Putative RNA viral sequences detected in an *Ixodes scapularis*-derived cell line

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Abstract

Ticks harbour various microorganisms, some of which act as pathogens of humans and animals. The recent advancement of genome sequencing technologies revealed that a wide range of previously unrecognised microorganisms exist in ticks. Continuous cell lines established from ticks could play a key role in the isolation of such microorganisms; however, tick cells themselves have been known to harbour symbiotic microorganisms. The present study aimed to characterise putative RNA viral sequences detected in the culture supernatant of one of the most frequently used tick cell lines, ISE6, which was derived from embryos of the blacklegged tick *Ixodes scapularis*. Viral particles purified from the culture supernatant were used for RNA extraction, followed by Illumina sequencing. The reads were *de novo* assembled and the resulting contigs were annotated by tBLASTx search. The results suggested that there were at least five putative viral sequences of four phylogenetically distinct lineages in ISE6 cells. The predominant viral sequence found in ISE6 cells, designated *I. scapularis* iflavirus, was a member of the family Iflaviridae, which is an arthropod-infecting virus group. We also identified L and M segments of the family Bunyaviridae, which could
not be classified into any of the five known genera, and a potential capsid protein related to Drosophila A virus. In addition to these previously unrecognised viruses, ISE6 was revealed to harbour a putative genome sequence of *I. scapularis*-associated virus-1, which was reported in a recent metagenomic study of *I. scapularis* itself. All the five putative viral sequences were detected by RT-PCR in both ISE6 cells and the culture supernatant. Electron microscopic analysis showed the existence of spherical virions with a varying diameter of 50 to 70 nm in the culture supernatant of ISE6 cells. Further studies are required to investigate the potential roles of ISE6-associated viruses in ticks.

**Keywords**

bunyavirus; iflavirus; ISE6; *Ixodes scapularis*; *Ixodes scapularis*-associated virus-1

**Introduction**
Ticks harbour various microorganisms including viruses, bacteria and protozoa, some of which act as pathogens of humans and animals. The recent advancement of genome sequencing technologies enabled microbial community analysis in a culture-independent manner (so-called metagenomic analysis) and revealed that a wide range of previously unrecognised microorganisms exist in ticks (Carpi et al., 2011; Nakao et al., 2013; Qiu et al., 2014; Tokarz et al., 2014). One of the requirements for post-metagenomic studies is the characterisation of individual microorganisms in terms of their potential pathogenicity to humans and animals, their influence on tick biology and their interaction with other microbial linages.

Tick cell lines are key to achieving the above-mentioned objective. First, tick cells are useful for isolation and molecular characterisation of microorganisms harboured by ticks (Bell-Sakyi et al., 2012; 2007). Of note, prolonged culture of some tick-borne pathogens in tick cells facilitates adaptation to mammalian systems (Bekker et al., 2002; Munderloh et al., 2004), indicating that tick cell lines can be better tools for the isolation of microorganisms than mammalian cells. Second, tick cell lines are useful to investigate the interaction between tick-borne microorganisms and ticks in vitro, and
can be valuable tools to evaluate the vector competence of ticks for pathogens (Bell-Sakyi et al., 2012). *In vitro* observation of the replication cycles and intracellular localisation of microorganisms may help with the study of their survival mechanisms in ticks and transmission dynamics in nature.

Since the establishment of the first tick cell line from *Rhipicephalus appendiculatus* (Varma et al., 1975), nearly 60 different cell lines originating from 16 different tick species are now available (Bell-Sakyi et al., 2012). Alberdi et al. (2012) screened for bacterial endosymbionts and endogenous viruses in over 50 tick cell lines. Despite the detection of virus-like particles by transmission electron microscopy (TEM), PCR assays amplifying some known tick-borne viruses failed to detect viral genome sequence in many of the cell lines (Alberdi et al., 2012), suggesting that uncharacterised viruses exist in tick cell lines. In fact, the authors observed reovirus-like particles in ISE6 cells by TEM, while PCR was negative for St Croix River virus (SCRV), the only previously characterised tick virus (Alberdi et al., 2012; Attoui et al., 2001).

