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Wildlife fleas and ticks in Wisconsin, USA: unrecognized vectors of bacterial pathogens

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ABSTRACT

Small wildlife species host flea and tick species that can also infest or transmit pathogens to domestic animals and humans, including *Anaplasma, Babesia, Bartonella, Borrelia, Ehrlichia,* and *Rickettsia* species. Despite their zoonotic potential, little is known regarding the prevalence, diversity, and epidemiology of these pathogens. Therefore, we aimed to survey the ectoparasites found on Eastern Cottontail Rabbits (rabbits), Eastern Grey Squirrels (squirrels), and Virginia Opossums (opossums) in south-central Wisconsin, and describe the prevalence of select pathogens. Ectoparasites were opportunistically collected from small mammals, then identified to the species level, pooled, washed, and DNA extracted for quantitative PCR (qPCR) to detect Anaplasmataceae, Apicomplexa, *Bartonella,* hemotropic *Mycoplasma,* and *Rickettsia.* To analyze the genomic diversity of uncharacterized *Bartonella*, three flea pools were subject to metagenomic sequencing. *Cediopsylla simplex* and *Haemaphysalis leporispalustris* were the most common ectoparasites on rabbits, while *Orchopeas howardi* was most common on squirrels and opossums. *Bartonella* species were detected in *C. simplex* pools (n = 52), most commonly two distinct *Bartonella alsatica*-like bacteria (38 %; 20/52). *Bartonella durdenii,* definitively identified by metagenomic sequencing, was detected in 42 % (13/31) of *O. howardi* pools from squirrels. From metagenomic sequencing, *B. alsatica*-like species displayed a 4.8 % dissimilarity rate while *B. durdenii* displayed a 0.4 % dissimilarity rate. Sequencing of one *B. alsatica*-like flea pool also identified phage-associated genes not found in the *B. alsatica* genome. *Rickettsia felis* (n = 1) and opossum-associated hemotropic *Mycoplasma* sp. (n = 2) were detected in *O. howardi* from opossums. *Rickettsia bellii* and *Anaplasma* sp. were detected in *Haemaphysalis leporispalustris* from rabbits. These findings reinforce the value of metagenomic sequencing, facilitating the correct identification of *B. durdenii* and identifying genes not found in the type strain, specifically phage related genes. Due to the known zoonotic potential of *B. alsatica,* further examination of *B. alsatica*-like and *B. durdenii* pathogenicity is warranted.

1. Introduction

Wildlife species serve as an abundant source of parasite and vector-borne pathogen maintenance and changes in the nature, frequency, and intensity of human-animal contact can increase pathogen spillover risk ((OHHLEP) et al., 2023). Among wildlife species, small mammals – including rabbits, squirrels, and opossums – are particularly abundant in urban and suburban areas, increasing interactions with humans and domestic animals. These wildlife species serve as hosts to known vectors of zoonotic pathogens including *Amblyomma americanum, Ctenocephalides felis, Dermacentor variabilis, Ixodes scapularis,* and *Haemaphysalis longicornis* (White et al., 2021). These flea and tick species vector numerous pathogens including *Anaplasma, Babesia, Bartonella, Borrelia,* and *Rickettsia* species (Moore et al., 2024; Rodino et al., 2020).

Wisconsin has a humid continental climate with warm/hot summers, similar to the surrounding Midwestern United States. Multiple zoonotic vector-borne diseases are common in the Upper Midwest, including *Babesia* and *Borrelia* that utilize sylvatic reservoir hosts (Krause, 2019; Mead et al., 2024). The urban, suburban, and rural areas surrounding south-central Wisconsin are inhabited by a vari-

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ety of small wildlife including the Eastern Cottontail Rabbit (*Sylvilagus floridanus,* herein referred to as rabbits), Eastern Grey Squirrel (*Sciurus carolinensis*, herein referred to as squirrels), and Virginia Opossum (*Didelphis virginiana,* herein referred to as opossums). As in other areas, these species commonly live among human developments, regularly interacting with humans and domestic animals.

Both rabbits and squirrels are broadly distributed on the North American continent and host to the species-specific *Cediopsylla simplex* and *Orchopeas howardi* fleas, respectively (Nelder et al., 2009; Rothschild and Ford, 1972). Diverse rabbit flea species (*Cediopsylla inaequalis, Spilopsyllus cuniculi*, *Xenopsylla cunicularis*, etc.) are broadly assumed to transmit *Bartonella alsatica,* although *ssrA* gene sequences of *B. alsatica* in the United States report limited homology (96.9 %) to European *B. alsatica* isolates (Márquez, 2015; Sato et al., 2020). Further investigation is necessary to determine if the *B. alsatica*-like bacteria in the United States is a new species. The squirrel is host to *Bartonella durdenii,* although the transmission mechanism or presence of *B. durdenii* in fleas is relatively unexplored (Bown et al., 2002; Nelder et al., 2009; Reeves et al., 2005). The epidemiology, genetic variation, and pathogenicity of these *Bartonella* species in animals and humans remain to be described.

In contrast, the opossum is host to diverse flea species including those associated with cats (*C. felis),* humans (*Pulex irritans),* and wildlife (*O. howardi, Xenopsylla cheopis)* (Bezerra-Santos et al., 2021). The opossum may play a role in the maintenance and dissemination of various vector-borne pathogens, including *Rickettsia felis* (reviewed by Bezerra-Santos et al.) (Bezerra-Santos et al., 2021; Brown and Macaluso, 2016).

Small wildlife species serve as reservoir hosts for multiple tick-associated pathogens and are often infested by generalist tick species, including *Amblyomma, Dermacentor,* and *Ixodes* ticks (Bezerra-Santos et al., 2021; Nelder et al., 2009; Schiff et al., 2025). Both rabbits and squirrels are also associated with more host-specific ticks: *Haemaphysalis leporispalustris* and *Ixodes marxi,* respectively (Kollars and Oliver, 2003). Multiple pathogenic bacterial species have been amplified from *H. leporispalustris,* including *Francisella tularensis* and *Rickettsia* species (Crandall et al., 2022; Roth et al., 2017; Sánchez-Montes et al., 2020).

The increasing number of human, domestic animal, and wildlife interactions associated with urban sprawl, the potential for zoonotic pathogen transmission, and the paucity of information regarding wildlife associated vectors was the rationale for this investigation of wildlife associated fleas and ticks in the Midwestern United States. We first aimed to determine the ectoparasites infesting small mammals in southcentral Wisconsin. We then determined the presence of select pathogens (Anaplasmataceae, Apicomplexa, *Bartonella,* hemotropic *Mycoplasma,* and *Rickettsia)* in fleas and ticks collected from small mammals. Our second aim was to map the presence of flea and tick infestation, and flea- and tick-borne pathogens and compare their presence in urban versus rural locations. Based upon our initial findings, our final aim was to more precisely characterize ambiguous *Bartonella* species utilizing Illumina metagenomic sequencing of flea pools. We hypothesized that rabbits and squirrels would be infested with their associated flea species (*C. simplex* and *O. howardi,* respectively), and that these flea species would be infected with specific host adapted *Bartonella* species.

