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BRIEF REPORT

Discovery of a novel iflavirus sequence in the eastern paralysis tick *Ixodes holocyclus*

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Abstract

Ixodes holocyclus, the eastern paralysis tick, is a significant parasite in Australia in terms of animal and human health. However, very little is known about its virome. In this study, next-generation sequencing of *I. holocyclus* salivary glands yielded a full-length genome sequence which phylogenetically groups with viruses classified in the *Iflaviridae* family and shares 45% amino acid similarity with its closest relative Bole hyalomma asiaticum virus 1. The sequence of this virus, provisionally named Ixodes holocyclus iflavirus (IhIV) has been identified in tick populations from northern New South Wales and Queensland, Australia and represents the first virus sequence reported from *I. holocyclus*.

Iflaviruses (classified within family *Iflaviridae*) are small, non-enveloped viruses found in a diverse range of arthropod species. These viruses comprise a mono-partite, single-stranded, positive-sense RNA genome of approximately 9-11 kb [1]. While historically, the majority of iflaviruses were associated with hosts of the class Insecta, recent metagenomic studies have identified multiple iflavirus-like sequences from ticks collected in China [2]. *Ixodes holocyclus*, a tick of the Ixodidae family, is native and endemic to the eastern coastal region of Australia [3, 4]. This tick is of significant veterinary importance primarily due to its ability to induce severe paralysis in domestic animals [4, 5]. However, the broad host range of this tick also makes it a potential vector of interest for bacterial and viral diseases of animals and humans, in particular association with a lyme-like disease reported in Australia [6]. While recent studies using next generation sequencing technologies have provided some insight into the bacterial diversity within *I. holocyclus*, there is little to no information about the virome of this ectoparasite [7].

In this study, next generation sequencing was performed on RNA extracted from the salivary glands of *I. holocyclus* ticks. Unfed ticks were collected in the Lismore-Nimbin area of northern New South Wales (NSW), Australia using the dragging method as described by Gladney (1978) [8] and were subsequently fed on laboratory rats for unrelated experiments. Analysis of sequencing data revealed a novel iflavirus-like sequence encoding a 2946 amino acid polyprotein with 45% identity to its closest relative, Bole hyalomma asiaticum virus 1 (BhaV-1) by blastp analysis [2]. Preliminary analysis of the genome organisation was performed using EBI tools HMMER and Interpro domain search programs [9, 10]. HMMER domain analysis revealed two picornavirus capsid protein (Rhv-like) domains (pfam: PF00073) followed by a cricket paralysis virus capsid protein-like (CRPV-like) domain (pfam: PF08762) suggesting

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Caitlin A. O'Brien and Sonja Hall-Mendelin contributed equally to the manuscript.

Depositories: The Genbank accession number for the genetic sequence of *Ixodes holocyclus* Iflavirus is KY020412.

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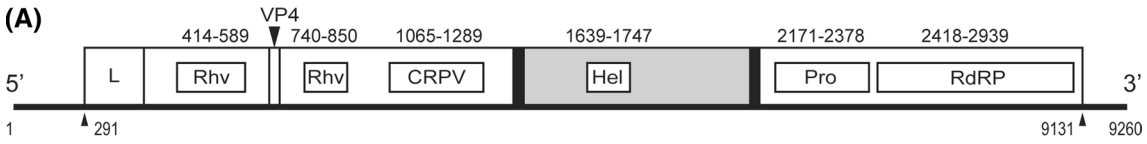
(A)



(B)

Sample type	Location	Date of collection	Identified by	Similarity to prototype (% nt)*
Salivary glands	Lismore-Nimbin area, NSW	2000 – 2002	NGS - Illumina	-
Salivary glands	Pinjarra Hills, QLD	2010	NGS – 454	97.9-98.9
Unfed adults	Suffolk park, NSW	2010	NGS - 454	97.0-99.0
Viscera	Brisbane, QLD	2012	NGS – Illumina	94.4-98.9
Salivary glands	Pooled QLD and NSW	2013	NGS – Illumina	98.9
Viscera	Pooled QLD and NSW	2013	NGS - Illumina	98.5-99

