

Molecular evolution of epizootic hemorrhagic disease viruses in North America based on historical isolates using motif fingerprints

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Abstract Epizootic hemorrhagic disease virus (EHDV) is an orbivirus of the Reoviridae family that has significant impact on wild and captive white-tailed deer. Although closely related to bluetongue virus that can cause disease in sheep and cattle, North American EHDV historically has not been associated with disease in cattle or sheep. Severe disease in cattle has been reported with other EHDV strains from East Asia and the Middle East. To understand the potential role of viral genetics in the epidemiology of epizootic hemorrhagic disease, a molecular characterization of North American EHDV strains from 1955 to 2012 was conducted via conventional phylogenetic analysis and a new classification approach using motif fingerprint patterns. Overall, this study indicates that the genetic make-up

of EHDV populations in North America have slowly evolved over time. The data also suggested limited reassortment events between serotypes 1 and 2 and introduces a new analysis tool for more detailed sequence pattern analysis.

Keywords Epizootic hemorrhagic disease · Molecular evolution · Motif fingerprint

Introduction

Epizootic hemorrhagic disease (EHD) in North American wild ruminants, caused by epizootic hemorrhagic disease virus (EHDV), was first reported in 1960 [1]. This first strain is known as EHDV New Jersey or serotype 1. Another strain was isolated in Alberta Canada and has been designated as serotype 2 [2]. These two EHDV serotypes have been circulating in North America since the initial isolations. The biting midge (*Culicoides sonorensis*) was shown to be capable of transmitting these two strains of EHDV [3, 4]. The EHDV genome consists of segmented double-stranded RNA, as in other members of the Family *Reoviridae*, genus *Orbivirus* [5]. Ten dsRNA segments encode for 7 structural and 4 non-structural proteins [6]. Segments L2 and M6 encode the outer capsid VP2 and VP5 that affect virus serum antibody neutralization. VP2 is the primary determinant of virus serotype. Underneath the capsid is the inner core containing VP3 and VP7 encoded by segments L3 and S7. Within the core are the minor structural proteins including the virus polymerase VP1 encoded by L1 as well as VP4 (M4) and VP6 (S9) [6]. The M5 gene segment is highly conserved between North American serotypes 1 and 2 [7] and encodes the NS1 protein that forms tubules in cells [8]. The NS2 gene that

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Table 2 Percent nucleotide and (predicted amino acid) sequence identities based on Muscle multiple sequence alignment

Genome segment (gene)	Total no. of sequences*	Type 1 Lowest % identity	Type 2 Lowest % identity	Type 1 and 2 Lowest % identity	All NA types Lowest % identity
L1 (VP1)	30 (13/15/2)	96.3 (98.3)	96.7 (98.8)	96.1 (98.3)	96.0 (98.3)
L2 (VP2)	37 (14/21/2)	96.5 (97.4)	96.1 (96.6)	48.8 (33.9)	44.2 (30.9)
L3 (VP3)	30 (13/15/2)	95.6 (98.0)	96.2 (99.3)	95.6 (98.0)	95.6 (98.0)
M4 (VP4)	30 (13/15/2)	97.0 (98.8)	95.0 (98.1)	94.8 (98.1)	94.8 (98.1)
M5 (NS1)	32 (14/16/2)	97.2 (98.0)	96.8 (98.7)	96.8 (98.0)	96.7 (98.0)
M6 (VP5)	32 (13/16/3)	96.8 (98.7)	95.6 (99.2)	64.8 (67.6)	63.6 (65.3)
S7 (VP7)	75 (20/53/2)	97.2 (98.8)	95.7 (98.6)	78.4 (93.4)	78.4 (93.4)
S8 (NS2)	33 (14/17/2)	95.9 (96.0)	93.9 (84.2)	93.5 (83.6)	92.9 (83.6)
S9 (VP6)	30 (13/15/2)	94.8 (93.0)	95.0 (93.9)	94.8 (93.1)	94.2 (92.2)
S10 (NS3)	101 (35/64/2)	94.9 (97.4)	93.8 (98.2)	93.4 (97.4)	93.4 (97.4)

* No. of sequences aligned Type1/Type2/Type 6

encodes the phosphorylated NS2 protein is also highly conserved between North American serotypes 1 and 2 [9]. The smallest gene segment S10 encodes two non-structural proteins NS3 and NS3a using two in frame start codons [10].

Previously, molecular evolution studies of North American EHD viruses have been done based on single genes and findings have varied. Initial studies indicated that the L2 and L3 genes of EHD viruses segregate into genetically distinguishable geographic groups [11, 12]. A subsequent study based on L2 and S10 revealed a more random distribution of genetic types [13], but a later study utilizing these same gene segments after an outbreak of disease again demonstrated a tendency for geographic genetic types or topotypes [14]. This discrepancy may be due to differences in how the comparisons were made and which viral gene segments were used for each study. To generate a more complete understanding of the molecular evolution of these viruses, we have generated a comparison of all ten genome sequences of representative strains from 1955 until 2012. Historically, only serotypes 1 and 2 were known to circulate in North America [5]; however, in 2006, serotype 6 was identified [15]. In this study, we determined sequences for EHDV 1 and 2 strains and analyze the data in conjunction with previously reported serotype 6 sequences.

Materials and methods

Virus and RNA purification

The viruses were obtained from collections maintained by the Arthropod-Borne Animal Diseases Research Unit (USDA, Agricultural Research Service, Manhattan, KS

and the Southeastern Cooperative Wildlife Disease Study (University of Georgia, Athens, GA) (Table 1). Typically, viruses were isolated from tissue (liver or spleen) using BHK or CPAE cell culture and RNA extracted as described previously [16]. The double-stranded RNA was further purified as described previously [17] and purity assessed with agarose gel electrophoresis and UV absorbance.

Whole genome amplification, sequencing, and phylogenetic analysis

The whole genome amplification was done as described previously [18] with modifications described in [16, 19, 20]. In the beginning of this project, an ABI 454 DNA sequencer (Life Technologies, Grand Island, NY) was used to generate the sequence data. Later during the study, an Ion Torrent PGM sequencer (Life Technologies, Grand Island, NY) was used. The sequences were assembled with the default parameters of the map to reference assembly and analyzed using Geneious 7.0 (Biomatters Ltd, New Zealand). Raw data already had trimmed regions annotated from the sequencer; however, additional trimming was performed to ensure removal of any residual primer regions by utilizing the re-trim sequences option. Once consensus sequences were created, additional 5' and 3' manual trimming was done based on consensus terminal sequences of 5'-GTTAAA.....CACTTAC-3'. Sequence alignments for each viral gene segment independently, and predicted proteins derived from their open reading frames were performed with Muscle and consensus phylogenetic trees constructed using a Jukes–Cantor Neighbor-joining bootstrap (1000 replicates) analysis (Geneious 8.0, Biomatters Ltd, New Zealand). The gene segments of the EHDV serotype 1 strain from Nigeria 1967 were used to root the phylogenetic trees.

