Genomic and antigenic characterization of the newly emerging Chinese duck egg-drop syndrome flavivirus: genomic comparison with Tembusu and Sitiawan viruses

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Duck egg-drop syndrome virus (DEDSV) is a newly emerging pathogenic flavivirus causing avian diseases in China. The infection occurs in laying ducks characterized by a severe drop in egg production with a fatality rate of 5–15 %. The virus was found to be most closely related to Tembusu virus (TMUV), an isolate from mosquitoes in South-east Asia. Here, we have sequenced and characterized the full-length genomes of seven DEDSV strains, including the 5′- and 3′-non-coding regions (NCRs). We also report for the first time the ORF sequences of TMUV and Sitiawan virus (STWV), another closely related flavivirus isolated from diseased chickens. We analysed the phylogenetic and antigenic relationships of DEDSV in relation to the Asian viruses TMUV and STWV, and other representative flaviviruses. Our results confirm the close relationship between DEDSV and TMUV/STWV and we discuss their probable evolutionary origins. We have also characterized the cleavage sites, potential glycosylation sites and unique motifs/modules of these viruses. Additionally, conserved sequences in both 5′- and 3′-NCRs were identified and the predicted secondary structures of the terminal sequences were studied. Antigenic cross-reactivity comparisons of DEDSV with related pathogenic flaviviruses identified a surprisingly close relationship with dengue virus (DENV) and raised the question of whether or not DEDSV may have a potential infectious threat to man. Importantly, DEDSV can be efficiently recognized by a broadly cross-reactive flavivirus mAb, 2A10G6, derived against DENV. The significance of these studies is discussed in the context of the emergence, evolution, epidemiology, antigenicity and pathogenicity of the newly emergent DEDSV.

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The GenBank/EMBL/DDBJ accession numbers for the genome sequences of seven strains of DEDSV are JQ920420–JQ920426. The GenBank/EMBL/DDBJ accession numbers for the ORF sequences of TMUV and STWV are JX477685 and JX477686, respectively.

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INTRODUCTION

Since April 2010, a severe duck disease has spread throughout the main duck-producing regions in China (Hu *et al.*, 2011; Su *et al.*, 2011). The infected ducks display anorexia accompanied by a heavy drop in egg production. As the disease progresses, some ducks exhibit an uncoordinated gait and loss of balance. Total mortality can range from 5 to 15 %. Consequently, this disease is a major concern for the Chinese poultry industry (Hu *et al.*, 2011). The aetiological agent was previously identified as a newly emerged flavivirus and designated Baiyangdian virus (BYD virus) (Su *et al.*, 2011). However, since we now recognize that a plant virus, barley yellow dwarf virus, already has the designation BYDV, we propose the alternative name duck egg-drop syndrome virus (DEDSV) for this important avian pathogen. Analysis of partial sequences of the envelope (E) and non-structural (NS) NS5 gene, demonstrated its close relationship with the Ntaya virus (NTAV) group in the genus *Flavivirus*, family *Flaviviridae*. Additionally, DEDSV was shown to be particularly closely related to Tembusu virus (TMUV) and Sitiawan virus (STWV) (Hu *et al.*, 2011; Su *et al.*, 2011). Other research groups have performed similar investigations and defined their newly isolated viruses as strains of TMUV or Tembusu-like virus (Cao *et al.*, 2011; Yan *et al.*, 2011).

Members of the genus *Flavivirus* in the family *Flaviviridae* are positive-sense, ssRNA viruses that infect humans and other vertebrates (Gould & Solomon, 2008). By taking into account the vector, vertebrate hosts, antigenic phylogenetic and biogeographical characteristics, the flaviviruses were divided into three groups: mosquito-borne viruses, tick-borne viruses and viruses with no known arthropod vector (Porterfield, 1980). For the mosquito-borne flaviviruses, they have been further subdivided into seven subgroups including the NTAV group (refer to the ICTV database – ICTV 2010, http://talk.ictvonline.org/files/ictv_documents/m/msl/1231.aspx).

The NTAV group currently comprises the following viruses: NTAV, Bagaza virus (BAGV), TMUV, Ilheus virus (ILHV) and Israel turkey meningoencephalomyelitis virus (ITV). Two other viruses, namely STWV and Rocio virus (ROCV), are considered to be subtypes of TMUV and ILHV, respectively. The NTAV group causes disease in several different hosts; ITV infects turkeys producing non-suppurative meningoencephalitis and myocardial necrosis (Guy & Malkinson, 2008). ILHV is not associated with epidemic disease but has been isolated sporadically from patients in Brazil, Trinidad, Panama, Argentina, Colombia and Ecuador, with acute febrile illness presenting as headache, myalgia and malaise (Shope, 2003). ROCV is the only member known to have caused at least two human epidemics of severe encephalitis between 1973 and 1990 in the south-eastern region of Brazil (Shope, 2003). STWV infects chickens causing encephalitis and growth retardation (Kono *et al.*, 2000). BAGV causes both human illness (Gould & Solomon, 2008) and avian infections in Africa and Spain (Agüero *et al.*, 2011; Digoutte, 1978; Traore-Lamizana *et al.*, 1994). TMUV and NTAV were isolated from mosquitoes. No known diseases in either human or animals have been recorded (Weissenböck *et al.*, 2010). To date, whole genomic sequences are available for only three members of the NTAV group, namely BAGV, ILHV and ROCV (Kuno & Chang, 2005, 2007; Medeiros *et al.*, 2007). Thus, their evolutionary characteristics and genetic relationships are currently inadequately defined.

The flavivirus genomic RNA contains a unique ORF, flanked by a type 1 capped 5′-terminal non-coding region (NCR) and a 3′-terminal NCR that, together, form specific secondary stem–loop structures required for RNA translation, replication and/or expression of biological traits most likely including pathogenetic determinants (Lindenbach & Rice, 2003; Rice *et al.*, 1985). The ORF encodes the viral proteins as a large polyprotein precursor that is co- and post-translationally processed by cellular and viral proteases into at least 10 separate products. The N-terminal region encodes three structural proteins: capsid (C), pre-membrane (prM) (which is post-translationally cleaved to produce pr and M protein) and E, followed by the non-structural proteins NS1 (soluble complement-fixing antigen), NS2A, NS2B, NS3 (serine protease/RNA helicase), NS4A, NS4B and NS5 (RNA-dependent RNA polymerase/methyltransferase) (Chambers *et al.*, 1990).

In this study, we have characterized the antigenicity of DEDSV in terms of its relationship with other mosquito-borne flaviviruses and sequenced the full-length genomes of seven strains of DEDSV (including their 5′- and 3′-NCR) and the ORF sequences of both TMUV and STWV. The close relationship between DEDSV and TMUV/STWV was confirmed. In detailed antigenic studies with representative human pathogenic flaviviruses, we demonstrate significant antigenic cross-reactivity with the implication that these DEDSV shared epitopes may indicate the potential for infection of human beings by DEDSV. At the same time, we also found that the broadly neutralizing mAb 2A10G6, which was originally raised against dengue virus (DENV) (Deng *et al.*, 2011), binds to a very high titre with DEDSV. We therefore propose DEDSV should be considered to be a new member of the NTAV group of flaviviruses, forming a subgroup together with TMUV and STWV.

