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Short Communication

Genomics and evolution of *Aedes*-borne flaviviruses

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We analysed the complete coding sequences of all recognized species of *Aedes*-borne flavivirus, including previously uncharacterized viruses within the yellow fever virus (YFV), Spondweni virus (SPOV) and dengue virus (DENV) groups. Two major phylogenetic lineages were revealed: one included the YFV and Entebbe bat virus groups, and the other included the DENV, SPOV and *Culex*-borne flavivirus groups. This analysis supported previous evidence that *Culex*-borne flaviviruses have evolved from ancestral *Aedes*-borne viruses. However, the topology at the junction between these lineages remains complex and may be refined by the discovery of viruses related to the Kedougou virus. Additionally, viral evolution was found to be associated with the appearance of new biological characteristics; mutations that may modify the envelope protein structure were identified for seven viruses within the YFV group, and an expansion of host–vector range was identified in the two major evolutionary lineages, which in turn may facilitate the emergence of mosquito-borne flaviviruses.

The genus *Flavivirus* includes 53 recognized species, many of which are human pathogens, and several other viruses yet to be documented as species. On the basis of phylogenetic evidence and taking into account their known vectors and natural vertebrate hosts, the species were divided into ecologically distinguishable groups that formed phylogenetically distinct lineages (Gaunt *et al.*, 2001; Gould *et al.*, 2003; Kuno *et al.*, 1998; Billoir *et al.*, 2000). The arthropod-borne flaviviruses are either tick-borne flaviviruses (TBFVs) composed of 12 species belonging to three different groups (Grard *et al.*, 2007) that cause encephalitis (tick-borne encephalitis virus) or haemorrhagic fever (Omsk hemorrhagic fever virus), or mosquito-borne flaviviruses (MBFVs) (27 species belonging to seven different groups; Thiel *et al.*, 2005) that include human pathogens which cause haemorrhagic disease [yellow fever virus (YFV) and dengue virus (DENV)] or encephalitis [West Nile virus (WNV), Japanese encephalitis virus and others]. Among these arboviruses, a number are denoted as emerging viruses with a high potential for global dispersion as illustrated by the spread of WNV in North America following its inadvertent introduction into this area in 1999. The genus also encompasses viruses with no known vector (NKV) (Porterfield, 1980) that include mammalian viruses (12 species belonging to three different groups) for which arthropod vectors have not been identified. During the past decade, continuous efforts have been made to improve the understanding of the phylogenetic relationships, to propose a reliable taxonomy and to develop molecular tools for diagnosis or viral surveillance. Complete open reading frame (ORF) sequences are now available for more than 30 flaviviruses species; however, except for the TBFVs (Grard *et al.*, 2007), the exhaustive characterization of the NKVs and MBFVs still needs to be achieved.

MBFVs are either mainly associated with *Culex* spp. mosquitoes [they are hereafter referred to as the '*Culex* group' i.e. Japanese encephalitis, Aroa, Kokobera and Ntaya virus groups as defined by the International Committee on Taxonomy of Viruses (ICTV)], or with *Aedes* spp. mosquitoes ['*Aedes* group' i.e. DENV, Spondweni virus (SPOV) and YFV groups as defined by

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are DQ859056–DQ859067.

Two supplementary figures are available with the online version of this paper.

the ICTV]. The phylogenetic lineage that encompasses *Aedes*-borne viruses also includes a clade of three viruses with NKV, isolated from bats, viz, Entebbe bat virus (ENTV), Yokose virus and Sokuluk virus (Gaunt *et al.*, 2001; Kuno *et al.*, 1998; Kuno & Chang, 2006; Tajima *et al.*, 2005) that constitute the ENTV group. However, among the viruses that are vectored by mosquitoes, this biological association with *Aedes* spp. is not absolute since some viruses have been isolated from *Culex* or *Phlebotomus* spp. (Table 1).

The current study focused on the *Aedes* group for which we report a comprehensive genetic characterization with complete ORF sequencing for 12 MBFVs corresponding to 10 species, including (Table 1): (i) species within the YFV group: *Wesselsbron virus* (WESSV), *Banzi virus* (BANV), *Edge Hill virus* (EHV), *Jugra virus* (JUGV), *Saboya virus* (SABV), Potiskum virus (POTV) (*Saboya* sp.), *Sepik virus* (SEPV), *Uganda S virus* (UGSV) and *Bouboui virus* (BOUV); (ii) species of the SPOV group: *Zika* (ZIKV) and Spondweni virus, previously recognized as *Zika virus* sp.; (iii) *Kedougou virus* (KEDV) currently included in the DENV group.

