DNA (gDNA) was extracted from the whole body of each thawed flea using a DNeasy Blood & Tissue Kit (Qiagen Inc.) following the methodology of Dergousoff and Chilton (2007). Flea gDNA samples were stored at -20°C. The methods used to amplify and sequence ~450 bp of 28S rRNA are the same as those described by Thoroughgood et al. (2021). PCR primers Sen-ITS2 (5'-GGGTCGATGAAGAACGCAGC-3') (Vobis et al. 2004) and ITSR (5'-TTTAGGGGGTAGTCTCACCTG-3') (Luchetti et al. 2007) were used to amplify (~430 bp) of the ITS-2 and part of the flanking regions (i.e., 5.8S and 28S rRNA genes) of individual fleas. These PCRs were conducted in 25 µl volumes containing 5x Phusion Green HF buffer (Fisher Scientific), 1.7 mM $MgCl_2$, 200 mM of each dNTP, 0.75 mM of each primer, 1.25 of Phusion HotStart II polymerase (Fisher Scientific) and 1.5 µl of flea gDNA. Negative (i.e., no gDNA) controls were used for each PCR run. The PCR conditions used were 94°C for 5 mins., then 30 cycles of 94°C for 60s, 55°C for 60s, and 72°C for 60s, and a final extension of 72°C for 5 minutes. 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 banding patterns of amplicons were visualized by UV transillumination. Amplicons were purified using shrimp alkaline phosphatase (0.014 U/µl) (New England BioLabs, Pickering,

Canada) and exonuclease I (0.27 U/µl) (Fisher Scientific) as described in Thoroughgood et al. (2021). These purified amplicons were subjected to automated DNA sequencing using primers Sen-ITS2 and ITSR in separate reactions. The identification of *N. inopina, O. rupestris, O. tuberculata, O. labis* and *O. bruneri* were based on comparisons with the 28S and/or ITS2 DNA sequences of previously morphologically identified specimens of each of these species (Thoroughgood et al. 2021, Chapter 2). Identification of the other fleas to the species (e.g. *Aetheca wagneri*) or genus/family level was based on a comparison of flea DNA sequences available on GenBank.

5.4 RESULTS

A total of 225 fleas were collected during this study, 151 from the urban site and 74 from the rural site (Table 5.2). There was no significant difference in flea prevalence between sites during spring ($\chi^2_1 = 1.57$, $p > 0.05$), summer ($\chi^2_1 = 3.79$, $p > 0.05$) or fall ($\chi^2_1 = 1.20$, $p > 0.05$). However, at the urban site, flea prevalence was significantly greater ($\chi^2_2 = 12.05$, $p < 0.001$) in spring than in summer and fall. In contrast, at the rural site, there was no significant difference in flea prevalence among seasons ($\chi^2_2 = 5.14$, $p > 0.05$). There were no significant differences in the mean intensity of fleas in burrows among seasons at the urban site ($H = 0.49$, $p > 0.05$) or the rural site ($H = 0.24$, $p > 0.05$), but the mean intensity of fleas at the urban site was significantly greater ($U = 2123.5$, $p < 0.001$) than at the rural site (Table 5.2). The number of fleas collected per swab ranged from one to 19 at the urban site and from one to five at the rural site. Most of the fleas (i.e., 95% at the urban site and 77% at the rural site) were collected during the spring and summer months (Table 5.2). No fleas were collected in October based on a sampling of 40

Table 5.2: Seasonal variation in the proportion of swabs from which fleas were collected (i.e., flea prevalence) and the mean intensity of fleas in the burrows of Richardson's ground squirrels at the two sites in Saskatchewan.

	Urban Site				Rural Site			
	No. of swabs	% of swabs with fleas	Total no. of fleas	Mean intensity of fleas	No. of swabs	% of swabs with fleas	Total no. of fleas	Mean intensity of fleas
Spring	360	13.9	72	1.4	300	9.7	33	1.1
Summer	360	9.2	71	2.2	400	5.5	24	1.1
Fall	160	3.1	8	1.6	160	5.6	17	1.9
Total	880	10.0	151	1.7	860	7.0	74	1.2

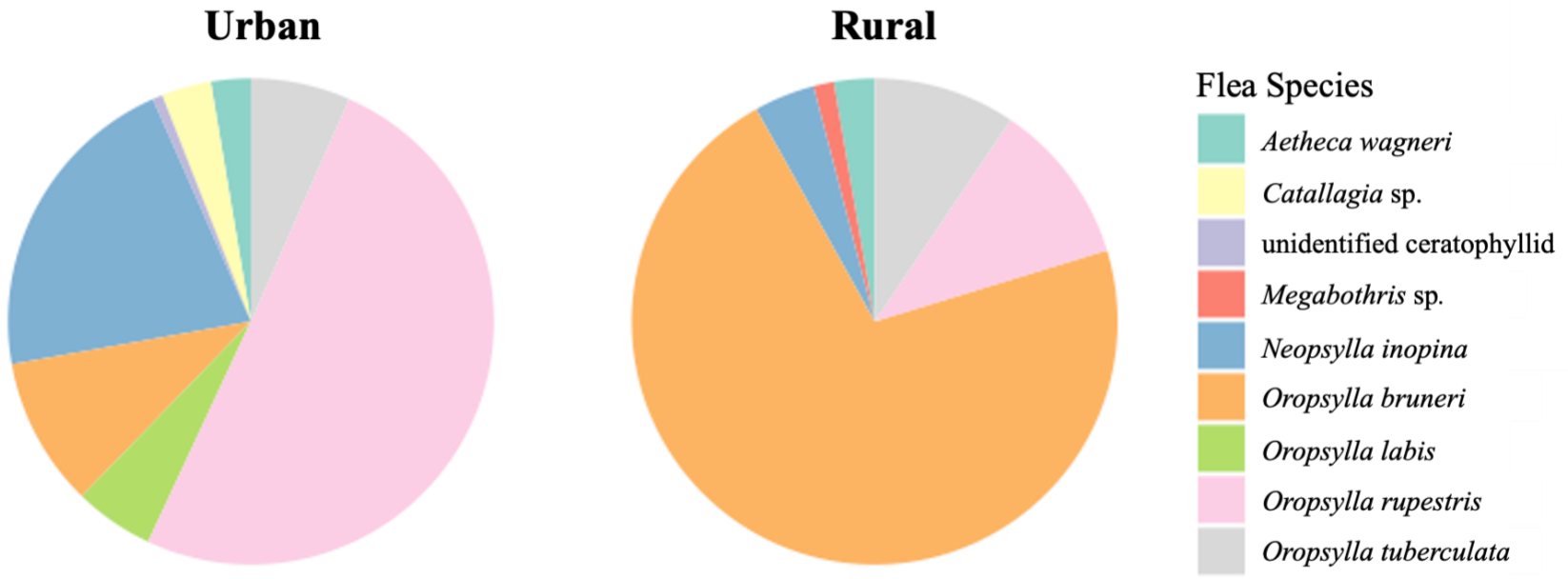
burrows at the urban site (i.e., 20 burrows on two different days). The final sampling date that yielded any fleas by the burrow swabbing technique was September 20th, 2019.

Nine species of fleas were detected in the burrows of Richardson's ground squirrels in this study (Table 5.3). Six species were easily identified at the species level, whereas three taxa could only be identified to genus (i.e., *Catallagia* sp., *Megabothris* sp., and an unidentified species within the family Ceratophyllidae). Only one specimen was collected for two of these three taxa (i.e., *Megabothris* sp. and the unidentified ceratophyllid), whereas the five specimens of *Catallagia*, all collected from the urban site, belonged to the same species based on their identical ITS-2 sequences. Multiple flea specimens (i.e., 6-84) were collected for each of the other six species (Table 5.3). *Oropsylla rupestris*, *O. bruneri* and *Neopsylla inopina* represented 187 (83%) of the 225 fleas collected. Of the nine species collected in this study, eight were found at the urban site, but only six species were present at the rural site (Table 5.3). Five species, *O. rupestris*, *O. bruneri*, *O. tuberculata*, *N. inopina* and *A. wagneri* were found at both sites. *Oropsylla labis*, *Catallagia* sp., and the unidentified ceratophyllid were only found at the urban site, while the single specimen of *Megabothris* sp. was collected from the rural site (Table 5.3). A comparison of the fleas collected in burrows from the two sites (Fig 5.2) showed that *O. rupestris* was the most abundant species (i.e., 50%) of all the fleas collected at the urban site, but only the second most abundant (15%) flea species at the rural site. In contrast, *O. bruneri,* was the most abundant flea at the rural site, representing 72% of the fleas collected, but was only the third most abundant (i.e., 10%) at the urban site. *Neopsylla inopina* accounted for 21% of the fleas collected from burrows at the urban site (Fig 5.2 and Table 5.3).

Table 5.3: Number of fleas collected from the burrows of Richardson's ground squirrels at the urban and rural sites sampled between April 30 and October 2, 2019.

Flea Species	Urban Site	Rural Site	Total
Oropsylla rupestris	76	8	84
Oropsylla bruneri	15	53	68
Neopsylla inopina	32	3	35
Oropsylla tuberculata	10	7	17
Oropsylla labis	8	0	8
Aetheca wagneri	4	2	6
Catallagia sp.	5	0	5
unidentified ceratophyllid	1	0	1
Megabothris sp.	0	1	1
Total	151	74	225

Figure 5.2: Comparison of the proportion of nine flea species collected from the burrows of Richardson's ground squirrels at the urban site (n = 151) and the rural site (n = 74) in central Saskatchewan.



There were also seasonal differences in the relative abundance of most flea species, and this also varied between sites (Fig 5.3 and Table 5.4). All four species of *Oropsylla* occurred in ground squirrel burrows throughout most of the sampling period, but there were some clear differences between species in their timing of occurrence. *Oropsylla bruneri* was collected in all three seasons at the rural site, whereas at the urban site, this species was only present in spring and summer. *Oropsylla labis* was only collected sporadically at the urban site in April, May, July and September. The peak period for *O. rupestris* was in July (urban site) or August (rural site), whereas *O. tuberculata* was more frequent in April and May at the urban site, and in May at the rural site. A majority of *N. inopina* were also collected in May (67% at the rural site and 72% at the urban site) with very few individuals in burrows after June. The single specimens of *Megabothris* sp. and unidentified ceratophyllid were collected in June and July, respectively. The small number of *A. wagneri* collected were detected in burrows from July to September. The five specimens of *Catallagia* sp. were collected from burrows in May, August, and September (Table 5.4).

Figure 5.3: Community structures of fleas in the burrows of Richardson's ground squirrels at the urban site and the rural site in central Saskatchewan during the different seasons.

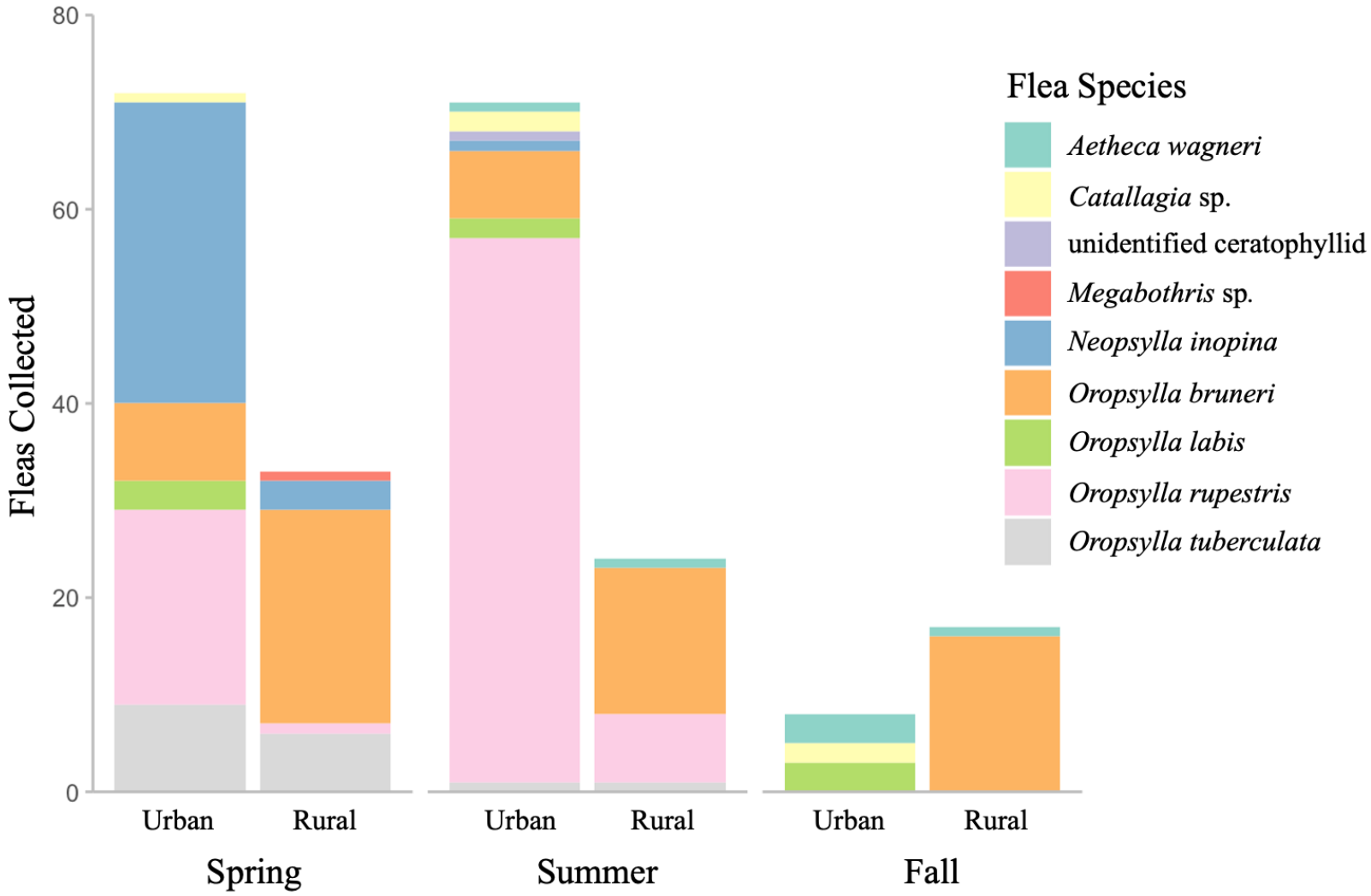


Table 5.4: Percentage of individuals of each flea species collected per month at each site.

Flea Species	Site (n*)	Spring			Summer		Fall
		April	May	June	July	August	September
Oropsylla rupestris	urban (76)	4	9	13	64	9	-
	rural (8)	-	-	13	13	75	-
Oropsylla bruneri	urban (15)	-	33	20	7	40	-
	rural (53)	-	32	9	9	19	30
Neopsylla inopina	urban (32)	22	72	3	-	3	-
	rural (3)	-	67	33	-	-	-
Oropsylla tuberculata	urban (10)	30	40	20	-	10	-
	rural (7)	-	86	-	14	-	-
Aetheca wagneri	urban (4)	-	-	-	-	25	75
	rural (2)	-	-	-	50	-	50
Oropsylla labis	urban (8)	12.5	25	-	25	-	37.5
Catallagia sp.	urban (5)	-	20	-	-	40	40
unidentified ceratophyllid	urban (1)	-	-	-	100	-	-
Megabothris sp.	urban (1)	-	-	100	-	-	-

* = number of individual fleas

5.5 DISCUSSION

Fleas that parasitize Richardson's ground squirrels generally belong to two families, the Ceratophyllidae and Ctenophthalmidae; however, some species in the Leptopsyllidae, Hystrichopsyllidae and Pulicidae have also been reported on this host species (Burgess 1955, Holland 1985, Galloway and Christie 1990, Lindsay and Galloway 1997, 1998, Hastriter 2023). They can also be separated into those species that are frequently found on *U. richardsonii* (i.e., "true parasites") and those that primarily occur on other host species but occasionally occur on Richardson's ground squirrels in areas where they coexist with these other host species (i.e., incidental parasites) (Holland 1985). In the present study, nine species of flea were detected in the burrows of Richardson's ground squirrels. Four of these, *A. wagneri*, an unidentified ceratophyllid, *Megabothris* sp. (Ceratophyllidae) and *Catallagia* sp. (Ctenophthalmidae), are incidental fleas of *U. richardsonii* (Holland 1985, Galloway and Christie 1990). *Aetheca wagneri* is primarily a parasite of mice (*Peromyscus* spp.); however, it has been documented on a wide range of mammalian hosts species (Hastriter 2023), including *U. richardsonii* (Galloway and Christie 1990, Lindsay and Galloway 1997). Although the species identity of the five specimens of *Catallagia* was uncertain, all had identical DNA sequences of the nuclear first internal transcribed spacer, suggesting they belong to a single species, the most likely of which is *C. decipiens*. This species is normally a parasite of small rodents (e.g., deer mice, voles, and jumping mice) but has been reported to parasitize *U. richardsonii* in Saskatchewan (Burgess 1955, Holland 1985). The single specimen of *Megabothris* collected could be one of three species reported in the province by Holland (1985); *M. asio* which parasitizes primarily meadow voles (e.g., *Microtus pennsylvanicus*), *M. lucifer* that occurs on a range of rodents (i.e., species of *Microtus* and *Peromyscus*) and *M. quirini* that is also associated with a variety of small rodents.

Of these, only *M. asio* has been reported on *U. richardsonii* in Saskatchewan (Holland 1985), but this species, along with *M. quirini*, have been recorded on Richardson's ground squirrels in Manitoba (Galloway and Christie 1990, Lindsay and Galloway 1997). Another species in the genus, *M. acerbus*, which is a common parasite of *Tamias striatus* (eastern chipmunks) (Holland 1985), has been reported on *U. richardsonii* in Manitoba (Galloway and Christie 1990) but is unlikely to occur in Saskatchewan given that eastern chipmunks do not occur in this province (Snyder 1982). The presence of different incidental flea species on *U. richardsonii* will vary significantly based on the relative abundance of any other mammalian hosts and their fleas that utilize the burrows of Richardson's ground squirrels. It is also possible that ground squirrels acquire incidental flea species if they explore the nests of small rodents in the same habitat.

Five of the nine taxa collected in the present study, *Oropsylla bruneri*, *O. labis*, *O. tuberculata*, *O. rupestris* (Ceratophyllidae), and *Neopsylla inopina* (Ctenophthalmidae), are known to parasitize Richardson's ground squirrels at many other locations throughout its broad distributional range (i.e., = "true parasites") (Burgess 1955, Holland 1985, Galloway and Christie 1990, Lindsay and Galloway 1997, 1998, Hastriter 2023). Another three species commonly found on Richardson's ground squirrels, *Thrassis bacchi*, *T. pandorae* (Ceratophyllidae), and *Rhadinopsylla fraterna* (Ctenophthalmidae) (Burgess 1955, Holland 1985, Galloway and Christie 1990, Lindsay and Galloway 1997, Hastriter 2023), were not detected in the burrows of *U. richardsonii* in the present study. This was surprising with respect to *R. fraterna* given that this species has been recorded on these hosts in southern Saskatchewan (Thoroughgood et al., unpublished data), and was the fourth most abundant flea collected from Richardson's ground squirrels over a three-year period in Darlingford, Manitoba (Lindsay and Galloway 1997). The three most abundant species at Darlingford were *O. rupestris*, *O. bruneri* and *N. inopina*

(Lindsay and Galloway 1997), which were also the three most abundant species found in the present study. Interestingly, Lindsay and Galloway (1997) did not detect *O. rupestris* on *U. richardsonii* at their other field sites (Perimeter and St. Norbert) located 120 km northeast of Darlingford (Lindsay and Galloway 1997). However, Darlingford is located on the west of the Manitoba escarpment (i.e., the transition from the western uplands to the eastern lowlands), whereas the other two sites are located 120 km to the northeast (Lindsay and Galloway 1997). *Oropsylla labis* and *O. tuberculata*, which accounted for 11% of fleas in the burrows of Richardson's ground squirrels in the present study, were not detected by Lindsay and Galloway (1997) on any of the almost 4,000 *U. richardsonii* they examined at Darlingford, St. Norbert and Perimeter. However, one female *O. labis* has been collected from a Richardson's ground squirrel near Coulter (southwestern Manitoba), while *O. tuberculata* has been recorded on *U. richardsonii* at several locations across Manitoba (Galloway and Christie 1990). Also, Galloway and Christie (1990) found that *O. tuberculata, O. rupestris, T. bacchi, N. inopina* and *R. fraterna* did not occur east of the Western Manitoba Uplands. This information, combined with the differences in presence/absence of some flea species between the urban and rural sites in central Saskatchewan, indicates that there is significant geographical variation in the presence/absence and relative abundance of the flea species that commonly parasitize *U. richardsonii.*

Several abiotic and biotic factors (e.g., soil type, precipitation levels, burrow microclimate, host diversity, density, body condition, immune status, heterozygosity, age, reproductive status and grooming behaviour, and interspecific competition among fleas) have been examined as possible factors influencing the diversity and/or abundance of rodent fleas at a given locality (Galloway and Christie 1990, Brinkerhoff et al. 2006, Friggens et al. 2010, Krasnov et al. 2010, Eads et al. 2016, Eads and Hoogland 2016, 2017, Russell et al. 2018,

Sackett 2018, Stark 2002). For example, variation in the relative abundance of fleas on prairie dogs has been linked to precipitation levels and the subsequent effects on soil moisture and vegetation growth, and reduced host fitness (Friggens et al. 2010, Eads and Hoogland 2017). During years of drought, when food sources (i.e., vegetation) are scarce, black-tailed prairie dogs have a reduced body condition and weakened immune system, and may spend less time grooming, resulting in an increased flea burden (Eads et al. 2016, Eads and Hoogland 2016). Although there were no major differences in climatic conditions (i.e., temperature and rainfall) or soil type between the two sites in our study, there were indeed differences in the vegetation, density of ground squirrel burrows, frequency of human activity, and the presence/absence of pavement, walking trails, irrigation, and drainage, all of which may contribute to differences in flea species present and/or their abundance at the two sites. Friggens and Beier (2010) showed that increased levels of human disturbance in urban sites compared to more remote rural sites can result in an increase in flea species diversity, and an increase in the prevalence and intensity of flea infestation on mammalian hosts. The results of the present study are in partial agreement with this in that more flea species were detected at the urban site, and the mean intensity of fleas in burrows was greater at the urban site, whereas flea prevalence did not differ between sites. The higher mean intensity of fleas in burrows at the urban site may also be a consequence of the higher density of host burrows, and possibly host individuals, at that site. The prevalence of fleas in the burrows of Richardson's ground squirrels at the urban site declined from spring (April-June), through summer (July-August) to the fall (September and October). This finding differs from the study by Liccioli et al. (2020), where they reported a decline in the prevalence of fleas in the burrows of black-tailed prairie dogs within Grasslands National Park in south-western Saskatchewan from April to August, followed by an increase in flea prevalence in September.

There appeared to be a similar pattern of flea prevalence at our rural site; however, the results of the statistical analyses showed that there was no significant difference in flea prevalence among seasons at this site. Fleas were collected from burrows up to either September 18 (urban site) or September 20 (rural site), but no fleas were collected from these dates until October 3 when sampling ended.

We did detect differences in the seasonal abundance of different flea species (i.e., "true parasites" of *U. richardsonii*) in burrows at both sites in central Saskatchewan. Although caution is advised in over interpreting flea abundance based on burrow swab data (Eads et al. 2021) because of the limitations of the sampling technique (e.g., failure to collect fleas in the deeper sections of a burrow; Eads 2017, Eads et al. 2021), there was some degree of concordance between our results and those studies that examined the seasonal activities of fleas on Richardson's ground squirrels (Lindsay and Galloway 1997, 1998). For example, the number of *N. inopina* in the burrows of Richardson's ground squirrels peaked in May at both sites of the present study which is consistent with the findings of Lindsay and Galloway (1997) who found that *N. inopina* had the highest abundance on Richardson's ground squirrels at three sites in Manitoba during May and June, and that their numbers then declined through to September. The occurrence of *N. inopina* in burrows at our sites was primarily from April to June, which is consistent with the statement by Bossard (2022) that this is a "spring flea". Lindsay and Galloway (1998) suggested that *N. inopina* may overwinter in ground squirrel burrows as nonreproductive adults or as adults and/or pupae within cocoons. In contrast to *N. inopina*, the peak abundance of *O. rupestris* in burrows occurred in July at the urban site and in August at the rural site, whereas Lindsay and Galloway (1997) found *O. rupestris* parasitized *U. richardsonii* at Darlingford (Manitoba) most often from June to early August. The relative abundance of *O.*

bruneri in ground squirrel burrows also appears to have two peaks, the first occurring in May at both the urban and rural sites, and then the peak in August at the urban site and in September at the rural site. This finding agrees with that of Lindsay and Galloway (1997) who found that *O. bruneri* had two peaks of infestation on *U. richardsonii* in May and August which were indicative of two generations of fleas per year. The first peak occurred on adult hosts, while the second peak involved fleas on juvenile hosts (Lindsay and Galloway 1997). This seasonal activity pattern of *O. bruneri* on hosts is also consistent with that on Franklin's ground squirrels (*Poliocitellus franklinii*) in Manitoba (Reichardt and Galloway 1994). Differences in seasonal abundance have also been reported previously by others for *O. tuberculata* and *O. labis*, species detected in the burrows of Richardson's ground squirrels in the present study. Cully et al. (1997) found that in the Moreno Valley of New Mexico (USA), *O. labis* primarily parasitizes Gunnison's prairie dogs in the summer, whereas *O. tuberculata* occurs on hosts in late fall-early spring. Similarly, Friggens et al. (2010) found that only *O. tuberculata* occurred on and in the burrows of Gunnison's prairie dogs within the Valles Caldera National Preserve (northern New Mexico) during spring and summer. Although our sample sizes for these two species were also relatively small (i.e., 8 *O. labis* and 18 *O. tuberculata*), *O. labis* was collected more often from the burrows of *U. richardsonii* during the summer and the fall, while *O. tuberculata* was found in burrows during spring and summer. Differences in seasonal abundance of *O. labis* and *O. tuberculata* in rodent populations in New Mexico and central Saskatchewan (a distance of > 2,400 km) is not unexpected given the major differences in environmental conditions, and the diversity, abundance and seasonal activity patterns of the different host species infested by these flea species. Changes in the microclimates of rodent burrows and nests, particularly the relative

humidity, is one important factor influencing the off-host survival of fleas and seasonal differences in their relative abundance (e.g., Stark 2002).