2. Material and methods

2.1. Sample collection

Ectoparasite collection was performed in compliance with University of Wisconsin-Madison IACUC protocol #V006461 and #V006749 and Wisconsin Department of Natural Resources Scientific Collectors Permit #SCPN-22–53. All ectoparasites were collected at the Dane County Humane Society's Wildlife Center (DCHS-WC) from March 21st, 2022, to March 7th, 2024. Animals were found injured, ill, or orphaned by members of the public and presented to DCHS-WC for rehabilitation. Animal weight, sex, species, and address of origin was recorded. Ectoparasites, collected opportunistically from live (conscious or anesthetized) or dead hosts, were placed in Ziploc bags and stored at –20 °C prior to shipment to North Carolina State University (NCSU). Fleas and ticks were identified to the species level according to previously published keys at the University of Wisconsin and NCSU (National Communicable Disease Center, 1969; Egizi et al., 2019).

2.2. Pooling

For hosts with less than 5 fleas, all fleas belonging to the same species from a single host were pooled. For hosts with more than 5 fleas, up to 3 pools were created with 5 fleas each. Ticks were pooled according to individual host, tick species, tick sex, engorged status, and life stage. If more than 5 ticks were assigned to an individual pool, ticks were split equally into multiple pools.

2.3. Molecular analysis

Pooled ectoparasites were washed twice in PBS and twice in ethanol as detailed previously (Manvell et al., 2022). Ectoparasites were then bead beat (Fisherbrand™ Bead Mill 4 Mini Homogenizer, Fisher Scientific, Waltham, MA, USA 02451) until the exoskeleton was visibly broken, approximately 12 s. DNA extraction was performed utilizing the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA) following the manufacturer's tissue extraction protocol. DNA concentration was determined spectrophotometrically (Nanodrop, Thermo Fisher Scientific, Waltham, MA, USA).

For flea and tick pools, quantitative real-time PCR (qPCR) assays for the detection of Anaplasmataceae, Apicomplexa, *Bartonella,* hemotropic *Mycoplasma,* and *Rickettsia* spp. were performed targeting the 16S rRNA (Moore et al., 2023; Tyrrell et al., 2020), 18S-5.8S intergenic spacer (Calchi et al., 2024), *ssrA* (Tyrrell et al., 2019), 16S rRNA (Manvell et al., 2021), and 23S-5S rRNA (Tyrrell et al., 2019) genes, respectively (Supplemental Table 1). Species identity was determined by NCBI BLAST search (https://blast.ncbi.nlm.nih.gov/) and was considered a definitive identity if the search returned a single species with >99 % homology.

Bartonella 16S-23S intergenic spacer (ITS) conventional PCR amplification was attempted for all samples positive for *Bartonella* DNA by the *ssrA* gene. One forward primer (BsppITS325s) and two reverse primers (BsppITS1000as and BsppITS1100as) were used for amplification and bidirectional Sanger sequencing as previously described (Supplemental Table 1) (Cherry et al., 2011, 2009). Bidirectional sequences were assembled, trimmed, and aligned in SnapGene (version 7.2.1) utilizing MAFFT alignment with default parameters. Phylogenetic trees were constructed to compare the obtained sequences to those retrieved from GenBank or ATCC utilizing PhyML 3.0 to with Smart Model Substitution (SMS) to determine the best substitution model (Lefort et al., 2017). The *B. durdenii* ITS sequence was obtained from the ATCC genome (ATCC-1452), as it is not available publicly. Trees were then visualized and annotated in iToL (https://itol.embl.de/) and Affinity Designer 2.

2.4. Data analysis

Data were analyzed and visualized in R (version 4.4.1). Geographic data was converted from address to longitude and latitude utilizing the Geocode by Awesome Tables plugin in Google Sheets.

Geographic data including host species and pathogen presence was visualized in ArcGIS Pro (version 3.1.0).

Infection rates were estimated as the minimum infection rate (MIR) and maximum likelihood estimate (MLE). MIR was calculated in R utilizing the number of infected pools divided by the total number of vectors included in all pools. MLE was calculated utilizing the PoolPrev function in the PoolTestR package (version 0.2.0). Flea infection rate was calculated by flea and *Bartonella* species. In instances where more than five flea pools of the same flea species were obtained from two host species, the *Bartonella* infection rate was compared utilizing a Fisher's Exact Test.

2.5. Metagenomic sequencing

In order to investigate the detection of undetermined *Bartonella* sp., three flea pools were submitted to Genewiz by Azenta Life Sciences (New Jersey, USA 07080) for Illumina metagenomic shotgun sequencing on the Novaseq X plus (California, USA 92122). *Bartonella* reads were not specifically enriched and all metagenomic sequences was analyzed. Read quality was assessed using FASTQC (version 0.12.1), and adapters trimmed utilizing Trimmomatic (version 0.36). As the genome is not publicly available for *Cediopsylla simplex* or *Orchopeas howardi,* it was not possible to exclude host (flea) genomic data. Reads were then aligned to a reference genome with Bowtie2 (version 2.3.2). All reads were aligned with either a *B. alsatica* (Accession ID NZ_CP058235.1) or *Bartonella silvicola* (ATCC BAA-1453) and *B. durdenii* (ATCC BAA-1452) genome based on the suspected *Bartonella* species from PCR reference gene analysis. After genome alignment with Bowtie2, samtools (version 1.20) was utilized to generate a consensus sequence.

Contigs were assembled utilizing metaSPAdes (version 3.15.3) with the default minimum contig length of 2000 base pairs. Contigs were then binned utilizing MetaBAT2 (version 1.7) and Metagenome Assembled Genomes (MAGs) phylogenetically classified with GTDB-Tk (version 2.3.2, release 207 and 214 data). Genome completeness from MAGs was assessed with CheckM (version 1.0.18) and annotated with Prokka (version 1.14.5). As CheckM is designed for prokaryotic genomes and limited information is available regarding wildlife flea genomic data, host (flea) genome completeness and redundancy was assessed with BUSCO against the insecta_odb12 dataset (Manni et al., 2021).

To align contigs with *Bartonella* genomic sequences, a custom BLAST database was generated containing 22 complete or chromosome level *Bartonella* reference genomes available on the NCBI Genome browser (https://www.ncbi.nlm.nih.gov/datasets/genome/) with a maximum of 2 scaffolds, as well as the *B. silvicola* (ATCC BAA-1453) and *B. durdenii* (ATCC BAA-1452) genomes from ATCC (Manassas, VA). The rBLAST package (version 0.99.2) in R was then utilized to align contigs with the reference genome database. For each *Bartonella* species, the percentage identity of aligned contigs was calculated as the aligned width (width of aligned contigs minus mismatches and open gaps) divided by the total width of contigs aligned to that *Bartonella* species.

As *Bartonella* bins were not recovered from all flea pools with MetaBAT2, Average Nucleotide Identity (ANI) was obtained by comparing all contigs that had >50 % identity to one or more *Bartonella* species to the reference strain with PyOrthoANI (version 0.7.0).

