MOLECULAR EPIDEMIOLOGY OF TICK RHABDOVIRUSES AND MOLECULAR IDENTIFICATION OF TICK SPECIES IN CANADA

Kenning Vic Tran

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Kenning Tran

Date of Defence: December 1, 2025

Dr. Nariman Shahhosseini	Adjunct Professor	Ph.D.
Dr. Trushar Patel	Professor	
Thesis Co-Supervisors		
Dr. Cameron Goater	Professor	Ph.D.
Thesis Examination Committee Member		
Dr. Dmytro Yevtushenko	Associate Professor	Ph.D.
Thesis Examination Committee Member		
Dr. Michael Gerken	Professor	Ph.D.
Chair, Thesis Examination Committee		

Abstract

Ticks are important vectors of pathogens that pose significant threats to human and animal health in Canada. With the onset of climate change, tick geographic ranges are changing and expanding in Canada, raising concerns for increased disease burden of tick-borne pathogens and highlighting the need for continued comprehensive surveillance of endemic tick species and circulating pathogens. This thesis reports the identification of two novel tick-rhabdoviruses circulating in *Dermacentor andersoni* and *Ixodes auritulus* tick populations, respectively, in western Canada, and the first documentation of *Ixodes scapularis*, the primary Lyme Disease vector of eastern Canada, found off migratory birds on Vancouver Island. These findings contribute to our understanding of tick ecology and pathogen transmission dynamics, informing future public health and wildlife disease management strategies and emphasize the importance of a comprehensive surveillance system in Canada.

Contribution of Authors

Chapter 2 is a collaborative manuscript prepared for submission to *Pathogens*. The work in this chapter was conducted in collaboration with Dr. Shaun Dergousoff's laboratory at the Lethbridge Research and Development Centre of Agriculture and Agri-Food Canada, and with Dr. Catherine A. Hogan's Parasitology Laboratory and Dr. Muhammad Morshed's Zoonotic and Emerging Pathogens Laboratory at the British Columbia Centre for Disease Control. I performed all pathogen testing, as well as the molecular and morphological identifications of ticks received from Peddar Bay and Rocky Point, British Columbia. Dr. Dergousoff provided additional support in confirming morphological species identifications. Dr. Hogan and Dr. Morshed contributed infection results and species identifications for samples collected from Nanaimo, British Columbia. Dr. Shahhosseini designed all steps of the study, from field collections to laboratory analyses, and provided direct supervision, training, and guidance during data interpretation and manuscript preparation. I drafted the initial manuscript, which was revised with feedback and edits from Dr. Dergousoff, Dr. Hogan, Dr. Morshed, and Dr. Shahhosseini.

Chapter 3 details unpublished work completed with the assistance of Dominic Czekay, Dr. Sarah-Jo Paquette, Dr. Ajay Poudel, and Dr. Maulik Badmalia. I conducted wet laboratory experiments and phylogenetic analyses. Dr. Shahhosseini designed all steps of the study, from field collections to laboratory analyses, and provided direct supervision, training, and guidance during data interpretation and manuscript preparation. I prepared the manuscript, incorporating feedback and edits from Dr. Shahhosseini.

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List of Abbreviations

Alberta	AB
American Dog-Tick Rhabdovirus-2	ADTR-2
British Columbia	BC
British Columbia Centre for Disease Control	BC CDC
Canadian Food Inspection Agency	CFIA
Crimean Congo hemorrhagic fever virus	CCHFV
Cytochrome c oxidase subunit I	COI
Deer tick virus	DTV
Genomic DNA	gDNA
Glycoprotein	G
Insect-specific viruses	ISVs
International Committee on Taxonomy of Viruses	ICTV
Large polymerase Protein	L
Lyme Disease	LD
Manitoba	MB
Matrix Protein	M
Maximum Likelihood	ML
Movement Proteins	MP
Next-generation sequencing	NGS
Nova Scotia	NS
Nucleoprotein	N
Ontario	ON

Phosphate buffered saline	PBS
Phosphoprotein	P
Powassan virus	POWV
Quebec	QC
RNA dependent RNA polymerase	RdRp
Rocky mountain spotted fever	RMSF
Rocky mountain tick Rhabdovirus	RMTR
Saskatchewan	SK
Tick borne encephalitis virus	TBEV
Tick borne pathogens	TBPs
Vancouver Island University	VIU

Chapter 1. Introduction

1.1 Background

Ticks are the second most important vector for transmission of pathogens to humans, livestock and wildlife [1]. Historically, Canada's harsh winters have limited the tick survival and distribution across the country. However, with the effects of climate change, human activity and land use change, it is expected that Canada will experience an increase of tick-borne diseases (TBD) as more and more ticks establish in new areas [2, 3]. At the time of writing, this range expansion has already caused significant disease and economic burden, as documented in the rising cases of human transmission of Lyme Disease (LD) in Canada. A 16-fold increase in diagnosed cases of LD has been observed from 2009-2019 [4]. Estimates of years past show that TBD burden of TBD causes a significant economic burden, with 3 billion USD spent from 2006 to 2010 [5]. With many endemic ticks of medical importance still not occupying their potential geographic range, and the onset of climate change and introduction of invasive species, the burden of TBDs is expected to rise throughout the 21st century in Canada [6].

Canada is currently endemic to 15 different tick-borne pathogens, including 11 bacteria, three viral, and one protozoan [7] . Fueled by both animal migration and a warming climate, ticks have been expanding into Canada northward at a rate of 35-55 km per year [8]. The northward expansion of ticks presents a two-pronged concern: facilitating the spread of endemic species into new regions and the introduction of new tick species, alongside the emergence of novel pathogens they may carry. Despite the growing concern over tick-borne pathogens, surveillance efforts in Canada remain limited. Most efforts have focussed on LD and its competent vectors in eastern Canada, leaving significant knowledge gaps regarding other endemic tick species and the pathogens they carry.

Acting as competent vectors capable of transmitting many viruses that inflict public health and livestock concerns [9-11], a comprehensive tick surveillance system is critical for providing key information for risk assessment and mitigation of potential outbreaks. While TBVs currently have low transmission risks in North America, the introduction of competent vectors, new viruses, and geographical expansion of already endemic viruses can have significant consequences to public health and the economy. Potential outbreaks of TBVs, such as African Swine Fever in Iowa, USA, have been estimated to cost up to 50 billion USD [12]. Currently, there are four major TBVs of public health concern transmitted in North America: Colorado tick fever virus, Powassan virus, Heartland virus and Bourbon virus [13-16]. Given the potential for substantial economic and public health consequences, establishing a robust tick surveillance system in Canada is essential to detect emerging threats early and inform targeted control efforts.

Surveillance of ticks is typically conducted through a combination of both passive and active surveillance systems to address shortcomings of both methods [17]. Passive surveillance, which involves accepting ticks submitted by the public, veterinarians, physicians, bird banding teams and reported cases of transmission, can provide information on emerging or changing risk [18]. However, passive surveillance greatly relies on public awareness and participation, potentially skewing reports in places of high population density. Active surveillance allows for detailed information on the incidence of disease along with risk factors, as well as the location and species of ticks in the geographical area surveyed [18]. However, with Canada being so wide and vast, difficulty arises with active surveillance since adequate surveillance is inherently labour-intensive. Therefore, a combination of both active and passive surveillance is recommended to overcome the challenges imposed by Canada's vast geography and uneven population distribution.

In addition to tracking tick populations, effective surveillance must also include the pathogens they carry. Accurate reporting of TBDs often depends on molecular diagnostic techniques, as clinical misdiagnosis is common due to the nonspecific and overlapping symptoms of many viral infections [19, 20]. This underreporting hampers timely public health responses. The complexity is further compounded by the fact that individual ticks can carry and transmit multiple pathogens simultaneously, increasing the risk of co-infections and complicating both diagnosis and surveillance [21].

As climate change progresses, the potential geographical expansion of ticks across Canada emphasizes the need for a comprehensive, standardized surveillance system. Surveillance systems are the first line of defence against arboviruses, providing insight into the true range of the disease and allowing for mitigation and prevention strategies [22, 23].

1.2 Tick Biology

There are over 900 different species of ticks in the world, breaking into two different main families: Argaside and Ixodidae [24]. All tick life cycles are comprised of 4 different stages: egg, larva, nymph, adult. After hatching from eggs, ticks must quest for a blood meal during each life stage to survive and complete their life cycle [25]. During the blood meal, ticks are able to transmit the virus they harbour to the host [25]. The geographic range and time of year of peak questing activity is dependent on the climate. With warmer winters and longer summers, ticks are becoming active latter in the year [26].

Concerningly, through their feeding behaviour, they have enabled themselves to hitchhike, travelling vast distances on their host throughout the blood meal (4-7 days) and enabling wide-spread dispersal. Avian-tick associations are particularly significant, as Canada contains four

different migratory bird pathways. Previous studies have documented migratory birds captured on Vancouver Island to be parasitized by *Amblyomma humerale*, a species native to South America, normally reported in regions over 5000km away [27]. Furthermore, migratory birds have been observed to bring ticks carrying *Borrelia burgdorferi,* the causative agent responsible for LD and introduce them to Canada from distant locations such as the United States, Central and South America [28]. New TBDs have been introduced into Canada from these migratory pathways. For example, *Babesia microti,* the causative agent of human pathogen Babesiosis, which was only endemic in northeastern and northern-Midwestern United States of America [29], has been introduced and established itself in Manitoba, creating a public health threat [30].

1.3 Morphological and Molecular Identification of Ticks

Primarily relying on morphological techniques to identify tick species is an unreliable and inefficient method due to large variability within larval, nymphal, and adult tick life stages [31]. To address these shortcomings, many studies incorporate molecular techniques, which increase confidence and accuracy of results by enabling rapid and accurate discrimination between species [32]. Accuracy of species identification is key for surveillance studies, as identifying the presence and establishment of known competent vectors of diseases is key in prevention and outbreak preparation [33].

Historically, molecular identification has relied on amplifying the mitochondrial cytochrome c oxidase subunit I (COI) gene as the standard DNA barcode, resulting in the largest sequence collection [31, 32]. The marker has demonstrated a sufficient “barcode gap”, powerful enough to distinguish morphologically similar presenting species. However, due to the lack of

comprehensive, widespread surveillance studies of Canada incorporating molecular identification, knowledge gaps exist in sequence coverage of Canadian ticks [31]. To address these gaps, we incorporated the mitochondrial 16S rDNA marker as an additional barcode, improving species-level resolution when COI data are incomplete or unavailable [33]`.

The use of molecular techniques for tick identification has gained traction in recent years, particularly as invasive tick species continue to expand their ranges and establish in new areas. For example, prior to the introduction of *Haemaphysalis longicornis*—a vector competent for Dabie bandavirus and *B. burgdorferi*—to the Americas, specimens collected prior to the widespread awareness of its introduction were frequently morphologically misidentified as native *Haemaphysalis* species [34].

1.4 Genome Organization of Rhabdoviridae

The Rhabdoviridae family comprises a diverse group of negative-sense, single-stranded RNA (−ssRNA) viruses within the order *Mononegavirales*. Members of this family possess a linear, non-segmented genome typically ranging from 10 to 16 kilobases (kb) in length. Despite displaying considerable genetic, host and ecological diversity across genera of the family, the overall genome architecture of rhabdoviruses is remarkably conserved [35].

The prototypical genome organization for *Rhabdoviridae* encodes for five core structural and enzymatic proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and the large polymerase protein (L), following the gene order 3′–N–P–M–G–L–5′ [36]. These genes are transcribed sequentially from the 3′ leader to the 5′ trailer region by the viral RNA-dependent RNA polymerase (RdRp), which initiates at the leader promoter and terminates

transcription at gene junctions defined by conserved intergenic signal, contributing to transcriptional attenuation [37].

1. **Nucleoprotein (N):** The N protein encapsulates the genomic RNA to form a ribonucleoprotein (RNP) complex. Functioning as the template for transcription and replication, playing a key role in genome protection and regulation of RNA synthesis [38].
2. **Phosphoprotein (P):** A non-catalytic cofactor of the L protein, P functions as a chaperone for newly synthesized N, maintains the polymerase in proximity to the RNP, and is often heavily phosphorylated, modulating interactions with host immune signalling pathways [39, 40].
3. **Matrix Protein (M):** M bridges the RNP complex and the viral envelope, coordinating viral assembly and budding. It is also implicated in the suppression of host gene expression and immune evasion through host nuclear export interference [41].
4. **Glycoprotein (G):** This transmembrane protein is responsible for host cell attachment and membrane fusion. It is a major determinant of host range, tropism, and virulence, and is the principal target of neutralizing antibodies in vertebrate-infecting rhabdoviruses [42].
5. **Large Polymerase (L):** The L protein harbors all enzymatic activities required for transcription and replication, including RNA polymerase, capping, methyltransferase, and polyadenylation functions. Its interaction with the P protein and RNP is critical for efficient viral RNA synthesis [43, 44].

In addition to the five canonical genes, many rhabdoviruses encode accessory genes, particularly within intergenic or alternative reading frames. These accessory genes can differ significantly between genera and species, often reflecting adaptations to specific hosts or

transmission vectors [36]. For instance, some plant rhabdoviruses include movement proteins (MPs) to facilitate cell-to-cell spread via plasmodesmata [45], while animal-infecting rhabdoviruses may harbor interferon antagonists or immune modulatory proteins [46].

1.5 Circulating Tick Rhabdoviruses

Recent advances in metagenomic and transcriptomic studies have unveiled a greater diversity in the genome organization of the Rhabdoviridae family, particularly among arthropod- and tick-associated rhabdoviruses [47]. Currently, the genus Alpharicinrhavirus has been recognized as a hard-tick-specific genus within the Rhabdoviridae family. Interestingly, this genus exhibits non-canonical gene organizations, bicistronic gene segments, and novel accessory proteins with unknown functions, suggesting lineage-specific evolutionary adaptations [48]. Alpharicinrhavirus have been identified in diverse geographic regions, including Thailand, China, Australia, Norway, and Trinidad. Due to the novelty of this genus, a significant knowledge gap of the pathogenic potential of these viruses remains.