(C)

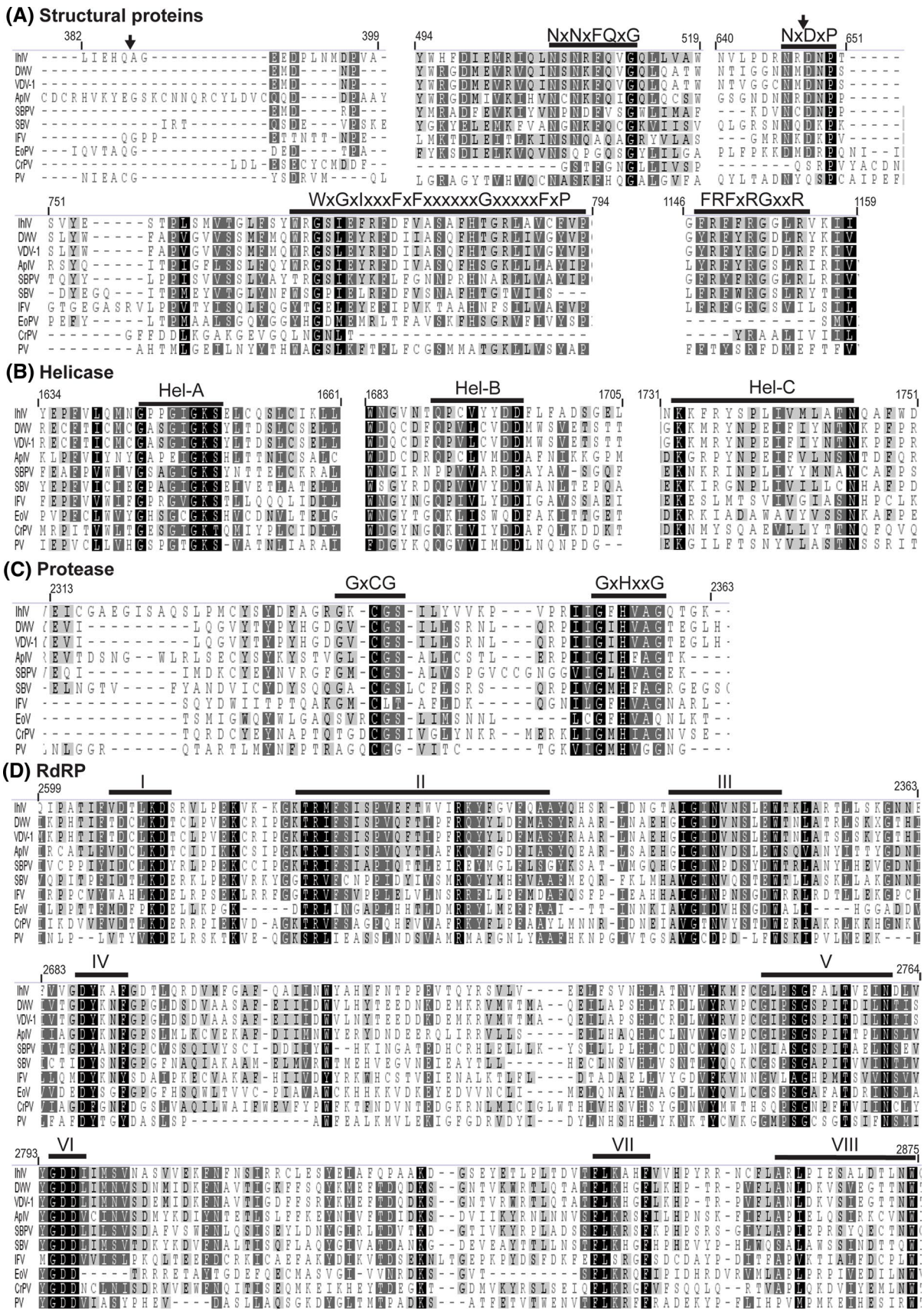
Sample type	Location	Date of collection	Positive samples/ Samples tested (%)	Similarity to prototype (% nt)
Bodies	Lismore-Nimbin area, NSW	2000	3/10 (30 %)	99.7
Bodies	Lismore-Nimbin area, NSW	2001	8/15 (53 %)	ND
Whole ticks	Moruya, NSW	2015	0/13 (0%)	-
Whole ticks	Tuross Heads, NSW	2015	0/2 (0%)	-
Whole ticks	Broulee, NSW	2015	0/2 (0%)	-
Whole ticks	Cape Conran, Victoria	2015	0/2 (0 %)	-

