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Diversity and host specificity of *Borrelia burgdorferi*'s outer surface protein C (*ospC*) alleles in synanthropic mammals, with a notable *ospC* allele U absence from mixed infections

Scarlet A. Shifflett,[1] Francisco C. Ferreira,[2] Julia González,[2] Alvaro Toledo,[2] Dina M. Fonseca,[2] Vincenzo A. Ellis[1]

AUTHOR AFFILIATIONS See affiliation list on p. 12.

ABSTRACT Interactions among pathogen genotypes that vary in host specificity may affect overall transmission dynamics in multi-host systems. *Borrelia burgdorferi*, a bacterium that causes Lyme disease, is typically transmitted among wildlife by *Ixodes* ticks. Despite the existence of many alleles of *B. burgdorferi*'s *sensu stricto* outer surface protein C (*ospC*) gene, most human infections are caused by a small number of *ospC* alleles ["human infectious alleles" (HIAs)], suggesting variation in host specificity associated with *ospC*. To characterize the wildlife host association of *B. burgdorferi*'s *ospC* alleles, we used metagenomics to sequence *ospC* alleles from 68 infected individuals belonging to eight mammalian species trapped at three sites in suburban New Brunswick, New Jersey (USA). We found that multiple allele ("mixed") infections were common. HIAs were most common in mice (*Peromyscus* spp.) and only one HIA was detected at a site where mice were rarely captured. *ospC* allele U was exclusively found in chipmunks (*Tamias striatus*), and although a significant number of different alleles were observed in chipmunks, including HIAs, allele U never co-occurred with other alleles in mixed infections. Our results suggest that allele U may be excluding other alleles, thereby reducing the capacity of chipmunks to act as reservoirs for HIAs.

KEYWORDS host specificity, pathogen interactions, *Peromyscus*, reservoir hosts, *Tamias striatus*

Host specificity (i.e., the diversity of host species a pathogen infects) is an important pathogen phenotype. Specialist pathogens infect a single host species or a few closely related host species while generalist pathogens infect multiple host species. Pathogen genotypes may exclude others from multiple-genotype (mixed) infections (1, 2). The exclusion of generalist pathogens by a specialist pathogen (3) may reduce the transmission rate of the generalist pathogens to other host species. Genotype exclusion may be direct (e.g., through resource competition) or indirect if antibodies to one pathogen genotype are effective at blocking infection by other pathogen genotypes, but not vice versa ["asymmetric cross reactivity" (4)]. In the case of human infections, specialists circulating among wildlife reservoirs could lower human infection risk by reducing the overall population sizes of generalists that can infect humans. Such a phenomenon, while theoretically possible, requires empirical support to prove that it exists in nature.

Lyme disease is caused by the bacterium *Borrelia burgdorferi* (in this study, *B. burgdorferi sensu stricto*; hereafter *B. burgdorferi*) and is the most common vector-borne infectious disease in the United States (5). *B. burgdorferi* is transmitted to vertebrate hosts, including humans, by blacklegged ticks (*Ixodes scapularis*) in the upper Midwest and northeastern United States and western blacklegged ticks (*Ixodes pacificus*) in the western United States (6). *B. burgdorferi* is maintained in a zoonotic life cycle between

Editor Guy H. Palmer, Washington State University, Pullman, Washington, USA

Address correspondence to Vincenzo A. Ellis, vaellis@udel.edu.

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Ixodes ticks and vertebrate hosts. Common mammalian hosts for *B. burgdorferi* in the upper Midwest and northeastern U.S. include deer mice (*Peromyscus maniculatus*), white-footed mice (*Peromyscus leucopus*), eastern chipmunks (*Tamias striatus*), masked shrews (*Sorex cinereus*), Northern short-tailed shrews (*Blarina brevicauda*), and eastern meadow voles [*Microtus pennsylvanicus*; (7–11)]. The white-footed mouse is considered the main reservoir host because they frequently host the immature stages of *I. scapularis* and infected mice have a high probability of transmitting the bacterium to feeding blacklegged ticks (8, 12, 13).

The most polymorphic gene in the *B. burgdorferi* genome is the outer surface protein C gene (*ospC*) (14–16), and it is expressed when *B. burgdorferi* migrates from the tick's midgut to its salivary glands while feeding on a vertebrate host (17, 18). It is targeted by the vertebrate host's immune system (19, 20) and is required to establish infection in vertebrate hosts (20–22). Infected hosts develop antibodies to *ospC* and are therefore unlikely to be reinfected by bacteria with the same *ospC* allele (23, 24). Moreover, *ospC*-mediated asymmetric cross-reactivity has been shown in *Borrelia afzelii*, a species in the Lyme disease group, and this cross-reactivity depends on the host species genotype (4). Isolates of *B. burgdorferi* belonging to the same *ospC* "major allele" group (hereafter, "alleles") can differ by up to 2% in their *ospC* gene sequences, but genetic distances between alleles are greater than 8% (15, 25). There are at least 28 distinct *ospC* alleles (26) which are generally named with single letters (i.e., alleles A, B, C, etc.).

While *B. burgdorferi* infects many vertebrate species, alleles vary in their host specificities, with some alleles infecting multiple host species and others restricted to one or a few host species (27). Moreover, only a subset of *B. burgdorferi* carrying *ospC* alleles (alleles A, B, K, and I) commonly causes disseminated infections in humans [i.e., infections that spread from the skin to the blood and cerebrospinal fluid (28, 29); these are known as human infectious alleles, hereafter, "HIAs"]. Brisson and Dykhuizen (27) examined the distribution of *ospC* alleles in four small mammal species in Millbrook, NY, and showed that different host species share some alleles while other alleles are species-specific. Among the hosts analyzed, eastern chipmunks harbored the most alleles (14), followed by the white-footed mouse (12), northern short-tailed shrew (11), and the eastern gray squirrel (8). Half of the 14 alleles identified infected all four hosts, but two alleles (alleles L and M) infected only two hosts each, and one allele (allele U) only infected eastern chipmunks. Based on surveys of ticks in the environment, all four host species in the study would likely have been exposed to all 14 *ospC* alleles, suggesting that differences in allele host specificity were not an artifact of incomplete sampling and instead represented actual differences in host specificity (27).

