THE UL31 TO UL35 GENE SEQUENCES OF DUCK ENTERITIS VIRUS CORRESPOND TO THEIR HOMOLOGS IN HERPES SIMPLEX VIRUS 1

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Summary. – Five ORFs in the genome of Duck enteritis virus (DEV) corresponding to UL31, UL32, UL33, UL34, and UL35 genes of Herpes simplex virus 1 (HSV-1) were amplified by a modified "targeted gene walking" PCR, cloned, and sequenced. UL33, UL34, and UL35 genes were oriented from the left to the right of genome, while UL31 and UL32 had an opposite orientation. A comparison of deduced amino acid sequences of the DEV ORFs with their alphaherpesvirus homologs showed well-conserved regions except for the UL34 and UL35 genes. Phylogenetic analysis revealed that DEV was closer to the genus *Mardivirus* than to any other genus of the subfamily *Alphaherpesvirinae.* Based on this evidence, we proposed to assign DEV to the subfamily *Alphaherpesvirinae*.

Key words: Duck enteritis virus; UL31, UL32, UL33, UL34, and UL35 gene sequences; phylogenetic analysis

Introduction

Duck viral enteritis is an acute and contagious disease affecting the members of the order *Anseriformes*. At first, it was detected in domestic ducks in Holland as early as 1923 (Baudet, 1923). China recorded its first outbreak in 1957 (Huang, 1959). The causative agent of the disease DEV or Anatid herpesvirus 1 is presented as an unassigned virus within the family *Herpesviridae* (Fauquet *et al*., 2005). The disease can lead to a vascular damage with tissue hemorrhaging, digestive mucosal eruptions, lymphoid organ lesions, and degeneration of the parenchymatous organs (Liebovitz, 1991). Birds recovered from the infection often turned into carriers of DEV and shed the virus. Reactivation of the latent DEV is involved in enteritis outbreaks in both domestic and migrating waterfowl that causes difficulties in monitoring and control of the disease (Shawky and Schat, 2002).

The genome of DEV is a linear double-stranded DNA that contains approximately 180 kb with a G+C content of 64.3%, the highest report for any avian herpesvirus (Gardner *et al*., 1993). The full genome sequence and organization of DEV has not been reported to date. The typical genome of an alphaherpesvirus is divided into unique long (UL) and unique short (US) segment that are flanked by internal and terminal repeats (IR and TR). Some genes of DEV located at the UL region including UL6 and UL22–UL30 containing 11 complete ORFs have been characterized (Plummer *et al*., 1998; Hansen *et al*., 1999; Li *et al*., 2006; Liu *et al.*, 2007). The arrangement and the transcription orientation of these genes are consistent with HSV-1 and Marek's disease virus type 1 (MDV-1) (McGeoch *et al*., 1988; Lee *et al*., 2000),

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Abbreviations: aa = amino acid; BoHV-1 = Bovine herpesvirus 1; DEV = Duck enteritis virus; EHV-1 = Equid herpesvirus 1; EHV-4 = Equid herpesvirus 4; HSV-1 = Herpes simplex virus 1; HSV-2 = Herpes simplex virus 2; HVT = Turkey herpesvirus; ILTV = Infectious laryngotracheitis virus; IR = internal repeat; MDV-1 = Marek's disease virus type 1; MDV-2 = Marek's disease virus type 2; NLS = nuclear localization signal; PRV = Pseudorabies virus; TR = terminal repeat; UL = unique long; US = unique short; VZV = Varicella-zoster virus

what suggested a similarity in the genome organization of DEV and alphaherpesviruses.

In this study, we presented the molecular characteristics of the genes encoding UL31, UL32, UL33, UL34, and UL35 proteins of DEV. We performed a phylogenetic analysis of 5 proteins deduced from UL31–UL35 genes to illustrate the evolutionary relationships between DEV and alphaherpesviruses, what would be helpful for classification of DEV into taxonomical scheme. Furthermore, the sequences of the genes UL31–UL35 were useful in PCR diagnosis of duck viral enteritis.

Materials and Methods

Virus strain. In this work, we worked with the strain DEV clone-03 that was used also for the preparation of commercial DEV vaccine in China. The virus was purified as described previously and was propagated in chicken embryo fibroblasts growing in DMEM (Gibco-BRL) (Li *et al*., 2006; Liu *et al*., 2007). When the cytopathic effect reached 80%, the infected cells were harvested and subjected to three freezing-thawing cycles and stored at -70°C until used.