Five of the nine flea species collected from the burrows of Richardson's ground squirrels in central Saskatchewan (i.e., *O. bruneri, O. labis, O. rupestris, O. tuberculata* and *A. wagneri*) are vectors of *Y. pestis* (Wilder et al. 2008, Eisen and Gage 2009, Hastriter 2023), and are also known to parasitize black-tailed prairie dogs (Holland 1985, Russell et al. 2018, Liccioli et al. 2020). Sylvatic plague in black-tailed prairie dogs in Grasslands National Park, the only population of this rodent species in Canada, was first detected in 2010 (Antonation et al. 2014). Given that populations of black-tailed prairie dogs can suffer significant mortality (>90%) because of infection with *Y. pestis* (Cully and Williams 2001), the Canadian population of *C. ludovicianus* is considered at great risk of exposure to sylvatic plague and local extinction (Liccioli et al. 2020). These prairie dogs in southwestern Saskatchewan coexist with Richardson's ground squirrels (Liccioli et al. 2020), hence there is potential for the exchange of fleas between these host species, particularly in their burrows. Our findings of seasonal changes in the relative abundance of *Oropsylla* species in the burrows of *U. richardsonii* provides foundational knowledge for assessing the potential risk of *Y. pestis* transmission to *C. ludovicianus* in southwestern Saskatchewan. Additional research is also needed to determine if the fleas parasitizing *U. richardsonii* and *C. ludovicianus* in Saskatchewan are hosts for other bacterial pathogens (e.g., *Rickettsia* and *Bartonella* spp.).

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5.7 TRANSITION STATEMENT

The results of the previous two chapters (Chapters 4 and 5), showed that 11 flea species, representing two families, occurred on Richardson's ground squirrels and/or were present in their burrows. I also demonstrated differences in flea species diversity and abundance between sites. Some of these flea species are vectors of pathogenic bacteria. This raises the question whether all fleas associated with Richardson's ground squirrels have the same bacterial species in their microbiomes? In the next chapter (Chapter 6), I examine the presence of three bacterial genera (e.g., *Bartonella*, *Rickettsia* and *Wolbachia*) within the fleas collected from the burrows of Richardson's ground squirrels at the urban and rural site in central Saskatchewan. The first objective is to determine if the bacterial genera reported in other flea species are also present in fleas associated with Richardson's ground squirrels. The second objective is to determine the relative abundance of these bacteria.

CHAPTER 6: EXAMINATION OF THE COMMUNITY COMPOSITION OF BACTERIA IN FLEAS COLLECTED FROM THE BURROWS OF RICHARDSON'S GROUND SQUIRRELS (*Urocitellus richardsonii*) IN AN URBAN AND RURAL SITE IN CENTRAL SASKATCHEWAN, CANADA[5]

6.1 ABSTRACT

Fleas are important vectors of a multitude of pathogens that can infect wildlife, domestic animals and humans. Knowledge of the presence and relative abundance of different bacterial species in fleas is important for understanding the risk of disease in vertebrates, including humans. The bacterial community of fleas has been demonstrated to be dominated by a single phylotype usually of one of three genera; *Rickettsia*, *Wolbachia*, or *Bartonella*. The objective of this study was to determine the frequency and quantity of *Rickettsia*, *Wolbachia*, and *Bartonella* in nine species of fleas associated with Richardson's ground squirrels using qPCR and conventional PCR. The results revealed that these fleas had a low bacterial load (log ratio to starting quantity of flea 28S DNA = 0.41 to 0.79) that was influenced by the collection location and the presence of a blood meal in fleas. The prevalence of *Rickettsia*, *Wolbachia*, and *Bartonella* in fleas was low based on the results of qPCR analyses. Sequences of the citrate synthase gene of *Bartonella* detected in the gDNA of *Oropsylla bruneri* were most similar to *B. washoensis*. Two distinct strains of *Wolbachia* were also isolated from *Oropsylla* and *Neopsylla inopina* using conventional PCR. Multiple bands were present when investigating the presence of *Wolbachia* using the surface protein suggesting the possibility of mixed infections. We also report the novel detection and characterization of a *Rickettsia*-like endosymbiont in three *Oropsylla* species and

[5] This chapter is in collaboration with C. Diyes and Neil Chilton.

Neopsylla inopina. Further investigation is required to understand the diversity and abundance of *Rickettsia*-like endosymbionts in Siphonaptera.

6.2 INTRODUCTION

In recent years, there has been an increasing interest in the diversity and community structure of bacteria within insects. The relationship between insects and bacteria in their digestive and reproductive tissues can be mutualistic (i.e., mutually beneficial to both organisms), beneficial for the bacterium with no negative or positive effect on the insect host (i.e., commensalism), or detrimental to the insect host (Dillon and Dillon 2004, Feldhaar and Gross 2009, Gündüz and Douglas 2009, Kaltenpoth and Flórez 2020, Duplais et al. 2021). Insects acquire bacteria by vertical transmission (e.g. from mother to offspring) and/or horizontal transmission (e.g. interaction with environment and other individuals) (Kikuchi et al. 2007, Kremer and Huigens 2011, Gonella et al. 2015, Onchuru et al. 2018). The microbiota of insects differs among species within each order (Jones et al. 2010, Colman et al. 2012, Vásquez et al. 2012, Hawlena et al. 2013, Hammer et al. 2014, Cohen et al. 2015, Alves et al. 2016). Differences in bacterial diversity and abundance within microbiomes may be related to the diet of the insect host (Colman et al. 2012, Jones et al. 2013, 2019, Yun et al. 2014, Chung et al. 2017, Zheng et al. 2021), and the spatial or temporal aspects of the insect hosts (Clay et al. 2008, Jones et al. 2010, 2012, Schmid et al. 2015, Muturi et al. 2018). The diversity within the bacterial community of an arthropod vector can be affected by the presence of a blood meal and the exposure to different host individuals and species (Wang et al. 2011, Lawrence et al. 2015). The ability of bacteria to colonize a host can be inhibited by the presence of other bacteria present within the community (Dillon et al. 2005, Cardoza et al. 2006, Deng et al. 2022).

Adult fleas are hematophagous insects that parasitize a variety of mammals and birds (Holland 1985, Whiting et al. 2008). During the egg, larval, and pupal stages, fleas are non-parasitic and feed on organic material within the burrows and nests of their hosts. Adult fleas are particularly susceptible to colonization by microbes through their midgut epithelia during a bloodmeal as they do not produce a peritrophic matrix (Erickson et al. 2009). This matrix is a protective membrane expected to shield the midgut epithelium from digestive enzymes, toxins, and some microbes (Greene et al. 2015). The adults of some flea species are important vectors of *Yersinia pestis, Rickettsia felis, R. typhi*, and *Bartonella henselae* (Eisen and Gage 2012). These bacteria are the causative agents of the plague, flea-borne spotted fever, murine typhus, and cat scratch disease, respectively (Eisen and Gage 2012). Fleas acquire infections of *Y. pestis, B. henselae*, and *R. typhi* by feeding on infected mammalian hosts (i.e., horizontal transmission) and have the potential to transmit them to susceptible hosts (Eisen and Gage 2012).

The bacterial communities of fleas exhibit similar species richness and diversity to the bacteria present in the blood of the mammalian host they parasitize (Cohen et al. 2015). This indicates that acquisition of a blood meal is a key factor determining the bacterial communities of fleas. Although most flea species are host generalists, vector specificity is exhibited by the composition of the bacterial community of the arthropod vector (Hawlena et al. 2013, Jones et al. 2015). For example, *Bartonella* spp. are the most dominant bacteria in the fleas *Orchopeas leucopus* and *Ctenophthalmus pseudagyrtes*, species that parasitize *Peromyscus leucopus* and *Microtus ochrogaster* (Hawlena et al. 2013). In contrast, the microbiota of the ticks *Ixodes scapularis* and *Dermacentor variabilis*, which feed on the same species of rodent host as *O. leucopus* and *C, pseudagyrtes*, are often dominated by bacterial endosymbionts, that includes

Rickettsia buchneri in *I. scapularis* and *Francisella*-like endosymbionts in *D. variabilis* (Hawlena et al. 2013).

Species diversity of bacteria in flea microbiomes is generally relatively low, with Proteobacteria representing the most prevalent and abundant taxa in bacterial communities (Jones et al. 2013). Low bacterial diversity in microbiomes may be a consequence of endosymbionts (i.e., bacterial mutualists) blocking the invasion of new bacterial species (Dillon and Dillon, 2004). Furthermore, the host's immune response and low nutrient availability in the blood can also result in a lower species diversity of bacteria in microbiomes (Graf et al. 2006, Worthen et al. 2006, Jones et al. 2010). For example, in three species of the genus *Oropsylla, O. hirsuta, O. montana,* and *O. tuberculata cynomuris,* there is a negative interaction between the relative abundance of two dominant groups of bacteria, *Bartonella* and Rickettsiales (Jones et al. 2012).

Bartonella, Rickettsia, and/or *Wolbachia* typically dominate the bacterial communities of fleas in North America (Jones et al. 2008, 2010, 2012, 2013, Hawlena et al. 2013, Lawrence et al. 2015, Vasconcelos et al. 2018). *Bartonella* are facultative parasites of red blood cells of a variety of mammals (Kosoy 2010, Kosoy et al. 2018). Different species and strains of *Bartonella* are hypothesized to be specific for certain groups of rodent hosts (Jardine et al. 2005). For example, thirty different strains of *B. washoensis* have been found in black-tailed prairie dogs (*Cynomys ludovicianus*) (Jardine et al. 2005, Bai et al. 2007), Richardson's ground squirrels (*Urocitellus richardsonii*) (Jardine et al. 2005, 2006b), and Franklin's ground squirrels (*Spermophilus franklinii*) (Jardine et al. 2005). *Bartonella washoensis* has also been associated with a case of human myocarditis in Washoe County, Nevada (Kosoy et al. 2003, Kosoy 2010). This bacterium has also been detected in *Oropsylla hirsuta* (Bai et al. 2007), a flea commonly

associated with *C. ludovicianus*, except for the population in southern Saskatchewan (Holland 1985). Although *Bartonella* has not been examined in fleas feeding on *U. richardsonii*, 48% of Richardson's ground squirrels in central Saskatchewan have been shown to be infected with *Bartonella* (Jardine et al. 2006a). Also, juvenile ground squirrels have a significantly greater prevalence of infection than adults (Jardine et al. 2006a). However, *Bartonella* infection varies throughout the year, with the peak infection occurring in July (Jardine et al. 2006a). Four genotypes of *Bartonella* have been detected in Richardson's ground squirrels collected near Saskatoon in Saskatchewan (Jardine et al. 2005). One of these genotypes (H) was found in 79% of infected Richardson's ground squirrels (Jardine et al. 2005).

Rickettsiales bacteria, belonging to two genera, *Rickettsia* and *Wolbachia*, can have a significant impact on the prevalence of *Bartonella* in fleas (Jones et al. 2008, 2012, Cohen et al. 2015, Vasconcelos et al. 2018). *Rickettsia* are gram-negative bacteria that are organized into four main groups; the basel ancestral group, the spotted fever group which includes the human pathogen, *Rickettsia rickettsii* ticks, the transitional group which includes *R. felis* transmitted by fleas, and the typhus group, that includes *R. typhi* which is transmitted by fleas and *R. prowazekki* which is vectored by lice to vertebrate hosts (Azad and Beard 1998, Eisen and Gage 2012, Blanton 2019). *Wolbachia* are intracellular bacteria of a wide variety of invertebrate hosts and are transmitted transovarially (Hilgenboecker et al. 2008, Werren et al. 2008, Kaur et al. 2021). It has been estimated that 66% of all insect species have a species-specific strain of *Wolbachia* (Hilgenboecker et al. 2008). Some *Wolbachia* strains are known to distort sex ratios, cause cytoplasmic incompatibility, and inhibit the colonization of disease pathogens in their insect host (Hilgenboecker et al. 2008, Werren et al. 2008). Differences in bacterial communities

of males and females of some species of *Oropsylla,* can be attributed to the presence of *Wolbachia* within the microbiota of the female fleas (Cohen et al. 2015).

Investigations of the bacteria present in Siphonaptera in North America has mainly focused on four flea species, the cat flea, *Ctenophthalmus felis* (Vasconcelos et al. 2018), and *Oropsylla tuberculata*, *Oropsylla hirsuta*, and *Oropsylla montana*, which are parasites of black-tailed prairie dogs and vectors of *Y. pestis* (see Jones et al. 2008, 2010, 2012, 2013, Erickson et al. 2009). Black-tailed prairie dogs and Richardson's ground squirrels, which coexist in Grasslands National Park in south-western Saskatchewan (Liccioli et al. 2020), share several flea species, including *O. labis*, *O. tuberculata, O. rupestris*, *Thrassis bacchi* and *Rhadinopsylla fraterna* (Holland 1985, Liccioli et al. 2020, Thoroughgood et al. 2021). Richardson's ground squirrels are considered to be reservoirs of *Y. pestis*; however, their role in plague epizootics is poorly understood (Fitzgerald 1970, Anderson and Williams 1997, Cully and Williams 2001). Despite the broad distribution of *U. richardsonii* (Michener and Koeppl 1985, Augustine et al. 2023), little is known of the bacteria present within fleas associated with these sciurid rodents in central Saskatchewan, except for studies on *Bartonella* conducted by Jardine et al. (2005, 2006a, 2006b). Studies on the bacterial communities within fleas associated with *U. richardsonii* are lacking. The objective of this chapter is to use PCR-based assays to determine the prevalence of bacteria in flea species associated with Richardson’s ground squirrels at an urban and rural site in central Saskatchewan. The focus is on bacterial genera commonly reported in fleas (i.e., *Bartonella*, *Rickettsia* and *Wolbachia* spp.). If these bacteria are present in fleas from one or both sites, real-time or quantitative PCR (qPCR) will be used to determine their relative abundance.

6.3 MATERIALS AND METHODS

6.3.1 Flea samples, DNA extraction and PCR assays

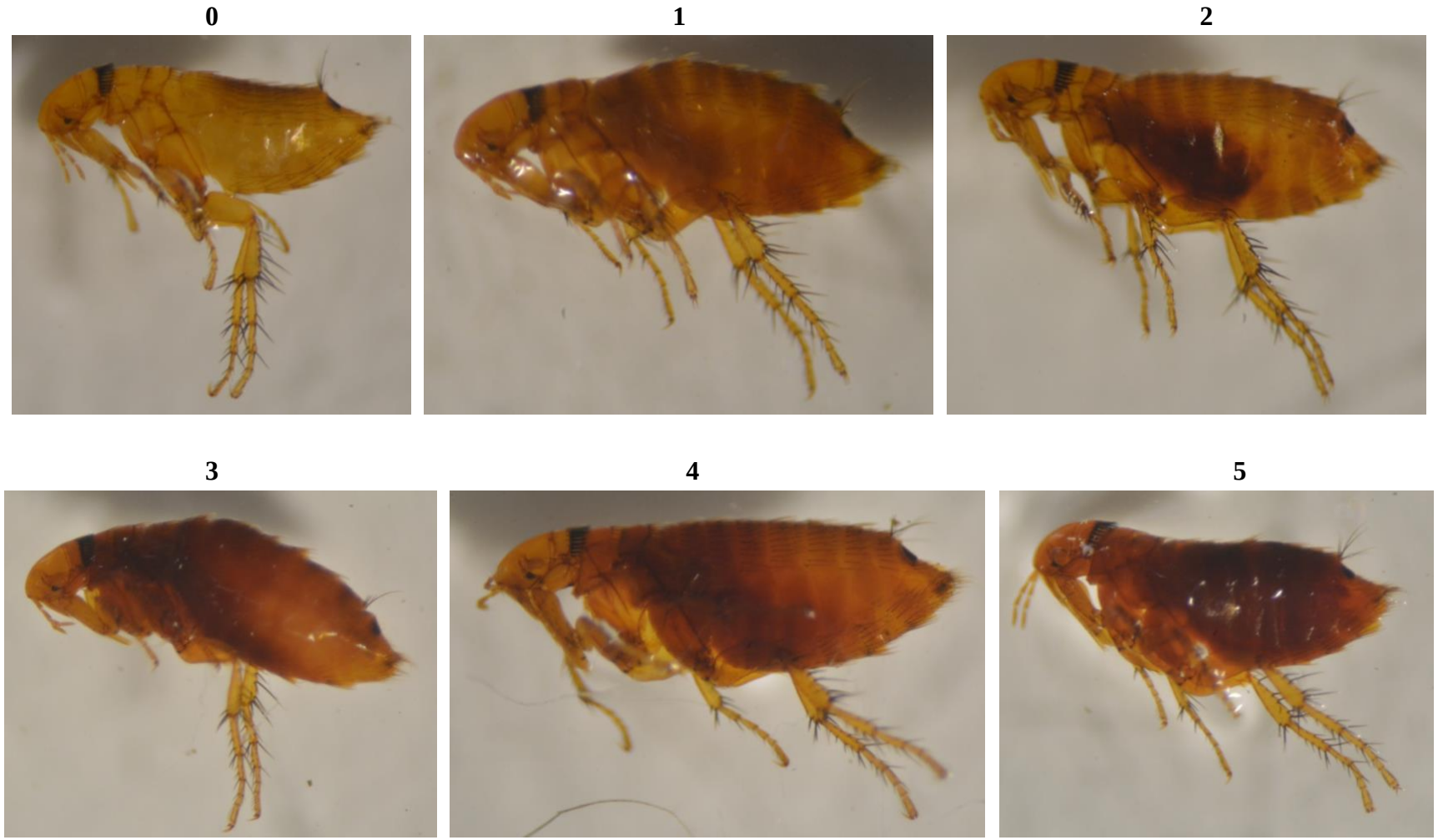
Fleas were collected from the burrows of Richardson's ground squirrels from an urban site, the University of Saskatchewan campus in Saskatoon (52°08'12.2"N 106°37'59.9"W), and from a rural site near Bradwell (51°52'54.2"N 106°14'26.1"W) as part of a seasonal activity pattern and diversity study conducted in 2019 (Chapter 5; Thoroughgood and Chilton 2024). Two hundred and twenty-five fleas collected in that study (i.e., from April 30th to September 20th) were used in the present study (Table 6.1). Microscopic examination of fleas was conducted to determine the presence and abundance of a blood meal. The following scale (0-5 scale) was used to define blood meal status; 0 = no obvious blood meal, 3 = an older blood meal based on presence of pigments, and 5 = a full fresh blood meal (Fig. 6.1). The species identity of each flea was determined, where possible, based on the DNA sequencing results of the nuclear 28S rRNA gene (Thoroughgood et al. 2021) or the nuclear second internal transcribed spacer (ITS-2) (Chapter 3). Genomic (g) DNA was extracted from the total body of each individual flea according to Dergousoff and Chilton (2007).

Several conventional PCR-based assays were conducted to detect if *Bartonella*, *Wolbachia*, and/or *Rickettsia* were present in the microbiomes of the 225 fleas. These bacterial genera are commonly detected in the microbiomes of other flea species (Jones et al. 2008, 2010, 2012, 2013, Hawlena et al. 2013, Lawrence et al. 2015, Vasconcelos et al. 2018). Real-time or quantitative PCR (qPCR) assays were used to determine the total bacterial load within individual fleas, and to quantify flea DNA in a gDNA sample. The latter was used as a common denominator in calculations of relative bacterial DNA among flea gDNA samples. Negative control (i.e., no gDNA) samples were included in each conventional and qPCR assay.

Table 6.1: Number of fleas collected from the burrows of Richardson's ground squirrel at two sites that were used to examine the bacteria in their microbiomes.

Flea Species	Urban Site	Rural Site	Total
Oropsylla rupestris	76	8	84
Oropsylla bruneri	15	53	68
Neopsylla inopina	32	3	35
Oropsylla tuberculata	10	7	17
Oropsylla labis	8	0	8
Aetheca wagneri	4	2	6
Catallagia sp.	5	0	5
Megabothris sp.	0	1	1
unknown ceratophyllid	1	0	1
Total	151	74	225

Figure 6.1: Representative images of the blood meal scale (0-5) in *Oropsylla rupestris*.



The methods used for agarose gel electrophoresis of amplicons produced by conventional PCR assay, and the purification of amplicons for DNA sequencing, were the same as those conducted as in Thoroughgood et al. (2021, Chapter 2). DNA (i.e., nucleotide and amino acid) sequences were manually aligned and analyzed using Geneious Prime 2021.2.2 (https://www.geneious.com). For protein coding genes, amino acid sequences were generated using bacterial genetic code and determined by the most readable reading frame. Blast searches (GenBank) were conducted on nucleotide and amino acid sequences to determine the identity of sequences. Phylogenetic analyses, using the neighbor-joining method in PAUP v4.0b10 (Swofford 2002), were conducted on the sequence data to determine the relationships of the bacterial taxa detected in fleas associated with Richardson's ground squirrels.

6.3.2 Flea 28S rRNA gene qPCR assay

The abundance of flea DNA in all 225 gDNA samples was assessed by qPCR of part (309 bp) of the nuclear 28S ribosomal RNA (rRNA) gene. The sequence of primers and probe (fluorescently labelled with a FAM reporter with a Tamra quencher) for this qPCR assay (Table 6.2) was designed based on an alignment of the 28S rRNA gene sequences of the flea species associated with Richardson's ground squirrels (Chapter 3). This qPCR assay was conducted in low-profile Axygen™ 96-well PCR microplates (Axygen Biosciences, CA, USA) or Bio-Rad 0.2 mL thin-wall 8 tube strips with optical flat 8-cap strips (Bio-Rad Laboratories, inc. France and USA). Real-time PCRs were performed using 20 μL reactions containing 10 μL of 2xPrimeTime Gene Expression Master Mix (Integrated DNA Technologies [IDT], Coralville, Iowa, USA), 500 nM of forward and reverse primers, 250 nM of the labelled probe (IDT), and 3 μL of template or water (the latter representing a negative control). The cycling conditions used

were: 95°C for 3 minutes, then 40 cycles of 95°C for 15 seconds and 54°C for 1 minute. A standard curve was developed using a serial dilution of the constructed linear double-stranded DNA sequence, from here on, referred to as a gBlock, which included the binding sites of the forward and reverse primers and a fluorescent probe (Table 6.2). This gBlock, and the others used in this study for other genetic markers (see below), were designed with the target (probe) sequences as well as an internal control, which were the primers and probes for the detection of the *Rickettsia* citrate synthase gene (*gltA*). Resuspension and serial dilution procedures follow those of Diyes (2024), where a gBlock was resuspended in TE buffer to create a 10 ng per μL solution. A ten-fold serial dilution from 10^8 to 10 times was prepared using 20 μL of gBlock and 180 μL of DNase & RNase free water. Four flea gDNA samples were duplicated on multiple qPCR plates for between run comparisons whereas seven flea samples were duplicated on the plate for in-run variation. Only qPCR runs using a gBlock with an efficiency of above 90% and a R^2 value near 1 were considered accurate for analysis.

6.3.3 Total bacteria load qPCR assay

Initially, the objective was to characterize all bacterial taxa in the microbiomes of several flea samples using next-generation sequencing (NGS) targeting the chaperonin-60 gene (*cpn60*). Although region(s) of the bacterial 16S ribosomal RNA (rRNA) were the conventional molecular target used in NGS to determine the bacterial diversity and structure of the microbiomes in a variety of vertebrate and invertebrates (Hawlena et al. 2013, Nelson et al. 2013, Godon et al. 2016), *cpn60* provides a greater resolution for the identification of bacteria at the species level (Links et al. 2012). This molecular target has been used to examine the bacterial communities in humans (Schellenberg et al. 2009, 2017, Dos Santos et al. 2023) and sheep

Table 6.2: Sequences of the primers, fluorescent probe and gBlock used for the flea 28S rRNA gene qPCR assay.

	Sequence (5’ – 3’)
Forward primer	TTCAGGACCCGTCTTGAAACAC
Reverse primer	AATAAGACGCCCCGGGATTG
Probe	Tamara-CTAGCATGTGCGCAAGTCATTGGG-FAM
gBlock*	GCTTGACGCTTCAGGACCCGTCTTGAAACACCTTAC GGGAGTACTAGCATGTGCGCAAGTCATTGGGGTACT ACTCAATCCCGGGGCGTCTTATTATGGAGTATATAG GACAACCGTTTATTTATAACCTGATAATTCGTTAGA TTTTACCGATAAATTTCTGCATATGATGTTTGAGTAA TGGCTG

*No. of copies in resuspended gBlock per 1 μL of stock = 5.22×10^8

(Umbach et al. 2023). Primers were adapted to target the *cpn60* region of bacteria commonly found in arthropod vectors, which included *Rickettsia* (GCP Diyes pers. Comm.). Unfortunately, no amplicons were produced during the initial PCR of the NGS analyses for gDNA samples derived from fed and unfed fleas; so NGS was abandoned following multiple attempts at different dilutions and extraction concentrations. Given this outcome, the total bacterial load in each flea gDNA sample was assessed by qPCR. A standard-curve for total bacterial load was determined using a gBlock targeting the 16S rRNA gene (Table 6.3). The same PCR conditions were used as in the flea qPCR assay (see section 6.3.2) except that the annealing temperature used was 57°C. As this assay was targeting the 16S rRNA gene, which is ubiquitous in all bacteria, there was an expectation that the "negative" control samples may also show some amplification due to bacteria present in the master mix.