Fig. 1 Neighbor-joining consensus phylogenetic trees using Jukes–Cantor distance model of 1000 boot-strapped iterations for representative EHDV genes encoding outer capsid virus structural proteins. **a** L2 (VP2) and **b** M6 (VP5)

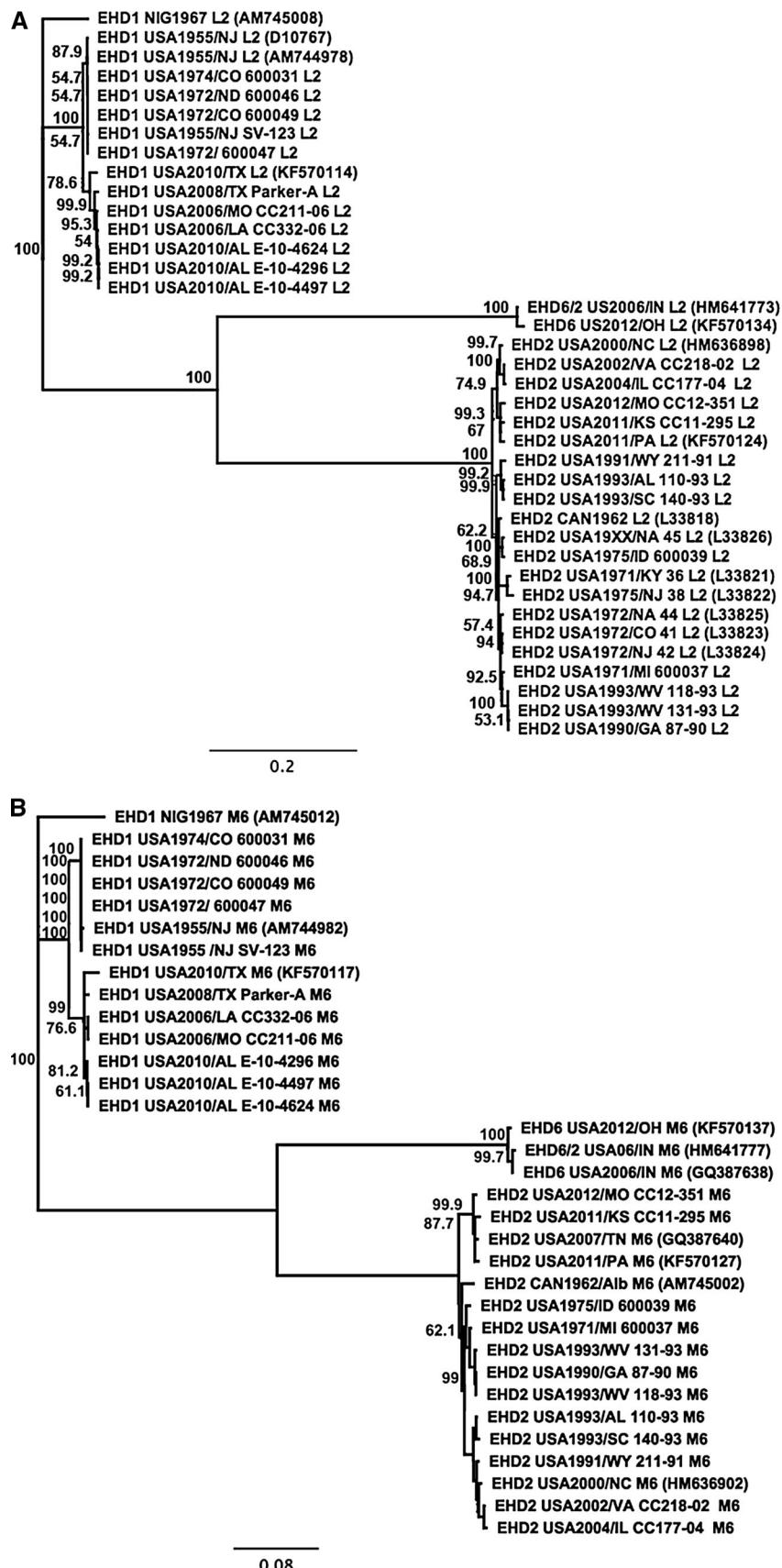


Fig. 2 Neighbor-joining consensus phylogenetic trees using Jukes–Cantor distance model of 1000 boot-strapped iterations for the EHDV genes encoding core virus structural proteins. L3 (VP3) and S7 (VP7)