Viral DNA extraction and PCR. Infected cell lysates were centrifuged at 10,000 rpm for 5 mins. The supernatant was used for extraction of viral DNA by the extraction kit (Invitrogen). A modified "targeted gene walking" PCR strategy was used to amplify the unknown sequence of DEV genome (Parker *et al*., 1991). The procedure for amplification of DEV genome fragment was summarized in Fig. 1. UL30 gene (Acc. No. EF203709) was selected as a starting point to amplify viral genome. Primers used in PCR amplification were listed in Table 1. PCR was carried out in a 25 μl reaction mixture as described previously (Li *et al*., 2006).

Cloning and sequencing. PCR products were analyzed by electrophoresis in 0.8% agarose and sequenced directly or after cloning into the pMD18-T vector (TaKaRa). Each region was sequenced from different PCR products at least three times.

Sequence and phylogenetic analysis. To search for ORFs, the full-length assembled sequence was analyzed by using the program of Gene Runner (version 3.00, Hasting Software, Inc.) on both strands. As a second approach to identify genes in DEV, the sequence was submitted to a GenBank for Blast search analysis. The deduced amino acid sequences of these ORFs were compared with their homologs in other alphaherpesviruses using the DNAStar program (version 7.0, DNAStar, Inc.). To assess the genetic relationship of DEV, phylogenetic analysis of 5 deduced proteins with their homologs was performed by using the Megalign program with the method of Clustal V (DNAStar). The reference strains and Acc. Nos. were as follows: Herpes simplex virus 1 (HSV-1) (X14112), Herpes simplex virus 2 (HSV-2) (NC_001798), Varicella zoster virus (VZV) (NC_001348), Bovine herpesvirus 1 (BoHV-1) (NC_001847), Equid herpesvirus 1 (EHV-1) (AY464052), Equid herpesvirus 4 (EHV-4) (NC_001844), Pseudorabies virus (PRV) (NC_006151), Marek's disease virus type 1 (MDV-1) (NC_002229), Marek's disease virus type 2 (MDV-2) (NC_002577), Turkey herpesvirus (HVT) (AF291866), Infectious laryngotracheitis virus (ILTV) (NC_006623).

Table 1. Primers used in PCR amplification

Primer name	Direction	Primer sequences
S1	Forward	5'-TTTAAAACGCTTTATTCCAG-3'
S2	Reverse	5'-GTTCTGCTGCTTCTAGGAGT-3'
S3	Forward	5'-GCATTTCAAATGCGTCATCG-3'
S4	Reverse	5'-TCGCTGTATTAGGTCCCTAC-3'
S5	Forward	5'-AATAGTATATTACGGCGGCT-3'
S6	Reverse	5'-CAAAACACACTAACCACATG-3'
S7	Forward	5'-TGAAAACAATGGCTGGAATC-3'
S8	Reverse	5'-GAGAACGATGAAGAGATAAG-3'
A1	Forward	5'-ACTGTGAGAGTGACGAAACC-3'
A2	Reverse	5'-ATTCTTCAAACGACGTTCAG-3'
A3	Forward	5'-CCTAGTCGCCAATCTTTTAG-3'
A4	Reverse	5'-GAAAGCGACATGGATTCGCC-3'

Results

Sequence and molecular characteristics of DEV UL31–UL35 genes

The assembled fragments containing 5 complete ORFs were designated as corresponding homologs of HSV-1, e.g. UL31, UL32, UL33, UL34, and UL35. These 5 genes of DEV were arranged collinearly to homologs in HSV-1 as followed: 5'-UL31-UL32-UL33-UL34-UL35-3' (Fig. 1c). UL31 and UL32 gene had the same transcription orientation, while the other three genes in the same transcription direction were opposite to the former gene cluster. UL31 and UL32 shared the same predicted poly (A) signal located at 89 to 94 nt downstream of the stop codon of UL31 gene. In addition, there were two potential poly (A) signals shared by UL33, UL34 and UL35 gene downstream of the stop codon of UL35 gene. The DNA sequence from UL31 to UL35 of DEV clone-03 genome is available from GenBank under the Acc. No. EF203708.

UL31 was composed of 933 nt encoding a protein of 310 aa with a M_r of 38 K. The 3'-terminus of DEV UL31 overlapped with the UL30 gene by 77 nt in a tail-to-tail direction. DEV UL31 protein showed an identity from 21.6% to 52.9% compared with 11 reference strains. It showed a closer relationship with VZV, but was far from ILTV. UL31 had four phosphorylation sites for protein kinase C at the 12, 40, 50, 164 aa, respectively. Multiple alignment of the UL31 protein with homologs of reference strains revealed four high-consensus regions (Fig. 2).