This paper reports viral genome sequences obtained from one of the most frequently used tick cell lines, ISE6, which was derived from embryos of the
blacklegged tick *Ixodes scapularis* (Kurtti et al., 1996). Initially, we recognised the existence of some virus sequences in the culture supernatant of ISE6 cells when this cell line was employed for isolation and characterisation of novel tick-borne viruses in field-collected ticks. High-throughput RNA sequencing analysis revealed that there are at least five putative virus sequences from four phylogenetically different virus lineages in this cell line.

**Materials and Methods**

**Cell line.**

The ISE6 cell line was kindly provided by the CEH Institute of Virology and Environmental Microbiology (Oxford, UK). The use of ISE6 cells was authorized by the University of Minnesota, the original provider of the cell line, under a material transfer agreement between the University of Minnesota and Hokkaido University. The cells were cultured in L-15B medium supplemented with 10% fetal calf serum, 5% tryptose phosphate broth (Sigma–Aldrich, St. Louis, MO, USA) and 0.1% bovine lipoprotein concentrate (MP Biomedicals, Irvine, CA, USA) at 32°C as described by
Virus purification.

A total of 200 mL of the culture supernatant of ISE6 cells was obtained at 7 days after the last passage. The supernatant was centrifuged at 190 g for 10 min at 4°C to remove cell debris. The supernatant was collected and the virus particles were pelleted through a 20% sucrose cushion at 28,000 rpm for 1.5 h with a Beckman SW41 rotor at 4°C. The pellet was re-suspended in phosphate-buffered saline and the purified virus was stored at –80°C until use.

TEM.

ISE6 culture supernatant and culture medium (as a negative control) were subjected to low-speed centrifugation followed by ultracentrifugation as described above. The pelleted was fixed with 0.25% glutaraldehyde in PBS for 1 week at 4°C. Fixed samples supplemented with 0.1% bovine serum albumin were adsorbed to collodion-carbon-coated copper grids and negatively stained for 30 s with 2% phosphotungstic acid solution (pH 5.8). Virus particles were observed under an H-7650 TEM (Hitachi, Tokyo, Japan) at 100 kV. For ultrathin sections, ISE6 cells were pelleted
by centrifugation at 190 g for 10 min at 4°C, and fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 30 minutes. The fixed pellet was washed with cacodylate buffer, postfixed with 2% osmium tetroxide in the cacodylate buffer for 1 hour at 4°C, dehydrated with a series of ethanol gradients followed by propylene oxide, embedded in Epon 812 Resin mixture (TAAB Laboratories Equipment Ltd., Aldermaston, UK), and polymerized at 60°C for 2 days. Ultrathin sections (70 nm) were stained with EM stainer (Nissin EM, Tokyo, Japan) and lead citrate and examined with an H-7650 TEM at 80 kV.

**High-throughput RNA sequencing.**

RNA extracted from the pellet using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) was subjected to double-stranded complementary DNA (cDNA) synthesis using the cDNA Synthesis Kit (M-MLV Version; TaKaRa Bio Inc., Shiga, Japan). The double-stranded cDNA was sequenced using the Illumina MiSeq v3 system (Illumina, San Diego, CA, USA) with paired-end reads (2 × 300 nucleotides (nt)) following the manufacturer’s protocol. The Illumina sequencing data were deposited in EMBL/GenBank/DDBJ under the accession number DRA003818.
PCR and Sanger sequencing.

