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Characterization of a Novel Flavivirus from Mosquitoes in Northern Europe That Is Related to Mosquito-Borne Flaviviruses of the Tropics▽

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A novel flavivirus was isolated from mosquitoes in Finland, representing the first mosquito-borne flavivirus from Northern Europe. The isolate, designated Lammi virus (LAMV), was antigenically cross-reactive with other flaviviruses and exhibited typical flavivirus morphology as determined by electron microscopy. The genomic sequence of LAMV was highly divergent from the recognized flaviviruses, and yet the polyprotein properties resembled those of mosquito-borne flaviviruses. Phylogenetic analysis of the complete coding sequence showed that LAMV represented a distinct lineage related to the *Aedes* sp.-transmitted human pathogenic flaviviruses, similarly to the newly described Nounané virus (NOUV), a flavivirus from Africa (S. Junglen et al., J. Virol. 83:4462-4468, 2009). Despite the low sequence homology, LAMV and NOUV were phylogenetically grouped closely, likely representing separate species of a novel group of flaviviruses. Despite the biological properties preferring replication in mosquito cells, the genetic relatedness of LAMV to viruses associated with vertebrate hosts warrants a search for disease associations.

The genus *Flavivirus* in the family *Flaviviridae* consists of 53 recognized virus species that are enveloped, positive-sense single-stranded RNA viruses. The virion consists of three structural proteins: capsid (C), membrane (M), and envelope (E). In addition, seven nonstructural proteins are present in infected cells (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). Based on their antigenic properties and vector associations, flaviviruses have been grouped into mosquito-borne, tick-borne, and no-known-vector viruses and have been isolated from vertebrates, bats, and rodents (15, 25). The grouping of flaviviruses according to their transmission mode is strongly supported by phylogenetic analyses of their genomic sequences (9, 18, 31).

Mosquito-borne flaviviruses are a large and divergent group of viruses that can be differentiated phylogenetically into those associated either with encephalitic disease and transmission by *Culex* spp. mosquitoes or with diseases with hemorrhagic complications and transmission by *Aedes* spp. (18). Seven groups of mosquito-borne flaviviruses, namely, the Aroa, dengue, Japanese encephalitis, Kokobera, Ntaya, Spondweni, and yellow fever virus groups are recognized (15, 25). These groups include important animal and human pathogens such as dengue virus (DENV), West Nile virus (WNV), Japanese encephalitis virus (JEV), and yellow fever virus (YFV).

Unclassified insect flaviviruses that have no recognized association with vertebrates have been isolated from a variety of mosquito species and also from mosquito cell lines. These insect flaviviruses do not appear to infect vertebrate cells and are not associated with human or animal disease. The cell fusing agent virus (CFAV), a tentative species in the genus *Flavivirus*, was the first of these insect viruses to be characterized (5, 40), Although CFAV was originally identified in cultured mosquito cells, it was later isolated from mosquitoes collected in Puerto Rico (7). This as-yet-unclassified insect flavivirus group now also includes Kamiti river virus (KRV) isolated in Kenya (10, 38) and a virus isolated from *Culex* spp. in Japan, designated culex flavivirus (22). In addition, related viral sequences or isolates have been recently reported from mosquitoes in Spain (1), the United States and Trinidad (26), and Mexico (14). Moreover, the identification of flaviviruslike sequences integrated within the genomes of *Aedes* mosquitoes further complicates the evolutionary history of the flaviviruses. These sequences, currently referred to as cell silent agent are genetically most closely related to CFAV and possibly share common evolutionary origin (11). Phylogenetically, the insect viruses form a divergent outgroup that may represent a primordial flavivirus lineage. Apart from the insect flaviviruses, the other recently discovered novel flaviviruses represent highly divergent lineages, such as Tamana bat virus (13), and Ngoye virus (20). Recently, a novel flavivirus, Nounané virus (NOUV) was isolated from a novel mosquito vector species, *Uranotaenia mashonaensis* in Côte d'Ivoire (23), and was shown to be phylogenetically related to the human pathogenic mosquito-borne flaviviruses.

Several arboviruses have been reported from Northern Europe including the flavivirus tick-borne encephalitis virus (24, 36) but, to date, no mosquito-borne flaviviruses have been isolated. Our aim was to screen for arboviruses in Finland by

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studying mosquitoes using virus isolation and subsequent arbovirus antigen detection, which resulted in the identification of a novel flavivirus. We present here the isolation and characterization of this isolate, designated Lammi virus (LAMV), and discuss the implications of our findings.