1.5 Thesis Objectives

Tick surveillance in Canada remains largely focused on *B. burgdorferi*, the causative agent of Lyme disease and its competent vectors. This narrow focus has contributed to a significant knowledge gap regarding the diversity and distribution of tick-borne arboviruses. Understanding the current distribution of tick species and the viruses they harbor is critical for anticipating and mitigating future outbreaks.

This thesis addresses these gaps through collaborative efforts involving veterinary clinics, bird banding networks, citizen scientists and active field surveillance to detect circulating tick-

borne viruses in key regions of Canada. The objectives of this thesis are: (1) to investigate the distribution of tick species parasitizing avian hosts on Vancouver Island, and (2) to identify and characterize tick-rhabdoviruses circulating in Canada.

Chapter 2 explores the tick–avian host relationship along the Pacific Flyway, with a focus on species distribution and *Borrelia* (Lyme disease) prevalence. Chapter 3 presents the detection of two novel tick-associated rhabdoviruses, including their preliminary phylogenetic placement and evolutionary relationships within the Rhabdoviridae family.

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Chapter 2: First Record of *Ixodes scapularis* on Migratory Birds on Vancouver Island, Western Canada

2.1 Foreword

This chapter consists of a manuscript prepared for and submitted to *Pathogens*. This work is the result of a joint collaboration between Dr. Shaun Dergousoff ,Dr. Catherine A. Hogan, Dr. Mohammad Morshed, Stefan Iwassa, Dr. Erin Fraser, and Dr. Nariman Shahhosseini. I conducted the wet laboratory experiments for samples collected from Peddar Bay and Rocky Point. Dr. Dergousoff aided in the morphological identification of a portion of the Peddar Bay and Rocky Point samples. The Nanaimo samples were processed and tested by Dr. Hogan's Parasitology Laboratory and Dr. Morshed's Zoonotic and Emerging Pathogens Laboratory at the Public Health Laboratory at the British Columbia Centre for Disease Control. I wrote the manuscript with input edits from Dr. Dergousoff, Dr. Hogan, Dr. Morshed, Dr. Fraser and Dr. Shahhosseini.

2.2 Abstract

Lyme disease (LD) is the most frequently reported vector-borne disease in Canada. The blacklegged tick, *Ixodes scapularis,* and the western blacklegged tick, *Ixodes pacificus,* are the primary vectors of *Borrelia burgdorferi*, the causative agent of LD in eastern-central and western Canada, respectively. Several studies have demonstrated the relationship between ticks and avian hosts as a significant concern, with birds serving both as vehicles for tick introduction into new environments and as amplifying hosts for some tick-borne pathogens (TBPs). This study investigates the occurrence of tick species found on migratory birds on Vancouver Island.

Tick samples were collected from avian hosts by the Rocky Point Bird Observatory and Vancouver Island University (VIU) at Rocky Point, Pedder Bay, and Buttertubs Marsh, Nanaimo, on Vancouver Island. Tick species were identified using morphological keys and confirmed through DNA sequence analysis of cytochrome c oxidase subunit I (COI) and 16S rDNA gene markers, with phylogenetic analyses supporting species assignments.

A total of 224 tick specimens belonging to three genera were collected from 21 bird species. *Ixodes auritulus* was the most prevalent species (n = 157; 70.09%), followed by *Ixodes spinipalpis* (n = 36; 15.63%), *Dermacentor andersoni* (n = 11; 4.91%), *I. scapularis* (n = 7; 3.57%), *I. pacificus* (n = 8; 3.57%), *Dermacentor variabilis* (n = 2; 0.89%), *Dermacentor albipictus* (n = 2; 0.89%), and *Haemaphysalis leporispalustris* (n = 1; 0.45%). All *Ixodes* ticks were tested for the presence of *B. burgdorferi* by amplifying the ospA surface protein gene. A total of 50 samples tested positive for the pathogen. Notably, *I. scapularis* ticks were identified on seven bird species: *Passerella iliaca*, *Melospiza lincolnii*, *Pipilo maculatus*, *Catharus ustulatus*, *Zonotrichia atricapilla, Troglodytes pacificus*, and *Turdus migratorius.*

This study provides the first record of *I. scapularis* infesting avian hosts west of the Rocky Mountains during southward fall migration, highlighting the potential for translocation and range expansion of this species facilitated by migratory birds. Such movement could increase the risk of *B. burgdorferi* transmission in regions previously considered low risk. Understanding the role of migratory birds in tick dispersal is critical for evaluating LD epidemiology and highlights the need for continued surveillance of tick-carrying migratory birds across Canada.

2.3 Introduction

Lyme disease (LD) is the most frequently reported vector-borne disease in Canada, responsible for causing a nationwide public health and economic burden [1, 2]. *Ixodes scapularis* and *Ixodes pacificus* ticks are the principal vectors of *Borrelia burgdorferi*, the causative agent of LD, in eastern-central and western Canada, respectively. These ticks serve as vectors of several other human and animal pathogens in North America, including *Anaplasma phagocytophilum*, *Babesia microti*, *Borrelia miyamotoi*, Powassan virus (POWV), deer tick virus (DTV) and *Ehrlichia muris*-like species [3-5]. Initially regarded as a minor pest upon its discovery in the 1970s, *I. scapularis* has undergone significant geographic range expansion and has established itself as one of the most important vectors for multiple human pathogens in the United States [6-8].

The range expansion of this species through eastern Canada is closely linked in part to climate change, which influences the distribution and survival of many tick species [9, 10]. Passive surveillance data indicate that *I. scapularis* has been detected throughout the country, spanning from Nova Scotia to British Columbia [11, 12]. However, its populations do not occur in all regions predicted to contain suitable habitat. Most modelling studies have focused on assessing the surrounding regions of already endemic areas, highlighting concerns of northward expansion and

westward expansion as far as Saskatchewan [9, 10]. The potential of a westward expansion for the species as far as British Columbia remains unclear, as few studies have investigated this area and shown suitability [13].

Bird migration is a crucial mechanism of dispersal for tick species that are associated with avian hosts. This parasitic relationship is of significant concern, as birds can facilitate the widespread dispersal of ticks and act as amplifying hosts for human and animal pathogenic microorganisms [14-16]. For instance, migratory birds captured in Canada have been documented to be infested with ticks native to South America during the spring migration season [17]. While the northward expansion of *I. scapularis* carrying *B. burgdorferi* has been well documented along the Atlantic and Mississippi flyways [16, 18], there is a notable lack of research on the Pacific Flyway [19]. The Pacific Flyway—the longest and most diverse migration pathway in North America—presents unique opportunities for new interactions between birds and ticks, potentially enabling the introduction of ticks from distant regions.

Identification of immature ticks poses a significant challenge due to limited distinguishing morphological features [20]. To address this, previous studies have demonstrated the utility of molecular markers—particularly the mitochondrial cytochrome c oxidase subunit I (COI) gene—for species-level identification of ticks. COI has been widely used for DNA barcoding because of its strong interspecific divergence and low intraspecific variation, providing a significant "barcode gap", allowing reliable discrimination between medically necessary and morphologically similar species such as *I. scapularis* and *I. pacificus* [21-23]. In addition, 16S rDNA of the large ribosomal subunit has been recognized as a complementary or alternative marker, offering robust support for species identification in tick systematics [24].

The aim of this study is to investigate tick-bird associations along the Pacific Flyway and the role of avian hosts in the dissemination of *B. burgdorferi* on Vancouver Island, western Canada. We aim to assess the prevalence of *B. burgdorferi* and its competent vectors, highlighting the public health implications of LD transmission influenced by bird migration. This work contributes to a deeper understanding of the potential for long-distance dispersal of ticks and tick-borne pathogens via avian hosts, with important implications for Lyme disease epidemiology in previously underrepresented regions.

2.4 Materials and Methods

2.4.1 Tick Collection from Avian Hosts and Morphological Identification

Ticks were collected by staff from the Rocky Point Bird Observatory and bird banding members from Vancouver Island University (VIU) at three locations on Vancouver Island (Figure 1): Pedder Bay, Rocky Point and Buttertubs West Banding Station. Sample collection was conducted from late April to October 2024 at Buttertubs West and from July to mid-October during the 2022–2024 period at Pedder Bay and Rocky Point. Birds were captured using mist nets as part of routine banding operations. Areas around the wings, legs and head of captured birds were quickly inspected for ticks and removed using fine-pointed tweezers, placed in plastic vials, and stored at -20°C to maintain RNA stability. Samples were subsequently shipped on ice packs to the Centre for Vector-Borne Diseases at the Canadian Food Inspection Agency in Lethbridge or the Public Health Laboratory at the British Columbia Centre for Disease Control in Vancouver. Specimens were identified to species level, using morphological keys [24], then stored at -70°C until further analysis. Observations of avian-tick interactions from this study were used to create a chord diagram constructed using Microsoft Power BI version 2.144.679.0 with the "Chord" plugin, using default settings.

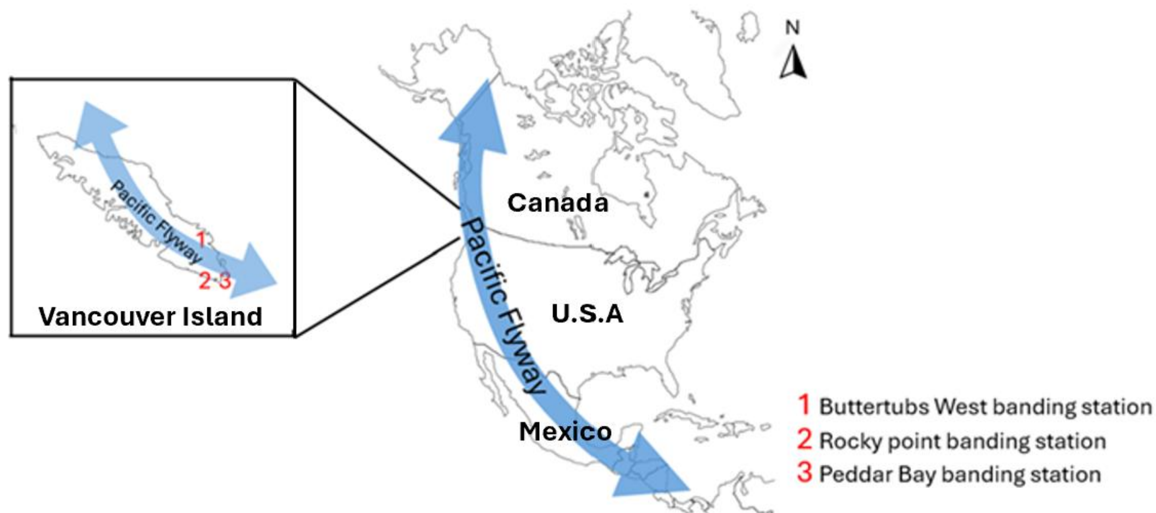

Figure 1. The Pacific Flyway migration route for birds in North America (right) [25], and the locations of bird banding and tick collection sites in British Columbia, Canada, from 2022 to 2024.

2.4.2 DNA Extraction from Ticks

Genomic DNA (gDNA) was extracted from individual ticks. Either the whole body or a single leg of each tick was placed in a 2 mL microcentrifuge tube with three 5 mm stainless steel beads and 500 µL of phosphate-buffered saline (PBS) [26]. Tick specimens were homogenized using a Qiagen TissueLyser II® (QIAGEN, Toronto, ON, Canada) set to 1,800 oscillations per minute for 10 minutes. Samples were then centrifuged at 15,000 rpm for 10 minutes at room temperature, and the supernatant was transferred to a new tube. gDNA was extracted and purified

using the QIAGEN DNeasy Blood and Tissue Kit (QIAGEN, Toronto, ON, Canada), following the manufacturer's protocol for purification of total DNA from insects [21].

2.4.3 Molecular Confirmation of Species Identification

To confirm initial morphological identifications of samples, DNA barcoding amplifying two different regions was preformed. A 658-bp fragment of the mitochondrial cytochrome c oxidase subunit 1 (COI) gene and a 454-bp fragment of the 16S rDNA gene. For COI PCR amplification, the following primers were used: Forward primer LCO1490–5'-GGTCAACAAATCATAAAGATATTGG-3' and reverse primer HC02198–5'-TAAACTTCAGGGTGACCAAAAAATCA-3' [27]. The reaction mixture contained 5 µL of 4X AllTaq Master Mix (QIAGEN, Toronto, ON, Canada), which contains AllTaq polymerase, dNTPs and $MgCl_2$, 0.5 µL each of forward and reverse primers (20 µM), 2 µL of DNA template, and 12µL of nuclease-free water. The thermocycler conditions were: initial denaturation at 95°C for 5 minutes, followed by 5 cycles of 94°C for 1 minute, 45°C for 1 minute, and 72°C for 1 minute, followed by 35 cycles of 94°C for 1 minute, 54°C for 1 minute, and 72°C for 1 minute, with a final extension at 72°C for 5 minutes [27].

For 16S rDNA PCR amplification, the following primers were used: Forward primer 16s F-5'-TTAAATTGCTGTRGTATT-3'and reverse primer 16s R1–5'-CCGGTCTGAACTCASAWC-3' [23]. The reaction mixture consisted of 5 µL of 4X AllTaq Master Mix (QIAGEN, Toronto, ON, Canada), 0.3 µL each of forward and reverse primers (20 µM), 5 µL of gDNA template, and 9.4 µL of water. Thermocycler conditions were: 94°C for 5 minutes, followed by 5 cycles of 94°C for 30 seconds, 49°C for 30 seconds, and 68°C for 30 seconds; 5 cycles at 94 °C for 30 s, annealing from 47 °C to 43 °C in 2 °C steps each for 30 s, 68

°C for 30 s; then 30 cycles at 94 °C for 30 s, 43 °C for 30 s, and 68 °C for 30 s, with a final extension at 68°C for 5 minutes [23].

2.4.4 Screening for *Borrelia burgdorferi*

All *Ixodes* species were tested for the presence of *Borrelia burgdorferi* by amplifying the 250 bp of OspA gene using the following primers: forward primer 3– 5'-TTCTGACGATCTAGGTCAAA-3' reverse primer 4– 5'-GCAGTTAAAGTTCCTTCAAG-3' [16]. The PCR mixture consisted of 5 µL of 4X AllTaq master mix (QIAGEN, Toronto, ON, Canada), 0.67 µL of each primer (20 µM), 10 µL of template, and 3.66 µL of water. Thermocycler conditions were: 94°C for 5 minutes, followed by 50 cycles of 94°C for 90 seconds, 55°C for 30 seconds, and 68°C for 110 seconds, with a final extension at 68°C for 5 minutes [16].

