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Virome Analysis of *Amblyomma americanum*, *Dermacentor variabilis*, and *Ixodes scapularis* Ticks Reveals Novel Highly Divergent Vertebrate and Invertebrate Viruses

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ABSTRACT

A wide range of bacterial pathogens have been identified in ticks, yet the diversity of viruses in ticks is largely unexplored. In the United States, *Amblyomma americanum*, *Dermacentor variabilis*, and *Ixodes scapularis* are among the principal tick species associated with pathogen transmission. We used high-throughput sequencing to characterize the viromes of these tick species and identified the presence of Powassan virus and eight novel viruses. These included the most divergent nairovirus described to date, two new clades of tick-borne phleboviruses, a mononegavirus, and viruses with similarity to plant and insect viruses. Our analysis revealed that ticks are reservoirs for a wide range of viruses and suggests that discovery and characterization of tick-borne viruses will have implications for viral taxonomy and may provide insight into tick-transmitted diseases.

IMPORTANCE

Ticks are implicated as vectors of a wide array of human and animal pathogens. To better understand the extent of tick-borne diseases, it is crucial to uncover the full range of microbial agents associated with ticks. Our current knowledge of the diversity of tick-associated viruses is limited, in part due to the lack of investigation of tick viromes. In this study, we examined the viromes of three tick species from the United States. We found that ticks are hosts to highly divergent viruses across several taxa, including ones previously associated with human disease. Our data underscore the diversity of tick-associated viruses and provide the foundation for further studies into viral etiology of tick-borne diseases.

Ticks (class Arachnida, subclass Acari) have been implicated as vectors in a wide range of human and animal diseases worldwide (1–10). Approximately 900 species of ticks have been described and taxonomically classified into three families: *Argasidae* (argasid or soft ticks), *Ixodidae* (ixodid, or hard ticks), and *Nuttalliellidae* (11). Their propensity for feeding on a wide array of hosts, expansive range, and long life cycle underscore the importance of tick surveillance for the presence of potential pathogens. Argasid and ixodid ticks combined transmit a greater diversity of viral, bacterial, and protozoan pathogens than any other arthropod vector (12). The worldwide incidence of tick-borne disease is increasing, due partly to increased frequency of endemic tick-borne diseases and partly to the discoveries of new tick-associated agents (13).

In the United States, bacterial agents are implicated in the majority of tick-borne disorders. Lyme disease, caused by *Borrelia burgdorferi*, represents the most frequently reported tick-borne illness (14). Other bacterial agents, such as *Anaplasma*, *Ehrlichia*, *Rickettsia*, and other *Borrelia* species, as well as the protozoan *Babesia* contribute to the overall spectrum of tick-borne disease (1, 6, 7, 14–16). Conversely, viral causes are diagnosed in only a fraction of tick-borne disease cases (14). Despite considerable insights into the diversity of tick bacteriomes, our understanding of tick-associated viruses is still limited. Traditional viral isolation and identification methods using tissue culture have isolated several tick-associated viruses but few have been characterized thus far. In comparison to bacterial and protozoan agents, the literature associated with tick-borne viruses in the Americas is also limited. Most of the literature to date on suspected viral tick-borne pathogens focuses on those found in Europe, Asia, and Africa (3, 17). Powassan and Colorado tick fever viruses have been historically recognized as the only human tick-borne viral pathogens in the United States (5, 18).

Recently, the Heartland virus, a new pathogenic tick-borne virus, was isolated from patients in Missouri and characterized by high-throughput sequencing (HTS) (19). Heartland virus was shown to be phylogenetically similar to severe fever with thrombocytopenia syndrome virus (SFTSV), a tick-borne virus isolated from ticks and humans in China in 2009 (10). The emergence of novel pathogenic tick-borne viruses as well as the dearth of data on tick viromes suggests a need for viral surveillance and discovery in ticks. Although it seems plausible that there are tick-borne viruses that have not been amenable to isolation via tissue culture, to our knowledge, no extensive culture-independent studies have been attempted to examine tick viromes. Such studies not only might identify viruses associated with acute disease but also could provide insights into the pathogenesis of more controversial chronic illnesses associated with tick bites (20, 21). Thus, to survey viral diversity, we examined the viromes of three human-biting ticks in the United States (*Amblyomma americanum*, *Dermacentor variabilis*, and *Ixodes scapularis*) by HTS. Our analysis and characterization identified eight new viruses and indicates that ticks carry a wide array of previously uncharacterized viral agents.

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FIG 1 Map of tick collection sites. (Map from StepMap.)

MATERIALS AND METHODS

Adult *I. scapularis*, *D. variabilis*, and *A. americanum* ticks were collected within a 2-square-mile area of Heckscher State Park (Suffolk County, NY) in April 2013 (Fig. 1). Ticks were pooled prior to nucleic acid extraction; two pools of *I. scapularis* ($n = 30$/pool), two pools of *D. variabilis* ($n = 30$/pool), and a single pool of *A. americanum* ($n = 25$) were prepared. A two-step purification and nuclease treatment protocol was followed prior to extraction to enrich for viral sequences and minimize host, bacterial, and fungal templates that could compete with the virus template in HTS (22). Each tick pool was homogenized in 500 μl of phosphate-buffered saline (PBS), followed by purification through a 0.45-μm filter. The filtrate (237 μl) was treated with 1.5 μl RNase A for 15 min, followed by Turbo DNase (7.5 μl), Benzonase (1.8 μl), and 2.7 μl of 1 M $MgCl_2$ for 45 min. All nuclease treatment was performed at room temperature. A 250-μl portion of the nuclease-treated filtrate was added to 750 μl of Nuclisens buffer, and total nucleic acid (TNA) was extracted using the EasyMag extraction platform (bioMérieux). TNA from each pool was eluted in a 35-μl volume.

Unbiased high-throughput sequencing. TNA (11 μl) from each tick pool was subjected to first- and second-strand cDNA synthesis with SuperScript III reverse transcriptase (Invitrogen) and Klenow fragment (New England BioLabs), respectively. Random primers (30 ng/μl) (Invitrogen) were used in both assays. An Ion Shear Plus reagent kit (Life Technologies) was used for double-stranded-cDNA fragmentation at 37°C for 25 min. Agencourt AMPure XP (Beckman Coulter) reagent (1.8× sample volume) was used for DNA purification. Ion Xpress adapters and unique Ion Xpress barcodes (Life Technologies) were ligated to fragmented material by using the Ion Plus fragment library kit (Life Technologies). Ligation was performed at 25°C for 15 min and 72°C for 5 min. Ligated and nick-repaired products were purified with Agencourt AMPure XP (0.85× sample volume) and amplified according to the manufacturers' instructions with a Platinum PCR Super Mix High Fidelity mixture from the Ion Plus fragment library kit. Amplified products were purified as described above for the ligation reaction. An Agilent high-sensitivity DNA kit was used for library quantitation on the Bioanalyzer 2100 instrument. The concentration of each barcoded library was approximately 50 nM. Librar-

ies were diluted to approximately 45 nM, and a pool of libraries in equimolar concentrations was prepared. An Ion OneTouch 200 template kit, v2 (Life Technologies), was used to bind barcoded libraries to Ion Sphere particles (ISPS). Emulsion PCR of DNA-linked ISPS was performed on the Ion OneTouch 2 instrument (Life Technologies). An Ion OneTouch ES instrument was used to isolate template-positive ISPS. An Ion PGM sequencing 200 kit, v2 (Life Technologies), was used for sequencing of template-positive ISPS, which were loaded on the Ion 316 chip for further processing on the Ion personal genome machine (PGM) system (Life Technologies). Approximately 600,000 reads were obtained for each library.

