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RAPID COMMUNICATION

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Inter-serotype reassortment among epizootic haemorrhagic disease viruses in the United States

Leyi Wang[1] | Saraswathi Lanka[1] | Debbie Cassout[1] | Nohra E. Mateus-Pinilla[2] | Ganwu Li[3] | William C. Wilson[4] | Dongwan Yoo[5] | Paul Shelton[6] | Richard L. Fredrickson[1]

[1]Department of Veterinary Clinical Medicine and the Veterinary Diagnostic Laboratory, College of Veterinary Medicine, University of Illinois, Urbana, Illinois

[2]Illinois Natural History Survey-Prairie Research Institute, University of Illinois Urbana-Champaign, Champaign, Illinois

[3]Department of Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, Iowa

[4]United States Department of Agriculture, Agricultural Research Service, Arthropod-borne Animal Diseases Research Unit, Manhattan, Kansas

[5]Department of Pathobiology, College of Veterinary Medicine, University of Illinois at Urbana-Champaign, Urbana, Illinois

[6]Illinois Department of Natural Resources, Division of Wildlife Resources, Springfield, Illinois

Correspondence
Leyi Wang, Department of Veterinary Clinical Medicine and the Veterinary Diagnostic Laboratory, College of Veterinary Medicine, University of Illinois, Urbana, IL.
Email: leyiwang@illinois.edu

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Abstract

First described in 1955 in New Jersey, epizootic haemorrhagic disease (EHD) causes a severe clinical disease in wild and domestic ruminants worldwide. Epizootic haemorrhagic disease outbreaks occur in deer populations each year from summer to late autumn. The etiological agent is EHD virus (EHDV) which is a double-stranded segmented icosahedral RNA virus. EHD virus utilizes point mutations and reassortment strategies to maintain viral fitness during infection. In 2018, EHDV serotype 2 was predominantly detected in deer in Illinois. Whole genome sequencing was conducted for two 2018 EHDV2 isolates (IL41747 and IL42218) and the sequence analyses indicated that IL42218 was a reassortant between different serotypes whereas IL41747 was a genetically stable strain. Our data suggest that multiple strains contribute to outbreaks each year.

KEYWORDS
epizootic haemorrhagic disease virus, reassortment, serotype

1 | INTRODUCTION

Epizootic haemorrhagic disease (EHD) is an insect-transmitted infectious disease in wild and domestic ruminants. The outbreak of EHD was first reported in 1955 in New Jersey of the United States (Shope, Macnamara, & Mangold, 1960). Following the initial identification, EHD has widely been found in many states in the USA. EHD virus (EHDV) is a non-enveloped icosahedral virus with a genome consisting of 10 segments of double-stranded RNA, coding for seven structural proteins (VP1 to VP7) and four non-structural proteins (NS1, NS2, NS3 and NS3a). Based on the antigenic analyses, EHDV was originally classified into eight serotypes; however, a further study proved that the serotype 3 was actually serotype 1, indicating that there are seven serotypes (Anthony et al., 2009). Genetic analysis has also been used to determine geographic genetic types (Shirafuji et al., 2017).

In the USA, two serotypes EHDV1 and EHDV2 have been circulating for many decades, whereas a new reassortant EHDV6 is also found endemic since 2006 (Allison et al., 2010; Ruder et al., 2017). Previous

studies tentatively concluded that VP2 and VP5 segments of US reassortant EHDV6 were derived from exotic Australia EHDV6 with remaining eight segments from endemic EHDV2 (Allison et al., 2010). Currently, US EHDV6 is found in several states (Ruder et al., 2017). In addition to infection in cervids, EHDV can also cause infection in bovids including cattle, bison, and yaks. Worldwide, infections in cattle were reported for four different serotypes of EHDV1, EHDV2, EHDV6 and EHDV7 in outbreaks and sporadic cases (Savini et al., 2011). All three serotypes of EHDV in deer (EHDV1, 2, and 6) and two serotypes in cattle (EHDV2 and 6) have been reported in the State of Illinois, indicating a complex situation of EDHV in Illinois (Schirtzinger et al., 2019). In the current study, we report the identification of EHDV and genetic characterization of two EHDV2 isolates in Illinois.

2 | MATERIALS AND METHODS

2.1 | EHDV detection

A real-time RT-PCR targeting NS3 (S10) of EHDV1 and EHDV2 developed previously was applied to all samples including different tissues and blood submitted directly by clients or collected by pathologists in our laboratory (Wilson et al., 2009). Since the NS3 gene of US reassortant EHDV6 is derived from EHDV2, the aforementioned test can also detect US EHDV6. Since 2018, regardless of positive or negative tested by NS3 real-time RT-PCR, other sets of real-time RT-PCR assays are used for screening all samples and typing positive samples (Maan et al., 2017).

2.2 | Virus isolation

Virus was isolated from spleen tissue samples of deer. EHD virus positive spleen tissues were homogenized in MEM, and centrifuged at 1,341 *g* for 20 min to remove debris. After centrifugation, the supernatant was filtered through 0.45 μM filter and used to inoculate BHK cells that were propagated in MEM with 10% calf serum. A real-time RT-PCR assay was used to monitor virus growth. After two passages, two EHDV isolates (IL41747 and IL42218), which were used in the present study, showed the Ct values of 14.

2.3 | Sequencing and phylogenetic analysis

Viral RNA was extracted using QIAamp One-For-All nucleic acid kit (QIAGEN) and the sequence-independent single-primer amplification (SISPA) method was used for metagenomic amplification (Wang, Stuber, Camp, Robbe-Austerman, & Zhang, 2016). A Nextera XT kit was used for library preparation followed by sequencing using MiSeq reagent kit v2 (500-cyles) on MiSeq as described previously (Wang et al., 2016). Raw FASTQ data were assembled using SPAdes software (Bankevich et al., 2012) and the generated FASTA file was used for megablast against the local nucleotide database. Phylogenetic analyses of sequences were performed using the neighbour-joining method with 1,000 bootstrap replicates in MEGA 7.0.26.