RESULTS

Full-genome sequencing and genome structures of DEDSV, TMUV and STWV

We have isolated seven strains of DEDSV (Table 1), all of which are pathogenic. The laying ducks infected with isolated DEDSV-JXSP showed analogous clinical manifestations, like a sudden decline of food uptake (Fig. S1a, available in JGV Online), severe egg drop (Fig. S1b) and haemorrhage of spleen and ovaries (Fig. S2), with those

Table 1. Viruses studied in this report

Name or ORF available	Source of isolation	Location of isolation	Sequence available
DEDSV-byd1	Egg-laying duck	Hebei Province, PR China	Full sequence
DEDSV-JXSP	Meat-type duck	Beijing Autonomous City, PR China	Full sequence
DEDSV-Duan	Egg-laying duck	Beijing Autonomous City, PR China	Full sequence
DEDSV-JX2	Egg-laying duck	Jiangxi Province, PR China	Full sequence
DEDSV-JS	Egg-laying duck	Jiangsu Province, PR China	Full sequence
DEDSV-goose	Goose	Beijing Autonomous City, PR China	Full sequence
DEDSV-pigeon	Pigeon	Beijing Autonomous City, PR China	Full sequence
TMUV MM1775	*Culex tritaeniorhynchus*	Kuala Lumpar, Malaysia	ORF
STWV	Broiler chicken	Perak state, Malaysia	ORF
TMUV ZJ-407	Breeder duck	Zhejiang Province, PR China	Full sequence
TMUV YY5	Shaoxing duck	Zhejiang Province, PR China	Full sequence
TMUV GH-2	Goose	Zhejiang Province, PR China	Full sequence
Duck flavivirus TA	Duck	Shandong Province, PR China	Full sequence

infected with DEDSV-byd1 we have performed previously (Su *et al.*, 2011). Seven strains of DEDSV and one strain each of TMUV and STWV were sequenced (Table 1). The whole genome of DEDSV, including the 5′- and 3′-NCRs, was 10990 nt in length with an ORF of 10278 nt flanked by 5′- and 3′-NCRs of 94 and 618 nt, respectively. The ORF encoded a polyprotein of 3425 aa. The ORFs for both TMUV and STWV were also 10278 nt, encoding 3425 aa. The 10 recognized flaviviral proteins (defined above) were identified. The lengths of these proteins and the specific positions of relevant genes are shown in Fig. 1(a).

The similarity between these DEDSV isolates was at least 99.7 %, confirming they are all strains of the same virus. As shown in Fig. 1(b), a phylogenetic tree was constructed based on the ORF nucleotide sequences. The DEDSV-JX2 lineage roots to the DEDSV major cluster. Notably, DEDSV-goose and DEDSV-pigeon share a clade with DEDSV-JS and DEDSV-JXSP. Moreover, the duck 'Tembusu virus' strains YY5, ZJ-407 and GH-2 recently identified by another group (Yun *et al.*, 2012), all of which were isolated from the same province in China, segregated into one cluster. All of the other strains including duck flavivirus strain TA (Liu *et al.*, 2012) formed another cluster and they were isolated from neighbouring provinces in China (Table 1).

TMUV and STWV showed 88.8 and 87.4 % nt sequence identity and 97 and 96.5 % aa sequence identity with DEDSV, respectively. The close relationship between TMUV and DEDSV strongly implies that DEDSV, like STWV, can be designated a subtype of TMUV.

Comparison with other flaviviruses

In order to study the phylogenetic relationship between DEDSV/TMUV/STWV and other members of the genus

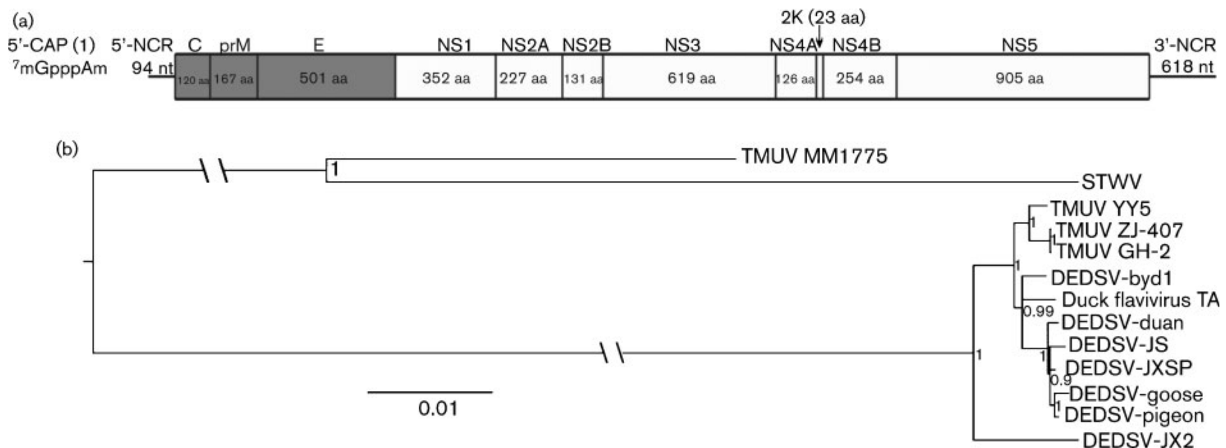

Fig. 1. DEDSV genome structures and the phylogenetic analysis of seven strains of DEDSV. (a) Genome structure, RNA elements and polyprotein constitutions. Structural proteins are highlighted in grey. (b) Bayesian phylogeny of the ORF nucleotide dataset of seven strains of DEDSV, TMUV strain MM1775 and STWV were used as outgroups. Posterior probabilities are shown. The tree is midpoint-rooted. Bar, 0.01 substitutions per site.