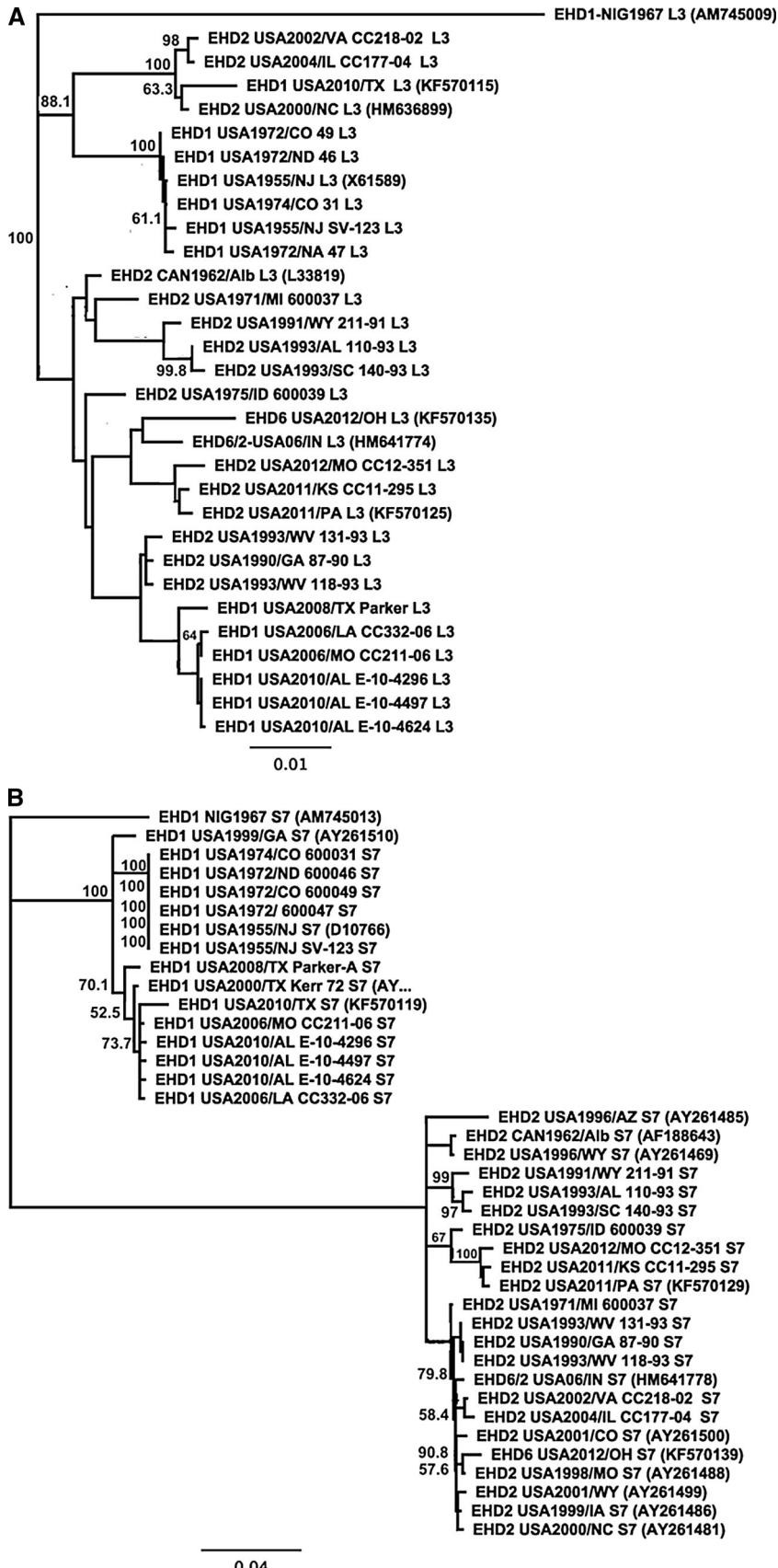


Fig. 3 Neighbor-joining consensus phylogenetic trees using Jukes–Cantor distance matrix of 1000 boot-strapped iterations for representative EHDV genes encoding inner virus structural proteins. **a** L1 (VP1), **b** M4 (VP4), and **c** S9 (VP6)

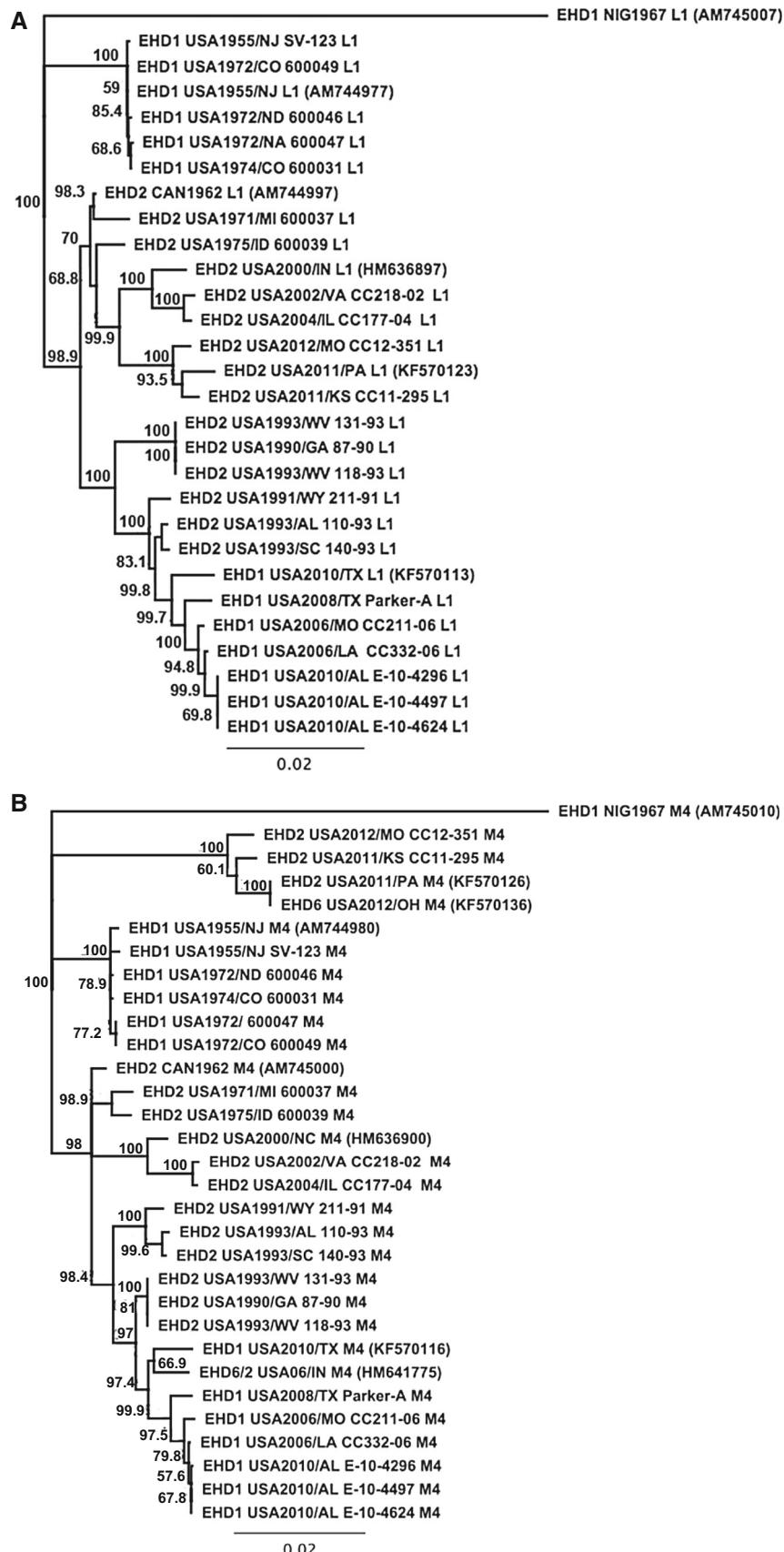
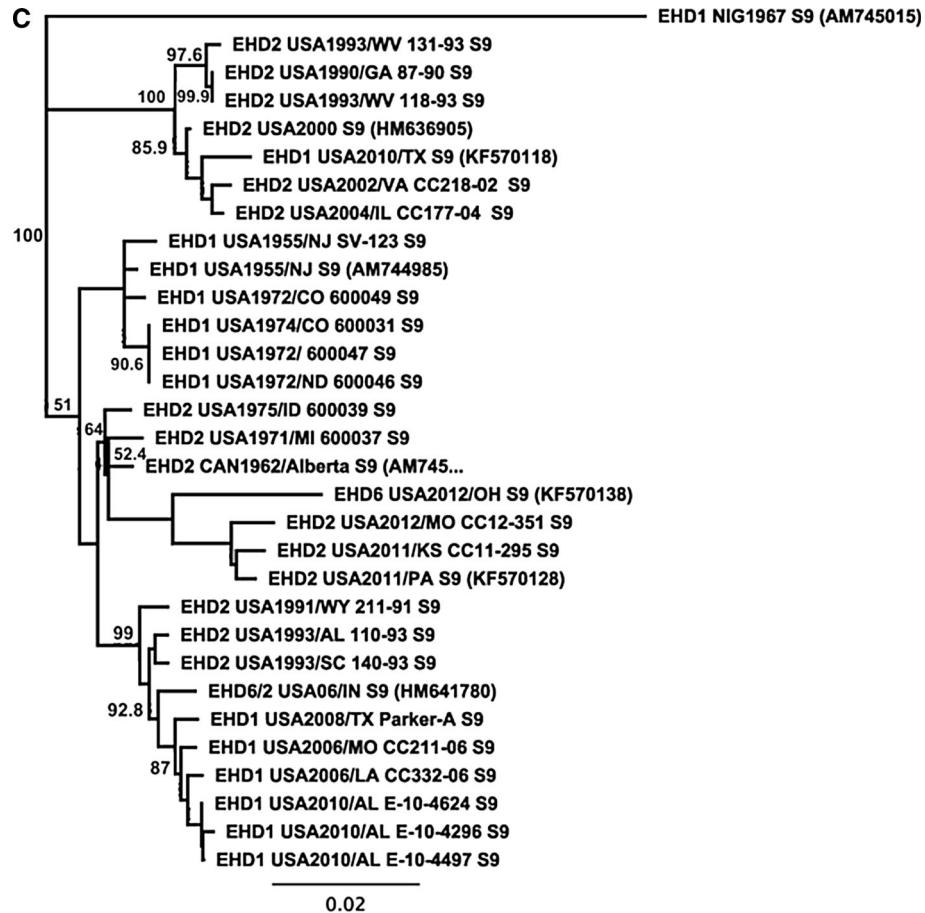