The 3'-terminus of UL32 gene overlapped with UL31 by 8 nt. UL32 gene encoded a protein of 597 aa that shared a strong homology with corresponding homolog of HSV-1 and MDV-1. Compared with the reference strains, protein UL32 showed the lowest identity of 29.6% with ILTV and

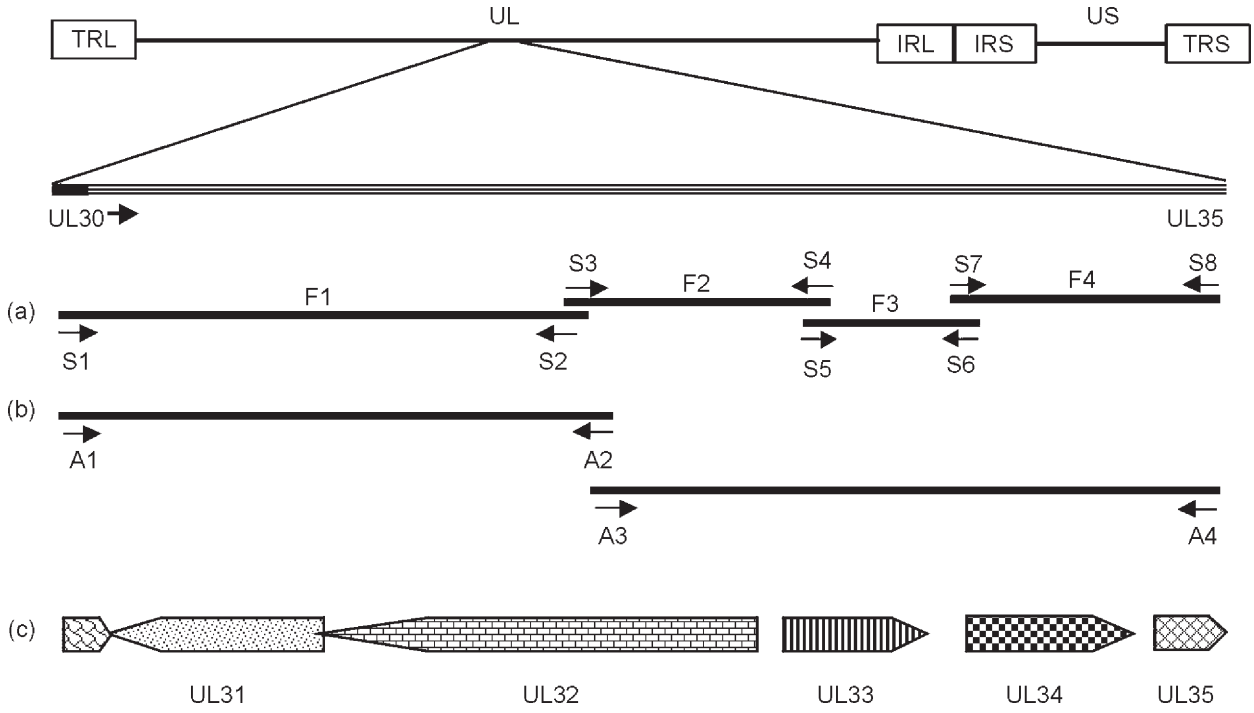


Fig. 1

PCR strategy and arrangement of the genes UL31 through UL35 in DEV genome

The genomic structure is adopted according to the HSV-1 prototype. (a) four fragments, F1, F2, F3, and F4 were amplified using primers S1S2, S3S4, S5S6, S7S8 and assembled to a form of continuous DNA fragment. (b) two pairs of specific primers (A1A2 and A3A4) spanning the amplified region were used to confirm the accuracy of the amplification. (c) transcription orientation of genes UL31 through UL35.