Viral RNA was extracted from the supernatant medium of infected cell cultures (C6/36), and RT-PCR and nucleotide sequencing were performed as previously reported (Grard *et al.*, 2007). Briefly, the first set of RT-PCRs targeted the envelope, NS3 and NS5 genes with previously reported degenerate primers (Crochu *et al.*, 2004; Gaunt & Gould, 2005; Moureau *et al.*, 2007) and a new set of primers was used for the amplification of the NS3 gene (NS3FR: 5′-TKICKICCIAYICKICCICKICKYTGIGCNGY-3′; NS3FS:5′-GGIGTIYTICAYACIATGTGGCAYGTIACN-3′). Specific primers were deduced for gap filling by long range RT-PCR (cMaster RT plus PCR System kit; Eppendorf) and amplification products were sequenced using the Long PCR Product Sequencing procedure as described elsewhere (Emonet *et al.*, 2007). The 3′ termini of the ORFs were amplified using NS5-specific forward primer and the degenerate reverse primer 3′UTR-MOS (5′-GGTCT-CCWMTAACCTCTAG-3′). The 5′ termini of the ORFs were amplified using virus-specific reverse primer and a set of forward primers deduced from 5′UTR alignment (sequences available on request). Final contiguous sequences were reconstructed using Sequencher 4.5 software (Gene Codes Corporation). A complete amino acid alignment was generated using K-align (Lassmann & Sonnhammer, 2005; available at the EMBL server - http://www.ebi.ac.uk/Tools/kalign/index.html) and highly divergent regions were removed manually. This resulted in a final 2304 aa alignment. A maximum-likelihood (ML) tree (Fig. 1) was then inferred from this alignment using the WAG model of amino acid substitution implemented in the TREE-PUZZLE program (Strimmer & von Haeseler, 1996). In addition, ML analysis was conducted from the deduced NS5 gene alignment (827 aa positions) using the same parameters (Supplementary Fig. S2 available in JGV Online). Results of sequence analysis were combined with available ecological characteristics of viruses such as geographical dispersal, host–vector associations and pathology compiled from the CRORA viral database (Centre collaborateur OMS de Reference et de Recherche sur les Arbovirus, Institut Pasteur de Dakar; http://www.pasteur.fr/recherche/banques/CRORA/), the International Catalogue of Arboviruses (Karabatsos, 1985) and the relevant published scientific literature (Table 1).

The global topology of the tree based on the complete amino acid sequences (Fig. 1) corresponds closely to those previously reported (Billoir *et al.*, 2000; Cook & Holmes, 2006; Grard *et al.*, 2007; Kuno & Chang, 2006; Medeiros *et al.*, 2007), i.e. an 'NS3-like' topology. The earliest divergence assigns the TBFVs and two NKVs groups (the Modoc and Rio Bravo virus groups) to one branch, and the MBFV groups together with the third NKV group (the ENTV group) to a second branch. The MBFVs were further divided into two lineages, viruses associated with *Aedes* transmission being distributed to both lineages. The first one includes viruses belonging to the YFV group and viruses of the ENTV group. The second lineage includes the DENV group (*Aedes* group), a large number of viruses transmitted by *Culex* mosquitoes (*Culex* group), but also *Aedes*-borne viruses, which are genetically closely related to *Culex*-borne flaviviruses, i.e. the SPOV group and KEDV (Fig. 1), corresponding closely with earlier publications (Gould *et al.*, 2003; Kuno *et al.*, 1998). Analysis of amino acid alignments of viruses in the *Aedes* group showed that the polyprotein putative cleavage sites, enzymic motifs in the NS3 and NS5 genes and the sequence of the fusion peptide were highly conserved.