3 | RESULTS AND DISCUSSION

During the past decade (2009–2018), University of Illinois Veterinary Diagnostic Laboratory received 745 cases for EHDV testing, of which 146 were positive for EHDV with a positive rate of 19.6% (Table S1). EHDV2 was the predominant serotype with 87.5% in 2018. Among 14 EHDV2 confirmed cases in 2018, three samples were randomly chosen for virus isolation, and IL41747 and IL42218 were successfully isolated. Viral RNA was extracted using QIAamp One-For-All nucleic acid kit, and metagenomic sequencing was conducted on MiSeq (Wang et al., 2016). After assembly and megablast, complete or near-complete genomic sequences were acquired for both isolates and deposited in the GenBank Database (Accession number: MK754239–MK754248 for IL41747 and MK754249–MK754258 for IL42218). The strains with complete sequences of all 10 segments available at GenBank were included for analysis in the present study.

It was intriguing that the phylogenetic analysis showed that IL41747 and IL42218 were distantly related to each other on all trees of different genes, especially structural genes (Figures 1–4, Figure S1, and Table S2), suggesting that multiple EHDV2 strains were co-circulating in the State of Illinois in 2018. A further observation revealed that IL41747 was closely related to the 2007 Indiana cattle strain CC372-07b, two 2011 strains (Pennsylvania strain 11-3174 and Kansas State strain CC11-295) and 2012 Ohio cattle strain 12-43618, which forms a small stable cluster in trees of all genes except NS2 (Figure S1). In the NS2 tree, IL41747 correlated with only three (CC372-07b, CC11-295, and 12-43618) but not with 11-3174. These data suggest that 2018 IL41747-like strains are genetically stable and slowly evolving in hosts.

In the present study, a further analysis of the phylogenetic trees for US reassortant EHDV6 revealed that its internal eight segments were not solely derived from endemic serotype 2 (Allison et al., 2010). Instead, a few segments (VP1 and NS1 for all five strains of US EHDV 6 and VP4 and VP6 for the original Indiana strain CC 304-06) were derived from endemic EHDV1 (Figures 1–4 and Figure S2). In addition, five US EHDV6 strains with complete genome sequences did not cluster together on the trees of VP4, VP6, and NS2 (Figures 2 and 3, Figure S1). In the VP4 and VP6 trees, CC 304-06 correlated with EHDV1 whereas the other four EHDV6 strains showed a close relatedness to EHDV2 (Figures 2 and 3). In the NS2 tree, CC 304-06, OV208 Florida, and three other strains formed three different lineages (Figure S1). These data indicate that US EHDV6 strains may have been generated through complex and multiple reassortment events.

Surprisingly, our data analysis revealed that the strain IL42218 was an inter-serotype reassortant with its VP2, VP5, NS1 and NS3 derived from EHDV2, VP4 and VP6 derived from EHDV1, and VP1, VP3, VP7 and NS2 derived from US EHDV6 (Figures 1–4, Figures S1 and S3). Thus, IL42218 is a multiple reassortant between EHDV1, EHDV2 and EHDV6. In addition, IL42218 had a 6-nucleotide insertion in the VP6 gene compared with other EHDV2 strains, making its VP6 two amino acids longer (Figure S4). This insertion was only found in other serotypes including endemic and foreign EHDV1