To examine the existence of putative viral sequences in the ISE6 cells and the culture supernatant, conventional PCR was performed on the cDNA generated from the total RNA extracted from both ISE6 cells and the culture supernatant. As a negative control, total RNA was extracted from the culture medium prior to use in maintenance of ISE6 cells. PCR was also performed using genomic DNA (gDNA) extracted from ISE6 cells to investigate whether the viral sequences are incorporated into the tick genome. Total RNA was extracted from the ISE6 cells and the culture supernatant/medium using, respectively, the NucleoSpin RNA II (TaKaRa Bio Inc.) and the QIAamp Viral RNA Mini Kit. The RNA was treated with DNase I (New England Biolabs, Ipswich, MA, USA) and subjected to cDNA synthesis using the PrimeScript II 1st strand cDNA Synthesis Kit (TaKaRa Bio Inc.). gDNA was extracted from ISE6 cells using the NucleoSpin Tissue kit (TaKaRa Bio Inc.). The PCR primers used in this study are listed in Table 1. All PCR reactions were performed in a total volume of 25 μL containing 12.5 μL of 2× Gflex PCR Buffer (TaKaRa Bio Inc.), 0.5 μL of each primer (10 μM), 0.5 μL of Tks Gflex DNA Polymerase (TaKaRa Bio Inc.) and 1.0 μL of
template cDNA or gDNA. Sanger sequencing of the amplified products was performed using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an ABI Prism 3130x genetic analyser (Applied Biosystems).

**Data analysis.**

After trimming low-quality reads and PhiX control reads, the resulting reads were *de novo* assembled using CLC Genomics Workbench (version 4.9). The contigs with high coverage (> ×100) were initially screened for homology to known viruses by local BLASTn and tBLASTx searches against the NCBI Reference Sequence (RefSeq) database (http://www.ncbi.nlm.nih.gov/) (e-value < 10⁻⁴). The potential viral sequences were then compared with the draft genome sequence of *I. scapularis* (GenBank no. NZ_ABJB010000000) to filter out sequences possibly derived from the tick genome. In the search for sequences of M and S segments of a novel bunyavirus, a custom database comprising the sequences of all known Bunyaviridae deposited in GenBank was employed.

**Multiple sequence alignment and phylogenetic analysis.**

The nucleotide sequences were aligned together with representative sequences
of other known relevant viruses available in GenBank using MUSCLE as implemented in MEGA (version 6) (Tamura et al., 2013). After manual modification of the alignments, phylogenetic trees were constructed using the neighbour-joining method with 1,000 bootstrap replicates, using MEGA.

Results

Detection of putative viral sequences.

Out of 5,316,590 raw sequence reads (average length = 289.6 nt) generated from Miseq, 4,873,495 (average length = 238.2 nt) were used for de novo assembly after trimming low-quality reads/bases and PhiX control reads. The number of contigs generated with high coverage (> ×100) was 84. A total of 19 contigs showed similarity with the reference sequences of the virus database by tBLASTx search. After comparison with the *I. scapularis* genome, four contigs were initially determined as virus sequences (Table 2). These four contigs were not annotated by BLASTn search, indicating that nucleotide homologies with viruses deposited in the RefSeq database are low.
TEM analysis showed the existence of spherical virions with diameters ranging from 50 to 70 nm in the culture supernatant of ISE6 cells. These virions had spikes on their surface and apparently lacked an envelope (Figures 1A and 1B). It was difficult to discriminate between virions by their size and surface structure observed by TEM. No similar structures were observed in the culture medium (Figures 1C and 1D). In ultrathin sections of ISE6 cells, numerous spherical virion-like structures were observed in the cytoplasm (Figures 1E and 1F).

**Iflaviruse.**

Approximately 20% of the reads were mapped to contig 1, which was predicted to be a member of the Iflaviruse genus by tBLASTx search (Table 2). This contig is composed of 9,252 nt, excluding the 3’-terminal poly(A), and contains a single open reading frame (ORF) encoding a polyprotein of 2,991 amino acids (aa) following an incomplete internal ribosome entry site. Six conserved domains associated with viral RNA replication were identified by InterProScan (Jones et al., 2014): three picornavirus capsid-like domains at aa positions 405–620, 698–903 and 1,071–1,288, a RNA
helicase domain at aa positions 1,650–1,820, a peptidase domain at aa positions 2,223–
2,423 and an RNA-dependent RNA polymerase (RdRp) domain at aa positions 2,471–
2,956 (Figure 2A), which are well conserved throughout the genus Iflavirus. The
phylogenetic analysis also grouped this novel virus, tentatively designated *Ixodes*
*scapularis* iflavirus (ISIV), into the genus Iflavirus (Figure 3). Because the aa sequence
identity between the capsid proteins of ISIV and the other iflaviruses is less than 90%
(data not shown), ISIV would constitute a novel species in the genus Iflavirus following
the criteria described elsewhere (Chen et al., 2012).