6.3.4 Detection of *Bartonella* by conventional PCR and by qPCR

The presence of *Bartonella* in fleas was assessed in all 225 fleas using a qPCR assay targeting a 301 bp region of the transfer messenger RNA (*ssrA* gene). There is a single-copy gene in *Bartonella* species of this transfer mRNA molecule in prokaryotes (Karzai et al. 2000, Diaz et al. 2012). This assay has been designed to detect over 30 species of *Bartonella* (Diaz et al. 2012). The same amplification conditions were used as those described in the flea qPCR assay (see section 6.3.2). A conventional PCR assay targeting the citrate synthase gene (*glt*A) (Table 6.4) was used to determine the specific species or strain of *Bartonella* present within 40 flea gDNA samples. These conventional PCRs were conducted in 25 μL mixtures containing 5X Phusion Green HF buffer (Fisher Scientific), 2.5 mM $MgCl_2$, 200 μM of each dNTP, 0.75 μM of each primer, 1.25 U of Phusion Hot Start II polymerase (Fisher Scientific), and 2 μL of DNA template or water for

Table 6.3: Sequences of the primers and fluorescent probe (from Abzazou et al. 2018), and gBlock (Diyes 2024) used in the qPCR assay to determine the total bacterial load in flea gDNA samples.

	Sequence (5’ – 3’)
Forward primer (1055f)	ATGGCTGTCGTCAGCT
Reverse primer (1329r)	ACGGGCGGTGTGTAC
Probe	Tamara-CAACGAGCGCAACCC-FAM
gBlock*	ATGGAGTATATAGGACAACCGTTTATTTATAACCTGA TAATTCGTTAGATTTTACCGATAAATTTCTGCATATGA TGTTTGAGTAATGGCTGTCGTCAGCTCATAGCATATG CAACGAGCGCAACCCCATATGTACACACCGCCCGTTG TCCCGAT

* No. of copies in resuspended gBlock per 1 μL of stock = 6.26×10^8

Table 6.4: Sequences of the qPCR primers, fluorescent probe and gBlock used for the *ssrA* qPCR assay (from Diaz et al. 2012), and the primers of the conventional PCR assay (from Gundi et al. 2012) used to amplify the gltA gene of *Bartonella*.

	Sequence (5' – 3')
***ssrA* qPCR assay**	
Forward primer	GCTATGGTAATAAATGGACAATGAAATAA
Reverse primer	GCTTCTGTTGCCAGGTG
Probe	Tamara-ACCCCGCTTAAACCTGCGACG -FAM
gBlock*	TTACGGCTATGGTAATAAATGGACAATGAAATAAGCTTG ACGCGACCCCGCTTAAACCTGCGACGGCCGATATCACCT GGCAACAGAAGCATGGAGGTATATAGGACAACCGTTTAT TTATAACCTGATAATTCGTTAGATTTTACCGATAAATTTC TGCATATGATGTTTGAGTAATGGCTG
Conventional *glt*A PCR assay	
CS443F	GCTATGTCTGCATTCTATCA
CS1210R	GATCYTCAATCATTTCTTTCCA

* No. of copies in resuspended gBlock per 1 μL of stock = 5.33×10^8

negative controls. The amplification conditions used were 95°C for five minutes, then 40 cycles of 95°C for 30 seconds, 60°C for 60 seconds and 72°C for 30 seconds, followed by a final extension at 72°C for five minutes.

6.3.5 Detection of *Wolbachia* by conventional PCR and by qPCR

Preliminary testing for the presence of *Wolbachia* in fleas involved targeting the 16S rRNA gene (Braig et al. 1998) and a surface protein gene (*wsp*) of this bacterial genus (O'Neill et al. 1992). This testing was conducted on 22 flea samples (i.e., from Chapters 2 and 4), representing *Oropsylla rupestris* (n=12), *O. tuberculata* (n=2), *Neopsylla inopina* (n=6), *O. bruneri* (n=1), and *Rhadinopsylla fraterna* (n=1). The objective was to determine which molecular target would be used for further screening of other flea gDNA samples. Conventional PCR analyses were conducted in 25 μL volumes containing 5X Phusion Green HF buffer (Fisher Scientific), 1.75 mM $MgCl_2$, 200 μM of each dNTP, 0.75 μM of each primer (Table 6.5), 1.25 U of Phusion Hot Start II polymerase (Fisher Scientific), and 1.5 μL of DNA template or water (= negative control). The amplification conditions used for *wsp* screening were an initial denaturing at 95°C for 5 minutes, followed by 35 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 60 seconds, and a final extension of 72°C for 5 minutes. The cycling conditions for the 16S rRNA gene were an initial denaturing at 95°C for 5 minutes, followed by 40 cycles of 95°C for 30 seconds, 52°C for 30 seconds, and 72°C for 120 seconds, and a final extension of 72°C for 5 minutes. Further screening was conducted on 216 flea samples representing the nine species from Table 6.1 targeting the *wsp* gene. Following the initial screening for *Wolbachia*, qPCR was conducted on 50 flea gDNA samples using primers and probes (Table 6.5) designed in this study

Table 6.5: Primer sequences for conventional PCR targeting the 16S rRNA gene (from Braig et al. 1998) and *wsp* (from O'Neill et al. 1992) of *Wolbachia*. Also included are the sequences of the primers, fluorescent probe and gBlock (designed in the present study) used in the qPCR assay to amplify the *Wolbachia wsp* gene.

	Sequence (5' – 3')
Conventional *Wolbachia* 16S gene qPCR assay	
Forward primer	CACAGAAGAAGTCCTGGCTAAC
Reverse primer	CGCCCTTTACGCCCAATAA
Conventional *Wolbachia wsp* assay	
Forward primer	TTGTAGCCTGCTATGGTATAACT
Reverse primer	ACATGAAAATCATACCTATTC
***Wolbachia wsp* qPCR assay**	
Forward primer	GGTGCATTTGGTTACAAAATG
Reverse primer	TGTAACTCCAGAAATCAAACTC
Probe	Tamara-ATTGAAGATATGCCTATCACTC-FAM
gBlock*	GCCGATATGGTGCATTTGGTTACAAAATGATGGAGTATA TTGAAGATATGCCTATCACTCCTTACGGAGTTTGAT TTCTGGAGATGGAGTATATAGGACAACCGTTTATT TATAACCTGATAATTCGTTAGATTTTACCGATAAAT TTCTGCATATGATGTTTGAGTAATGGCTG

*No. of copies in resuspended gBlock per 1 μL of stock = 5.27×10^{10}

targeting a ~300 bp fragment of the *Wolbachia wsp* gene based on the sequence alignment (Table 6.5).

6.3.6 Detection of *Rickettsia* by conventional PCR and by qPCR

All 225 flea gDNA samples were screened for the presence of *Rickettsia* using the qPCR assay of Wölfel et al. (2008) which targets the rickettsial citrate synthase-encoding gene (*gltA*) (Table 6.6). This assay detects both *Rickettsia felis* (a member of the spotted fever group of *Rickettsia*) and *R. typhi* (a member of the typhus group of *Rickettsia*), species recorded in fleas of several species, such as *Ctenocephalides felis* (Reif et al. 2008, Nogueras et al. 2013, Thepparit et al. 2013), *Xenopsylla ramesis* (Rzotkiewicz et al. 2015), and *Xenopsylla cheopsis* (Leulmi et al. 2014). All flea gDNA samples that had CT values of 3-40 for the *gltA* qPCR assay were subjected to a conventional nested PCR (nPCR) assay designed to amplify part (434 bp) of the rickettsial 17 kDa surface antigen gene (Heise et al. 2010) (Table 6.6). Also included in these PCR analyses were no gDNA (i.e., negative) controls and a positive control sample, the gDNA of an adult Rocky Mountain wood tick, *Dermacentor andersoni*, known to contain the DNA of *Rickettsia peacockii*. PCRs were performed in a 25 µL solution volume containing 200 µM of each dNTP, 2.5 mM $MgCl_2$, 0.5 U of Phusion Hot Start II polymerase (Fisher Scientific), 50 pmol (2 µM) of primers, and 2 µL of template gDNA. The cycling conditions used were: 95ºC for 5 min, followed by 35 cycles of 95ºC for 60 s, 54ºC for 60 s, and 72ºC for 60 s, with a final extension at 72ºC for 5 min.

Table 6.6: Sequences of the qPCR primers and probe (= PanRick assay of Wölfel et al. 2008) used to amplify the rickettsial citrate synthase-encoding gene (*gltA*).

	Sequence (5' – 3')
PANRICK qPCR assay	
Forward primer	ATAGGACAACCGTTTATTT
Reverse primer	CAAACATCATATGCAGAAA
Probe	Tamara- CCTGATAATTCGTTAGATTTTACCG -FAM
Conventional PCR assay targeting 17KDa gene	
Forward primer (17K-5)	GCTTTACAAAATTCTAAAAACCATATA
Reverse primer (17K-3)	TGTCTATCAATTCACAACTTGCC

Note: the gBlock used for this assay was the same as that shown in Table 6.5 of *Wolbachia*.

6.3.7 Detection of novel *Rickettsia*-like endosymbionts by conventional PCR

Sequence analyses of one *Rickettsia* 17 kDa sequence produced from one rural adult *O. rupestris* revealed the presence of a *Rickettsia*-like endosymbiont (RLE) (6.3.6). To further describe the *Rickettsia*-like endosymbionts in fleas, conventional PCR assays were conducted on several specimens of *O. rupestris* and *N. inopina*. These assays targeted the citrate synthase gene, the 16S rRNA gene, the cytochrome oxidase A gene, and the RLE 17 kDa antigen gene (Table 6.7). PCRs were carried out in a 25 µL solution volume containing 200 µM of each dNTP, 2.5 mM $MgCl_2$, 0.5 U of Phusion Hot Start II polymerase (Fisher Scientific), 50 pmol (2 µM) of primers, and 2 µL of template gDNA or 2 µL of water for negative controls. The cycling conditions for cytochrome oxidase A and 16S rRNA genes were 98°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, with a final extension at 72°C for 10 min. The cycling conditions for the 17 kDa gene were: 98°C for 5 min, followed by 35 cycles of 98°C for 30 s, 54°C for 30 s, and 72°C for 120s, with a final extension at 72°C for 10 min. The conditions for the citrate synthase gene followed that for the 17 kDa gene except that the denaturing, annealing, and extension times were increased from 30 s to 60 s.

Table 6.7: Sequences of the PCR used to investigate *Rickettsia*-like endosymbionts

	Sequence (5’ – 3’)
Citrate Synthase gene (*gltA*) – Pilgrim (2020)	
RiGltA405-F	GATCATCCTATGGCA
RiGltA1193-R	TCTTTCCATTGCCCC
Cytochrome oxidase A gene (*coxA*) – Pilgrim (2020)	
RiCoxA317_F	ATAGGTGCACCGGATATGGC
RiCoxA1409_R	CCGATAGATGATACCATATTCCA
16S rRNA gene – Pilgrim (2020)	
Ri170-F	GGGCTTGCTCTAAATTAGTTAGT
Ri1500_R	ACGTTAGCTCACCACCTTCAGG
17 kDa gene – Pilgrim (2020)	
Ri17kD_F	TCTGGCATGAATAAACAAGG
Ri17kD_R	ACTCACGACAATATTGCCC
17 kDa gene – Pilgrim et al. (2017)	
Ri_Meg17KD_F	GGCATGAATAAACAAGGT
Ri_Meg17KD_R	GAGTATACTCACGACAATATT

6.4 RESULTS

6.4.1 Flea 28S rRNA gene

As fleas are blood feeding arthropods, we assessed the presence and estimated the existing blood meal according to a 0-5 scale (Fig. 6.1) in our samples collected from burrows of Richardson's ground squirrels (Table 6.8). To assess the flea DNA in each of the samples, primers were designed based on the 28S rRNA alignment of flea species collected from Richardson's ground squirrels (Chapter 3). A standard curve was generated based on the DNA concentration of the standard curve and the cycling threshold of the gBlock control (Fig. 6.2). The starting quantity of DNA was significantly affected by the presence of a blood meal ($W = 1993$, $p = 0.0029$) and the location of sampling ($W = 7337$, $p = 2.8 \times 10^{-6}$). The cycling threshold (CT) values ranged from 15.2 – 21.9 (Table 6.9).

6.4.2 Total bacterial load in fleas

To assess the bacterial load in the flea samples, the starting quantity of the 16S rRNA gene was determined based on the standard curve derived from DNA concentration of the gBlock and the cycling threshold (Fig. 6.3A). The CT value of samples ranged from 20.73 to 35.15 while the negative control ranged from 36.72-39.67 (Table 6.10). The presence of a blood meal did have an effect on the starting quantity of total bacteria ($W = 2141$, $p = 0.014$). Fleas from the rural site had a significantly higher total bacterial load on average in comparison to fleas from the urban site ($W = 3349$, $p = 3.6 \times 10^{-6}$). The ratio of total bacteria DNA to flea DNA on the log transformed scale ranged from 0.11 – 3.64 (Table 6.11).

Table 6.8: Blood meal presence and assigned rating of the blood meal score according to the scale (0-5) in Figure 6.1 for all flea samples (nine species) taken from burrows of Richardson's ground squirrels (see Chapter 5; Thoroughgood and Chilton 2024).

		Blood Meal Present			Blood Meal Scale					
Species	**Local**	**No**	**Yes**	**Unknown**	**0**	**1**	**2**	**3**	**4**	**5**
Aetheca wagneri	Rural	1	0	1	1	0	0	0	0	0
Aetheca wagneri	Urban	1	1	2	1	1	0	0	0	0
Catallagia sp.	Urban	0	5	0	0	3	1	1	0	0
unknown ceratophyllid	Urban	0	1	0	0	0	1	0	0	0
Megabothris sp.	Rural	0	1	0	0	0	0	0	0	1
Neopsylla inopina	Rural	0	3	0	0	0	0	2	1	0
Neopsylla inopina	Urban	2	29	0	2	8	6	8	3	5
Oropsylla bruneri	Rural	5	46	2	5	4	16	8	7	11
Oropsylla bruneri	Urban	2	13	0	2	1	4	1	4	3
Oropsylla labis	Urban	3	3	2	3	0	0	1	1	1
Oropsylla rupestris	Rural	1	7	0	1	2	2	1	2	0
Oropsylla rupestris	Urban	13	63	0	13	18	16	13	7	9
Oropsylla tuberculata	Rural	1	6	0	1	1	0	1	2	2
Oropsylla tuberculata	Urban	2	8	0	2	0	1	2	2	3

Figure 6.2: Representative standard curve for flea 28S rRNA gene qPCR assay.

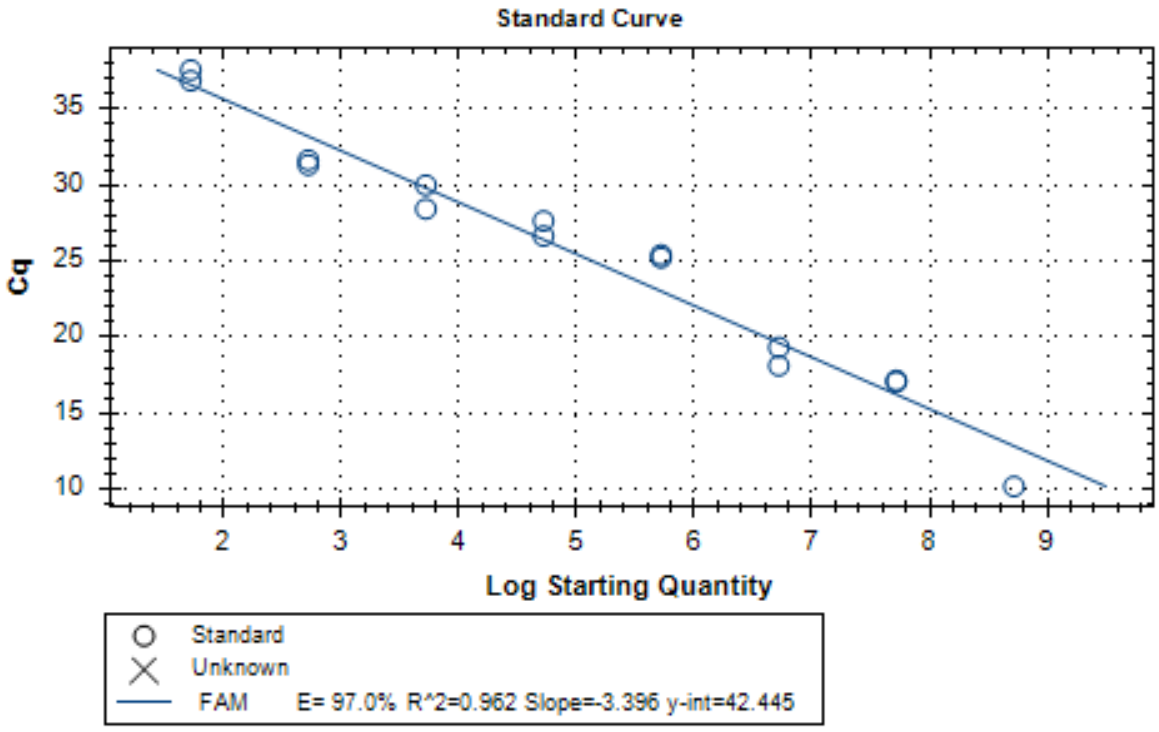
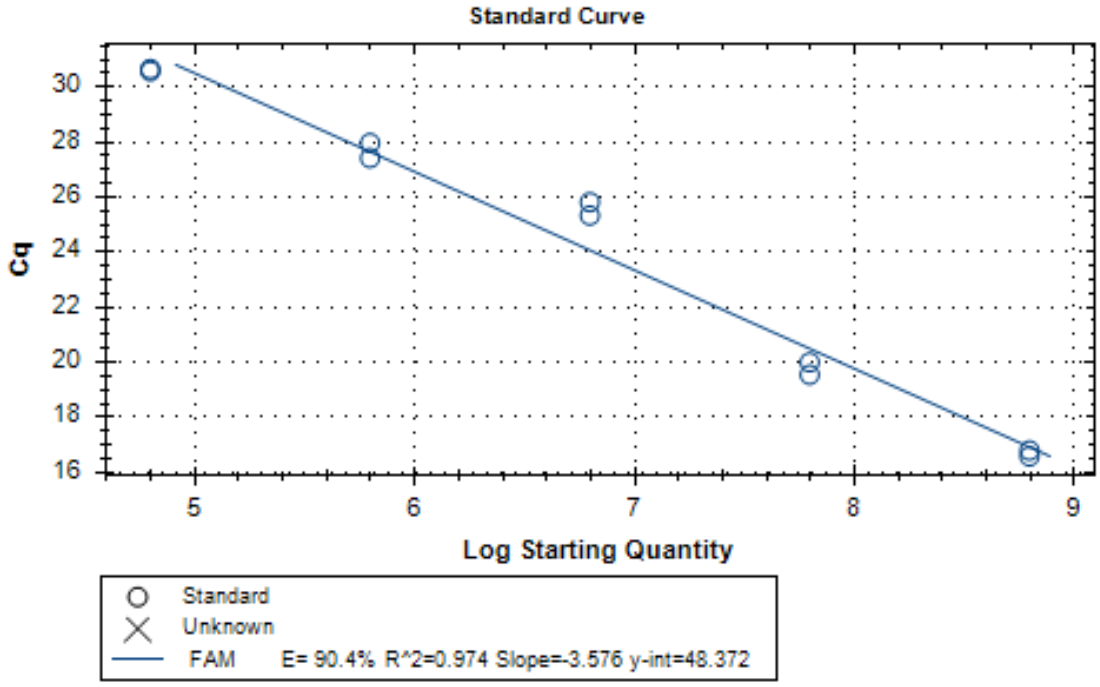


Table 6.9: Range and mean CT values for flea qPCR 28S RNA gene assay for nine flea species collected from the urban and rural sites.

Species	**Site**	**n**	**Mean**	**Min**	**Max**
Aetheca wagneri	Urban	4	18.6	17.8	20.0
A. wagneri	Rural	2	20.4	19.9	21.0
Catallagia sp.	Urban	5	19.7	18.3	21.2
unknown ceratophyllid	Urban	1	16.4	16.4	16.4
Megabothris sp.	Rural	1	19.0	19.0	19.0
Neopsylla inopina	Rural	3	17.5	17.0	18.1
N. inopina	Urban	32	17.6	15.2	21.4
Oropsylla bruneri	Urban	15	17.6	15.8	19.8
O. bruneri	Rural	53	18.1	15.7	21.9
O. labis	Urban	8	18.0	15.4	20.6
O. rupestris	Urban	76	17.9	15.6	21.5
O. rupestris	Rural	8	17.9	16.4	19.8
O. tuberculata	Rural	7	18.0	17.1	18.9
O. tuberculata	Urban	10	18.7	17.1	20.0

Figure 6.3: Representative standard curves for A) total bacterial load and B) *Bartonella* qPCR assays.

A)



B)

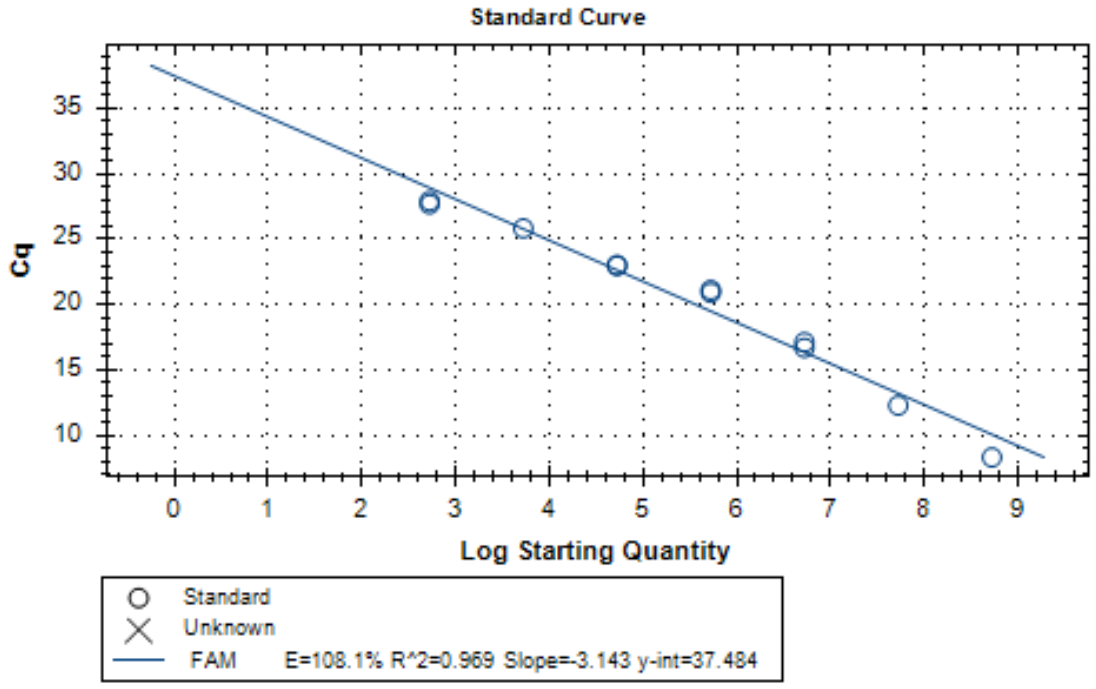


Table 6.10: CT values of total bacteria qPCR assays targeting the 16S rRNA gene in the gDNA samples of the nine flea species collected from burrows of Richardson's ground squirrels.

		CT Value				
Species	**Local**	**20-25**	**25-30**	**30-35**	**35-40**	**NA**
Aetheca wagneri	Urban	0	1	2	1	0
A. wagneri	Rural	0	1	1	0	0
Catallagia sp.	Urban	0	0	5	0	0
unknown ceratophyllid	Urban	1	0	0	0	0
Megabothris sp.	Rural	0	0	1	0	0
Neopsylla inopina	Urban	6	22	4	0	0
N. inopina	Rural	1	2	0	0	0
Oropsylla bruneri	Urban	0	6	9	0	0
O. bruneri	Rural	6	28	17	0	2
O. labis	Urban	0	2	6	0	0
O. rupestris	Urban	28	30	18	0	0
O. rupestris	Rural	6	2	0	0	0
O. tuberculata	Urban	1	6	3	0	0
O. tuberculata	Rural	0	2	5	0	0

NA = no amplification. Negative control CT values ranged from 36.72 to 39.67.

Table 6.11: Mean, median, and (range) of copy numbers of flea 28S and total bacteria 16S rRNA gene. Ratio is the log transformed value of total bacteria 16S rRNA gene per 10 000 flea 28S rRNA genes.