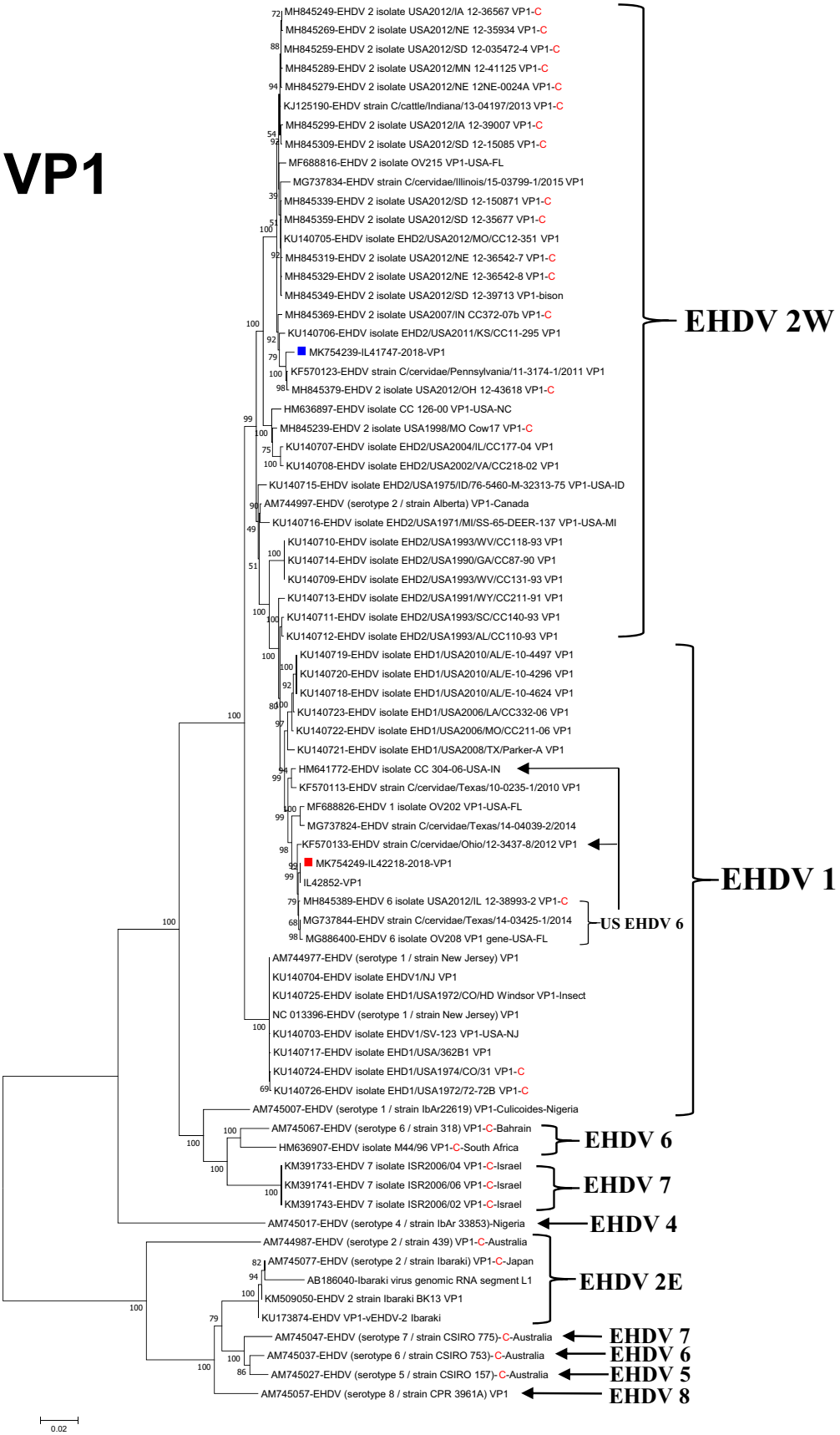

FIGURE 1 Phylogenetic tree analysis of VP1 and VP2 sequences of EHDV strains including IL41747 (indicated with a blue square) and IL42218 (indicated with a red square). The sequences acquired from GenBank were labeled with their accession numbers and non-Cervidae host information was provided with C representing cattle. Serotype information is provided [Colour figure can be viewed at wileyonlinelibrary.com]

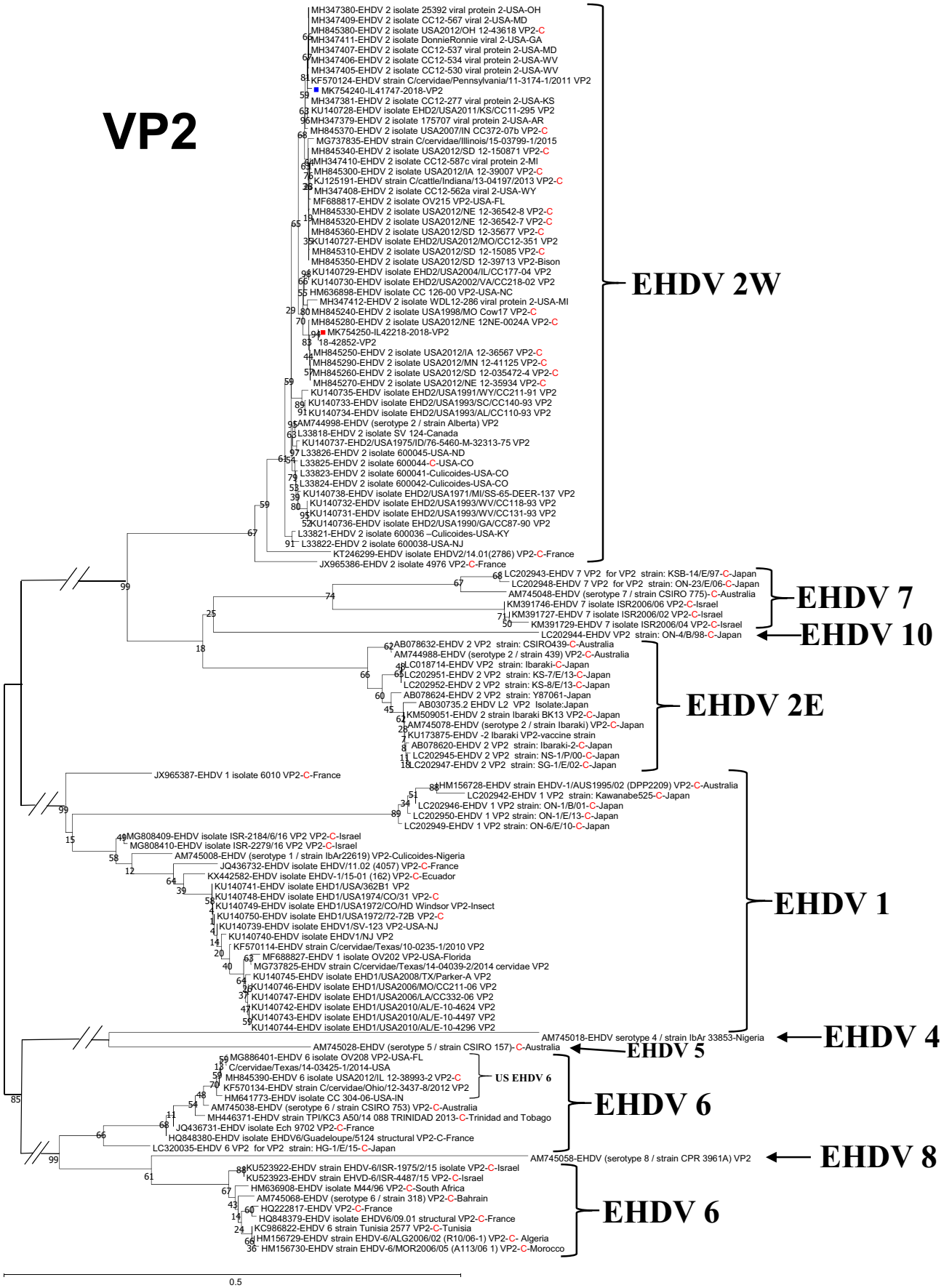

FIGURE 1 (Continued)