The demultiplexed reads were preprocessed by trimming primers and adapters, length filtering, and masking of low-complexity regions (WU-BLAST 2.0). The remaining reads were subjected to a homology search using BLASTn against a database consisting of ribosomal and genomic metazoan sequences. Following the processing, the remaining reads amounted to 116,946 (pool 1) and 131,676 (pool 2) reads for *I. scapularis*, 30,353 (pool 1) and 29,334 (pool 2) reads for *D. variabilis*, and 297,175 reads for the *A. americanum* pool.

The host-subtracted reads were assembled using the Newbler assembler (454, v2.6). Contigs and singletons were then subjected to a homology search against the entire GenBank database using BLASTn and the viral GenBank database using BLASTx. Contigs and singletons with similarity to viral sequences from the BLASTx analysis were again subjected to a homology search against the entire GenBank database to correct for biased E values. For potential viral candidates, close relatives were used to identify low-homology regions in the genome from BLASTx; gaps were filled in by PCR using primers specific to the assembled sequence. Genome termini were obtained by using a 5′ and 3′ rapid amplification of cDNA ends (RACE) kit (Clontech Laboratories). The final genome sequences were verified by classical dideoxy sequencing using primers designed to generate overlapping PCR products.

Tick screening. To determine the authenticity and prevalence of viral sequences in ticks, we used cDNA generated from adult *I. scapularis* ticks for a previous study as the template for PCR (23). The ticks were collected in 2008 from four separate locations in New York State: two in Suffolk County (Heckscher State Park and Fire Island) and two in Westchester County (Kitchawan Nature Preserve and Blue Mountain Reservation) (Fig. 1). cDNA from individual ticks was screened by PCR for each novel virus identified by HTS. cDNAs from individual virus-positive ticks were used to generate viral genomic sequence equivalent to the sequences obtained by HTS. The sequences were obtained by generating overlapping PCR products using HTS sequences as a reference for primer design and sequence assembly. All PCR products were verified by dideoxy sequencing. All genome assemblies and alignments were performed with Geneious v 6.1 and Mega 5.2 (24) programs. Phylogenetic trees were constructed with Mega 5.2 using the maximum-likelihood method with 1,000 bootstrap replications. Amino acid trees were generated using the JTT matrix-based model. Nucleotide trees were generated using the Jukes-Cantor model.

Nucleotide sequence accession numbers. The sequence of Powassan virus was deposited under GenBank accession number KJ746872. Sequences of the L and S segments of South Bay virus were deposited in GenBank under accession numbers KJ746877 to KJ746878 (originating from tick pools) and KM048320 to KM048321 (individual tick H38). The S and L sequences of the blacklegged tick phlebovirus from individual *I. scapularis* ticks were deposited under accession numbers KM048313 to KM048316, and the sequences originating from tick pools were deposited under accession numbers KJ746873 to KJ746876. The L and S sequences of the American dog tick phlebovirus were deposited under accession numbers KJ746901 to KJ746902 (originating from tick pools) and KM048311 to KM048312 (individual tick H6). The complete 6,507-nt L ORF of the *Mononegavirales*-like virus was deposited under accession number KJ746903 (originating from tick pools) and KM048317 (individual tick FI3). The sequences of *I. scapularis*-associated virus 1 (accession number KM048318) and tetravirus-like virus (KM048322) were obtained from individual ticks. The sequence of *I. scapularis*-associated virus 2 was obtained from tick pools and deposited under accession number KM048319.

TABLE 1 Viral sequences identified in *I. scapularis* and *D. variabilis* by high-throughput sequencing

Pool	Identified viral sequences
I. scapularis pool 1	Nairovirus, phlebovirus,[a] invertebrate-like virus, powassan virus
I scapularis pool 2	Nairovirus, phlebovirus,[a] invertebrate-like virus,[a] mononegavirus
D. variabilis pool 1	Phlebovirus, tetravirus-like virus
D. variabilis pool 2	Phlebovirus, tetravirus-like virus

[a] Sequences from two distinct viruses were identified.

RESULTS

Analysis of HTS data revealed the presence of eight previously uncharacterized viruses (Table 1). The most diverse virome was observed in *I. scapularis*, where, in addition to Powassan virus, we identified sequences representing six novel viruses. Bunyavirus-like sequences represented more than 50% of the total filtered reads in both *I. scapularis* pools and were similar to viruses in the genera *Nairovirus* and *Phlebovirus*. Two viruses were identified in each pool of *D. variabilis*; one virus was similar to phleboviruses, and the other displayed limited homology to insect viruses. Although the pool of *A. americanum* generated the most reads of the five analyzed tick pools, a rhabdovirus was the lone virus identified in this tick species. The complete genome characterization of this virus, named Long Island tick rhabdovirus (LITRV), has been reported (25).

Arthropod genomes frequently contain integrated fragments of archaic RNA viral genomes (26–28). The genome of *I. scapularis* contains numerous sequences of viral origin, designated endogenous viral elements (EVEs) (26). To test for the authenticity of the novel sequences obtained by HTS, we compared results of reverse transcription-PCR (RT-PCR) to those of DNA PCR as an additional indication of the template source, i.e., authentic viral nucleic acid versus tick genomic DNA. Consistent with a model where these novel virus sequences represent authentic RNA viruses and not EVEs, products were obtained with RT-PCR but not with PCR.

Powassan virus. Two lineages of Powassan virus (POWV; family *Flaviviridae*, genus *Flavivirus*) circulate in the United States: lineage I, isolated mainly from *Ixodes cookei*, and lineage II (also known as deer tick virus), detected predominately in *I. scapularis* (29). Human infection with viruses from either lineage has been linked with Powassan encephalitis, a severe, potentially life-threatening neurological illness (29, 30). POWV lineage II has been detected in *I. scapularis* throughout the Northeast (23, 31, 32). In our HTS study, approximately 0.8% of all filtered reads from *I. scapularis* pool 1 were from POWV lineage II. Assembly of all reads and contigs from this pool resulted in assembly of 98% of the 10.8-kb POWV genome. Nucleotide comparison of the complete polyprotein coding sequence (GenBank accession number KJ746872) indicated that the nucleotide sequence of this virus (designated strain LI-1) is 99.4% identical to that of isolate NSF001 (GenBank accession number HM440559), obtained from Nantucket, MA, in 1996, and clustered with viruses in a subclade of POWV lineage II isolated from the Northeast (Fig. 2) (32). The