			Flea 28S rRNA gene			Total Bacteria 16S rRNA gene			
Species	**no.**	**Local**	**Mean**	**Median**	**Range**	**Mean**	**Median**	**Range**	**ratio**
Aetheca wagneri	4	Urban	2.06×10^8	2.26×10^8	6.78×10^7 - 3.06×10^8	4.42×10^5	2.30×10^4	3.19×10^3 - 1.72×10^6	1.33
Aetheca wagneri	2	Rural	5.65×10^7	5.65×10^7	$(3.62 - 7.68) \times 10^7$	5.05×10^5	5.05×10^5	1.01×10^4 - 1.00×10^6	1.95
Catallagia sp.	5	Urban	1.18×10^8	8.11×10^7	3.05×10^7 - 2.20×10^8	4.64×10^4	2.69×10^4	$(1.10 - 9.69) \times 10^4$	0.59
unidentified ceratophyllid	1	Urban	7.78×10^8	7.78×10^8		3.92×10^6	3.92×10^6		1.70
Megabothris sp.	1	Rural	1.26×10^8	1.26×10^8		1.61×10^4	1.61×10^4		0.11
Neopsylla inopina	32	Urban	1.68×10^9	1.10×10^9	7.48×10^7 - 6.80×10^9	3.34×10^6	1.94×10^6	6.53×10^4 - 1.20×10^7	1.30
Neopsylla inopina	3	Rural	1.35×10^9	1.42×10^9	8.28×10^8 - 1.81×10^9	5.33×10^6	2.75×10^6	2.53×10^5 - 1.30×10^7	1.60
Oropsylla bruneri	15	Urban	4.55×10^8	3.43×10^8	7.65×10^7 - 1.15×10^9	6.98×10^4	3.87×10^4	3.78×10^3 - 4.02×10^5	0.18
Oropsylla bruneri	53	Rural	3.05×10^8	2.22×10^8	1.61×10^7 - 1.09×10^9	1.33×10^8	1.59×10^7	5.74×10^3 - 2.64×10^9	3.64
Oropsylla labis	8	Urban	4.11×10^8	2.39×10^8	4.54×10^7 - 1.51×10^9	2.45×10^5	7.80×10^4	6.42×10^3 - 1.02×10^6	0.78
Oropsylla rupestris	76	Urban	1.13×10^9	8.05×10^8	4.92×10^7 - 5.09×10^9	9.15×10^6	1.01×10^6	1.14×10^4 - 9.05×10^7	1.91
Oropsylla rupestris	8	Rural	1.27×10^9	1.10×10^9	2.66×10^8 - 2.88×10^9	1.02×10^7	7.54×10^6	1.25×10^5 - 2.27×10^7	1.90
Oropsylla tuberculata	10	Urban	1.96×10^8	1.62×10^8	7.05×10^7 - 4.92×10^8	7.03×10^5	1.89×10^5	9.60×10^3 - 5.37×10^6	1.56
Oropsylla tuberculata	7	Rural	2.81×10^8	2.65×10^8	$(1.48 - 4.86) \times 10^8$	1.25×10^5	4.75×10^4	3.58×10^4 - 5.44×10^5	0.64

6.4.3 Detection and genetic characterization of *Bartonella* in fleas

Standard curves were generated for the *Bartonella ssrA* gBlock to determine the quantity of *Bartonella ssrA* in each flea gDNA sample (Fig. 6.3B). The negative control for this assay regularly had amplification at CT 32.98 due to an unknown source of contamination. Samples were considered negative if their amplification occurred at CTs values greater than their corresponding negative control samples. The CT values for the flea gDNA samples using the *Bartonella ssrA* assay ranged from 18.5 to 38.27, with a starting quantity of 1.93 $x10^7$ to 72.9 per sample (Table 6.12). The starting quantity of *Bartonella* was not influenced by the presence of a blood meal within the sample ($W = 3197$, $p = 0.5035$).

Of the 40 samples tested using conventional PCR techniques (Table 6.13), seven *O. bruneri* and one *O. tuberculata* produced *gltA* amplicons that could be sent for sequencing. Only six of the seven *O. bruneri* gDNA samples produced clean *gltA* sequences of 749 base pairs. Three different *gltA* sequence types (A-C) were obtained for the 6 samples. Sequence "A" was detected in one flea from the rural site. It differs by a single transitional mutation at location 307 in the sequence alignment (Fig. 6.4) when compared to sequence "B". Sequence "B" was detected in the gDNA of four fleas from the rural site. Sequence "C", detected in the gDNA of a single flea from the urban site, differed at 16 positions (i.e.15 transitional and two transversional mutational changes) when compared to sequence "A". The nucleotide sequences of "A" and "B" were 93% and 92% identical, respectively, to the *gltA* sequence of *B. washoensis* (GenBank accession number MN229489). Both sequence types also had a 92% sequence similarity to the *gltA* sequence of *B. gliris* (CP123962), and 91% sequence similarity to the *gltA* sequence of *B. henselae* (KY913624). Sequence "C" was 92% identical to the *gltA* sequence of *B. gliris* (CP123962) and had 91% sequence similarity to the *gltA* sequences of *B. washoensis*

(MN229489) and *B. henselae* (KY913624). The *gltA* amino acid sequence of "A" and "B" were identical. There was one difference in the amino acid sequences of "A" and "B" when compared to "C". This difference (Arginine to Histidine) occurred at position 53 of the sequence alignment (Fig. 6.5). The closest match in amino acid sequences for "A" and "B" was the *gltA* amino acid sequence of six to *B. gliris* (CP123962), but they differed at six positions in the sequence alignment. The closest match for "C" was also with *B. gliris* (CP123962), but it differed at 5 positions in the sequence alignment. Despite this outcome, the results of the phylogenetic analyses of the amino acid sequence data showed that the three *gltA* sequence types isolated from *O. bruneri* formed a clade with *B. washoensis* (Fig. 6.6). However, the support for this clade was not strong (i.e., bootstrap value of 73%).

Rodent associated *Bartonella* infections have been investigated in Saskatchewan, Canada with the citrate synthase gene (Jardine et al. 2005). Figure 6.7 shows the neighbour-joining tree produced from a phylogenetic analysis based on the 381 bp alignment of the *gtlA* nucleotide sequences in Jardine et al. (2005) and the three sequence types (genotypes) obtained in the present study. All three sequence types belonged to a clade with strong bootstrap support (93%) that contained sequences from *B. washoensis* isolated from a Californian ground squirrel and *Bartonella* isolated from Franklin's ground squirrels, thirteen-lined ground squirrels, Richardson's ground squirrels and an eastern chipmunk. However, there was strong statistical support (i.e., bootstrap value of 93%) that the three *Bartonella* genotypes isolated from *O. bruneri* did not belong to the clade containing *B. washoensis* and the *Bartonella* from the eastern chipmunk. *Bartonella* genotypes "A" and "B" from *O. bruneri* at the urban site were identical in sequence to a *Bartonella* genotype that was previously isolated from Franklin's ground squirrels and thirteen-lined ground squirrels (AY584570; Kosoy et al. 2004, Jardine et al. 2005, 2006b).

Table 6.12: *Bartonella* qPCR results which targeted the *ssrA* gene of all gDNA flea-positive samples. The starting quantity is based on the CT value and DNA concentration of gBlock. Ratio is the log transformed value of *Bartonella ssrA* gene copies per 10 000 total bacteria 16S rRNA gene copy.

		CT Value				Starting Quantity			
Species	**Local**	**0-25**	**25-30**	**30-35**	**35-40**	**Mean**	**Min**	**Max**	**Ratio**
Aetheca wagneri	Rural	0	0	2	0	5.30×10^3	4.53×10^3	6.06×10^3	2.02
Aetheca wagneri	Urban	0	3	0	0	1.32×10^4	9.48×10^3	1.85×10^4	2.47
Megabothris sp.	Rural	0	1	0	0	5.35×10^4			4.52
Neopsylla inopina	Rural	1	0	0	0	3.43×10^5			2.81
Neopsylla inopina	Urban	0	2	0	0	6.63×10^4	3.35×10^4	9.90×10^4	2.30
Oropsylla bruneri	Rural	1	7	16	0	8.10×10^5	1.92×10^2	1.93×10^7	1.79
Oropsylla bruneri	Urban	0	0	6	0	5.48×10^3	3.80×10^3	8.05×10^3	2.89
Oropsylla labis	Urban	0	1	0	0	3.83×10^4			3.19
Oropsylla rupestris	Rural	0	1	2	0	5.14×10^3	3.75×10^3	7.67×10^3	0.70
Oropsylla rupestris	Urban	0	14	19	12	1.79×10^4	7.29×10^1	2.60×10^5	1.29
Oropsylla tuberculata	Rural	0	0	4	0	5.01×10^3	3.27×10^3	7.21×10^3	2.60
Oropsylla tuberculata	Urban	0	2	3	0	2.30×10^4	4.32×10^3	6.43×10^4	2.51

Table 6.13: The number of fleas of each species tested for *Bartonella* using qPCR and conventional PCR techniques, and the number that were positive for each assay.

Species	Site	*ssRNA* qPCR		Conventional *gltA* PCR	
		No. of samples tested	Positive	No. of samples tested	Positive
Aetheca wagneri	Rural	2	2	0	-
Aetheca wagneri	Urban	4	3	0	-
Catallagia sp.	Urban	5	0	0	-
unknown ceratophyllid	Urban	1	0	0	-
Megabothris sp.	Rural	1	1	1	0
Neopsylla inopina	Rural	3	1	0	-
Neopsylla inopina	Urban	32	2	0	-
Oropsylla bruneri	Rural	53	24	18	6
Oropsylla bruneri	Urban	15	6	3	1
Oropsylla labis	Urban	8	1	0	-
Oropsylla rupestris	Rural	8	3	0	-
Oropsylla rupestris	Urban	76	45	14	0
Oropsylla tuberculata	Rural	7	4	1	0
Oropsylla tuberculata	Urban	10	5	3	1

Figure 6.4: Alignment of *Bartonella* sequences (types A, B and C) for the citrate synthase gene (*gltA*) detected within the gDNA of six specimens of *Oropsylla bruneri* (5 from the rural sites and 1 from the urban site). Dots represent the same nucleotide as in the sequence of type A.

```
                          10         20         30         40         50         60
                           |          |          |          |          |          |
A (rural; n=1) TGATTCCATTGATATTACAGATCCTCAACAGAGAATGATCGCTTCTATTCGTCTCATTTC
B (rural; n=4) ............................................................
C (urban; n=1) ...C..............T.........................................

                          70         80         90        100        110        120
                           |          |          |          |          |          |
A              AAAGGTTCCAACTCTTGCCGCCATGGCGTATAAATATAGTATTGGGCAGGCATTTGTTTA
B              ............................................................
C              ...A........................................................

                         130        140        150        160        170        180
                           |          |          |          |          |          |
A              TCCGCGAAATGATTTGAGTTATGCTGCCAATTTCCTCCGTATGTGTTTTTCTGTTCCTTG
B              ............................................................
C              ..........................................A.................

                         190        200        210        220        230        240
                           |          |          |          |          |          |
A              TGAAGAATATAAAATCAATCCAGTGCTTACTCAAGCTATGGATCGAATTTTTATTCTCCA
B              ............................................................
C              ............................................................

                         250        260        270        280        290        300
                           |          |          |          |          |          |
A              TGCAGATCATGAACAAAATGCTTCTACATCCACTGTACGTCTTGCTGGGTCATCGGGAGC
B              ............................................................
C              ..........................G.................................

                         310        320        330        340        350        360
                           |          |          |          |          |          |
A              TAATCCGTTTGCATGCATCGCAGCAGGTGTTGCGTGCCTTTGGGGACCAGCTCATGGTGG
B              ......A.....................................................
C              ..................................A.........................

                         370        380        390        400        410        420
                           |          |          |          |          |          |
A              AGCCAATGAAGCATGCCTAAAAATGTTACAAGAAATAGGTTCCGTCAAAAGAATTCCTGA
B              ............................................................
C              ...................................................T........

                         430        440        450        460        470        480
                           |          |          |          |          |          |
A              ATTCATTGCTCGTGCAAAAGATAAAAATGATCCTTTCCGTCTTATGGGTTTTGGCCATCG
B              ............................................................
C              .........G..................................................T.....

                         490        500        510        520        530        540
                           |          |          |          |          |          |
A              TGTCTATAAAAATTATGATCCACGCGCAAAAATCATGCAACAAACCTGTCATGAAGTCTT
B              ............................................................
C              ............................................................
```

Figure 6.4: continued

```
                    550         560         570         580         590         600
                     |           |           |           |           |           |
A          AAAAGAACTTAATATTCAAGATGATCCACTTCTTGATATTGCTATAGAACTTGAAAATAT
B          ............................................................
C          ...........................................................C..

                    610         620         630         640         650         660
                     |           |           |           |           |           |
A          TGCCTTGAATGATGAATACTTTGTTGAAAAAAGGCTTTATCCGAATGTTGATTTCTATTC
B          ............................................................
C          ....C.A.....................................C.....................

                    670         680         690         700         710         720
                     |           |           |           |           |           |
A          TGGCATTACACTAAAAGCTTTAGGCTTTCCAACTGAAATGTTTACTGTTCTTTTTGCATT
B          ............................................................
C          ........................T...................................

                    730         740
                     |           |
A          AGCACGCAGTGTCGGCTGGGTCGCGCAAT
B          .............................
C          ...G.........................
```

Figure 6.5: Alignment of the *gltA* amino acid sequences for the *Bartonella* in the six specimens of *O. bruneri* (types A, B and C), a human isolate of *B. washoensis* (GenBank accession number MN229489), *B. gliris* (accession number CP123962), and *B. henselae* (accession KY913624). Dots indicate same amino acid as in sequence of the *Bartonella* in the rural *O. bruneri* specimen S178.

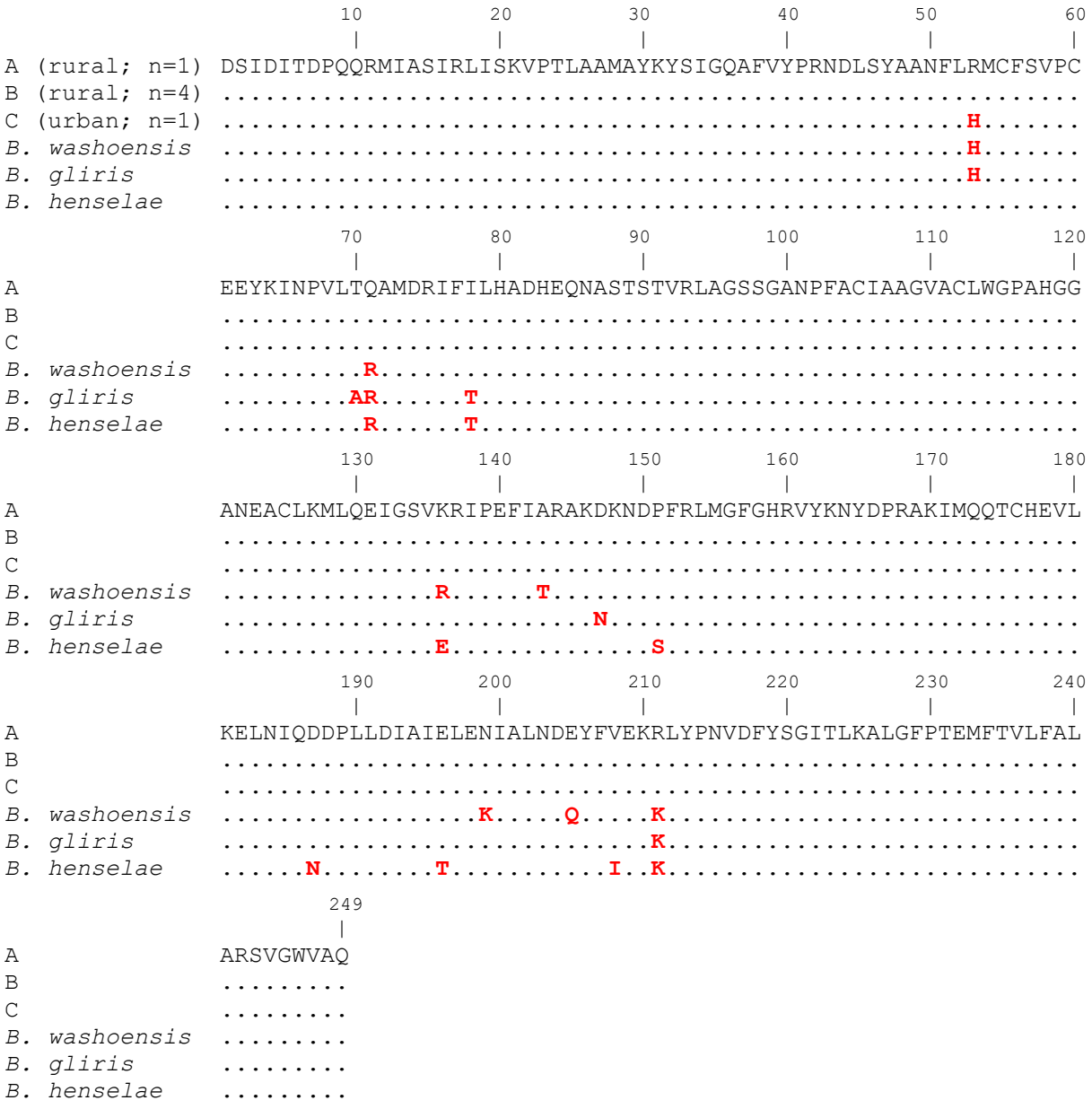


Figure 6.6: Neighbour-joining tree depicting relationships of *Bartonella* based on the amino acid sequence of the citrate synthase gene. The number above each branch indicates the bootstrap value.

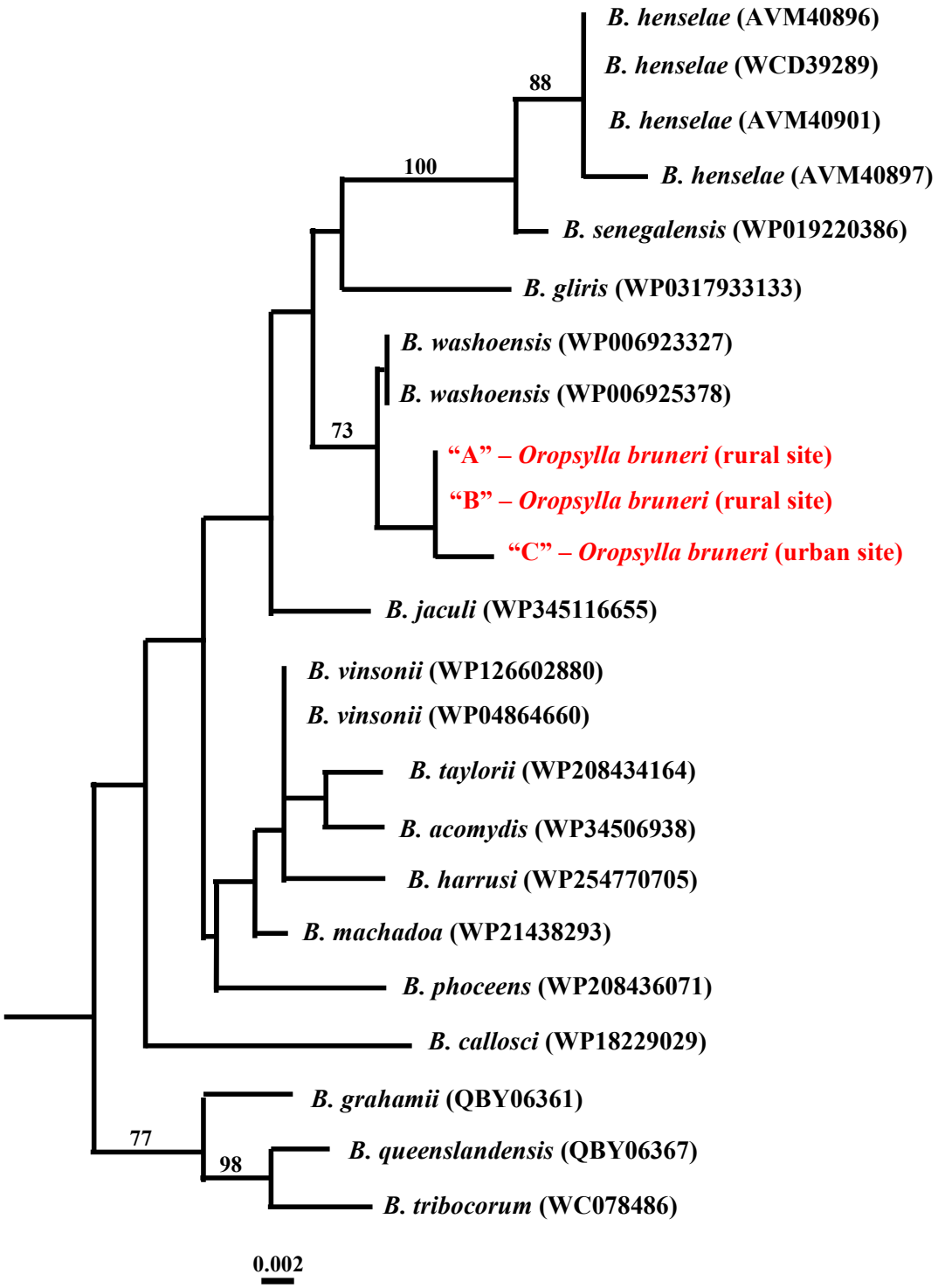
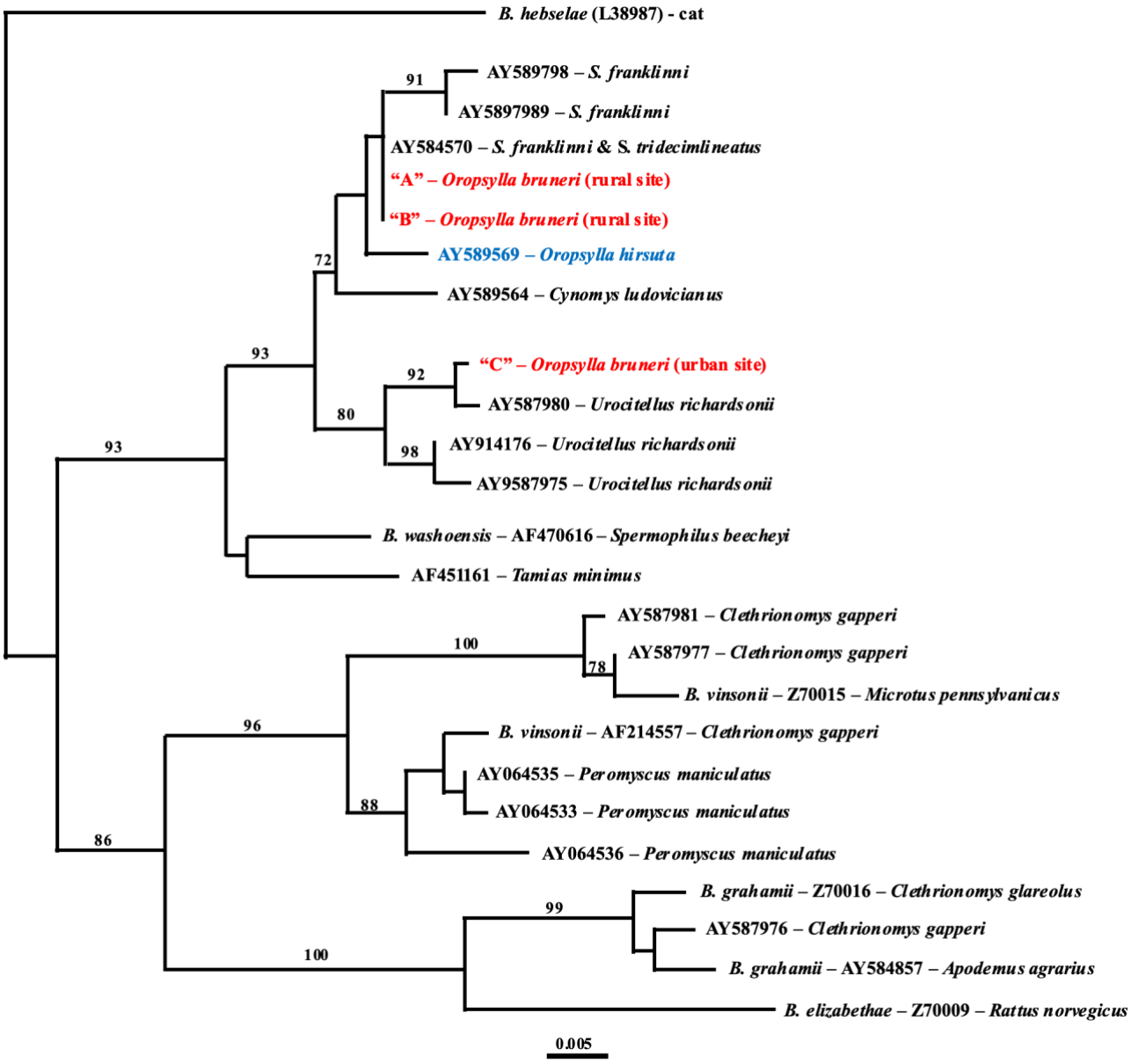


Figure 6.7: Neighbour-joining tree depicting the relationship of *Bartonella* spp. based on the nucleotide sequences of the citrate synthase gene. Data are derived from Jardine et al. (2005) [accession numbers shown] with the incorporation of the sequences from the three sequence types isolated from *Oropsylla bruneri* samples (indicated in red). The number above each branch indicates the bootstrap value.

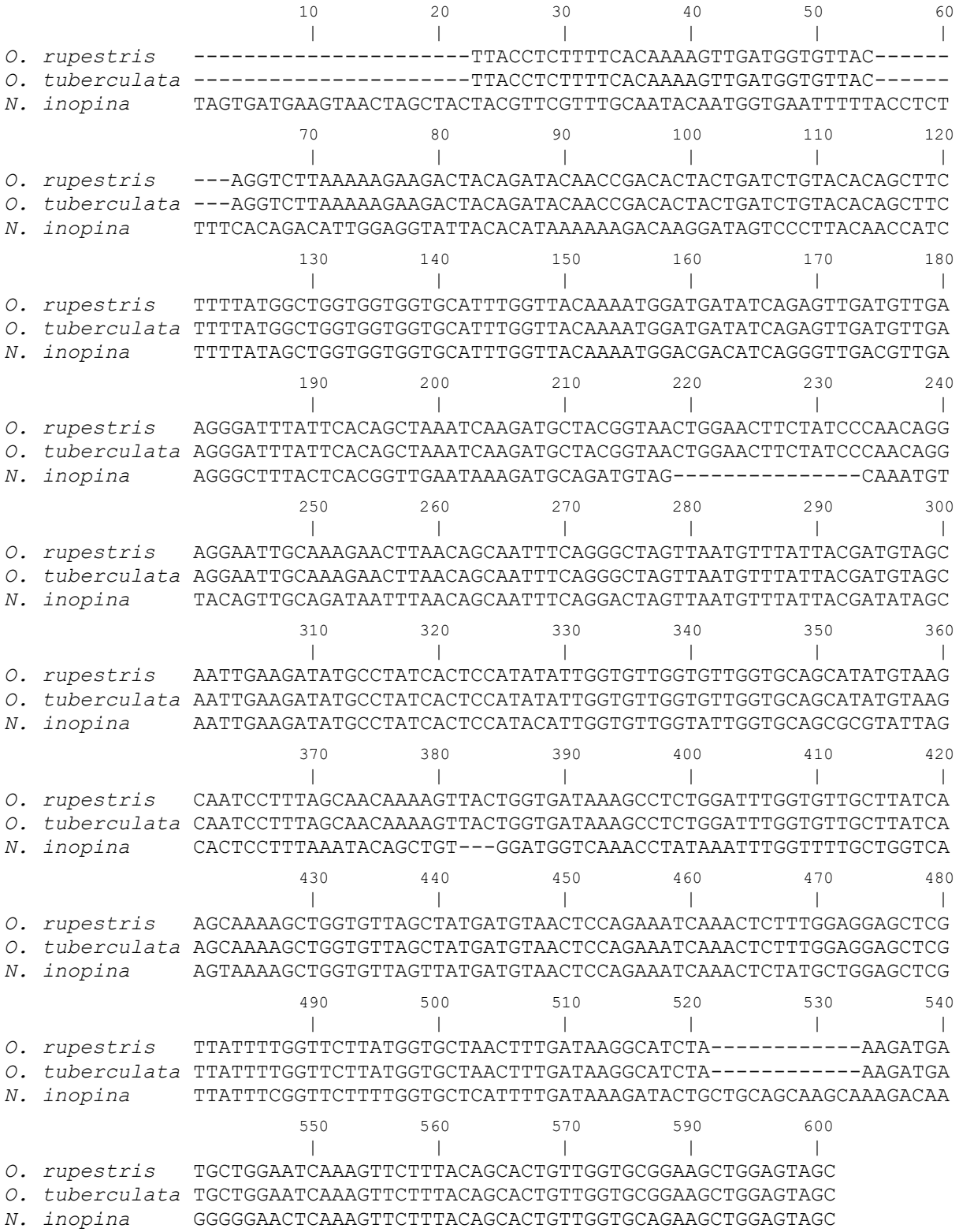


They belonged to a clade with 72% bootstrap support that included other isolates from Franklin's ground squirrels and thirteen-lined ground squirrels, and from *O. hirsuta* parasitizing a black-tailed prairie dog. In contrast, genotype "C" from *O. bruneri* at the urban site belonged to a clade, with 80% bootstrap support, that contained *Bartonella* isolated from Richardson's ground squirrels. Genotype C differed by one nucleotide substitution when compared to a *Bartonella* genotype isolated from Richardson's ground squirrels (AY587980.1; Jardine et al. 2005).