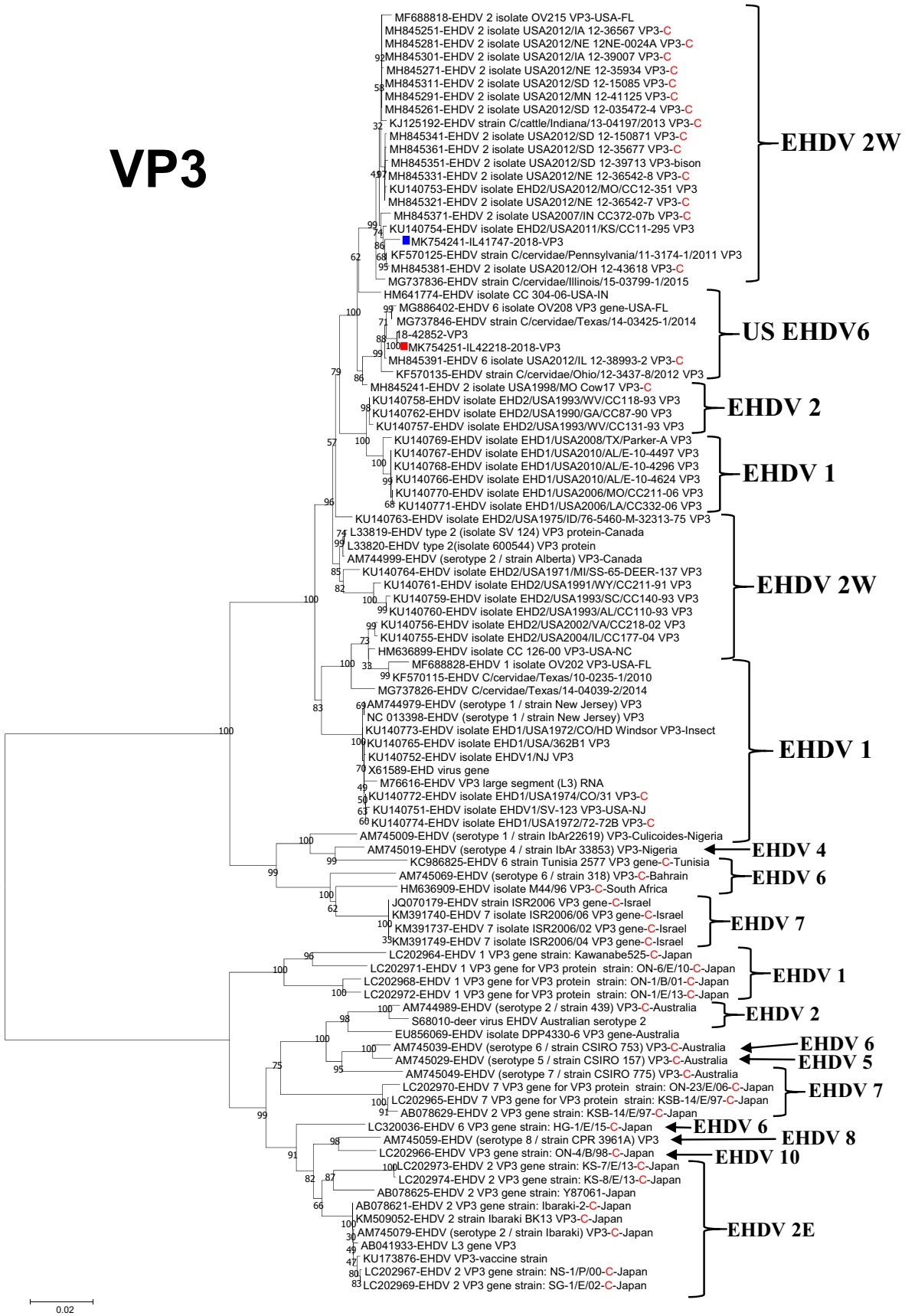

FIGURE 2 Phylogenetic tree analysis of VP3 and VP4 sequences of EHDV strains including IL41747 (indicated with a blue square) and IL42218 (indicated with a red square). The sequences acquired from GenBank were labelled with their accession numbers and non-Cervidae host information was provided with C representing cattle. Serotype information is provided [Colour figure can be viewed at wileyonlinelibrary.com]