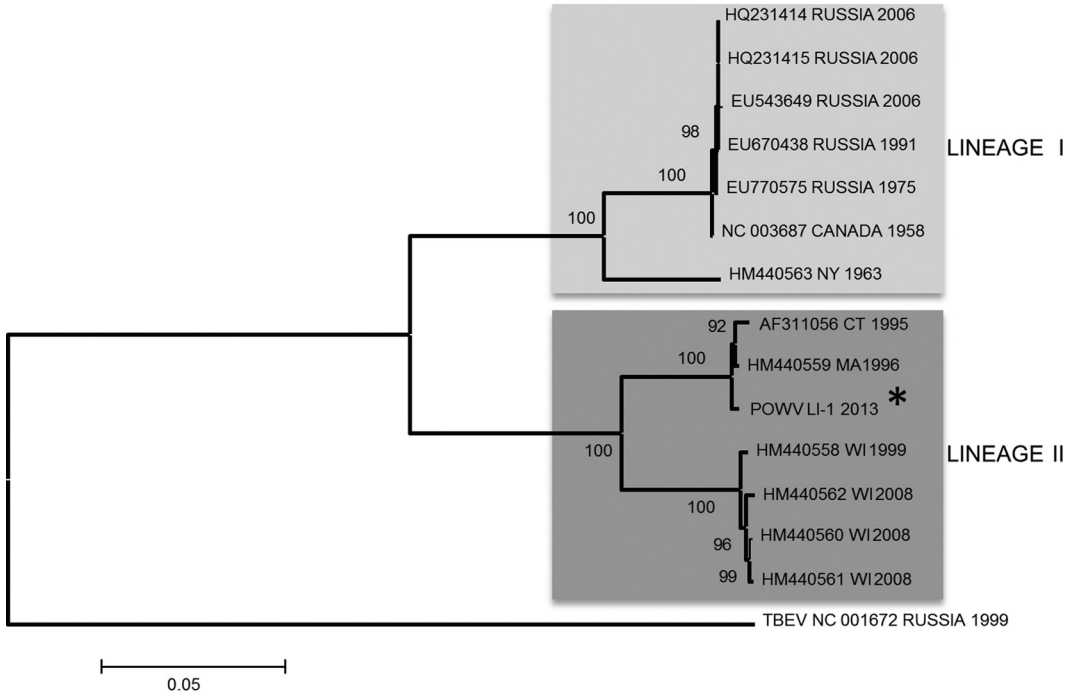

FIG 2 Maximum-likelihood phylogenetic tree based on the nucleotide sequence of the complete POWV polyprotein. The accession number, geographical location, and year of isolation/detection are indicated for each isolate. The sequence generated in this study is indicated by an asterisk. Tick-borne encephalitis virus (TBEV) is shown as an outgroup.

nucleotide identity to the subclade of lineage II made up of isolates from the midwestern United States was 93.4%.

Nairovirus. The genus *Nairovirus* (family *Bunyaviridae*) is comprised of 37 tick-borne viruses. Its genome consists of three segments of negative-sense single-stranded RNA, designated small (S), medium (M), and large (L), that encode the nucleocapsid protein (N), the envelope glycoproteins (Gn and Gc), and an RNA-dependent RNA polymerase (L), respectively (33). Both *I. scapularis* pools contained multiple contigs with sequences similar to viruses in the genus *Nairovirus* by BLASTx. Assembly of these *Nairovirus*-like contigs, using CCHFV as a reference genome, provided >90% coverage of the S and L segments. The complete segments were obtained by overlapping PCR and 3′ and 5′ RACE. The assembled sequences showed low similarity to other members of the genus, suggesting that this virus, provisionally named South Bay virus (SBV) after its geographic location, represents a novel nairovirus species. We also obtained the complete S and L sequences from an individual SBV-positive tick (designated SBV H38). Comparison of the genomic nucleotide sequences obtained from HTS and SBV H38 indicated that they were 97% and 99% identical in the S and L segments, respectively. SBV reads were the predominant viral reads obtained from both *I. scapularis* pools and accounted for 25% of filtered reads from pool 1 and 41% of reads from pool 2. However, despite an exhaustive bioinformatics analysis, we were unable to identify any contigs or reads with any similarity to *Nairovirus* M segments. Attempts at virus isolation by inoculation of *I. scapularis* SBV PCR-positive pools in Vero, Cos7, C6/36, and 297 cell lines were unsuccessful.

To ascertain whether the SBV sequences represented authentic viral reads, we screened *I. scapularis* DNA and corresponding cDNA for the presence of SBV. Partial S and L sequences were amplified in SBV-positive tick cDNA but were absent from the corresponding genomic DNA. Additionally, we found that *I. scapularis* genome contains an integrated *Nairovirus* N gene open reading frame (ORF) (accession number XM_002414099), although it was not transcribed in any tick sample. This sequence was more similar to SBV than to other nairovirus sequences, likely representing an ancestral integration of an SBV-like nairovirus into the *I. scapularis* genome.

Conserved genus-specific sequences at the termini of each segment are a feature of all bunyaviruses. In nairoviruses, the 5′-terminal sequence typically consists of UCUCAAAAGA. We found that the sequences of SBV L and S termini were consistent with the nairovirus sequence with the exception of a single nucleotide change at the fifth position (UCUC*U*AAAGA).

Phylogeny and L segment analysis. Of the 37 described viruses assigned to the genus *Nairovirus*, only seven are completely sequenced, and there are no published sequence data for 21 putative members of the genus. For the remaining nine viruses, the only available sequence data consist of a short (<450-nt) fragment within the L segment (34). This region is part of the polymerase catalytic domain and is highly conserved, with no nucleotide insertions or deletions in any of the 16 nairoviruses analyzed to date. Thus, we used the amino acid sequence from this region to determine the phylogenetic relationships of SBV to the rest of the *Nairovirus* genus. SBV contained a 7-amino acid (aa) insertion in this region and had <45% identity to the next closest virus, distinguishing SBV from other nairoviruses (Table 2). SBV did not cluster with any of the described nairovirus serogroups but fell outside all described viruses in this genus (Fig. 3).

The L segment of SBV is 13,892 nucleotides (nt) long and contains a 13,611-nt ORF that encodes a 4,536-aa protein. This rep-

TABLE 2 Percent identity comparison of SBV to other nairoviruses within a conserved fragment of the L protein

Virus	% identity to:															
	South Bay	Farallon	Raza	Punta Salinas	Abu Hammad	Abu Mina	Qalyub	Bandia	Tillamook	Finch Creek	Erve	Kupe	Dugbe	Hazara	CCHFV	NSD Ganjam
South Bay																
Farallon	43.2															
Raza	41.7	96.6														
Punta Salinas	41.7	89.5	89.5													
Abu Hammad	38.7	71.2	69.0	67.8												
Abu Mina	44.7	71.2	71.2	66.7	86.6											
Qalyub	41.7	64.3	61.9	65.5	70.1	70.1										
Bandia	38.7	64.3	64.3	65.5	71.2	69.0	94.9									
Tillamook	43.2	67.8	67.8	63.1	65.5	69.0	67.8	69.0								
Finch creek	44.7	69.0	69.0	64.3	65.5	70.1	70.1	71.2	95.7							
Erve	37.1	59.5	59.5	61.9	55.6	56.9	51.7	54.3	58.2	59.5						
Kupe	37.1	64.3	63.1	61.9	60.7	64.3	58.2	60.7	69.0	70.1	65.5					
Dugbe	34.0	60.7	59.5	60.7	60.7	61.9	54.3	59.5	65.5	67.8	66.7	95.7				
Hazara	40.2	64.3	63.1	59.5	63.1	64.3	64.3	65.5	71.2	74.5	69.0	85.7	82.8			
CCHFV	37.1	65.5	61.9	60.7	61.9	60.7	60.7	60.7	70.1	69.0	66.7	87.6	84.7	86.6		
NSDV Ganjam	37.1	61.9	60.7	61.9	59.5	63.1	61.9	64.3	70.1	72.3	69.0	92.2	89.5	89.5	88.5	
NSDV	37.1	61.9	60.7	61.9	59.5	63.1	61.9	64.3	70.1	72.3	69.0	92.2	89.5	89.5	88.5	100.0

resents the longest known nairovirus open reading frame, over 600 codons longer than the L ORF of Dugbe virus. Comparison of SBV to L proteins from other nairoviruses revealed that SBV does not cluster with any of the previously sequenced viruses and contains only 28% amino acid identity to the next closest virus (Erve virus) (Fig. 4A; Table 3). In SBV, the catalytic polymerase domain is located within the region between amino acid residues 2600 and 3200 and contains all known viral RNA polymerase motifs (pre-motif A and motifs A to E) (35).

