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

Ryo Nakao[1,2], Keita Matsuno[3,4], Yongjin Qiu[5], Junki Maruyama[6], Nao Eguchi[6], Naganori Nao[6], Masahiro Kajihara[6], Kentaro Yoshii[7], Hirofumi Sawa[4,8,9,10], Ayato Takada[4,6,9], Chihiro Sugimoto[4,5,9]

[1]Unit of Risk Analysis and Management, Hokkaido University Research Center for Zoonosis Control, Sapporo, Japan

[2]Laboratory of Parasitology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan

[3]Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan

[4]Global Institution for Collaborative Research and Education, Hokkaido University, Sapporo, Japan

[5]Division of Collaboration and Education, Hokkaido University Research Center for Zoonosis Control, Sapporo, Japan

17 [6]Division of Global Epidemiology, Hokkaido University Research Center for Zoonosis

18 Control, Sapporo, Japan

19 [7]Laboratory of Public Health, Department of Disease Control, Graduate School of

20 Veterinary Medicine, Hokkaido University, Sapporo, Japan

21 [8]Division of Molecular Pathobiology, Hokkaido University Research Center for

22 Zoonosis Control, Sapporo, Japan

23 [9]Department of Disease Control, School of Veterinary Medicine, University of Zambia,

24 Lusaka, Zambia

25 [10]Global Virus Network, Baltimore, USA

26

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

59 Ticks harbour various microorganisms including viruses, bacteria and
60 protozoa, some of which act as pathogens of humans and animals. The recent
61 advancement of genome sequencing technologies enabled microbial community
62 analysis in a culture-independent manner (so-called metagenomic analysis) and revealed
63 that a wide range of previously unrecognised microorganisms exist in ticks (Carpi et al.,
64 2011; Nakao et al., 2013; Qiu et al., 2014; Tokarz et al., 2014). One of the requirements
65 for post-metagenomic studies is the characterisation of individual microorganisms in
66 terms of their potential pathogenicity to humans and animals, their influence on tick
67 biology and their interaction with other microbial linages.
68 Tick cell lines are key to achieving the above-mentioned objective. First, tick
69 cells are useful for isolation and molecular characterisation of microorganisms
70 harboured by ticks (Bell-Sakyi et al., 2012; 2007). Of note, prolonged culture of some
71 tick-borne pathogens in tick cells facilitates adaptation to mammalian systems (Bekker
72 et al., 2002; Munderloh et al., 2004), indicating that tick cell lines can be better tools for
73 the isolation of microorganisms than mammalian cells. Second, tick cell lines are useful
74 to investigate the interaction between tick-borne microorganisms and ticks *in vitro*, and

75 can be valuable tools to evaluate the vector competence of ticks for pathogens
76 (Bell-Sakyi et al., 2012). *In vitro* observation of the replication cycles and intracellular
77 localisation of microorganisms may help with the study of their survival mechanisms in
78 ticks and transmission dynamics in nature.

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

89 This paper reports viral genome sequences obtained from one of the most
90 frequently used tick cell lines, ISE6, which was derived from embryos of the

91 blacklegged tick *Ixodes scapularis* (Kurtti et al., 1996). Initially, we recognised the
92 existence of some virus sequences in the culture supernatant of ISE6 cells when this cell
93 line was employed for isolation and characterisation of novel tick-borne viruses in
94 field-collected ticks. High-throughput RNA sequencing analysis revealed that there are
95 at least five putative virus sequences from four phylogenetically different virus lineages
96 in this cell line.
97
98 **Materials and Methods**
99 **Cell line.**
100 The ISE6 cell line was kindly provided by the CEH Institute of Virology and
101 Environmental Microbiology (Oxford, UK). The use of ISE6 cells was authorized by
102 the University of Minnesota, the original provider of the cell line, under a material
103 transfer agreement between the University of Minnesota and Hokkaido University. The
104 cells were cultured in L-15B medium supplemented with 10% fetal calf serum, 5%
105 tryptose phosphate broth (Sigma–Aldrich, St. Louis, MO, USA) and 0.1% bovine
106 lipoprotein concentrate (MP Biomedicals, Irvine, CA, USA) at 32°C as described by

107 Munderloh and Kurtti (1989).

108 **Virus purification.**

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

115 **TEM.**

116 ISE6 culture supernatant and culture medium (as a negative control) were
117 subjected to low-speed centrifugation followed by ultracentrifugation as described
118 above. The pelleted was fixed with 0.25% glutaraldehyde in PBS for 1 week at 4°C.
119 Fixed samples supplemented with 0.1% bovine serum albumin were adsorbed to
120 collodion-carbon-coated copper grids and negatively stained for 30 s with 2%
121 phosphotungstic acid solution (pH 5.8). Virus particles were observed under an H-7650
122 TEM (Hitachi, Tokyo, Japan) at 100 kV. For ultrathin sections, ISE6 cells were pelleted

123 by centrifugation at 190 g for 10 min at 4°C, and fixed with 2.5% glutaraldehyde in 0.1
124 M cacodylate buffer (pH 7.4) for 30 minutes. The fixed pellet was washed with
125 cacodylate buffer, postfixed with 2% osmium tetroxide in the cacodylate buffer for 1
126 hour at 4°C, dehydrated with a series of ethanol gradients followed by propylene oxide,
127 embedded in Epon 812 Resin mixture (TAAB Laboratories Equipment Ltd.,
128 Aldermaston, UK), and polymerized at 60°C for 2 days. Ultrathin sections (70 nm) were
129 stained with EM stainer (Nissin EM, Tokyo, Japan) and lead citrate and examined with
130 an H-7650 TEM at 80 kV.

131 **High-throughput RNA sequencing.**

132 RNA extracted from the pellet using the QIAamp Viral RNA Mini Kit
133 (Qiagen, Hilden, Germany) was subjected to double-stranded complementary DNA
134 (cDNA) synthesis using the cDNA Synthesis Kit (M-MLV Version; TaKaRa Bio Inc.,
135 Shiga, Japan). The double-stranded cDNA was sequenced using the Illumina MiSeq v3
136 system (Illumina, San Diego, CA, USA) with paired-end reads (2 × 300 nucleotides
137 (nt)) following the manufacturer's protocol. The Illumina sequencing data were
138 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

155 template cDNA or gDNA. Sanger sequencing of the amplified products was performed
156 using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems,
157 Foster City, CA, USA) and an ABI Prism 3130x genetic analyser (Applied Biosystems).