Flavivirus, we selected 39 representative flaviviruses, the ORF sequences of which are known. The GenBank accession numbers and acronyms for these viruses are listed in Table S1. The phylogenetic analysis was therefore based on the ORF-encoded sequences of 42 flaviviruses using Bayesian methods (Fig. 2a). Under these circumstances, DEDSV-byd1 was placed in the TMUV and STWV lineage supporting our previous, but less extensive analysis based on the E and NS5 protein sequences (Su *et al.*, 2011). Recently, a whole-genome sequence of DEDSV-like virus (strain TMUV-JS804) isolated from a DEDS outbreak in China was deposited in GenBank (NCBI accession no. NC_015843.1). This strain showed 99.6 % nt identity to DEDSV-byd1 and clustered with DEDSV-byd1 (Fig. 2a). Nevertheless, despite this close similarity JS804 had a shorter 3′-NCR (544 nt compared with 618 nt for our isolates). We suspect that this is the same virus causing DEDS in China but its genomic sequence was not fully completed. The phylogenetic analysis also illustrates the recognized cluster segregation, with 100 % posterior probability among the mosquito-borne, tick-borne and no-known vector flavivirus groups (Gaunt *et al.*, 2001; Porterfield, 1980). On the basis of their phylogenetic, ecological and virus–vector relationships, the mosquito-borne flaviviruses were subdivided into *Culex* spp.-associated and *Stegomyia* spp. -associated groups (Gaunt *et al.*, 2001). DEDSV-byd1 falls in the *Culex* spp.-associated group. Therefore, whilst the natural arthropod vector of DEDSV-byd1 has not been identified, the phylogenetic data and their correlation with vector–host associations support the interpretation that DEDSV is most likely transmitted by ornithophilic *Culex* spp. mosquitoes. This should be confirmed experimentally in the future.

The phylogenetic tree (Fig. 2b) based on genomic sequence data of 39 representative flaviviruses shows that DEDSV clusters with a group of viruses that diverged from the Japanese encephalitis virus (JEV) serocomplex virus cluster. These two clades were previously shown to be primarily *Culex* spp.-associated viruses, i.e. viruses that tend to be ornithophilic and anthropophilic in their host-feeding preference (Gaunt *et al.*, 2001; Gould *et al.*, 2003). The tree also shows that these two clades are rooted by Old World viruses.

Analysis of the cleavage sites, glycosylation sites and conserved cysteine residues

The predicted hexapeptide cleavage sites within the polyproteins of DEDSV-byd1, TMUV and STWV were compared together with their closest relative, BAGV, as shown in Table S2. The three most closely related viruses, as in the phylogenetic tree, have only slight differences at the junctions, VirC/AnchC, E/NS1, NS1/NS2A, NS3/NS4A and NS4B/NS.

To clarify the differences between these viruses, comparative alignments were prepared between the four viruses based on the entire genome sequence, the ORF, the polyprotein and each individual protein (Table 2). These analyses confirm that DEDSV-byd1, TMUV and STWV are more closely related to each other than they are to BAGV. This conclusion is based on the evidence that the structural and non-structural proteins are the same length and the nucleotide identities between them are higher. Moreover, STWV is closer than DEDSV-byd1 to TMUV. These viruses are distinct from BAGV in the lengths of C, NS2A, NS2B and NS4A and the polyprotein of BAGV has 3426 aa, while the other three have 3425 aa.

The number of potential *N*-linked glycosylation sites (N-LGlyS) in the prM, E and NS1 proteins of DEDSV-byd1, TMUV and STWV were 2-2-3, 2-2-2 and 2-1-2, respectively, whereas BAGV has 1-1-3. No glycosylation site was predicted to exist in the hydrophobic domains in the viruses studied. Disulfide bonds formed by cysteine residues are important for the stabilization and conformation of the proteins. For prM, the conserved six cysteine residues clustered in the pr domain. This is similar to other flaviviruses. The pattern of 12 cysteine residues found in all mosquito-borne flaviviruses in the E and NS1 genes is also conserved in the three flaviviruses.

Functional and structural motifs/modules

Many motifs/modules conserved in the flaviviruses (Gao *et al.*, 1993) are also found in the DEDSV-byd1, TMUV and STWV. In each E monomer of flaviviruses, three structural domains are discernible. Domain II is involved in dimerization (Modis *et al.*, 2004). It contains a highly conserved fusion peptide at its tip, i.e. aa residues 98–110, which binds to the membrane of the host cell during fusion (Bakonyi *et al.*, 2004). This fusion loop is also shared by DEDSV-byd1, TMUV and STWV. Domain III has been implicated in receptor binding. As shown in Table 3, a putative integrin-binding motif (a tripeptide sequence of aa 388–390) is encoded by RGD in JEV, Usutu virus, Murray Valley encephalitis virus and Alfuy virus (Bakonyi *et al.*, 2004; Hurrelbrink *et al.*, 1999; May *et al.*, 2006; Sumiyoshi *et al.*, 1987), RGE in West Nile virus (WNV) (Castle *et al.*, 1985) and Kunjin virus (Coia *et al.*, 1988) and RGP in St. Louis encephalitis virus (SLEV) (Ciota *et al.*, 2007). However, the corresponding sequence is TGE in BAGV (Kuno & Chang, 2007), TGP in ROCV (Medeiros *et al.*, 2007) and QEN in ILHV (Kuno & Chang, 2005). We identified the sequence as SGK in DEDSV-byd1, TMUV and STWV. In the N-terminal third of the NS3, the catalytic triad (H47, D75 and S135) and the proposed substrate-binding pocket of the trypsin-like serine protease (Gly133, Ser135, Gly136, Gly148, Leu149 and Gly153) (Valle & Falgout, 1998) were also found to be conserved in the three viruses, as well as the RNA helicase motif D285-E286-A287-H288 in the C-terminal third of the protein (Gorbalenya *et al.*, 1989). Similar to other flaviviruses, a homologous G667-D668-D669 motif common to RNA-dependent RNA polymerase (Rice *et al.*, 1985) was also found in all DEDSV-byd1, TMUV and STWV in the C-terminal third of the NS5 protein.

Secondary structures of the NCRs

The MFOLD program was used to predict the secondary structure (SS) of the viral terminal region of the DEDSV