2.4.5 DNA Sequencing and GenBank Submission

The resulting PCR products were analyzed using a QIAxcel DNA Fast Analysis Kit for the QIAxcel Advanced Instrument (QIAGEN, Toronto, ON, Canada). To determine the size of the amplicons, QX DNA Size Marker (50 - 1500 bp) was run alongside QX Alignment Marker (15 - 1000 bp). COI and 16S rDNA amplicons of the expected size were purified using a PCR product clean-up kit (Zymogen Research, USA) and submitted for Sanger sequencing using only one primer — either the forward or the reverse — per sample (Eurofins Scientific, Louisville, Kentucky). Sequences were visually inspected, trimmed and edited using Geneious Prime v2024.08 [28]. To confirm species identification, COI sequences generated in this study were validated using the BOLD Identification Tool (via the validated Canadian Arthropod Library), and the NCBI GenBank database using the BLAST tool with the Megablast algorithm. All DNA sequences generated in this study were deposited to GenBank under accession numbers PV653371

to PV653415, PV163173 to PV163296. A subset of OspA sequences were chosen representing different years of collection and host species and submitted to GenBank, corresponding to the following accession numbers: PV804968-PV804982.

2.4.6 Evolutionary Relationship of *I. scapularis* and *I. pacificus*

Initial identification of *I. scapularis* and *I. pacificus* specimens was based on established morphological criteria [25]. Since early life stages are challenging to identify morphologically, and several specimens were damaged, taxonomic assignments were subsequently validated using phylogenetic analyses of mitochondrial COI and 16S rDNA markers. Species clustering patterns were assessed to evaluate the phylogenetic distinctiveness and geographic structure of *I. scapularis* and *I. pacificus* populations.

To investigate species boundaries and evolutionary relationships, mitochondrial gene sequences were analyzed using phylogenetic methods. In addition to sequences generated in this study, a curated dataset of publicly available sequences was compiled from GenBank (Supplementary Table 1 and Table 2). Representative sequences from additional tick species—including *I. spinipalpis*, *I. auritulus*, *D. albipictus*, *D. variabilis*, and *D. andersoni*—were retrieved from GenBank (Supplementary Table 1 and Table 2) and included to serve as outgroups and comparative references. *Ixodes ricinus* was selected as the primary outgroup to root the tree. Care was taken to include at least one sequence per species, location, and year of collection to capture spatial and temporal diversity.

Separate multiple sequence alignments were performed for the COI (658 bp) and 16S rDNA (454 bp) genes using the ClustalW algorithm. Phylogenetic trees for each marker were constructed using the Neighbor-Joining (NJ) method based on Tamura-Nei genetic distances to

elucidate species clustering patterns. All alignments and phylogenetic analyses were conducted using Geneious v2024.08 [28].

2.4.7 Tracing *I. scapularis* Origins on Migratory Birds: An Attachment-Duration and Flight-Behavior Appporximation

To estimate the potential geographic origin of *I. scapularis* found on migratory birds, we defined species- and stage-specific tick attachment durations based on published literature [15, 29]. Reported attachment durations vary according to tick life stage: larvae typically remain attached for 3 to 5 days and nymphs for 4 to 6 days. For mapping purposes, we used average attachment durations of 4 days for larvae and 5 days for nymphs. These values reflect biologically realistic durations under natural conditions and are consistent with previous field and laboratory studies [30, 31].

To estimate the maximum potential distance a tick could have traveled on a bird, we combined these average attachment durations with known species-specific migratory behavior. For each bird species, we used literature-based values for the average migration distance flown per night [32-39] and estimated the number of flight nights that could occur within the tick attachment period. These estimates allowed us to calculate a realistic maximum potential migration distance during which a tick could have been transported, thus narrowing the potential geographic origin of ticks collected from birds during migration stopovers (Table 1).

Table 1. Potential maximum migration distances of the seven bird species hosting *Ixodes scapularis* under natural migration conditions.

Species	Migration Distance Category	Average Distance per Flight Day (Range in km)	Tick Life Stage	Average Tick Attachment Duration (Days)	Flight Nights During Tick Attachment	Potential Maximum Distance Range Travelled by Birds During Tick Attachment (km)
Passerella iliaca	Medium	150 (100–200)	Nymph	5	2–3	300–450
Pipilo maculatus	Short	100 (50–150)	Nymph	5	1–2	100–200
Catharus ustulatus	Long	350 (300–400)	Nymph	5	3–4	1,050–1,400
Troglodytes pacificus	Short	100 (50–150)	Larva	4	2–3	200–300
Melospiza lincolnii	Medium	150 (100–200)	Larva	4	2–3	300–450
Zonotrichia atricapilla	Medium	150 (100–200)	Nymph	5	3–4	450–600
Turdus migratorius	Medium	200 (100–300)	Nymph	5	3–4	600–800

2.5 Results

2.5.1 Prevalence of Tick Species

A total of 224 tick specimens were collected, identified morphologically, and further confirmed through molecular analysis, representing eight different species. Among these, *I. auritulus* was the most prevalent species (n = 157; 70.09%), followed by *I. spinipalpis* (n = 36; 16.07%), *D. andersoni* (n = 11; 4.91%), *I. pacificus* (n = 8; 3.57%), *I. scapularis* (n = 7; 3.12%), *D. variabilis* (n = 2; 0.89%), *D. albipictus* (n = 2; 0.89%), and *H. leporispalustris* (n = 1; 0.45%). Among the collected specimens, 23 were adults, 99 were larvae, and 102 were nymphs (Table 2).

Table 2. Tick Species and Life Stage Composition observed from Migratory Birds at Three Vancouver Island Stopover Sites (2022–2024)

Tick species	Year of collection			Months of collection						Stage of development			
	2022	2023	2024	Apr	May	Jul	Aug	Sep	Oct	Larvae	Nymph	Adult	Total
I. auritulus	26	20	111	6	0	4	22	100	25	63	80	14	157 (70.1%)
I. spinipalpis	6	0	30	12	5	2	8	7	2	22	14	0	36 (15.6%)
I. pacificus	1	0	7	0	4	1	3	0	0	6	2	0	8 (3.6%)
I. scapularis	0	7	0	0	0	0	0	4	3	2	5	0	7 (3.12%)
D. andersoni	0	11	0	0	0	0	0	5	6	5	0	6	11 (4.9%)
D. variabilis	0	2	0	0	0	0	0	1	1	0	2	0	2 (0.9%)
D. albipictus	1	1	0	0	0	0	0	1	1	1	1	0	2 (0.9%)
H. leporispalustris	0	0	1	0	0	0	1	0	0	0	1	0	1 (0.4%)

2.5.2 Bird Species Infested by Ticks

Eight tick species were found on 21 different bird species. Among all bird species, individual tick infestation was most frequently observed on *Passerella iliaca* (n=54; 24.11%) followed by *Pipilo maculatus* (n=24; 10.71%), *Melospiza melodia* (n=22; 10.27%), *Troglodytes pacificus* (n=21; 9.38%), *Catharus ustulatus* (n=19; 8.48%), *Melospiza lincolnii* (n=14; 5.80%), *Zonotrichia atricapilla* (n=12; 5.36%), *Junco hyemalis* (n=11; 4.91%), *Catharus guttatus* (n=9; 4.02%), *Troglodytes aedon* (n=8; 3.57%), *Cardellina pusilla* (n=6; 2.68%), *Turdus migratorius* (n=5; 2.23%), *Leiothlypis celata* (n=4; 1.79%), *Geothlypis trichas* (n=3; 1.34%), *Zonotrichia albicollis* (n=3; 1.34%), *Thryomanes bewickii* (n=3; 1.34%), *Haemorhous purpureus* (n=2; 0.89%), *Geothlypis tolmiei* (n=2; 0.89%), *Passerculus sandwichensis* (n=1; 0.45%), *Setophaga petechia* (n=1; 0.45%), and *Piranga ludoviciana* (n=1; 0.45%). Age class (juvenile vs adult) of the

captured birds was not recorded during this study. As a result, we cannot confirm whether these ticks were acquired locally or transported during migration.

Seven of these bird species were infested with *I. scapularis* larvae and nymphs. In total, two *I. scapularis* larvae and five nymphs were found, with a single *I. scapularis* sample collected from each of the following bird species: *P. iliaca*, *P. maculatus*, *C. ustulatus*, *Z. atricapilla*, *M. lincolnii*, *T. pacificus*, and *T. migratorius* (Figure 2).

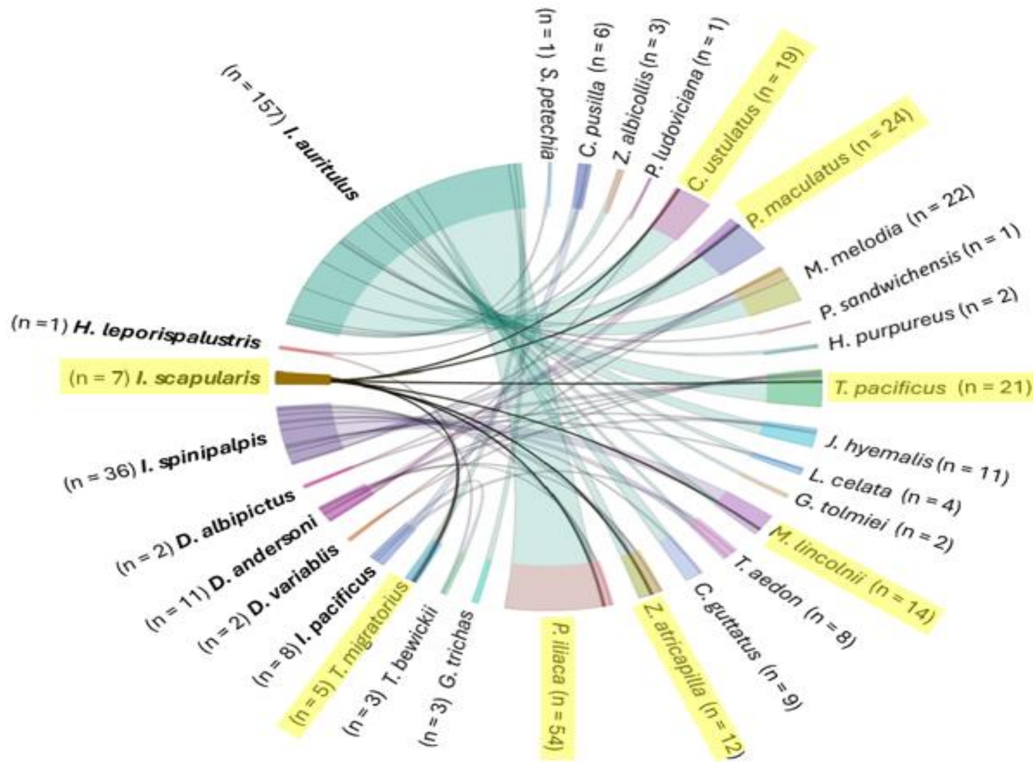

Figure 2. Chord diagram illustrating observed occurrences of avian host-tick associations of ticks collected from 2022-2024 created using Microsoft Power BI (version 2.144.679.0) with the "Chord" custom visual, ribbon sizes depicted are proportional to observed interactions. Highlighted are observed *Ixodes scapularis* avian host interactions.

2.5.3 Prevalence of *B. burgdorferi* Spirochetes in Ticks

All *Ixodes* ticks were tested for the presence of *B. burgdorferi*. Out of 208 ticks screened, 50 (24%) tested positive for *B. burgdorferi* DNA. The infected ticks included 42 *I. auritulus*, 3 *I. pacificus*, 1 *I. scapularis*, and 4 *I. spinipalpis* (Table 3). All ticks positive for *B. burgdorferi* originated from Rocky Point and Pedder Bay and included 22 larvae and 20 nymphs. Of the amplified partial sequences of the OspA gene, all sequences showed consistency amongst each other (~3% divergence amongst each other) and showed >97% identity to other submitted OspA sequences in GenBank (EU081296, AY654920, CP132452).

Table 3. Prevalence of *Borrelia burgdorferi* in *Ixodes* spp. collected from Pedder Bay, Rocky point and Buttertubs West Banding Station migratory bird observatories on Vancouver Island from 2022 to 2024.

Year	Species	Life stage	Positive	Negative	Total	% Positive
2022	*I. auritulus*	Adult	6	8	26	42.3%
		Nymph	5	4		
		Larvae	0	3		
	I. spinipalpis	Nymph	1	0	6	50%
		Larvae	2	3		
	I. pacificus	Nymph	0	1	1	0
2023	*I. auritulus*	Nymph	1	11	20	5%
		Larvae	0	8		
	I. scapularis	Nymph	0	2	7	14.3%
		Larvae	1	4		
2024	*I. auritulus*	Nymph	13	46	111	27%
		Larvae	17	35		
	I. spinipalpis	Nymph	1	11	30	10.8%
		Larvae	3	15		
	I. pacificus	Nymph	1	0	7	42.8%
		Larvae	2	4		

2.5.4 Species Delineation by Phylogenetic Tree

To assess species boundaries and evolutionary relationships among tick specimens, phylogenetic analyses were performed using NJ trees based on 658 bp of the COI gene (Figure 3A) and 454 bp of the 16S rDNA gene (Figure 3B). A total of 33 sequences generated from this study were included in the construction of NJ trees (11 *I. scapularis*, 6 *I. spinipalpis*, 6 *I. auritulus*, 3 *I. pacificus*, 2 *D. variabilis*, 2 *D. albipictus* and 3 *D. andersoni*) and representatives from other

tick species: *I. spinipalpis*, *I. auritulus*, *D. albipictus*, *D. variabilis*, and *D. andersoni*. Sequences were obtained from specimens collected across various regions of North America over multiple years. To ensure a representative sequence consistency within species of sequences generated from this study, a subset of sequences was evaluated, confirming minimal intraspecific variation. During the construction of the NJ trees, we selected sequences generated over multiple years and locations to demonstrate consistency among sample sequences within each species.