158 **Data analysis.**

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

169 **Multiple sequence alignment and phylogenetic analysis.**

170 The nucleotide sequences were aligned together with representative sequences

171 of other known relevant viruses available in GenBank using MUSCLE as implemented
172 in MEGA (version 6) (Tamura et al., 2013). After manual modification of the
173 alignments, phylogenetic trees were constructed using the neighbour-joining method
174 with 1,000 bootstrap replicates, using MEGA.
175
176 **Results**
177 **Detection of putative viral sequences.**
178 Out of 5,316,590 raw sequence reads (average length = 289.6 nt) generated
179 from Miseq, 4,873,495 (average length = 238.2 nt) were used for *de novo* assembly
180 after trimming low-quality reads/bases and PhiX control reads. The number of contigs
181 generated with high coverage (> ×100) was 84. A total of 19 contigs showed similarity
182 with the reference sequences of the virus database by tBLASTx search. After
183 comparison with the *I. scapularis* genome, four contigs were initially determined as
184 virus sequences (Table 2). These four contigs were not annotated by BLASTn search,
185 indicating that nucleotide homologies with viruses deposited in the RefSeq database are
186 low.

187 **TEM.**

188 TEM analysis showed the existence of spherical virions with diameters
189 ranging from 50 to 70 nm in the culture supernatant of ISE6 cells. These virions had
190 spikes on their surface and apparently lacked an envelope (Figures 1A and 1B). It was
191 difficult to discriminate between virions by their size and surface structure observed by
192 TEM. No similar structures were observed in the culture medium (Figures 1C and 1D).
193 In ultrathin sections of ISE6 cells, numerous spherical virion-like structures were
194 observed in the cytoplasm (Figures 1E and 1F).

195 **Iflavirus.**

196 Approximately 20% of the reads were mapped to contig 1, which was
197 predicted to be a member of the Iflavirus genus by tBLASTx search (Table 2). This
198 contig is composed of 9,252 nt, excluding the 3'-terminal poly(A), and contains a single
199 open reading frame (ORF) encoding a polyprotein of 2,991 amino acids (aa) following
200 an incomplete internal ribosome entry site. Six conserved domains associated with viral
201 RNA replication were identified by InterProScan (Jones et al., 2014): three picornavirus
202 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

219 *Ixodes scapularis* bunyavirus (ISBV), all the contigs (n = 3,075), including those with
220 low coverage, were compared with all the sequences of known Bunyaviridae deposited
221 in GenBank. This successfully recovered the M segment (contig 5 (coverage = ×93)),
222 while the S segment was not detected. Contig 5 is 4,321 nt in length, encoding a single
223 coding sequence of the putative glycoprotein precursor (1,350 aa) (Figure 2B), which is
224 also grouped together with Wenzhou Shrimp Virus 2 in phylogenetic analysis (Figure
225 4B).

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

227 Contig 3 was initially associated with turnip rosette virus (NC_004553.3) in
228 the genus Sobemovirus by tBLASTx search against the RefSeq database. A further
229 similarity search against all sequences deposited in GenBank revealed that this sequence
230 has the highest similarity (98% nucleotide identity) with the partial genome sequence of
231 ISAV-1 isolate K13 (KM048318) in the same genus, which was recovered from
232 field-captured *I. scapularis* through a metagenomic study (Tokarz et al., 2014). The
233 contig sequence encodes a putative protease (540 aa) and RdRp (313 aa), both of which
234 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

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

257 To our surprise, we recovered the sequences of L and M segments of one or
258 more putative viruses of the family Bunyaviridae from the culture supernatant of ISE6
259 cells (Table 2). Several members of this virus family have been recognised as tick-borne
260 viruses of medical and veterinary importance. The family is currently divided into five
261 genera (Orthobunyavirus, Hantavirus, Nairovirus, Phlebovirus and Tospovirus) based
262 on antigenic, structural and genetic characteristics (Plyusnin et al., 2012). As evidenced
263 by the finding of Gouléako virus in mosquitoes in Côte d'Ivoire (Marklewitz et al.,
264 2011), bunyaviruses that cannot be classified into any of the five known genera have
265 also come to attention. Recent metagenomic studies of arthropod-borne viruses led to
266 the discovery of many such unclassified bunyaviruses (Ballinger et al., 2014; Webster et

267 al., 2015). Based on the sequences found, the closest relative of ISBV is Wenzhou
268 Shrimp Virus 2, which is one of the unclassified bunyaviruses found in a metagenomic
269 study of Chinese shrimps (Li et al., 2015), and thus its phylogenetic position is not
270 clearly understood. Despite an intensive search using a custom database comprising the
271 sequences of all known Bunyaviridae deposited in GenBank, we could not find the
272 sequence of the potential S segment of ISBV. Similarly, Li et al. (2015) failed to detect
273 the sequence of putative S segment of Wenzhou Shrimp Virus 2 in the deep sequencing
274 data.

275 The predominant putative virus found in ISE6 cells was a member of the
276 family Iflaviridae, which is an arthropod-infecting virus group with a single-stranded
277 RNA genome. Along with the advancement of next-generation sequencing techniques,
278 an increasing number of iflaviruses are being reported in various arthropod species even
279 in laboratory colonies (Liu et al., 2015); however, there is no report to date on the
280 detection of this virus group from ticks. Some iflaviruses have occasionally been
281 associated with adverse effects on the survival of arthropod hosts (Martin et al., 2012;
282 Silva et al., 2015; Valles and Hashimoto 2009). For example, deformed wing virus,

283 which is an iflavirus commonly infecting the European honeybee (*Apis mellifera*)
284 asymptomatically with low viral loads (de Miranda and Genersch, 2010), causes
285 morphological deformity and reduces the life span with elevated virus loads when
286 transmitted by the ectoparasitic mite *Varroa destructor* (de Miranda and Fries, 2008;
287 Dainat et al., 2012). Further studies are required to investigate the role of ISIV *in vivo*
288 and to assess the prevalence of ISIV and its related viruses in natural populations of
289 ticks.

290 In addition to the previously unrecognised viruses, ISE6 was revealed to
291 harbour partial sequence of the putative virus ISAV-1, which was recently discovered in
292 *I. scapularis* collected at Heckscher State Park in the United States (Suffolk County,
293 NY) (Tokarz et al., 2014), where the authors identified a partial genome sequence (2.8
294 kb) of ISAV-1 encompassing the majority of a putative protease and an RdRp without
295 information on its capsid protein. A recent metagenomic study reported the detection of
296 a virus closely related to ISAV-1, designated *Humaita-Tubiacanga* virus (HTV), in a
297 laboratory colony of the mosquito *Aedes aegypti* (Aguiar et al., 2015). The authors
298 detected a sequence corresponding to the RdRp of HTV together with one viral segment

299 (1,609 nt) predicted to encode a protein with a coat domain, with the highest similarity
300 to a capsid protein of DAV. As supported by further evidence of small RNA profiles and
301 simultaneous detection by RT-PCR in individual mosquitoes, the authors proposed that
302 the capsid protein related to DAV is associated with the RdRp of HTV. Likewise, we
303 detected one virus sequence encoding a putative viral capsid protein with the highest
304 similarity to DAV in ISE6 cells, while a sequence corresponding to the RdRp of DAV
305 was absent. These facts collectively support the possible association of ISAV-1 with a
306 capsid protein related to DAV.