Host specificity of *B. burgdorferi ospC* alleles has received limited attention since Brisson and Dykhuizen (27), and it remains to be determined whether the results of their study hold for other locations in the range of *B. burgdorferi*. Furthermore, *ospC* alleles exhibit non-random patterns of association in ticks (30), but little is known about their interactions in vertebrate hosts. Specifically, it is unclear whether interactions among *ospC* alleles of differing host specificities can have impacts on human infection risk, for example, through the exclusion of HIAs by specialists like allele U. To address these questions, we quantified *ospC* diversity and prevalence in an extensive collection of small- and medium-sized mammals from three sub-urban sites in New Jersey. We tested (i) if any *ospC* alleles were more specialized or generalized than expected by chance, (ii) if mammalian species hosted a higher or lower diversity of *ospC* alleles than expected by chance, and (iii) if alleles are randomly associated in mixed infections. We also quantified the prevalence of *B. burgdorferi* among mammalian host species. These investigations allowed us to better understand host specificity in this widespread and common vector-borne disease.

MATERIALS AND METHODS

Sample collection

We trapped 249 individuals of 11 small and medium mammal species between May and September 2021 at three locations on the Rutgers University campus in New Brunswick, New Jersey, using Sherman and Tomahawk live traps. The study sites were Goat Farm (40.48 latitude, −74.44 longitude), Rutgers Gardens (40.47, −74.42), and University Inn (40.48, −74.43). We collected a 2- mm ear biopsy from individuals of 10 of the 11 species in the field using a sterile ear punch (Fisherbrand Animal Ear Punch). For Northern short-tailed shrews, we removed the tip of their tails using sterilized surgical scissors. We stored each biopsy in 200 µL of RNAlater (Thermo Fisher Scientific, Waltham, MA, USA) at 4°C for 1 to 3 days, followed by long-term storage at −20°C until DNA extraction. Trapping effort was similar across all sites although trapping effort for small mammals was higher at the University Inn than at the other two sites (31).

The three sampling sites are in urbanized areas of New Jersey. Natural vegetation at the sites was primarily deciduous forest dominated by oak trees (*Quercus* spp.) and huckleberry shrubs (*Gaylussacia* spp.). Very few small mammals were captured at the University Inn site, despite more intense sampling efforts (31), providing a natural experiment to test the dependency of allele diversity and composition on host species diversity and composition.

Molecular screening of mammal tissue for *B. burgdorferi* and *ospC* sequencing

RNAlater was first removed to extract DNA from each sample, and the ear tissue was rinsed twice with 400 µL of 1× phosphate buffered saline solution (PBS). Next, five 2.8 mm stainless steel beads and 200 µL of 1× PBS were added to the sample tube. Tubes were transferred to a TissueLyser (Qiagen, Germantown, MD, USA), and ear samples were disrupted for 2 min at the maximum oscillation frequency (30 Hz). This step was repeated until the mixture was homogeneous. Half of the homogenate (100 µL) was stored in a −80°C freezer for future use, and DNA was extracted from the other half using a DNeasy Blood and Tissue kit (Qiagen, Germantown, MD, USA) following the manufacturer's protocol.

We tested each sample in duplicate for *B. burgdorferi* using a quantitative PCR (qPCR) protocol (32). Individuals were considered positive for *B. burgdorferi* if at least one replicate tested positive. The qPCR protocol included one primer set and one probe to target *B. burgdorferi*. We combined 10 µL of TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), 0.18 µL of a 10 µM solution of each primer (fliD-Fwd and fliD-Rev), 0.05 µL of a 10 µM solution of the probe (fliD), and 7.18 µL of ultrapure water in each reaction. Primer and probe sequences were taken from Hojgaard et al. (32). Each well in the qPCR plate was brought up to 20 µL with the addition of 2 µL of DNA extract. The qPCR thermal profile protocol was 50°C for 2 min followed by 2 min at 95°C for the initial stage, followed by 40 cycles of 95°C for 1 second and 60°C for 20 seconds. The qPCR reactions were performed on an Applied Biosystems QuantStudio 3 Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA, USA). *B. burgdorferi* prevalence was determined by qPCR infection status.

The *ospC* gene of *B. burgdorferi* was amplified for all positive qPCR samples using a PCR protocol developed by Di et al. (33). The 25 µL PCR reaction included 12.5 µL of master mix (DreamTaq Hot Start Master Mix 2X, Thermo Fisher Scientific, Waltham, MA, USA), 1.25 µL of a 10 µM solution of each primer (Oc-Fwd and Oc-Rev), 7 µL of ultrapure water, and 3 µL of DNA template. The PCR thermal profile protocol used one cycle of initial denaturation (2 min at 95°C), followed by 35 cycles of denaturation (30 s at 95°C), annealing (30 s at 52°C), and extension (1 min at 72°C), and a final extension at 72°C for 10 min. All PCRs and qPCRs included at least one negative control (ultrapure water) and two positive controls (DNA from a *B. burgdorferi*-infected tick and white-footed mouse). We visualized PCR amplification through gel electrophoresis with 1.5% agarose gels.

We used the nested PCR protocol of Bunikis et al. (34) to amplify *ospC* in samples that did not amplify with the Di et al. (33) protocol, but were infected according to qPCR. For the Bunikis et al. (34) PCR, our first (i.e., outer) PCR was a 25 µL reaction containing 12.5 µL of master mix (DreamTaq Hot Start Master Mix 2X, Thermo Fisher Scientific, Waltham, MA, USA), 1.25 µL of a 10 µM solution of each primer (*ospC*_F and *ospC*_R), 7 µL of ultrapure water, and 3 µL of DNA extract. The thermal profile for the outer PCR reaction was the same as the single-round PCR of Di et al. (33) described above. The nested (i.e., inner) 25 µL reaction contained 12.5 µL of master mix, 1.25 µL of a 10 µM solution of each primer (*ospC*_Fn and *ospC*_Rn), 8 µL of ultrapure water, and 2 µL of PCR amplicon from the first reaction. The PCR thermal profile for the nested reaction was one cycle of initial denaturation (2 min at 95°C), followed by 30 cycles of denaturation (30 s at 95°C), annealing (30 s at 52°C), and extension (1 min at 72°C), and a final extension at 72°C for 10 min. All PCRs included at least one negative control (ultrapure water; also, negative controls from the first reaction were run in the nested reaction and confirmed negative in the gel) and two positive controls and visualized as before.