In addition to the polymerase domain, the nairovirus L proteins may contain several other protein motifs, such as ovarian tumor domain (OTU), topoisomerase domain, zinc finger motif, and a leucine zipper motif (35, 36). The OTU domain represents a family of cysteine proteases, and it is present in the N termini of all seven sequenced nairovirus L proteins. In CCHFV, this domain may function in modulation of interferon response in host cells (37). We found no evidence of the presence of a functional OTU domain in SBV; the conserved catalytic residues (D37, C40, and H151 in CCHFV) were absent, and overall, the SBV N-terminal region of the L showed little similarity to other nairoviruses. The N-terminal topoisomerase motif, zinc finger motif, and leucine zipper motifs were also absent.

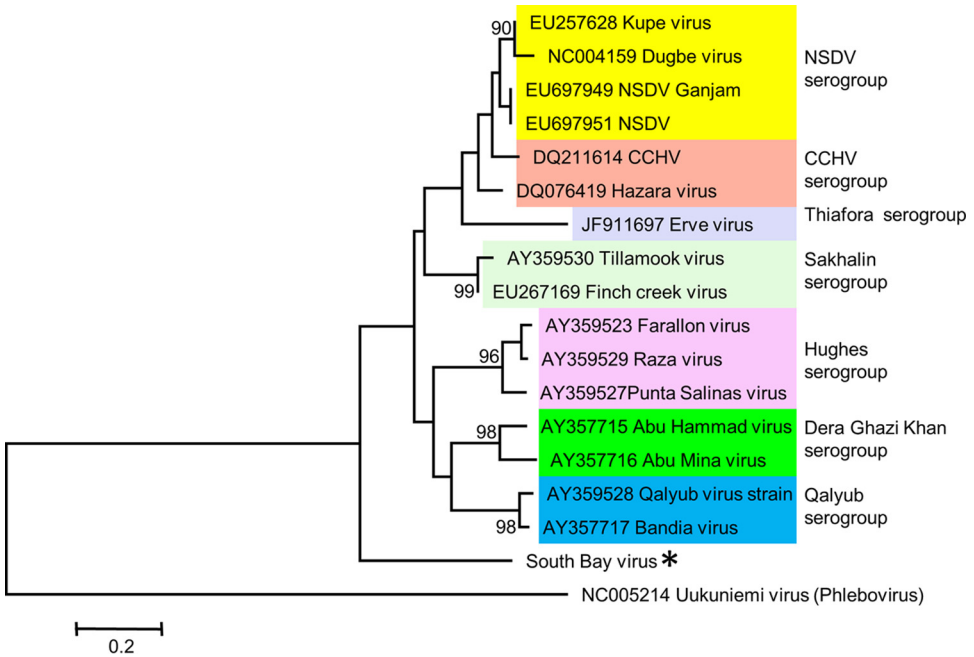

FIG 3 Maximum-likelihood phylogeny of all available nairovirus sequences, based on a conserved 150-amino-acid fragment of the L protein. Accession numbers are provided next to the virus names. The asterisk indicates the virus characterized in this study.

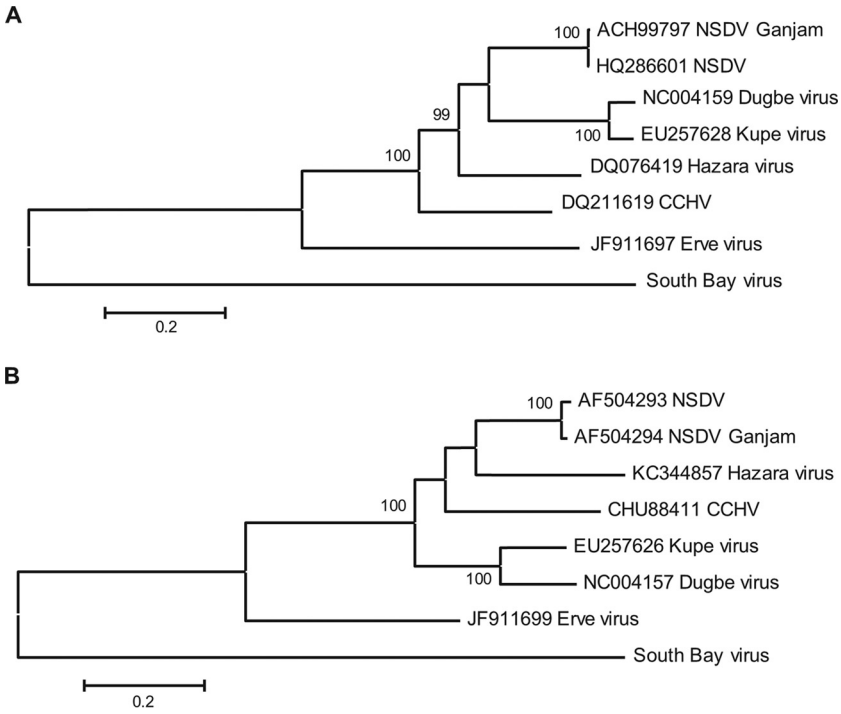

FIG 4 Maximum-likelihood phylogeny based on the complete L (A) and N (B) proteins of all nairoviruses with available genomic sequence.

Analysis of the S segment. SBV has an unusually long S segment, comprising 5,536 nucleotides (nt) and containing a 1,664-nt ORF encoding a putative N protein. The N protein has a number of important functions in negative-sense single-stranded RNA viruses. It binds to genomic RNA, forming ribonucleoprotein complexes that associate with the polymerase for viral RNA synthesis. In bunyaviruses, N proteins are also associated with immune response modulation. In SBV, the N ORF begins at position 865 from the 3′ end of the segment and is predicted to encode a 547-aa N protein, representing the second largest *Nairovirus* N. Only Erve virus, encoding a 630-aa protein, contains a longer nucleocapsid sequence (38). The amino acid identity of the SBV N protein to the other seven sequenced *Nairovirus* N proteins ranged between 18.8% and 21.6% (Table 2; Fig. 4B). Despite the overall high degree of divergence from other nairoviruses, we also identified a highly conserved domain present at the C-terminal part of the protein. This domain represents the only area of high conservation among all eight viruses. Out of 32 aa in this region, 11 were conserved among all nairoviruses (Fig. 5). Two recent analyses of the crystal structure of the CCHFV N protein revealed a number of residues essential for N function. Several of the implicated residues are present within this conserved domain. Carter et al. analyzed the interaction of CCHFV N with RNA and identified five residues (K90, K132, Q300, K411, and H456) essential for RNA binding (39). We found a high degree of conservation of these residues between SBV and other nairoviruses (Fig. 5). An independent analysis by Guo et al. revealed that the CCHFV N possesses metal-dependent DNA endonuclease activity and implicated seven potential active-site residues (40). Four of these (R384, E387, H453, and Q457) are conserved in all nairoviruses, including SBV, with both H453 and Q457 being part of the conserved C-terminal domain. Another residue, K411, was one of the residues implicated as also being essential for RNA binding by Carter et al. (39). For the remaining two residues, South Bay and Erve viruses represent the only viruses with amino acid variations; for residue R298, SBV contains a Y and Erve virus a K, and for Y374, both viruses contain an H.