307 An initial scan for potential virus sequences detected 19 contigs. Because the
308 rest of the virus-like sequences showed high similarity with draft genome sequences of *I.*
309 *scapularis*, we suspected that they are integrated in the genome of *I. scapularis* and thus
310 did not include them in the further analysis. It is, however, possible that some of them
311 were derived from endogenous viruses infecting the *I. scapularis* Wikel strain used in
312 the whole genome sequencing project (Gulia-Nuss et al., 2016). As reported elsewhere,
313 some arthropods have acquired a number of virus-like sequences in their genomes
314 during evolution (Ballinger et al., 2014; Katzourakis and Gifford, 2010; Liu et al., 2010).

315 It is therefore important to perform paleovirological analysis of arthropods with a
316 process of *in silico* identification of endogenous virus elements incorporated into the
317 host genome.

318 Considering the high abundance of viral sequences associated with ISIV in
319 the sequencing data, the majority of the virions observed in the culture supernatant by
320 TEM might be expected to be ISIV (Figure 1). However, the morphological features of
321 the particles were different from the virion properties of the genus Iflavirus, which are
322 approximately 30 nm in diameter with no distinctive surface structures (Chen et al.,
323 2012). Since ISAV-1 was only reported from a metagenomic study (Tokarz et al., 2014),
324 the morphological features of ISAV-1 are completely unknown. The size of the only
325 characterised tick virus, SCRV, is approximately 55 nm in diameter (Attoui et al., 2001),
326 which is comparable to that of the virions observed in this study (between 50 and 70 nm
327 in diameter). It is unfortunate that we could not differentiate the virions with a varying
328 diameter due to the similarity of their surface structures and the resolution limits of
329 TEM. Further studies using immune-electron microscopy are required to determine the
330 morphological structure of each virion in ISE6 cells.

331 The role of viruses in tick cells is totally unknown. There is some evidence in
332 mosquito cell lines that some persistently infecting viruses, for example C6/36
333 densovirus (Parvoviridae), cell-fusing agent virus (Flaviviridae) and *Aedes*
334 *pseudoscutellaris* reovirus (Reoviridae), modulate exogenous viruses' replication and
335 vice versa, possibly in part through regulation of host antiviral immune responses
336 (Morazzani et al., 2012). Hence, the experimental outcomes using ISE6 cells, which
337 might contain several putative endogenous viruses, should be carefully considered.
338 Because we characterised the putative viral sequences in ISE6 cells, it becomes possible
339 to determine the genome sequences of the viruses isolated in ISE6 cells by removing the
340 sequencing reads from putative viruses by *in silico* analysis. In fact, we have determined
341 whole genomes of previously uncharacterized bunyaviruses, which can be propagate in
342 ISE6 cells but not in mammalian cell lines (manuscript in preparation). It might be
343 worth trying to generate virus-free cell lines by interfering with replication of putative
344 viruses in ISE6 cells based on siRNA silencing technology (Bell-Sakyi and Attoui,
345 2013).

346 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.

Acknowledgements

We acknowledge Dr. Ulrike Mundrloh of the University of Minnesota for kindly giving permission to use ISE6 cells. This work was supported by JSPS KAKENHI Grant-in-Aid for Young Scientists (B) (25850195) and (A) (15H05633), and for Scientific Research on Innovative Areas (16H06431) and Japan Science and

Technology Agency (JST)/Japan International Cooperation Agency (JICA) within the framework of the Science and Technology Research Partnership for Sustainable Development (SATREPS).

References

Aguiar, E.R.G.R., Olmo, R.P., Paro, S., Ferreira, F.V., de Faria, I.J.D.S., Todjro, Y.M.H., Lobo, F.P., Kroon, E.G., Meignin, C., Gatherer, D., Imler, J.-L., Marques, J.T., 2015. Sequence-independent characterization of viruses based on the pattern of viral small RNAs produced by the host. Nucleic Acids Res. 43, 6191–6206.

Alberdi, M.P., Dalby, M.J., Rodriguez-Andres, J., Fazakerley, J.K., Kohl, A., Bell-Sakyi, L., 2012. Detection and identification of putative bacterial endosymbionts and endogenous viruses in tick cell lines. Ticks Tick Borne Dis. 3, 137–146.

Attoui, H., Stirling, J.M., Munderloh, U.G., Billoir, F., Brookes, S.M., Burroughs, J.N., de Micco, P., Mertens, P.P., de Lamballerie, X., 2001. Complete sequence

characterization of the genome of the St Croix River virus, a new orbivirus isolated from cells of *Ixodes scapularis*. J. Gen. Virol. 82, 795–804.

Ballinger, M.J., Bruenn, J.A., Hay, J., Czechowski, D., Taylor, D.J., 2014. Discovery and evolution of bunyavirids in arctic phantom midges and ancient bunyavirid-like sequences in insect genomes. J. Virol. 88, 8783–8794.

Bekker, C.P.J., Bell-Sakyi, L., Paxton, E.A., Martinez, D., Bensaid, A., Jongejan, F., 2002. Transcriptional analysis of the major antigenic protein 1 multigene family of *Cowdria ruminantium*. Gene 285, 193–201.

Bell-Sakyi, L., Attoui, H., 2013. Endogenous tick viruses and modulation of tick-borne pathogen growth. Front. Cell Infect. Microbiol. 3, 25.

Bell-Sakyi, L., Kohl, A., Bente, D.A., Fazakerley, J.K., 2012. Tick cell lines for study of Crimean-Congo hemorrhagic fever virus and other arboviruses. Vector Borne Zoonotic Dis. 12, 769–781.

Bell-Sakyi, L., Zweygarth, E., Blouin, E.F., Gould, E.A., Jongejan, F., 2007. Tick cell lines: tools for tick and tick-borne disease research. Trends Parasitol. 23, 450–457.