**Bunyavirus.**

Contig 2 (9,147 nt) was assigned as a putative viral sequence related to the L
segment of a bunyavirus (Table 2). Contig 2 encodes a single ORF (2,978 aa), which
comprises a bunyavirus RdRp domain at aa positions 880–1,636 (Figure 2B). This
putative viral sequence was clustered together with an unclassified virus, Wenzhou
Shrimp Virus 2 (LC094424) which was recognised in RNA deep sequencing of shrimps
(*Penaeus monodon* and *Exopalaemon carinicauda*) (Li et al., 2015) in a phylogenetic
tree (Figure 4A). To detect the M and S segments of the virus tentatively designated
Ixodes scapularis bunyavirus (ISBV), all the contigs (n = 3,075), including those with low coverage, were compared with all the sequences of known Bunyaviridae deposited in GenBank. This successfully recovered the M segment (contig 5 (coverage = ×93)), while the S segment was not detected. Contig 5 is 4,321 nt in length, encoding a single coding sequence of the putative glycoprotein precursor (1,350 aa) (Figure 2B), which is also grouped together with Wenzhou Shrimp Virus 2 in phylogenetic analysis (Figure 4B).

Ixodes scapularis-associated virus-1 (ISAV-1).

Contig 3 was initially associated with turnip rosette virus (NC_004553.3) in the genus Sobemovirus by tBLASTx search against the RefSeq database. A further similarity search against all sequences deposited in GenBank revealed that this sequence has the highest similarity (98% nucleotide identity) with the partial genome sequence of ISAV-1 isolate K13 (KM048318) in the same genus, which was recovered from field-captured I. scapularis through a metagenomic study (Tokarz et al., 2014). The contig sequence encodes a putative protease (540 aa) and RdRp (313 aa), both of which showed the highest similarities (97%) with those encoded by ISAV-1.
Unclassified virus. We identified that contig 4 of 1,485 nt encodes a putative viral capsid protein (411 aa) with similarity to an unclassified positive-strand RNA virus, Drosophila A virus (DAV). We could not find other fragments of this virus among all the contigs, including those with low coverage (<×100).

PCR. PCR was performed on the cDNA generated from the total RNA extracted from ISE6 cells, the culture supernatant, and the culture medium and gDNA extracted from ISE6 cells. All the putative viral sequences were detected in both ISE6 cells and the culture supernatant but not in the culture medium (Figure 5). When gDNA was included as a template, no amplification was observed except for the reaction with primers amplifying tick mitochondrial 16S rRNA gene (rDNA). Sanger sequencing of the PCR products confirmed the specific amplification of each target (data not shown).

Discussion

The present study demonstrated that at least five distinct putative viral
sequences existed in a tick cell line, ISE6. In comparison with bacterial detection, where universal primers are available to amplify variable regions of bacterial 16S rDNA, a search for previously uncharacterised viruses has been hampered by the lack of proper detection tools, and little is known about viruses infecting ticks and tick cell lines (Alberdi et al., 2012; Attoui et al., 2001). The use of high-throughput sequencing technology sheds light on these poorly characterised viruses.

To our surprise, we recovered the sequences of L and M segments of one or more putative viruses of the family Bunyaviridae from the culture supernatant of ISE6 cells (Table 2). Several members of this virus family have been recognised as tick-borne viruses of medical and veterinary importance. The family is currently divided into five genera (Orthobunyavirus, Hantavirus, Nairovirus, Phlebovirus and Tospovirus) based on antigenic, structural and genetic characteristics (Plyusnin et al., 2012). As evidenced by the finding of Gouléako virus in mosquitoes in Côte d’Ivoire (Marklewitz et al., 2011), bunyaviruses that cannot be classified into any of the five known genera have also come to attention. Recent metagenomic studies of arthropod-borne viruses led to the discovery of many such unclassified bunyaviruses (Ballinger et al., 2014; Webster et
al., 2015). Based on the sequences found, the closest relative of ISBV is Wenzhou Shrimp Virus 2, which is one of the unclassified bunyaviruses found in a metagenomic study of Chinese shrimps (Li et al., 2015), and thus its phylogenetic position is not clearly understood. Despite an intensive search using a custom database comprising the sequences of all known Bunyaviridae deposited in GenBank, we could not find the sequence of the potential S segment of ISBV. Similarly, Li et al. (2015) failed to detect the sequence of putative S segment of Wenzhou Shrimp Virus 2 in the deep sequencing data.