TABLE 3 Identity of South Bay virus N and L protein sequences to those of other nairoviruses

Virus	% identity to South Bay virus in protein (segment)	
	N (S)	L (L)
Erve virus	21.5	25
CCHFV	18.8	25.6
Hazara virus	21.6	25.5
NSDV	21.6	25.9
NSDV Ganjam	21.0	25.8
Kupe virus	19.6	26.0
Dugbe virus	19.0	26.0

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                  *** **    *    **     ***    *
NSDV        451   SEHLLHQSLVGKRSPFQNAYNIRGNATSIQII   482
NSDV        451   SEHLLHQSLVGKRSPFQNAYNIRGNATSIQII   482
Kupe        452   SEHLLHQSLVGKRCHFQNAYKVKGNATNVEIV   483
Erve        444   SEHLFHQILVGKRTAYQNAFQVKGNATDTKIV   475
Hazara      454   SEHLLHQSLVGKRSPFQNAYLIKGNATNINII   485
Dugbe       452   TEHLLHQSFVGKRCPTQNAYKVRGNATNVNII   483
CCHV        451   SEHLLHQSLVGKQSPFQNAYNVKGNATSANII   482
South Bay   470   SEHLFHQFLLNKRSPFQNTHRLEGNALNVEIV   501
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FIG 5 Alignment of the conserved C-terminal domain present in all *Nairovirus* N proteins. Conserved residues are indicated by asterisks. Residues implicated in N function are indicated in bold. The amino acid position numbers within the N protein are displayed for each virus.

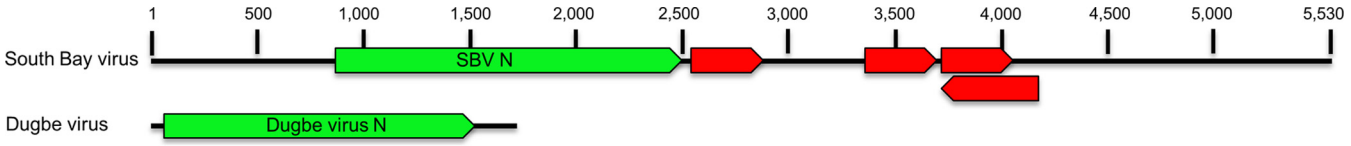

FIG 6 Comparison of the S segments of SBV and Dugbe virus. The locations of the N ORFs are shown in green. Putative additional SBV ORFs are shown in red. Numbers indicate nucleotide positions within the segment.

The S segment of SBV contains an additional 3,028 nt of sequence following the termination of the N ORF (Fig. 6). This portion of the segment does not display homology to any known sequence and contains several short potential ORFs, the longest consisting of 441 nt, though none contain any identifiable domains. The terminal 1,400 nt of the S segment do not contain any potential ORF longer than 170 nt.

Prevalence of SBV in ticks. To determine the prevalence and distribution of SBV in ticks, we used cDNA generated from individual ticks in 2008 from four locations in two New York State counties (Fig. 1). These samples were previously screened for the presence of multiple tick-borne pathogens (23). We detected SBV in all four locations, with a total of 20 out of 100 ticks being positive for the virus (Table 4). The highest number of SBV-positive ticks originated from Heckscher State Park (12 out of 26). The SBV sequences were genetically homogeneous, even at different collection sites. Partial S and L sequences obtained from all SBV-positive ticks displayed 98 to 100% nucleotide identity among all viruses.

Phlebovirus. The genus *Phlebovirus* is comprised of over 70 viruses isolated primarily from ticks, mosquitoes, and sand flies (41). We identified multiple contigs with homology to phleboviruses by BLASTx in each pool of *I. scapularis* and *D. variabilis*. The contigs originating from each tick species were assembled separately to a reference phlebovirus genome (Precarious Point virus). Initial sequence comparison indicated that both viruses likely represented new phlebovirus species, as they were distinct from each other and other phlebovirus sequences in GenBank. We provisionally named the *I. scapularis*-associated virus the blacklegged tick phlebovirus (BTPV) and the *D. variabilis*-associated virus the American dog tick phlebovirus (ADTPV).

We obtained complete S and L segments of both viruses; as was the case with the nairovirus, we were unable to identify any sequences with similarity to phlebovirus M segments. The typical phlebovirus segment terminal sequence, ACACAAAG (5′ end), was identified at the termini of both viruses, with the exception of a single nucleotide change at the 3′ end of the BTPV L segment (ACACAA*U*G).

During the genome assembly phase of BTPV, we identified two distinct putative BTPV S and L segments, suggesting the presence of multiple genotypes of this virus. This was confirmed by PCR using cDNA from both tick pools and individual *I. scapularis* ticks as the template and sequencing of PCR products. The two genotypes, designated BTPV-1 and BTPV-2, were 12% and 15% divergent in the nucleotide sequences of the S and L segments, respectively.

PCR analysis of individual ticks indicated that BTPV and ADTPV were highly prevalent in their tick hosts (Table 4). Out of 24 adult *I. scapularis* from Heckscher State Park, 12 (50%) were positive by PCR for BTPV-1 or BTPV-2 (Table 4). We screened 12 *D. variabilis* ticks collected on Fire Island, NY, approximately 16 miles southeast of Heckscher State Park, and identified 9 ticks positive for ADTPV (75%). We obtained complete S and L sequences from two individual *I. scapularis* ticks (designated BTPV-1 H12 and BTPV-2 H5, accession numbers KM048313 to KM048316) and ADTPV sequences from one individual *D. variabilis* (ADTPV H6, accession numbers KM048311 and KM048312). The nucleotide sequence of all segments from individual ticks were 97% to 99% identical to the sequence obtained by HTS.

S segment. A typical *Phlebovirus* S segment is approximately 1,700 nt in length and codes for a nucleocapsid (N) and a nonstructural protein (NSs) in an ambisense orientation (42). The lengths of the putative S segments of BTPV-1 and BTPV-2 were >2,400 nt and contained unusually long ORFs of 1,581 and 1,578 nt, respectively (Fig. 7). These would encode putative 526- and 525-aa proteins. The N-terminal portions of these putative proteins displayed no homology to any known protein, whereas the C-terminal 250-aa portion exhibited similarity to other *Phlebovirus* N proteins. Comparison of the C-terminal portions of BTPV-1 and BTPV-2 N proteins revealed that they were 91% identical to each other and <26% identical to other *Phlebovirus* N proteins. We identified ORFs corresponding to the typical genomic orientation of the *Phlebovirus* NSs ORF; however, these were unusually short and displayed no homology to any reported NSs protein.

The S segment of ADTPV also contained an atypically long ORF of 1,209 nt encoding a putative 402-aa protein. Similar to BTPV, the characteristic *Phlebovirus* N protein was identified only

TABLE 4 Prevalence of novel viral sequences in individual ticks

Location	No. of positive ticks/total (%)						
	SBV	BTPV	*I. scapularis* mononegavirus	ISAV1	ISAV2	ADTPV	Tick-borne tetravirus-like virus
Heckscher State Park	12/26 (46)	12/24 (50)	0/27		0/2		
Kitchawan Nature Preserve	5/25 (20)			5/25 (20)	0/35		
Fire Island	1/24 (4)		1/20 (5)		0/23	9/12 (75)	1/9 (11)
Blue Mountain Reservation	2/25 (8)				0/10		
Total	20/100 (20)	12/24 (50)	1/47 (2)	5/25 (20)	0/70	9/12 (75)	1/9 (11)

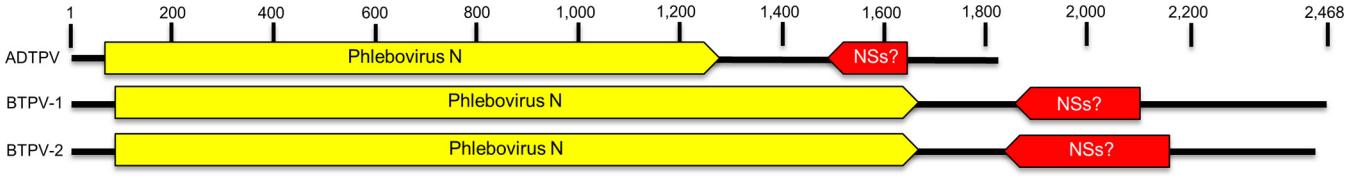

FIG 7 Schematic of the S segments of BTPV-1, BTPV-2, and ADTPV. The putative N ORFs are shown in yellow. The putative ORFs corresponding to the genomic location of the NSs are displayed in red.

within the C-terminal 250-aa sequence of this protein. A putative NSs ambisense ORF was identified, although it was shorter than the ORFs identified in BTPV (151 nt). The amino acid identity of the putative N of ADTPV was 24% to those of BTPV-1 and BTPV-2 and <34% to other *Phlebovirus* N proteins (Table 5).