393 Black, W.C., Piesman, J., 1994. Phylogeny of hard- and soft-tick taxa (Acari: Ixodida)
394 based on mitochondrial 16S rDNA sequences. Proc. Natl. Acad. Sci. U.S.A. 91,
395 10034–10038.
396 Carpi, G., Cagnacci, F., Wittekindt, N.E., Zhao, F., Qi, J., Tomsho, L.P., Drautz, D.I.,
397 Rizzoli, A., Schuster, S.C., 2011. Metagenomic profile of the bacterial communities
398 associated with *Ixodes ricinus* ticks. PLoS ONE 6, e25604.
399 Chen, Y.P., Nakashima, N., Christian, P.D., Bakonyi, T., Bonning, B.C., Valles, S.M.
400 and Lightner, D., 2012. Family *Iflaviridae*, in: Andrew, M.Q.K., Adams, M.J.,
401 Carstens, E.B., Lefkowitz, E.J. (Eds.), Virus taxonomy, Ninth report of the
402 International Committee on Taxonomy of Viruses. Elsevier Academic Press, San
403 Diego, CA, pp. 846–849.
404 Dainat, B., Evans, J.D., Chen, Y.P., Gauthier, L., Neumann, P., 2012. Dead or alive:
405 deformed wing virus and *Varroa destructor* reduce the life span of winter
406 honeybees. Appl. Environ. Microbiol. 78, 981–987.
407 de Miranda, J.R., Fries, I., 2008. Venereal and vertical transmission of deformed wing
408 virus in honeybees (*Apis mellifera* L.). J. Invertebr. Pathol. 98, 184–189.

409 de Miranda, J.R., Genersch, E., 2010. Deformed wing virus. J. Invertebr. Pathol. 103
410 Suppl 1, S48–61.
411 Gulia-Nuss, M., Nuss, A.B., Meyer, J.M., Sonenshine, D.E., Roe, R.M., Waterhouse,
412 R.M., Sattelle, D.B., la Fuente, de, J., Ribeiro, J.M., Megy, K., Thimmapuram, J.,
413 Miller, J.R., Walenz, B.P., Koren, S., Hostetler, J.B., Thiagarajan, M., Joardar, V.S.,
414 Hannick, L.I., Bidwell, S., Hammond, M.P., Young, S., Zeng, Q., Abrudan, J.L.,
415 Almeida, F.C., Ayllón, N., Bhide, K., Bissinger, B.W., Bonzon-Kulichenko, E.,
416 Buckingham, S.D., Caffrey, D.R., Caimano, M.J., Croset, V., Driscoll, T., Gilbert,
417 D., Gillespie, J.J., Giraldo-Calderón, G.I., Grabowski, J.M., Jiang, D., Khalil,
418 S.M.S., Kim, D., Kocan, K.M., Koči, J., Kuhn, R.J., Kurtti, T.J., Lees, K., Lang,
419 E.G., Kennedy, R.C., Kwon, H., Perera, R., Qi, Y., Radolf, J.D., Sakamoto, J.M.,
420 Sánchez-Gracia, A., Severo, M.S., Silverman, N., Šimo, L., Tojo, M., Tornador, C.,
421 Van Zee, J.P., Vázquez, J., Vieira, F.G., Villar, M., Wespiser, A.R., Yang, Y., Zhu,
422 J., Arensburger, P., Pietrantonio, P.V., Barker, S.C., Shao, R., Zdobnov, E.M.,
423 Hauser, F., Grimmelikhuijzen, C.J.P., Park, Y., Rozas, J., Benton, R., Pedra, J.H.F.,
424 Nelson, D.R., Unger, M.F., Tubio, J.M.C., Tu, Z., Robertson, H.M., Shumway, M.,

425 Sutton, G., Wortman, J.R., Lawson, D., Wikel, S.K., Nene, V.M., Fraser, C.M.,
426 Collins, F.H., Birren, B., Nelson, K.E., Caler, E., Hill, C.A., 2016. Genomic
427 insights into the *Ixodes scapularis* tick vector of Lyme disease. Nat. Commun. 7,
428 10507.
429 Jones, P., Binns, D., Chang, H.Y., Fraser, M., Li, W., McAnulla, C., McWilliam, H.,
430 Maslen, J., Mitchell, A., Nuka, G., Pesseat, S., Quinn, A.F., Sangrador-Vegas, A.,
431 Scheremetjew, M., Yong, S.Y., Lopez, R., Hunter, S., 2014. InterProScan 5:
432 genome-scale protein function classification. Bioinformatics 30, 1236–1240.
433 Katzourakis, A., Gifford, R.J., 2010. Endogenous viral elements in animal genomes.
434 PLoS Genet. 6, e1001191.
435 Kurtti, T.J., Munderloh, U.G., Andreadis, T.G., Magnarelli, L.A., Mather, T.N., 1996.
436 Tick cell culture isolation of an intracellular prokaryote from the tick *Ixodes*
437 *scapularis*. J. Invertebr. Pathol. 67, 318–321.
438 Li, C.X., Shi, M., Tian, J.H., Lin, X.D., Kang, Y.J., Chen, L.J., Qin, X.C., Xu, J.,
439 Holmes, E.C., Zhang, Y.Z., 2015. Unprecedented genomic diversity of RNA
440 viruses in arthropods reveals the ancestry of negative-sense RNA viruses. Elife 4.

441 Liu, H., Fu, Y., Jiang, D., Li, G., Xie, J., Cheng, J., Peng, Y., Ghabrial, S.A., Yi, X.,
442 2010. Widespread horizontal gene transfer from double-stranded RNA viruses to
443 eukaryotic nuclear genomes. J. Virol. 84, 11876–11887.
444 Liu, S., Chen, Y., Bonning, B.C., 2015. RNA virus discovery in insects. Curr. Opin.
445 Insect Sci. 8, 54–61.
446 Marklewitz, M., Handrick, S., Grasse, W., Kurth, A., Lukashev, A., Drosten, C.,
447 Ellerbrok, H., Leendertz, F.H., Pauli, G., Junglen, S., 2011. Gouleako virus isolated
448 from West African mosquitoes constitutes a proposed novel genus in the family
449 *Bunyaviridae*. J. Virol. 85, 9227–9234.
450 Martin, S.J., Highfield, A.C., Brettell, L., Villalobos, E.M., Budge, G.E., Powell, M.,
451 Nikaido, S., Schroeder, D.C., 2012. Global honey bee viral landscape altered by a
452 parasitic mite. Science 336, 1304–1306.
453 Morazzani, E.M., Wiley, M.R., Murreddu, M.G., Adelman, Z.N., Myles, K.M., 2012.
454 Production of virus-derived ping-pong-dependent piRNA-like small RNAs in the
455 mosquito soma. PLoS Pathog. 8, e1002470.