Both COI and 16S rDNA consensus tree phylogenies resolved well-supported monophyletic clades corresponding to recognized species. In the COI tree (Figure 3A), each species, including *D. variabilis*, *D. andersoni*, *D. albipictus*, *I. auritulus*, *I. spinipalpis*, *I. pacificus* and *I. scapularis*, clustered into distinct lineages. Tree robustness was evaluated using bootstrapping of 1000 replicates. There was strong statistical support for a clade comprising the seven *I. scapularis* samples from B.C. (highlighted in red) and *I. scapularis* from eastern North America (e.g., Quebec, Maine, Nova Scotia), indicating minimal mitochondrial divergence across geographical regions. *Ixodes pacificus* sequences similarly formed a cohesive clade, independent of geographic origin or collection year.

The 16S rDNA tree (Figure 3B) corroborated these findings. All species formed discrete, well-supported clades, including the B.C. *I. scapularis* samples, which again clustered closely with northeastern U.S. reference sequences. This pattern was consistent for *I. pacificus*, as well as other species such as *I. spinipalpis* and *I. auritulus*, which also formed distinct monophyletic groups.

Together, these findings demonstrate strong species-level resolution across tick taxa and confirm the utility of both COI and 16S rDNA markers for robust phylogenetic inference.

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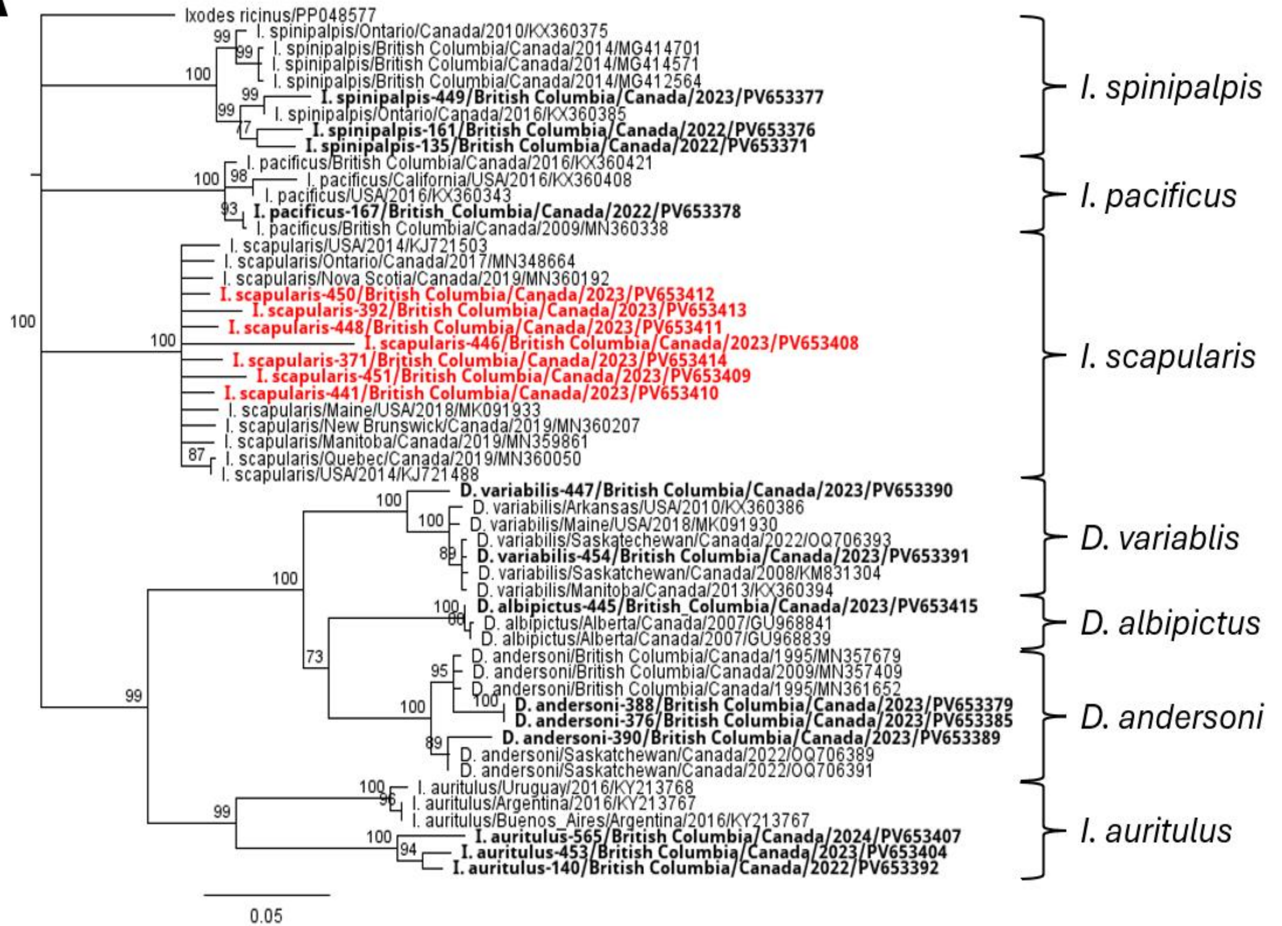

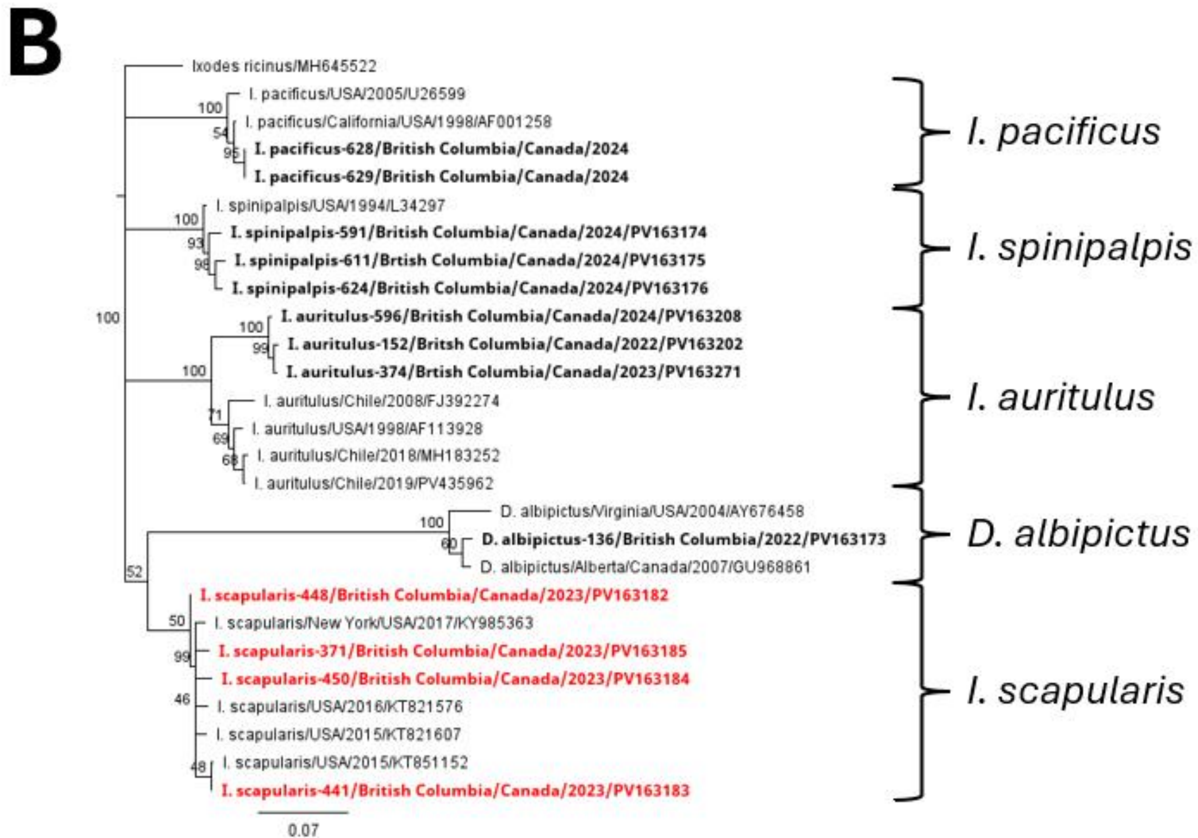

Figure 3. Neighbour-joining phylogenetic tree depicting evolutionary relationships based on partial COI (A) and 16S rDNA (B) gene sequences generated in this study and retrieved from GenBank. Sequences were selected to represent varying collection years. All sequences generated from this study are shown in bold, with *Ixodes scapularis* sequences highlighted in bold red. *Ixodes ricinus* was used as the outgroup to root both trees.

2.5.5 Approximation of Possible Geographic Origins of *I. scapularis* Based on Bird Migration and Tick Attachment

To estimate the likely geographic origins of *I. scapularis* found on migratory birds on Vancouver Island, we modeled the maximum potential flight distances by incorporating tick attachment durations and species-specific migratory behaviors. The model integrates average daily flight ranges and typical attachment periods for larval and nymphal ticks, yielding biologically realistic estimates of how far a bird could have transported a tick prior to capture. Importantly, the

inference is based not only on distance modeling but also on known migratory routes, which inherently consider geographic constraints such as the Rocky Mountains. Together, these factors provide an ecologically grounded framework for inferring plausible source regions of the ticks.

The resulting map (Figure 4) depicts a southward migratory corridor in fall extending inland across B.C. The orange point indicates the mid-point between sampling sites on southern Vancouver Island where *I. scapularis* ticks were collected (Rocky Point and Peddar Bay). The three shaded zones represent modeled potential origin areas, with the darkest zone (closest to the sampling sites) corresponding to short-distance migration within 1–2 flight nights, and lighter gradients representing mid- and long-distance possibilities over up to 4–5 nights. The geographic extent of these zones—when considered alongside known migratory behavior and natural barriers— suggests that *I. scapularis* ticks found in this study were likely dispersed from areas west of the Rocky Mountains, including central and coastal regions of B.C. This provides evidence for prior westward translocation of *I. scapularis* into B.C, and secondary southward dispersal by avian hosts during their fall migration. These findings highlight the role of bird migration in facilitating the continued range expansion of this medically important tick species.

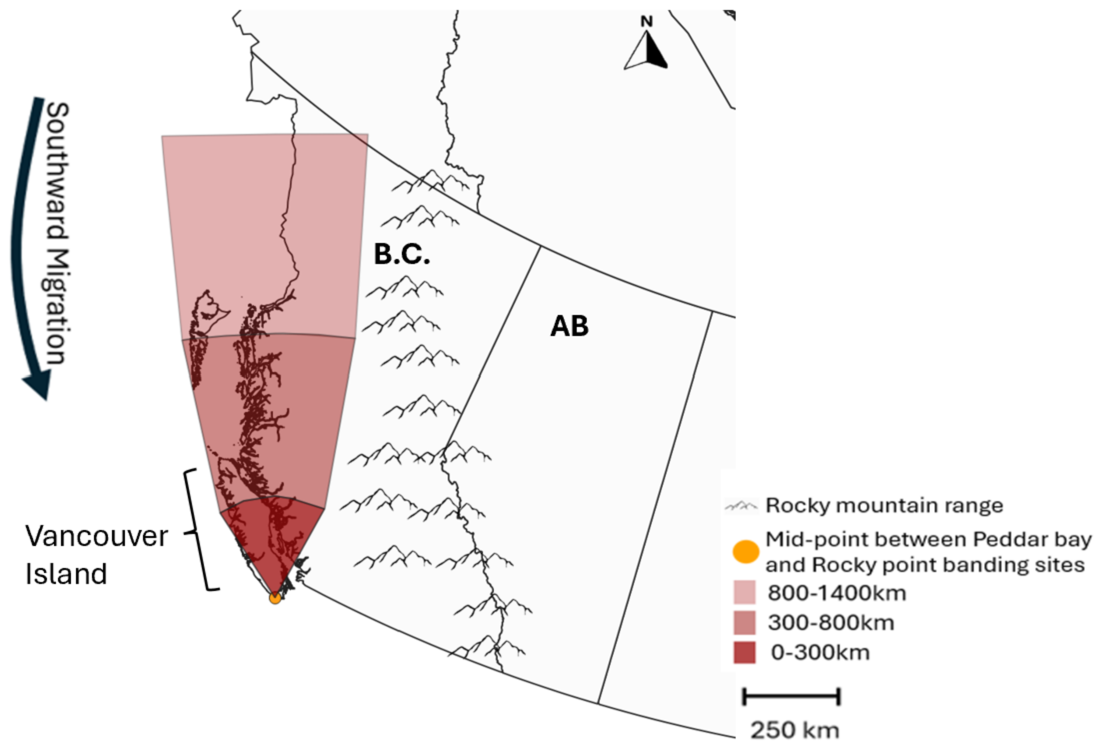

Figure 4. Modeled potential geographic origin zones of *Ixodes scapularis* collected from migratory birds on Vancouver Island, British Columbia. Concentric zones represent estimated dispersal distances based on typical tick attachment duration and bird-specific migratory behavior. Zones correspond to maximum travel distances of 0–300 km (darkest red), 300–800 km (medium red), and 800–1,400 km (lightest red) from the midpoint between the two tick-positive bird capture sites. These ranges reflect realistic migration potential during average larval and nymphal tick attachment periods (4–5 days). The orange point marks the sampling location on southern Vancouver Island. Map created using QGIS software (version 3.42.2).

2.6 Discussion

Although only a few *I. scapularis* ticks were detected on migratory birds in this study, these observations highlight the potential for long-distance transport of vectors. International examples where avian hosts infested with ticks played roles, such as the spread of Crimean-Congo hemorrhagic fever virus (CCHFV) in Spain [41,42]. The distribution of Severe Fever with

Thrombocytopenia Syndrome virus (SFTSV) cases correlates with migratory bird routes in China, demonstrating that birds can transport ticks over long distances [43]. These findings emphasize the importance of monitoring for exotic vector species and invasive pathogens in Canada. At the time of writing, *I. scapularis* remains rare on the west coast of North America, with no established populations reported to date [12,23]. Recent passive surveillance reports from neighbouring regions of Vancouver Island, in the Pacific Northwest region of the U.S.A., indicate that *I. scapularis* is one of the most frequently reported non-endemic ticks on the west coast through passive surveillance, with most instances linked to a history of travel to endemic areas of North America [44]. However, in BC, *I. scapularis* ticks have been rarely detected from passive surveillance over the last 20 years, most occurrences being attributed to a history of travel to endemic areas as well [11, 23]. Our study presents the first record of *I. scapularis* in Western Canada associated found parasitizing both migratory and non-migratory birds.