We cleaned the PCR product of amplified samples using AMPure XP beads (Beckman Coulter, Horsham, PA, USA), and diluted them to 0.2 ng/µL to build Illumina libraries with the Illumina Nextera XT DNA Library Prep kit (Illumina, San Diego, CA, USA) using the i7 and i5 unique dual index adapters from the Nextera XT Index kit (Illumina, San Diego, CA, USA). Libraries were cleaned with AMPure XP beads, pooled in equimolar ratios, and sent to the University of Delaware's Genotyping and Sequencing Center at the Delaware Biotechnology Institute for sequencing. Libraries were sequenced on an Illumina Miseq using a 500-cycle nano sequencing kit (Illumina, San Diego, CA, USA). The sequencing was paired-end with 251 cycles per read.

Bioinformatic analysis

ospC alleles were identified from the short-read sequences following the protocol described by Di et al. (33) with modifications as presented in Shifflett et al. (35). Briefly, reads were trimmed, checked for quality control, and mapped to the FASTA *ospC* reference file in Di et al. (33). Depth of coverage was then calculated at each base pair position across the reference sequences. Alleles were considered present if all nucleotide positions were sequenced to a depth of coverage of at least 5× between nucleotide positions 200 and 550 of the reference (the more variable gene region). We also quantified the proportion of unmapped reads in each sample using SAMtools stats (36). One sample (R3) had reads incompletely mapping to several alleles, suggesting a possible novel allele formed through recombination (37); however, we could not recover a sequence using Sanger sequencing. Five additional samples showed unrecovered alleles due to sequence reads not mapping to the reference file. Samples with unresolved alleles were not included in the *ospC* analyses.

Statistical analyses

We removed recaptures from all *ospC* and prevalence analyses. We also removed the single captured eastern cottontail (*Sylvilagus floridanus*) from all *ospC* analyses. While the majority of *Peromyscus* mice were likely white-footed mice (*P. leucopus*), we refer to them as *Peromyscus* spp. to account for potential misidentification between the white-footed mouse and the deer mouse (*P. maniculatus*). We tested whether *B. burgdorferi* prevalence varied across sites using a Fisher's exact test and across species using a Pearson's χ^2 test in R v. 4.2.1 (38).

We calculated the host specificity of *ospC* alleles as (i) the number and (ii) the Gini-Simpson diversity index of host species of each allele infected. We also quantified the diversity of *ospC* alleles found in hosts by calculating (i) the number and (ii) the Gini-Simpson diversity index of *ospC* alleles that infected each host species. We calculated the Gini-Simpson index using the diversity function (with argument "index = simpson") in the R package vegan v.2.6-2 (39). To test if allele host specificity and allele diversity per host differed from a random expectation, we randomly shuffled *ospC* alleles

among infected individuals of all host species 9,999 times. For each randomization, we calculated a randomized allele host specificity and allele diversity per host species and used those values to create a random distribution to compare with each measured value. We then calculated two-tailed *P*-values for each measured value to determine if they were more extreme than expected by chance.

We classified individuals with mixed infections as those having more than one sequenced *ospC* allele. We used the cor.test function in R v. 4.2.1 (38) to correlate the frequency of *ospC* alleles found in mixed infections with allele frequencies found in single infections. A positive correlation would be consistent with the random association of alleles in mixed infections (35). We also calculated the frequency of mixed infections across host species.

We used the Chao1 estimator (40) to determine the expected number of *ospC* alleles across all sampling sites and the number of alleles expected to infect each host species. We used the estimateR function in the R package vegan v.2.6-2 (39) based on allele frequencies to calculate the Chao1 estimator.

RESULTS

Prevalence

We tested 249 individual hosts (272 including recaptures) across 11 species for *B. burgdorferi* and found 81 infections by qPCR; after removing recaptures (23 individuals), there were 68 qPCR infections (27.3%). Three host species were not infected despite substantial sampling (Virginia opossums, *Didelphis virginiana*, *n* = 11; groundhogs, *Marmota monax*, *n* = 8; raccoons, *Procyon lotor*, *n* = 32; Fig. 1; the lone eastern cottontail was also not infected) despite hosting nymphal blacklegged ticks potentially infected with *B. burgdorferi*. *B. burgdorferi* prevalence varied across host species with sample sizes greater than five ($\chi^2 = 46.99$, df = 8, $P < 0.005$), even when removing the three uninfected