456 Munderloh, U.G., Kurtti, T.J., 1989. Formulation of medium for tick cell culture. Exp.
457 Appl. Acarol. 7, 219–229.
458 Munderloh, U.G., Lynch M.J., Herron, M.J., Palmer, A.T., Kurtti, T.J., Nelson, R.D.,
459 Goodman, J.L., 2004. Infection of endothelial cells with *Anaplasma marginale* and
460 *A. phagocytophilum*. Vet. Microbiol. 101, 53–64.
461 Nakao, R., Abe, T., Nijhof, A.M., Yamamoto, S., Jongejan, F., Ikemura, T., Sugimoto,
462 C., 2013. A novel approach, based on BLSOMs (Batch Learning Self-Organizing
463 Maps), to the microbiome analysis of ticks. ISME J. 7, 1003–1015.
464 Plyusnin, A., Beaty, B., Elliott, R.M., Goldbach, R., Kormelink, R., Lundkvist, A.,
465 Schmaljohn, C.S., Tesh, R.B., 2012. Family *Bunyaviridae*, in: Andrew, M.Q.K.,
466 Adams, M.J., Carstens, E.B., Lefkowitz, E.J. (Eds.), Virus taxonomy, Ninth report
467 of the International Committee on Taxonomy of Viruses. Elsevier Academic Press,
468 San Diego, CA, pp. 725–741.
469 Qiu, Y., Nakao, R., Ohnuma, A., Kawamori, F., Sugimoto, C., 2014. Microbial
470 population analysis of the salivary glands of ticks; a possible strategy for the
471 surveillance of bacterial pathogens. PLoS ONE 9, e103961.

472 Silva, L.A., Ardisson-Araujo, D.M.P., Tinoco, R.S., Fernandes, O.A., Melo, F.L.,
473 Ribeiro, B.M., 2015. Complete genome sequence and structural characterization of
474 a novel iflavirus isolated from *Opsiphanes invirae* (Lepidoptera: Nymphalidae). J.
475 Invertebr. Pathol. 130, 136–140.
476 Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6:
477 Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol. 30, 2725–
478 2729.
479 Tokarz, R., Williams, S.H., Sameroff, S., Sanchez Leon, M., Jain, K., Lipkin, W.I.,
480 2014. Virome analysis of *Amblyomma americanum*, *Dermacentor variabilis*, and
481 *Ixodes scapularis* ticks reveals novel highly divergent vertebrate and invertebrate
482 viruses. J. Virol. 88, 11480–11492.
483 Valles, S.M., Hashimoto, Y., 2009. Isolation and characterization of *Solenopsis invicta*
484 virus 3, a new positive-strand RNA virus infecting the red imported fire ant,
485 *Solenopsis invicta*. Virology 388, 354–361.

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 some arboviruses. J. Med. Entomol. 11, 698–706.

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., Haddrill, P.R., Obbard, D.J., 2015. The discovery, distribution, and evolution of viruses associated with *Drosophila melanogaster*. PLoS Biol. 13, e1002210.

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.

502

503 **Figure 2. Predicted genome organisations of *Ixodes scapularis* iflavirus (A) and**

504 ***Ixodes scapularis* bunyavirus (B).**

505 *De novo* assembled contigs were subjected to an ORF search, and the putative amino

506 acid sequences were used for a conserved domain search with InterProScan. Each

507 domain is indicated by a grey bar, and the name is shown beneath the bar.

508

509 **Figure 3. Phylogenetic tree of the genus Iflavirus.**

510 The full-length sequence of the *Ixodes scapulari*s iflavirus (contig 1) (shown

511 underlined) was used for phylogenetic analysis with the other iflaviruses. Drosophila C

512 virus in the family Dicistroviridae was deployed as an outgroup. Bootstrap probabilities

513 higher than 70% are indicated near the branches. The scale bar indicates 0.5 changes per

514 nucleotide position.

515

516 **Figure 4. Phylogenetic trees of the family Bunyaviridae.**

517 The full-length sequences of the *Ixodes scapulari*s bunyavirus (contigs 2 and 5) (shown

518 underlined) were used for phylogenetic analyses with the other bunyavirus L (A) and M
519 segments (B), respectively. Bootstrap probabilities higher than 70% are indicated near
520 the branches. The scale bars indicate 0.5 changes per nucleotide position.
521
522 **Figure 5. PCR amplification of each putative viral sequence and tick**
523 **mitochondrial 16S rRNA gene.**
524 PCR was performed on the cDNA generated from the total RNA extracted from ISE6
525 cells (1), the culture supernatant (2), and the culture medium (3) and gDNA extracted
526 from ISE6 cells (4). ISIV, ISB_L, ISB_M, ISAV-1, ISUV, and 16S rDNA represent PCR
527 reactions with primers amplifying *Ixodes scapularis* iflavirus, L segment of *Ixodes*
528 *scapularis* bunyavirus, M segment of *Ixodes scapularis* bunyavirus, *Ixodes scapularis*
529 associated virus-1, unclassified virus related to Drosophila A virus, and tick
530 mitochondrial 16S rRNA gene (rDNA), respectively. All the five putative viral
531 sequences were detected in both ISE6 cells and the culture supernatant while only tick
532 mitochondrial 16S rDNA was amplified from gDNA. Lane M shows a 100-bp DNA
533 marker.

534

535 **Table 1. Oligonucleotide primers used for PCR amplification of each putative viral**

536 **sequence and tick mitochondrial 16S rRNA gene sequence.**

537

538 **Table 2. Summary of putative partial viral genome sequences identified in ISE6**