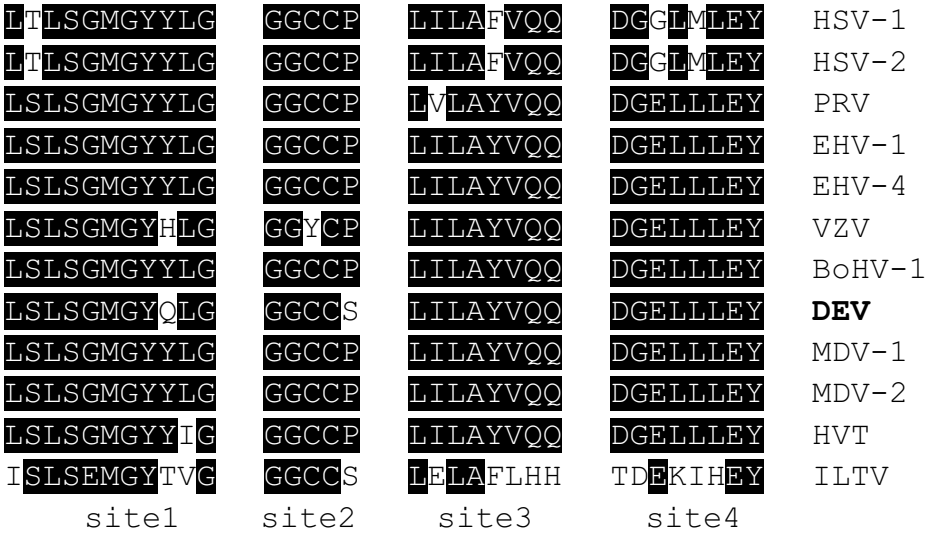


Fig. 2

Multiple alignment of 4 sites in UL31 homologs of DEV and examined alphaherpesviruses

Black areas = identical aa.

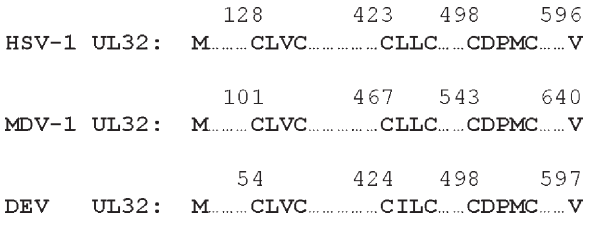


Fig. 3

Conserved cysteine rich zinc-binding structure in UL32 homologs of HSV-1, MDV-1 and DEV

CXXC or CXXXC sequences were shown with numbers indicating the aa position.

the highest of 44.6% with EHV-1. The C-X-X-C or C-X-X-X-C motif was a cysteine rich, zinc-binding structure that was conserved in the UL32 protein among alphaherpesviruses (Fig. 3). In addition, UL32 possessed five potential N-linked glycosylation sites at the 11, 184, 203, 211, 356 aa, respectively. Seven protein kinase C phosphorylation sites were predicted at the 62, 112, 174, 177, 213, 410, 554 aa, respectively.

DEV UL33 gene was a 408 bp continuous ORF encoding a protein of 135 aa with a M_r of 14.7 K. A non-coding sequence 132 bp was found between the genes UL32 and UL33. DEV UL33 protein showed an identity from 30.8% to 46.3% in relation to the reference strains. It demonstrated the highest identity with BoHV-1 and the lowest with MDV-1. The deduced protein of DEV UL33 had significant amino acid identity with 11 counterparts

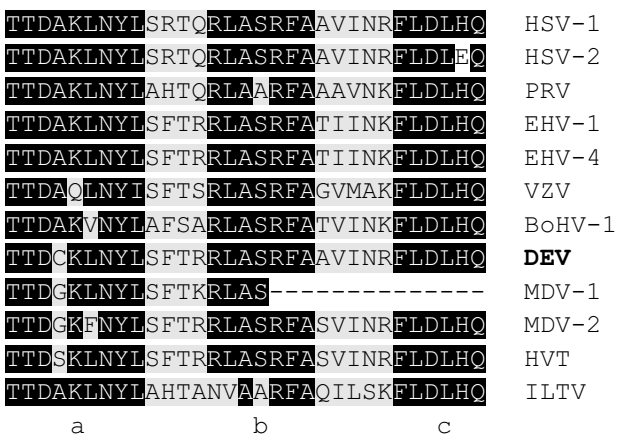


Fig. 4

Three conserved regions (a, b, c) in UL33 protein of DEV and examined alphaherpesviruses

Black areas = conserved regions; gray areas = variable regions.

in the subfamily *Alphaherpesvirinae* with three conserved domains (Fig. 4).

DEV UL34 gene encompassed 830 nt that encoded a protein of 276 aa with a M_r of 30.0 K. The UL33 and UL34 gene had the same transcription orientation and was separated by 209 nt non-coding sequences. UL34 possessed three phosphorylation sites for protein kinase C at the 169, 201, 234 aa near the C-terminus. Multiple alignment of DEV UL34 with 11 alphaherpesvirus strains revealed the lowest identity with ILTV of 14.9% and the highest with BoHV-1 of 37.7%.