Fig. 3 continued



Motif fingerprint discovery

All proteins entries from Genbank assigned to the *Orbivirus* genus were used for this analysis. Each protein was divided into position-independent and non-overlapping subsequences of twelve amino acids long. This motif library was scanned across every known proteome covering more than 283,000 taxonomies assigning organism strain, serotype, species, family, and superfamily. Two amino acid substitutions were used as cut-off to be considered a homology hit of membership to each of these taxonomic levels. With the scanning process, we classified three types of motif fingerprints (MF): *MF-Type I* are segments specific to a given viral taxonomical group (e.g., species, sub-types or strain); *MF-Type II* are shared by the host and pathogen only, which might have been co-opted by the pathogen to affect immune signaling, regulatory or metabolic pathways; and *MF-Type III* are non-specific segments shared in more than two species. Only *MF-Type I* specific to EHDV and stored in an object relation database were used in the subsequent clustering analysis [21].

Clustering of MF matrix

We developed a simplified representation of the presence of a MF in the EHDV viral genome by generating a MF event matrix (MFEM) where each column (g) represents a MF occurrence (1) or absence (0), and each row represents a particular EHDV MF event (n) in a given strain. To determine the distance between viral entries (i.e., genus, species and strain), the MFEM = $g \times n$ array was bi-clustered using the Pearson correlation coefficient (r) between g . Since the number of g values contained in each sequence is uneven, we used a hierarchical clustering algorithm. For the final formation of the cluster, we weighed the distance between n as the average distance where the number of MF in each EHDV genome. Let x and y be n -component vectors for which we want to calculate the degree of association. For pairs of quantities (x_i, y_i) , $i = 1, \dots, n$, the correlation coefficient r is given by the formula

$$r = \frac{\sum_{i=1}^n (x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum_{i=1}^n (x_i - \bar{x})^2 \sum_{i=1}^n (y_i - \bar{y})^2}},$$