Within the YFV group, seven viruses (EHV, BOUV, BANV, UGSV, JUGV, POTV and SABV) have a common ancestral root. Surprisingly, analysis of the conserved cysteines in the envelope (E) protein revealed that these viruses shared two major C→S mutations that abolished one of the six disulphide bridges, normally characteristic of the flavivirus envelope glycoprotein. Supplementary Fig. S1 (available in JGV Online) shows an alignment of E protein sequences and the position where these mutations occurred, i.e. cysteines C-60 and -121 (DENV2 envelope amino acid sequence numbering). These residues are usually paired to form one of the three disulphide bridges (bond b on Supplementary Fig. S1) that forms part of the structure of the envelope domain II, which supports the fusion peptide. No compensatory mutation at other positions of the envelope gene could be identified. The five remaining disulphide bonds are defined by the following paired cysteines: 3–30 (bond a), 74–105 (bond c), 92–116 (bond d), 185–285 (bond e) and 302–333 (bond f). Other viruses with non-conserved cysteines in the E gene have been previously identified, but they are all 'atypic' flaviviruses: (i) the mammalian virus Tamana bat virus with the C→Y mutation at position 121 that also abolishes the disulphide bond b, and (ii) the three mosquito viruses: Cell fusing agent virus, Kamiti River virus and *Culex* flavivirus (Hoshino *et al.*, 2007) where the two cysteines of the

disulphide bond a are missing. Such mutations may be associated with significant modifications of the envelope conformation, and therefore may have an influence on viral entry into cells and/or the antigenic properties of the E protein.

Finally, by combining the phylogenetic relationships with the ecological characteristics (Table 1) we have extended the knowledge of the original host–vector associations for these seven viruses. Their vertebrate hosts include monkeys, rodents, antelope (BOUV), wallabies (EHV), and also birds (UGS) and bats (JUGV). BOUV, EHV and UGSV are mainly (but not exclusively) associated with *Aedes* spp. mosquitoes. SABV transmission is clearly associated with *Phlebotomus* spp. (Ba *et al.*, 1999; Fontenille *et al.*, 1994; Traore-Lamizana *et al.*, 2001) and BANV transmission is associated with *Culex* spp. (McIntosh, 1984a). The latter virus is the only one of this group for which human cases have been reported. Hence, this lineage is characterized by an increasing diversity of arthropod vectors, which may in turn facilitate the infection of a greater number of possible vertebrate hosts, including humans.

Taking into account the phylogenetic relationships, the ecological characteristics and the specific properties of the envelope protein, we propose that EHV, BOUV, BANV, UGSV, JUGV, POTV and SABV should be incorporated into a new taxonomic group, designated, the 'Edge Hill virus' group, which is distinct from the YFV group. Although EHV is found in Australia, the lineages most closely related to EHV are found in Africa. As pointed out by Gould *et al.* (2003), among the eight MBFVs species most closely related to EHV (i.e. the monophyletic lineage including both the YFV and EHV groups) only SEPV and JUGV are found outside Africa. Hence, under the most parsimonious scenario, the geographical origin of the EHV group would be most likely assigned to Africa, EHV being secondarily exported to Australia.

According to this taxonomic proposal and until many more viruses have been identified, the YFV group may be limited to YFV, SEPV and WESSV (Fig. 1). WESSV is a human and veterinary pathogen transmitted by *Aedes* spp. mosquitoes in Africa and Asia. SEPV has been responsible for only one human case and one sheep infection in Australia. It has only rarely been isolated from arthropod vectors and has never been associated with *Aedes* spp. mosquitoes. The genetic distance between SEPV and WESSV (12 % aa p-distance) is in agreement with their taxonomic assignment to two different species.