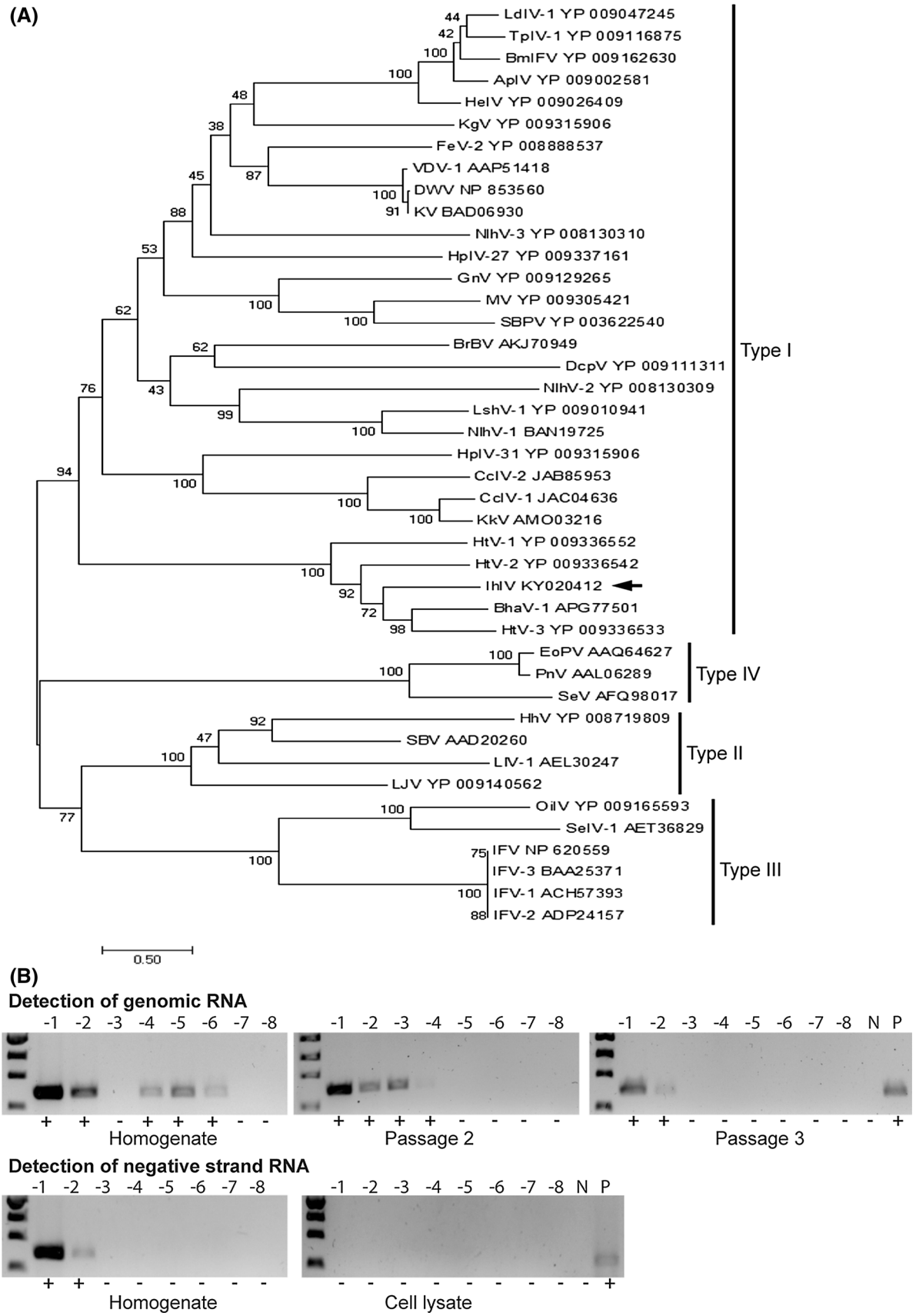
Fig. 1 a) A schematic of the IhIV genome. b) Summary of IhIV sequences found by next generation sequencing in *I. holocyclus* collected in various locations. *Nucleotide identities to prototype IhIV (isolate 1) across contigs from 216–8838 bp in length. c) Summary of IhIV prevalence in *I. holocyclus* ticks collected in Victoria and NSW identified by RT-PCR