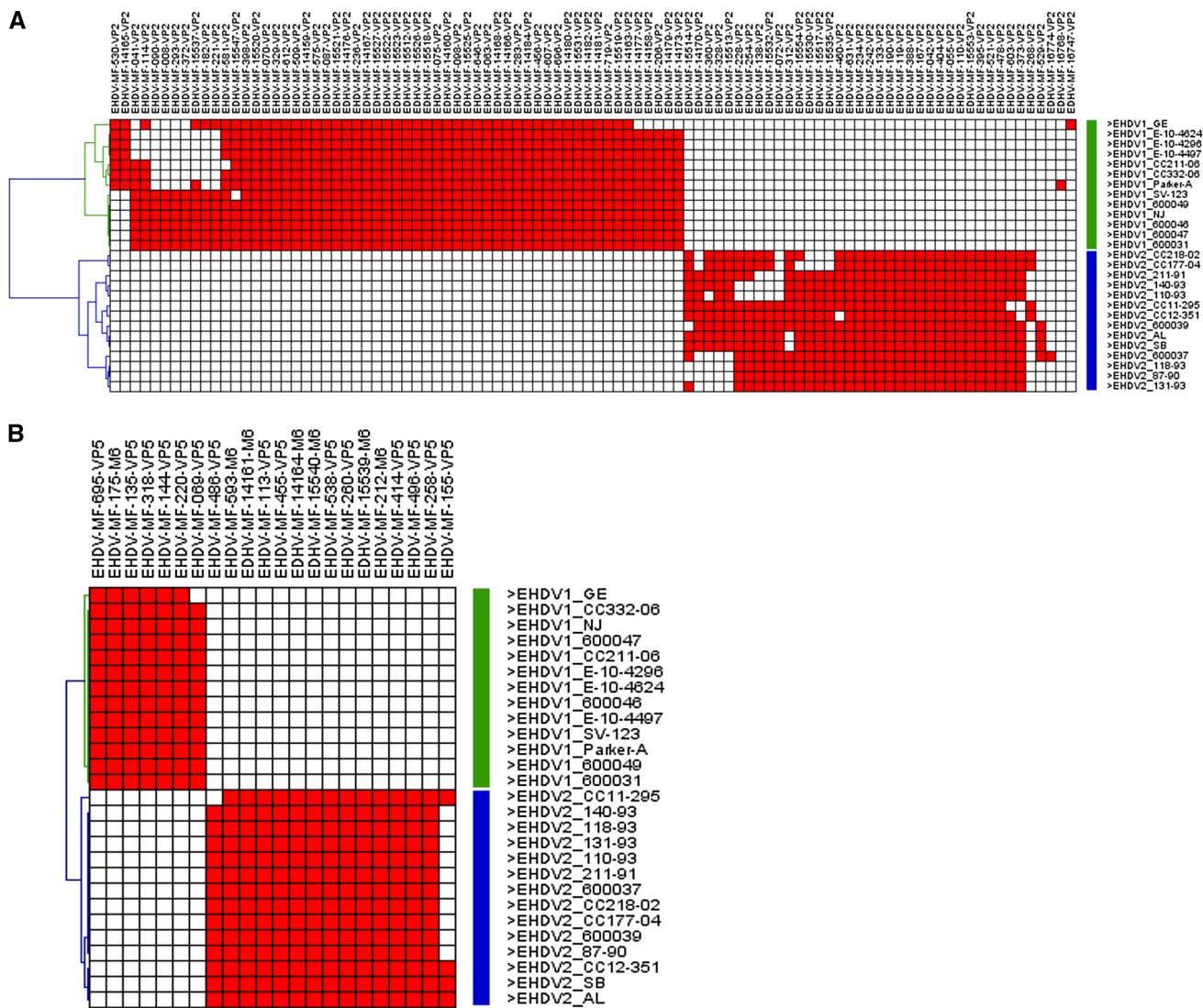


Fig. 4 Motif fingerprinting for representative EHDV genes encoding outer capsid virus structural proteins. **a** L2 (VP2) and **b** M6 (VP5). Red indicates the presence of peptide motif, and white indicates the absence of the motif. Green indicates EHDV1 and blue indicates EHDV2

where \bar{x} and \bar{y} are the means of the vectors of x and y , respectively.

Results

The study generated twenty-four total genome sequences for EHDV types 1 and 2. During the course of the study, the technology used changed from initially the ABI 454 DNA sequencer to the Ion Torrent PGM sequencer when the latter became available. There were no conclusive differences due to artifacts of assembly depending on technology; however, that was not the scope of this project. A few apparent artifacts were realized during this study. First and most obvious are the read lengths and depth of

coverage. The 454 data, while having longer read lengths [50–804 base pair (bp)], did not have as even coverage throughout the genome (8–1755). The PGM had shorter read lengths (18–549 bp), but a more uniform depth (40–5724) throughout the genome. Sections in the largest segments (VP1, VP2) were always more difficult to resolve with both technologies. Without using a reference sequence to build the assemblies, it was easier to assemble full-length genes using data from the 454 than the PGM. Comparisons of the sequences from six strains for each of the serotypes 1 and 2 were generated using the two technologies. There was no evidence that the sequence and/or assembly technology used affected the sequence data. The majority of the sequence data was generated using the Ion Torrent PGM sequencer.

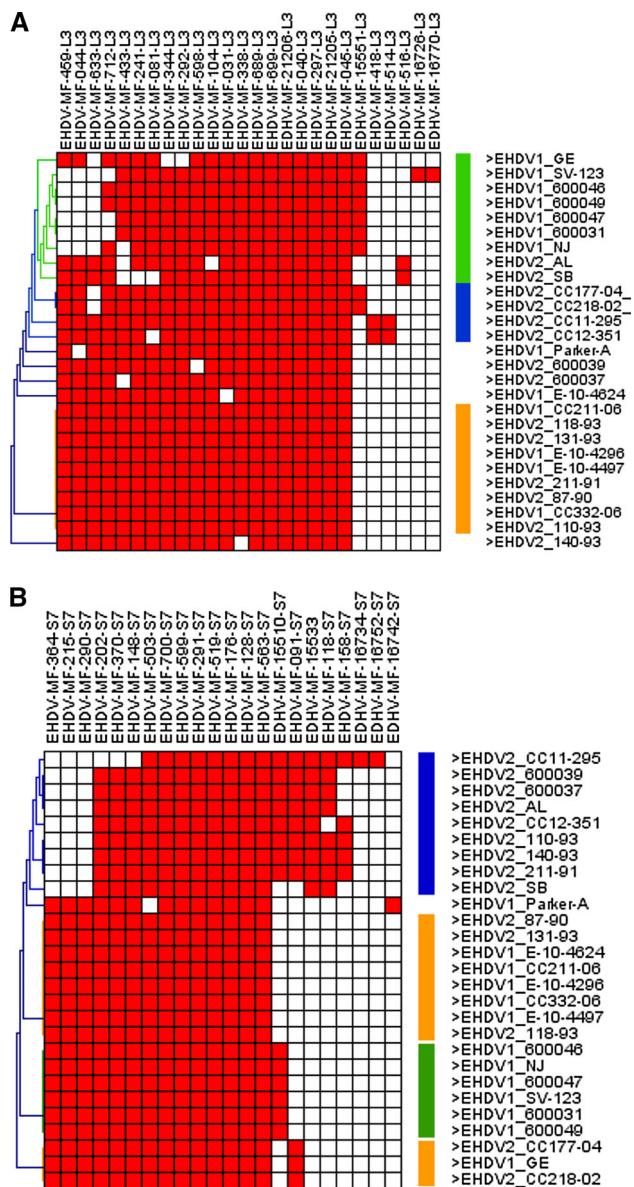


Fig. 5 Motif fingerprinting for the EHDV genes encoding core virus structural proteins. L3 (VP3) and S7 (VP7). Red indicates the presence of peptide motif, and white indicates the absence of the motif. Green indicates EHDV1 and blue indicates EHDV2, and orange indicates ambiguous serotype designation due to sequence conservation

The complete sequence of twenty-four isolates generated in this study was compared to all sequences available in Genbank, and thus, the number of sequences available per genome sequence varied from 30 to 101 as shown in Table 2. Since this study was designed to compare serotypes 1 and 2 and there are limited sequence data available for serotype 6, sequence identity comparison with serotype 6 is not reported here. Sequences available for

serotype 6 were included in the phylogenetic analysis, however, for a more complete representation of North American EHDV. A phylogenetic analysis of the ten segments concatenated into a “genome-wide” artificial single-stranded genomes was conducted but found not to be informative since the serotype specific genes drove the analysis. Therefore, the data analyses reported here are based on individual genome segments.

The complete virus genomes of twelve strains of EHDV-1 were determined (Table 1). Since there were identical strains within the dataset, the range of lowest genetic variability is reported in Table 2. Within EHDV-1 serotype, the lowest sequence % identity for all segments was 94.8–97.2 % on the nucleotide level and 93–98.8 % on the predicted amino acid level (Table 2). The genomes of twelve strains of EHDV-2 were determined (Table 1) and compared with known North American EHDV-2 sequences. The range of lowest genetic variability within this serotype for all segments was 93.8–96.8 % on the nucleotide level and 84.2–99.3 % on the predicted amino acid level (Table 2). As would be expected, the greatest sequence variability between the two serotypes was noted with the L2 and M6 gene segments, at 48.8 and 64.8 % nucleotide identity, respectively. The lowest percent nucleotide identity for all North American serotypes (i.e., including serotype 6) was 44.2 % (L2) and 63.6 % (M6). Both the L2 and M6 genes segregate into distinct clades in phylogenetic analysis (Fig. 1). The recently introduced serotype 6 L2 (VP2) and M6 (VP5) genes are more closely related to serotype 2.