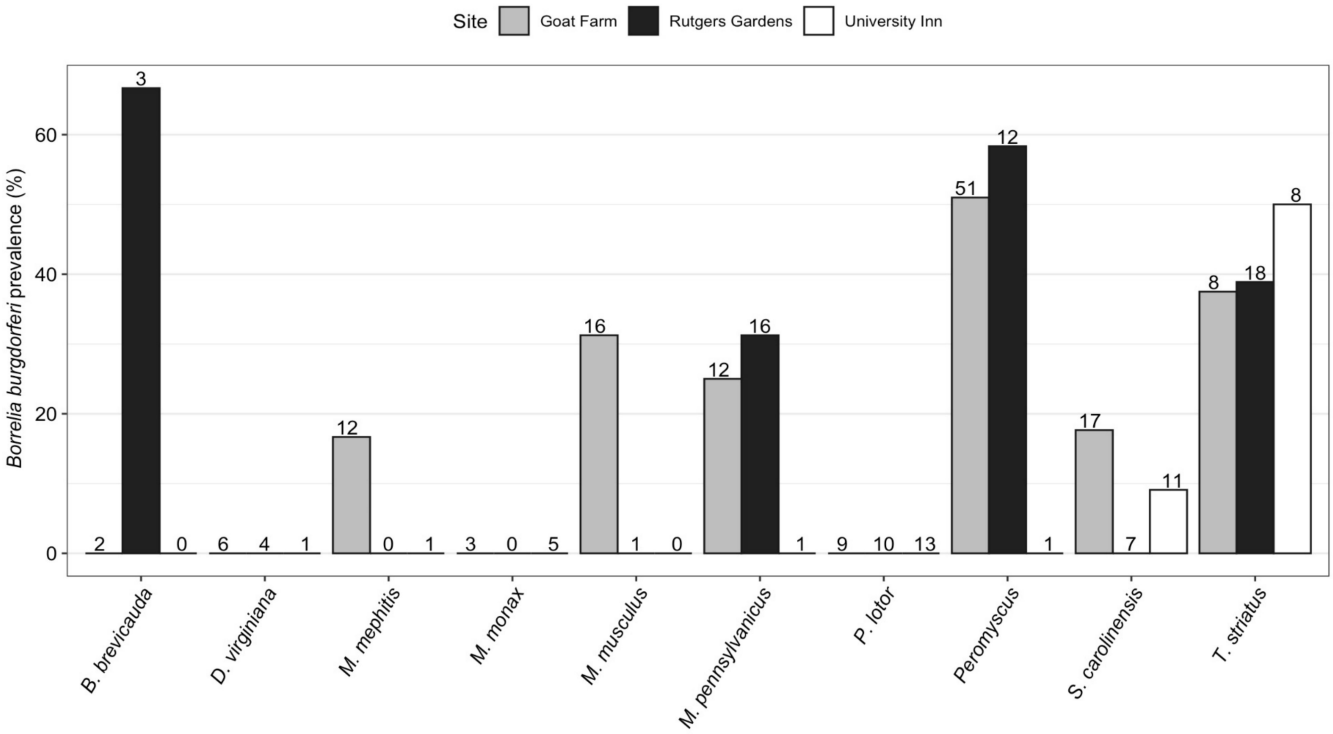

FIG 1 *B. burgdorferi* prevalence (as determined by qPCR) across sites (Goat Farm, Rutgers Gardens, University Inn) and host species (*B. brevicauda*, northern short-tailed shrew; *D. virginiana*, Virginia opossum; *Mephitis mephitis*, striped skunk; *M. monax*, groundhog; *Mus musculus*, house mouse; *M. pennsylvanicus*, eastern meadow vole; *P. lotor*, raccoon; *Peromyscus*, white-footed/deer mice; *Sciurus carolinensis*, eastern gray squirrel; *T. striatus*, eastern chipmunk). The number of captured individuals of each species at each site is shown above its corresponding bar.

species (χ^2 = 20.1, df = 5, P < 0.005), and reached the highest levels in *Peromyscus* spp. (51.6%) and eastern chipmunks (41.2%). Among recaptured individuals, those infected with *B. burgdorferi* were also infected at subsequent captures. Furthermore, three uninfected individuals were infected upon recapture a month later; 10 individuals remained uninfected between captures.

B. burgdorferi prevalence among all individuals, irrespective of host species, varied among the three sites (Fisher's exact test P = 0.047). Prevalence was highest at the Goat Farm site (30.9%), which also had the highest sample size (n = 136; Table S1). University Inn, the site with almost no *Peromyscus* spp. or eastern meadow voles (Fig. 1), had the smallest sample size and prevalence (n = 41, 12.2%). Two host species (eastern chipmunks and *Peromyscus* spp.) had sufficient sample sizes at more than one site to compare *B. burgdorferi* prevalence. However, prevalence did not vary among sites for either species (eastern chipmunks, n = 3 sites in the comparison, Fisher's exact test P = 0.901; *Peromyscus* spp., n = 2, Fisher's exact test P = 0.752).

ospC allele diversity

We sequenced *ospC* in 70 infected individuals (13 recaptures) and identified 14 alleles across six species and three sites (Fig. 2). HIAs were found at the greatest relative frequencies (Fig. 2; Tables 1 and S1), with allele B occurring in 25 individuals (27.8% of all infected individuals), K in 17 (18.9%), and A in 15 (16.7%). Alleles B, K, and A (all HIAs) made up the majority of alleles at Rutgers Garden (62.5% of all alleles) and Goat Farm (68.5%; Table S1). University Inn had the lowest allele diversity of the sites (only alleles U, T, and I were found), with one HIA found (Table 1). University Inn also had only one individual *Peromyscus* spp., which was sampled twice 33 days apart. We estimated the number of *ospC* alleles across the study sites using the Chao1 estimator as 14.6 ± 1.18 s.e., suggesting we recovered almost all (14/14.6) of the allele diversity.

While eastern chipmunks had half the sample size (14 infected individuals) of *Peromyscus* spp. (28 infected individuals), they were infected with more unique *ospC* alleles (12) than *Peromyscus* spp. (8). The eastern meadow vole had six infected individuals with five unique *ospC* alleles. Eastern gray squirrels showed the lowest number of unique *ospC* alleles (1), which was likely due to only finding two infected individuals. The striped skunk had three *ospC* alleles (B, A, and C) in two infections.

Host specificity of *ospC* alleles and diversity of *ospC* alleles in host species

Host specificity did not differ from a random expectation for any allele except U, which was more specialized than expected by chance. Allele U only infected eastern