To identify *Bartonella* reference genes in contigs, a custom BLAST database was created with the contigs for each sample. rBLAST was then utilized to align *Bartonella* reference genes (*gltA, ssrA, ftsZ,* 16S rRNA, 23S rRNA, and *rpoB*) with a reference gene library curated for *B. alsatica, B. durdenii,* and *B. silvicola* from reference genomes. For reference genes with a single contig with high coverage (>70 %), percentage alignment was calculated in R.

Contigs that did not align with the reference genome (*B. alsatica* or *B. durdenii*) were selected for further analysis. As Prokka annotation was only performed for binned reads, all open reading frames longer than 150 nucleotides were extracted using the NCBI ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/) and compared to published reference sequences (refseq_protein database) using the BLASTp algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The best alignment was recorded for analysis and visualization in R utilizing the gggenes package.

3. Results

In total, 759 fleas and 123 ticks were collected from 67 mammalian hosts: 39 rabbits, 16 squirrels, 9 opossums, 2 red foxes (*Vulpes vulpes*), and 1 bobcat (*Lynx rufus*). A majority of the hosts had experienced physical trauma (76 %; 51/67), however most were alive at the time of intake (94 %, 63/67).

The number of infested hosts, ectoparasite species, number of total ectoparasites, and range of ectoparasites per host are displayed in Table 1 for each host species. The tick life stage, sex, and engorged status is displayed in Table 2.

All hosts were sampled from south-central Wisconsin (Fig. 1).

3.1. Flea-borne pathogen detection

Flea pools were formed by pooling up to 5 fleas of the same species from the same host (Table 1). In total, DNA was extracted from 105 flea pools for pathogen detection. The genus *Bartonella* weas the most common pathogen detected across all flea species (36 %, 38/105; Fig. 2a). *Bartonella* was most common in *C. simplex* pools (43 %, 25/58) from rabbits and squirrels with a diversity of species detected including two *B. alsatica*-like genotypes, *B. henselae, B. koehlerae*-like, and *B. vinsonii* subsp. *berkhoffii* (Fig. 2a, Table 3). The *B. alsatica*-like 1 *ssrA* sequence displays 98.75 % (158/160 base pairs) alignment to *Bartonella* previously reported in Colorado, USA (GenBank Accession ID MN654368.1; Sato et al., 2020). While the *B. alsatica*-like 2 *ssrA* sequence displays only 92.55 % (149/161 base pairs) alignment to the same sequence. *Bartonella* detection was also common in *O. howardi* pools with 28 % (13/46) of flea pools infected with a *Bartonella* species with no close alignments of the *ssrA* gene on GenBank. This *Bartonella* spp. was significantly more common in *O. howardi* pools from squirrels (42 %, 13/31) than opossums (0/15; Fisher's Exact Test, $p = 0.0037$). Alignment of the *Bartonella* ITS (16S-23S) is depicted in Fig. 2b. The *O. howardi Bartonella* spp. was originally determined to be *B. silvicola,* due to alignment of the *Bartonella* ITS region. Utilizing metagenomic sequencing, the *Bartonella* spp. was determined to be *B. durdenii*. At the time of PCR testing and manuscript preparation, *B. durdenii* did not have a *ssrA* or ITS amplicon available in GenBank. Representative *B. alsatica* like-1 (PV612472, PV612359), *B. alsatica* like-2 (PV612473, PV612360), and *B. durdenii* (PV612474, PV612361) *ssrA* and ITS (16S-23S rRNA) sequences were deposited in GenBank.

Only a few other flea-borne pathogens were detected among the flea pools. A species similar to '*Candidatus* Mycoplasma haemodidelphidis' (97.42 %, Accession ID AF178676) was detected in two *O. howardi* pools from a single opossum. *Rickettsia felis* was detected in a single *O. howardi* pool from an opossum. The geographic location of flea-borne pathogen detection is available in Supplementary Fig. 1.

Wolbachia DNA was amplified from a majority of *O. howardi* pools (80 %, 37/46), as well as a small number of *C. simplex* (4 %, 2/58) by qPCR targeting Anaplasmataceae. All *Wolbachia* sequences aligned perfectly with *W. pipientis* over the amplified region (Accession ID AP028950.1; 135/135, 100 %).

Table 1
Ectoparasite species infesting small mammalian hosts in south-central Wisconsin, USA.

Host Species	Number of Infested Hosts	Ectoparasite Species	Total Number of Ectoparasites	Range (ectoparasites per host)	Number of Ectoparasite Pools
Bobcat (*Lynx rufus*)	1	*Cediopsylla simplex*	3	N/A	1
Eastern Cottontail Rabbit (*Sylvilagus floridanus*)	1	*Amblyomma americanum*	4	N/A	4
	39	*Cediopsylla simplex*	272	1–40	52
	5	*Haemaphysalis leporispalustris*	113	1–21	43
	1	*Ixodes scapularis*	3	N/A	2
Eastern Grey Squirrel (*Sciurus carolinensis*)	2	*Cediopsylla simplex*	2	1–1	2
	16	*Orchopeas howardi*	217	1–50	31
Red Fox (*Vulpes vulpes*)	2	*Cediopsylla simplex*	8	N/A	2
Virginia Opossum (*Didelphis virginiana*)	1	*Ctenocephalides felis*	1	N/A	1
	1	*Cediopsylla simplex*	6	N/A	1
	2	*Dermacentor variabilis*	2	1–1	2
	1	*Haemaphysalis leporispalustris*	1	N/A	1
	7	*Orchopeas howardi*	246	3–159	15

The number of hosts infested with each ectoparasite species by host and ectoparasite species. The total number of ectoparasites collected by host and ectoparasite species, and the range of fleas per host is included. The range includes only infested hosts. N/A= Not applicable.

Table 2
Number of ticks collected from small mammal hosts by species, lifestage, sex, and engorged status.

Host Species	Number of Ectoparasites	Number of Pools	Ectoparasite Species	Lifestage	Sex	Engorged
Eastern Cottontail Rabbit (*Sylvilagus floridanus*)	3	3	*Amblyomma americanum*	Larva	–	–
	1	1		Nymph	–	–
	1	1	*Haemaphysalis leporispalustris*	Larva	–	–
	46	21		Nymph	–	–
	36	11		Adult	Male	–
	3	2		Adult	Female	Yes
	28	8		Adult	Female	No
	1	1	*Ixodes scapularis*	Adult	Male	–
	1	1		Adult	Female	Yes
Virginia Opossum (*Didelphis virginiana*)	1	1	*Haemaphysalis leporispalustris*	Adult	Male	–
	2	2	*Dermacentor variabilis*	Adult	Male	–

N/A has been substituted for "-" to increase table readability.

3.2. Tick-borne pathogen detection

Tick pools were formed by pooling ticks of the same species, sex, and life stage from the same host. DNA was extracted from 52 tick pools for pathogen detection (Table 2). *Rickettsia bellii* (274/274 bp, 100 %, GenBank Accession ID CP015010.1) was the most common bacteria detected in *H. leporispalustris* tick pools (20 %, 9/44). The MIR for *R. bellii* infection in *H. leporispalustris* was calculated to be 33 % (1/3) for engorged female adults, 11.4 % (4/35) for adult males, 10.3 % (3/29) for engorged nymphs, and 3.7 % (1/27) for unengorged female adults.