6.4.4 Detection and genetic characterization of *Wolbachia* in fleas

During preliminary screening of flea gDNA samples with *wsp*, 17 samples had a single band of ~600 bp on agarose gels (data not shown). Of these, purified amplicons from five *O. rupestris*, one *O. tuberculata*, and five *N. inopina* were subjected to DNA sequencing. The results showed that there were no differences in *Wolbachia wsp* sequences among the five *O. rupestris* samples. Similarly, there were no differences in *Wolbachia wsp* sequences among the five *N. inopina* samples. In addition, the *Wolbachia wsp* sequence in the *O. tuberculata* sample was identical to *wsp* sequences in *O. rupestris* (Fig. 6.8). In contrast, the *Wolbachia wsp* sequence in *N. inopina* differed significantly from that in the two species of *Oropsylla*. The sequences were aligned over a length of 601 bp. The *wsp* sequence in *N. inopina* had an additional 22 bp at the 5' end, a 9 bp insertion at alignment positions 55-63, a 15 bp deletion at alignment positions 219-233, a 3 bp deletion at alignment positions 382-384, and a 12 bp insertion at alignment positions 522-533 (Fig 6.8). In addition, there are 58 transitional and 69 transversional mutational changes when the *Wolbachia wsp* sequences are compared between *N. inopina* and the two *Oropsylla* species. Results of a blast search revealed that *wsp* sequence of *Wolbachia* in the two *Oropsylla* species had the greatest similarity (534/548 bp; 99%) to the *wsp*

Figure 6.8: Alignment of the *wsp* sequences of *Wolbachia* in *Oropsylla rupestris*, *O. tuberculata* and *Neopsylla inopina*.



sequence of a *Wolbachia* endosymbiont in *Oropsylla silantiewi* (MF045789; Fig. 6.9). The *wsp* sequence of *Wolbachia* in *Neopsylla inopina* had the greatest similarity (545/546 bp) to a *Wolbachia* endosymbiont of *Paradoxopsyllus repandus* (KX3845018; Fig. 6.10).

The amino acid sequence of the *wsp* gene was identical in the *O. rupestris* and *O. tuberculata* samples (Fig. 6.11). The *wsp* amino acid sequence of the *Wolbachia* in the two *Oropsylla* species differed at position 77 from the *O. silantiewi* endosymbiont, where an alanine in the *O. rupestris* and *O. tuberculata* sequences is a threonine in the *O. silantiewi*. In comparison, *N. inopina* and *P. repandus* had one difference in their amino acid sequence at location 4 (Val to Glu; Fig. 6.11).

As the amino acid sequence isolated from *Oropsylla* species and *N. inopina* differed by 34%, we decided to run the phylogenetic analysis of the genera separately. The phylogenetic relationship of the *Wolbachia* isolated from *Oropsylla is* depicted in a neighbour-joining (nj) tree based on *wsp* nucleotide sequence (Fig. 6.12). There was total statistical support (i.e., bootstrap value of 100%) of the *Wolbachia* in *O. rupestris* and *O. tuberculata* forming a clade with the *Wolbachia* in *O. silantiewi.* The nj tree also depicts this clade as being the sister taxon to a clade, with 93% bootstrap support, that contains *Wolbachia* from species of hymenopterans (i.e., bees and wasps), hemipterans (i.e. bugs), a dipteran (flies), a coleopteran (i.e., beetles) and arachnids (i.e., spiders). These clade groups form a clade with strong statistical support (i.e., bootstrap value of 93%) with *Wolbachia* from the flea, *Archaeopyslla erinacie*.

The nj tree depicting the phylogenetic relationships of the *Wolbachia* in *N. inopina* based on *wsp* nucleotide sequence data is shown in Figure 6.13. There was total statistical support (i.e., bootstrap value of 100%) of the *Wolbachia* in *N. inopina* forming a clade with the *Wolbachia* in the flea, *P. repandus*. There was no evidence of a sister taxa relationship between this clade, and

Figure 6.9: Alignment of *wsp* sequences of *Wolbachia* in *Oropsylla rupestris* and *O. tuberculata* (combined as they are identical) with the *wsp* sequence of the *Wolbachia* endosymbiont in *O. silantiewi* (GenBank accession number MF045789). Dots indicate the same nucleotide as in the sequence of the *Wolbachia* in *O. rupestris* and *O. tuberculata.*

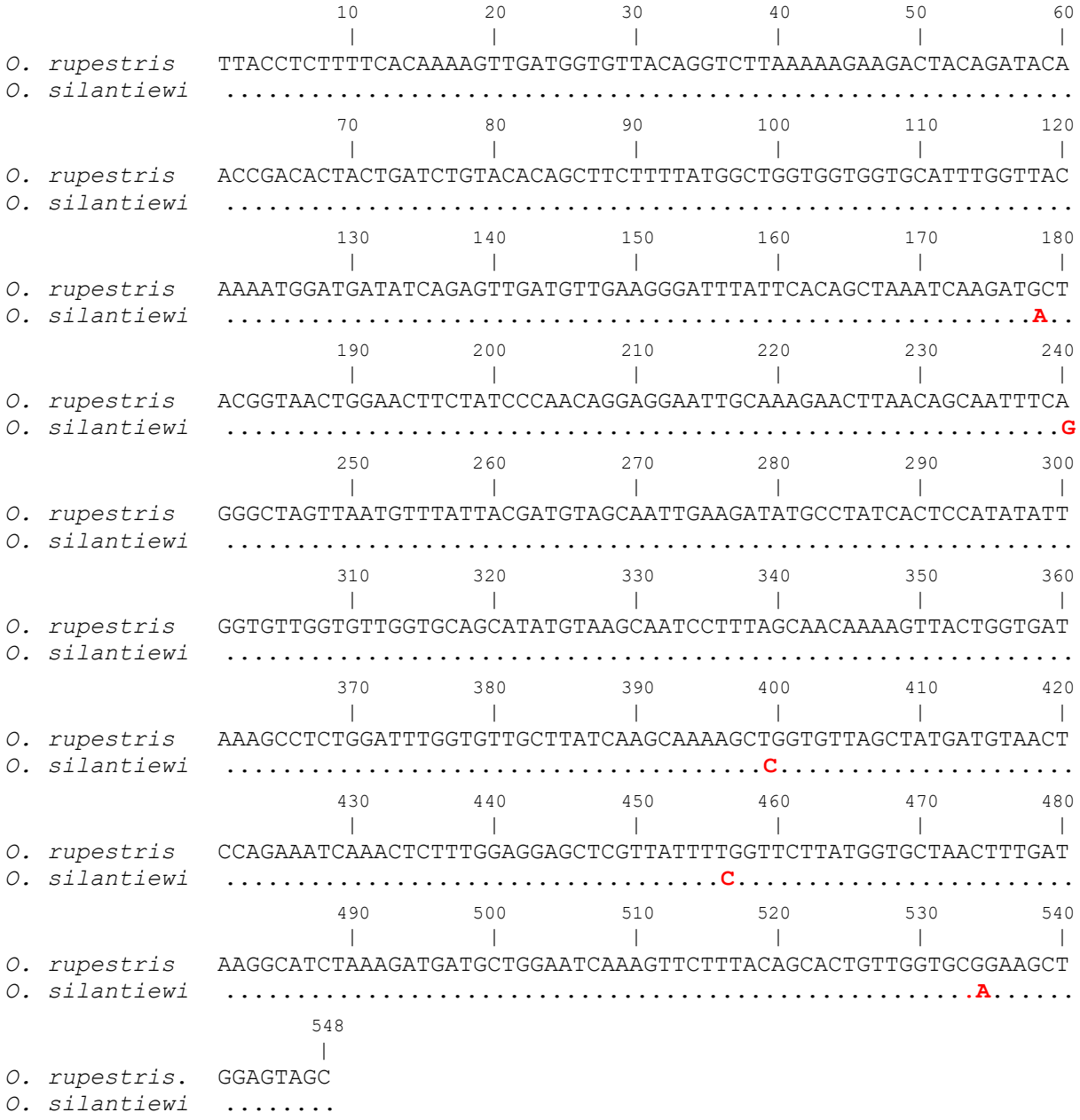


Figure 6.10: Alignment of the *wsp* sequences of *Wolbachia* in *Neopsylla inopina* and a *Wolbachia* endosymbiont in *Paradoxopsyllus repandus* (GenBank accession number KX385018). Dots indicate the same nucleotide as in the sequence of the *Wolbachia* in *N. inopina*.

```
                        10          20          30          40          50          60
                         |           |           |           |           |           |
N. inopina      AGTGATGAAGTAACTAGCTACTACGTTCGTTTGCAATACAATGGTGAATTTTTACCTCTT
P. repandus     ..........A..........................................................

                        70          80          90         100         110         120
                         |           |           |           |           |           |
N. inopina      TTCACAGACATTGGAGGTATTACACATAAAAAAGACAAGGATAGTCCCTTACAACCATCT
P. repandus     ............................................................

                       130         140         150         160         170         180
                         |           |           |           |           |           |
N. inopina      TTTATAGCTGGTGGTGGTGCATTTGGTTACAAAATGGACGACATCAGGGTTGACGTTGAA
P. repandus     ............................................................

                       190         200         210         220         230         240
                         |           |           |           |           |           |
N. inopina      GGGCTTTACTCACGGTTGAATAAAGATGCAGATGTAGCAAATGTTACAGTTGCAGATAAT
P. repandus     ............................................................

                       250         260         270         280         290         300
                         |           |           |           |           |           |
N. inopina      TTAACAGCAATTTCAGGACTAGTTAATGTTTATTACGATATAGCAATTGAAGATATGCCT
P. repandus     ............................................................

                       310         320         330         340         350         360
                         |           |           |           |           |           |
N. inopina      ATCACTCCATACATTGGTGTTGGTATTGGTGCAGCGCGTATTAGCACTCCTTTAAATACA
P. repandus     ............................................................

                       370         380         390         400         410         420
                         |           |           |           |           |           |
N. inopina      GCTGTGGATGGTCAAACCTATAAATTTGGTTTTGCTGGTCAAGTAAAAGCTGGTGTTAGT
P. repandus     ............................................................

                       430         440         450         460         470         480
                         |           |           |           |           |           |
N. inopina      TATGATGTAACTCCAGAAATCAAACTCTATGCTGGAGCTCGTTATTTCGGTTCTTTTGGT
P. repandus     ............................................................

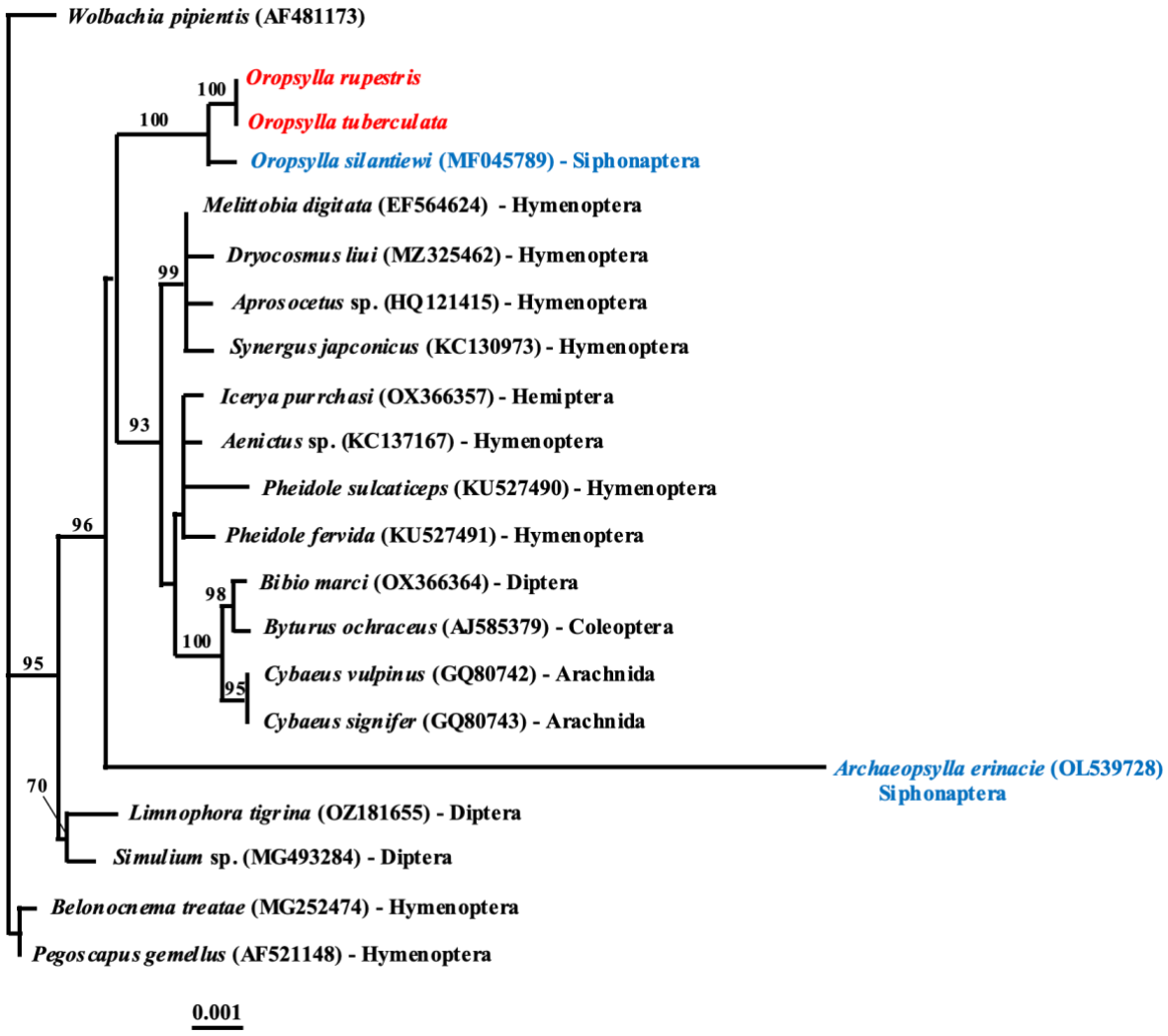
                       490         500         510         520         530         540
                         |           |           |           |           |           |
N. inopina      GCTCATTTTGATAAAGATACTGCTGCAGCAAGCAAAGACAAGGGGGAACTCAAAGTTCTT
P. repandus     ............................................................

                    546
                     |
N. inopina      TACAGC
P. repandus     ......
```

Figure 6.11: Alignment of amino acid sequences of *wsp* for a *Wolbachia* endosymbiont in *O. silantiewi* (GenBank accession number MF045789), the *Wolbachia* in *Oropsylla rupestris* and *O. tuberculata*, a *Wolbachia* endosymbiont in *Paradoxopsyllus repandus* (accession number KX385018) and the *Wolbachia* in *Neopsylla inopina*. Amino Acids in blue are conserved for all 5 taxa.

```
                          10         20         30         40         50         60
                           |          |          |          |          |          |
O. silantiewi  ----------FAYNGEILPLFTKVDGVTGLKKKTTDTTDTTDLYTASFMAGGGAFGYKMD
O. rupestris   ----------------LPLFTKVDGVTGLKKKTTDTTDTTDLYTASFMAGGGAFGYKMD
O. tuberculata ----------------LPLFTKVDGVTGLKKKTTDTTDTTDLYTASFMAGGGAFGYKMD
P. repandus    SDEETSYYVRLQYNGEFLPLFTDIGGITHKKDKDSP-------LQPSFIAGGGAFGYKMD
N. inopina     SDEVTSYYVRLQYNGEFLPLFTDIGGITHKKDKDSP-------LQPSFIAGGGAFGYKMD
                          70         80         90         100        110        120
                           |          |          |          |          |          |
O. silantiewi  DIRVDVEGIYSQLNQDTTVTGTSIPTGGIAKNLTAISGLVNVYYDVAIEDMPITPYIGVG
O. rupestris   DIRVDVEGIYSQLNQDATVTGTSIPTGGIAKNLTAISGLVNVYYDVAIEDMPITPYIGVG
O. tuberculata DIRVDVEGIYSQLNQDATVTGTSIPTGGIAKNLTAISGLVNVYYDVAIEDMPITPYIGVG
P. repandus    DIRVDVEGLYSRLNKDADVANVT-----VADNLTAISGLVNVYYDIAIEDMPITPYIGVG
N. inopina     DIRVDVEGLYSRLNKDADVANVT-----VADNLTAISGLVNVYYDIAIEDMPITPYIGVG
                          130        140        150        160        170        180
                           |          |          |          |          |          |
O. silantiewi  VGAAYVSNPLATKVTGDKASGFGVAYQAKAGVSYDVTPEIKLFGGARYFGSYGANFDK--
O. rupestris   VGAAYVSNPLATKVTGDKASGFGVAYQAKAGVSYDVTPEIKLFGGARYFGSYGANFDK--
O. tuberculata VGAAYVSNPLATKVTGDKASGFGVAYQAKAGVSYDVTPEIKLFGGARYFGSYGANFDK--
P. repandus    IGAARISTPLNTAVDG-QTYKFGFAGQVKAGVSYDVTPEIKLYAGARYFGSFGAHFDKDT
N. inopina     IGAARISTPLNTAVDG-QTYKFGFAGQVKAGVSYDVTPEIKLYAGARYFGSFGAHFDKDT
                          190        200
                           |          |
O. silantiewi  --ASKDDAGIKVLYSTVGAEAGV
O. rupestris   --ASKDDAGIKVLYSTVGAEAGV
O. tuberculata --ASKDDAGIKVLYSTVGAEAGV
P. repandus    AAASKDKGELKVLYS
N. inopina     AAASKDKGELKVLYSTVGAEAGV
```

Figure 6.12: Neighbour-joining tree depicting the phylogenetic relationships of the *Wolbachia* in *Oropsylla* based on *wsp* nucleotide sequence data. The number above each branch indicates the bootstrap value.



any other clade of *Wolbachia*, including the clade containing the *Wolbachia* from the flea *A. erinacie*. The nj tree places the clade containing the *Wolbachia* in *N. inopina* with a clade containing *Wolbachia* in coleopterans but there was no statistical support for this placement.

The nj tree depicting the phylogenetic relationships of the *Wolbachia* in *N. inopina* based on *wsp* nucleotide sequence data is shown in Figure 6.13. There was total statistical support (i.e., bootstrap value of 100%) of the *Wolbachia* in *N. inopina* forming a clade with the *Wolbachia* in the flea, *P. repandus*. There was no evidence of a sister taxa relationship between this clade, and any other clade of *Wolbachia*, including the clade containing the *Wolbachia* from the flea, *A. erinacie*. The nj tree places the clade containing the *Wolbachia* in *N. inopina* with a clade containing *Wolbachia* in coleopterans but there was no statistical support for this.

A total of eight fleas had a single band at ~ 800 bp for the *Wolbachia* 16S rRNA assay (data not shown). We were only able to get clean sequence from one *O. bruneri* and three *O. rupestris*. There was no intraspecific difference within the three *O. rupestris* samples. There is one gap in the sequence, 13 transitions, and 2 transversions between the *Oropsylla* spp. sequences (Fig. 6.14). The *Wolbachia* in *O. rupestris* and *O. bruneri* belonged to two different clades. The *Wolbachia* in *O. bruneri* belonged to a clade with total statistical support (i.e., bootstrap value of 100%), that contained *Wolbachia* isolates from five insect orders, the Coleoptera, Diptera, Hymenoptera, Lepidoptera, and Siphonaptera (Fig. 6.15). The latter was represented by the human flea, *Pulex irritans*, a species in the family Pulicidae. The *Wolbachia* in *O. rupestris* was found external to this clade, but no sister taxon relationship could be established in this analysis.

Figure 6.13: Neighbour-joining tree depicting the phylogenetic relationships of *Wolbachia* in *Neopsylla inopina* with other *Wolbachia* based on the nucleotide sequences of surface protein. Sequences from fleas are in two separate clades with *N. inopina* grouping with *Paradoxopsyllus repandus* and distinctly from samples isolated from Coleoptera. The number above each branch indicates the bootstrap value.

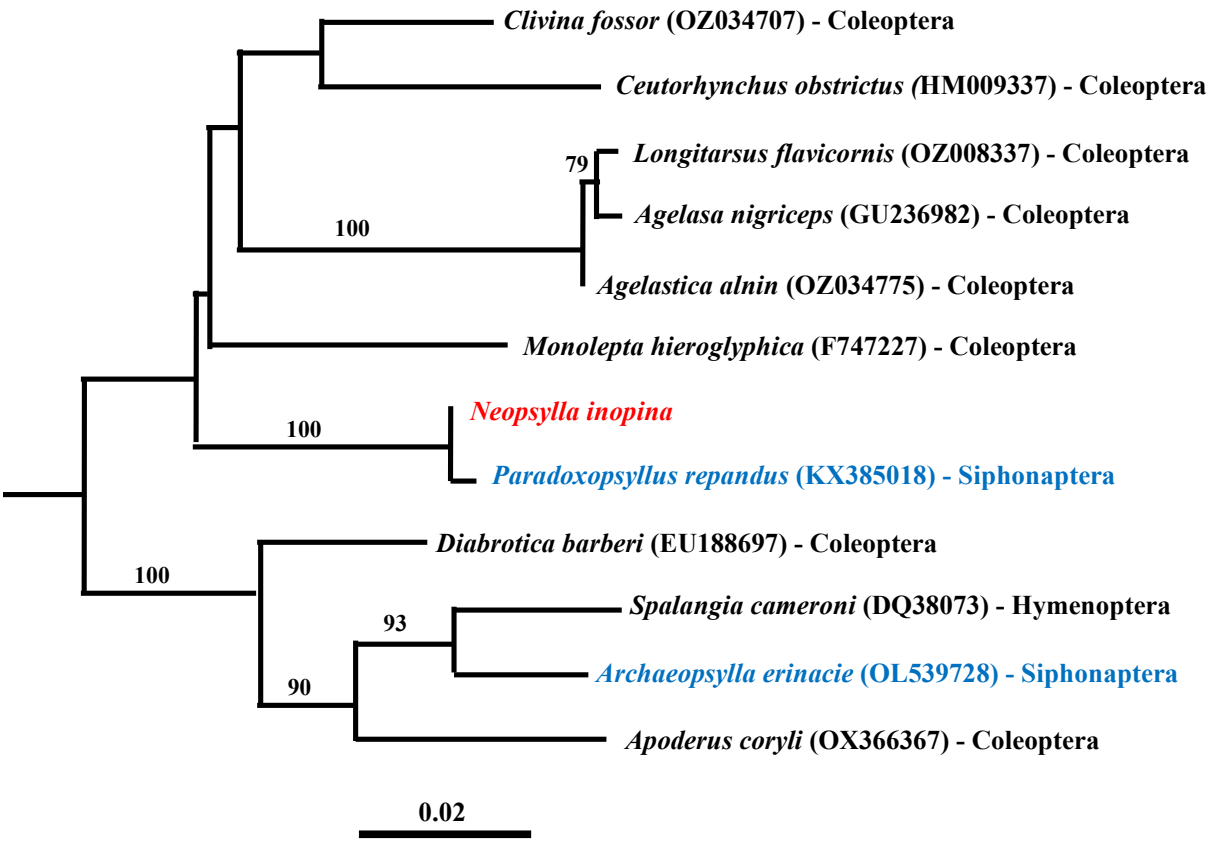


Figure 6.14: Alignment of 16S rRNA gene sequences of *Wolbachia* in *Oropsylla rupestris* and *O. bruneri*. Dots represent the same nucleotide as in the sequence of *Wolbachia* in *O. rupestris*.

```
                         10         20         30         40         50         60
                          |          |          |          |          |          |
O. rupestris    TAGTGGCAGACGGGTGAGTAATATATAGGAATCTACCTAGTAGTACGGAATAATTGTTGG
O. bruneri      ..................G.G.....................................

                         70         80         90        100        110        120
                          |          |          |          |          |          |
O. rupestris    AAACGACAACTAACACCGTATACGCCCTACGGGGGAAAAATTTATTGCTATTAGATGAGC
O. bruneri      .....G.......T.............................................

                        130        140        150        160        170        180
                          |          |          |          |          |          |
O. rupestris    CTATATTAGATTAGCTAGTTGGTAGAGTAATAGCCTACCAAGGCAATGATCTATAGCTGA
O. bruneri      ......................G.....................................

                        190        200        210        220        230        240
                          |          |          |          |          |          |
O. rupestris    TCTGAGAGGATGATCAGCCACACTGGAACTGAGATACGGTCCAGCCTCCTACGGGAGGCA
O. bruneri      ............................................A................

                        250        260        270        280        290        300
                          |          |          |          |          |          |
O. rupestris    GCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCTATGCCGCATGAGTGAAG
O. bruneri      ............................................C................

                        310        320        330        340        350        360
                          |          |          |          |          |          |
O. rupestris    AAGGCCTTTGGGTTGTAAAGCTCTTTTAGTGAGGAAGATAATGACGGTACTCACAGAAAA
O. bruneri      ............................................................G.

                        370        380        390        400        410        420
                          |          |          |          |          |          |
O. rupestris    AGTCCTGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGAGGGCTAGCGTTATTCGG
O. bruneri      ............................................................

                        430        440        450        460        470        480
                          |          |          |          |          |          |
O. rupestris    AATTATTGGGCGTAAAGGGCGCGTAGGCTGATTAATAAGTTAAAAGTGAAATCCCAAGGC
O. bruneri      ..........................G.....G.............................

                        490        500        510        520        530        540
                          |          |          |          |          |          |
O. rupestris    TTAACCTTGGAATTGCTTTTAAAACTGTTAATCTAGAGATTGAAAGAGGATAGAGGAATT
O. bruneri      .C........................C.................................

                        550        560        570        580        590        600
                          |          |          |          |          |          |
O. rupestris    CCTAGTGTAGAGGTAAAATTCGTAAATATTAGGAGGAACACCAGTGGCGAAGGCGTCTAT
O. bruneri      ..............G.............................................

                        610        620        630        640        650        660
                          |          |          |          |          |          |
O. rupestris    ............................................................
O. bruneri      ............................................................
```

Figure 6.14 continued

```
                          670         680         690         700         710         720
                           |           |           |           |           |           |
O. rupestris    GGTAGTCCACGCTGTAAACGATGAATGTTAAATATGGGGAGT-TTACTTTCTGTATTACA
O. bruneri      ....................................A...T..................

                          730         740         750         760         770         780
                           |           |           |           |           |           |
O. rupestris    GCTAACGCGTTAAGCATTCCGCCTGGGGACTACGGTCGCAAGATTAAAACTCAAAGGAAT
O. bruneri      ............A............................................................

                          790         800         810         820         830         840
                           |           |           |           |           |           |
O. rupestris    TGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAAAACC
O. bruneri      ............................................................

                          850
                           |
O. rupestris    TTACCACTCCTTG
O. bruneri      ............
```

Figure 6.15: Neighbour-joining tree depicting the phylogenetic relationships from *Oropsylla* with *Wolbachia* isolates from a variety of insects based on the nucleotide sequences of the 16S rRNA gene. The number above each branch indicates the bootstrap value.