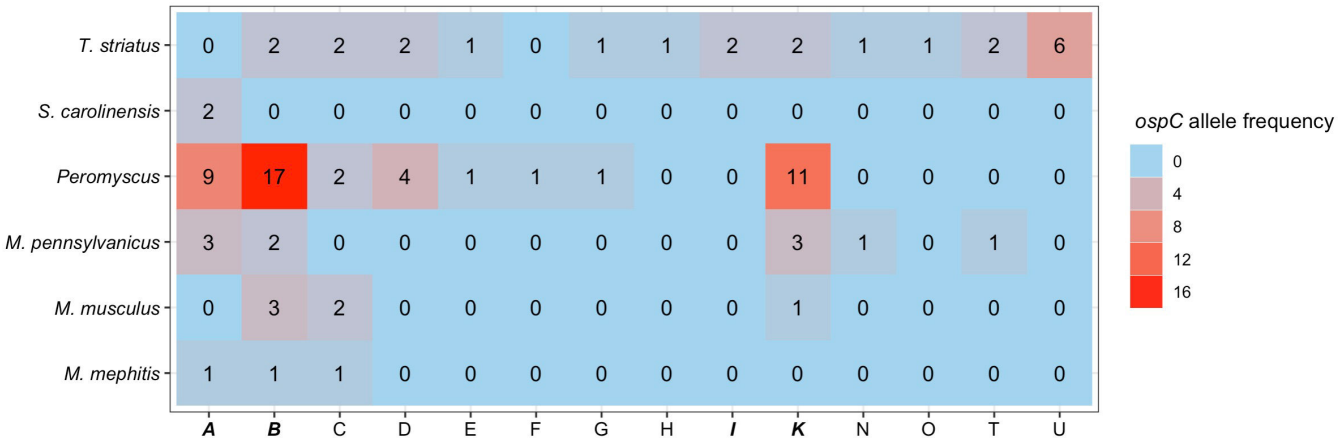

FIG 2 Heatmap showing *ospC* allele frequencies across host species (*T. striatus*, eastern chipmunk; *S. carolinensis*, eastern gray squirrel; *Peromyscus*, white-footed/deer mice; *M. pennsylvanicus*, eastern meadow vole; *M. musculus*, house mouse; *M. mephitis*, stripped skunk). Higher allele frequencies are redder and lower frequencies are bluer. Human infectious alleles (A, B, I, and K) are italicized and in bold font.

TABLE 1 Distribution of *ospC* alleles across sites[a]

Site	Sample size	qPCR infections	*B*	*K*	*A*	C	D	U	T	E	G	*I*	N	F	H	O
Goat Farm	136	42	***16***	***10***	***11***	6	5	1	0	1	1	***0***	0	1	1	1
Rutgers Garden	71	21	***9***	***7***	***4***	1	1	3	2	1	1	***1***	2	0	0	0
University Inn	41	5	***0***	***0***	***0***	0	0	2	1	0	0	***1***	0	0	0	0

[a]The total number of individuals sampled (all species) and the number of individuals infected with *B. burgdorferi* as determined by qPCR are shown for each site. The number of each unique *ospC* alleles found in each site is also shown. Human infectious alleles (A, B, I, and K) are italicized.

chipmunks (Fig. 2) but was expected to infect about three host species by chance (Table 2). The frequencies of alleles F, H, and O were too low (one of each) for those alleles to be included in the analysis. We repeated this analysis using the Gini-Simpson index instead of number of host species per allele and found the same results (Table S2).

While eastern chipmunks appeared to be the only host species infected by a specialized *ospC* allele (U), they also were infected with more *ospC* alleles than expected by chance (12 alleles observed, *ca.* nine expected; Table 3). Furthermore, the species with the highest prevalence (*Peromyscus* spp.) was infected by fewer alleles than expected by chance (eight observed, *ca.* 11 expected). The number of alleles infecting the other host species did not differ from a random expectation (Table 3). We repeated this analysis using the Gini-Simpson index instead of number of *ospC* alleles per host species with similar results (Table S3).

Mixed infections

After removing recaptures, leaving 57 infected hosts with recovered *ospC* alleles, 18 (31.6%) had mixed infections (i.e., more than one allele present). The mean number of alleles present in mixed infections (also referred to as "allelic multiplicity") was 2.83 ± 1.29 SD with two individuals (a *Peromyscus* spp. and an eastern chipmunk) each having six alleles (Fig. 3). Most mixed infections occurred at Goat Farm where 12 individuals had more than one allele and infections with two alleles occurred most frequently. Similarly, Rutgers Garden had six individuals with mixed infections ranging from two to six alleles per infection. University Inn did not have mixed infections. Overall, *Peromyscus* spp. had the greatest number of mixed infections (11) followed by eastern chipmunks (3) and eastern meadow voles (2), but there was not a significant difference in mixed infection frequency among species with a sample size of at least five infections (Fisher's exact test $P = 0.659$). We did not find any mixed infections with more than two alleles in striped skunks or in house mice (Fig. 3). The mean number of *ospC* alleles within mixed infections was greatest in eastern chipmunks (4.00 ± 1.00 s.e.), followed by eastern meadow voles (3.00 ± 1.00 s.e.) and *Peromyscus* spp. (2.64 ± 0.36 s.e.). The relative frequency of *ospC* alleles in single infections was positively correlated with the relative frequency of alleles in mixed infections ($r = 0.71$, $P = 0.005$; Fig. S1). However, allele U occurred in 15.4% of single infections but did not occur in any mixed infections and appeared as an outlier in the analysis (Fig. S1). Thus, while eastern chipmunks had the highest average number of alleles per mixed infection compared to the other hosts, they had far fewer mixed

TABLE 2 Observed and expected host specificity of *ospC* alleles[a]

	A	*B*	C	D	E	G	*I*	*K*	N	T	U
Observed	***4***	***5***	4	2	2	2	***1***	***4***	2	2	1
Mean expected	***4.26***	***4.94***	3.22	3.02	1.67	1.67	***1.66***	***4.42***	1.66	2.13	3.02
SD expected	***0.85***	***0.77***	0.83	0.81	0.47	0.47	***0.47***	***0.84***	0.47	0.64	0.81
P-value	***1***	***1***	0.70	0.52	1	1	***0.67***	***1***	1	1	0.03

[a]Host specificity ("Observed") was quantified as the number of host species an *ospC* allele infected. The number of host species an allele was expected to infect is the mean host specificity across 9,999 randomizations ("Mean expected"); the standard deviation of the mean is also presented ("SD expected") and the *P*-value indicating whether the observed host specificity differed from the random expectation. Human infectious alleles (A, B, I, and K) are italicized.

TABLE 3 Observed and expected *ospC* allele diversity in infected vertebrate host species (*M. mephitis*, striped skunk; *M. pennsylvanicus*, eastern meadow vole; *M. musculus*, house mouse; *Peromyscus*, white-footed/deer mice; *S. carolinensis*, eastern gray squirrel; *T. striatus*, eastern chipmunk)[a]

	M. mephitis	*M. pennsylvanicus*	*M. musculus*	*Peromyscus* spp.	*S. carolinensis*	*T. striatus*
Observed	3	5	3	8	1	12
Mean expected	2.59	5.82	4.26	11.44	1.85	8.68
SD expected	0.54	1.14	0.91	1.19	0.36	1.32
P-value	1	0.78	0.39	0.01	0.30	0.03

[a]Allele diversity ("Observed") was quantified as the number of unique *ospC* alleles found infecting each host species. The expected number of alleles was found by averaging the expected values across 9,999 randomizations ("Mean expected"); the standard deviation of the mean is also presented ("SD expected") and the *P*-value indicating whether the *ospC* allele diversity in a host species differed from the random expectation.

infections than *Peromyscus* spp., because allele U, the allele that is specialized on eastern chipmunks and is relatively common, did not occur in mixed infections (Table 4; Fig. S1).