The predominant putative virus found in ISE6 cells was a member of the family Iflaviridae, which is an arthropod-infecting virus group with a single-stranded RNA genome. Along with the advancement of next-generation sequencing techniques, an increasing number of iflaviruses are being reported in various arthropod species even in laboratory colonies (Liu et al., 2015); however, there is no report to date on the detection of this virus group from ticks. Some iflaviruses have occasionally been associated with adverse effects on the survival of arthropod hosts (Martin et al., 2012; Silva et al., 2015; Valles and Hashimoto 2009). For example, deformed wing virus,
which is an iflavivirus commonly infecting the European honeybee (*Apis mellifera*) asymptomatically with low viral loads (de Miranda and Genersch, 2010), causes morphological deformity and reduces the life span with elevated virus loads when transmitted by the ectoparasitic mite *Varroa destructor* (de Miranda and Fries, 2008; Dainat et al., 2012). Further studies are required to investigate the role of ISIV *in vivo* and to assess the prevalence of ISIV and its related viruses in natural populations of ticks.

In addition to the previously unrecognised viruses, ISE6 was revealed to harbour partial sequence of the putative virus ISAV-1, which was recently discovered in *I. scapularis* collected at Heckscher State Park in the United States (Suffolk County, NY) (Tokarz et al., 2014), where the authors identified a partial genome sequence (2.8 kb) of ISAV-1 encompassing the majority of a putative protease and an RdRp without information on its capsid protein. A recent metagenomic study reported the detection of a virus closely related to ISAV-1, designated *Humaita-Tubiacanga* virus (HTV), in a laboratory colony of the mosquito *Aedes aegypti* (Aguiar et al., 2015). The authors detected a sequence corresponding to the RdRp of HTV together with one viral segment
(1,609 nt) predicted to encode a protein with a coat domain, with the highest similarity to a capsid protein of DAV. As supported by further evidence of small RNA profiles and simultaneous detection by RT-PCR in individual mosquitoes, the authors proposed that the capsid protein related to DAV is associated with the RdRp of HTV. Likewise, we detected one virus sequence encoding a putative viral capsid protein with the highest similarity to DAV in ISE6 cells, while a sequence corresponding to the RdRp of DAV was absent. These facts collectively support the possible association of ISA V-1 with a capsid protein related to DAV.

An initial scan for potential virus sequences detected 19 contigs. Because the rest of the virus-like sequences showed high similarity with draft genome sequences of *I. scapularis*, we suspected that they are integrated in the genome of *I. scapularis* and thus did not include them in the further analysis. It is, however, possible that some of them were derived from endogenous viruses infecting the *I. scapularis* Wikel strain used in the whole genome sequencing project (Gulia-Nuss et al., 2016). As reported elsewhere, some arthropods have acquired a number of virus-like sequences in their genomes during evolution (Ballinger et al., 2014; Katzourakis and Gifford, 2010; Liu et al., 2010).
It is therefore important to perform paleovirolological analysis of arthropods with a process of \textit{in silico} identification of endogenous virus elements incorporated into the host genome.