539 **cells.**

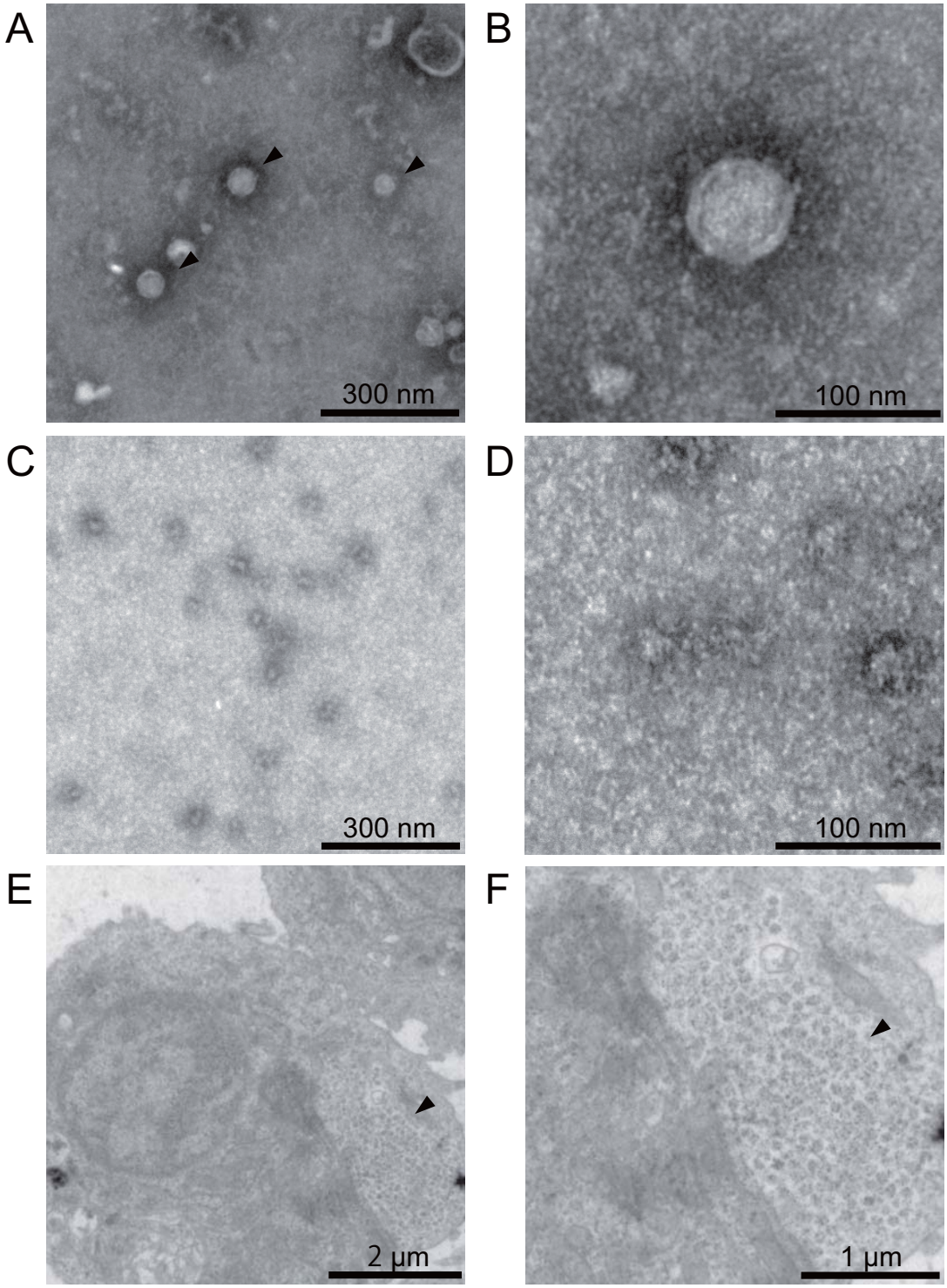

Fig. 1

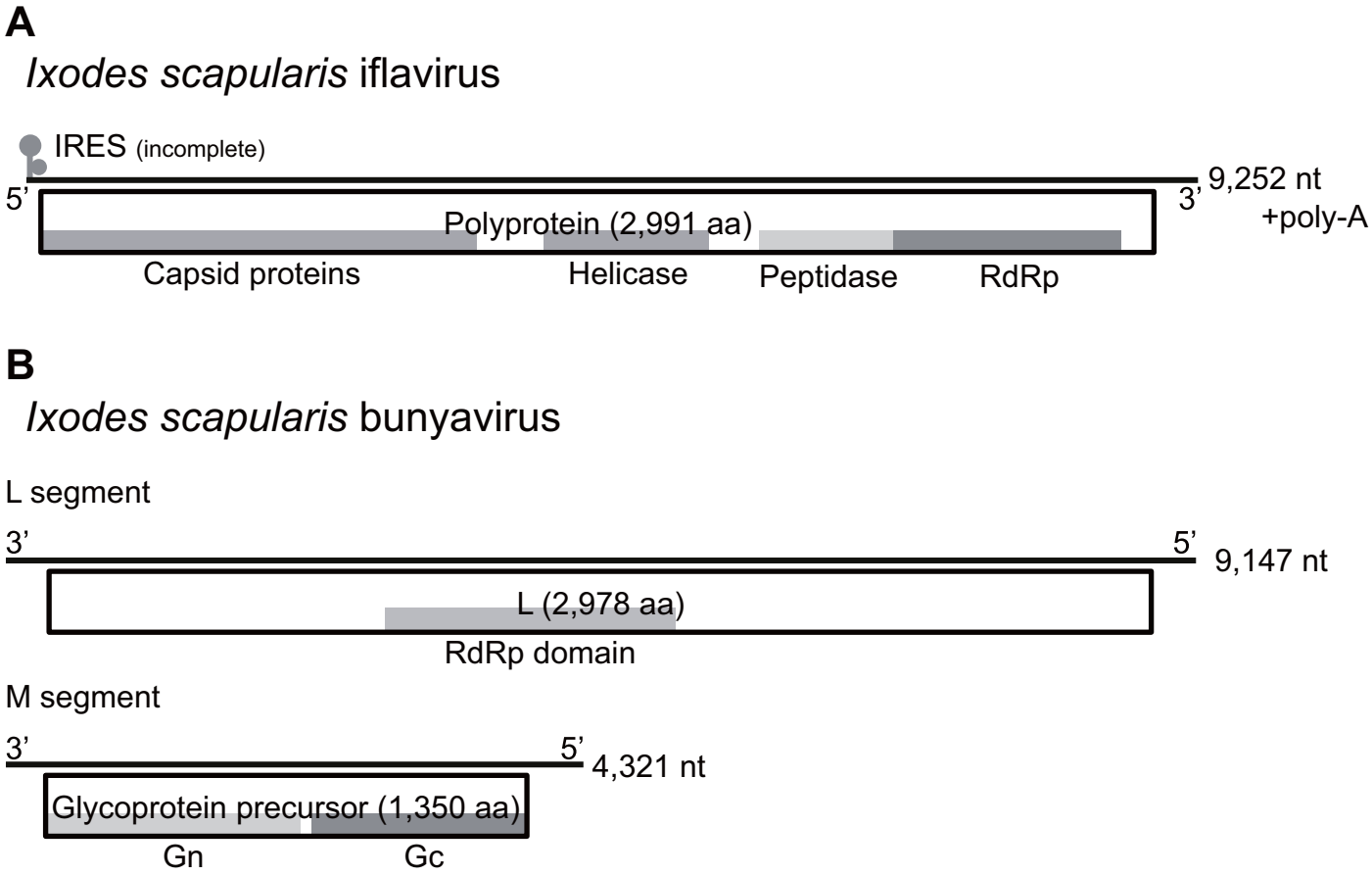
A
Ixodes scapularis iflavirus
IRES (incomplete)
5'
3'
9,252 nt
+poly-A
Polyprotein (2,991 aa)
Capsid proteins
Helicase
Peptidase
RdRp
B
Ixodes scapularis bunyavirus
L segment
3'
5'
9,147 nt
L (2,978 aa)
RdRp domain
M segment
3'
5'
4,321 nt
Glycoprotein precursor (1,350 aa)
Gn
Gc