Recapture infection dynamics

We define recaptures in this study as individuals who were captured at least 20 days after their previous capture. Two recaptures maintained the same *ospC* allele between captures (Fig. 4). For example, one *Peromyscus* spp. was infected with allele A both in August and September. The only recaptured and infected eastern chipmunk was infected with allele U in July and September. However, several recaptured individuals had dynamic *ospC* allele compositions (Fig. 4). Some *ospC* alleles may also have been undetected at some points. For example, one *Peromyscus* spp. was captured four times and was infected by allele A at the first, third, and fourth captures, but not at the second capture.

DISCUSSION

The prevalence of *B. burgdorferi* varied among host species (Fig. 1). Furthermore, we identified 14 unique *ospC* alleles among the infections (Fig. 2), approximately the full diversity of alleles expected in the sample. While the host specificity of most alleles did not differ from a random expectation, allele U (a non-HIA) appeared specialized

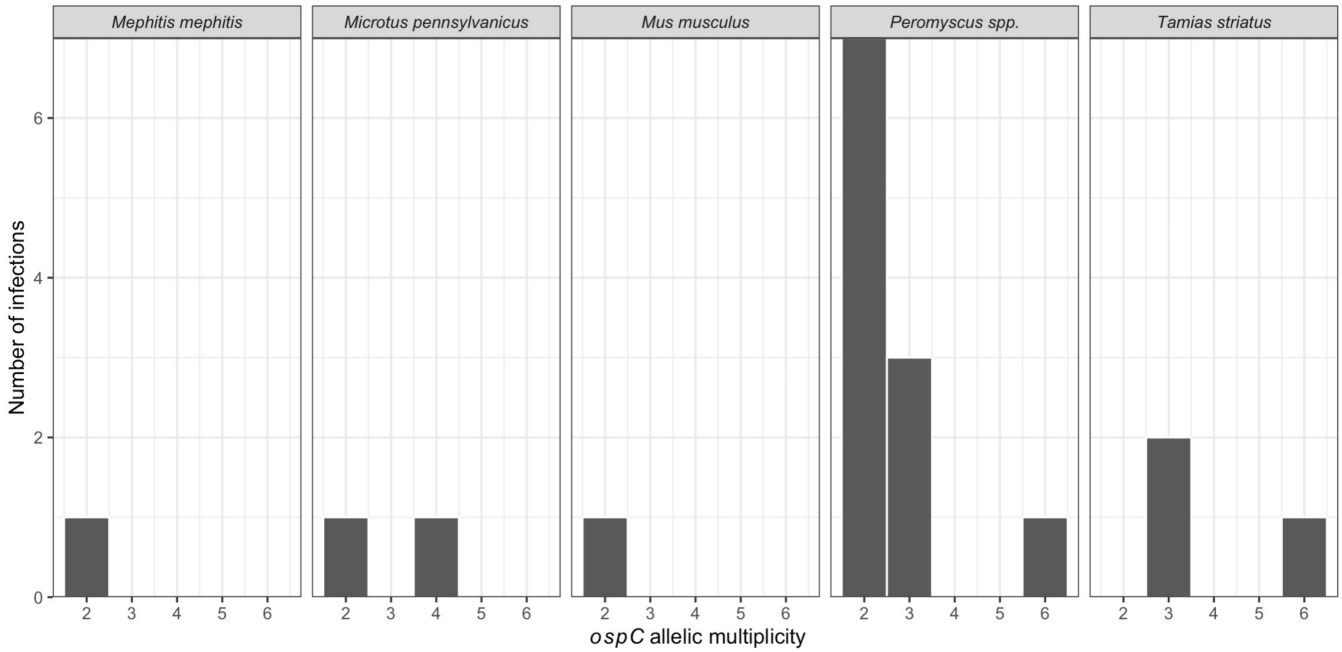

FIG 3 Frequency of mixed allele infections by host species (*M. mephitis*, stripped skunk; *M. pennsylvanicus*, eastern meadow vole; *M. musculus*, house mouse; *Peromyscus spp.*, white-footed/deer mice; *T. striatus*, eastern chipmunk). The number of *ospC* alleles within a mixed infection is represented by the x-axis ("*ospC* allelic multiplicity") and the frequencies are shown on the y-axis.

TABLE 4 *ospC* allele composition in infected eastern chipmunks (*T. striatus*) separated by site[a]

Site	ID	U	*B*	C	D	*I*	*K*	T	E	G	H	N	O	*A*	Total unique alleles
Goat Farm	R123	0	***0***	0	0	***0***	***0***	0	0	0	0	0	1	***0***	1
	R131	1	***0***	0	0	***0***	***0***	0	0	0	0	0	0	***0***	1
	R193	0	***0***	1	1	***0***	***0***	0	0	0	1	0	0	***0***	3
Rutgers Gardens	R166	0	***1***	1	0	***0***	***1***	0	0	0	0	0	0	***0***	3
	R176	1	***0***	0	0	***0***	***0***	0	0	0	0	0	0	***0***	1
	R262	0	***1***	0	1	***1***	***1***	0	0	1	0	1	0	***0***	6
	R72	1	***0***	0	0	***0***	***0***	0	0	0	0	0	0	***0***	1
	R88	0	***0***	0	0	***0***	***0***	0	1	0	0	0	0	***0***	1
	R89	1	***0***	0	0	***0***	***0***	0	0	0	0	0	0	***0***	1
	R90	0	***0***	0	0	***0***	***0***	1	0	0	0	0	0	***0***	1
University Inn	R147	0	***0***	0	0	***1***	***0***	0	0	0	0	0	0	***0***	1
	R148	0	***0***	0	0	***0***	***0***	1	0	0	0	0	0	***0***	1
	R36	1	***0***	0	0	***0***	***0***	0	0	0	0	0	0	***0***	1
	R58	1	***0***	0	0	***0***	***0***	0	0	0	0	0	0	***0***	1