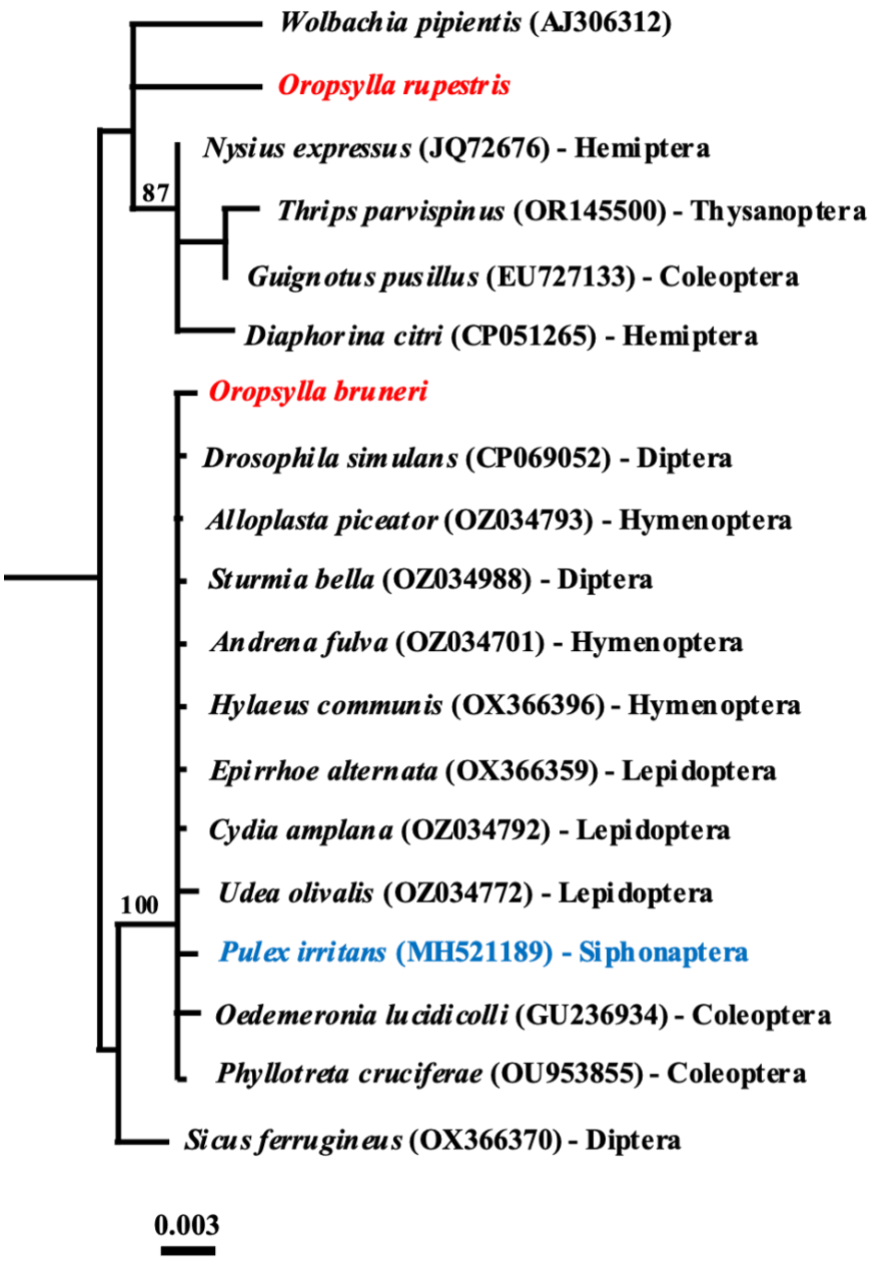


Table 6.14: *Wolbachia* screening using *wsp* primers. Samples were considered positive if there was an amplicon of ~600 bp in size. Additional bands were observed with ~1300 bp, 1150 bp, 900 bp, and 350 bp sizes.

				Additional Bands (bp)			
Species	Local	Tested	Positive	1300	1150	900	350
Aetheca wagneri	Rural	2	0	0	0	0	0
A. wagneri	Urban	4	0	0	0	0	0
Catallagia sp.	Urban	5	0	0	2	0	0
unknown ceratophyllid	Urban	1	0	0	0	0	0
Megabothris sp.	Rural	1	0	0	0	0	0
Neopsylla inopina	Rural	3	0	3	0	0	0
N. inopina	Urban	32	6	30	0	0	0
Oropsylla bruneri	Rural	53	47	0	27	5	31
O. bruneri	Urban	15	8	0	1	0	7
Oropsylla labis	Urban	8	8	0	0	0	0
O. rupestris	Rural	8	0	6	5	0	0
O. rupestris	Urban	76	0	52	15	13	0
O. tuberculata	Rural	7	4	0	2	0	0
O. tuberculata	Urban	10	7	1	3	0	0

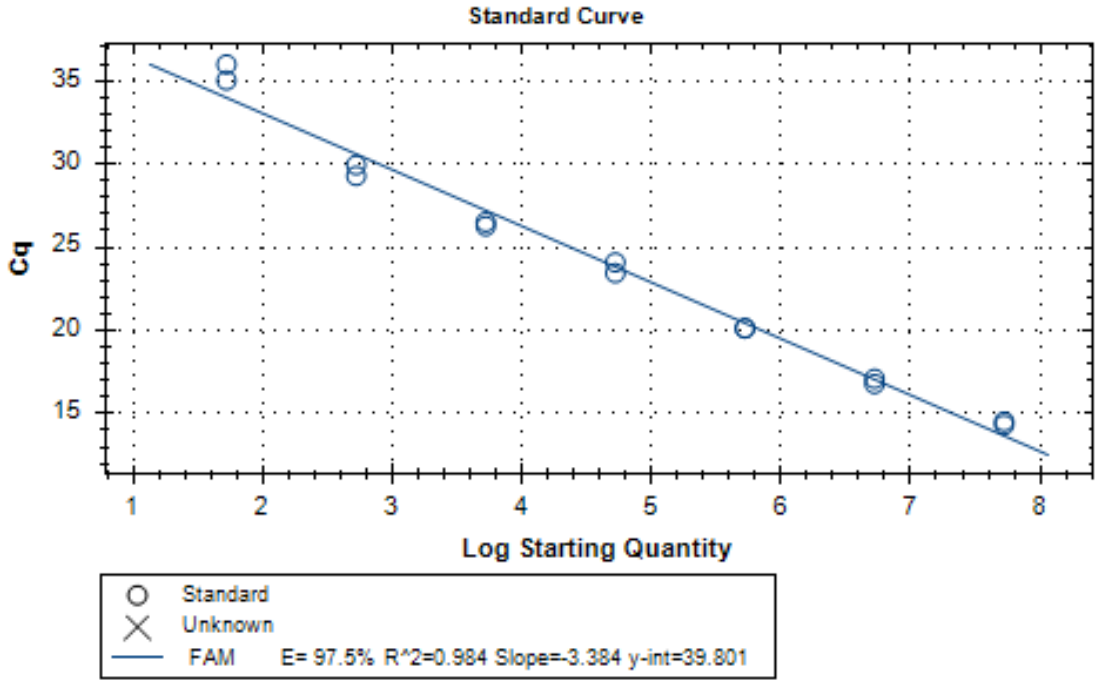
All but nine *O. rupestris* were screened for *Wolbachia* using the *wsp* primer set. Samples that had a band around 600 bp were considered positive. *Wolbachia* was only found in *N. inopina*, *O. bruneri*, *O. labis*, and *O. tuberculata* (Table 6.14). Additional bands ranging from 350 to 1300 bp were found in several species that included *N. inopina*, *O. bruneri*, *O. rupestris*, *O. tuberculata*, and *Catallagia* sp. (Table 6.14). Standard curves were generated as previously discussed by the gBlock (Fig. 6.16A). However, we failed to detect *Wolbachia* using the qPCR protocol in 50 samples that had tested positive with conventional PCR (Table 6.15). The only sample that had sufficient amplification was a *Catallagia* sp. that had an amplicon fragment at ~1150 bp.

6.4.5 Detection of *Rickettsia* in fleas

Quantitative screening for *Rickettsia* species was conducted in 133 samples (Table 6.16). Of these samples, six *O. bruneri* samples had a cycling threshold above 15 and were considered positive. An additional 26 samples had amplification between 35.76-40 cycles (Table 6.17) and were considered negative based on the calculated starting quantity (Fig. 6.16B). Thirty samples were screened using the *Rickettsia* 17 kDa primer pair (Table 6.16). Though four samples had amplicons of 300 bp, clean sequence was only produced in a single rural *O. bruneri* sample. This sequence had a 98% identity to a *Rickettsia*-like endosymbiont from *Neurigona lineata* (Fig. 6.17) with one transition and five transversions.

Figure 6.16: Representative standard curves for the A) *Wolbachia* and B) *Rickettsia* qPCR assays using their respective gBlocks.

A)



B)

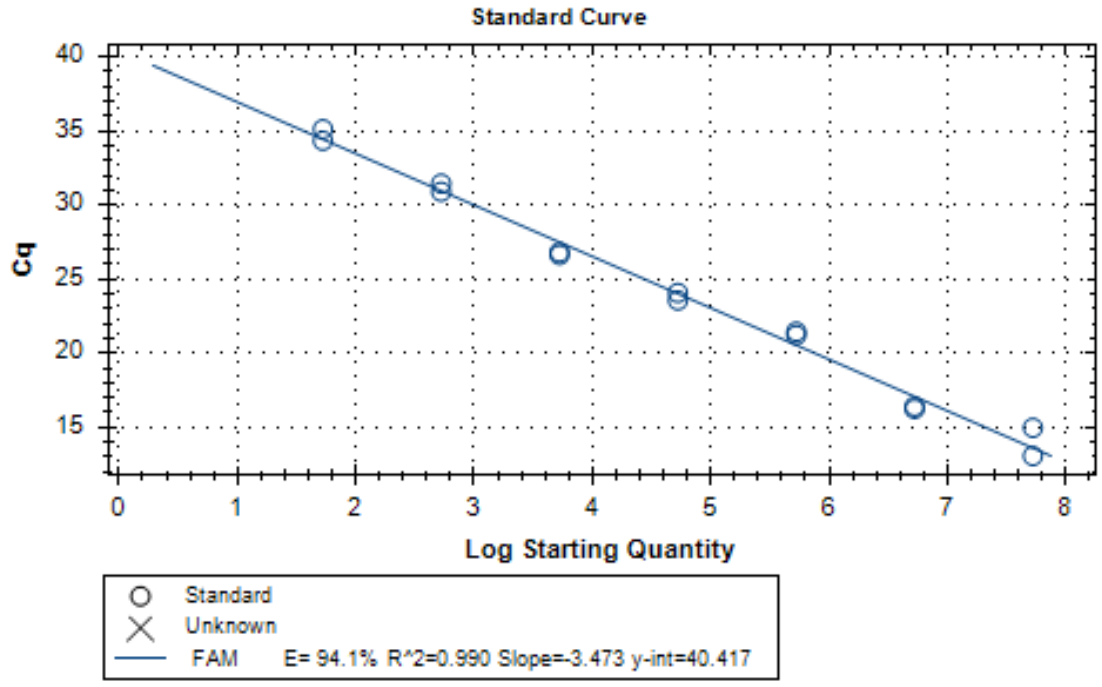


Table 6.15: The CT values for the *wsp Wolbachia* qPCR assay conducted on individual fleas of each species.

		CT Values		
Species	**Site**	**0**	**>15**	**39**
Aetheca wagneri	Urban	0	0	0
A. wagneri	Rural	0	0	0
Catallagia sp.	Urban	0	1	0
unknown ceratophyllid	Urban	1	0	0
Megabothris sp.	Rural	1	0	0
Neopsylla inopina	Urban	0	0	0
N. inopina	Rural	0	0	0
Oropsylla bruneri	Urban	15	0	0
O. bruneri	Rural	7	0	1
O. labis	Urban	7	0	0
O. rupestris	Urban	0	0	0
O. rupestris	Rural	0	0	0
O. tuberculata	Urban	10	0	0
O. tuberculata	Rural	7	0	0

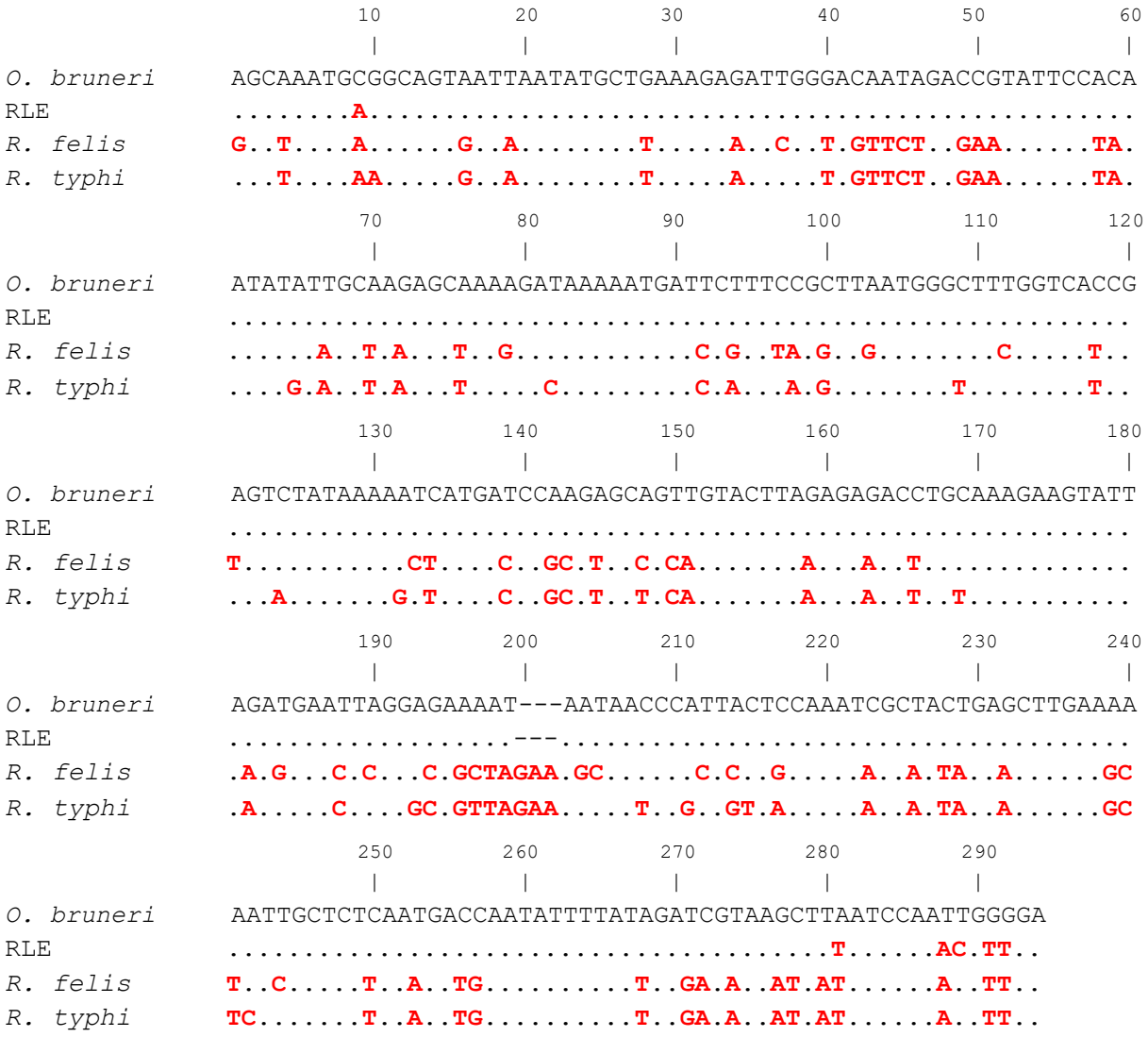
Table 6.16: Samples per species used for screening of *Rickettsia* using qPCR to detect the *gltA* gene and conventional PCR techniques targeting the 17 kDa gene in nine species of fleas collected from burrows of Richardson's ground squirrels.

Species	Local	qPCR no. of samples tested	qPCR +ve	Conventional PCR no. of samples tested	Conventional PCR +ve
Aetheca wagneri	Rural	0	0	1	0
A. wagneri	Urban	0	0	1	0
Catallagia sp.	Urban	1	0	2	0
unknown ceratophyllid	Urban	1	0	0	0
Megabothris sp.	Rural	1	1	0	0
Neopsylla inopina	Rural	0	0	0	0
N. inopina	Urban	0	0	3	1
Oropsylla bruneri	Rural	53	14	7	1
O. bruneri	Urban	15	3	2	0
O. labis	Urban	0	0	0	0
O. rupestris	Rural	0	0	3	0
O. rupestris	Urban	45	12	9	2
O. tuberculata	Rural	7	1	2	0
O. tuberculata	Urban	10	1	0	0

Table 6.17: CT values for *Rickettsia* targeting the *gltA* gene. No amplification occurred between 14.6 to 35.76 cycles.

		CT Values		
Species	**Local**	**0**	**>15**	**30-40**
Aetheca wagneri	Urban	0	0	0
A. wagneri	Rural	0	0	0
Catallagia sp.	Urban	1	0	0
unknown ceratophyllid	Urban	1	0	0
Megabothris sp.	Rural	0	0	1
Neopsylla inopina	Urban	0	0	0
N. inopina	Rural	0	0	0
Oropsylla bruneri	Urban	12	1	2
O. bruneri	Rural	39	5	9
O. labis	Urban	0	0	0
O. rupestris	Urban	33	0	12
O. rupestris	Rural	0	0	0
O. tuberculata	Urban	9	0	1
O. tuberculata	Rural	6	0	1

Figure 6.17: Alignment of *Rickettsia* 17 kDa antigen gene sequences for the bacterium within *Oropsylla bruneri* and a *Rickettsia*-like endosymbiont (RLE) (GenBank accession number JQ925596), *R. felis* (accession number JF448469), and *R. typhi* (accession number KU167056).



6.3.6 Genetic characterization of novel *Rickettsia*-like endosymbiont (RLE) in fleas.

To investigate the identity of the *Rickettsia*-like endosymbiont in randomly selected flea samples, we conducted conventional PCR assays targeting the RLE *coxA, gltA,* and 16S rRNA gene (Table 6.18). There was no intraspecific variation in 16S rRNA sequences of RLEs in the gDNA of *O. rupestris* and *N. inopina* (Fig. 6.18). However, there was one bp insertion, 20 transitions and 8 transversions when comparing the RLEs from the two flea species. The neighbour-joining tree depicting the phylogenetic relationships between the RLEs from *Oropsylla* and *N. inopina* based on the 16S rRNA sequence data are shown in Figure 6.19. Although there was limited resolution of the RLE from flea and other insects, the RLEs from *Oropsylla* formed a clade with strong statistical support (i.e., bootstrap value of 86) to the exclusion of the RLE in *N. inopina* indicating that they represent different species.

Clean sequences for the *coxA* gene were only available for RLEs from four gDNA samples of *Oropsylla rupestris.* All four sequences were identical to one another, and had a 99% sequence identity to the 16S rDNA sequences of a *Candidatus tisiphia* endosymbiont (Fig. 6.20), RLE of the spider, *Parasteatoda lunata* (GeBank accession number OZ035003). Only one clean RLE sequence was generated for citrate synthase gene (Fig. 6.21). The RLE sequence was from a *O. bruneri.* This sequence was a 100% identical to the *gltA* sequences isolated from *Neurigona lineata* (Genbank accession number JQ925596) and *Troxochrus scabriculus* (DQ231485). The *gltA* sequences of the RLE from *O. bruneri* only had 71-72% identity to the *gltA* sequences of *Rickettsia typhi* and *R. felis*.

Table 6.18: Samples tested for RLE using conventional PCR for either *coxA*, *gltA*, the RLE 17 kDa antigen gene and the 16S rRNA gene. Multiple primer pairs were used for 16S rRNA gene to encompass the entire sequence.

Gene:		**CoxA**		**Citrate Synthase**		**17 kDa**		**16S rRNA gene**					
Primer Pairs								**Ri170/1500**		**700/mid R**		**midF/1500**	
		Tested	**+ve**	**Tested**	**+ve**	**Tested**	**+ve**	**Tested**	**+ve**	**Tested**	**+ve**	**Tested**	**+ve**
Species	**Site**												
Catallagia sp.	Urban	4	3	1	0	2	0	0	0	2	0	1	0
unknown ceratophyllid	Urban	1	1	0	0	0	0	0	0	0	0	1	0
Megabothris sp.	Rural	1	1	1	0	1	0	0	0	1	0	1	1
Neopsylla inopina	Rural	1	1	0	0	0	0	0	0	0	0	0	0
N. inopina	Urban	5	5	5	5	4	0	0	0	4	0	5	4
Oropsylla bruneri	Rural	6	2	5	0	3	0	2	0	3	0	0	0
O. bruneri	Urban	4	3	1	0	1	0	0	0	1	0	1	0
O. labis	Urban	5	5	4	0	2	0	0	0	2	0	3	1
O. rupestris	Rural	1	0	1	0	0	0	0	0	0	0	0	0
O. rupestris	Urban	10	7	8	6	3	1	6	5	3	1	2	1
O. tuberculata	Rural	5	3	5	0	3	0	0	0	3	0	2	2
O. tuberculata	Urban	4	3	2	0	0	0	0	0	0	0	0	0

Figure 6.18: Alignment of RLE 16S rRNA gene between *Oropsylla rupestris* samples (S080, S104, S123, and S133) and *Neopsylla inopina* samples (S074, S027, and S026).