the N-terminus of the polyprotein encodes the structural proteins. HMMER and interpro domain searches identified an RNA helicase (pfam: PF00910), protease (IPR009003) and RNA-dependent RNA polymerase (RdRP) (pfam: PF00680) domain at the C-terminus (Fig. 1a). This genome organisation is consistent with other published iflaviruses except for the absence of 3' polyadenylation which may be due to the inability to confidently elucidate the extreme 3' and 5' termini of the genome by next generation sequencing [11]. The sequence was tentatively assigned the name Ixodes holocyclus iflavirus (IhIV).

Analysis of transcriptome data obtained from the RNA of additional *I. holocyclus* ticks collected in Queensland and northern NSW, identified highly similar IhIV sequences in salivary gland and viscera samples of engorged ticks taken from bandicoots, cats and dogs, as well as unfed adults collected from the ground (Fig. 1b). Generation of these libraries has been described in detail previously [12]. In order to investigate the prevalence of IhIV in the Lismore-Nimbin region of NSW, *I. holocyclus* bodies in pools of 5-10 ticks were homogenised in L-15B300 media with additives as described by Munderloh and Kurtti (1989) [13] using stainless steel beads. RNA was extracted from homogenates using the Machery-Nagel Viral RNA isolation kit. An RT-PCR assay using primers targeting the predicted helicase region of the IhIV genome was developed to screen these RNA samples (IhIV_F: 5'-TCCGAGAGTGCTAATTCGTCG-3', IhIV_R: 5'-CGTCGGTGAAGAGCATTACG-3'). This analysis showed that IhIV was present in 30% (3/10) of the pools of ticks collected in December 2000, and 53% (8/15) of pools collected in February 2001 (Fig. 1c). To investigate the presence of IhIV in other regions, 19 pools of 1-5 ticks collected from infested dogs by veterinary practices in southern New South Wales (Moruya, Tuross Heads and Broulee) and Cape Conran, Victoria in 2015 were tested by RT-PCR, with all being negative (Fig. 1c). Limited distribution has been reported previously for other insect-specific viruses [14]. However, as this testing was only performed on a total of 27 ticks, further testing of a larger sample size is warranted.

Fig. 2 Amino acid alignment of IhIV with related viruses of the Picornavirales order: IhIV (KY020412), DWV (NP_853560), VDV-1 (AAP51418), ApIV (YP_009002581), SBPV (YP_003622540), SBV (AAD20260), IFV (NP_620559), EoV (AAQ64627), CrPV (AKA63265), PV (ALI31819) showing conserved motifs in the a) structural proteins with conserved cleavage sites depicted by arrows, b) helicase, c) protease and d) RdRP domains


(A) Structural proteins
NxNxFQxG
NxDxP
WxGxIxxxFxFxxxxxxGxxxxxFxP
FRFxRGxxR
(B) Helicase
Hel-A
Hel-B
Hel-C
(C) Protease
GxCG
GxHxxG
(D) RdRP
I
II
III
IV
V
VI
VII
VIII
IhIV
DWV
VDV-1
ApIV
SBPV
SBV
IFV
EoPV
CrPV
PV