The second lineage of the MBFV group includes DENV in a basal position (although this is not strongly supported), followed by clustering of ZIKV, SPOV and KEDV and then viruses associated with *Culex* transmission. This branching pattern suggests that viruses transmitted by *Culex* spp. mosquitoes evolved from an ancestral lineage associated with *Aedes* spp. mosquitoes, as was previously suggested from the NS5 nucleotide sequence data (Gould *et al.*, 2001, 2003). The complete ORF phylogeny also suggests two possible taxonomic reassessments. SPOV is currently recognized as a member of the species *Zika virus*. Both viruses circulate in Africa but ZIKV was also isolated in Asia and Oceania. They are transmitted by *Aedes* spp. mosquitoes but ZIKV was presumably associated with primates (Wolfe *et al.*, 2001; CRORA, 2005a) and can induce human epidemics (Lanciotti *et al.*, 2008; ProMED-mail, 2007) whereas, SPOV was associated with sporadic human cases (Burke & Monath, 2001; McIntosh, 1984b; Wolfe *et al.*, 1982; CRORA, 2005b) and despite serological evidence of infection of cattle, sheep and goats (Burke & Monath, 2001) its reservoir host remains unknown. SPOV and ZIKV differ by 25 % aa p-distance. By comparison, WESSV and SEPV, which are classified as two different species, differ by 12 % aa p-distance. Hence, it seems reasonable to propose the creation of the species *Spondweni virus*. The second taxonomic revision concerns KEDV, which is currently a member of the DENV group. In previously reported phylogenies based on the NS5 gene, KEDV was associated with the DENV lineages (Gaunt *et al.*, 2001; Kuno *et al.*, 1998) or in a basal position in the second lineage (Gould *et al.*, 2001; Kuno & Chang, 2005). However, the phylogeny we inferred from the complete amino acid data suggested that KEDV is most closely related to the SPOV group, although this was not strongly supported (Fig. 1), and indeed, in the NS5 ML phylogeny (Supplementary Fig. S2) KEDV appeared to be separated from both the DENV and SPOV groups. An analysis of amino acid distances in the NS5 gene showed that, by comparison with ZIKV which is clearly related to *Culex*-borne flaviviruses, KEDV is similar to viruses of both the *Culex* group and the DENV group, again showing that its phylogenetic position is ambiguous (Fig. 2). It is therefore possible that KEDV belongs to a distinct group of viruses for which other members remain to be discovered. In addition KEDV circulates in Africa but its vertebrate host is unknown and to date only one human case has been reported (CRORA, 2005c). As previously suggested (Kuno & Chang, 2007) it seems appropriate to consider KEDV to be a distinct group from the DENV group. The phylogenetic relationships inferred here for the other members of the *Aedes* group are in agreement with their current taxonomic status.

In conclusion, the hypothesis that the *Culex*-associated flaviviruses evolved from ancestral *Aedes*-associated viruses seems robustly confirmed by phylogenetic analysis of complete amino acid sequences. However, the topology at the junction between both groups is complex and may be refined in the future by the full-length characterization of more *Culex*-associated viruses and possibly the discovery of new viruses related to SPOV, ZIKV or KEDV. Viral evolution in the mosquito-borne flavivirus group is associated with the appearance of a number of new biological characteristics: firstly, significant modifications of the envelope protein were identified in the EHV group, which may modify the folding of this protein. Secondly, viral evolution was associated with an enlargement of the