To complement morphological identification and strengthen species-level confirmation, molecular characterization was conducted using two mitochondrial markers (COI and 16S rDNA). Phylogenetic analyses revealed that the *I. scapularis* specimens collected in B.C formed a strongly supported monophyletic clade with reference sequences from eastern North America, distinctly separated from *I. pacificus* and other related taxa. This concordance between morphological and molecular data provides robust evidence for the accurate identification of *I. scapularis* in western Canada, supporting its presence beyond its traditionally recognized range. Few studies have evaluated the potential and suitability for a west-coast establishment of *I. scapularis*, as most research has focussed on eastern North America. Whether western Canada provides a suitable environment for tick establishment remains unclear. Currently, *I. pacificus* is the primary vector responsible for *B. burgdorferi* transmission in B.C. However, *I. pacificus* is not widely distributed

across BC and is primarily restricted to the coastal areas of the southwestern region [12]. Of particular concern, studies have shown that *I. scapularis* exhibits a higher transmission efficiency for *B. burgdorferi* and a greater prevalence of infection compared to *I. pacificus* (Couper, Yang et al. 2020). *I. scapularis* thrives in deciduous and mixed forests and frequently interacts with human-modified environments, such as untrimmed lawns and ornamental shrubbery in urban areas [46].

Millions of birds migrate in and out of Canada using the Pacific Flyway [47], which remains underrepresented in tick surveillance studies. Migratory birds serve as important dispersal agents for ticks, particularly *I. scapularis*, facilitating the spread of LD [19]. During migration, birds make stopovers in grassy and wooded areas, providing opportunities for ticks to detach or attach to ground-foraging avian hosts. There is well-documented evidence that birds carrying multiple *I. scapularis* have contributed to the establishment of tick populations in Rondeau Provincial Park and Turkey Point [48,49].

Ticks collected in this study reflect both the southward fall migration and northward spring migration between North America and wintering grounds in Central and South America, and vice versa. A comparison of tick species collected in 2024, during both spring and fall migrations, showed consistency, with only endemic ticks being identified. The only difference in collection between the seasons was the observation of one *H. leporispalustris* tick collected during the fall. Interestingly, only ticks captured during the fall migration season were observed to be carrying *B. burgdorferi*. A total of eight different bird species were found to be parasitized by ticks during the spring migration versus a total of 18 different bird species observed during the fall migration. The discrepancy in observed tick and bird species in 2024 between seasons may be attributed to the differing experience levels of the field teams involved in sample collection, a factor previously

noted in an earlier study by Ogden, et al. [16]. Additionally, six bird species observed during fall but not during spring are considered year-round residents (*T. migratorius, T. bewickii, T. pacificus, H. purpureus, M. melodia, P. maculatus*).

Notably, this study reports the first finding of *I. scapularis* ticks of various life stages parasitizing birds on Vancouver Island during the fall migration. In this study, *I. scapularis* was observed only in 2023, parasitizing both migratory and non-migratory birds during the fall migration. These findings suggest that *I. scapularis* ticks were acquired in B.C rather than transported from distant endemic regions on the east coast, given the maximum potential distance a tick could travel on migratory birds is approximately 1,400 km, along with the direction of the Pacific Flyway used by birds during fall migration.

The detection of *I. scapularis* on migratory birds during fall migration on Vancouver Island raises important questions about the geographic origin of this medically necessary tick in western Canada. Given the limited attachment duration of ticks (4–5 days) and the realistic migratory range of bird hosts during that window (≤1,400 km), it is highly improbable that *I. scapularis* was transported directly from the eastern provinces to Vancouver Island during fall migration. Instead, a more parsimonious explanation is that *I. scapularis* has already been introduced into parts of central or northern B.C. via earlier east-to-west translocation events, potentially facilitated by human travel or the movement of domestic animals.

In the current study, birds likely acquired ticks locally within B.C. and subsequently carried them southward toward Vancouver Island as part of their natural migratory route. This suggests a two-step process: (1) potential long-range dispersal of *I. scapularis* into western Canada, followed by (2) secondary redistribution by birds within the region. These findings underscore the

importance of regional surveillance, not only to detect new introductions but also to monitor local tick dispersal dynamics facilitated by migratory birds.

The unique geography of Vancouver Island creates migration bottlenecks, which can influence bird movement patterns. Additionally, the Rocky Mountains act as a barrier restricting the east-west bird migration. Based on known migration patterns and tick-host attachment durations, the maximum distance traveled by *I. scapularis* ticks travelling via avian host was estimated at 1,400 kilometers from the collection site. However, the absence of data on engorgement level and the possibility of attachment shortly before capture introduce uncertainty about the precise location of tick acquisition. These unanswered questions highlight the need for future active surveillance in Western Canada. Notably, one of the *I. scapularis* specimens tested positive for *B. burgdorferi*, confirming the presence of LD-associated spirochetes in a tick transported by birds.

It is critical to continue monitoring medically important ticks on migratory birds to identify additional avian species involved in tick dispersal and to quantify their contributions to the range expansion of tick species of concern into western Canada and the U.S. Additionally, conducting targeted environmental surveys along migratory routes can facilitate the early detection of newly established tick populations. Future investigations should focus on the ecological and environmental factors, such as suitable habitat, climate and host availability, that could allow small, localized populations to establish in British Columbia. Previous studies have shown that pockets of favorable conditions can support the establishment of *I. scapularis* populations in unexpected areas [50]. Public education efforts in newly infested areas may also be necessary, particularly since *I. scapularis* has now been documented in regions beyond its traditional range.

Given its potential role in the increasing risk of LD transmission in northwestern North America, proactive surveillance and mitigation efforts are imperative.

2.7 Conclusion

In conclusion, we present the first molecularly confirmed record of *Ixodes scapularis* ticks parasitizing both migratory and non-migratory birds on Vancouver Island. This finding is noteworthy given that *I. scapularis* is not currently considered established in the region, yet the detection of this species on avian hosts demonstrates a viable pathway for its introduction via migratory flyways. Concerningly, one of the seven ticks tested positive for *Borrelia burgdorferi*, the causative agent of Lyme disease, underscoring the potential for long-distance dispersal of both vector and pathogen. These results raise important public and animal health concerns, particularly regarding the role of birds in facilitating the spread of medically significant tick species into coastal British Columbia. These findings prompt future work of continued surveillance along the pacific flyway, monitoring ticks being translocated and spread via avian hosts as well as active surveillance to investigate the possibility of an established colony in BC.

2.8 Supplementary Data

Table 2.4 Metadata of sequences retrieved from GenBank used to construct Neighbour-Joining phylogenetic trees based on a 658 bp fragment of the COI gene.

Species	Accession Number	Year	Location
D. variabilis	OQ706393	2022	Beaver Creek, Saskatchewan, Canada
D. variabilis	KX360394	2013	Riding Mountain National Park, Manitoba, Canada
D. variabilis	KX360386	2010	Arkansas, USA
D. variabilis	KM831304	2008	Grasslands National Park, Saskatchewan, Canada
D. variabilis	MK091930	2018	Maine, Portland, USA
D. andersoni	MN357679	1995	Oliver, British Columbia, Canada
D. andersoni	OQ706391	2022	Beaver Creek, Saskatchewan, Canada
D. andersoni	MN361652	1995	Oliver, British Columbia, Canada
D. andersoni	MN357409	2009	Vaseux Lake, British Columbia, Canada
D. andersoni	OQ706389	2022	Beaver Creek, Saskatchewan, Canada
D. albipictus	GU968841	2007	Dillberry, Alberta, Canada
D. albipictus	GU968839	2007	Oyen, Alberta, Canada
I. auritulus	KY213768	2016	Rocha, Uruguay
I. auritulus	KY213767	2016	Buenos Aires, Argentina

I. spinipalpis	KX360385	2016	Ontario, Canada
I. spinipalpis	MG414701	2014	Vancouver Island, British Columbia, Canada
I. spinipalpis	MG414571	2014	Vancouver Island, British Columbia, Canada
I. spinipalpis	MG412564	2014	Vancouver Island, British Columbia, Canada
I. pacificus	MN360338	2009	Victoria, British Columbia, Canada
I. pacificus	KX360343	2016	USA
I. pacificus	KX360408	2016	California, USA
I. scapularis	KJ721503	2014	USA
I. scapularis	MN360050	2019	St. Sylvestre, Quebec, Canada
I. scapularis	MN359861	2019	Winnipeg, Manitoba, Canada
I. scapularis	MK091933	2018	Cumberland, Maine, Canada
I. scapularis	MN348664	2017	South Frontenac, Ontario, Canada
I. scapularis	MN360192	2019	Nova Scotia, Canada
I. scapularis	MN360207	2019	Saint John, New Brunswick, Canada

Table 2.5 Metadata of sequences retrieved from GenBank used to construct Neighbour-Joining phylogenetic trees based on a 454-bp fragment of 16s rDNA.

Species	Accession Number	Year	Location
D. albipictus	GU968861	2007	Dillberry, Alberta, Canada
D. albipictus	AY676458	2004	Virginia, USA

I. auritulus	FJ392274	2008	Chile
I. auritulus	MH183252	2018	Chile
I. auritulus	PV435962	2019	Chile
I. auritulus	AF113928	1998	USA
I. pacificus	AF001258	1997	Sonoma County, California, USA
I. pacificus	U26599	1995	USA
I. spinipalpis	L34297	1994	USA
I. scapularis	KT851152	2015	USA
I. scapularis	KY985363	2017	USA
I. scapularis	KT821607	2015	USA

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Authors' Contributions

Conceived and designed the study: NSH. Data collection: NSH, KT, DC, SJP, SJD, SI, MM, CH, EF. Analyzed the data: NSH, KT. Wrote the manuscript: NSH, KT. Contributed to manuscript revision: NSH, KT, DC, SJP, SJD, SI, MM, CH, EF. All authors read and approved the final manuscript.

Conflict of Interests

The authors declare that they have no competing interests.

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Chapter 3. Genomic Characterization of Newly Discovered Rhabdoviruses in Tick Species in Canada

3.1 Foreword

This chapter is based on unpublished work conducted with the aim of preparing a manuscript for submission to the journal *Viruses*. The research was carried out in collaboration with Dr. Sarah-Jo Paquette, Dr. Ajay Poudel, Dr. Maulik Badmalia, and Dominic Czekay. I performed all wet laboratory experiments and analyzed the data. I drafted the manuscript and incorporated edits from Dr. Nariman Shahhosseini. All work in this chapter, from experimental design to data interpretation, was conducted under the direct guidance, training, and supervision of Dr. Shahhosseini.

3.2 Abstract

Climate change affects the global distribution of tick species that vector pathogens, creating new habitats suitable for tick establishment and resulting in an increase of tick-borne diseases (TBDs) in areas of Canada currently considered low risk. Other factors include global trade, human and domestic animal movement, and most notably, the four avian migration routes that contribute to the potential introduction of new vectors and tick-borne viruses into Canada. Continued surveillance of circulating tick species and TBVs through both passive and active collection can provide an effective "early warning" detection system for emerging pathogens.

In this study, a total of 745 tick samples were collected using a combination of passive and active surveillance from British Columbia (BC), Alberta (AB), Saskatchewan (SK), Manitoba (MB), Ontario (ON), Quebec (QC) and Nova Scotia (NS) between 2019 and 2024. Samples were identified using both morphological and molecular techniques and tested for the presence of Rhabdoviridae viral RNA using a pan RT-PCR. We report the first detection of two novel tick-rhabdoviruses in Canada: Rocky Mountain Tick Rhabdovirus (RMTR), found in *Dermacentor andersoni*, and Coastal Avian Tick Rhabdovirus (CATR), found in *Ixodes auritulus*.

Based on preliminary phylogenetic analysis using Maximum Likelihood (ML) trees of a fragment of the block III gene of the L protein, both novel viruses cluster with distinct members of the Alpharicinrhavirus genus. The detection and phylogenetic analyses of these novel tick-rhabdoviruses significantly enhance our current knowledge of the genetic diversity, classification, and evolution of the Rhabdovirus family. Further studies are warranted to elucidate the ecological roles and potential pathogenicity of both RMTR and CATR.

3.3 Introduction

The family Rhabdoviridae is one of the largest and most diverse virus families, with members that pose concerns to both human and animal health. Many members can infect a wide range of organisms, including, but not limited to, fish, birds, reptiles, mammals, insects, and plants [1]. Rhabdoviruses are negative-sense, single-stranded RNA viruses, ranging from 10 kb to 16 kb. They are enveloped viruses with a lipid bilayer derived from the host cell membrane, containing glycoprotein spikes that facilitate attachment and entry into the host cell [2, 3]. All members share a conserved set of five canonical structural and functional proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and RNA-dependent RNA polymerase (L).

Within the Rhabdoviridae family, the genus Alpharicinrhavirus comprises recently discovered rhabdoviruses found exclusively in hard ticks. Tick-borne rhabdoviruses have been discovered in multiple countries worldwide, including Iran, the USA, Norway, and China [4-7]. Members of Alpharicinrhavirus are largely uncharacterized and understudied, primarily because they were discovered through metagenomic sequencing rather than traditional isolation methods. The potential pathogenicity of these viruses to vertebrates remains unclear. Currently, three tick-rhabdoviruses have been identified as circulating in North America: American Dog Tick Rhabdovirus 1 and 2, detected in *Dermacentor variabilis,* and the Long Island tick Rhabdovirus, found in *Amblyomma americanum* [8, 9]. Prior to this study, no tick-rhabdoviruses had been documented in Canada. However, as climate change progresses, tick-borne arboviruses are expected to expand and establish across multiple Canadian provinces [10].