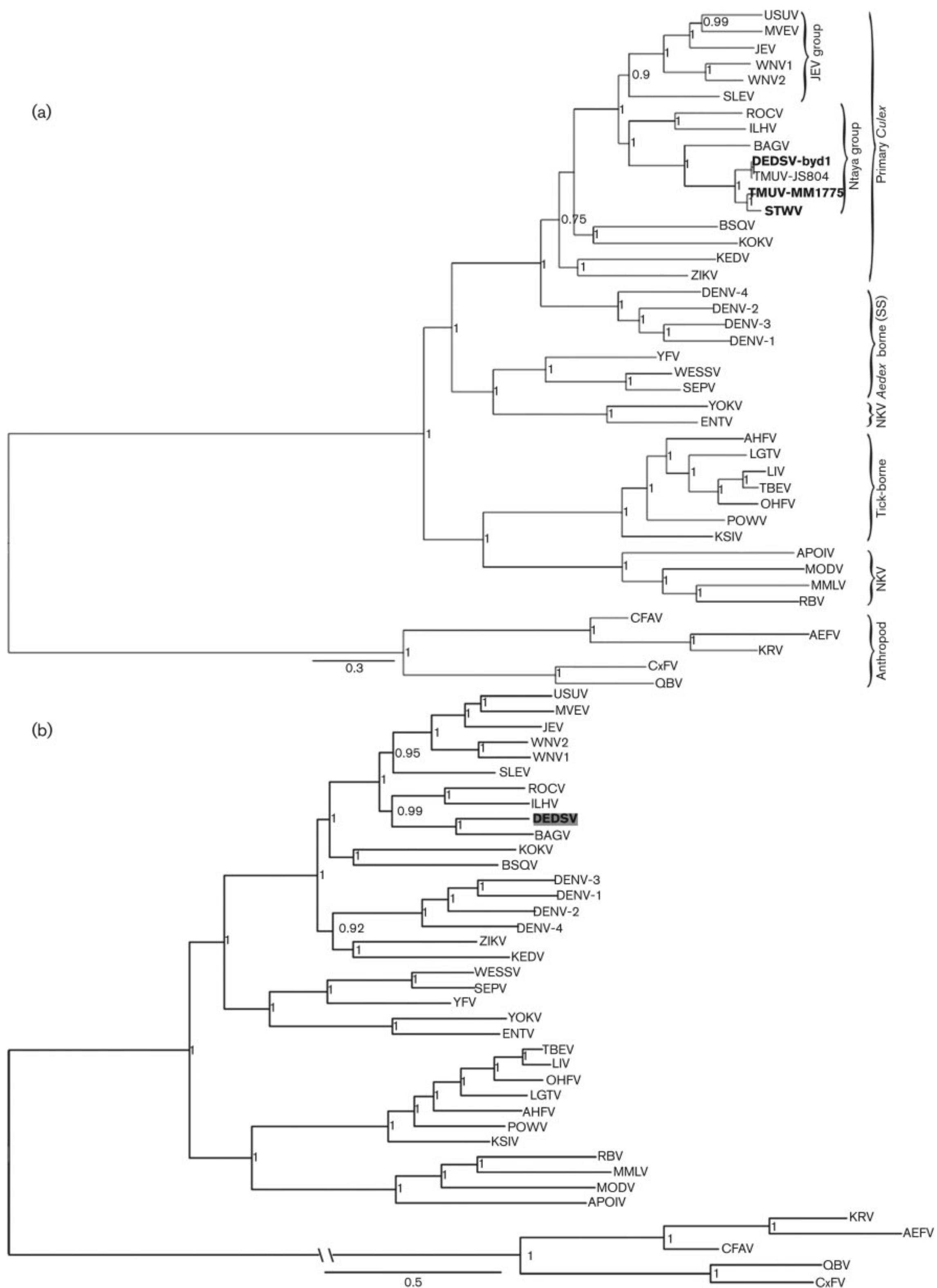

Fig. 2. Bayesian phylogeny of representative flaviviruses with DEDSV, TMUV and STWV. Posterior probabilities are shown for all the clades. (a) Phylogenetic tree was based on the ORF sequence dataset. Those flavivirus sequences that are published for the first time in the current study, namely DEDSV strain byd1, TMUV strain MM1775 and STWV, are highlighted in bold. All horizontal branch lengths are drawn to scale; bar, 0.3 substitutions per site. (b) Phylogenetic tree was based on the complete genome sequences. DEDSV is highlighted in grey. All horizontal branch lengths are drawn to scale; bar, 0.5 substitutions per site. NKV, No known vector flaviviruses; SS, secondary loss NKV flaviviruses. See Table S1 for virus abbreviations and GenBank accession numbers.

Table 2. Comparison of the genes or genomic regions of the four flaviviruses in the NTAV group

Numbers in parentheses represent the percentage amino acid or nucleotide identity with DEDSV, respectively.

Gene or genomic region	DEDSV	BAGV	TMUV	STWV
5′-NCR	94 nt	94 nt (74.7)	–	–
C	120 aa	122 aa (67.2)	120 aa (95.8)	120 aa (93.3)
prM	167 aa	167 aa (81.4)	167 aa (97.6)	167 aa (95.2)
E	501 aa	501 aa (83.6)	501 aa (97.0)	501 aa (96.8)
NS1	352 aa	352 aa (78.4)	352 aa (94.0)	352 aa (93.2)
NS2A	227 aa	226 aa (63.9)	227 aa (92.5)	227 aa (92.1)
NS2B	131 aa	132 aa (95.6)	131 aa (97.0)	131 aa (97.0)
NS3	619 aa	619 aa (85.6)	619 aa (98.7)	619 aa (98.2)
NS4A	126 aa	126 aa (82.5)	126 aa (96.8)	126 aa (95.2)
2K	23 aa	23 aa (87.0)	23 aa (95.6)	23 aa (95.6)
NS4B	254 aa	253 aa (78.7)	254 aa (98.0)	254 aa (98.0)
NS5	905 aa	905 aa (85.9)	905 aa (97.8)	905 aa (98.0)
Polyprotein	3425 aa	3426 aa (81.3)	3425 aa (97.0)	3425 aa (96.5)
ORF	10278 nt	10281 nt (71.8)	10278 nt (88.8)	10278 nt (87.4)
3′-NCR	618 nt	566 nt (64.5)	–	–
Total length of genome	10990 nt	10941 nt (71.4)	–	–

genomic RNA. First, we predicted the SSs of 5′-terminal sequence and 3′-terminal sequence separately (Fig. 3a). Stem–loop A (SLA), stem–loop B (SLB) and the capsid hairpin (cHP) were as defined for other flaviviruses (Gritsun *et al.*, 2006). Both *in vivo* and *in vitro* data supported the idea that SLA, present in the 5′-UTR, functions as the promoter element for the viral polymerase (Villordo & Gamarnik, 2009). SLA is essential for the viral NS5 protein binding to the viral RNA, while the SLB and cHP are dispensable (Dong *et al.*, 2007). The 3′-terminal sequence has a conserved long stem–loop (3′-SL) structure, which is predicted to be conserved in all flavivirus genomes. The structure has a conserved pentanucleotide CACAG in the anthropod-borne flaviviruses (Brinton *et al.*, 1986; Hahn *et al.*, 1987; Wengler & Castle, 1986), which is critically important for viral RNA replication, it is located in the bulge at the top of the 3′-SL in DEDSV. The conserved sequence (CS)1 is located just upstream of the 3′-SL. The 5′-CS is 12 nt long in the C gene. As already known, cyclization of the viral genome is crucial for viral genome replication. The predicted SS of the cyclized viral RNA is shown in Fig. 3(b). As previously reported in other flaviviruses, the 5′-CS and 3′-CS are involved in genome cyclization and form double-stranded structures. The complementarity rather than the sequence per se is essential for RNA synthesis. Regarding the upstream AUG region (UAR) found conserved in other mosquito-borne flaviviruses, a functionally homologous sequence for DEDSV could not be found, this is also the case for BAGV (Kuno & Chang, 2007).