Table 1. Viruses included in the phylogenetic study

Viruses genetically characterized in the current study												
Virus species	***Banzi virus***	***Bouboui virus***	***Edge Hill virus***	***Jugra virus***	***Saboya virus***		***Uganda S virus***	***Wesselsbron virus***	***Sepik virus***	***Zika virus***		***Kedougou virus***
Virus name	Banzi virus	Bouboui virus	Edge Hill virus	Jugra virus	Saboya virus	Potiskum virus	Uganda S virus	Wesselsbron virus	Sepik virus	Zika virus	Spondweni virus	Kedougou virus
Abbreviation	BANV	BOUV	EHV	JUGV	SABV	POTV	UGSV	WESSV	SEPV	ZIKV	SPOV	KEDV
Strain	SAH 336	DAK AR B490	YMP 48	P-9-314	Dak AR D4600	IBAN 10069	ORIGINAL	SAH-177 99871-2	7148	MR 766	SM-6 V-1	Dak AR D1470
GenBank accession no.	DQ859056	DQ859057	DQ859060	DQ859066	DQ859062	DQ859067	DQ859065	DQ859058	DQ859063	DQ859059	DQ859064	DQ859061
ICTV current status	←Yellow fever virus group→									←Spondweni virus group→		Dengue virus group
Suggested modification	←Edge Hill virus group→							←Yellow fever virus group→		*Zika* species	*Spondweni* species	Kedougou virus group
Mutated envelope*	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No	No	No
Main arthropod vector†	*Culex rubinotus* (90)	*Ae. africanus* (30)	*Ae. vigilax* (12)	?	Phlebotomines (80)	?	*Ae. longipalpis* (45)	*Ae. vexans* (50)	?	*Ae. furcifer* (100)	*Ae. circumluteolus* (20)	*Ae. minutus* (25)
								Ae. dalzieli (20)		*Ae. furcifer taylori* (40) *Ae. africanus* (80) *Ae. dalzieli* (49)		*Ae. dalzieli* (20)
Other possible arthropod vector (isolation)	3 *Culex* spp.	*Anopheles paludis*	*Culex annulirostris*	*Ae.* sp.	*Ae. vittatus*		6 *Ae.* spp.	18 *Ae.* spp.	*Armigeres* sp.	2 *Anopheles* spp.	4 *Ae.* spp.	4 *Ae.* spp.
	Mansonia africana	*Eretmapodites gr inornatus*	*Anopheles meraukensis*	*Uranotaenia* sp.	*Ae. africanus*		*Culex duttoni*	4 *Culex* spp.	*Mansonia* sp.	10 *Ae.* spp.	2 *Mansonia* spp.	
		6 *Ae.* spp.			*Rhipicephalus evertsi evertsi*			2 *Mansonia* spp.	*2 Ficalbia* spp.	*Mansonia* sp.	*Culex neavi*	
		2 *Culex* spp.						*Culex duttoni*		2 *Eretmapodites* spp.	1 *Eretmapodites* sp.	
Vertebrate host†	Sentinel hamster (3)	Monkeys (3): unprecised; *Cercopithecus nictitans*; *Papio papio*	Wallabies (2)	Bat (1): *Cynopterus brachyotis*	Rodents (19): *Tatera kempi*; *Jaculus jaculus*; *Arvicanthis niloticus*; *Mastomys* spp.; *Mus musculus*	Giant rat (1): *Cricetomys gambianus*	Bird (1): *Saxicola rubetra*	Sheep, goat, cattle (fever, abortion)	Sheep (1)	Monkeys (3): *Cercopithecus aethiops*; *Erythrocebus patas*; *Rhesus sentinelle*	?	?
	Mastomys natalensis (1)	Rodent (1)					Sentinel mice (2)					
		Antelope (1)										
Geographical distribution	Africa	Africa	Australia	Malaysia	Africa	Africa	Africa	Africa, Thailand	New Guinea	Africa, Asia, Oceania	Africa	Africa
Human cases	2 (fever)	?	?	?	?	?	Suspected during chikungunya epidemic‡	Many (fever, pain, anorexia)	1 (headache)	Many (fever, pain, rash)	5 (fever, pain)	1 (undescribed)

Table 1. cont.

Other species and viruses included in the genetic analysis								
Mosquito-borne flaviviruses			**Tick-borne flaviviruses**			**Viruses with no known vector**		
Dengue virus type 1	DENV1	U88536	*Kadam virus*	KADV	DQ235146	*Rio Bravo virus*	RBV	AF144692
Dengue virus type 2	DENV2	AF038403	*Meaban virus*	MEAV	DQ235144	*Modoc virus*	MODV	AJ242984
Dengue virus type 3	DENV3	M93130	*Saumarez Reef virus*	SREV	DQ235150	*Apoi virus*	APOIV	AF160193
Dengue virus type 4	DENV4	M14931	*Tyuleniy virus*	TYUV	DQ235148	*Montana myotis leukoencephalitis virus*	MMLV	AJ299445
Yellow fever virus	YFV	AF094612	*Royal Farm virus*	RFV	DQ235149			
Yellow fever virus (strain 17 D)	YFV17D	X03700	*Karshi virus*	KSIV	DQ235147	**Tentative species in the genus**		
Japanese encephalitis virus	JEV	M18370	*Gadgets Gully virus*	GGYV	DQ235145	Cell fusing agent virus	CFAV	M91671
Murray Valley encephalitis virus	MVEV	AF161266	*Powassan virus*	POWV	L06436			
St. Louis encephalitis virus	SLEV	DQ359217	Deer tick virus	DTV	NC_003218			
West Nile virus	WNV	M12294	*Kyasanur forest disease virus*	KFDV	AY323490			
Kunjin virus	KUNV	D00246	Alkhurma hemorrhagic fever virus	AHFV	AF331718			
Usutu virus	USUV	NC_006551	*Omsk hemorrhagic fever virus*	OHFV	AY323489			
Ilheus virus	ILHV	AY632539	(strain Bogoluvovska)					
Rocio virus	ROCV	AY632542	*Langat virus*	LGTV	AF253419			
Bagaza virus	BAGV	AY632545	*Tick-borne encephalitis virus*§					
Aroa virus			Louping ill virus	LIV	Y07863			
Iguape virus	IGUV	AY632538	Western tick-borne encephalitis	WTBEV	U27495			
Bussuquara virus	BSQV	AY632536	virus (strain Neudoerfl)					
Kokobera virus	KOKV	AY632541	Turkish sheep encephalitis virus	TSEV	DQ235151			
Entebbe bat virus	ENTV	AY632537	Eastern tick-borne encephalitis virus	ETBEV	AB062064			
Yokose virus	YOKV	NC_005039	(strain Sofjin)					