The UL35 gene was the smallest ORF characterized in this study. It encoded a protein of 117 aa with a M_r of 12.8 K. DEV UL35 showed a weak level of identity with the reference strains from 14.5% with ILTV to 34.2% with MDV-2.

Phylogenetic analysis of DEV in relation to alphaherpesviruses

Phylogenetic trees were constructed from alignments of 5 deduced proteins (Fig. 5). In general, the phylogenetic trees of the examined 5 proteins were different. From the view of UL31, DEV formed a cluster with VZV, but showed a relatively close relationship with the genus *Mardivirus*. DEV UL32 formed a single cluster with closer relationship to the genera *Mardivirus* and *Simplexvirus*. From the point of view of UL34 and UL35, DEV was closer to the genus *Mardivirus,* but formed a single branch. DEV UL33 showed a distance from the four genera of the subfamily *Alphaherpesvirinae* and formed a single branch.

Discussion

In this paper, we reported partial genomic sequences of UL31, UL32, UL33, UL34, and UL35 genes of DEV. The arrangement and transcription orientation of the five genes were collinear with their homologs in HSV-1 and MDV-1. Phylogenetic analysis of the 5 proteins deduced from these genes showed that DEV was close to the genus *Mardivirus,* but formed a single branch that was consistent to our previous report about genetic relationship of DEV based on DEV UL22–UL30 proteins (Li *et al*., 2006; Liu *et al.*, 2007). Considered these two points, DEV should be classified in the subfamily *Alphaherpesvirinae*.

Every one of five DEV proteins has its homolog in corresponding protein of examined alphaherpesviruses, but they exhibit a different level of conservation. Some conserved motifs were considered as signatures for identification of the protein function. The HSV-1 UL31 protein was identified as a nuclear phosphoprotein with a nuclear localization signal (NLS) KERRR in its N-terminus (Chang and Roizman, 1993). Although DEV UL31 had four conserved regions that were common amongst alphaherpesviruses, it did not contain the