[a]Unique identification numbers associated with each eastern chipmunk individual (ID) are presented. *ospC* alleles appear in descending order according to their relative frequencies in eastern chipmunks. The total number of unique *ospC* alleles found in each individual and each site are shown. Human infectious alleles (A, B, I, and K) are italicized.

on eastern chipmunks, as shown previously (27). Eastern chipmunks were infected with more alleles than expected by chance, while *Peromyscus* spp., which were most frequently infected by HIAs, were infected by fewer alleles than expected by chance (Table 3). Of all infections with recovered *ospC* alleles, *ca*. 32% were mixed allele infections, and the frequency at which alleles were found in mixed infections was positively correlated with the frequency at which those alleles were found in single infections (Fig. S1), consistent with the random assembly of mixed infections. However, despite the high diversity of *ospC* alleles infecting eastern chipmunks, the specialized allele U only occurred in single infections, suggesting it may exclude other alleles from mixed infections, plausibly through indirect mechanisms involving the host immune system. If this is true, allele U (or alleles of other genes in linkage disequilibrium with allele U) may reduce the overall frequency of HIAs in areas that have eastern chipmunks, and especially in areas where eastern chipmunks are the main reservoir of *B. burgdorferi*, as we observed in University Inn.

Competitive exclusion between coinfecting parasites has been demonstrated in multiple disease systems (41–46). Within the Lyme disease system, Devevey et al. (43) investigated experimental mixed infections between *ospC* alleles A, K, and N in mice and found the *ospC* allele causing the initial infection to have the greatest fitness, regardless of identity, suggesting a "priority effect." Furthermore, they found no evidence of cross-reactivity of *ospC* specific antibodies (43) and hypothesized that resource competition may underlie the negative interactions between *ospC* alleles. However, Nouri et al. (4) found different results in a similar experimental system with *ospC* of *B. afzelii*. Those authors found that the degree of *ospC* antibody cross-reactivity was stronger in one mouse strain than in others. We hypothesize that allele U is able to exclude other *ospC* alleles in eastern chipmunks, although it is unclear how this could happen. Devevey et al. (43) hypothesized that competitive exclusion of *ospC* alleles by the priority effect mechanism is most likely to occur in areas with low prevalence of *B. burgdorferi*, for example, in areas where the pathogen has only recently begun to occur. The generally high prevalence and *ospC* allele diversity we report confirm that *B. burgdorferi* is well established in our area. While indirect host immune system effects may underlie the distribution of allele U, plausibly through asymmetric antibody cross-reactivity in eastern chipmunks, our data are observational only. Furthermore, culturing the bacteria in infected tissue could be conducted to determine whether infections are active (47). Experimental investigations, along with a greater sample size across a larger geographic distance, are needed to better understand how allele U (or, as previously

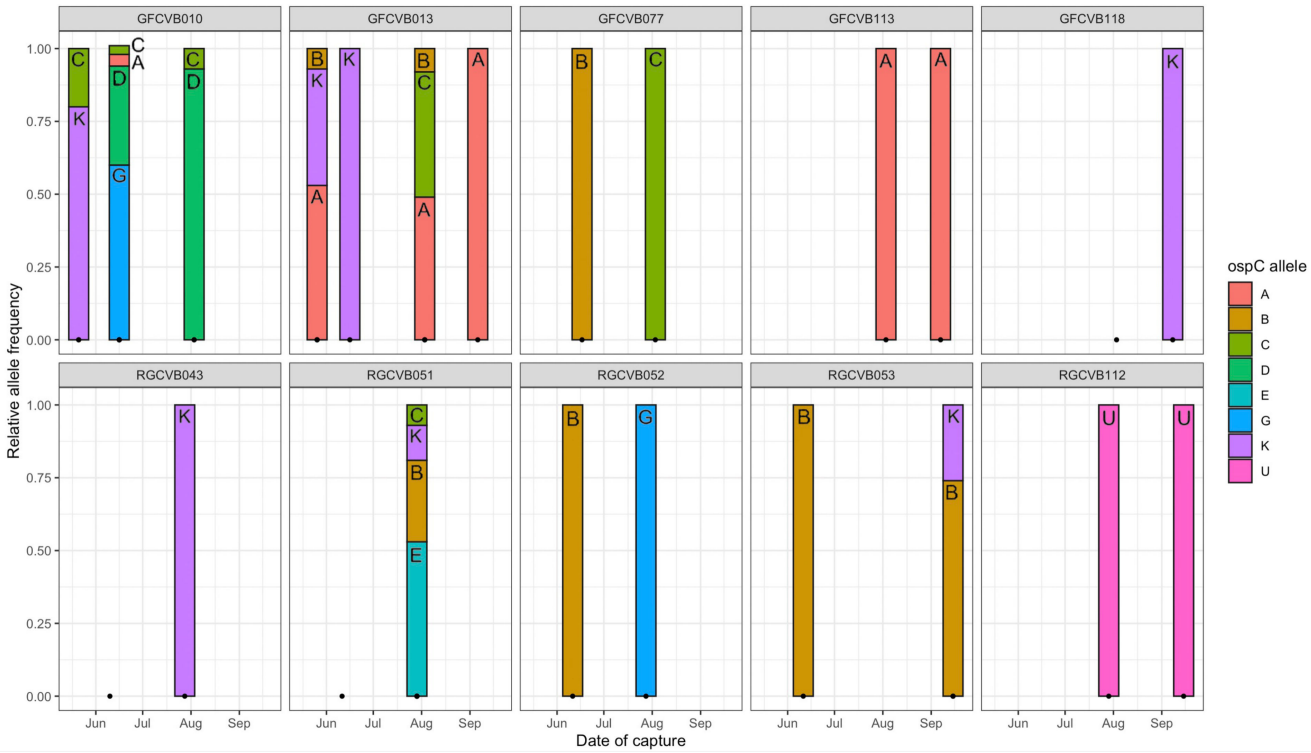

FIG 4 *ospC* allele compositions in *B. burgdorferi*-infected small mammals captured multiple times (June–September 2021). Only recaptured individuals infected with *B. burgdorferi* at least once are included. All but one individual (RGCVB112) are *Peromyscus* spp. (white-footed/deer mice); RGCVB112 is an eastern chipmunk. Relative allele frequencies within infections are shown on the y-axis (allele identity is labeled and has a unique color) and capture date on the x-axis; individuals are represented by separate panels in the figure. A point is shown on the x-axis for each of the capture dates, a point without a bar indicates the individual was not infected upon capture.

mentioned, alleles of other genes in linkage disequilibrium with allele U) is able to prevent mixed infections and specialize on eastern chipmunks.