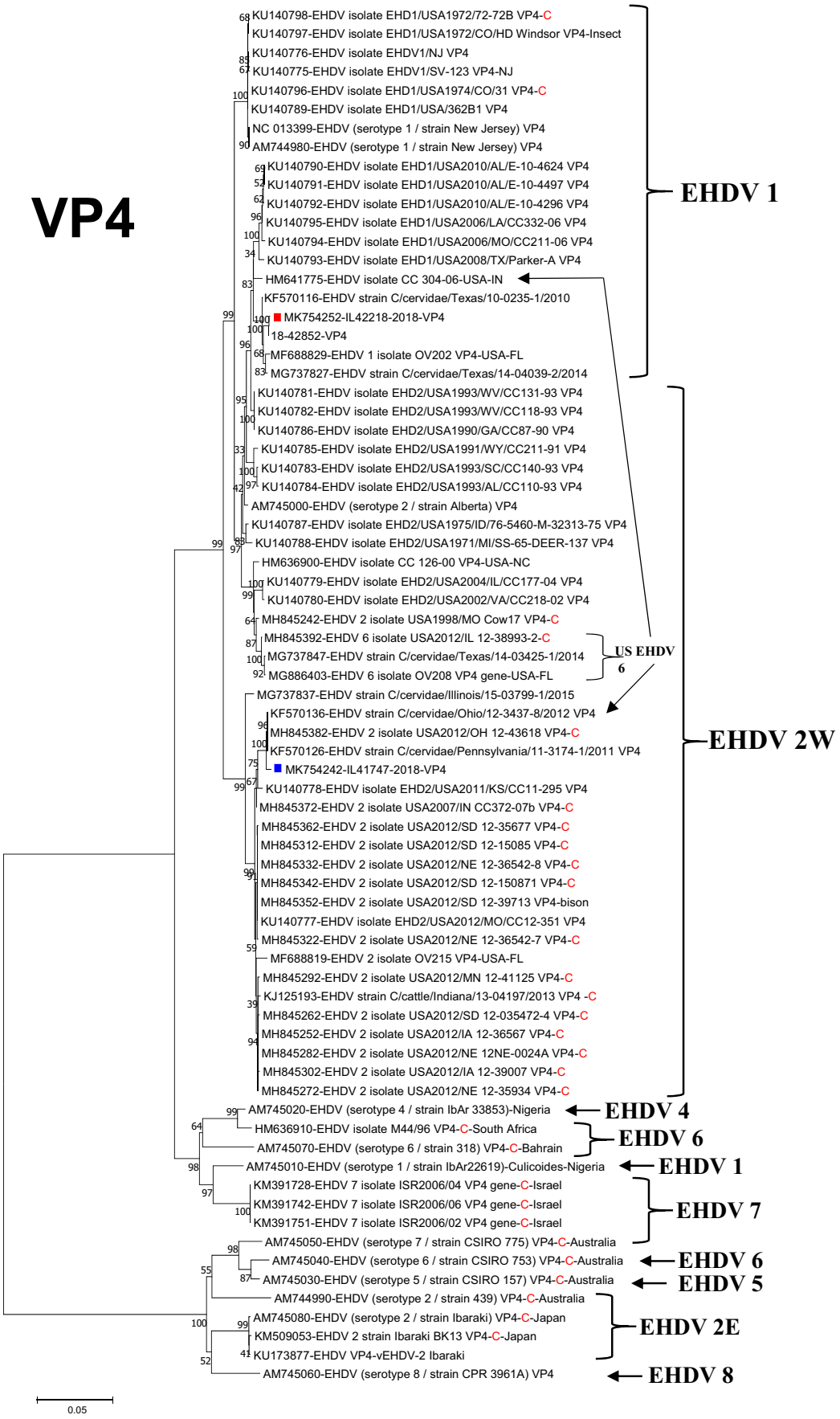

FIGURE 2 (Continued)