Fig. 2

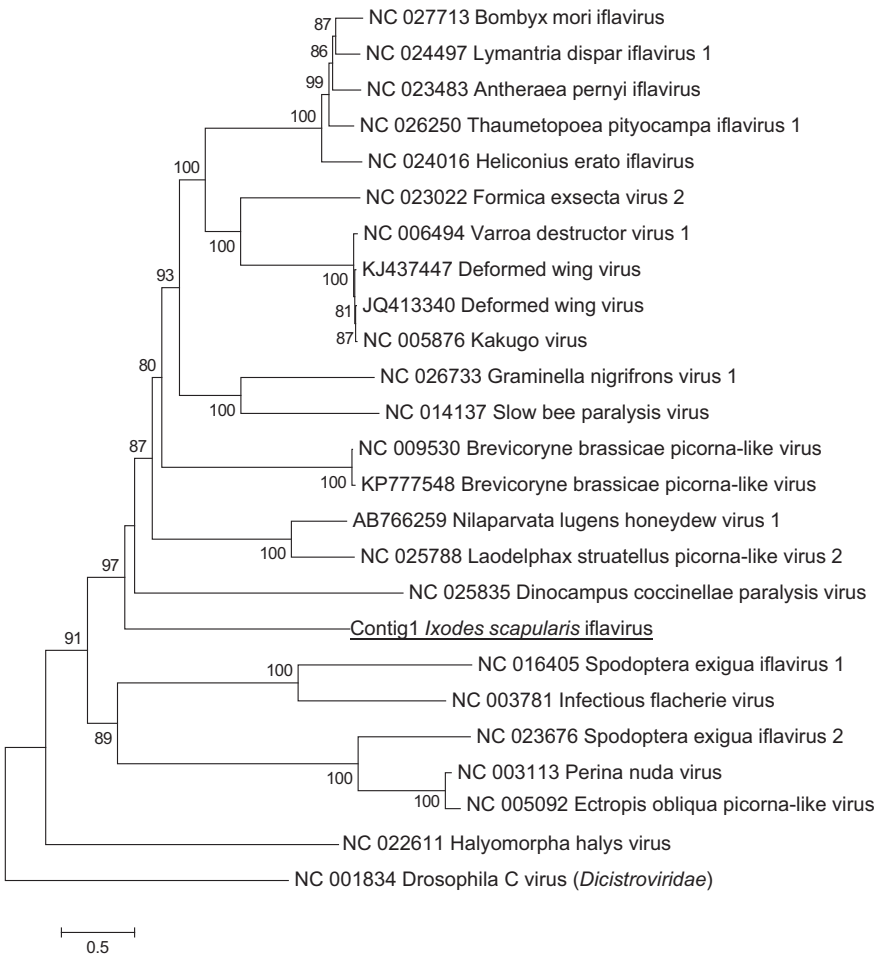
NC 027713 Bombyx mori iflavirus
NC 024497 Lymantria dispar iflavirus 1
NC 023483 Antheraea pernyi iflavirus
NC 026250 Thaumetopoea pityocampa iflavirus 1
NC 024016 Heliconius erato iflavirus
NC 023022 Formica exsecta virus 2
NC 006494 Varroa destructor virus 1
KJ437447 Deformed wing virus
JQ413340 Deformed wing virus
NC 005876 Kakugo virus
NC 026733 Graminella nigrifrons virus 1
NC 014137 Slow bee paralysis virus
NC 009530 Brevicoryne brassicae picorna-like virus
KP777548 Brevicoryne brassicae picorna-like virus
AB766259 Nilaparvata lugens honeydew virus 1
NC 025788 Laodelphax struatellus picorna-like virus 2
NC 025835 Dinocampus coccinellae paralysis virus
Contig1 Ixodes scapularis iflavirus
NC 016405 Spodoptera exigua iflavirus 1
NC 003781 Infectious flacherie virus
NC 023676 Spodoptera exigua iflavirus 2
NC 003113 Perina nuda virus
NC 005092 Ectropis obliqua picorna-like virus
NC 022611 Halyomorpha halys virus
NC 001834 Drosophila C virus (Dicistroviridae)
0.5

Fig. 3

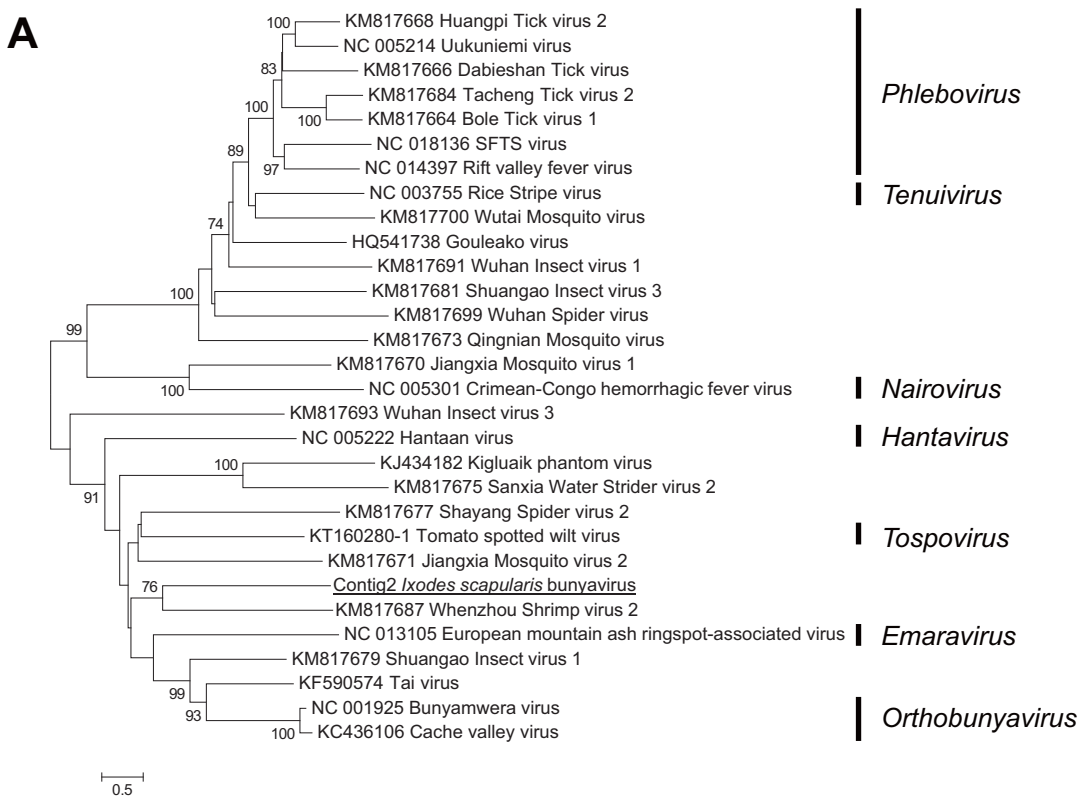
A
KM817668 Huangpi Tick virus 2
NC 005214 Uukuniemi virus
KM817666 Dabieshan Tick virus
KM817684 Tacheng Tick virus 2
KM817664 Bole Tick virus 1
NC 018136 SFTS virus
NC 014397 Rift valley fever virus
NC 003755 Rice Stripe virus
KM817700 Wutai Mosquito virus
HQ541738 Gouleako virus
KM817691 Wuhan Insect virus 1
KM817681 Shuangao Insect virus 3
KM817699 Wuhan Spider virus
KM817673 Qingnian Mosquito virus
KM817670 Jiangxia Mosquito virus 1
NC 005301 Crimean-Congo hemorrhagic fever virus
KM817693 Wuhan Insect virus 3
NC 005222 Hantaan virus
KJ434182 Kigluaik phantom virus
KM817675 Sanxia Water Strider virus 2
KM817677 Shayang Spider virus 2
KT160280-1 Tomato spotted wilt virus
KM817671 Jiangxia Mosquito virus 2
Contig2 Ixodes scapularis bunyavirus
KM817687 Whenzhou Shrimp virus 2
NC 013105 European mountain ash ringspot-associated virus
KM817679 Shuangao Insect virus 1
KF590574 Tai virus
NC 001925 Bunyamwera virus
KC436106 Cache valley virus
Phlebovirus
Tenuivirus
Nairovirus
Hantavirus
Tospovirus
Emaravirus
Orthobunyavirus
0.5
B
KM817707 Huangpi Tick virus 2
NC 005220 Uukuniemi virus
NC 014396 Rift Valley fever virus
NC 018138 SFTS virus
KM817710 Qingnian Mosquito virus
KM817716 Shuangao Insect virus 3
HQ541737 Gouleako virus
NC 003754 Rice Stripe virus
KM817728 Wutai Mosquito virus
KM817709 Jiangxia Mosquito virus 2
KM817708 Jiangxia Mosquito virus 1
KJ434183 Kigluaik phantom virus
NC 005219 Hantaan virus
KM817720 Whenzhou Shrimp virus 2
Contig5 Ixodes scapularis bunyavirus
NC 005300 Crimean-Congo hemorrhagic fever virus
KF590573 Tai virus
NC 013106 European mountain ash ringspot-associated virus
KT160281 Tomato spotted wilt virus
KM817714 Shuangao Insect virus 1
NC 001926 Bunyamwera virus
KC436107 Cache Valley virus
0.5