Despite increasing global detection of tick-borne rhabdoviruses like those in the genus Alpharicinrhavirus, research in Canada has primarily focused on bacterial pathogens, leaving tick-

borne viruses understudied [11]. Recently, virus discovery has been aided by advancements in sequencing technologies and applications. The genetic diversity of the family Rhabdoviridae has been greatly expanded due to these advancements, especially in the context of vectors [12, 13]. This progress has contributed to the increasing recognition of new genera within the family, which now includes 56 recognized genera and 454 species, as listed by the International Committee on Taxonomy of Viruses (ICTV). Continued tick collection through both passive and active surveillance remains a critical component of public and animal health monitoring, offering an early warning for the emergence and spread of tick-borne diseases (TBDs) [14]. This approach provides valuable insight into the introduction and distribution of medically important tick species in Canada.

Understanding the ecological role of arthropods is critical to complement surveillance efforts, as many rhabdoviruses replicate within their arthropod hosts rather than merely using them as passive carriers [12]. Arthropods may actively shape rhabdovirus diversity, as they share a deep evolutionary history with these viruses [5]. This long-standing relationship, combined with many species expanding their geographic ranges due to climate change [15], underscores their potential to affect human and animal health. As climate change progresses, Canada is expected to experience an increase in TBDs, resulting in strain on the economy and public health [15]. Understanding the genetic history, evolution, and molecular interactions between Rhabdoviruses and their hosts can provide valuable insights for predicting and mitigating potential spillover events [16].

The *Dermacentor andersoni* tick is a three-host tick of both public health and veterinary concern. At the time of writing, the species is found in the Rocky Mountain regions of western North America, particularly in British Columbia (BC), Alberta (AB) and Saskatchewan (SK) in Canada [17, 18]. *D. andersoni* ticks are competent vectors for multiple pathogens, including Rocky

Mountain Spotted Fever (RMSF), tick paralysis, anaplasmosis, Colorado tick Fever [19-22]. Notably, this species is well documented to parasitize a wide range of hosts, including domestic animals, wildlife, and humans [19, 23-25].

Although the *Ixodes auritulus* tick remains understudied regarding its natural history, it is well documented along the coastal areas of Canada, particularly BC, and commonly parasitizing birds [17]. *I. auritulus* ticks have been found along the Pacific Flyway, in North America, Central, and South America [26-28]. While this species has not been documented to parasitize mammals other than passerines, it is involved in the enzootic maintenance cycle of *Borrelia burgdorferi* in BC [29].

In this study, we report the first detection of two novel tick rhabdoviruses in Canada: Rocky Mountain tick rhabdovirus (RMTR) in *D. andersoni* and Coastal avian tick rhabdovirus (CATR) in *I. auritulus*. We present their phylogenetic relationships to other rhabdoviruses and describe their genetic characteristics. In addition, we provide data from a passive surveillance effort spanning multiple Canadian provinces that document tick species diversity and distribution. These findings highlight the role of domestic animals, birds, and human activity in facilitating tick movement and underscore broader trends in the geographic expansion of ticks across Canada.

3.4 Materials and Methods

3.4.1 Tick Collection and Morphological Identification

Ticks were collected using a combination of active and passive surveillance methods from a total of 23 different locations from six provinces in Canada: BC, AB, SK, MB, ON, QC, and NS. Host-seeking, or questing, ticks collected via active surveillance were collected from the field via the dragging or flagging method [30, 31]. Active surveillance sites were selected based on ease of accessibility and the reported populations of *D. andersoni* ticks, following their known environmental requirements. Passive surveillance was accomplished through a network of veterinarian clinics and bird banding teams spanning Canada, underscoring the role of animal movement, transporting ticks to new geographic locations. Observations of host-tick interactions from this study were illustrated using a chord diagram constructed using Microsoft Power BI version 2.144.679.0 with the "Chord" plugin, using default settings.

Ticks were removed using fine-pointed tweezers, placed in plastic vials, and stored in -20°C freezers until shipped on ice packs to the Centre for Vector-Borne Diseases at the Canadian Food Inspection Agency (CFIA) in Lethbridge for further analysis. Upon arrival, specimens were identified at the species level using the morphological key of Canadian tick species [17] and stored at -70°C for arbovirus testing (Figure 1).

Figure 3.1 Map of tick collection sites across Canada. Tick collection took place from 2019 to 2024**.** Sites highlighted in red represent passive surveillance locations, including veterinary clinics and migratory bird banding stations. Sites highlighted in blue indicate locations where ticks were collected using flagging or dragging methods.

3.4.2 Homogenization, DNA and RNA Extraction

DNA was extracted from either leg or whole-body homogenates, while RNA extraction was performed exclusively using whole-body homogenate. For this process, three 5 mm stainless steel beads and 500 µL of PBS were added to 2 ml tubes containing the tick specimens. The samples were homogenized using a Qiagen TissueLyser II® (QIAGEN, Toronto, ON, Canada) at 1,800 oscillations per minute for 10 minutes. Following homogenization, the samples were

centrifuged at 15,000 rpm for 10 minutes, and the resulting supernatant was transferred to a new tube.

DNA extraction was performed on the supernatant from both whole-body and leg homogenates using the QIAGEN DNeasy Blood and Tissue Kit (QIAGEN, Toronto, ON, Canada) according to the manufacturer's protocol [32]. For RNA extraction, the supernatant from whole-body homogenates was used, using the QIAGEN QIAamp Viral RNA kit (QIAGEN, Toronto, ON, Canada) according to the manufacturer's instructions [33].

3.4.3 Tick Identification Confirmation Using Molecular Techniques

Samples that were difficult to morphologically identify due to engorgement or damage were subjected to further identification using multiple DNA barcoding regions, including a 671-bp portion of the mitochondrial COI gene and a 454-bp portion of the 16S rDNA gene.

For COI PCR amplification, the following primers were used: TTAAATTGCTGTRGTATT (forward) and CCGGTCTGAACTCASAWC (reverse). The reaction mixture contained 5 µL of AllTaq polymerase mix, 0.5 µL each of forward and reverse primers (20 µM), 2 µL of DNA template, and 12 µL of water, for a total volume of 20 µL. Thermocycler conditions were: initial denaturation at 95°C for 5 minutes, followed by 5 cycles of 94°C for 1 minute, 45°C for 1 minute, and 72°C for 1 minute, followed by 35 cycles of 94°C for 1 minute, 54°C for 1 minute, and 72°C for 1 minute, with a final extension at 72°C for 5 minutes [34].

For 16S PCR amplification, the following primers were used: AACTAGGATTAGATACCCT (forward) and GCAGTTAAAGTTCCTTCAAG (reverse). The reaction mixture consisted of 5 µL of AllTaq polymerase master mix, 0.3 µL each of forward and

reverse primers (20 μM), 5 μL of DNA template, and 9.4 μL of water, bringing the total volume to 20 μL. Thermocycler conditions were: 94°C for 5 minutes, followed by 5 cycles of 94°C for 30 seconds, 49°C for 30 seconds, and 68°C for 30 seconds; then 5 cycles of 94°C for 30 seconds, 47°C for 30 seconds, and 68°C for 30 seconds; followed by 5 cycles of 94°C for 30 seconds, 45°C for 30 seconds, and 68°C for 30 seconds; and finally 30 cycles of 94°C for 30 seconds, 43°C for 30 seconds, and 68°C for 30 seconds, with a final extension at 68°C for 5 minutes [35].

3.4.4 Pan RT-PCR Detection of Tick-Borne Rhabdovirus RNA

Extracted RNA from a total of 745 whole-tick homogenates was subjected to a pan-Rhabdovirus RT-PCR to detect the presence of rhabdovirus RNA. Detection of tick-borne rhabdoviruses employed the following primers: AAT AAA TCA TAA CCA DMC BTT TTG YCK YAR RCC TTC (forward) and AAT AAA TCA TAA RAA GGY AGR TTT TTY KCD YTR ATG (reverse) to amplify the block III region of the L gene [36]. The reaction mixture consisted of 5 μL of RNA template, 6.25 μL of 2X reaction mix, 0.375 μL of enzyme mix, and 0.4375 μL of each forward and reverse primers (20 μM), bringing the final reaction volume to 12.5 μL. The thermocycler conditions were as follows: an initial incubation at 60°C for 1 minute, followed by reverse transcription at 50°C for 45 minutes and initial denaturation at 94°C for 2 minutes, followed by 45 cycles of denaturation at 94°C for 15 seconds, annealing at 55°C for 30 seconds, and extension at 68°C for 30 seconds. A final extension was performed at 68°C for 7 minutes [37]. Initially, extracted RNA samples were pooled in groups of five for testing. Positive pools were subsequently opened and tested again for individual confirmation of viral RNA presence. PCR products of the expected band size were sent for Sanger sequencing for further confirmation.

3.4.5 Bioinformatics Analysis and Phylogenetic Relationships

All sequences were analyzed using Geneious Prime 2024.0.4 and visually inspected. To investigate evolutionary relationships with other Rhabdoviridae, rhabdovirus sequences generated in this study were compared with established rhabdovirus sequences obtained from NCBI (Supplementary Table 1) and aligned using the ClustalW algorithm in Geneious Prime 2024.0.4. A Maximum Likelihood (ML) phylogenetic tree with Tamura-Nei genetic distances was generated in MEGA 12 to illustrate the resulting evolutionary and genetic relationships. Robustness of the tree was evaluated using a 1,000 bootstrap replicates [38].

3.4.6 Data Availability

The resulting PCR products were analyzed using a QIAxcel DNA Fast Analysis Kit for the QIAxcel Advanced Instrument (QIAGEN, Toronto, ON, Canada) . To determine the size of the amplicons, a QX DNA Size Marker (50 - 1500 bp) was run alongside a QX Alignment Marker (15 - 1000 bp) to identify amplicons at specified bp sizes for COI, block III region of the Rhabdoviridae family and 16S. Samples displaying a band at the anticipated position were purified using a PCR product clean-up kit (Zymogen Research, USA) and subsequently submitted for Sanger sequencing (Eurofins Scientific, Louisville, Kentucky). All DNA sequences generated in this study were deposited to GenBank under accession numbers PV653371 to PV653415 and PV163173 to PV163296.

3.5 Results

3.5.1 Species Identification and Host-Associated Dispersal Pathways

A total of 745 tick specimens were collected and identified through morphology and further confirmed with molecular techniques, belonging to eleven different species. *D. andersoni* was the most prevalent species (n = 371; 49.8%), followed by *I. auritulus* (n = 151; 20.3%), *D. variabilis* (n = 80; 10.7%), *Ixodes scapularis* (n = 53; 7.1), *Hemaphysalis leporispalustris* (n = 43; 5.8%), *Ixodes spinipalpis* (n = 18; 2.4%), *Ixodes cookei* (n = 8; 1.1%), *I. kingi* (n = 6; 0.8%), *I. pacificus* (n = 6; 0.8%), *D. albipictus* (n = 5; 0.7%), *Rhipicephalus sanguineus* (n = 4; 0.5%) (Table 1).

A total of 335 *Dermacentor andersoni* ticks were collected via active surveillance in Alberta, specifically at Alexander Wilderness Park, Chin Lakes, and Waterton National Park. All other ticks were obtained through passive surveillance. Of these, 16 *D. andersoni* were collected from domestic animals. All 151 *I. auritulus* were collected from avian hosts, while *D. variabilis* included 19 ticks from humans, 2 from birds and 59 from domestic animals. Eight *I. scapularis* ticks were recovered from birds and 45 from domestic animals. All 43 *H. leporispalustris* were collected from avian hosts in British Columbia and Ontario. Similarly, all *I. spinipalpis* were collected from birds. *I. cookei* and *I. kingi* were exclusively recovered from domestic animals. *I. pacificus* included 2 ticks from birds and 4 from domestic animals, while 3 *D. albipictus* were collected from birds and 2 from domestic animals. Finally, all 4 *Rhipicephalus sanguineus* ticks were collected from domestic animals (Figure 2).

Notably, *R. sanguineus* and *I. cookei* were detected outside their typically reported ranges. A cluster of four *R. sanguineus* ticks was collected from a dog at a veterinary clinic in Vancouver, British Columbia. Additionally, six *I. cookei* ticks were recovered in two separate instances from

the same veterinary clinic in Fort Macleod, AB: one tick from a dog in 2022 and five ticks from another dog in 2023 (Table 1).

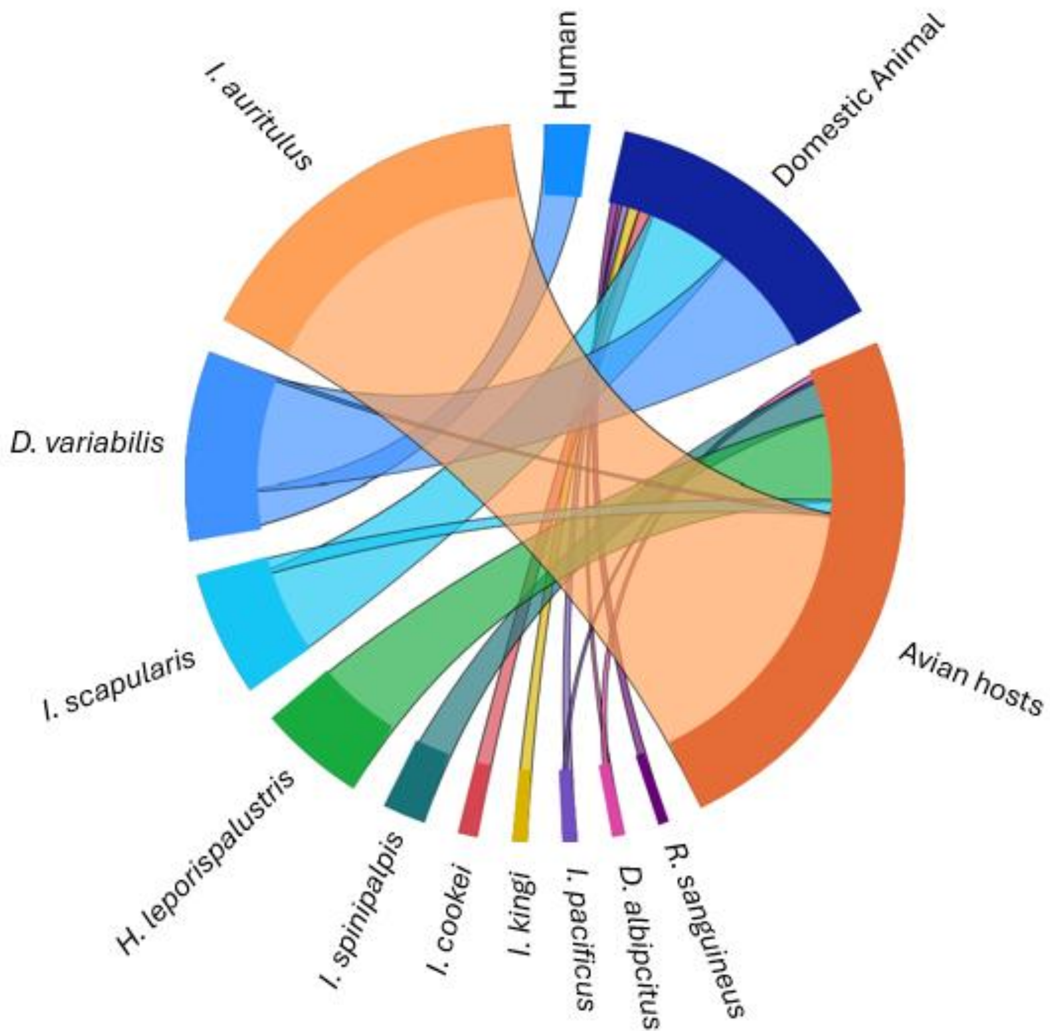

Figure 3.2 Chord diagram depicting tick-host associations observed and gathered through passive surveillance from 2019-2024, created using Microsoft Power BI (version 2.144.679.0) with the "Chord" custom visual, ribbon sizes depicted are proportional to observed interactions.