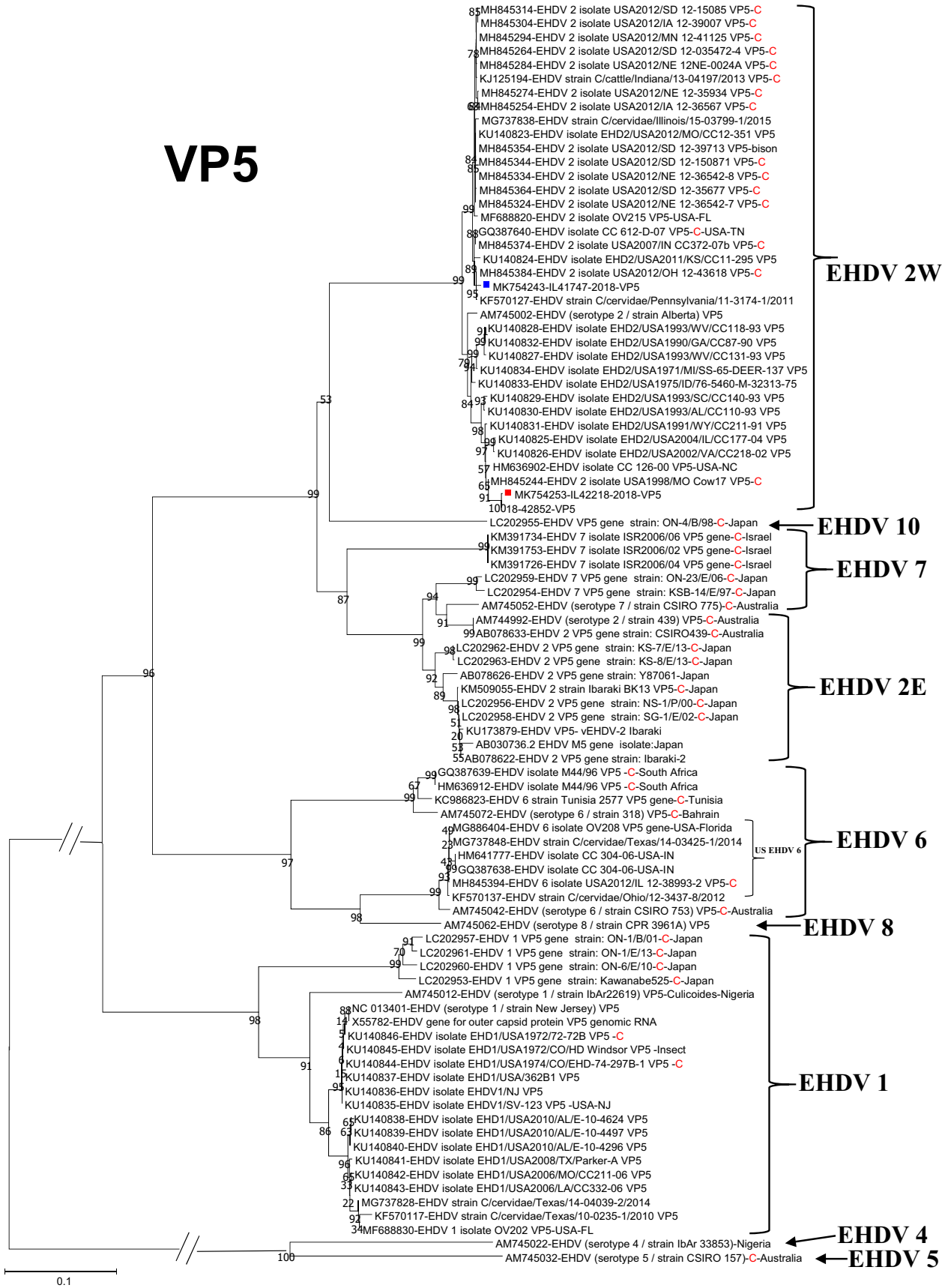

FIGURE 3 Phylogenetic tree analysis of VP5 and VP6 sequences of EHDV strains including IL41747 (indicated with a blue square) and IL42218 (indicated with a red square). The sequences acquired from GenBank were labeled with their accession numbers and non-Cervidae host information was provided with C representing cattle. Serotype information is provided [Colour figure can be viewed at wileyonlinelibrary.com]

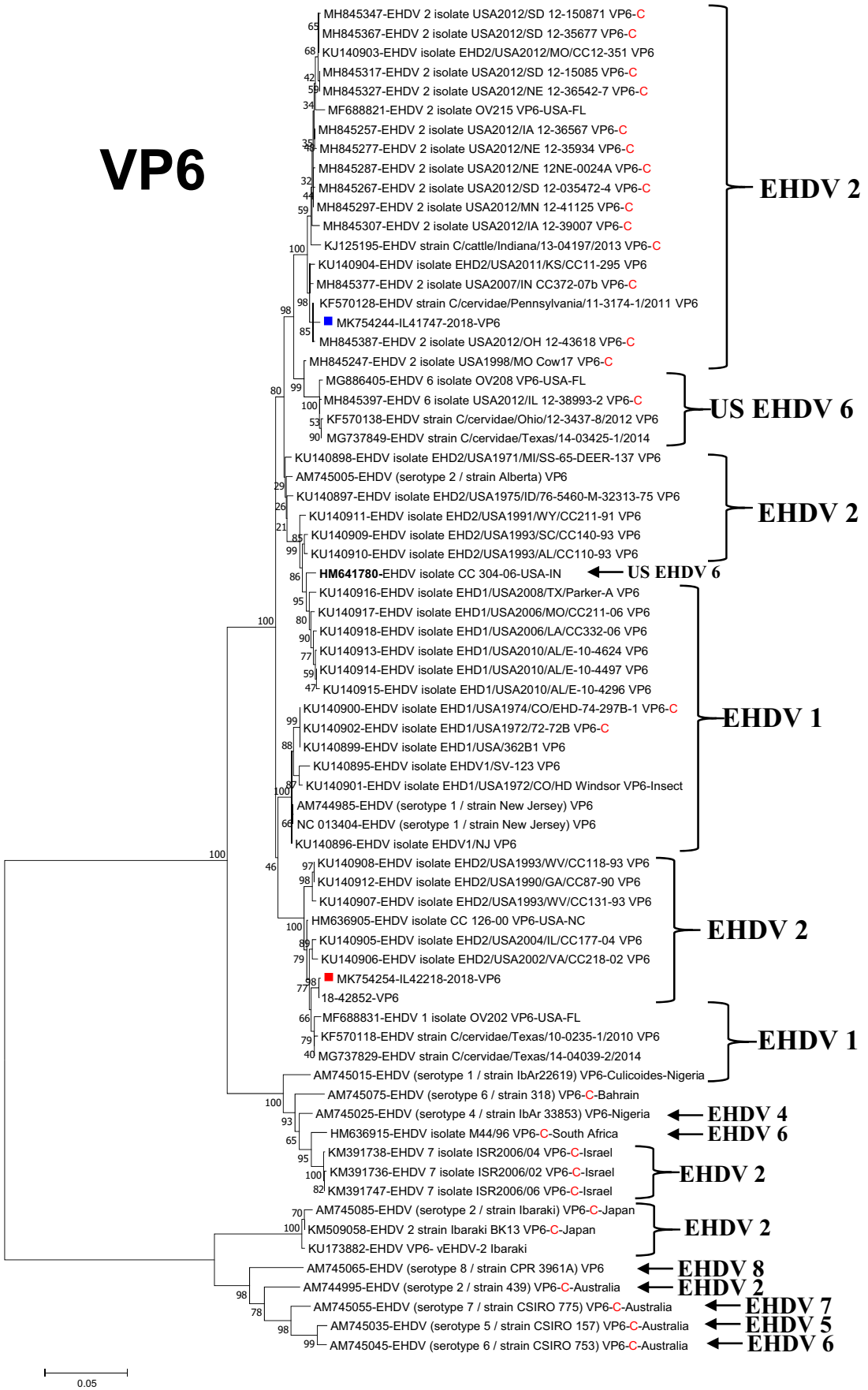

FIGURE 3 (Continued)