*Refers to the existence of the C→S mutations that abolish one of the six disulphide bridges in the E protein.

†The numbers in parentheses refer to the numbers of isolates that we could identify from the literature. *Ae.*, *Aedes*.

‡UGSV was isolated from human blood samples taken during a Chikungunya virus epidemic in Tanzania; its identification was performed retrospectively and remains doubtful (Woodall, 1985).

§Species and subtypes definitions according to Grard *et al.* (2007).

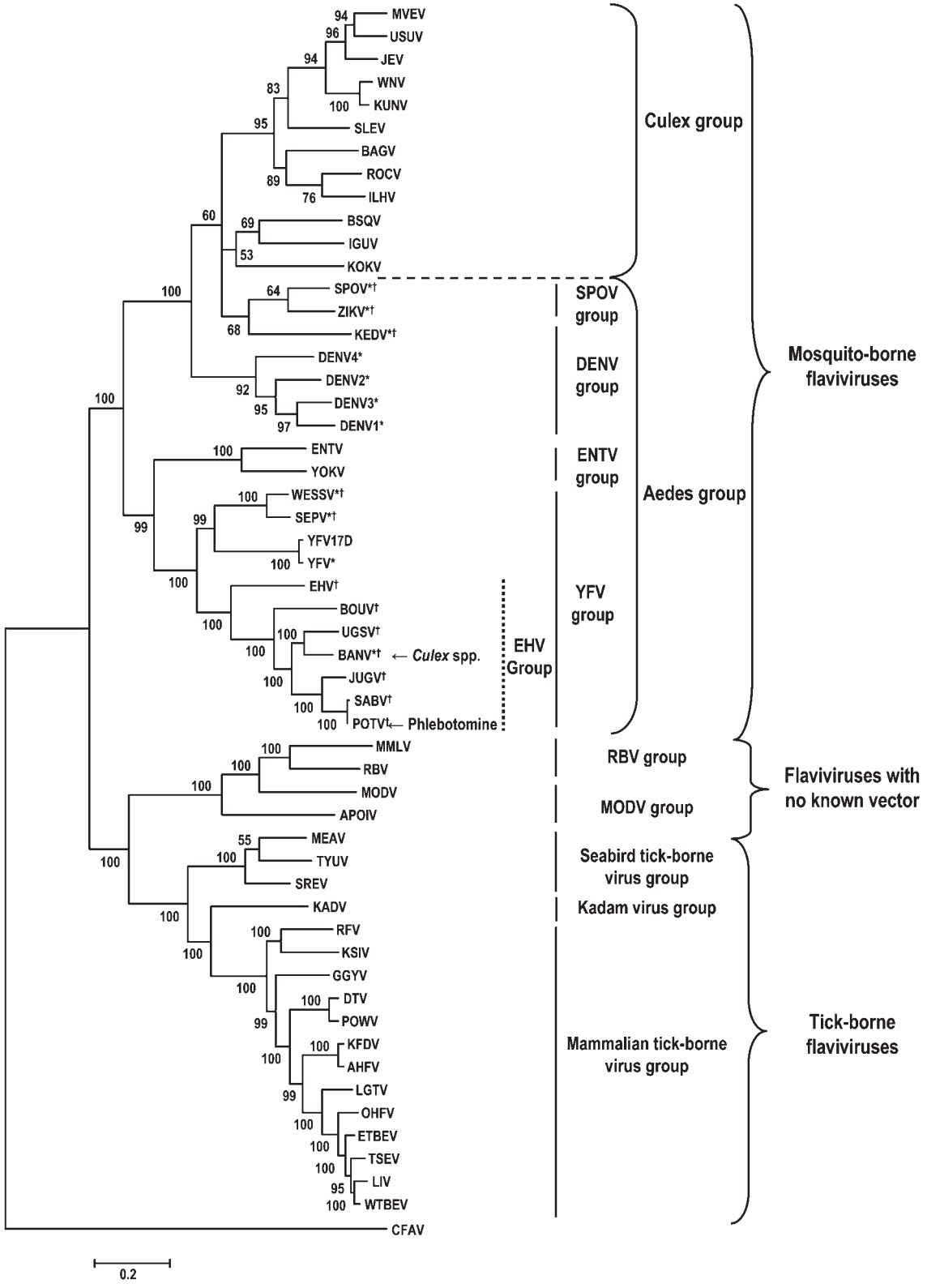

Fig. 1. ML (quartet puzzling) tree computed from the complete amino acid sequence alignment after the removal of the highly divergent regions. Quartet resampling values (shown next to each branch) resulted from 25 000 quartet puzzling steps. Branch lengths are drawn to a scale of amino acid substitutions per site. '*' Viruses of the *Aedes* group associated with human cases. '†' Sequences determined during this study.

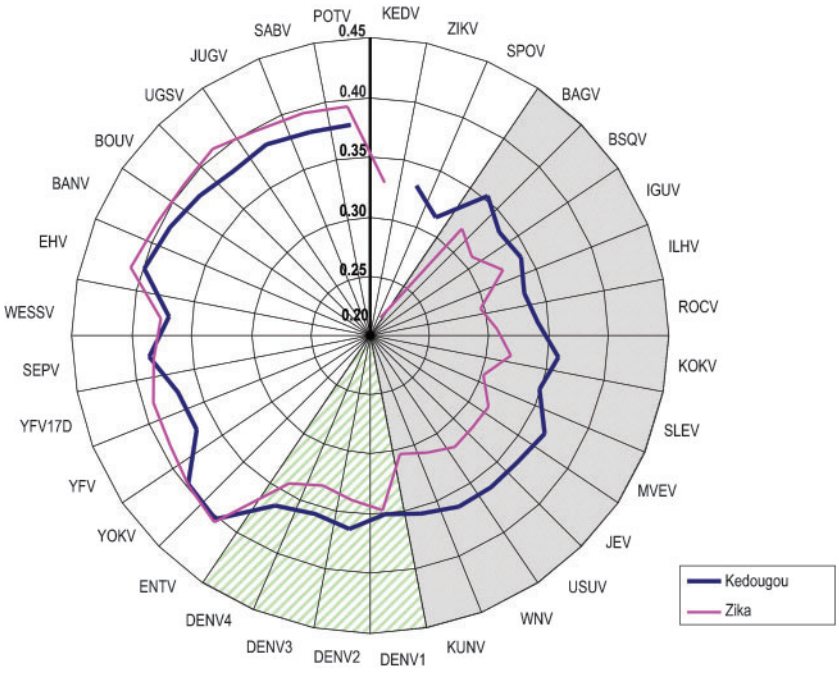

Fig. 2. Pairwise amino acid distances in the NS5 gene between KEDV and other MBFVs are reported and compared with distances between ZIKV and other MBFVs. Grey panel: viruses of the *Culex* group; green hatched panel: viruses of the DENV group.

vector range in the two major evolutionary lineages. This propensity to adapt to new arthropod vectors may prove to be a crucial point regarding the potential for emergence of mosquito-borne flaviviruses in the future. This is perhaps best exemplified by WNV, which has been isolated from more than 60 different mosquito species in North America (http://www.cdc.gov/ncidod/dvbid/westnile/mosquitoSpecies.htm; accessed 21 April 2009), possibly partly explaining why this virus has been so successful in the New World. Moreover, mosquito-borne YFV, WNV and St. Louis encephalitis virus have all been isolated from ticks in the wild (Burke & Monath, 2001), maybe reflecting evolutionary remnants equivalent to those reported on the basis of studies of flavivirus untranslated regions (Gritsun & Gould, 2006). Human activities (travel, displacement of both human and mosquito populations, modification of ecosystems, deforestation) and also climate change offer increasing possibilities for unanticipated ecological cycles and natural hosts, and thus for virus adaptation to mosquitoes that could enhance transmission success to humans.

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