Both our study and that of Brisson and Dykhuizen (27) show that *ospC* allele U specializes on eastern chipmunks. The fact that allele U does not occur in mixed infections and reaches a higher prevalence on its host species than any other allele (Fig. 2) is consistent with the hypothesis that specialists are more effective pathogens than generalists. Such a result has been shown elsewhere (48–52), but see Hellgren et al. (53) and Bedhomme et al. (54) for counterexamples.

The possible exclusion of HIAs in eastern chipmunks by allele U may be an important aspect of the transmission dynamics of the Lyme disease system. HIAs commonly occur in mixed infections, with eastern chipmunks having, on average, more alleles in mixed infections than any other vertebrate host [also shown in Brisson and Dykhuizen (27)], supporting the importance of this species in maintaining *ospC* allele diversity. However, under our hypothesis of allele exclusion, without the presence of allele U, eastern chipmunks would likely host more HIA infections. This would lead to more HIA-infected ticks in the environment, thereby increasing human infection risk. While the degree to which allele U could mitigate human infection risk is unclear, primarily because HIAs are maintained at high levels by *Peromyscus* spp., the reduction may not be negligible and will depend on relative mammalian host species population sizes.

Peromyscus spp. had the highest prevalence (Fig. 1) and were most frequently infected by HIAs (Fig. 2), consistent with other studies (27, 55, 56) and with the hypothesis that they are major drivers of Lyme disease risk in humans (7, 8). Between their high prevalence of infection (52% in this study) and high population sizes (57), especially in human-modified landscapes (57–59), *Peromyscus* spp. appear to be important for maintaining *B. burgdorferi* infections that can be transmitted to feeding ticks. The

importance of *Peromyscus* spp. to the maintenance of HIAs in the environment is highlighted by an unplanned natural experiment in our study design. The University Inn site had almost no *Peromyscus* spp. and, likely as a consequence, University Inn also only had a single HIA infection (the infection was in an eastern chipmunk).

We found *ospC* allele composition to be dynamic within *Peromyscus* spp. across time based on our recapture data (Fig. 4). Our results are similar to Swanson and Norris (60) who reported variation in *ospC* allele composition over time in *P. leucopus* in Maryland. Our results suggest that *Peromyscus* spp. may clear *B. burgdorferi* infections of certain alleles over short time periods (Fig. 4).

Eastern gray squirrels were infrequently infected by *B. burgdorferi* (four infections in 35 individuals sampled). This is consistent with other studies that have shown eastern gray squirrels to have a lower prevalence than other small mammals (27, 56). While *B. burgdorferi* infections and *ospC* alleles have been heavily studied in house mice within a laboratory setting (61–64), data are limited for free-ranging house mouse populations. We found a limited number of *ospC* alleles (3) in free-ranging house mice and 5/17 house mice infected with *B. burgdorferi* (29.41% prevalence). Three species with substantial sampling (raccoons, Virginia opossums, and groundhogs) were not infected with *B. burgdorferi* (Fig. 1). *B. burgdorferi* has been identified in engorged larval ticks removed from raccoons and Virginia opossums, suggesting they can be infected (56). However, previous work screening raccoon ear tissue for *B. burgdorferi* also failed to find infected individuals (65). *B. burgdorferi* infection intensity may be too low for accurate PCR amplification in these hosts and may only be detectable by screening engorged ticks. Alternatively, differences in prevalence in these hosts may result from spatial or temporal variation in prevalence and may not depend on surveillance methodology.

Our study provides new insights into the host specificity of *B. burgdorferi ospC* alleles in mammals. The observational data we collected are consistent with the hypothesis that *ospC* allele U reduces the overall frequency of HIAs through exclusion from mixed infections in eastern chipmunks and thereby reduces human Lyme disease infection risk. Experimental tests of this hypothesis are warranted. Understanding the ecology of pathogen interactions in the wild may lead to novel avenues of research for mitigating the effects of Lyme disease and other zoonotic diseases.

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AUTHOR AFFILIATIONS

[1]Department of Entomology and Wildlife Ecology, University of Delaware, Newark, Delaware, USA
[2]Center for Vector Biology, Department of Entomology, Rutgers University, New Brunswick, New Jersey, USA

PRESENT ADDRESS

Francisco C. Ferreira, Department of Entomology, Texas A&M University, College Station, Texas, USA
Julia González, Department of Entomology, Texas A&M University, College Station, Texas, USA

AUTHOR ORCIDs

Francisco C. Ferreira http://orcid.org/0000-0002-2034-4121
Julia González http://orcid.org/0000-0003-1517-4559
Vincenzo A. Ellis http://orcid.org/0000-0002-7193-9408

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DATA AVAILABILITY

All genetic and metadata are available on DataDryad: https://doi.org/10.5061/dryad.dr7sqvb54. This includes raw sequence reads, SAMtools coverage data, coverage data graphs, metadata, and R code.

ETHICS APPROVAL

All mammal sampling was conducted under a scientific collection permit from the New Jersey Division of Fish and Wildlife (permit no. SC 2021-01) and approval from the Rutgers University Institutional Animal Care and Use Committee (IACUC ID# AMEND202100256).

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Figure S1 (IAI00244-23-s0001.docx). Positive correlation between *ospC* allele relative frequencies in single infections (i.e., one allele in an infection) and mixed infections (more than one allele present in an infection).
Tables S1 to S3 (IAI00244-23-s0002.xlsx). Tables fully describing the dataset and host specificity analyses.

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