The L3 gene that encodes the inner core VP3 protein exhibited a high degree of conservation both within and between serotypes at 95–96 and 98–99.3 % nucleotide identity and predicted amino acid sequence identity, respectively (Table 2). Surprisingly, the S7 that encodes the other inner core protein VP7 displayed a lower sequence conservation between serotypes 1 and 2 (78.4 % identity) than the L3. Phylogenetic analysis revealed no real separation between serotypes for the L3 gene, whereas the S7 grouped according to serotype (Fig. 2).

Gene segments L1, M4, and S9 that encode the inner structural proteins are highly conserved like the L3 based on the lowest percent nucleotide and predicted amino acid identity, and are approximately the same both within and between serotypes (Table 2). There are sub-clades that are serotype specific but larger clades contain both the serotypes 1 and 2. The larger clades tend to be temporally associated but not geographically (Fig. 3).

The gene segments that encode the non-structural proteins also display similar % identity with and between North American serotypes (Table 2). The phylogenetic

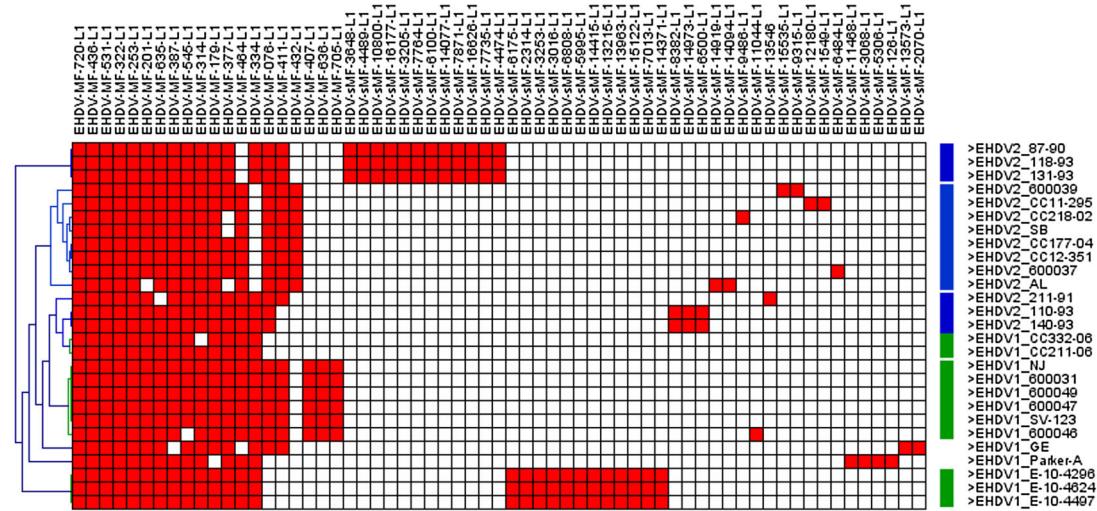
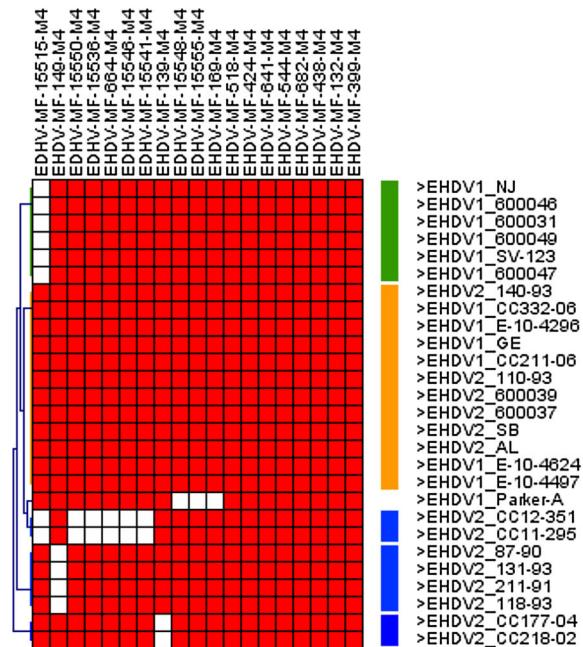
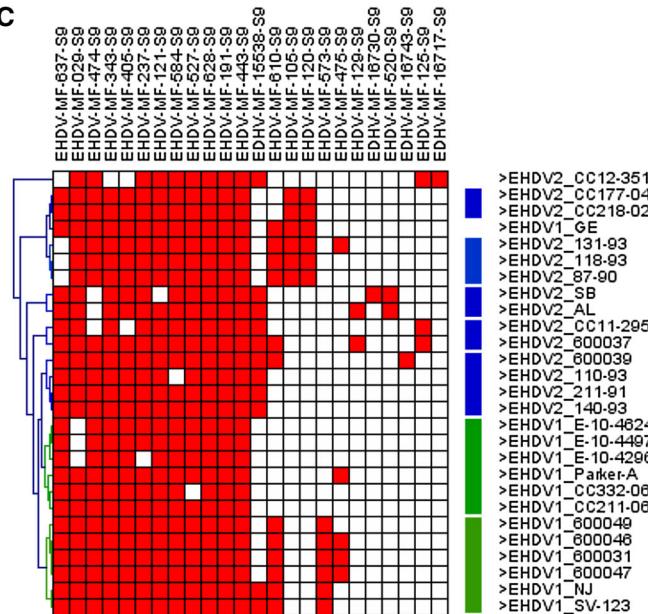
A**B****C**

Fig. 6 Motif fingerprinting for representative EHDV genes encoding inner virus structural proteins. **a** L1 (VP1), **b** M4 (VP4), and **c** S9 (VP6)

analysis showed clades that are primarily one serotype but with occasional alternate serotype included.

Motif fingerprint discovery analysis was performed to potentially identify sequence variations not detectable by more traditional phylogenetic analysis. Not only did this new method confirm the phylogenetic interpretation, but also the specificity or conservation of each motif fingerprint allowed us to rapidly identify regions in the viral genome that discriminate different serotypes and topotypes at a high resolution level. Examples of the analysis are shown in Figs. 4–7. Furthermore, our analysis established that approximately 40 % of the genome of EHDV can be

represented as *MF-Type I*, while the rest of the genome as *MF-Type II* and *MF-Type III*.