Considering the high abundance of viral sequences associated with ISIV in the sequencing data, the majority of the virions observed in the culture supernatant by TEM might be expected to be ISIV (Figure 1). However, the morphological features of the particles were different from the virion properties of the genus Iflavirus, which are approximately 30 nm in diameter with no distinctive surface structures (Chen et al., 2012). Since ISAV-1 was only reported from a metagenomic study (Tokarz et al., 2014), the morphological features of ISAV-1 are completely unknown. The size of the only characterised tick virus, SCRV, is approximately 55 nm in diameter (Attoui et al., 2001), which is comparable to that of the virions observed in this study (between 50 and 70 nm in diameter). It is unfortunate that we could not differentiate the virions with a varying diameter due to the similarity of their surface structures and the resolution limits of TEM. Further studies using immune-electron microscopy are required to determine the morphological structure of each virion in ISE6 cells.
The role of viruses in tick cells is totally unknown. There is some evidence in mosquito cell lines that some persistently infecting viruses, for example C6/36 densovirus (Parvoviridae), cell-fusing agent virus (Flaviviridae) and *Aedes pseudoscutellaris* reovirus (Reoviridae), modulate exogenous viruses’ replication and vice versa, possibly in part through regulation of host antiviral immune responses (Morazzani et al., 2012). Hence, the experimental outcomes using ISE6 cells, which might contain several putative endogenous viruses, should be carefully considered. Because we characterised the putative viral sequences in ISE6 cells, it becomes possible to determine the genome sequences of the viruses isolated in ISE6 cells by removing the sequencing reads from putative viruses by *in silico* analysis. In fact, we have determined whole genomes of previously uncharacterized bunyaviruses, which can be propagate in ISE6 cells but not in mammalian cell lines (manuscript in preparation). It might be worth trying to generate virus-free cell lines by interfering with replication of putative viruses in ISE6 cells based on siRNA silencing technology (Bell-Sakyi and Attoui, 2013).

In conclusion, the present study reports five putative viral sequences from
four phylogenetically different virus lineages in ISE6 cells. Unfortunately, since we could not test the presence of these putative viral sequences in ISE6 cells maintained in other laboratories, we cannot rule out the possibility that the ISE6 cells used in this study had been contaminated with the putative viruses during passage in our laboratory or the provider’s laboratory. Considering the increasing importance of tick cell lines in a wide range of research on ticks and tick-borne pathogens, a comprehensive search for tick-associated viruses not only in ISE6 cells but also in other tick cell lines is required. Expanding knowledge of the diversity of tick-borne pathogens as well as tick-associated viruses may lead to better understanding of the tripartite interaction between pathogenic microorganisms, non-pathogenic microorganisms and ticks.

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References


Miller, J.R., Walenz, B.P., Koren, S., Hostetler, J.B., Thiagarajan, M., Joardar, V.S.,

Hannick, L.I., Bidwell, S., Hammond, M.P., Young, S., Zeng, Q., Abrudan, J.L.,

Almeida, F.C., Ayllón, N., Bhide, K., Bissinger, B.W., Bonzon-Kulichenko, E.,

Buckingham, S.D., Caffrey, D.R., Caimano, M.J., Croset, V., Driscoll, T., Gilbert,

D., Gillespie, J.J., Giraldo-Calderón, G.I., Grabowski, J.M., Jiang, D., Khalil,

S.M.S., Kim, D., Kocan, K.M., Koči, J., Kuhn, R.J., Kurtti, T.J., Lees, K., Lang,

E.G., Kennedy, R.C., Kwon, H., Perera, R., Qi, Y., Radolf, J.D., Sakamoto, J.M.,

Sánchez-Gracia, A., Severo, M.S., Silverman, N., Šimo, L., Tojo, M., Tornador, C.,


J., Arensburger, P., Pietrantonio, P.V., Barker, S.C., Shao, R., Zdobnov, E.M.,

Hauser, F., Grimmelikhuijzen, C.J.P., Park, Y., Rozas, J., Benton, R., Pedra, J.H.F.,

Nelson, D.R., Unger, M.F., Tubio, J.M.C., Tu, Z., Robertson, H.M., Shumway, M.
Sutton, G., Wortman, J.R., Lawson, D., Wikel, S.K., Nene, V.M., Fraser, C.M.,


Varma, M.G., Pudney, M., Leake, C.J., 1975. The establishment of three cell lines from
the tick *Rhipicephalus appendiculatus* (Acari: Ixodidae) and their infection with

Webster, C.L., Waldron, F.M., Robertson, S., Crowson, D., Ferrari, G., Quintana, J.F.,
Brouqui, J.-M., Bayne, E.H., Ben Longdon, Buck, A.H., Lazzaro, B.P., Akorli, J.,
Haddrell, P.R., Obbard, D.J., 2015. The discovery, distribution, and evolution of