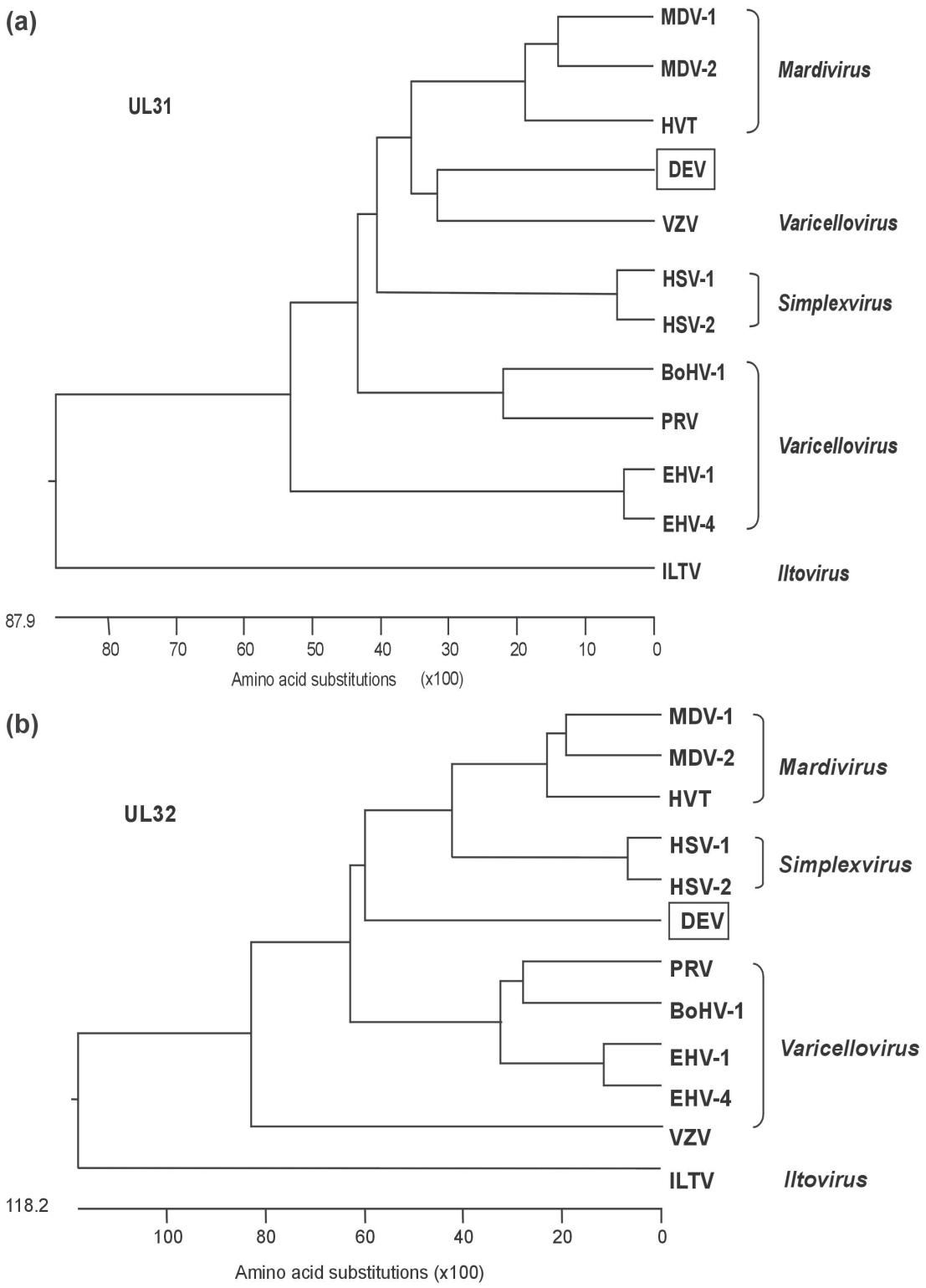
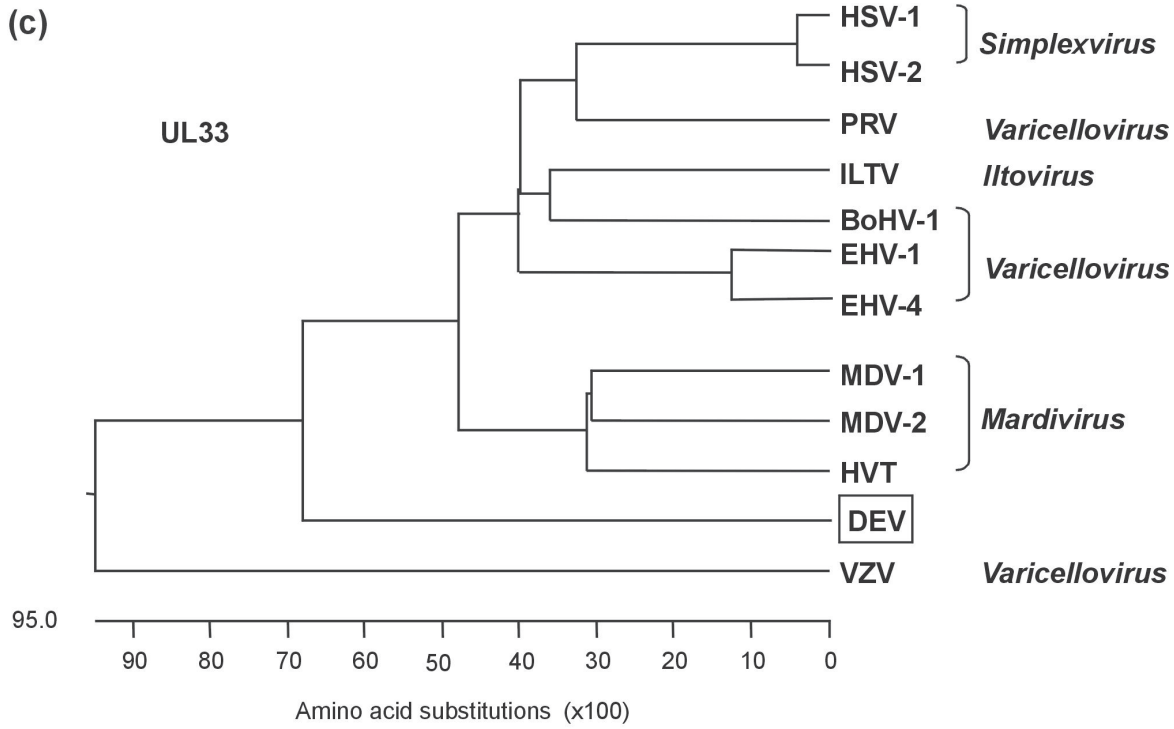