Discussion

In the US, EHD in white-tailed deer has historically been associated with EHDV serotypes 1 and 2 [5]. In 2006, serotype 6 was first isolated in the US [15]. Prior to this new isolation of EHDV-6, we had already initiated this project to determine the whole genomes of twenty-nine

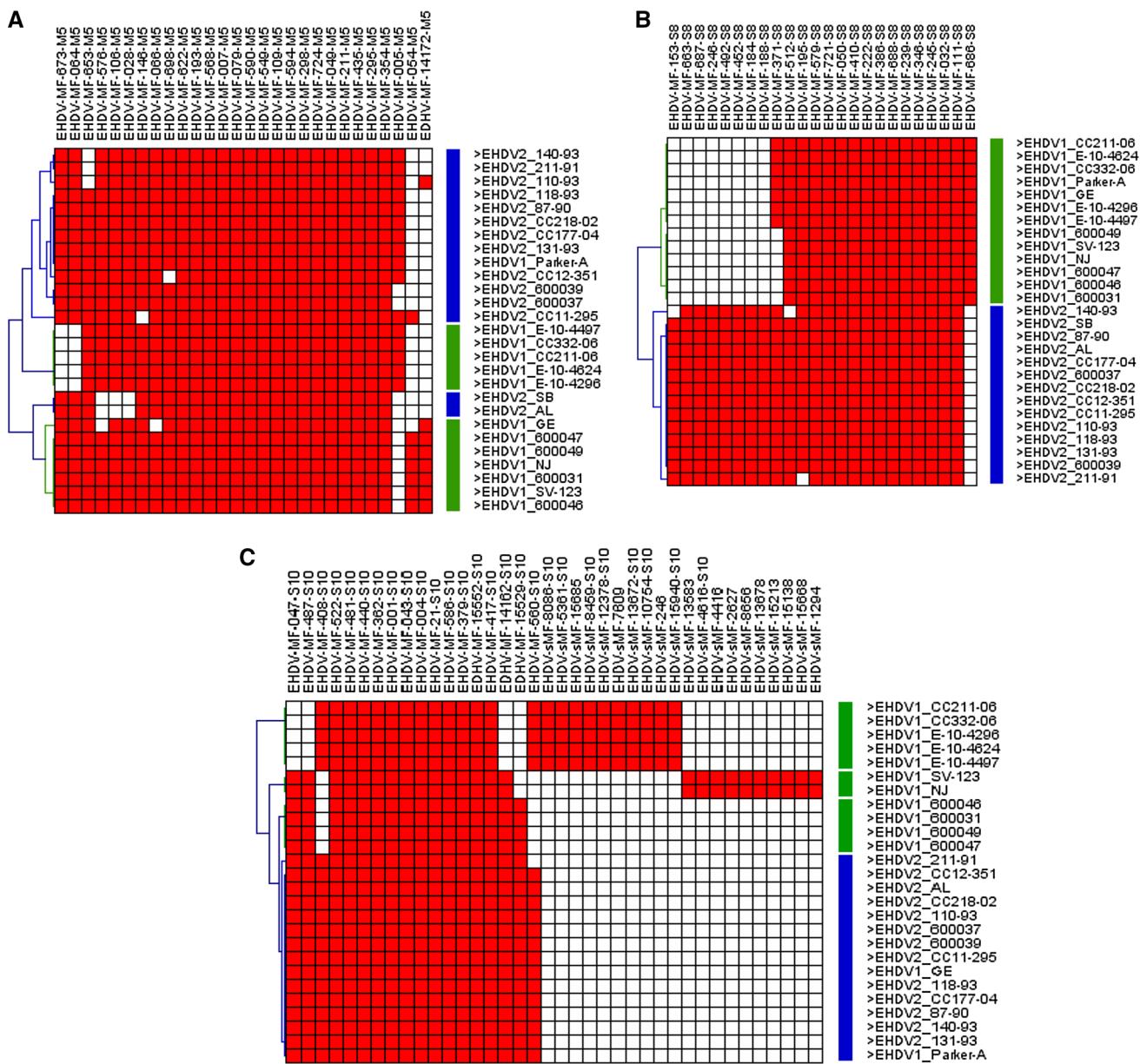


Fig. 7 Motif fingerprinting for representative EHDV genes encoding inner virus structural proteins. **a** M5 (NS1), **b** S8 (NS2), and **c** S10 (NS3)

strains of EHDV-1 and EHDV-2 in order to provide a historical perspective of EHDV in North America.

The L2 segment encodes the outer capsid protein VP2 that determines serotype [22]. As seen in previous studies focused on the L2 genetic variation [11], there is only around a 4 % genetic variation between strains of the same serotype but just under 50 % nucleotide identity between serotypes (Table 1). Perhaps not surprisingly, the M6 gene encoding the other outer capsid protein VP5 [22] also grouped phylogenetically according to serotype (Fig. 1b). What was unexpected was the lack of reassortment between EHDV-1 and EHDV-2 S7 gene segment (Fig. 1b) based on previous studies with the related Bluetongue virus

[23, 24]; however, this observation was reported previously [25]. The recently introduced EHDV-6 [15] appears to have an S7 gene closely related to EHDV-2 (Fig. 2b). The S7 encodes the inner core protein VP7 that is believed to stabilize the inner capsid structure composed of VP3 and VP7 [22]. Although the L3 genes did tend to group according to serotype, there is a clade that contains both the serotypes 1 and 2 (Fig. 2a). The genes encoding the inner structural proteins (L1, M4, and S9) all tend to have sub-clades according to serotype, but larger clades are composed of both serotypes (Fig. 3). The larger genes encoding for the non-structural proteins (M5 and S8) also displayed a similar phylogenetic pattern as the inner structural proteins

Fig. 8 Neighbor-joining consensus phylogenetic trees using Jukes–Cantor distance matrix of 1000 boot-strapped iterations for representative EHDV genes encoding inner virus structural proteins. **a** M5 (NS1), **b** S8 (NS2), and **c** S10 (NS3)