CS sequences in 3′-NCR play a role in viral RNA replication (Alvarez *et al.*, 2005). The organization, names of the structures and/or domains used in this study follow the nomenclatures established earlier (Chambers *et al.*, 1990; Hahn *et al.*, 1987; Proutski *et al.*, 1997). Besides the CS1, some other conserved sequences in the 3′-NCR were also identified (Table 4). Five CSs were found with the pattern ImRCS3-CS3-RCS2-CS2-CS1, where the ImCS3 describes the imperfectness of the conserved sequence differing from the corresponding consensus sequences in three or more bases. However, the CS organization of BAGV is CS1-CS2-CS3-RCS3 (Kuno & Chang, 2007). The CS pattern of DEDSV is different from BAGV but is the same as the JEV group.

Antigenic cross-reactivity with representative flaviviruses

Indirect ELISAs were carried out to investigate the cross-reactivity of immune sera from DEDSV-infected ducks, with other representative flaviviruses. Except for TMUV and BAGV, all of the viruses were standardized at 1000 p.f.u. ml^{-1} in the ELISA experiments. After coating plates with these viruses, the antiserum against DEDSV-byd1 was tested at a 1 : 200 dilution. The duck sera exhibited high levels of cross-reactivity with representative flaviviruses although the level of cross-reactivity was lower than the homologous test with DEDSV, as the positive control antigen (Fig. 4a). Whilst TMUV and BAGV appear to show lower readings with DEDSV when compared with the other representative flaviviruses, this probably reflects the fact that for TMUV and BAGV, the viruses were not adapted to tissue culture cells and thus, cell culture preparations were not available. For these two viruses, the antigen was prepared in suckling mouse brains. Nevertheless, TMUV and BAGV clearly show significant cross-reactivity with the DEDSV antiserum in this ELISA test.

Fig. 4(b)–(f) present the data with DEDSV-byd1 as the coating antigen, all of the mouse antisera against other representative flaviviruses could recognize the DEDSV-byd1, but the strongest level of cross-reactivity was observed with JEV and DENV. Also of significant interest was the

Table 3. Conserved regions within the precursor polyprotein of DEDSV, TMUV and STWV

Conserved element	E		NS3			NS5
	Receptor-binding domain	Integrin-binding domain	Catalytic triad	Substrate-binding pocket	RNA helicase motif	RNA-dependent RNA polymerase motif
Conserved sequence*	E599† D682	R680 G681 D682	H1550 D1578 S1638	G1636 S1638 G1639 G1651 L1652 G1654	D1788 E1789 A1790 H1791	G3196 D3197 D3198
DEDSV/TMUV/STWV sequence	N594 K676	S674 G675 K676	H1545 D1573 S1633	G1631 S1633 G1634 G1646 L1647 G1649	D1783 E1784 A1785 H1786	G3187 G3188 G3189

*Conserved sequences refer to the JEV serogroup viruses.
†The numbers represent the amino acid position in the polyprotein.

observation that the broadly cross-reactive DENV-2-derived mAb 2A10G6, which recognizes an epitope within the ^{98}DRXW^{101} motif localized in the fusion loop of the E protein (Deng *et al.*, 2011), also exhibited high binding activity to DEDSV-byd1. Compared with DENV-2, DEDSV-byd1 shows similar reactivity with the mAb. The comparative sequence alignment data confirmed that the conserved motif does exist in DEDSV-byd1 (Fig. 4g).

Neutralization profiles of 2A10G6 against representative flaviviruses

To investigate the neutralization potential of mAb 2A10G6 to the DEDSV, the neutralization activities of 2A10G6 against various flaviviruses including DEDSV were assessed using a standard plaque reduction neutralization assay. The results (Fig. 4i) demonstrated that 100 μg 2A10G6 ml^{-1} potently neutralized DENV-1–4, yellow fever virus (YFV) and WNV on BHK-21 cells with different neutralization potential. However, no neutralization activity of 2A10G6 was observed for JEV, TBEV and DEDSV.

DEDSV replication and growth were also tested in the mosquito cell line C6/36 and the results showed that the virus can grow in this cell line with high titre, but failed to produce any obvious cytopathic effect (CPE).

DISCUSSION

In 2010, a highly contagious duck disease spread widely and quickly in the duck-farming regions of China. The disease caused severe decreases in egg production and deaths in many birds, leading to huge economic losses (Hu *et al.*, 2011; Su *et al.*, 2011). Previously, we identified the pathogen as a new TMUV-related flavivirus, initially named as BYDV (Su *et al.*, 2011), which we have now renamed DEDSV. Entire genome sequencing, phylogenetic analysis and antigenic cross-reactivity studies were undertaken to characterize DEDSV in this study. In the light of its close relationship with TMUV and STWV, we have also carried out sequencing of the ORFs of TMUV and STWV for comparative analysis.

Seven strains of DEDSV were sequenced, including both the 5′- and 3′-NCRs, and their comparative genome alignments revealed high levels of identity. Phylogenetic analyses based on the whole genomes of 39 flaviviruses and the polyproteins of 42 viruses demonstrated the close relationships between DEDSV and members of the NTAV group viruses, but a particularly close relationship with TMUV.

In conclusion, analysis of the phylogenetic data (Fig. 2) provides us with a plausible explanation for the evolutionary origins and dispersal of DEDSV. First, the phylogenetic cluster that contains DEDSV exclusively contains Old World viruses of either African or Asian origin, all of which diverged from a sister group of New World viruses represented by ILHV and ROCV. In turn, the JEV-related viruses diverged from this DEDSV-related cluster which comprised Old World viruses and, in this tree, a single New