An *Anaplasma* species showing high identity to an uncultured *Anaplasma* sp. previously detected in *Haemaphysalis qinghaiensis* from China (243/244 bp, 99.6 % GenBank Accession ID OR792785) was detected in four *H. leporispalustris* pools (9 %, 4/44). The MIR for *Anaplasma* sp. infection in *H. leporispalustris* was calculated to be 5.7 % (2/35) in adult males, 3.7 % (1/27) in unengorged adult females, and 3.5 % (1/29) in engorged nymphs.

Bartonella alsatica-like 2 DNA was amplified from one engorged adult female *H. leporispalustris* pool from a rabbit. *Bartonella vinsonii* subsp. *berkhoffii* (167/167 bp, 100 %, GenBank Accession ID CP003124.1) was detected in one engorged nymphal *H. leporispalustris* pool from a rabbit. *Babesia divergens*-like MO1 (585/586 bp, 99.32 %, GenBank Accession ID PQ184854.1) was detected in a single engorged adult female *I. scapularis*. The geographic location of tick-borne pathogen detection is available in Supplementary Fig. 1.

3.3. Metagenomics

Three pooled flea samples were selected for Illumina shotgun sequencing on the basis of qPCR results: one pool of *O. howardi* infected with an unidentified *Bartonella* species and two pools of *C. simplex* from rabbits (*C. simplex* 1 containing *B. alsatica*-like 1 and *C. simplex* 2 containing *B. alsatica*-like 2), both infected with distinct, uncharacterized *B. alsatica*-like bacteria.

Illumina shotgun sequencing yielded 19.6–21.7 million reads per sample prior to trimming and 18.7–20.6 million reads per sample after trimming. Alignment of the *O. howardi Bartonella* sp. pool to *B. durdenii* resulted in the lowest dissimilarity rate (0.53 %) and highest number of mapped reads and coverage (Fig. 3a-c). When the two *B. alsatica*-like were aligned to *B. alsatica,* there was lower coverage and an increased dissimilarity rate (4.88 %). Binning with MetaBAT2 resulted in two bins from known flea-associated bacte-

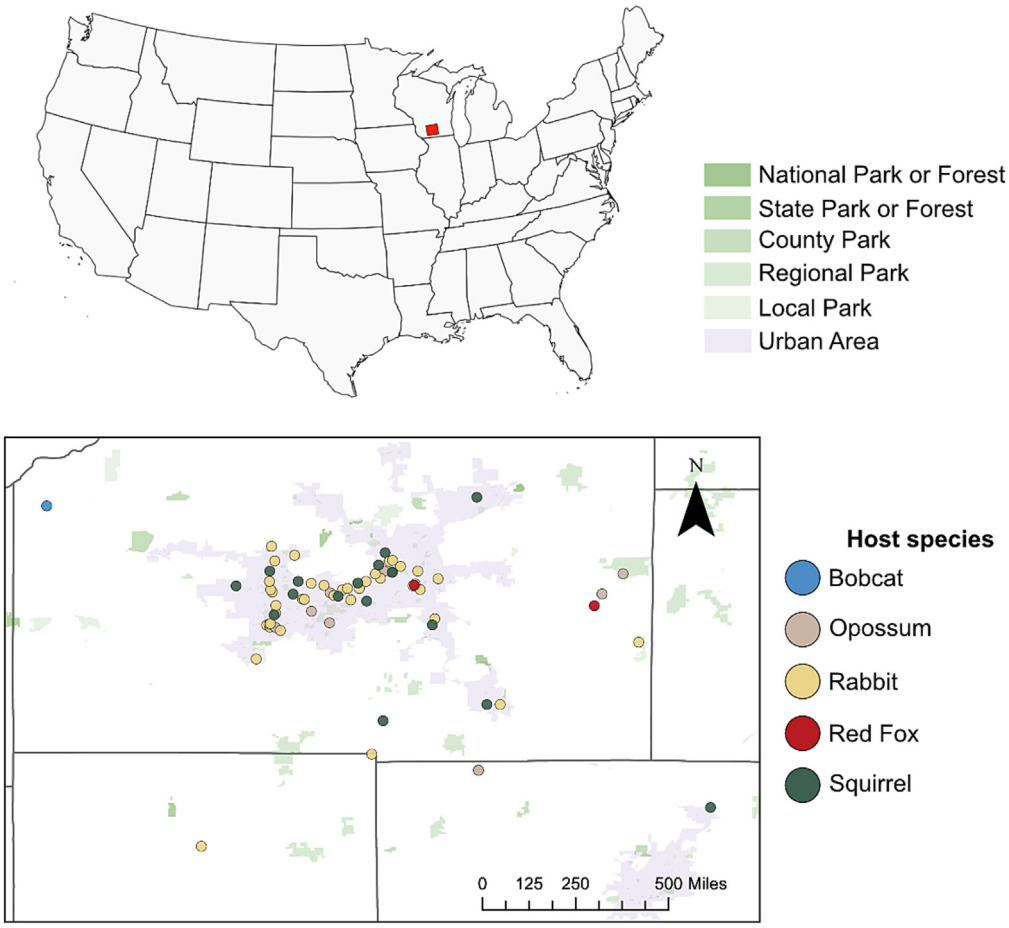


Fig. 1. Geographic location of host sampling and flea- and tick-borne pathogen detection. Reported location of origin for flea-infested animals sampled in the present study.

ria: *Bartonella* and *Wolbachia* in the *O. howardi* sample (Table 4). Other bins detecting host-related reads were detected in all samples and displayed an estimated 47.9–54.6 % completeness with limited duplication (0.13–0.34 %) (Supplemental Table 2). No flea-associated bacterial genera were identified by binning in the *C. simplex* samples, requiring further analysis to determine the presence and identity of *Bartonella* reads in *C. simplex* samples.

Due to the limited binning of *Bartonella* species, contigs assembled from trimmed reads were aligned with a curated *Bartonella* genome database. This analysis confirmed that the *Bartonella* detected in the *O. howardi* sample is *B. durdenii,* and not *B. silvicola* as was originally proposed following *Bartonella* ITS PCR alignment.

Considering all contigs over 2,000 base pairs and at least 50 % alignment to one or more *Bartonella* spp., the *O. howardi* pool possessed 106 *Bartonella*-aligned contigs with an Average Nucleotide Identity (ANI) of 99.45 % to *B. durdenii* utilizing PyOrthoANI. Utilizing rBLAST, the highest percentage of contigs aligned (100 %, 106/106 contigs) and percent identity of aligned contigs (99.58 %, 1,756,689/1,764,119 base pairs) was to *B. durdenii* (Fig. 4a, Supplemental Table 3). *Cediopsylla simplex* 1 had 151 *Bartonella*-aligned contigs with a 92.82 % ANI to *B. alsatica.* The percentage of contigs aligned was highest for *B. alsatica* (96.69 %, 146/151 contigs) as was the percent identity of aligned contigs (94.46 %, 399,293/422,696 base pairs; Supplemental Table 3). *Cediopsylla simplex* 2 had the lowest quantity of *Bartonella* contigs with 13 *Bartonella*-aligned contigs displaying an 93.75 % ANI to *B. alsatica*. Like *C. simplex* 1, the percentage of contigs aligned was highest for *B. alsatica* (100 %, 13/13 contigs) as was the percent identity of aligned contigs (93 %, 34,701/37,312 base pairs; Supplemental Table 3).