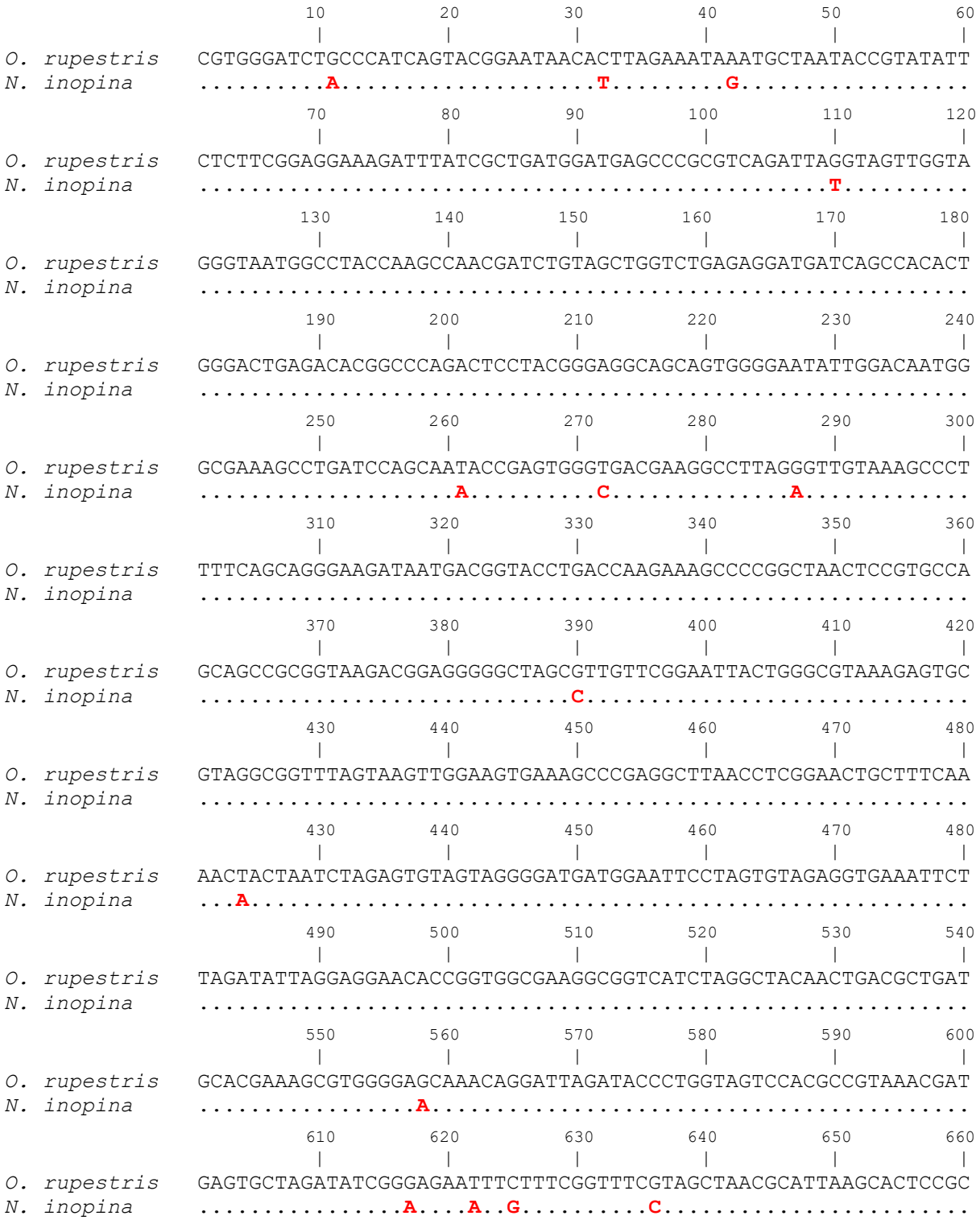


Figure 6.18: continued

```
                          670         680         690         700         710         720
                           |           |           |           |           |           |
O. rupestris    CTGGGGAGTACGGTCGCAAGATTAAAACTCAAA-GGAATTGACGGGGGCTCGCACAAGCG
N. inopina      ....................A.........A............T...........

                          730         740         750         760         770         780
                           |           |           |           |           |           |
O. rupestris    GTGGAGCATGCGGTTTAATTCGATGTTACGCGAAAAACCTTACCAACCCTTGACATGGTG
N. inopina      ............................................................

                          790         800         810         820         830         840
                           |           |           |           |           |           |
O. rupestris    GTCGCGGGAAGCAGAGATGCATCCCTTCAGTTCGGCTGGACCACACACAGGTGTTGCATG
N. inopina      ............................................................

                          850         860         870         880         890         900
                           |           |           |           |           |           |
O. rupestris    GCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCA
N. inopina      ................A...........................................CT.

                          910         920         930         940         950         960
                           |           |           |           |           |           |
O. rupestris    TTCTTATTTGCCAGCGGGTAATGCCGGGAACTATAAGGAAACTGCCGGTGATAAGCCAGA
N. inopina      ............................................................G..

                          970         980         990        1000        1010        1020
                           |           |           |           |           |           |
O. rupestris    GGAAGGTGGGGACGATGTCAAGTCATCATGGCCCTTATGGGTTGGGCTACACGCGTGCTA
N. inopina      .........A..................................................

                         1030        1040        1050        1060        1070        1080
                           |           |           |           |           |           |
O. rupestris    CAATGATATCCACAGAGGGAAGCAAGACGGTGACGTGGAGCAAATCCCTAAAAGATATCT
N. inopina      .....GC.....................................................

                         1090        1100        1110        1120        1130        1140
                           |           |           |           |           |           |
O. rupestris    CAGTTCGGATTGCTCTCTGCAACTCGAGAGCATGAAGTTGGAATCGCTAGTAATCGCGGA
N. inopina      .......A....T.................A..............................

                         1150        1160        1170
                           |           |           |
O. rupestris    TCAGCATGCCGCGGTGAATACGTTCTCGGGCCTTGTACA
N. inopina      ...................................
```

Figure 6.19: Neighbour-joining tree depicting the phylogenetic relationships of RLEs based on the nucleotide sequences of the 16S rRNA gene. The number above each branch indicates the bootstrap value.

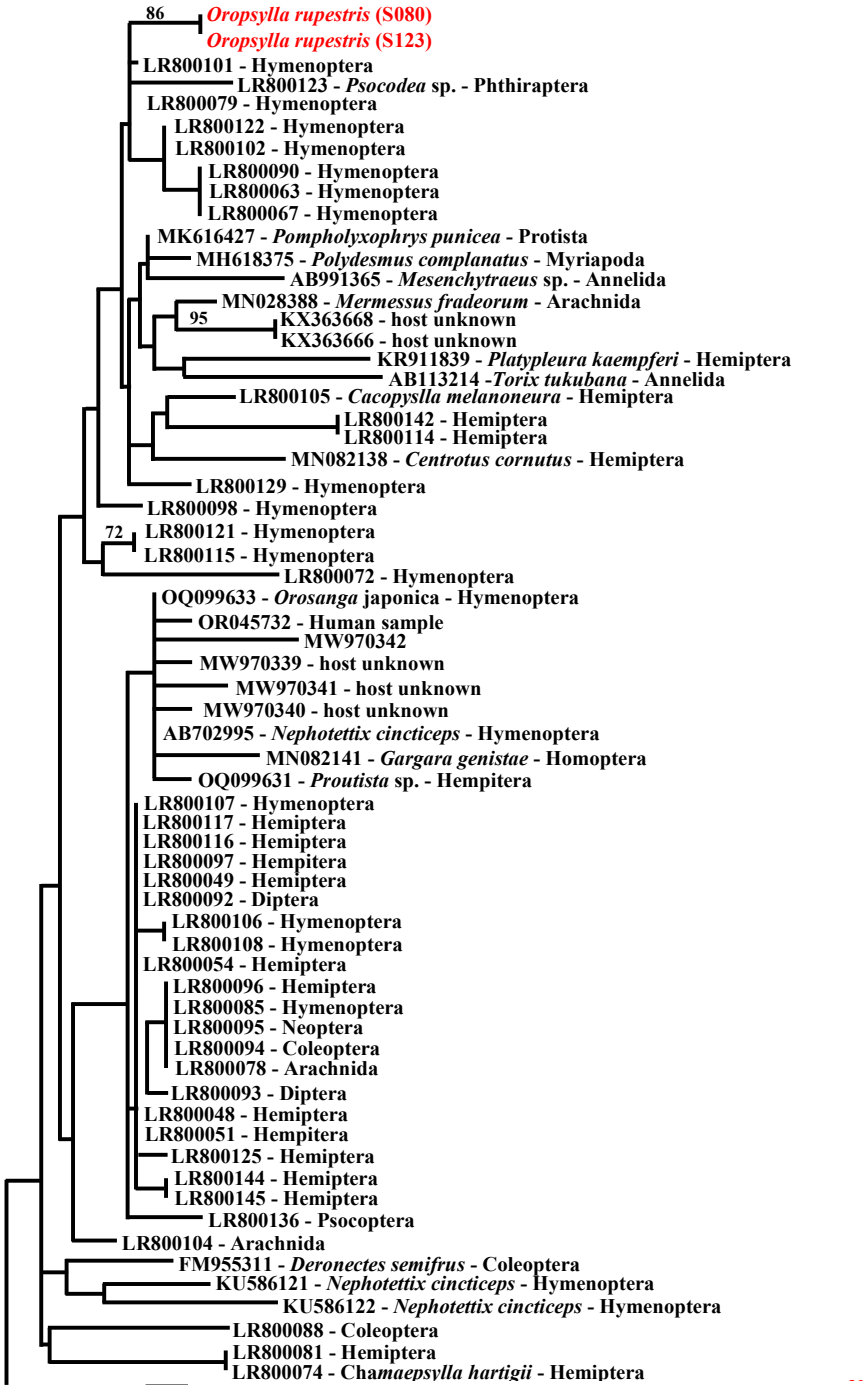


Figure 6.19: Continued

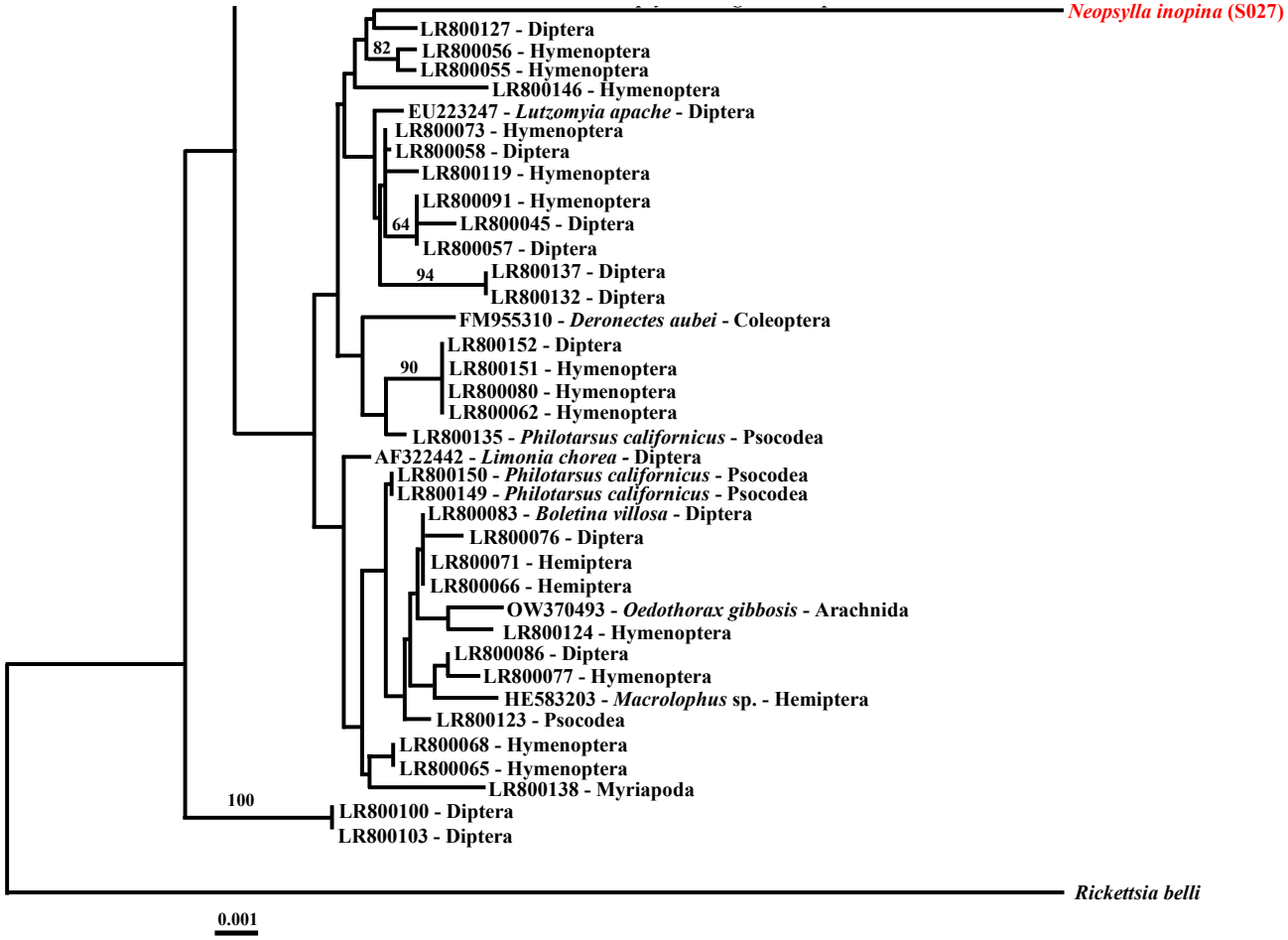


Figure 6.20: Alignment of *coxA* isolated from *Oropsylla rupestris* (OR RLE) in comparison to *Rickettsia*-like endosymbiont of *Zavrelimyia* sp. (GenBank accession number LR812144), RLE of *Cimex lectularius* (accession number CP084572), and *Candidatus tisiphia* endosymbiont of *Parasteatoda lunata* (accession number OZ035003).

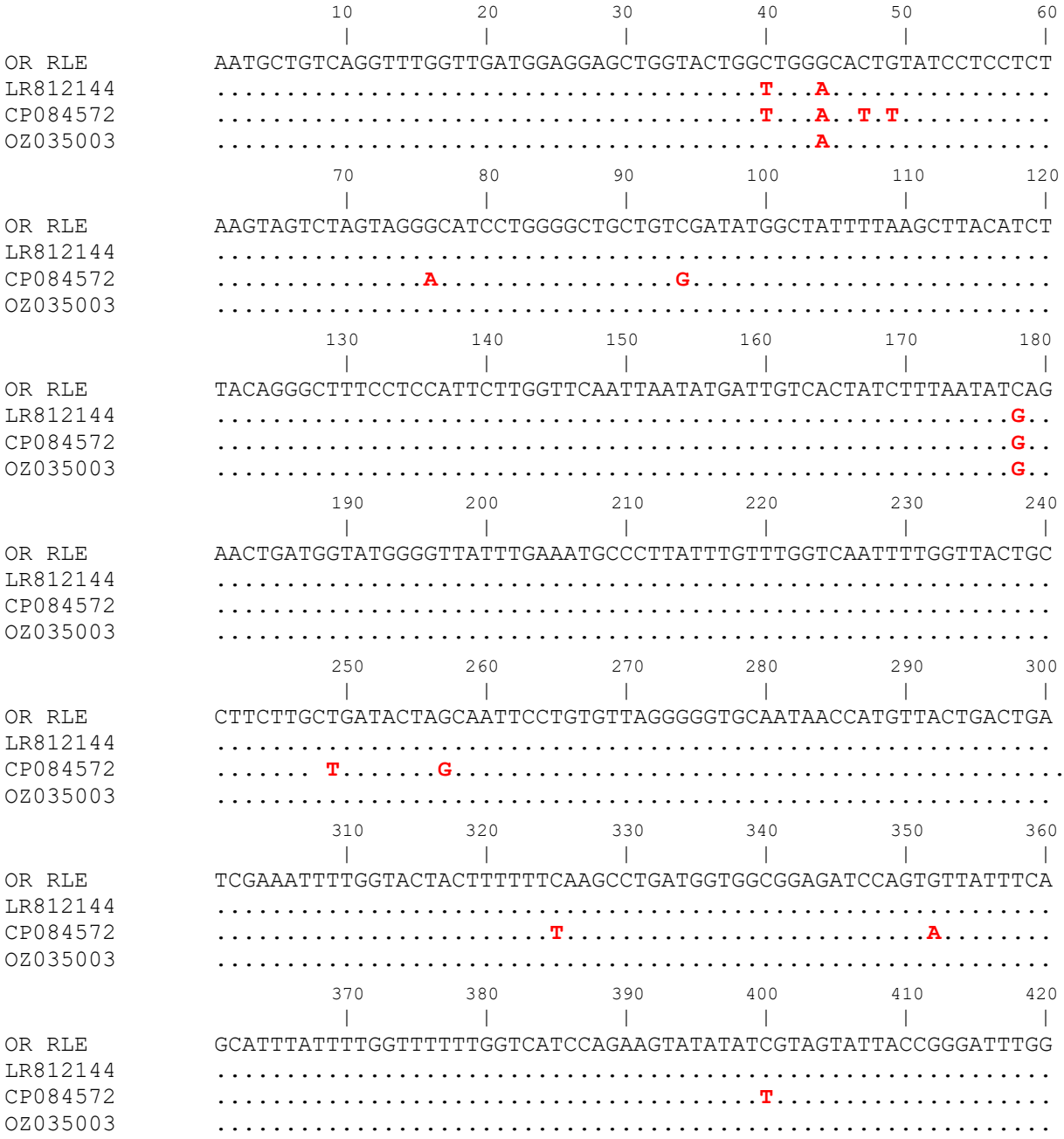


Figure 6.20: continued

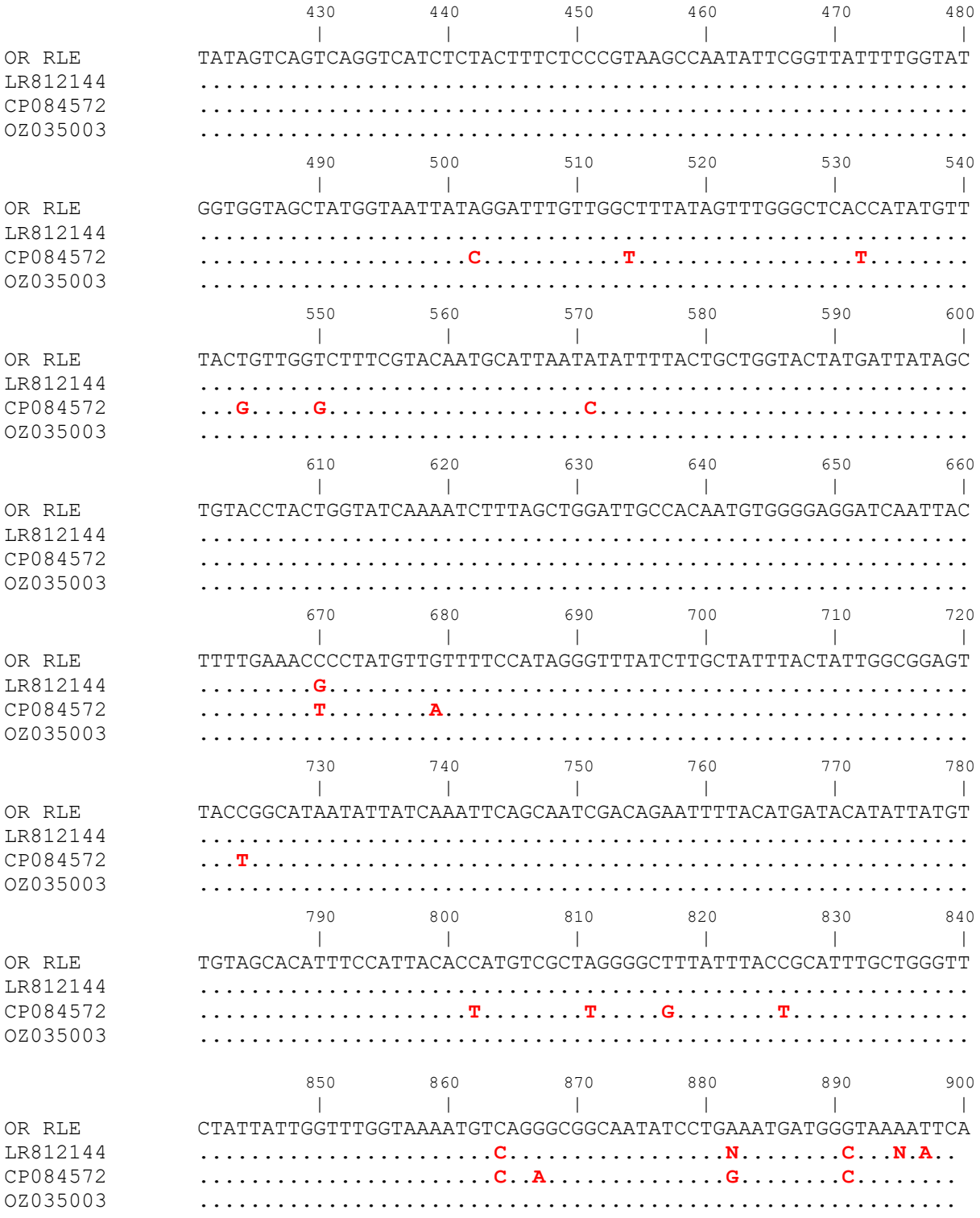


Figure 6.20: continued

```
                          910         920         930         940         950         960
                           |           |           |           |           |           |
OR RLE          TTTTTGGATTACCTTTATCGGCGTTAATTTGACATTCTTTCCTCAGCATTTTCTAGGATT
LR812144        .......G.............T..................????????????????????
CP084572        .......G..........T..T.............T..C............T.G.....
OZ035003        ..................T..T.....................................

                          970         980
                           |           |
OR RLE          AGCGGGTATGCCAAGAAGAATACCG
LR812144        ?????????????????????????
CP084572        ...T..A...................
OZ035003        ...T......................
```