L segment. The complete L segments of both BTPV-1 and BTPV-2 both had overall lengths of 6,733 nt and contained 6,624-nt ORFs that would encode a 2,207-aa L protein. The nucleotide sequence identity of BTPV-1 and BTPV-2 L ORFs was 84% (95.6% amino acid identity). The length of the ADTV L segment was 6,600 nt, with a 6,537-nt ORF encoding a 2,178-aa protein. The ADTV L protein was 29.6% and 29.2% identical to the L proteins of BTPV-1 and BTPV-2, respectively (Table 5).

Phylogenetic analysis. Phleboviruses have traditionally been classified into two groups consisting of sand fly/mosquito-borne and tick-borne viruses (41). At least three phylogenetic clusters of tick-borne phleboviruses have been identified, each comprised of several potential species: the Uukuniemi group, the Bhanja group, and the STFS group (19, 43, 44). We found that BTPV does not cluster with any of these groups and forms a separate monophyletic clade outside all tick-borne and sand fly/mosquito-borne phleboviruses, similar to Gouleako and Cumuto viruses. ADTPV is more similar to viruses within the Uukuniemi group but forms a distinct monophyletic clade outside this group (Fig. 8).

***Mononegavirales*-like virus.** In sequences originating from pool 2 of *I. scapularis*, we identified contigs with weak homology by BLASTx to the L protein RNA-dependent RNA polymerase domain (RdRp) of viruses within the order *Mononegavirales*. This order represents five families (*Bornaviridae*, *Filoviridae*, *Nyamiviridae*, *Paramyxoviridae*, and *Rhabdoviridae*) of monopartite negative single-strand viruses with comparable ORF organization (45). We obtained the complete 6,507-nt L ORF of this virus, which encodes a putative 2,168-aa protein, and identified a *Mononegavirales* RNA-dependent RNA polymerase (RdRp) mRNA-capping domain and a virus-capping methyltransferase domain, characteristic of a *Mononegavirales*-like virus L protein. Homology searches revealed this virus to be very distant from other *Mononegavirales* (Fig. 9). The greatest similarity was observed with the Nyamanini and Midway viruses (17% amino acid identity). Nyamanini virus (originally isolated in 1957 in South Africa) and Midway viruses (isolated in 1966 on islands in the Pacific) have frequently been isolated from argasid ticks and form a distinct lineage in the order *Mononegavirales* (46). Along with

TABLE 5 L and N protein comparison of ISPV and ADTV to selected phleboviruses

	% amino acid identity					
	L			N[a]		
Virus	BTPV-1	BTPV-2	ADTPV	BTPV-1	BTPV-2	ADTPV
BTPV-1		95.6	29.6		91.1	24.3
BTPV-2	95.6		29.2	91.1		23.6
ADTPV	29.6	29.2		24.3	23.6	
Murre virus	30.2	30.2	36.7	24.0	22.1	29.3
Forecariah virus	25.5	25.3	25.9	22.9	23.7	28.0
Precarious Point virus	30.6	30.8	37.5	25.2	23.3	33.2
Zaliv Terpenia virus	30.4	30.4	37.4	24.4	23.7	29.4
Uukuniemi virus	30.2	30.2	37.3	24.0	23.6	30.2
Chize virus	30.8	30.8	37.1	23.6	22.5	31.4
Grand Arbaud virus	30.8	31.1	36.3	22.1	21.4	29.8
Manawa virus	30.4	30.2	36.9	22.3	21.9	32.2
SFTS virus	27.2	26.8	30.0	23.7	23.7	25.8
FinV707 virus	30.1	30.2	36.8	25.2	23.7	30.2
RML-105355 virus	30.3	30.3	36.7	22.2	23.0	29.7
Heartland virus	27.0	26.8	29.9	22.3	21.2	25.8
Bhanja virus	25.3	25.4	26.0	22.9	23.7	28.3
Palma virus	25.2	25.3	25.8	23.7	24.4	27.6
Lone Star virus	25.3	24.9	26.3	20.3	20.3	28.3
Rift Valley fever virus	28.4	27.9	30.8	24.5	23.7	31.0
Gouleako virus	22.7	22.7	23.3	19.4	19.4	16.9

[a] For N, only the C-terminal 250-amino-acid region of BTPV and ADTPV was analyzed.