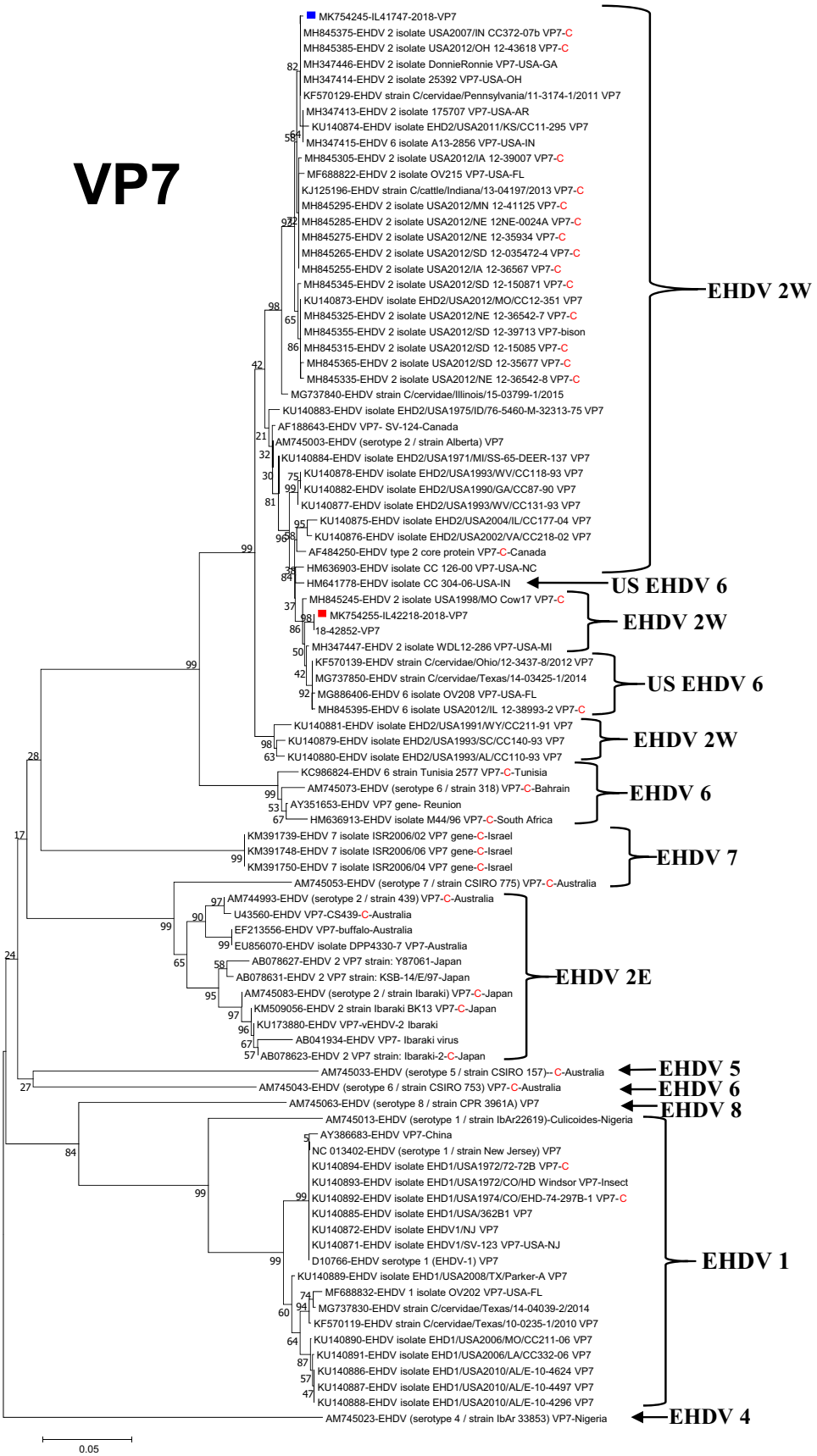

FIGURE 4 Phylogenetic tree analysis of VP7 and NS1 sequences of EHDV strains including IL41747 (indicated with a blue square) and IL42218 (indicated with a red square). The sequences acquired from GenBank were labelled with their accession numbers and non-Cervidae host information was provided with C representing cattle. Serotype information is provided [Colour figure can be viewed at wileyonlinelibrary.com]