Figure 6.21: Alignment of the citrate synthase sequence of the RLE from *Oropsylla bruneri* (S084) with those of two *Rickettsia*-like endosymbionts isolated from *Neurigona lineata* (Genbank accession number JQ925596) and *Troxochrus scabriculus* (DQ231485), and *Rickettsia typhi* (MH325382) and *Rickettsia felis* (OM936910).

```
                    10         20         30         40         50         60
                     |          |          |          |          |          |
S084          AGCAAATGAGGCAGTAATTAATATGCTGAAAGAGATTGGGACAATAGACCGTATTCCACA
JQ925596      ............................................................
DQ231485      ............................................................
MH325382      ...T..... A.....G..A........T.....A.....T.GTTCT..GAA......TA.
OM936910      G..T..... A..G.....A........T.....A..C..T.GTTCT..GAA......TA.

                    70         80         90        100        110        120
                     |          |          |          |          |          |
S084          ATATATTGCAAGAGCAAAAGATAAAAATGATTCTTTCCGCTTAATGGGCTTTGGTCACCG
JQ925596      ............................................................
DQ231485      ............................................................
MH325382      ....G.A..T.A...T.....C.........C.A..TA.G....A...T........T..
OM936910      ......A..T.A...T..G............C.G..TA.G........T..C.....T..

                   130        140        150        160        170        180
                     |          |          |          |          |          |
S084          AGTCTATAAAAATCATGATCCAAGAGCAGTTGTACTTAGAGAGACCTGCAAAGAAGTATT
JQ925596      ............................................................
DQ231485      ............................................................
MH325382      ...A.......G.T....C..GC.T..T.CA.......A...A..T..T...........
OM936910      T..T........CT....C...C.T..C.CA.......A...A..T..............

                   190        200        210        220        230        240
                     |          |          |          |          |          |
S084          AGATGAATTAGGAGAAAA---TAATAACCCATTACTCCAAATCGCTACTGAGCTTGAAAA
JQ925596      ................---.........................................
DQ231485      ................---.........................................
MH325382      .A.....C....GC.GTTAGAA.....T..G..GT.A.....A..A.TA..A......GC
OM936910      .A.G...C.C...C.GCTAGAA..C..T..GC.TT.G.....A..A.TA..A......GC

                   250        260        270        280
                     |          |          |          |
S084          AATTGCTCTCAATGACCAATATTTTATAGATCGTAAGCTTTATCCAA
JQ925596      ...............................................
DQ231485      ...............................................
MH325382      TC.......T..A..TG..........T..GA.A..AT.A.......
OM936910      T..C.....T..A..TG..........T..GA.A..AT.A.......
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6.5 DISCUSSION

Initially we set out to investigate the bacterial community of fleas with next-generation sequencing techniques. As we were unsuccessful at producing viable amplicons from this approach, we pivoted to a qPCR approach to assess the total bacterial load within the fleas as well as three key bacterial genera *Bartonella*, *Wolbachia* and *Rickettsia*. There has been an increasing interest in characterizing the bacterial community of insects, particularly disease vectors and how their bacterial community relates to disease transmission. Studies on the bacterial communities of fleas have found that they have a low species diversity and are typically dominated by a single phylotype (i.e., taxon), that belongs to one of three genera; *Rickettsia, Wolbachia* or *Bartonella* (Jones et al. 2008, 2010, 2012, 2013, Hawlena et al. 2013, Lawrence et al. 2015, Vasconcelos et al. 2018). The bacterial community of rodent blood is dominated by flea-borne bacteria (Cohen et al. 2015), indicating that a large portion of the bacterial community in fleas is obtained when they acquire a blood meal. There have been varying results on whether the location, year, or the flea species examined affect the composition of the bacterial community (Jones et al. 2008, 2010, 2012, 2015, Lawrence et al. 2015).

We found that the location of collection and presence of a blood meal significantly influenced the starting quantity concentration of total bacteria. Jones et al. (2010) found that the bacterial communities of *O. tuberculata, O. hirsuta*, and an unidentified flea species collected from black-tailed prairie dogs varied among host colonies and years rather than on the flea species examined or from individual hosts. Most of the bacterial community studies of *Oropsylla* species have focused on those associated with black-tailed prairie dogs in Colorado, USA (Jones et al. 2008, 2010, 2012, 2013). However, in Saskatchewan, black-tailed prairie dogs and Richardson's ground squirrels coexist and share a number of flea species (Liccioli et al. 2020).

As the role of Richardson's ground squirrels play in plague epizootics is still unclear, it is important to understand the composition of the bacterial community of fleas that parasitize Richardson's ground squirrels and that could transfer to black-tailed prairie dogs.

The bacterial load in the species examined in this study had a wide range (95.7 - 7.92 x 10^7) and was significantly lower than expected. The low level of bacterial load is one possible reason why our attempts to characterize the bacterial community of these samples using next-generation techniques was unsuccessful. To our knowledge, determination of the total bacterial load has not been quantified in fleas. Previous studies that focused on the transmission efficiency of *Yersinia pestis* by species of *Oropsylla* have quantified the bacterial load of *Y. pestis* after feeding inoculation (Wilder et al. 2008, Jones et al. 2013, Boegler et al. 2016). In these studies, fleas were fed blood artificially inoculated with *Y. pestis* at varying concentrations to determine the transmission efficiency at different time periods post feeding. Boegler et al. (2016) reported a median value ranging from 28 to 1.0 x10^6 cfu of *Y. pestis* per *O. montana*, while Wilder et al. (2008) found *O. tuberculata* had a median range of 1.52 x10^6 to 6.65 x10^6 cfu bacterial load in fleas. The low bacterial load of fleas may allow for easy colonization of *Y. pestis*. The midgut of fleas is considered a harsh environment that could prevent the colonization; however, the midgut epithelia in fleas is vulnerable to bacterial colonization as these particular insect hosts lack a peritrophic matrix (Erickson et al. 2009). Despite the low bacterial load detected in our flea samples, we set out to investigate the load of species of bacteria that commonly dominate the bacterial community of fleas in North America (Cohen et al. 2015, Jones et al. 2010, 2012).

A variety of *Bartonella* species have been isolated from a variety of rodents (Kosoy et al. 1997, Jardine et al. 2006b) including black-tailed prairie dogs (Bai et al. 2008, Rubio et al. 2014) and Richardson's ground squirrels (Jardine et al. 2005, 2006a). We expected to find a high

prevalence of *Bartonella* in fleas associated with Richardson's ground squirrels, as previous studies had reported 48% prevalence of *Bartonella* in these mammals (Jardine et al. 2006b). Forty-three percent of fleas were found to be positive using a qPCR analysis; however, only 8 gDNA samples were confirmed positive using a conventional PCR assay. As Jardine et al. (2006a) investigated the prevalence of *Bartonella* in the host's blood, it is possible that the concentration of *Bartonella* in host blood is higher, and therefore more detectable, than in parasitizing fleas. The low prevalence is similar to the prevalence of *Bartonella* (13.1%) from *Oropsylla* spp. fleas collected from black-tailed prairie dog colonies in Colorado (Stevenson et al. 2003). The quantity of a *Bartonella* infection may be below the limit of detection in our flea samples. Stevenson et al. (2003) theorized that *Bartonella* were capable of persisting in previously fed fleas after the digestion of a fresh blood meal. Our results support this as the presence of blood meal did not influence the starting quantity of *Bartonella* in the flea samples. Two distinct citrate synthase sequences of *Bartonella* were detected in *O. bruneri* samples. The results of the phylogenetic analyses placed one of the two sequence genotypes in a clade with *Bartonella* isolates from Franklin's ground squirrels and thirteen-lined squirrels, whereas the second genotype was placed in a clade with *Bartonella* isolates from Richardson's ground squirrels. *Oropsylla bruneri* is considered a true flea of Franklin's ground squirrels and can additionally parasitize Richardson's ground squirrels (Holland 1985, Lewis 2002).

Based on the results of conventional PCR results for *Wolbachia wsp* assay, 36% of individual fleas had amplicons of the expected size compared to pre-screening *wsp* sequences. Additional bandings were observed at approximately 1300 bp (41%), 1150 bp (69%), 900 bp (20%), and 350 bp (69%) which may represent non-target amplification or different strains of *Wolbachia*. Multiple bands and poor sequencing results may be an indication of infection by

multiple strains (Kajtoch et al. 2019). Sequences of the *wsp* gene for the *Wolbachia* in *N. inopina* were similar to a *wsp* sequence of *Wolbachia* isolated from the flea, *Paradoxopsyllus repandus*, in China (Yin et al. 2019). The *Wolbachia* detected in *Oropsylla* had a similar *wsp* sequence to *Wolbachia* isolated from *Oropsylla silantiewi*. *Wolbachia* endosymbionts of fleas have been grouped into a distinct clade (Kaur et al. 2021); however, our results do not support this grouping as the 16S rRNA gene sequence from *Oropsylla bruneri* had the highest identity to group A (containing *Wolbachia* from Arthropods) whereas the 16S sequence from *Oropsylla rupestris* was in a separate clade closer to group B (also containing *Wolbachia* from Arthropods). In comparison, the sequences from the *wsp* gene had *Oropsylla* species forming a clade with group A, whereas the *wsp* sequence from *N. inopina* formed a separate clade within group A however, closer to sequences isolated from Coleoptera. Further investigation is required to assess the diversity of *Wolbachia* within fleas, and the ecological and evolutionary implications of *Wolbachia* infection in fleas.

Given the low prevalence of *Bartonella* in fleas, we expected a large number of fleas to be PCR-positive for *Rickettsia* as *Rickettsia* and *Bartonella* appear to have a negative interaction based on co-occurrence data (Jones et al. 2012). Rickettsiales have been previously detected in *Oropsylla* species associated with black-tailed prairie dogs using next-generation sequencing (Jones et al. 2008, 2012). However, in this study, only 4.5% of the samples screened were positive for *Rickettsia* based on qPCR. The results of sequencing the *gltA* amplicon of one flea suspected to be infected with *Rickettsia*, revealed that the bacterium was not a *Rickettsia* species commonly associated with fleas (i.e. *R. felis* or *R. typhi*), but belonged to a group of *Rickettsia*-like endosymbionts found in other group of insects, that is, arthropods not infected with species of *Rickettsia*.

Rickettsia-like endosymbionts have only been recently described. Their discovery occurred after some researchers realized that the primers they were using to amplify the mitochondrial cytochrome oxidase I gene of insects, were not amplifying this gene, but the cytochrome oxidase A gene of this new group of rickettsiales bacteria inside the insect host that they subsequently defined as "*Rickettsia*-like endosymbionts" (Pilgrim 2020). We have found two novel *Rickettsia*-like endosymbionts in two different families of fleas based on our sequence results. As far as we are aware, *Rickettsia*-like endosymbionts have not previously been described in fleas; although some RLEs in fleas may have been identified incorrectly as taxa within the genus *Rickettsia* (e.g., Lawrence et al. 2015). The discovery of RLEs in fleas, especially given that they are potential hosts for *R. felis* or *R. typhi*, raises a number of interesting ecological and evolutionary questions. For example, how frequent are these RLEs in fleas? How many species of fleas parasitizing sciurid rodents are hosts for RLEs? Are these RLEs transmitted transovarially as are many bacterial endosymbionts? Are RLEs host specific? Finally, what ecological role(s) do RLEs play in the microbiomes of fleas? The ecological role of RLEs in other insects is relatively unknown but they are considered to be mutualists in the highland midge, *Culicoides impunctatus*, because they reside in the fat bodies of their insect host (Pilgrim et al. 2020). Nonetheless, further investigation is required to address the ecological and evolutionary questions that have arisen given the discovery of at least two distantly-related RLEs in two species of flea associated with Richardson's ground squirrels.

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CHAPTER 7: GENERAL DISCUSSION

Richardson's ground squirrels are important reservoir host for *Yersinia pestis*, the causative agent of plague, and many of the flea species that parasitize them, for example, *Oropsylla rupestris, O. bruneri, O. labis, O. tuberculata* and *A. wagneri*, are vectors of *Y. pestis* (Wilder et al. 2008, Eisen et al. 2009). Research has been conducted on the flea communities of Richardson's ground squirrels in the aspen parkland (Galloway and Christie 1990, Lindsay and Galloway 1997, 1998, Waterman et al. 2014) and on the prevalence and diversity of *Bartonella* in Richardson's ground squirrels in Saskatoon, Saskatchewan (Jardine et al. 2005, 2006a, 2006b). However, these studies have not incorporated bacterial diversity with the diversity of fleas parasitizing Richardson's ground squirrels. The goal of this thesis was to provide context on the prevalence and abundance of fleas that parasitize Richardson's ground squirrels in Saskatchewan as well as the bacterial communities in flea microbiomes. Additionally, I investigated how the relationships among fleas (i.e., vectors and parasites), bacteria (i.e., pathogens and mutualistic endosymbionts), and rodents (i.e., hosts for the fleas and bacteria within them) can be used as a model system to investigate macroecology concepts in a microscale. As some flea species are known vectors of *Y. pestis*, I set out to develop a molecular tool to differentiate multiple species of fleas found parasitizing black tailed prairie dogs and Richardson's ground squirrels in Grasslands National Park (Chapter 2, Thoroughgood et al. 2021), a location where *Y. pestis* has been known to occur (Antonation et al. 2014, Liccioli et al. 2020). Once I was able to identify the species of fleas, additional molecular markers (Chapter 3) were examined to determine their effectiveness to differentiate among flea species. Using a molecular approach for flea identification (Chapters 2 and 3) allowed me to examine community structure and seasonal

activity patterns of fleas (Chapters 4 and 5), and determine the bacteria present in the flea microbiomes (Chapter 6).

The community structure and seasonal activity patterns of fleas in Canada have been relatively neglected, particularly in Saskatchewan. To assess the community structure and vector specificity, I began by collecting fleas from Richardson's ground squirrels from Saskatchewan (near Moose Jaw) and Alberta (near Lethbridge) (Chapter 4). I investigated what characteristics of the host influenced the number of fleas on hosts and the community composition of fleas on these hosts. Evidence of seasonality patterns of fleas was observed during the investigations reported in Chapter 4; however, hosts were collected at random (i.e., chance sampling), and sampling was not conducted in a systematic manner over the time when hosts are active above ground. Therefore, I conducted a controlled collection experiment to determine the seasonal activity patterns of the flea species associated with Richardson's ground squirrels (Chapter 5, Thoroughgood and Chilton 2024) and investigate the community composition of the internal bacteria to determine if the bacteria reflected any seasonal patterns or vector specificity (Chapter 6).

7.1 FLEAS ON RICHARDSON'S GROUND SQUIRRELS

Fleas exhibited aggregation within Richardson's ground squirrel populations with 54% of hosts being parasitized by one of more fleas. The number of individuals hosts in a population that were infested decreased as the number of fleas parasitizing the host increased. For a given species of parasites, the abundance on/in individual of a host population typically follows a Poisson distribution (i.e. aggregation). However, a negative binomial distribution is more reflective of parasite abundance on/in hosts (Poulin 1993, Poulin 2013). I found that sex and

length of host, as well as the year and province of collection, to be strong predictors of the abundance of fleas parasitizing *U. richardsonii*. Richardson's ground squirrels exhibit cohort emergence and immergence patterns based on age and sex (Michener 1983, 1984). Male Richardson's ground squirrels were more likely to be parasitized by fleas than female ground squirrels (Chapter 4). Adult males are active above ground from mid-February or mid-March to June or early July, while juvenile males are active from end of May or June to September or early October (Michener 1983, 1984, Lindsay and Galloway 1997, Jardine et al. 2006a). In contrast, adult female *U. richardsonii* are active from March to July to early August, while juvenile females are above ground from end of May or June to August or early September (Michener 1983, 1984, Lindsay and Galloway 1997, Jardine et al. 2006a). The period of above ground activity of adult hosts corresponds to the peak activity period of *Neopsylla inopina* and *O. tuberculata*, while the juveniles are active during the time when *O. rupestris* are most active (Chapter 5). Uniquely, *O. bruneri* has two peaks in abundance, one when adult hosts are active and the second, during the activity period of juveniles. Jardine et al. (2006a) found that *U. richardsonii* adults were significantly more likely to be parasitized by fleas than juveniles; however, as their study was conducted mainly in a rural area near Saskatoon, and is similar to our rural site (Chapter 5), this may be due to a high prevalence of *O. bruneri* in their samples which peak in May and September when adults are still present (Michener 1983, 1984).

Eleven species of flea, *O. rupestris*, *O. bruneri*, *O. labis*, *O. tuberculata*, *Megabothris* sp. 1 (probably = *M. asio*), *Megabothris* sp. 2 (= *M. quirini*), *Aetheca wagneri*, *Catallagia* sp., *N. inopina*, *Rhadinopsylla fraterna* and an unknown species of ceratophyllid, were found to be associated with Richardson's ground squirrels. According to Holland (1985), the only *Catallagia* species known to occur in Saskatchewan is *C. decipens* (Holland 1985, Liccioli et al. 2020);

however, *C. borealis* has been collected on a Franklin's ground squirrel in Bird Hill Park, Manitoba (Galloway and Christie 1990). All 11 flea species collected during my study have previously been reported in Saskatchewan and Alberta (Holland 1985) and collected from Richardson's ground squirrels in Manitoba (Galloway and Christie 1990).

Oropsylla rupestris is considered to be the "Richardson's ground squirrel flea" and is likely to have a close evolutionary history with its host (Holland 1985). Therefore, it was not surprising that *O. rupestris* was the most abundant flea species on Richardson's ground squirrels in Saskatchewan (59%) and Alberta (93%) during 2019. This flea species also comprised 50% of the fleas collected on burrow swabs from the urban site (Saskatoon). *Oropsylla rupestris* was also the most dominant species collected from Richardson's ground squirrels in Grasslands National Park (Liccioli et al. 2020) in southern Saskatchewan, and near Winnipeg, Manitoba (Lindsay and Galloway 1997). *Oropsylla tuberculata* was the most abundant species on *U. richardsonii* collected in Saskatchewan during 2017 and 2018 (i.e., 49% and 53% of fleas collected, respectively). In contrast, *O. bruneri* comprised 72% of the fleas collected from burrows at the rural site in Saskatchewan. Although *O. bruneri* is commonly associated with Franklin's ground squirrels, Galloway and Christie (1990) argued that this flea species may be a habitat specialist rather than the Franklin's ground squirrel flea given that they collected *O. bruneri* throughout the range of Richardson's ground squirrels, Franklins ground squirrels, and thirteen-lined grounds in Manitoba.

Liccioli et al. (2020) found that Richardson's ground squirrels in Grasslands National Park were parasitized mainly by *O. rupestris* (74%) and *O. tuberculata* (23%). These species were also the most abundant species collected from swabs of black-tailed prairie dog and Richardson's ground squirrel burrows in Grasslands National Park (16% and 78% respectively;

Liccioli et al. 2020). Lindsay and Galloway (1997) found that Richardson's ground squirrels were mainly parasitized by *O. rupestris* (49%) and *O. tuberculata* (43%) in aspen parkland. These two species of flea are host generalists and exploit a variety of mammal hosts including black-tailed prairie dogs, arctic ground squirrels, Franklin's ground squirrels, and thirteen-lined ground squirrels (Holland 1985, Galloway and Christie 1990, Thoroughgood and Chilton unpublished data). Fleas that are exploit a broad range of hosts (= host generalists) can reach a higher abundance on their preferred host species (Krasnov et al. 2004).

The composition of the flea community in burrows at the rural site (near Bradwell, SK) was expected to be similar to the flea diversity and abundance on Richardson's ground squirrels Moose Jaw (SK) because of similarities in vegetation. However, the community structure of the burrows in the rural site was predominately *O. bruneri* rather than *O. rupestris* and *O. tuberculata* were the predominant species on Richardson's ground squirrels from Moose Jaw. Although swabbing of the burrows of *U. richardsonii* provides insights of the diversity and relative abundance of different fleas that may occur on individuals of the host population, this approach has its limitations. For example, Eads et al. (2021) determined that fleas collected on swabs of the burrows of white-tailed prairie dogs in Utah, as well as black-tailed prairie dogs in New Mexico and Montana, represented less than 26% of the fleas present on black-tailed prairie dogs and white-tailed prairie dogs from that area. Similarly, *Megabothris* sp. 2 (= *M. quirini*) and *R. fraterna* were collected from Richardson's ground squirrels but were absent on swabs taken from the burrows of *U. richardsonii*. This lack of detection may be due to difference in presence in host burrows, light sensitivity, and on-host duration of the flea species (Eads 2017, Eads et al. 2021). In contrast, we detected *Catallagia* sp. and an unknown species of ceratophyllid flea in the burrows of Richardson's ground squirrels that were not detected on any

host individual. The flea communities in this thesis were collected from northern mixed grass prairie ecoregion; however, the on-host community was collected from agricultural farmland while the off-host communities were from managed grass areas. Additional environmental factors (e.g., precipitation, soil acidity and composition) between the locations may influence the flea species prevalence and abundance.

The seasonal activity patterns of fleas on Richardson's ground squirrels (Chapter 4) and in their burrows (Chapter 5) were relatively similar (Table 7.1). Simultaneous collection of hosts and burrow swabs would need to be performed to encompass all fleas parasitizing Richardson's ground squirrels in these areas as burrow swabs do not provide a full representation of the community of on-host fleas (Eads 2017, Eads et al. 2021). The on-host activity of fleas can be influenced the reproductive status of the flea as well as by the host's reproduction status, grooming behaviour, and/or hair type (Richardt and Galloway 1994, Stark 2002, Eads 2017, Eads et al. 2021). For example, the undercoat of Franklin's ground squirrels is thick in spring and fall whereas in the summer their guard hair is sparse and wiry which may affect different species ability and duration of fleas to remain on the host (Richardt and Galloway 1994).

Table 7.1: Peak in abundance of the four most prevalent species of fleas from Richardson's ground squirrels (Chapter 4) and burrow swabs (Chapter 5).

Flea Species	Host		Burrow Swab	
	Alberta	Saskatchewan	Urban site	Rural site
O. rupestris	June	July	July	August
O. bruneri	-	April/June	May/August	May/September
O. tuberculata	June	April	May	May
N. inopina	May	April	May	May

7.2 BACTERIA IN FLEAS OF RGS

The bacterial communities of some flea species have been examined (see Jones et al. 2008, 2010, 2012, 2013, Hawlena et al. 2013, Lawrence et al. 2015, Vasconcelos et al. 2018). However, these studies have focused mainly on flea species that are vectors of *Y. pestis* and parasitize black-tailed prairie dogs. In Chapter 6, I investigated the bacterial community of fleas associated with Richardson's ground squirrels near the northern limit of the host's range. Overall, there was an extremely low bacterial load in all flea specimens. Next-generation sequencing of the 16S rRNA gene of from prairie dogs have relatively low read counts ranging from 305 to 4279 sequences per flea (Jones et al. 2010, 2013, 2015). This markedly different from studies on cat fleas (*Ctenocephalides felis*), where 61 000 to 218 500 sequence reads were obtained per flea in the study by Lawrence et al. (2015) or a mean read count of 10 000 in the study of Vasoncelos et al. (2018). These studies found that the bacterial communities of fleas were dominated by a single phylotype (i.e., Proteoalphabacteria such as *Rickettsia*, *Bartonella*, and/or *Wolbachia*). Jones et al. (2013) found that the bacterial diversity in fleas decreased following an infection of *Y. pestis*. They speculated that *Y. pestis* manipulates the bacterial community and out competes all other bacteria in the flea microbiome (Jones et al. 2013). However, the appropriation of the flea microbiome by *Y. pestis* may be due to rodent fleas not containing large numbers of bacteria in microbiome; hence, *Y. pestis* could easily colonize and dominate the bacterial community.

The microbiomes of fleas such as, *Ctenocephalides felis, Ctenophthalmus calceatus cabirus, Ctenophthalmus pseudagyrtes, Dinopsyllus lypusus, Orchopeas leucopus, Oropsylla hirsuta, O. montana, O. tuberculata, Stivalius torvus, Xenopsylla brasiliensis, X. cheopis, X. nubica,* are dominated by species of *Bartonella*, *Wolbachia*, or *Rickettsia* (Jones et al. 2008, 2010, 2012, 2013, Hawlena et al. 2013, Lawrence et al. 2015, Vasconcelos et al. 2018). Given

this, I decided to assess the prevalence and abundance of these genera within the 11 flea species associated with Richardson's ground squirrels. This had not been investigated previously. I found that relatively few fleas were infected with *Bartonella* (43% RT-PCR, 15% conventional PCR), *Wolbachia* (36% conventional PCR), and/or *Rickettsia* (4.5% RT-PCR, 3.3% conventional PCR), and that a majority of the fleas were not infected by these bacteria.

I was only able to detect *Bartonella* in six *O. bruneri* using a PCR assay designed to amplify the citrate synthase gene (g*ltA*). There was three different *gltA* sequences that most closely related to *B. washoensis*. Sequences "A" (from the rural site in August 2019) and "B" (from the rural site from May till July) were 100% identical to *B. washoensis* isolated from Franklin's ground squirrels and thirteen-lined ground squirrels (AY584570; Jardine et al. 2005). While sequence "C" (from the urban site in June) was more similar to *B. washoensis* isolated from Richardson's ground squirrels (AY587980l; Jardine et al. 2005). Jardine et al. (2006b) found that the prominent genotype of *Bartonella* changed throughout the active period of Richardson's ground squirrels and peaked in July. For example, the dominate genotype detected in Richardson's ground squirrels in Saskatoon, SK was genotype H of *B. washoensis* (accession AY914176). This bacterium increased in prevalence from 56% in April to over 80% in June through September (Jardine et al. 2005, 2006a). They also found that male Richardson's ground squirrels were significantly more likely to be infected by *Bartonella* in a univariate model (Jardine et al. 2006a). However, this association was no longer significant when age, year and month of sampling were incorporated into the model (Jardine et al. 2006a). Additionally, juvenile Richardson's ground squirrels had twice the levels of *Bartonella* infections compared to adults (Jardine et al. 2006a) which corresponds to the activity period of *O. bruneri* and *O. rupestris*. *Bartonella* is hypothesized to be specific to the host rodent, however, the bacteria

genera could instead be limited by the species of fleas parasitizing the host and their ability to transmit the bacterium. An alternative hypothesis would be that *Bartonella* infections are hindered by the vector competency and specificity rather than the host specificity of the bacterium. If *Bartonella* was host-specific, a specific genotype should be present throughout the entire activity period and not fluctuate based on the vector species present. Further analysis is required to determine the vector specificity of fleas associated with different *Bartonella* strains and the vector competency of these fleas to transmit *Bartonella*.

Flea species-specific strains of *Wolbachia* were detected in *N. inopina*, *O. rupestris*, and *O. tuberculata* using a conventional PCR assay. Although *Wolbachia* in fleas is thought to be a distinct clade from strains associated with other arthropods (Kaur et al. 2021), the results reported in this thesis demonstrate that the *Wolbachia* strains detected in different flea species belong to multiple clades within the genus. Taxa within this bacterial genus are known to cause cytoplasmic incompatibility (Sinkins 2004, Beckmann et al. 2017), distort sex ratios (Jiggins et al. 2000, Hurst et al. 2001), and pathogen resistance (Moreira et al. 2009, Kambris et al. 2010) in their arthropod hosts. In mosquitoes, certain strains of *Wolbachia* prevent the colonization and transmission of *Plasmodium falciparum* (i.e., the protozoan parasite that cause malaria) to susceptible human hosts (Moreira et al. 2009, Bourtzis et al. 2014). The functional role of *Wolbachia* and/or the effect of the fitness of their flea hosts is unknown, hence further studies are needed to address this gap in our understanding of the importance of bacteria in the microbiomes of fleas.

The novel detection of *Rickettsia*-like endosymbionts (RLEs) in fleas in the present study represents an important discovery. Fleas have previously only been linked to *Rickettsia felis* and *R. typhi* (Eisen and Gage 2012). *Rickettsia*-like endosymbionts were initially described as the

Torix group of *Rickettsia* isolated from *Torix* leeches (Kikuchi et al. 2002, Kikuchi and Fukatsu 2005). The prevalence and abundance of RLEs in arthropods has only recently been investigated (Pilgrim et al. 2021). This novel detection requires a deep investigation into the abundance and prevalence in fleas. The role that RLEs play in their arthropod hosts is not well understood except for the RLEs in highland midges. In these insects, RLEs were assumed to be endosymbiotic mutualists (Pilgrim et al. 2020). RLEs may therefore play a pivotal role in the providing nutrients to their hosts, as is the case of some endosymbionts in ticks, where these bacteria provide essential vitamins for their invertebrate host (Olivieri et al. 2019, Duron and Gottlieb 2020, Kolo and Raghavan 2023). RLEs may also influence the composition of the bacterial communities in flea. For example, Jones et al. (2010) noted that *Bartonella* and Rickettsiales bacteria may have a negative interaction between each other based on co-occurrence data. RLEs may play a role at preventing the colonization of vertically transmitted bacteria, however this requires further investigation.

7.3 FUTURE WORK

The work of this thesis was restricted to eleven species of fleas associated with Richardson's ground squirrels in the Canadian prairies. Although I was able to differentiate these species using several molecular markers, further exploration is required to determine if these markers can also be used to distinguish among other flea species that parasitize other host species. It is particularly important to have a reliable method to distinguish those species that transmit *Y. pestis* to endangered species like black-tailed prairie dogs and flea species that are non-vectors. Further investigation into the diversity and seasonal activity of flea species on Richardson's ground squirrels across their distribution in Canada (i.e., Alberta, Saskatchewan

and Manitoba) and the United States (i.e., Montana, North Dakota, South Dakota, and Minnesota) (Michener and Koeppl 1985, Augustine et al. 2023). Such investigations could provide insight as to which environmental factors (e.g., soil acidity, precipitation, and temperature), and if the relative abundance or density of suitable host species influences the diversity and abundance of the flea communities in an area. Given that *Bartonella* was only detected in *O. bruneri* poses an interesting question into the vector competency and specificity of the bacteria in this genus within fleas that parasitize rodents. The detection of multiple strains of *Wolbachia* and *Rickettsia*-like endosymbionts raises many questions on the diversity, prevalence, and abundance of these species in fleas and the role they play in the microbiome. Furthermore, the results obtained thus far suggests that there is low bacterial diversity and abundance in flea microbiomes. This raises a number of interesting questions that could be investigated; such as, how do the 11 species of flea examined in the present study acquire the bacteria in their microbiomes (i.e., by vertical and/or horizontal transmission)?, what are the differences in the bacterial communities in the blood of the different host species that these flea parasitize?, and do only some bacterial species present in the blood of mammalian hosts able to establish an infection in the flea species imbibing that blood?

7.4 CONCLUSION

In this thesis, I investigated the diversity and abundance of fleas on Richardson's ground squirrels in Saskatchewan and Alberta. These ground squirrels are mainly parasitized by four main species (*O. rupestris*, *O. bruneri*, *O. tuberculata* and *N. inopina*) which differ in their seasonal patterns of activity. Development of molecular techniques to differentiate and identify the eleven species fleas associated with Richardson's ground squirrels allowed me to determine

the bacteria in the flea microbiomes. There was limited diversity and a low abundance of bacteria in fleas; however, the composition and species of the bacterial community may be specific to the flea host. Further studies are needed to investigate the functional roles of specific strains of *Wolbachia* and *Rickettsia*-like endosymbionts (RLEs) in flea microbiomes, particularly the RLEs, since their presence in fleas appears not have been previously reported in the scientific literature.

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