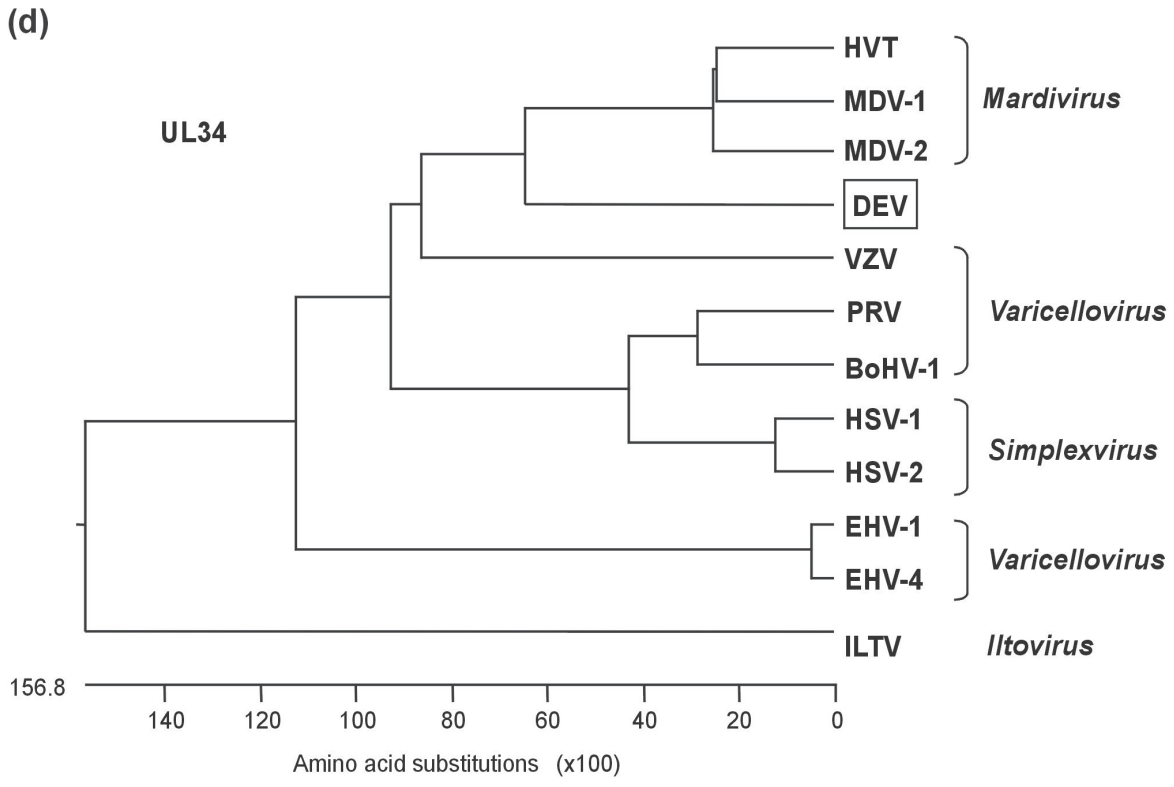
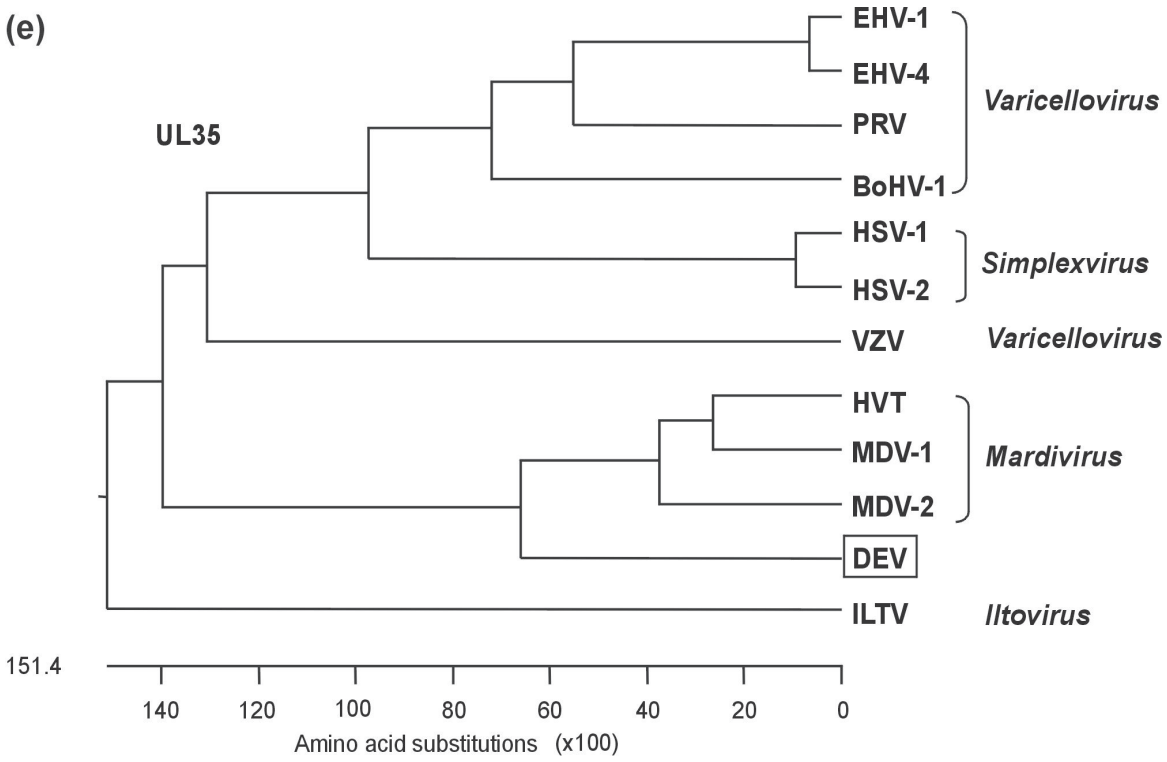