Alignment of *O. howardi* pool contigs to *B. durdenii* and *B. silvicola* reference genes, and sequences from PCR displayed high coverage and alignment of *B. durdenii* sequences (Fig. 4b). Contigs with perfect coverage and alignment to PCR amplicons (*ssrA* and ITS targets) were also identified. Only the *Bartonella* 16S, 16S-23S, and 23S reference sequences were obtained from *C. simplex* 1 with 98.22 % (5,415/5,513 bp) alignment to *B. alsatica.*

The alignment of all contigs from *C. simplex* 1 to *Bartonella* spp. showed that six contigs did not align with *B. alsatica* but instead aligned most closely with *Bartonella machadoae* (GCF_022559585.1), *B. vinsonii* (GCF_900638635.1), or *Bartonella krasnovii* (NZ_CP031844.2). Upon further analysis these contigs contain mostly phage related proteins, the two longest contigs are annotated in Fig. 5a-b. The four additional contigs are visualized in Supplementary Fig. 2 and include GH36-type glycosyl hydrolase domain-containing protein, transglycosylase SLT domain-containing protein, five phage related proteins, and six hypothetical proteins.

4. Discussion

This study documented multiple zoonotic or potentially zoonotic vector-borne pathogens in ectoparasites collected from small mammal wildlife species in Wisconsin. These wildlife species serve as hosts to phylogenetically diverse flea and tick species. Rabbits

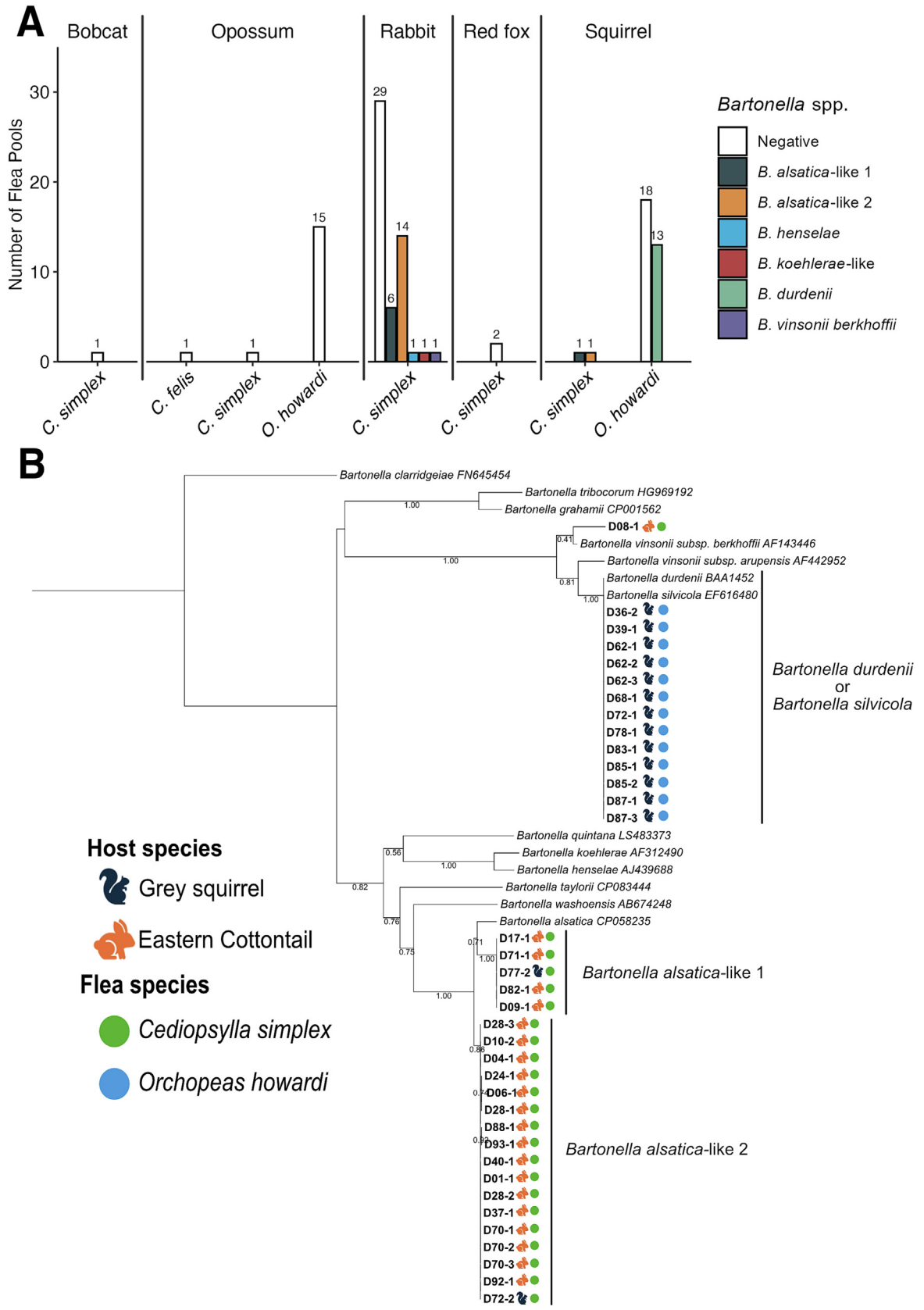


Fig. 2. Flea species and *Bartonella* species 16S-23S rRNA (ITS) sequences detected in flea pools. (A) *Bartonella* species detected in flea pools. (B) Phylogenetic tree aligning the detected *Bartonella* species (in bold) to published *Bartonella* species (in italics) utilizing Maximum Likelihood with substitution model HKY+G+I. The phylogenetic tree is rooted to *Bartonella clarridgeiae* (492 bp). Sample IDs are split into the host number (D##) and sample number (–#).

Table 3
Flea-borne pathogen pool infection rate, Minimum Infection Rate (MIR), and Maximum Likelihood Estimate (MLE).

Flea species	Host species	*Bartonella* species	Infected Pools (# infected pools/# pools)	MIR (# infected pools/# fleas in pools)	MLE (95 % CI)
Cediopsylla simplex	Rabbit or squirrel	*B. alsatica*-like 1	11.5 % (6/52)	3.6 % (7/192)	3.9 % (1.7–7.4 %)
Cediopsylla simplex	Rabbit or squirrel	*B. alsatica*-like 2	26.9 % (14/52)	7.3 % (14/192)	8.3 % (4.8–13.1 %)
Orchopeas howardi	Squirrel	*B. durdenii*	41.9 % (13/31)	9.3 % (13/140)	11.4 % (6.4–18.2 %)

For *Bartonella* spp. detected in multiple flea samples from a given host species, the flea species, *Bartonella* spp., and multiple infection rate estimates are reported.