Table 3.1 Summary of tick species identified of samples collected from the years 2019 to 2024.

Tick Species	Total (n)	Provinces Detected	Years Collected
D. andersoni	371	AB, SK	2019-2024
I. auritulus	151	BC	2022-2024
D. variabilis	80	AB, BC, MB, NS, SK	2022-20224
I. scapularis	53	BC, MB, NS, ON	2023-2024
H. leporispalustris	43	BC, ON	2024
I. spinipalpis	18	BC	2023-2024
I. cookei	8	AB, NS	2023-2024
I. kingi	6	AB	2022-2023
I. pacificus	6	BC	2022-2024
D. albipictus	5	AB, BC, NS	2022-2023
R. sanguineus	4	BC	2023

3.5.2 Rhabdoviridae prevalence

All 745 ticks collected in this study were screened for the presence of Rhabdoviridae RNA. Samples that tested positive were exclusively from *D. andersoni* and *I. auritulus* ticks. *D. andersoni* ticks were collected from the provinces of BC, AB and SK and detected as early as 2022. In contrast, *I. auritulus* samples were collected in BC and Rhabdoviridae RNA was detected only in 2024.

Of the 371 *D. andersoni* ticks collected, 82 (22.1%) tested positive for rhabdovirus RNA, with detection only in adult ticks (Table 2). Infection prevalence in areas where active surveillance was conducted—such as Chin Lake and Waterton National Park—was 36/148 (24%) and 45/146 (30.8%), respectively. A total of 151 *I. auritulus* ticks were collected, all of which were from migratory birds found on Vancouver Island. Twelve samples (7.9%) tested positive, and detection was limited to ticks collected in 2024. Our assay targets conserved genomic regions and was

optimized using known positive controls, providing confidence in detecting typical infections in field-collected ticks [4, 37]. However, because cryptic infections—such as those with very low viral loads or highly divergent viral variants—may fall below the detection threshold of any molecular assay, we cannot completely rule out their presence.

Table 3.2 Infection prevalence of *Dermacentor andersoni* and *Ixodes auritulus* ticks with Rocky Mountain Tick Rhabdovirus (RMTR) and Coastal Avian Tick Rhabdovirus (CATR), respectively, by year of collection.

Virus	Tick Species	Province	Year of collection	Number of Ticks	Infected ticks	Prevalence
RMTR	*D.andersoni*	AB	2020	2	0	0
			2022	93	4	4.3%
			2023	177	44	24.9%
			2024	73	33	45.2%
		BC	2023	20	0	0
			2024	3	0	0
		SK	2019	2	0	0
			2022	2	1	50%
	Total			371	82	22.1%
CATR	*I. auritulus*	BC	2022	26	0	0
			2023	20	0	0
			2024	105	12	11.4%
	Total			151	12	7.9%

3.5.3 Phylogenetic Relationship of Newly Discovered Rhabdoviruses

To investigate the evolutionary relationships and history of both novel tick viruses, a 471-bp fragment of the block III region of the L gene was used to construct a ML phylogenetic tree using the Tamura-Nei model with 1,000 bootstrap replicates. Highly divergent members of the family- such as Cytorhabdovirus, Dichorhabdovirus, Nucleorhabdovirus and Novirhabdovirus- were excluded from the ML tree to improve phylogenetic resolution.

When compared to sequences in GenBank using the BLASTn algorithm, both rhabdovirus sequences generated in this study showed the highest similarity to American dog tick rhabdovirus-2, detected in *D. variabilis* ticks in the United States, with approximately 96% query coverage and 72% nucleotide identity. Given the notable divergence of these tick-rhabdoviruses from other members of the Rhabdoviridae family, we consider both viruses to represent novel species.

The resulting ML tree revealed that both novel tick rhabdoviruses cluster with recognized members of the Alpharicinrhavirus genus, suggesting close evolutionary relationships. Precise placement within the genus remains tentative due to the use of a partial gene fragment, but clustering is consistent with other recognized Alpharicinrhavirus members. Members of Alpharicinrhavirus are currently split into two distinct clades due to the construction of a phylogenetic tree based on partial sequences. (Figure 3).

This preliminary phylogenetic analysis shows consistent clustering patterns for other already recognized Alpharicinrhavirus members. However, members currently classified within the genus are divided into two distinct clades—one clustering with CATR and *Norway mononegavirus-1* and the other with RMTR and the rest of the members comprising the Alpharicinrhavirus genus—highlighting potential unresolved relationships within the genus.

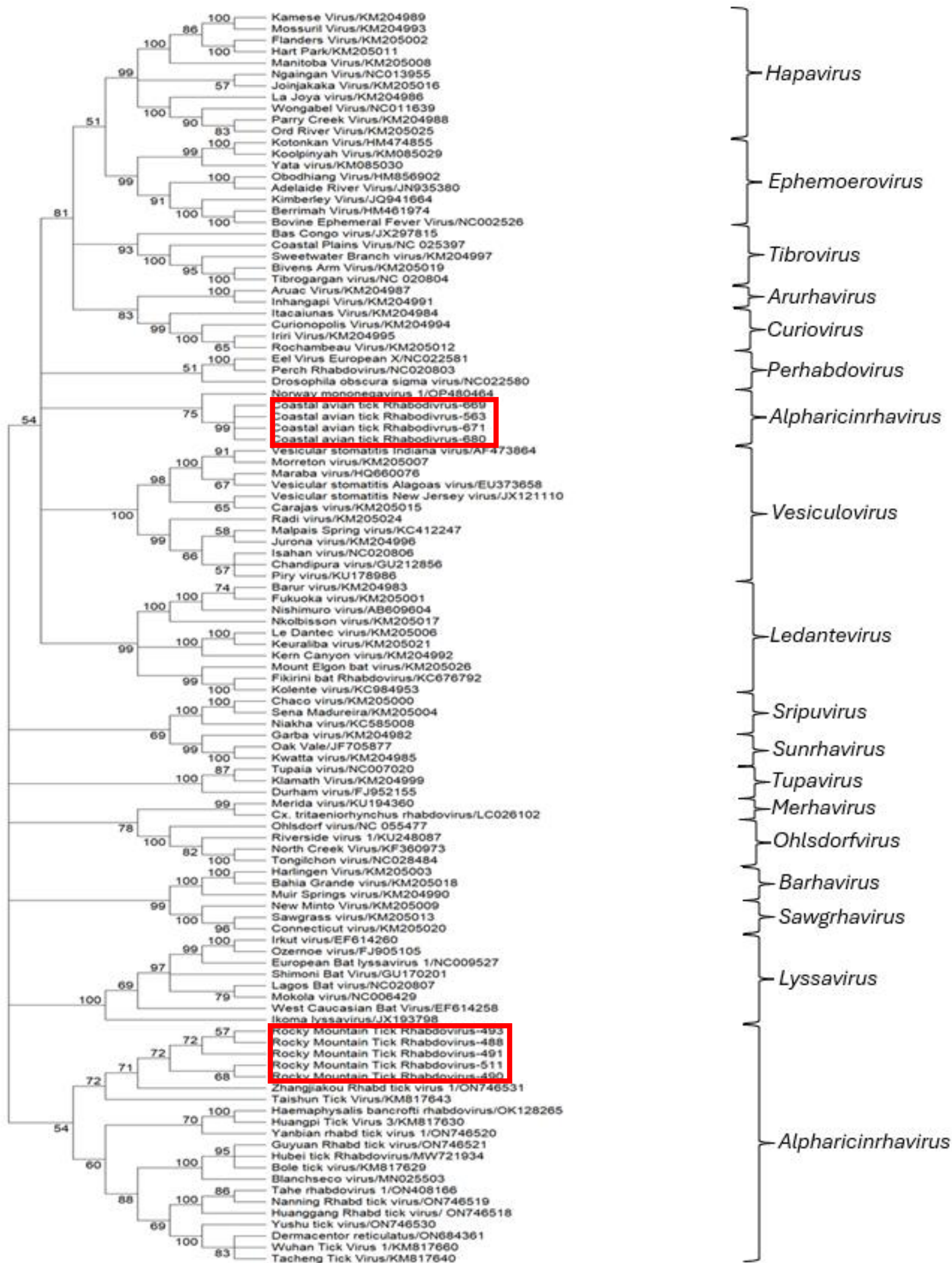

Figure 3.3 Maximum Likelihood phylogenetic tree showing the evolutionary relationships of CATR and RMTR in the context of representative members of other genera within the

Rhabdoviridae family. The tree was constructed using a fragment of block III of the L gene, which encodes the RNA-dependent RNA polymerase (RdRp) protein. Sequences were aligned using ClustalW, and the phylogeny was inferred using the Maximum Likelihood method under the Tamura-Nei nucleotide substitution model. Node support values represent bootstrap percentages based on 1,000 replicates. Taxon labels include GenBank accession numbers where available.

3.6 Discussion

The disease burden of tick-borne pathogens in Canada remains relatively low but is expected to increase due to the geographic expansion and introduction of new tick-borne pathogens (TBPs) into the country. Five prominent TBPs currently circulate in Canada—*Borrelia burgdorferi* (Lyme disease), *Anaplasma phagocytophilum* (anaplasmosis), *Babesia microti* (babesiosis), Powassan virus, and *Borrelia miyamotoi*—all of which are projected to increase in prevalence and disease burden [39]. To mitigate potential outbreaks, continued comprehensive surveillance of tick populations and TBVs in Canada is essential [40].

In this study, we collected a total of 745 ticks from 23 sampling locations across the following provinces: BC, AB, SK, MB, ON, and NS. Our study also examined how domestic animals travel and migratory birds contribute to tick dispersal and geographic range expansion. Notably, *R. sanguineus* ticks and *I. cookei* ticks were submitted by veterinary clinics in Vancouver, BC and Fort MacLeod, AB, respectively. Both species were found parasitizing domestic dogs. *R. sanguineus* is not considered endemic to Canada; however, it is frequently reported on domestic animals with a history of travel to tropical areas or from prolonged presence in veterinary clinic environments [17]. *I. cookei* ticks are endemic to eastern Canada, previously only reported from Newfoundland to southeastern Manitoba [17]. Importantly, these ticks are of public health concern as they are known to parasitize humans and are capable vectors of the Powassan virus [41].

While *I. cookei* was detected twice in the same location off dogs in Alberta over the course of a year, we cannot confirm local establishment based on these findings alone. Since these ticks were collected from domestic animals, the possibility of prior travel as the source of introduction cannot be ruled out. Moreover, this tick's reliance on medium-sized mammal hosts such as groundhogs [17] raises uncertainty, as these hosts may be absent or occur at low abundance in the region. These findings highlight the importance of continued surveillance to monitor potential range expansions and detect emerging tick populations before they become established.

A key observation from our collaboration with the Ontario bird banding team focused on the potential northward expansion of the invasive, self-reproducing, and Lyme disease–competent tick *H. longicornis* via avian hosts. From this study, we report no detection of a northward expansion of *H. longicornis* into Canada via avian hosts in Ontario. However, given the limitations inherent to bird banding procedures and the restriction to a single sampling site, these findings likely underestimate the true tick prevalence on avian hosts along the Mississippi flyway, as ticks may frequently go undetected during handling [42].

Our findings report the detection of two novel tick-rhabdoviruses in *D. andersoni* in Canada: RMTR in *D. andersoni* and CATR in *I. auritulus*. Preliminary BLASTn analysis of PCR-amplified fragments of the block III region of the L gene revealed that both novel tick rhabdoviruses show the highest similarity to American Dog-Tick Rhabdovirus-2 in (ADTR-2), previously detected in *D. variabilis* in the USA. ADTR-2 remains unclassified within the Alpharicinrhavirus genus due to the absence of a publicly available full-length nucleotide sequence.

To investigate the evolutionary and phylogenetic relationships of these novel viruses within the Alpharicinrhavirus genus, we constructed a maximum likelihood (ML) tree using a partial 471-bp

fragment of the L gene from representative members of the Rhabdoviridae family (Figure 2). Because clustering based on such a partial sequence may not accurately reflect full-length genome relationships, members of the Alpharicinrhavirus genus were split into two distinct clusters in our tree, which contrasts with the ICTV-recognized grouping of this genus. Furthermore, the preliminary phylogenetic analysis showed that both novel rhabdovirus sequences from this study fall within the Alpharicinrhavirus genus, but no conclusions can be drawn regarding their relative similarity to any specific virus or their precise evolutionary placement. This highlights that, without full-length genome sequences—or at minimum the complete L gene segment—any assertions about similarity or relatedness of these novel viruses are preliminary and should not be overinterpreted.