(A)
LdIV-1 YP 009047245
TpIV-1 YP 009116875
BmIFV YP 009162630
ApIV YP 009002581
HeIV YP 009026409
KgV YP 009315906
FeV-2 YP 008888537
VDV-1 AAP51418
DWV NP 853560
KV BAD06930
NlhV-3 YP 008130310
HpIV-27 YP 009337161
GnV YP 009129265
MV YP 009305421
SBPV YP 003622540
BrBV AKJ70949
DcpV YP 009111311
NlhV-2 YP 008130309
LshV-1 YP 009010941
NlhV-1 BAN19725
HpIV-31 YP 009315906
CcIV-2 JAB85953
CcIV-1 JAC04636
KkV AMO03216
HtV-1 YP 009336552
HtV-2 YP 009336542
IhIV KY020412
BhaV-1 APG77501
HtV-3 YP 009336533
EoPV AAQ64627
PnV AAL06289
SeV AFQ98017
HhV YP 008719809
SBV AAD20260
LIV-1 AEL30247
LJV YP 009140562
OiIV YP 009165593
SeIV-1 AET36829
IFV NP 620559
IFV-3 BAA25371
IFV-1 ACH57393
IFV-2 ADP24157
Type I
Type IV
Type II
Type III
0.50
(B)
Detection of genomic RNA
-1 -2 -3 -4 -5 -6 -7 -8
+ + - + + + - -
Homogenate
-1 -2 -3 -4 -5 -6 -7 -8
+ + + + - - - -
Passage 2
-1 -2 -3 -4 -5 -6 -7 -8 N P
+ + - - - - - - - +
Passage 3
Detection of negative strand RNA
-1 -2 -3 -4 -5 -6 -7 -8
+ + - - - - - -
Homogenate
-1 -2 -3 -4 -5 -6 -7 -8 N P
- - - - - - - - - +
Cell lysate

◂**Fig. 3** a) Maximum likelihood analysis of iflavirus amino acid sequences. Labels depict virus abbreviations and corresponding Genbank accession numbers. Nodes are labelled with bootstrap support values. The position of IhIV is highlighted with an arrow. b) Detection of the positive-sense and negative-sense RNA in serial ten-fold dilutions of *I. holocyclus* homogenate or ISE6 cell supernatant and lysate. Dilutions are represented in log form above each gel. N is no-template control; P is positive control. Positive and negative results are indicated with a "+" and "–", respectively

The full polyprotein of IhIV was aligned against selected sequences from viruses classified in the *Picornavirales* order using the MAFFT alignment tool via the CIPRES gateway [15–17]. Examination of the predicted polyprotein identified three conserved motifs reported for the major virion proteins (NxNxFQxG, WxGxIxxxFxFxxxxxxxFxP and FRFxRGxR) (Fig. 2a) [18]. Two conserved cleavage sites were also identified by this alignment, a glutamine-alanine (Q/A) cleavage at the predicted leader peptide-structural polyprotein junction and an arginine-aspartic acid (R/D) cleavage in the NxDxP motif which is conserved at the VP4/VP1 junction in deformed wing virus, slow bee paralysis virus and Brevicoryne brassicae virus (Fig. 2b) [19–21]. This alignment suggests that VP4 is likely to be encoded second in the structural polyprotein, consistent with the genome organisation of other iflaviruses [19–22]. The non-structural polyprotein of IhIV contains all conserved motifs found in other iflaviruses including three in the helicase (Hel-A, B, C), eight in the RdRP (I-VIII) and two in the protease (CxGC and GxHxxG) domains (Fig. 2b-d) [20, 23]. The cleavage sites of IhIV appear to be quite variable, similar to that observed for Ectropis obliqua virus [24]. IhIV does not contain any DxExNPGP or IExNPGP 2A-like motifs [24–26].**Fig. 2** Amino acid alignment of IhIV with related viruses of the Picornavirales order: IhIV (KY020412), DWV (NP_853560), VDV-1 (AAP51418), ApIV (YP_009002581), SBPV (YP_003622540), SBV (AAD20260), IFV (NP_620559), EoV (AAQ64627), CrPV (AKA63265), PV (ALI31819) showing conserved motifs in the a) structural proteins with conserved cleavage sites depicted by arrows, b) helicase, c) protease and d) RdRP domains