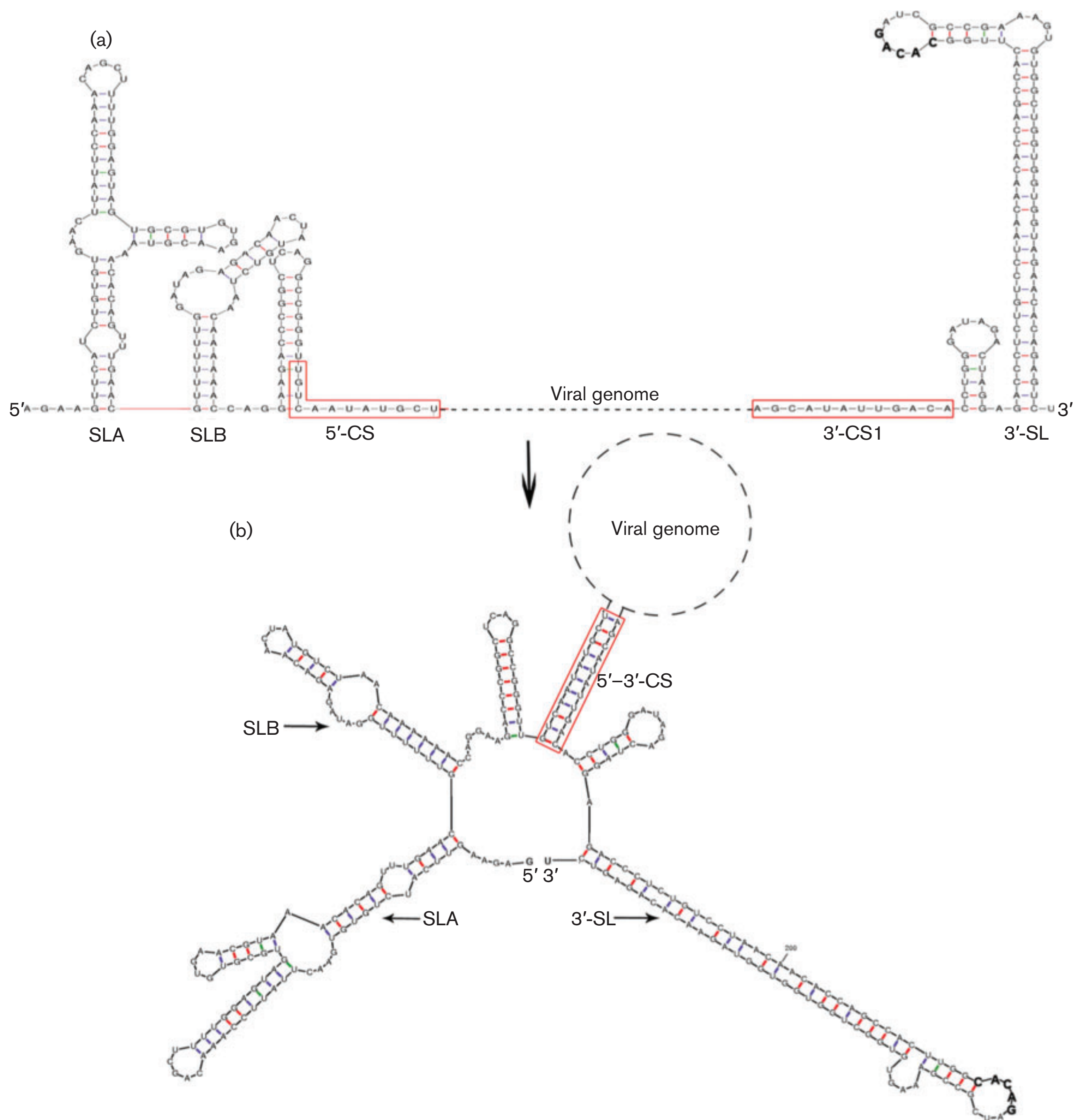

Fig. 3. Predicted secondary structure of the 5′- and 3′-terminal sequences of DEDSV. (a) Secondary structures of the 5′-terminal and 3′-terminal sequences were computed separately. (b) Shows the changes upon hybridization of the complementary sequences, which results in RNA cyclization. The folding pattern was generated by the MFOLD program (energy level=−170.4). CS, Conserved sequences; SLA, stem–loop A; SLB, stem–loop B; cHP, capsid region hairpin; 3′-SL, 3′-long stem–loop.

World virus (SLEV), the lineage of which clearly diverged from the Old World viruses. Thus, the most likely evolutionary scenario for DEDSV and indeed for TMUV is that they represent Old World lineages, probably of African origin (cf BAGV) that were dispersed into Asia via migratory birds and ornithophilic *Culex* spp. mosquitoes.

The genome strategy of DEDSV is the same as that of all the other mosquito-borne flaviviruses. Likewise, the distribution of cysteine residues in C, prM and E are identical to the other flaviviruses. However, the presence of the predicted N-LGlyS of DEDSV is different from that of the other NTAV group viruses. Currently, we do not know the function of these sites for DEDSV; nevertheless, in the absence of experimental data, there have been controversial results. Winkler *et al.* (1987) reported that non-glycosylated viruses were antigenically indistinguishable from glycosylated viruses. In contrast, other groups reported that glycosylation was essential for the virus, as viral replication, virulence, maturation or release of viral-like particles were perturbed in non-glycosylated variants

Table 4. Conserved sequences (CSs) in the 3′-NCRs of DEDSV compared with BAGV

Region	Sequence
CS1	
Consensus sequence*	ASCATATTGACRCCWGGGAWAAGAC
DEDSV	AGCATATTGACACCTGGGA–TAGAC
BAGV	AGCATATTGACACCTGGGA–GAGAC
CS2	
Consensus sequence*	GGWCTAGAGGTTAGWGGAGACCC
DEDSV	GGACTAGAGGTTAGAGGAGACCC
BAGV	GGACTAGAGGTTAGAGGAGACCC
RCS2	
Consensus sequence*	GGWCTAGAGGTTAGWGGAGACCC
DEDSV	GGACTAGAGGTTAGAGGAGACCC
BAGV†	
CS3	
Consensus sequence*	YCCCAGGWGGACTGGGTDAMCAAAKSBR
DEDSV	CCCCAGGAGGACTGGGAAAACAAAGCCA
BAGV	CCCCAGGTGGACTGGGTAAACAAAGCCG
RCS3	
Consensus sequence*	YCCCAGGWGGACTGGGTDAMCAAAKSBR
DEDSV	CCCCAGGAGGACTGGGTTAACAAATCTG
BAGV	CCCCAGGAGGACTGGGTCACCAAAGTGT

*Consensus sequences of CS1, CS2 and CS3 are based on the sequences of YFV, DENV complex viruses and JEV complex viruses. Solid line indicates a gap created artificially for alignment purposes. The following single letter codes are used; B, C, G or T; D, A, G or T; K, G or T; M, A or C; R, A or G; S, C or G; W, A or T; Y, C or T.
†The 3′-NCR of BAGV does not contain this sequence.

(Beasley *et al.*, 2005; Crabtree *et al.*, 2005; Goto *et al.*, 2005; Li *et al.*, 2006). Further studies will be needed to resolve these issues.

DEDSV shares many conserved motifs with other flaviviruses, including the catalytic triad motif, the substrate-binding motif and the RNA helicase motif in the NS3 and NS5 proteins. However, with regard to the RGX (D, E or T) motif in the E protein, which has been reported to be conserved among the JEV group, the sequence is SGK in DEDSV, TMUV and STWV. Notably, the corresponding sequences of BAGV, ILHV and ROCV are TGE, QEN and TGP, respectively. The motif has been postulated to be involved in receptor binding when viruses interact with the host cells. Therefore, this observation presumably has a significant impact on host specificity of these viruses and may partly explain why DEDSV has emerged recently to cause egg-drop syndrome in China.

At least one base-pairing (rather than the sequences per se) between the 5′–3′-terminal sequences is essential for viral RNA cyclization (Villordo & Gamarnik, 2009), which is critical for genome replication and translation (Khromykh *et al.*, 2001). DEDSV has one such conserved base-pairing as is the case for the other viruses in the NTAV group. For the CS in the 3′-NCR, we know that CS1 is necessary for the replication of the genome, but CS2 and CS3 are dispensable. DEDSV has an ImRCS3-CS3-RCS2-CS2-CS1 pattern, differing from the other viruses in the NTAV group, but is the same as that of the JEV group viruses. In this respect, DEDSV is more closely related to the JEV group viruses than to the other NTAV group viruses. Clearly, the secondary structures of the CSs require further studies to elucidate their functions.