**Legends to Figures and Tables**

**Figure 1.** Electron micrographs of virions observed in the supernatant and
ultrathin section of ISE6 cells.

(A) Virions purified from the culture supernatant of ISE6 cells, (B) enlarged image of a
virion, (C) cell culture medium only, and (D) enlarged image of culture medium only
were observed by an H-7650 transmission electron microscope (Hitachi, Tokyo, Japan)
at 100 kV. (E) Ultrathin section of an ISE6 cell and (F) enlarged image of virion-like
structures in the ISE6 cell. The arrowheads indicate the virions.
Figure 2. Predicted genome organisations of *Ixodes scapularis* iflavirus (A) and *Ixodes scapularis* bunyavirus (B).

*De novo* assembled contigs were subjected to an ORF search, and the putative amino acid sequences were used for a conserved domain search with InterProScan. Each domain is indicated by a grey bar, and the name is shown beneath the bar.

Figure 3. Phylogenetic tree of the genus Iflavirus.

The full-length sequence of the *Ixodes scapularis* iflavirus (contig 1) (shown underlined) was used for phylogenetic analysis with the other iflaviruses. Drosophila C virus in the family Dicistroviridae was deployed as an outgroup. Bootstrap probabilities higher than 70% are indicated near the branches. The scale bar indicates 0.5 changes per nucleotide position.

Figure 4. Phylogenetic trees of the family Bunyaviridae.

The full-length sequences of the *Ixodes scapularis* bunyavirus (contigs 2 and 5) (shown
underlined) were used for phylogenetic analyses with the other bunyavirus L (A) and M segments (B), respectively. Bootstrap probabilities higher than 70% are indicated near the branches. The scale bars indicate 0.5 changes per nucleotide position.

Figure 5. PCR amplification of each putative viral sequence and tick mitochondrial 16S rRNA gene.

PCR was performed on the cDNA generated from the total RNA extracted from ISE6 cells (1), the culture supernatant (2), and the culture medium (3) and gDNA extracted from ISE6 cells (4). ISIV, ISB_L, ISB_M, ISAV-1, ISUV, and 16S rDNA represent PCR reactions with primers amplifying *Ixodes scapularis* iflavirus, L segment of *Ixodes scapularis* bunyavirus, M segment of *Ixodes scapularis* bunyavirus, *Ixodes scapularis* associated virus-1, unclassified virus related to Drosophila A virus, and tick mitochondrial 16S rRNA gene (rDNA), respectively. All the five putative viral sequences were detected in both ISE6 cells and the culture supernatant while only tick mitochondrial 16S rDNA was amplified from gDNA. Lane M shows a 100-bp DNA marker.
Table 1. Oligonucleotide primers used for PCR amplification of each putative viral sequence and tick mitochondrial 16S rRNA gene sequence.

Table 2. Summary of putative partial viral genome sequences identified in ISE6 cells.
Ixodes scapularis iflavirus

A

Ixodes scapularis iflavirus

<table>
<thead>
<tr>
<th>L segment</th>
<th>M segment</th>
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<tbody>
<tr>
<td>3′ → 5′</td>
<td>3′ → 5′</td>
</tr>
<tr>
<td>9,252 nt</td>
<td>9,147 nt</td>
</tr>
<tr>
<td>IRES (incomplete)</td>
<td>RdRp domain</td>
</tr>
<tr>
<td>Polyprotein (2,991 aa)</td>
<td>Glycoprotein precursor (1,350 aa)</td>
</tr>
<tr>
<td>Capsid proteins</td>
<td>Gn, Gc</td>
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<td>Helicase</td>
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<td>Peptidase</td>
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<tr>
<td>RdRp</td>
<td></td>
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</table>