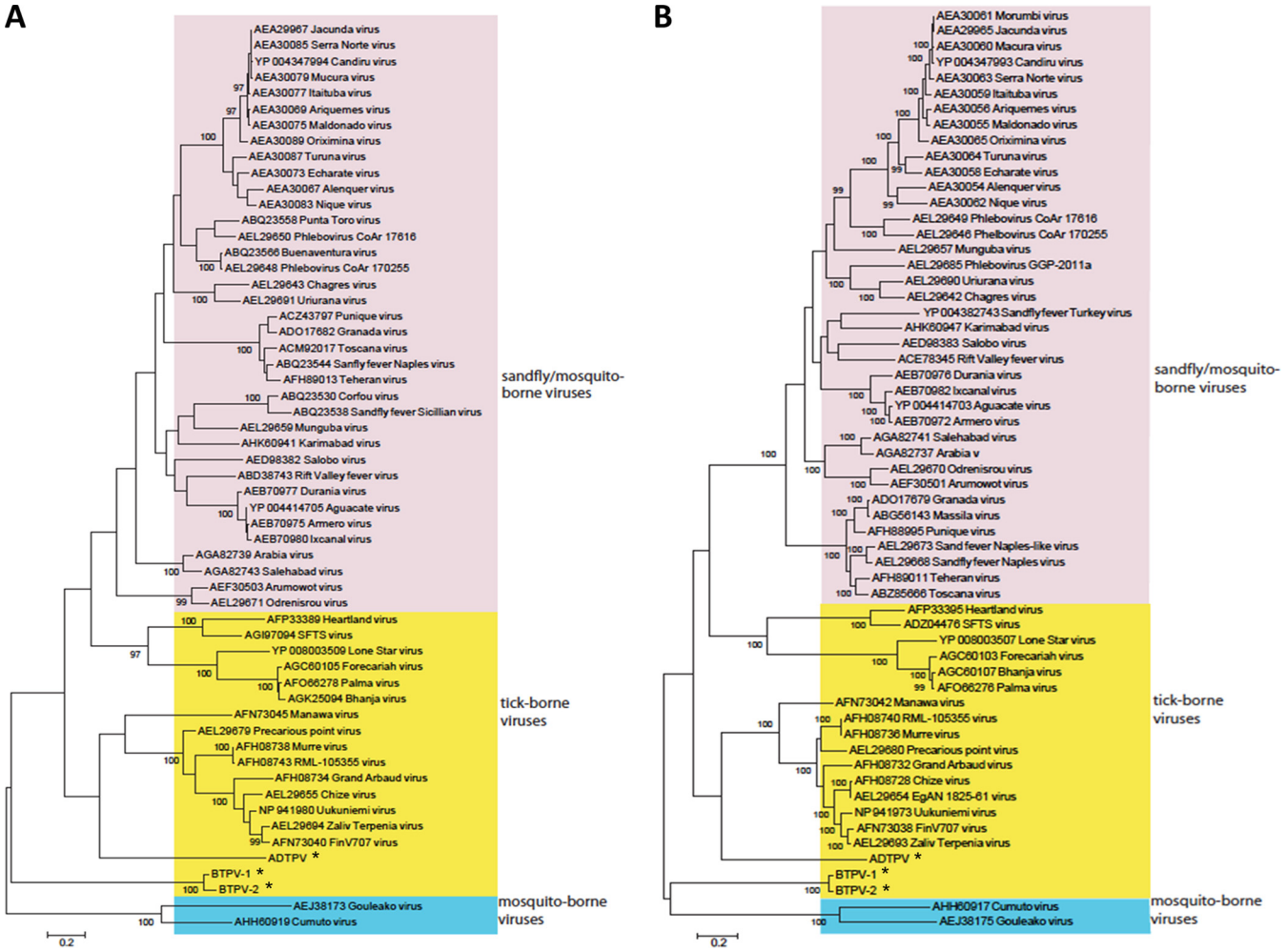

FIG 8 Maximum-likelihood phylogeny of BTPV-1, BTPV-2, and ADTPV estimated from amino acid sequences for phlebovirus N (A) and L (B). Accession numbers for every virus in the tree are indicated. Asterisks designate the viruses characterized in this study.

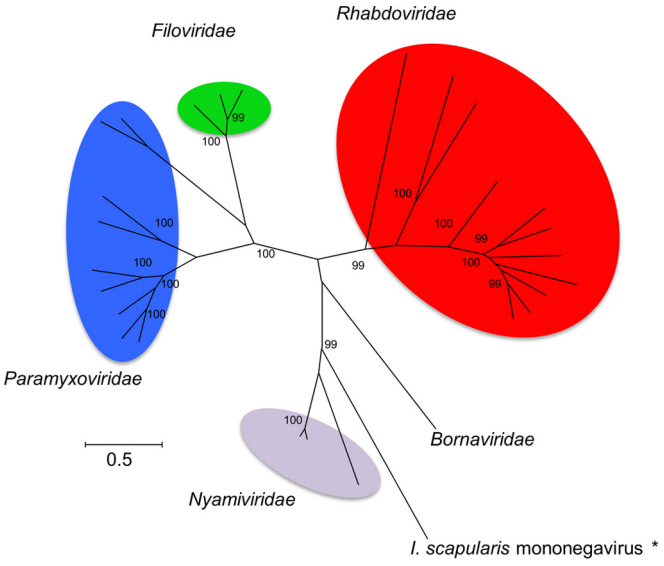

FIG 9 Phylogeny of the novel *I. scapularis*-associated mononegavirus (indicated by the asterisk) to viruses within *Mononegaviridae*. Each terminal branch represents a single type species representative of a genus.

soybean cyst nematode virus, both viruses are now included in *Nyamiviridae*, a new *Mononegavirales* family (47).

To determine the prevalence of this virus, we screened 47 individual tick samples by PCR (Table 4). We detected one positive tick (FI3; accession number KM048317). The complete nucleotide sequence of the FI3 L ORF was 99.2% identical to the assembled sequence (99% amino acid identity). This result, in concert with the HTS detection in only one tick pool with low genome coverage, suggests that this virus has a low prevalence in tick populations, at least within the geographical area surveyed.

Invertebrate/plant viruses. In addition to vertebrate viruses, we identified sequences with similarity to viruses associated with plants and arthropods. Two distinct invertebrate-like viruses were identified in *I. scapularis* pools, designated ISAV-1 (*Ixodes scapularis*-associated virus 1), present in both pools, and ISAV-2, identified in only one pool. We recovered partial genomes of both viruses (2.8 kb for ISAV-1 and 2.3 kb for ISAV-2), encompassing the majority of a putative protease and RdRp. Analysis of a 255-aa conserved portion of the RdRp indicated that these viruses were 50% identical to each other, and limited homology to described viruses was observed, with the highest similarity being to invertebrate viruses in the genus *Sobemovirus* (<25% amino acid simi-

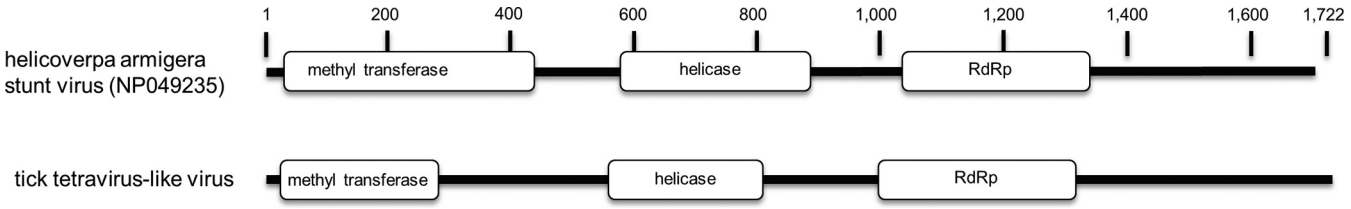

FIG 10 Comparison of the tetravirus-like virus replicase from *D. variabilis* to the *Helicoverpa armigera* stunt virus replicase. Numbers indicate amino acid positions.

larity). This genus represents positive-sense single-stranded RNA viruses that infect plants, with various arthropods being implicated as vectors (45). We identified ISAV-1 in 5 out of 20 ticks; however, none of these were positive for ISAV-2. We screened an additional 50 samples but were unable to identify any ISAV-2-positive ticks.

Both pools of *D. variabilis* contained sequences with low similarity (<20%) to a wide range of invertebrate single-stranded positive-sense viruses. We recovered a 5.3-kb segment of this virus encoding a putative >1,700-aa replicase. One out of nine *D. variabilis* ticks tested was positive for this virus, with 96.8% nucleotide and 99.3% amino acid identity to the HTS sequence (accession number KM048322). The N-terminal 1,300 aa of this protein contained the methyltransferase, helicase, and RdRp domains (Fig. 10). No putative domain could be identified within the >400-aa C-terminal portion. This genome organization was similar to viruses within the *Omegatetravirus* genus (family *Alphatetraviridae*) (http://ictvonline.org/virusTaxonomy.asp). We found limited amino acid identity (19%) across the N-terminal 1,300-aa portion of the replicase of this virus with a representative of this genus (*Helicoverpa armigera* stunt virus). Omegatetraviruses, isolated from moths (order Lepidoptera), have a bipartite genome. The RNA1 segment (approximately 5.3 kb in length) encodes a replicase containing all aforementioned domains, and the RNA2 segment (approximately 2.5 kb in length) encodes the capsid (45). We identified sequences for a putative RNA2 segment that encodes a protein with low (11% amino acid) identity to *Omegatetravirus* capsids. Further classification of this new virus was challenging due to high sequence divergence in combination with a limited number of available *Alphatetraviridae* sequences. Although we tentatively include this virus in the *Alphatetraviridae*, due to dissimilarity in sequence and host association, it likely represents a new family of arthropod-associated viruses.

DISCUSSION

In this study, we analyzed the viromes of *I. scapularis*, *D. variabilis*, and *A. americanum*, tick species frequently associated with pathogen transmission in the United States (14). In addition to exploring viral diversity, our work was aimed at uncovering viruses with potential relevance for human disease. We identified several novel viruses with genetic similarities to pathogens of humans and livestock. While these data do not allow conclusions regarding transmissibility, these discoveries can lay the foundation for such future work.