An alignment of 42 iflavirus sequences over 1236 amino acids covering the replicase proteins (aa 1711–2946 of IhIV) was performed using MAFFT. A maximum likelihood phylogenetic tree was constructed using the LG substitution matrix with 1000 bootstrap replicates in MEGA7 (v7.0.14) (Fig. 3a) [27]. The resulting phylogenetic tree supports the recent proposal for four clusters within the *Iflaviridae* family and shows that IhIV groups in the type I cluster which contains the prototype member infectious flacherie virus (IFV) [28]. IhIV forms a clade with its closest relative BhaV-1 and Hubei tick viruses -1, -2 and -3 which were recently identified in tick samples from China [2].

Attempts were made to culture IhIV using the *I. scapularis* cell line (ISE6) [29]. One pool of 5 ticks which was positive for IhIV sequence by RT-PCR was inoculated onto ISE6 cells, incubated for 9 days at 34 °C and subjected to two further blind passages. RT-PCR was performed on RNA extracted from ten-fold dilutions of the homogenate and supernatants collected at passages 2 and 3. This analysis revealed that IhIV sequence could be detected in homogenate diluted out to 10^{-6}. However, at passage 2, positive RT-PCR results could only be detected out to 10^{-4} and by passage 3 only dilutions 10^{-1} and 10^{-2} were positive, indicating a drop in viral titre over successive passaging (Fig. 3b). RT-PCR targeting the negative-sense genomic RNA was performed on RNA from homogenate and inoculated ISE6 cell lysate to detect the production of double-stranded replicative intermediates. While negative-strand RNA was detected in the homogenate at dilutions 10^{-1} and 10^{-2}, no negative-sense RNA was detected in the ISE6 lysate (Fig. 3c). Collectively these results indicate that IhIV does not grow in the ISE6 cell line, however the presence of negative-sense RNA in homogenate suggests viral replication within *I. holocyclus* ticks.

Seventy species of ticks are known in Australia [30]. Twenty-two of these are from the genus *Ixodes*. The two main lineages of the genus *Ixodes* are thought to be the "Australasian *Ixodes*" which comprises 28 species, including *I. holocyclus*, and the "other *Ixodes*" which contains the other 220 or so species of the *Ixodes* world, including *I. scapularis* [31]. Thus, *I. holocyclus* and *I. scapularis* are phylogenetically distant; which may explain why, a virus of *I. holocyclus*, could not infect *I. scapularis*-derived cells. Alternatively, the lack of replication in the ISE6 cell line of IhIV may reflect a tissue-restricted tropism, with a recent study showing that the ISE6 cell line is mostly "neuron-like" [32]. Recently, a study has identified a number of viral sequences including that of a new iflavirus, *Ixodes scapularis* iflavirus, from the ISE6 cell line [33]. The presence of a persistent viral infection may cause the ISE6 cell line to be refractory to infection with IhIV. IhIV sequence was found in both the bodies and salivary glands of *I. holocyclus* ticks which could potentially allow for horizontal transmission of this virus, consistent with findings of transmission for other iflaviruses [1, 18].

While attempts to culture IhIV were unsuccessful, sequence analysis has demonstrated the presence of one long, uninterrupted genome encoding a complete polyprotein with motifs characteristic of an iflavirus. Furthermore, testing for IhIV by RT-PCR in *I. holocyclus* samples demonstrated the presence of double-stranded RNA, an important intermediate in the replication of RNA viruses, and a relatively low prevalence in the ticks we studied suggesting that the sequence is not integrated in the host genome [34, 35]. This data suggests the IhIV sequence is likely to represent the genome of a functional virus rather than an endogenous viral element in the *I. holocyclus* genome [34–36]. The presence of IhIV sequence in both blood-fed

and unfed ticks suggests that IhIV is likely to be a virus of the tick, rather than a virus present in the blood meal from an infected animal and fits with the classification of iflaviruses as 'arthropod-only' viruses [1]. This sequence is the first viral sequence to be identified and characterised from the eastern Australian paralysis tick *I. holocyclus*.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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