The cross-reactivity experiments illustrated significant shared antigenic relationships between DEDSV and JEV, WNV, DENV, YFV and TBEV. All of the representative flaviviruses that were included in this analysis are pathogenic for human beings, consequently the potential threat of DEDSV to human health cannot be overlooked, especially when taking into consideration the similarities with the JEV group analysed above. The broad neutralization mAb 2A10G6 can react with DEDSV at a high degree, but has no neutralization activity.

In conclusion, we have characterized an important newly emerging avian arboviral pathogen. We have assigned a new name to this virus to avoid potential confusion with the plant virus BYDV. DEDSV is a new and highly pathogenic member of the NTAV group of mosquito-borne flaviviruses and most-closely related to TMUV and STWV. From the evidence presented above, we propose that these three viruses, isolated from distinct and distant localities, should be grouped together to form a new subgroup in the NTAV group. On the basis of phylogenetic, phylogeographic and ecological data, these three viruses are most probably

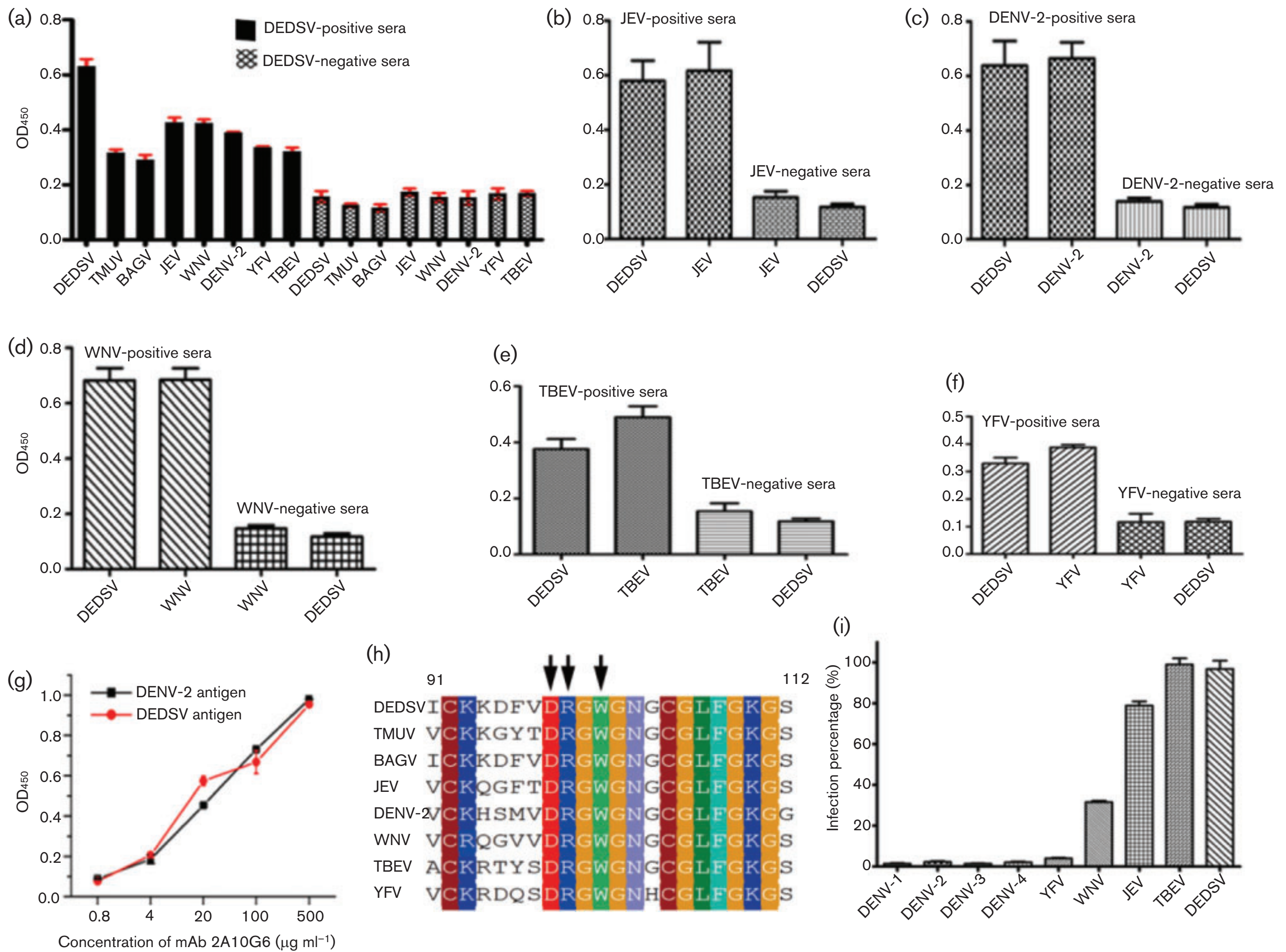

Fig. 4. Cross-reactivity patterns of DEDSV with representative flaviviruses determined by ELISA. (a) Coating representative flaviviruses, antisera against DEDSV collected from post-infection ducks was tested at a 200-fold dilution compared with DEDSV-negative control. Coating with DEDSV, then incubated with mouse polycolonal JEV (b), DENV-2 (c), WNV (d), TBEV (e) and YFV (f) sera at a 100-fold dilution. (g) mAb 2A10G6 reactivity with DEDSV compared with DENV-2 in concentration gradients. (h) Sequence alignment of DEDSV and representative flaviruses in the fusion loop region. The epitope recognized by 2A10G6 is also conserved in DEDSV. (i) Neutralization profiles of the broadly cross-neutralization mAb 2A10G6 against various flavivirus strains. The neutralization activities were assessed using a standard plaque reduction neutralization assay as described in the text.

descendant lineages of an African virus, which is transmitted between avian species via *Culex* spp. mosquitoes.

METHODS

Virus isolation and identification. Five DEDSV strains were isolated from the brains and ovaries of ducks presenting with DEDS (Su *et al.*, 2011). Two additional strains were isolated from the brain tissue of a pigeon and a goose. The RNA of all of these DEDSV strains had been identified by RT-PCR assay as described previously (Su *et al.*, 2011). TMUV MM1775 was originally identified in 1955 (Berge, 1975; Institute for Medical Research Federation of Malaya, 1957). The virus had been isolated from *Culex tritaeniorhynchus* collected on the outskirts of Kuala Lumpar, Malaysia by inoculation of suckling mouse brains. STWV was obtained as inactivated RNA through the courtesy of Dr Yuji Kono (Kono *et al.*, 2000).