Fig. 5

Phylogenetic analysis of the five proteins deduced from UL31 (a), UL32 (b), UL33 (c), UL34 (d), and UL35 (e) genes of DEV and examined alphaherpesviruses


(c)
UL33
HSV-1
HSV-2
PRV
ILTV
BoHV-1
EHV-1
EHV-4
MDV-1
MDV-2
HVT
DEV
VZV
Simplexvirus
Varicellovirus
Iltovirus
Varicellovirus
Mardivirus
Varicellovirus
95.0
90 80 70 60 50 40 30 20 10 0
Amino acid substitutions (x100)


(d)
UL34
HVT
MDV-1
MDV-2
DEV
VZV
PRV
BoHV-1
HSV-1
HSV-2
EHV-1
EHV-4
ILTV
Mardivirus
Varicellovirus
Simplexvirus
Varicellovirus
Iltovirus
156.8
140 120 100 80 60 40 20 0
Amino acid substitutions (x100)



NLS signature motif in the nuclear protein. Therefore, there was no reason to define UL31 as a nuclear protein. Characterization of this protein needs further experiments to identify its role.

MDV-1 UL32 is a membrane glycoprotein gp82 (Wu *et al*., 1997). DEV UL32 protein contained five predicted N-linked glycosylation sites, which suggested that UL32 might be a membrane glycoprotein like its homolog MDV-1 UL32. In addition, UL32 was used as a target gene to develop a PCR assay for detection of DEV and could be differentiated properly from other avian herpesviruses (Pritchard *et al*., 1999).

The HSV-1 UL33 is one of the six proteins including UL6, UL15, UL17, UL28, and UL32 required for cleaving the concatemeric DNA into unit-length genomes and packaging into preformed capsids (Reynolds *et al*., 2000). When any of these proteins is absent, the viral DNA will not cleave and empty capsids will accumulate in the nuclei of infected cells (Beard and Baines, 2004). Further experimental evidence is required to evaluate the role of DEV UL33 protein in the virus replication.

The HSV-1 UL34 gene product is a membrane-associated phosphoprotein. It contained a phosphorylation site recognized by the viral protein kinase (Purves *et al*., 1991). DEV UL34 protein contained 3 phosphorylation sites for protein kinase C, what suggested that DEV UL34 might be a homolog of the HSV-1 phosphoprotein. In addition, DEV UL34 protein contained a hydrophobic stretch of 22 residues (250–271 aa) near C-terminus, what was a common feature in herpesviruses (Yamauchi *et al*., 2001). HSV-1 UL34 protein is a tail-anchored type II membrane protein that has no N-terminal signal peptide sequence, but contains a hydrophobic segment near the C-terminus that orients the N-terminus in the cytoplasm (Kutay *et al*., 1993). Therefore, the DEV UL34 protein may be a tail-anchored type II membrane protein.

HSV-1 UL35 was designated as the protein VP26. DEV UL35 showed a lower homology with the corresponding homolog of the reference strains. HSV-1 UL35 is a capsid protein that is not essential for replication in cell culture (Desai *et al*., 1998). We could not predict the function of DEV UL35, because of its low identity with homologs of other herpesviruses. The DEV UL35 protein needs more experimental data and evidences to determine its role.

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