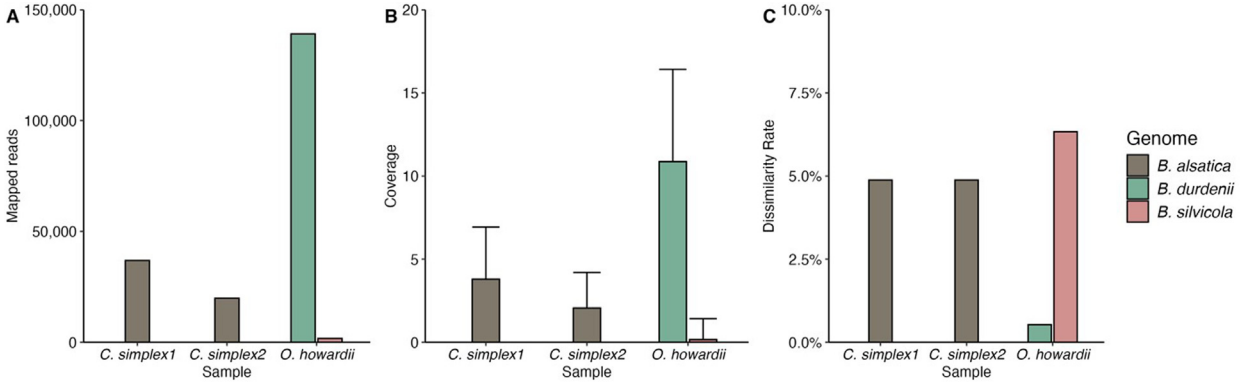


Fig. 3. Illumina read alignment to reference genome(s) utilizing Bowtie2. Number of mapped reads (A), coverage (B), and dissimilarity rate (number of mismatches, insertions, and deletions/number of aligned bases) (C) for three samples submitted for metagenomic sequencing.

Table 4
Overview of assembly metrics, gene prediction, and genome quality evaluation for draft bins associated with flea-associated bacteria from *Orchopeas howardi*.

Identifier	*D62_1*	*D62_2*
Source	*Orchopeas howardi*	*Orchopeas howardi*
Classification	***Wolbachia* sp.**	***Bartonella* sp.**
Total length (bp)	1,125,380	1,628,696
Number of contigs	113	93
Longest contig (bp)	46,782	105,462
Shortest contig (bp)	2,061	2,034
GC content (%)	34.1	38.6
No. of features◇	2,168	3,000
No. of genes ◇	1,065	1,483
No. of rRNAs◇	3	0
No. of tRNAs◇	34	33
No. tmRNAs◇	1	1
Draft Classification (MIMAG)	High-quality draft	Medium-quality draft
Marker lineage*	Rickettsiales	Rhizobiales
Completeness* (%)	95.73	99.0
Contamination* (%)	0.43	0.54
Closest placement^	*Wolbachia* sp. GCF_018141665.1	None
Closest placement average nucleotide identity (ANI)^	96.73	None

◇ Parameters based on Prokka annotation.
* Parameters based on CheckM estimation.
^ Parameters based on GTDB-Tk annotation.

were most often infested with *C. simplex* fleas and *H. leporispalustris* ticks, but were also occasionally host to ticks (*A. americanum, I. scapularis)* classically associated with zoonotic pathogen transmission. Squirrels were host to both *O. howardi* and *C. simplex* fleas, while opossums were host to diverse flea and tick species (*C. simplex, C. felis, D. variabilis, H. leporispalustris, O. howardi).*

In this study, *Bartonella* species were the most common pathogens detected in flea species. *B. durdenii* was most common in *O. howardi* (9.3 % MIR) from squirrels, while *B. alsatica*-like 1 (3.6 % MIR) and *B. alsatica*-like 2 (7.3 % MIR) were common in *C. simplex* from rabbits and squirrels. Other domestic animal or zoonotic *Bartonella* species (*B. henselae, B. koehlerae*-like, and *B. vinsonii* subsp. *berkhoffii*) were each detected in one *C. simplex* pool from different rabbit hosts. *Rickettsia bellii* and *Anaplasma* sp. DNA were amplified from ticks.

Diverse rabbit flea species (*C. simplex, Cediopsylla inaequalis, Spilopsyllus cuniculi,* and *Xenopsylla cunicularis*) are associated with, and assumed to transmit, *B. alsatica* in Europe and the United States (Márquez, 2015; Moore et al., 2023; Sato et al., 2020). Similar to the PCR and metagenomic alignment data reported herein, other reports of *B. alsatica* from the United States display relatively poor alignment with *B. alsatica* isolates from Europe. For example, a *Bartonella* sp. with 96.9 % DNA sequence alignment to *B. alsatica* was obtained from *C. inaequalis* fleas removed from the desert cottontail rabbit (*Syvilagus audubonii*) in Colorado, USA (Sato et al., 2020). Based upon the high error rate (4.88 %) when aligning metagenomic shotgun sequences to the published *B. alsatica* genome, the limited alignment of metagenomic assembled contigs (92.82–93.75 % ANI), and the comparison of *ssrA* or ITS PCR amplicons to *B. alsatica*, we propose these two bacteria (*B. alsatica*-like 1 and *B. alsatica*-like 2) are novel *Bartonella* species, distinct from the *B. alsatica* found in Europe.

In Europe, *B. alsatica* has been associated with human diseases in multiple case reports documenting lymphadenopathy and endocarditis in infected patients (Angelakis et al., 2008; Jeanclaude et al., 2009; Puges et al., 2019; Raoult et al., 2006). As *C. simplex* have been found infesting domestic cats and given the close association of rabbits and squirrels to human developments and domestic animals, further epidemiological and experimental investigation is necessary to understand the role of *C. simplex* in sustaining diverse *Bartonella* species in nature (Moore et al., 2023). The detection of diverse *Bartonella* spp. in *C. simplex* in this study is not unprecedented, as *B. vinsonii* subsp. *berkhoffii* and *Bartonella*