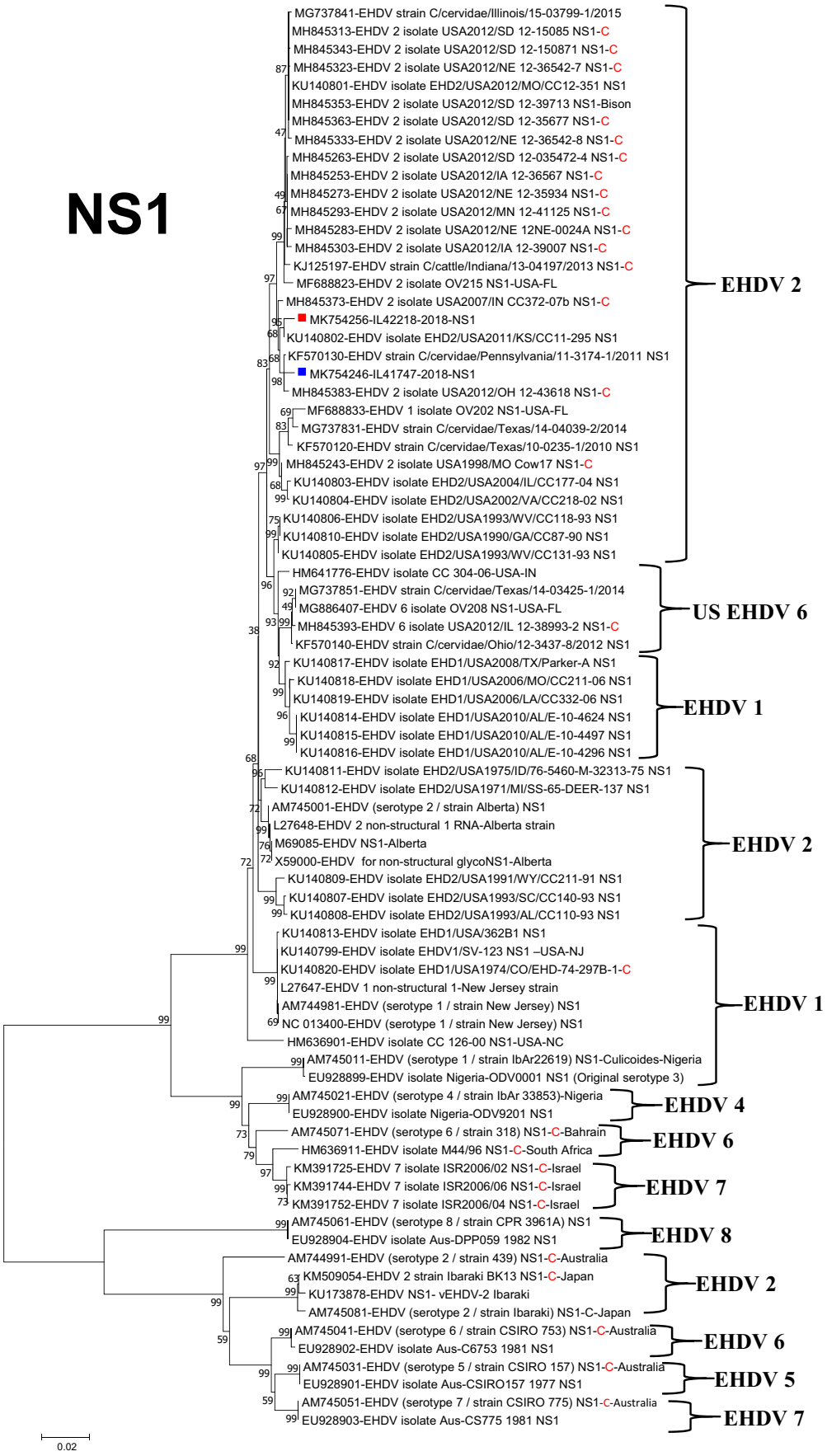

FIGURE 4 (Continued)

and other foreign types (EHDV4, EHDV6 and EHDV7). Analyses of two other 2018 clinical strains showed that they clustered together with IL42218, indicating that IL42218-like reassortants were predominant in Illinois in 2018. It should be noted that, due to an early start codon (before the insertion region) for NS4 protein encoded by VP6 gene observed in IL42218, 6-nt insertion did make its NS4 protein 2-aa longer but have no effect on NS4 expression of two other IL42218-like strains (IL42852 and IL42760; Figure S4).

Unlike the sequencing of few segments, whole genome sequencing (WGS) of all 10 segments of EHDV allows analysis of genetic changes and reassortment, providing a complete picture of virus evolutions. In our study, in addition to sequencing of two isolates, direct sequencing of two clinical samples with lower Ct values was also conducted, and complete or near-complete genomes of 7 or 9 segments were obtained. Previous studies used viral isolates instead of clinical samples for WGS of EHDV (Anbalagan, Cooper, Klumper, Simonson, & Hause, 2014; Schirtzinger et al., 2019; Wilson et al., 2016). Therefore, our and those studies suggest that the bottleneck for WGS of EHDV is a need for virus isolation. Further optimization of WGS protocols is warranted to determine circulating virus genetic populations in infected animals.

Segmented RNA viruses have a tendency for genetic reassortment in nature. Reassortment in bluetongue virus is flexible and a high percentage of reassortants carrying heterologous VP2 and VP5 protein genes are identified during in vitro experiments (Shaw et al., 2013). In contrast, EHDV as a member virus in the same family as bluetongue virus appears to require homologous reassortment (VP2 and VP5), such as US EHDV6. A previous study indicates that EHDV1 and EHDV2 also need homologous reassortment for VP7 (Anbalagan et al., 2014). In the current study, we show that IL42218 is an inter-serotype reassortant. This inter-serotype reassortant IL42218 strain has a very similar component of internal genes to those of US EHDV6. It remains unclear whether US EHDV6 has evolved from IL42218-like virus, or vice versa. Future studies are needed in vitro and in vivo to characterize the triple reassortant IL42218 strain, including growth curve comparisons and gene segment swapping using a reverse genetics tool. Five US EHDV6 strains form different lineages on the phylogenetic trees, raising the question as to whether more recent strains result from a continual reassortment between the original reassortant 2006 Indiana strain and EHDV1/2, or through multiple and parallel reassortment events.

With regards to how EHDV is maintained and spread in nature, a genomic characterization study suggests that multiple strains instead of a single strain are introduced and spread during outbreaks (Crum, Mead, Jackwood, Phillips, & Stallknecht, 2018). In the present study, we show that at least two different EHDV2 strains were co-circulating in Illinois in 2018, supporting the theory that multiple strains may be involved in EHD outbreaks. Multiple strains during outbreaks may contribute to virus reassortment between and within serotypes. Since a low evolution rate is observed for vector-borne EHDV (Jenkins, Rambaut, Pybus, & Holmes, 2002; Murphy, Howerth, MacLachlan, & Stallknecht, 2005), viral reassortment may play a critical role in the EHDV evolution.

In summary, our data suggest that the reassortant IL42218 and genetically stable IL41747 strains contributed to 2018 outbreaks in the State of Illinois. Continued WGS and analysis of EHDV is needed to monitor viral evolution.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ORCID

Leyi Wang https://orcid.org/0000-0001-5813-9505
Ganwu Li https://orcid.org/0000-0002-6370-9573

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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