Cell culture and virus propagation. DEDSV, at the third passage in embryonated duck eggs, was stored at −80 °C. For cell culture passage, BHK-21 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10 % FCS, 100 IU penicillin ml^{-1} and 100 mg streptomycin ml^{-1}. Cell monolayers were inoculated with stored infectious allantoic fluid, diluted in tenfold steps. The cultures were incubated at 37 °C in a 5 % CO_2 atmosphere and checked daily for CPE. When approximately 75 % of the cells showed CPE, the cultures were harvested, diluted and used to infect fresh cells. Virus from the third passage was used for virus genome amplification. The records show that TMUV MM1775 had been cultivated in suckling mouse brains for at least 11 sequential passages.

The infectivity assay of DEDSV to the C6/36 cell line. DEDSV-byd1 (1×10^5 p.f.u. ml^{-1}) was first added into C6/36 cell monolayers (1×10^6 cells per well) in six-well plates, then incubated at 33 °C for 1 h. The supernatants were removed and RPMI 1640 cultures with

2 % FBS were added, afterwards, the cultures were incubated at 33 °C under 5 % CO_2 atmosphere and checked daily for CPE.

Preparation of virus RNA, RT-PCR and sequencing. Viral RNA was extracted directly from the third passage of cell cultures for DEDSV or from mouse brain for TMUV and STWV using the QIAamp Viral RNA Mini kit (Qiagen) following the manufacturer's protocol. cDNA was synthesized using the Reverse Transcription System for RT-PCR (Promega). After reverse transcription, PCR was performed using the Phusion High-fidelity DNA Polymerase kit (NEB). Primers used for amplifying and sequencing the ORF regions (about 10 400 bp long) were described previously (Su *et al.*, 2011). Temperature cycles used were one cycle of heat denaturation at 94 °C, 5 min; 30 cycles of three temperature changes (94 °C for 1 min, 53 °C for 1 min and 72 °C for 1 min) for amplification and one cycle for final extension at 72 °C for 10 min. The amplicons were electrophoresed on a 1 % agarose gel. 5′- and 3′-ends of the genome were amplified using 5′-RACE and 3′-RACE, respectively. 5′-RACE was conducted according to the methods described previously (Freeman *et al.*, 2009). 3′-Ends were first poly(A) tailed and then amplified using 3′-RACE kits, according to the manufacturer's instructions (Invitrogen). The amplicons were sequenced using dideoxynucleotide cycle sequencing and products were run on an automated sequencer (Sunbiotech). For TMUV MM1775 and STWV, sequencing procedures were carried out as described previously (Grard *et al.*, 2007).

Multiple sequence alignment and phylogenetic analysis. Multiple sequence alignment and phylogenetic analysis were performed as described previously (Cook *et al.*, 2012). Briefly, alignments were conducted via MUSCLE embedded in MEGA5 (Tamura *et al.*, 2011). For the analysis of 40 representative flaviviral complete genome sequences and 42 representative flaviviral ORF nucleotide sequences, alignment was submitted to the GBlocks program, which objectively eliminates poorly aligned positions and divergent regions (Edgar, 2004). The best-fit models of nucleotide substitution were selected by using jModelTest (Posada, 2008). Phylogenetic analyses were conducted using these selected models under the Bayesian Markov chain Monte Carlo method implemented in MrBayes v3.2.0 (Huelsenbeck & Ronquist, 2001), with a minimum of 10 million generations and a burn-in of 10 %.

Determination of cleavage sites, glycosylation sites, cysteine residues and conserved motifs. For the polyproteins of DEDSV, TMUV and STWV, potential cleavage sites were determined by using the SignalP-NN program (http://www.cbs.dtu.dk/services) as described previously. Glycosylation sites were predicted by the NetNGlyc program (http://www.cbs.dtu.dk/services), and the cysteine residues were found by Protean [v. 7.1.0(44)] included in the LaserGene Program (DNA Star). Conserved motifs were localized by alignment using MEGA software (Tamura *et al.*, 2011).

Prediction of the genome secondary structure. The secondary structures in the 5′- and 3′-terminal sequences and cyclization between 5′- and 3′-terminal regions were implemented with the MFOLD program (Zuker, 2003). The first 150 nt of the genome including the entire 5′-NCR and the 5′-CS in the C gene and the last 110 nt of the 3′-NCR were used for the analysis, because the domains for cyclization of the genome were included (Villordo & Gamarnik, 2009).

Serology (indirect ELISA). In order to determine whether or not serum from DEDSV-infected ducks can cross-react antigenically with other representative flaviviruses, including TMUV, BAGV, JEV, WNV, DENV, YFV and TBEV, an indirect ELISA was performed as described previously (Deng *et al.*, 2011). All experiments involving the use of live TBEV and WNV were performed under the BSL-3 containment facilities. The strain names of the representative flaviviruses are listed as follows: JEV stain Beijing-01 (Hashimoto *et al.*, 1988), WNV strain Chin-01 (Li *et al.*, 2010), DENV-2 strain D2-43 (Liu *et al.*, 2010), YFV strain 17D (Galler *et al.*, 1998) and TBEV strain Senzhang (Si *et al.*, 2011).

Briefly, all of the tested flaviviruses except TMUV and BAGV were standardized at 1000 p.f.u. ml^{-1}. Flavivirus antigens were prepared from culture supernatants of the infected mosquito C6/36 cells or infected suckling mouse brain suspensions and were inactivated for 30 min at 56 °C. Subsequently, 96-well microtitre plates were coated overnight at 4 °C with these heat-inactivated antigens (1 : 100 dilution) in a pH 9.6 carbonate buffer. Plates were washed five times with PBST and blocked for 2 h at 37 °C with PBST containing 5 % skimmed milk. Plates were rinsed five times in PBST and then incubated with serum from DEDSV-infected ducks. Duck serum from non-infected birds was used as the control (in triplicate) for 1 h at 37 °C. Plates were washed five times and then incubated with peroxidase-conjugated goat anti-duck IgG (1 : 500) (KPL) for 1 h at 37 °C. Plates were washed five times and then incubated with TMB substrate (Promega). The reaction was stopped by the addition of 1 M H_2SO_4, and emission (450 nm) was read using a microplate reader (Beckman). Independently, cross-reactivities between DEDSV and different flavivirus-specific antibodies or the flavivirus cross-reactive mAb 2A10G6 were determined by ELISA.

2A10G6 (100 μg ml^{-1}) was pre-incubated (1 h, 37 °C) with approximately 200 p.f.u. of various flavivirus strains and the mixtures were added to BHK-21 cell monolayers in a 12-well plate and incubated at 37 °C for 1 h. Then mixtures were removed and 1 ml of 1.0 % (w/v) LMP agarose (Promega) in 2× DMEM plus 4 % (v/v) FBS was layered onto BHK-21 cells. After further incubation at 37 °C for 4–5 days, the wells were stained with 1 % (w/v) crystal violet dissolved in 4 % (v/v) formaldehyde to visualize the plaques. The percentage of plaque reduction was calculated as described previously (Deng *et al.*, 2011).

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