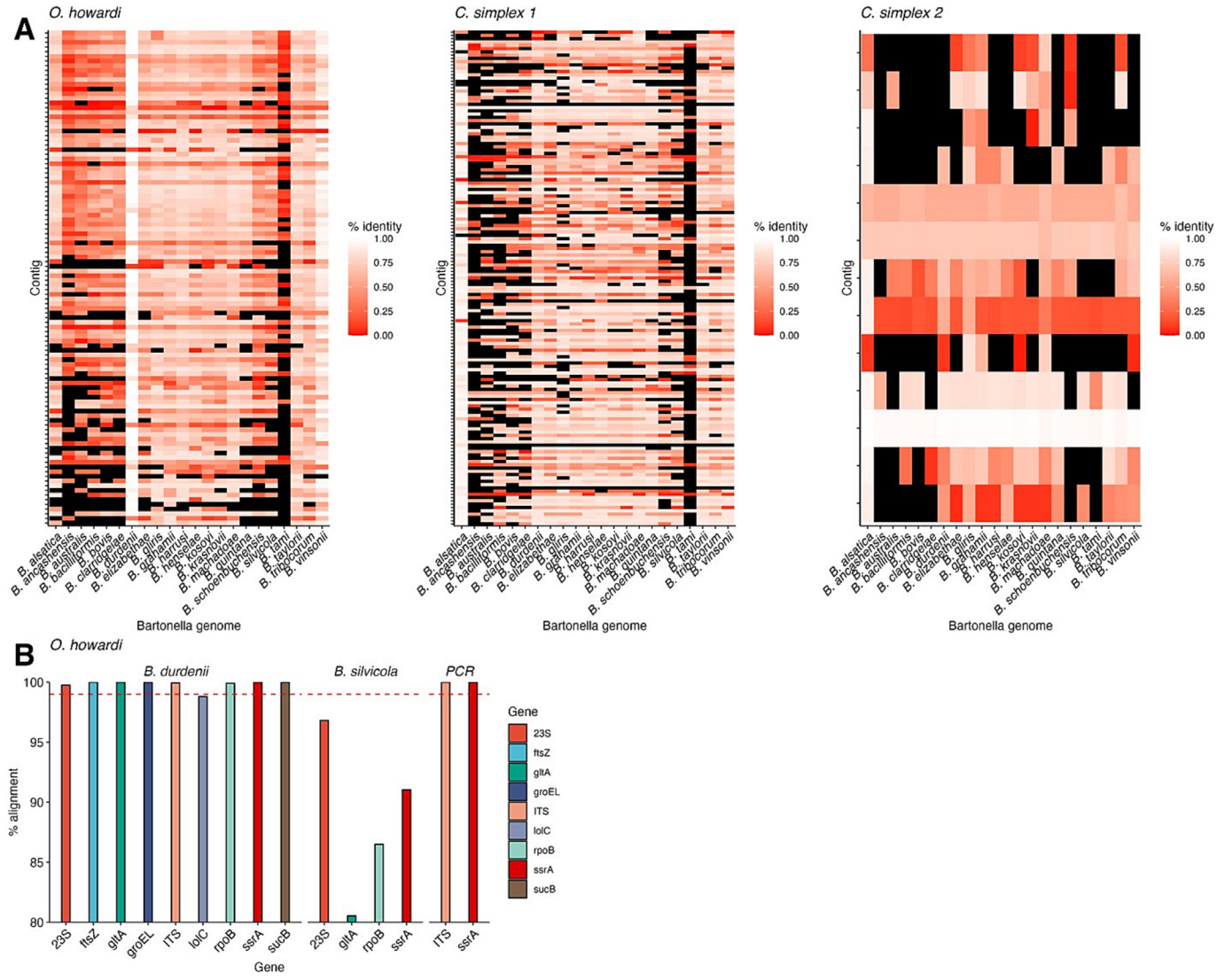
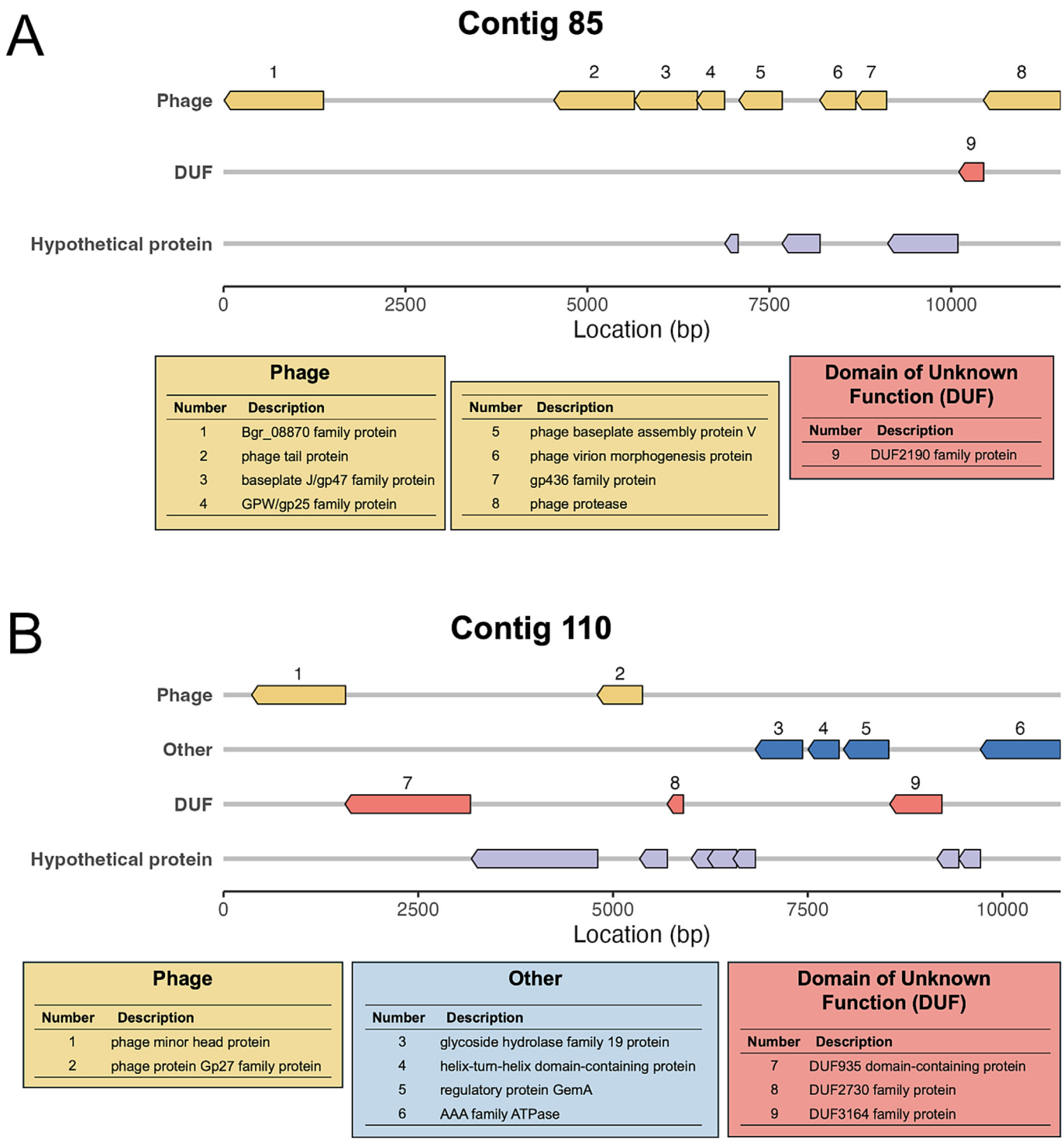


Fig. 4. Alignment of *Bartonella* contigs to *Bartonella* reference genome database and *Bartonella* reference genes. Heatmaps display alignment of various contigs to *Bartonella* reference genomes (A). Longer contigs are at the top, while shorter contigs are at the bottom. A complete lack of alignment is displayed as black. Comparison to select reference genes with >70 % coverage (B) is displayed as the total percentage alignment (y-axis). Alignment is shown to reference genomes and PCR performed in the present manuscript. The red dashed line indicated 99 % alignment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

rochalimae have been detected in *Euhoplopsyllus glacialis* and *C. inaequalis* from *Sylvilagus audubonii* (the desert cottontail rabbit) (Sato et al., 2020). To date, the vector or vectors that transmit *B. vinsonii* subsp. *berkhoffii* have not been clearly defined (Álvarez-Fernández et al., 2018; López-Pérez et al., 2017). The diversity of rabbit and rabbit flea species worldwide presents an intriguing opportunity to study the role of host, vector, or pathogen diversity in the host-vector-pathogen exchange.