Fig. 4

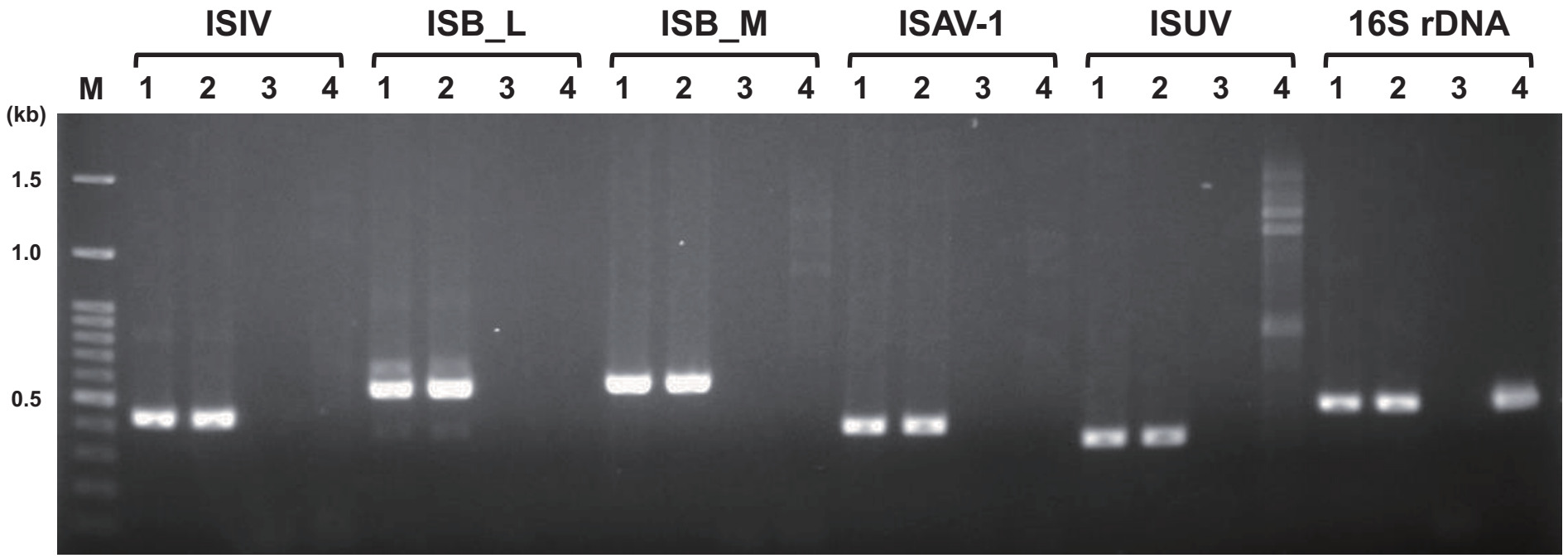
ISIV
ISB_L
ISB_M
ISAV-1
ISUV
16S rDNA
M 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
(kb)
1.5
1.0
0.5

Fig. 5

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

Primer name	Sequence (5' to 3')	Target	Amplicon size (bp)	Reference
ISIV_F	GGGGCGTGGTAAATGTGGTA	*Ixodes scapularis* iflavirus	437	This study
ISIV_R	ACACTCCGCGATGAAAGGTT			
ISB_L_F	GCATTGTGTCCAGCTCTTCA	*Ixodes scapularis* bunyavirus, L segment	547	This study
ISB_L_R	GTCTCCAGAAACAGGCTTGC			
ISB_M_F	CTGGCACAATCCTTTTGGTT	*Ixodes scapularis* bunyavirus, M segment	571	This study
ISB_M_R	TTCTCTCTCAGCCCTGGTGT			
ISAV-1_F	AACGTGTACGGAATGCACCT	*Ixodes scapularis* associated virus-1, RdRP	383	This study
ISAV-1_R	GTTTGGCCAGAACTTGGCAG			
ISUV_F	GACCACGGACACCAGTTTCT	Unclassified virus, capsid precursor	338	This study
ISUV_R	GGTCAGACAGATAGGTCGCC			
16S+1	CTGCTCAATGATTTTTTAAATTGCTGTGG	Tick mitochondrial 16S rRNA gene	460	Black and Piesman, 1994
16S−1	CCGGTCTGAACTCAGATCAAGTA			

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

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

[a]Contig size without polyA.