Bunyavirus-like sequences predominated in our HTS data. The known *Bunyaviridae* comprise more than 350 viruses, with many of those being only recently discovered and characterized (10, 19, 48–50). Relatively few nairoviruses have been discovered; Kupe and Finch Creek viruses were the only newly characterized members of this genus reported within the last decade (51, 52). In this study, we demonstrate molecular evidence of a new nairovirus present in ixodid ticks in North America. SBV is the first New World nairovirus with available coding region sequences of the S and L segments. With more than 70% amino acid divergence from other nairoviruses for these segments, it is the most divergent of all nairoviruses analyzed to date. Based on the amino acid analysis of the polymerase region, SBV forms a distinct phylogenetic lineage outside all currently described nairoviruses and does not cluster into any of the nairovirus serogroups.

Our findings indicate that the genus *Nairovirus* is significantly more diverse than previously appreciated. An earlier phylogenetic analysis of nairoviruses indicated the presence of two main phylogenetic clades, with one clade being isolated exclusively from ixodid ticks and the other from argasid ticks (34). The phylogeny is more complex, as SBV represents a previously unidentified, genetically distinct nairovirus clade. The current nairovirus classification scheme, based on a very short sequence fragment and serology, is tentative and is further confounded by the lack of complete genome sequence data for any argasid tick-isolated nairovirus. Future genome sequencing of genetically unclassified nairoviruses, especially those isolated from argasid ticks, may uncover viruses similar to SBV that will aid in the establishment of a more accurate classification system for this genus. Interestingly, during the preparation of the manuscript, a new divergent nairovirus was reported from bats in France, although the genome sequence of this virus was not available for comparison with that of SBV (53).

The detection of SBV in all four surveyed areas suggests that it may have a broad geographical distribution, perhaps throughout the range of *I. scapularis*, comparable to *B. burgdorferi* and other pathogens vectored by this tick. Other nairoviruses have been shown to have broad geographical distributions and have been isolated throughout the range of their hosts.

In addition to SBV, we identified viruses in *I. scapularis* and *D. variabilis* representing novel clades of tick-borne phleboviruses. Historically, phleboviruses were classified into tick-borne or sand fly/mosquito-borne groups based on vector, genomic, and serological relationships (41). The discovery of these highly divergent viruses along with the recent characterization of the atypical Gouleako and Cumuto viruses underscores the potential need to reassess this classification scheme (54, 55). Additionally, in conjunction with the recent identification of *A. americanum* as a host of Heartland and Lone Star viruses, our data also suggest that considerable diversity of phleboviruses may exist in tick species within the United States (56–58).

We discovered that all novel bunyaviruses had high infection rates in ticks. Twenty percent of all ticks from four different loca-

tions were infected with SBV. The rates of infection with BTPV and ADTPV were also high, although the analysis was limited to ticks from a single site. While these rates are higher than is typically reported for arboviruses, nairovirus infection rates of up to 20% have been reported in ticks (59). The highest rate of infection with SBV was observed in ticks from Heckscher State Park, which was also the origin of the ticks used for the high-throughput sequencing study described here. In contrast, Powassan virus was detected in less than 2% of these same ticks (23). This suggests that the infection rate of *I. scapularis* with SBV may be significantly higher than with Powassan virus, at least within the geographical range analyzed. Possible explanations for the high prevalence of these viruses may be the result of a high assortment of susceptible viremic hosts resulting in proficient horizontal transmission of the virus to uninfected ticks. The viruses may also be efficiently transmitted by nonviremic hosts, or by "cofeeding" of infected and uninfected ticks. In addition, as shown for some arboviruses, these viruses may be transstadially (maintained throughout life stages) and transovarially transmitted to progeny, resulting in the tick vector's also playing a role of a long-term reservoir in tick populations (3).

We do not know whether SBV, ADTPV, and BTPV are specific to their tick hosts. Some tick-borne viruses can be isolated from more than one tick species. CCHFV, for example, has been isolated from over 30 tick species and multiple genera throughout Europe, Africa, and Asia, though it is likely that not all of them represent true vectors (9). Although the HTS analysis suggests tick host specificity, we cannot rule out the possibility that other tick species may serve as hosts of these viruses.

The L segment organization of SBV, ADTPV, and BTPV is comparable to that of the L segments of other nairo- and phleboviruses. Conversely, the organization of the presumed S segments of these viruses is at least partially inconsistent with their putative genome affiliation. In these viruses, the S segments are considerably longer and, in the case of SBV, contain a large portion of sequence lacking identifiable ORFs. The N ORFs in ADTPV and BTPV are longer than typical *Phlebovirus* N ORFs, while the putative NSs ORFs are considerably shorter than typical *Phlebovirus* NSs ORFs and may not encode functional proteins. Although this genome arrangement is uncharacteristic, there is a precedent for unconventional *Phlebovirus* S segments, illustrated by the lack of an NSs ORF in Gouleako virus (54). Presumably, continued molecular surveillance will lead to the discovery of other bunyaviruses with atypical genome organization.

Another notable characteristic of these viruses is the lack of recognizable glycoprotein-coding segment. Although we recovered >90% of the L and S segments for SBV and both phleboviruses by HTS and dispelled the possibility of viral integration, we were unable to identify any sequences with similarity to *Bunyaviridae* M segments. In addition, we did not identify any large contigs (>300 nt) containing an uninterrupted ORF that was confirmed by PCR to be present in individual S and L segment-positive ticks but absent in virus-negative ticks. We also performed consensus PCR with numerous primers targeting *Phlebovirus* and *Nairovirus* M segments, without success. To investigate if the depth of sequencing was adequate to detect bunyavirus M segments, we selected a new pool of four adult *I. scapularis* ticks positive for both SBV and BTPV by PCR and analyzed them by Illumina HiSeq sequencing. Despite achieving a 10-fold increase in reads and nearly 100% coverage of S and L segments for both viruses, we again were unable to identify any sequences representative of potential M segments. We have considered various explanations for this confounding result. A complicated secondary structure of the M segments of these viruses may inhibit efficient cDNA synthesis and interfere with amplification (and detection). While it is plausible, we acknowledge such a confounding effect has not been reported for HTS analysis of other bunyaviruses. Alternatively, these viruses may not have a typical bunyavirus-like M segment. While it is incompatible with current understanding of bunyaviruses, the viruses uncovered here may represent viral lineages that do not have a typical bunyaviral M segment and employ other means for cellular entry. Finally, we also cannot exclude the possibility that the S and L segments of these viruses may exist in an episome-like form in tick cells. As such, they may not form an infectious virion but may instead use transovarial transmission or some other means as a vehicle for their dissemination to new hosts.

Our analysis also uncovered a novel mononegavirus in *I. scapularis*, although due to the limited sequence data obtained by HTS, we were limited to the analysis of the L ORF. Our data indicate that this virus may be a novel member of the family *Nyamiviridae*. Of the three viruses in this family, Nyamanini and Midway viruses display a high degree of similarity and are part of the genus *Nyavirus*, while the more divergent soybean cyst nematode virus is a representative of a yet-unnamed genus in the *Nyamiviridae* (47, 60; http://ictvonline.org/virusTaxonomy.asp). The virus uncovered in our study appears to be the most genetically diverse of this group and presumably constitutes a new genus within the *Nyamiviridae*, although a complete genetic characterization will be required to fully determine its taxonomy.

The results of our study indicate that ticks harbor a wide array of highly diverse viruses. One constraint of our study was the limited geographical distribution of the sampled ticks, as our HTS analysis focused on specimens collected from a single location. While we anticipate that many of the viruses uncovered by our HTS analysis are distributed along the range of their presumed tick hosts, we speculate that other agents remain to be discovered, and analysis of ticks from diverse geographical areas would reveal greater viral diversity of tick-borne viruses. We expect that when additional HTS data from diverse arthropod vectors become available, they will have a profound impact on viral taxonomy and will allow more precise elucidation of evolutionary relationships.

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