Bartonella durdenii was commonly detected in *O. howardi* pools, specifically from squirrels (42 %, 13/31). Originally reported in 2002, *B. durdenii* is closely associated with squirrels and *O. howardi* in the United Kingdom, where both are considered invasive species (Bown et al., 2002). The potential for *B. durdenii* to cause disease in squirrels, domestic animals, or humans is unknown. One case report suggested an unknown squirrel associated *Bartonella* sp. might have contributed to the migratory joint pain, mental decline, and eventual death of one patient (Breitschwerdt et al., 2009). Other *Bartonella* associated with diverse squirrel species, such as *Bartonella washoensis* which utilizes the Eurasian Red Squirrel (*Sciurus vulgaris*) and California Ground Squirrel (*Spermophilus beecheyi*) as reservoir hosts, are known to cause endocarditis or febrile illness in human patients (Bown et al., 2002; Kosoy et al., 2003; Loewenich et al., 2019).

The addition of metagenomic sequencing to further investigate the detection of *Bartonella* in *O. howardi* fleas was critical to accurately identifying *B. durdenii.* The *ssrA* and ITS (16S-23S rRNA) regions targeted for PCR aligned only with *B. silvicola* when searching GenBank with the nucleotide BLAST algorithm due to a lack of *B. durdenii* reference sequences in GenBank. The perfect alignment of the *B. durdenii* and *B. silvicola* ITS sequence is surprising and reinforces the value of amplifying multiple genetic targets. Therefore, if relying only on amplification of a limited number of reference genes, this species would have been misidentified as *B. silvicola.* With the increasing availability and decreasing cost of advanced sequencing technologies, metagenomic sequencing will continue to shed light on the diversity of bacteria, pathogens, and genes in the microbiome of vector species. Metagenomic sequencing also facilitated the detection of phage genes in *B. alsatica*-like 1 that are not present in *B. alsatica* (Accession ID NZ_CP058235.1) (Fig. 5-a-b).



Phage

Number	Description
1	Bgr_08870 family protein
2	phage tail protein
3	baseplate J/gp47 family protein
4	GPW/gp25 family protein
5	phage baseplate assembly protein V
6	phage virion morphogenesis protein
7	gp436 family protein
8	phage protease

Domain of Unknown Function (DUF)

Number	Description
9	DUF2190 family protein

Phage

Number	Description
1	phage minor head protein
2	phage protein Gp27 family protein

Other

Number	Description
3	glycoside hydrolase family 19 protein
4	helix-turn-helix domain-containing protein
5	regulatory protein GemA
6	AAA family ATPase

Domain of Unknown Function (DUF)

Number	Description
7	DUF935 domain-containing protein
8	DUF2730 family protein
9	DUF3164 family protein

Fig. 5. Detection of phage associated genes in *Cediopsylla simplex* 1 sample not found in *Bartonella alsatica*. Annotation of contigs from *C. simplex* sample 1 that align to *Bartonella* spp. but not the selected reference genome (*Bartonella alsatica*), suggesting the presence of a phage in the *Bartonella* sp. from *C. simplex* 1. Graph was created in R utilizing gggenes.

For ticks, bacteria in pathogenic genera were detected only in *H. leporispalustris,* likely due to the small sample size of other collected tick species. The detection of *R. bellii* in *H. leporispalustris* has been previously reported and *R. bellii* DNA has been amplified from jackrabbit (*Lepus californicus*) tissues (Backus et al., 2023; Sánchez-Montes et al., 2020). *Rickettsia bellii* is not considered pathogenic to animals or humans, although the development of eschars in an experimental guinea pig infection model suggests some degree of pathogenicity (Scola et al., 2009).

Detection of an uncultured *Anaplasma* sp. in *H. leporispalustris* was unexpected. Limited reports associate *H. leporispalustris* and *Anaplasma* species (Goethert and Telford, 2003). *Haemaphysalis leporispalustris* was assumed to vector *Anaplasma bovis* to rabbits on Nantucket Island, MA (Goethert and Telford, 2003). Therefore,

further research should determine the association of *H. leporispalustris* with additional *Anaplasma* species, including the cat, dog, horse, and human pathogen, *Anaplasma phagocytophilum* (Schiff et al., 2025; Yabsley et al., 2006).

Limitations associated with this research study included the opportunistic collection of fleas and ticks from wildlife species, which resulted in under sampling from rural areas, limiting the authors from assessing pathogen infection or ectoparasite infestation in these areas. It is also critical to note that the detection of pathogen DNA within a hematophagous ectoparasite species is not sufficient evidence to indicate that the ectoparasite species is a competent host or vector for a given pathogen species. Further research and experimental infection are necessary to determine host and vector competence. Given the limited geographic region sampled, the ability to translate these findings to other geographic locations is unknown. Additionally, as demonstrated by the metagenomic identification of *B. durdenii*, the use of limited phylogenetic marker genes for the assignment of other pathogen species may be insufficient to determine the species, strain, or true phylogenetic alignment.

5. Conclusions

This study documented a diversity of flea and tick species infesting small mammals in Wisconsin, including *C. simplex* and *O. howardi* fleas infesting rabbits, squirrels, and opossums, and *H. leporispalustris* infesting rabbits. These small mammals were also occasional hosts to ticks that bite domestic animals or humans, such as *A. americanum, D. variabilis,* and *I. scapularis.* PCR and/or metagenomic sequencing identified multiple under-studied *Anaplasma, Bartonella,* and *Rickettsia* species associated with small wildlife species. With the addition of metagenomic shotgun sequencing, *B. durdenii* was definitively identified and multiple phage genes found in a *B. alsatica*-like bacterium that are not found in *B. alsatica.* Due to the continued encroachment of humans into lands occupied by wildlife species, domestic animals and humans will continue to be exposed to wildlife associated ectoparasites and pathogens. Further research is necessary to explore the pathogenicity of the *Bartonella* spp. and unnamed *Anaplasma* sp. associated with wild small mammal species. Additionally, comparative genomic and pathogenicity studies should shed light on the role of sylvatic reservoirs in the maintenance of domestic animal or human associated *Bartonella* species identified in *C. simplex (B. henselae, B. koehlerae,* and *B. vinsonii* subsp. *berkhoffii).*

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CRediT authorship contribution statement

Charlotte O. Moore: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Caroline V. Andrews:** Writing – review & editing, Investigation, Data curation. **Erin M. Lemley:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Conceptualization. **Michelli Inacio Gonçalves Funnicelli:** Writing – review & editing, Software, Methodology, Formal analysis. **Marcos Rogério André:** Writing – review & editing, Software, Methodology, Formal analysis. **Edward B. Breitschwerdt:** Writing – review & editing, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization. **Erin Lashnits:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2025.08.004.

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