B

Ixodes scapularis bunyavirus

L segment

3′ → 5′
9,147 nt
L (2,978 aa)
RdRp domain

M segment

3′ → 5′
4,321 nt
Glycoprotein precursor (1,350 aa)
Gn, Gc

Fig. 2
Table 1. Oligonucleotide primers used for PCR amplification of each putative viral sequence and tick mitochondrial 16S rRNA gene sequence.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’ to 3’)</th>
<th>Target</th>
<th>Amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISIV_F</td>
<td>GGGCGTGGTAAATGTGGTA</td>
<td><em>Ixodes scapularis</em> iflavivirus</td>
<td>437</td>
<td>This study</td>
</tr>
<tr>
<td>ISIV_R</td>
<td>AACAATCCCGGATGAAAGGTT</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>ISB_L_F</td>
<td>GCATTGTGCAGCCTCTCTCA</td>
<td><em>Ixodes scapularis</em> bunyavirus, L segment</td>
<td>547</td>
<td>This study</td>
</tr>
<tr>
<td>ISB_L_R</td>
<td>GTCTCCAGAAACAGGCTTGC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ISB_M_F</td>
<td>CTGGCACAATCCTTTTGTT</td>
<td><em>Ixodes scapularis</em> bunyavirus, M segment</td>
<td>571</td>
<td>This study</td>
</tr>
<tr>
<td>ISB_M_R</td>
<td>TTCTCTCTCAGCCCTGGTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ISAV-1_F</td>
<td>AACGTGTACGGAATGCACCT</td>
<td><em>Ixodes scapularis</em> associated virus-1, RdRP</td>
<td>383</td>
<td>This study</td>
</tr>
<tr>
<td>ISAV-1_R</td>
<td>GTTTGCCAGAAACTGGCCAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ISUV_F</td>
<td>GACCACGGACACCAGTTTCT</td>
<td>Unclassified virus, capsid precursor</td>
<td>338</td>
<td>This study</td>
</tr>
<tr>
<td>ISUV_R</td>
<td>GGTCAGACAGATAGGTCGCC</td>
<td></td>
<td></td>
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<tr>
<td>16S+1</td>
<td>CTGCTCAATGATTTTTTTTAAAATTGCTGG</td>
<td>Tick mitochondrial 16S rRNA gene</td>
<td>460</td>
<td>Black and Piesman, 1994</td>
</tr>
<tr>
<td>16S−1</td>
<td>CCGTCTGACTCAGGATCAAGTA</td>
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</tbody>
</table>
**Table 2. Summary of putative partial viral genome sequences identified in ISE6 cells.**

<table>
<thead>
<tr>
<th>Contig ID</th>
<th>Length (bp)</th>
<th>Average coverage</th>
<th>Virus family</th>
<th>Provisional virus name</th>
<th>Closest relative (aa identity)</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9,252(^a)</td>
<td>24,785</td>
<td><em>Iflaviridae</em></td>
<td><em>Ixodes scapularis</em> iflavirus</td>
<td><em>Formica exsecta</em> virus 2 (25%)</td>
<td>LC094426</td>
</tr>
<tr>
<td>2</td>
<td>9,147</td>
<td>172</td>
<td><em>Bunyaviridae</em></td>
<td><em>Ixodes scapularis</em> bunyavirus, L segment</td>
<td>Wenzhou Shrimp Virus 2 (25%)</td>
<td>LC094424</td>
</tr>
<tr>
<td>3</td>
<td>2,686</td>
<td>934</td>
<td>Unassigned</td>
<td><em>Ixodes scapularis</em>-associated virus-1, peptidase and RdRp</td>
<td><em>Ixodes scapularis</em>-associated virus-1 (98%)</td>
<td>LC094964</td>
</tr>
<tr>
<td>4</td>
<td>1,485</td>
<td>378</td>
<td>Unassigned</td>
<td>Unclassified virus, capsid precursor</td>
<td>Drosophila A virus (36%)</td>
<td>LC094965</td>
</tr>
<tr>
<td>5</td>
<td>4,321</td>
<td>93</td>
<td><em>Bunyaviridae</em></td>
<td><em>Ixodes scapularis</em> bunyavirus, M segment</td>
<td>Wenzhou Shrimp Virus 2 (25%)</td>
<td>LC094425</td>
</tr>
</tbody>
</table>

\(^a\)Contig size without polyA.