3.7 Conclusion

In conclusion, our surveillance study of Canadian ticks investigated the movement of ticks via domestic animals and avian hosts, as well as the circulation of tick rhabdoviruses in Canada. We report, for the first time, the molecularly confirmed detection of *Ixodes cookei* ticks in Alberta, recovered from canine hosts in a veterinary clinic. Additionally, we identified two distinct novel tick rhabdoviruses in Canadian ticks. Given their significant genetic divergence from published and ICTV-recognized members of the *Rhabdoviridae* family, we provisionally name these viruses CATR and RMTR, after the common names of their respective vectors.

Phylogenetic analysis, based on a fragment of the block III region of the L protein and ML tree construction, suggests that these viruses share evolutionary relationships with members of the *Alpharicinrhavirus* genus. Our ML trees also indicate possible unresolved evolutionary relationships within the genus, as evidenced by inconsistent clustering patterns. To improve

phylogenetic resolution, future work should employ next-generation sequencing (NGS) to obtain full-length L protein sequences for comprehensive analysis.

3.8 Study Limitations, Mitigation Strategies, and Constraints to Execution

I acknowledge that the evolutionary analysis in this study remains incomplete. Because only a 471-bp fragment of the L gene was available for analysis, phylogenetic resolution was insufficient to determine the evolutionary relationship of novel tick viruses detected in this study with confidence. Furthermore, the absence of full-length L gene sequences limits formal classification. According to ICTV guidelines, clustering and taxonomic assignments of rhabdoviruses should be based on either full-length genome sequences or, at a minimum, the complete L gene segment to ensure accurate phylogenetic placement. To resolve this limitation, next-generation sequencing (NGS) would be required to obtain full-length genome sequences. This step was not undertaken during my study due to external logistical constraints beyond my control, including operational disruptions and other resource priorities at CFIA that took precedence throughout my time at CFIA, which prevented the implementation of NGS at this stage.

3.9 Acknowledgements

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Creek Veterinary Service, Edgewater Companion Animal Clinic, Lomsnes Veterinary Hospital, Big Rock Animal Clinic, VCA Canada Centre Animal Hospital, Dakota Veterinary Hospital, Macleod Veterinary Hospital, Côte-des-Neiges Veterinary Clinic, Truro Veterinary Clinic, Greenwood Animal Hospital, and Basinview Animal Hospital.

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3.10 Supplementary data

Table 3.3 Metadata of Rhabdoviridae sequences retrieved from GenBank used for construction of Maximum Likelihood phylogenetic tree.

Accession number	Isolation Year	Country	Host
GU212856	2004	India	Homo sapien
KC412247	1985	USA	*Aedes campestris*
KM204996	1962	Brazil	*Haemagogus sp.*
NC020806	1977	Iran	*Phlebotomus papatasi*
HM566195	2008	Madagascar	Mosquito
KM205026	1964	Kenya	Bat
KM205024	1982	Italy	*Phlebotomus perfiliewi*
JX121110	1989	USA	Insect
FJ872827	1972	France	*Sprivivirus esox*
KU178986	1960	Brazil	Piry virus
KM205015	1983	Brazil	*Lutzomyia sp.*
AF473864	1998	USA	Equine
KM205007	1986	Colombia	*Lutzomyia sp.*
HQ660076	2010	Brazil	Mouse
EU373657	1961	Trinidad	Mites
EU373658	1964	Brazil	Mule
NC008514	2016	China	*Siniperca chuantsi*
NC022581	2009	Netherlands	*Anguilla anguilla*
NC020803	1981	France	*Perca fluviatilis*
KC676792	2011	Kenya	*Hipposideros commersoni*
KC984953	1985	Guinea	*Amblyomma variegatum/Hipposideros jonesi*
NC022580	2007	United Kingdom	*Drosophila obscura*

KM205006	1965	Senegal	Homo sapiens
KM205021	1968	Senegal	*Tatera kempi*
KM204992	1956	USA	*Myotis yumanesis*
AB609604	2011	Japan	Wild Boar
KM205001	1982	Japan	*Culicoides punctatus*
KM205017	1965	Cameroon	*Eretmapodites leucopous*
KM204983	1962	India	*Rattus wroughtoni*
KM205019	1982	USA	*Culicoides insignis*
NC020804	1976	Australia	*Culicoides brevitarsis*
KM204997	1982	USA	*Culicoides insignis*
NC025397	1981	Australia	Bovine
KM204982	1970	Central African Republic	*Corythornis cristata*
JF705877	1993	Australia	*Anopheles annulipes*
KM204985	1964	Suriname	*Culex sp.*
JX297815	2009	Democratic Republic of Congo	Homo sapien
KM204987	1955	Trinidad and Tobago	*Trichoprosopon theobaldi*
JQ941664	1980	Australia	*Bos taurus*
HM461974	1981	Australia	*Bos taurus*
NC002526	2000	Sudan	Mosquito
HM856902	2010	Sudan	Mosquito
JN935380	1981	Australia	Cattle
KM085030	1969	Central African Republic	*Mansonia uniformis*
HM474855	1967	Nigeria	*Culicoides sp.*
KM085029	1985	Australia	*Bos taurus*

KM204988	1973	Australia	*Culex annulirostris*
KM205025	1976	Australia	*Culex annulirostris*
NC011639	2018	Australia	*Culicoides austropalpalis*
KM204986	1958	Panama	*Culex dunni*
KM205016	1966	Papua New Guinea	*Culicinae sp.*
NC013955	2009	Australia	Marcopod
KM205002	1961	USA	*Culiseta melanura*
KM205011	1955	USA	*Culex tarsalis*
KM205008	1977	Canada	*Culex tarsalis*
KM204989	1967	Uganda	*Culex annulioris*
KM204993	1959	Mozambique	*Culex sitiens*
KM205013	1964	USA	*Dermacentor variablis*
KM205020	1978	USA	*Ixodes dentatus*
KM205009	1972	USA	*Hemaphysalis leporispalustris*
FJ985749	2004	Cote d'Ivoire	*Culex sp.*
NC040664	2001	Iran	*Hyalomma anatolicum*
KM204990	1976	USA	*Aedes sp.*
KM205003	2001	USA	*Culex salinarius*
KM205018	1974	USA	*Ochlerotatus sollicitans*
KU194360	2007	Mexico	*Culex quinquefasciatus*
LC026102	2015	Japan	*Culex tritaeniorhynchus*
KM204999	1962	USA	*Microtus montanus*
NC007020	2004	USA	Moribund tree shrew
FJ952155	2009	USA	Bird
KF360973	1997	Australia	*Culex sitiens*
NC028484	2012	South Korea	*Culex bitaeniorhynchus*

NC055477	2012	Germany	*Ochlerotatus cantans*
KU248087	2014	Hungary	Mosquitoes
OK128265	2020	Australia	*Haemaphysalis bancrofti*
KM817630	2012	China	*Haemaphysalis doenitzi*
MF141072	2014	Norway	*Ixodes ricinus*
ON408171	2021	China	*Ixodes persulcatus*
OK128265	2020	Australia	*Haemphysalis bancrofti*
KM817630	2012	China	*Haemaphysalis doenitzi*
MN025503	2017	Trinidad/Tobago	*Amblyomma ovale*
KM817629	2012	China	*Hyalomma asiaticum*
MW721934	2019	China	*Haemaphysalis longicornis*
ON684361	2012	Croatia	*Dermacentor reticulatus*
KM817640	2013	China	*Dermacentor marginatus*
ON746530	2018	China	Cattle
KM817660	2012	China	*Rhipicephalus microplus*
ON408166	2020	China	*Haemaphysalis japonica*
ON746518	2019	China	Cattle
ON748519	2020	Russia	*Cladonia stellaris*
KM204995	1982	Brazil	*Lutzomyia sp.*
KM205012	1973	French Guiana	*Coquillettidia albicosta*
KM204994	1985	Brazil	*Culicoides sp.*
KM204984	1984	Brazil	*Culicoides sp.*
GQ375258	2009	France	*Drosophila melanogaster*
ON748531	2020	Russia	*Cladonia stellaris*
KM817643	2013	China	*Haemaphysalis hystricis*
KC994644	2009	Peru	Mosquitoes
JX193798	2009	Tanzania	*Civettictis Civetta*
EF614258	2007	Russia	Bat
NC006429	1996	France	Shrew

GU170201	2009	Kenya	*Hipposideros commersoni*
NC020807	1985	Senegal	*Eidolon helvum*
NC009527	1968	Germany	*Eptesicus serotinus*
EF614260	2003	Russia	Bat
FJ905105	2007	Russia	Homo sapien

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Chapter 4: Conclusions

4.1 Overview

This thesis investigated tick surveillance in Canada, focusing on two critical research gaps: tick-borne viruses (TBVs) and host-mediated dispersal patterns. The findings from these studies have important implications for public health and expand our understanding of TBVs in Canada. Notably, we report the first documentation of the eastern Canada primary vector for Lyme Disease (LD), *Ixodes scapularis* parasitizing avian hosts on Vancouver Island [1, 2]. Highlighting the role of migratory birds in introducing medically important ticks to new regions exemplifies the constant need for surveillance along flyways. As well as the discovery and phylogenetic characterization of two novel tick-rhabdoviruses: Coastal Avian Tick Rhabdovirus (CATR) and Rocky Mountain Tick Rhabdovirus (RMTR). The discovery of this novel tick Rhabdovirus demonstrates that Canadian tick populations harbour previously undescribed viral diversity, underlining the importance of continued viral surveillance for anticipating potential emerging threats.

Using an integrative approach combining the strengths of both morphological and molecular identification [3, 4], this work provides the first evidence of *I. scapularis* parasitizing both migratory and non-migratory birds during the fall migration of 2023 (Chapter 3). This raises concerns about the potential for westward expansion and establishment of *I. scapularis* populations via avian-mediated dispersal, which could elevate the risk of TBDs such as LD and Powassan virus in previously unaffected areas [5-7]. Alarmingly, one of the seven *I. scapularis* collected in this study tested positive for *Borrelia burgdorferi*, the causative agent of LD. The results from this study remain inconclusive regarding whether established colonies of *I. scapularis* exist in western Canada; however, this major finding underscores the need for continued surveillance and proactive public health measures.

Lastly, we conducted Canada-wide surveillance of tick populations, which not only facilitated the detection of *Ixodes cookei* in Alberta for the first time [2] but also enabled the investigation of Rhabdoviridae RNA presence in Canadian ticks (Chapter 4). This surveillance led to the discovery of two novel viruses circulating in *Ixodes auritulus* and *Dermacentor andersoni* ticks. Sequences were compared to other members of the family using the BLASTn algorithm, revealing significant divergence between both novel rhabdoviruses and recognized members of the Rhabdoviridae family, as defined by the International Committee on Taxonomy of Viruses (ICTV). We provisionally named these viruses after the common names of their respective vectors: CATR in *I. auritulus* and RMTR in *D. andersoni*.

Using a Maximum Likelihood (ML) phylogenetic tree, we examined the evolutionary and genetic relationships of these novel tick rhabdoviruses in the context of the broader Rhabdoviridae family. It is important to note that this analysis was based on a partial 471-bp fragment of the L gene, and therefore, any evolutionary or similarity-based conclusions should be interpreted cautiously. Preliminary findings indicate that both viruses exhibit evolutionary relationships with the Alpharicinrhavirus genus; however, members of this genus appear to be split into two distinct clusters in our tree, which is inconsistent with the ICTV-recognized monophyly of Alpharicinrhavirus. Consequently, no assertions can be made regarding the closest relative or detailed evolutionary placement of RMTR and CATR and claims about vertebrate host range or pathogenic potential based on partial sequences are not scientifically supported. Full-length genome sequences, or at minimum complete L gene segments, will be required to resolve these phylogenetic relationships accurately.

4.2 Future Directions

The detection of *I. scapularis* ticks on avian hosts on Vancouver Island presents a significant public health concern and warrants further investigation into the potential for westward expansion of this species. Avian hosts can aid in the dissemination of these ticks, as previously documented with northward expansions in Ontario [11]. Future work should focus on active surveillance along the Pacific Flyway, particularly engaging in flagging and dragging at key migratory bird stopover sites, to assess the frequency and scale of tick introductions. Continued monitoring of ticks parasitizing avian hosts, paired with pathogen screening, will help clarify the role of migratory birds in transporting *I. scapularis* and potentially zoonotic pathogens, such as *B. burgdorferi*. Unfortunately, few studies have investigated whether biomes exist in British Columbia (BC) where the necessary requirements for the establishment of *I. scapularis* colonies to occur [12, 13]. Modelling focussing specifically on BC, evaluating the habitat suitability and climate could provide further active surveillance sampling sites, supporting early identification of regions at risk for tick establishment. Longitudinal studies to evaluate overwintering success and reproductive capacity of *I. scapularis* in western habitats would provide valuable insight into establishment risk. These efforts are essential for anticipating shifts in LD risk and informing public health preparedness in BC and other western provinces. Especially since the native principal vector of western Canada, *Ixodes pacificus* does not show high suitability for most of BC, being restricted mostly to areas surrounding large bodies of water [14]. It is an important question *of I. scapulari*s will be able to establish in areas considered low risk due to *I. pacificus's* inability to establish.

In parallel, the discovery of two novel rhabdoviruses in Canadian tick populations highlights the need for expanded surveillance of TBVs. Future work should include metagenomic

sequencing of tick viromes across diverse host species and geographic regions to better characterize the diversity, abundance, and ecological dynamics of rhabdoviruses and other RNA viruses. Given the high sequence divergence observed and the lack of known vertebrate infection, these novel viruses may represent insect-specific viruses (ISVs). Investigating the prevalence and biology of ISVs in ticks will be crucial in determining their role in shaping the tick virome, their interactions with other pathogens, and their impact on vector competence. Experimental studies on viral replication and transmission dynamics, particularly whether these viruses can infect vertebrate hosts or are restricted to the tick, will be critical for assessing their zoonotic potential. Phylogenetic and evolutionary analyses should also be expanded to include full-length sequences of the L gene or complete genomes, as partial sequences do not allow for a reliable determination of evolutionary relationships or similarity between novel viruses. Strengthening national and global databases with more tick-associated viral genomes will improve taxonomic resolution and support early detection of emerging TBVs. Together, these efforts can contribute to a more comprehensive understanding of virus-vector-host relationships and help anticipate future public and veterinary health threats.

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