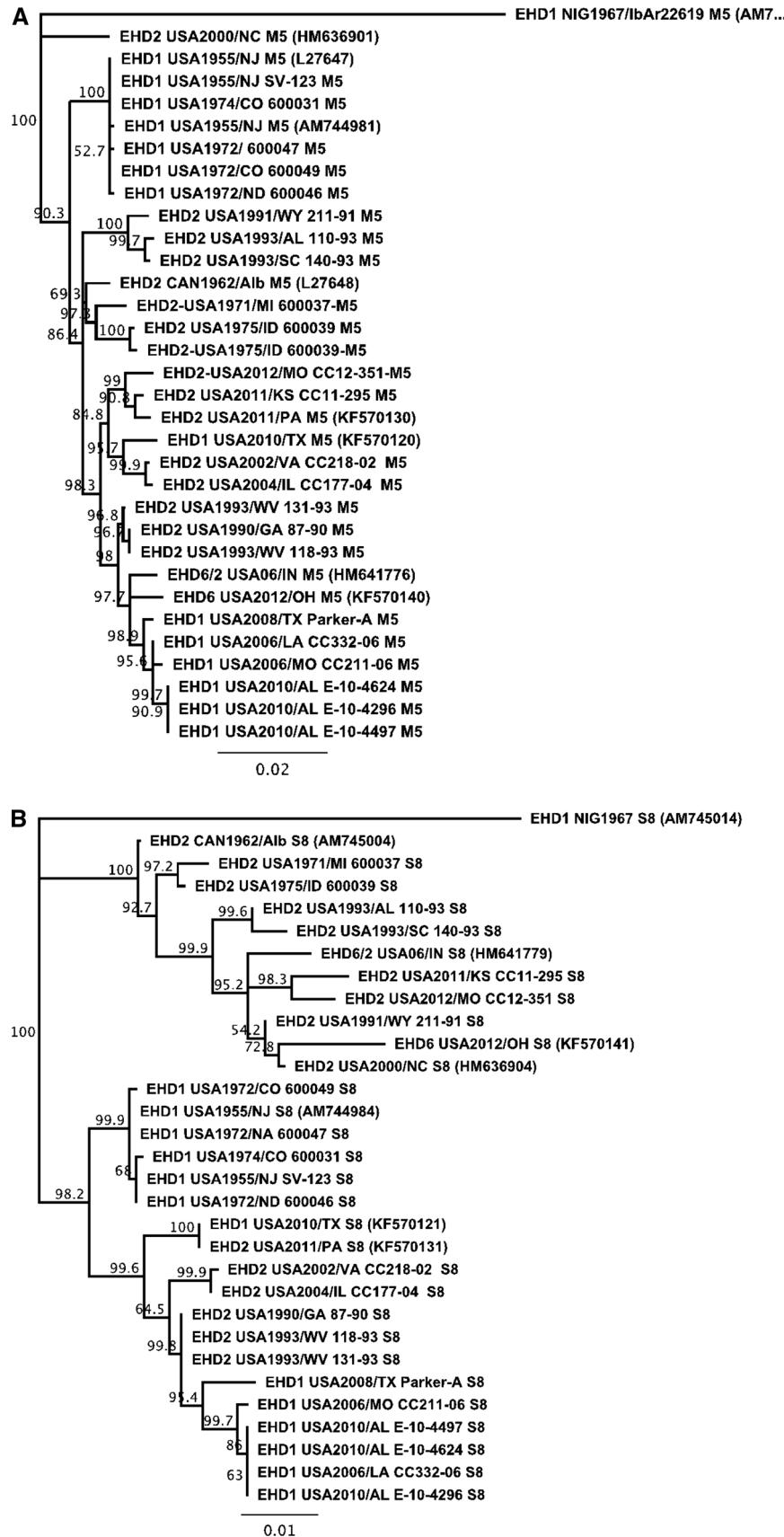


Fig. 8 continued



(Fig. 8a, b). Although to some extent the smallest gene S10 encoding the NS3 displayed a similar phylogenetic pattern, there were clades that contained both the serotypes 1 and 2. These phylogenetic observations are likely more representative of virus circulating at the time of isolation than separation based on serotype designation. Reassortment is likely less common in regions of epidemic transmission where EHDV-2 is the more commonly isolated serotype and concurrent outbreaks of both serotypes are rare. In 2012, there was isolation of EHDV-2 from cattle with clinical disease [26]. Ongoing efforts will determine the sequence of these viruses, as well as their relationship to known serotypes. While it is clear that the viruses are evolving genetically over time, it remains unclear in which, and indeed, whether these genetic changes are affecting virus phenotype. Unfortunately, cattle infection is generally subclinical, and white-tailed deer are very susceptible to disease [27]. Thus, it is problematic to investigate genetic determinants of virulence using available molecular technologies such as reverse genetics [28].

Whole genome amplification and next-generation sequencing is providing new insights into the

epidemiology of orbiviral diseases. This technology was used to understand EHDV molecular evolution. The data clearly indicate the potential to determine the genetic origin of new strains isolated from previously naïve regions. However, methodologies employing traditional sequencing and phylogenetic analysis, by contrast, clearly lack the breadth and depth that can be brought to associate traits in the specific serotypes with specific genomic regions. To address this limitation, we introduce the motif fingerprinting approach as a new tool to map genetic changes at different levels of resolution with practical implications in diagnostics, microbial discrimination, and vaccine development. These 12-amino acid long motif fingerprints named *MF-Type I* are specific to EHDV and can be mapped to their corresponding 36-nucleotide genomic signature. The analysis of different segments of EHDV using *MF-Type I* forms a binary pattern that clearly discriminates serotypes and strains and at higher resolution than phylogenetic trees. The specificity to EHDV and conservation of each motif fingerprint across many strains shows that this approach can quickly identify genome regions that can be used to design and develop new and

specific detection assays including primers and probes. Although some inconsistencies in the classification were observed due to the conservation of the viral proteins and the limited number of viral genomes, it is evident that as more orbiviral sequence data becomes available our approach will provide greater insights of the evolution and spread of EHDV over time. As shown in Fig. 5a and b, the motif fingerprint analysis of the L2 and M6 regions, which encode proteins that affect virus serum neutralizing antibodies, our approach mapped regions that are unique and conserved across different strains and serotypes. This enhances the selection of targets for antibody production and rational vaccine development. Because of their specificity, as motif fingerprint and genomic signature libraries continue to grow, this approach can be used to scan very large datasets of genomic and unbiased metagenomic information and detect EHDV. It is important to clarify that while our analysis focuses in the discrimination using *MF-Type I*, regions of the viral genome (*MF-Type II* and *MF-Type III*) can be associated with adaptation; however, because of their lack of specificity to EHDV and limited resolution, they were excluded from this study.

This manuscript provides a foundation of genetic information to understand the molecular evolution of EHDV and introduces new tools to further delineate genetic differences and similarities. Monitoring of genetic changes among new isolates will continue to provide insights into the molecular epidemiology of EHDV.

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Compliance with ethical standards

Conflict of interest The corresponding author and a majority of the authors do not have conflicts of interest; however, the Motif Fingerprinting software utilized was developed by co-authors from Orion Integrated Biosciences Inc.

Human and animal rights This article does not contain any studies with human participants or animals performed by any of the authors.

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