MATERIALS AND METHODS

Mosquito collection and virus isolation. Mosquitoes (Diptera; Culicidae) were collected in 2004 using hand nets in July at Konnevesi Research Station (N62° 37′, E026°21′) in Central Finland ($n \approx 2,000$) and in August at Lammi Biological Station (N61°04′, E25°08′) in Southern Finland ($n \approx 1,200$). The mosquitoes were frozen briefly at −20°C, sealed individually in tubes, and transported on dry ice to storage at −70°C. In addition, mosquito larvae were collected from both sites (total $n \approx 700$). The larvae were allowed to hatch in the laboratory prior to processing. Individual mosquitoes were homogenized in 100 μl of Dulbecco phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin and antibiotics (100 IU of penicillin/ml and 100 μg of streptomycin/ml) using sterile sand. Aliquots of 50 μl from approximately 24 individual mosquito homogenates were combined to form a pool. The pools were filtered through syringe-driven 0.45-μm-pore-size filters prior to inoculation of cells. The virus isolations were carried out in 25-cm^2 bottles using cultured mosquito (*Aedes albopictus* C6/36, ATCC CRL-1660) and mammalian (Vero E6, ATCC CRL-1586) cells. The C6/36 cells were maintained in Leibovitch L-15 medium supplemented with 2% heat-inactivated fetal calf serum, glutamine, 100 IU of penicillin/ml, and 100 μg of streptomycin/ml at 27°C, and the Vero E6 cells were maintained in minimal essential medium supplemented with 2% fetal calf serum, glutamine, and antibiotics at 37°C in 5% CO_2. Freshly confluent monolayers of both cell lines were rinsed with PBS prior to infecting with 200 μl of mosquito pool filtrates for 1 h. After the incubation, fresh medium was added. The cells were observed for cytopathic effects (CPE), and the cells and supernatant media were harvested when disruption of ca. 50% of the monolayer was observed. If no CPE were observed, the cells were harvested after 14 days and studied by immunofluorescence assay (IFA). The supernatant media were stored at −70°C for further studies.

IFA. The cells from virus isolation experiments were washed twice in PBS and then dried on 10-well microscope slides, fixed in cold acetone for 7 min, and stored at −70°C until used. IFA screening of the slides was performed with flavivirus and Sindbis virus (family *Togaviridae*; genus *Alphavirus*) immunoglobulin G (IgG)-positive human sera and mouse polyclonal antibodies against Californian serogroup viruses (family *Bunyaviridae*; genus *Orthobunyavirus*). Additional tests were performed using the pan-flavivirus group, E-protein-specific monoclonal antibodies HB-112 (21) and 813 (19). All appropriately diluted antibodies were incubated on the IFA test slides for 1 h at 37°C, washed three times in PBS, and washed once in distilled water prior to incubation with appropriate fluorescein isothiocyanate-labeled secondary antibodies (Jackson Immunoresearch/Dako) for 30 min. After incubation with the secondary antibodies the slides were washed as described above, and the cells were examined for the presence of fluorescent antigen by using a fluorescence microscope.

HI test. The ability of LAMV to agglutinate goose erythrocytes was tested directly on Tween/ether-inactivated supernatant medium obtained from infected and control uninfected C6/36 cell monolayers. Once it had been established that LAMV did agglutinate goose erythrocytes, different pH were tested for finding the optimal conditions for hemagglutination inhibition (HI) tests. The antigen was then subjected to HI tests using the sera of patients known to have immunity to tick-borne encephalitis virus (TBEV), DENV, or WNV at pH 6.0 according to standard protocols (6).

In vitro culture of LAMV. Primary chicken embryo fibroblasts and cultured cells of different mammalian species including human (SH-SY5Y, HEK293, and HeLa), mouse (Neuro 2A), hamster (BHK-21), porcine (PK-15), and monkey (Vero and Vero E6) cells were infected with the LAMV isolate, incubated at 37°C, and observed for the appearance of CPE. The presence of LAMV antigen in the infected cells was analyzed with flavivirus IgG-positive human sera by IFA on days 7 and 14 postinfection.

Virus amplification in mice. The supernatant medium obtained from C6/36 cells infected with LAMV, and the original filtered homogenates from the positive mosquito pool were used in an attempt to infect 2- to 3 day-old NMRI mice intracerebrally. The mice were observed for signs of disease and sacrificed on day 14 of the experiment. These animal experiments were carried out according to the animal experiment regulations of the University of Helsinki under the license ESLH-2006-03286/Ym-23 (STU 466 A). The RNA was extracted from the brain tissues by using TriPure Isolation Reagent (Roche Applied Science) according to manufacturer's instructions and studied for the presence of LAMV RNA by using a partial NS5 gene-amplifying PCR (39).

Ultracentrifugation and electron microscopy. To obtain concentrated virus material for further analysis, confluent C6/36 cells were infected with LAMV for 7 days at room temperature. Supernatant media were collected, filtered through a 0.22-μm-pore-size filter, and ultracentrifuged at 28,000 rpm for 3 h and 45 min at 4°C using an SW28 rotor. The sedimented pellets were dissolved overnight in TEN buffer (0.05 M Tris-HCl, 0.001 M EDTA [pH 7.5]) at 4°C. Electron microscopy (EM) was performed on the sedimented virus mounted on copper grids after negative staining with 2% KPTA (tungstophosphoric acid, with the pH adjusted to 6.0 with potassium hydroxide) using a JEOL JEM-1400 transmission electron microscope.

Genome amplification and sequencing. The genomic RNA was extracted from the supernatant medium of infected cells or from sedimented virus by using the QIAamp viral RNA minikit (Qiagen) according to the manufacturer's instructions. Complete open reading frame amplification and sequencing was initiated using published oligonucleotides targeted to conserved areas of the flavivirus genome (2, 17, 29, 39). Based on the sequences obtained, new LAMV-specific primers were designed and used to produce overlapping amplicons for the assembly of the complete open reading frame sequence. The oligonucleotides used in the amplification and sequencing of the complete genome are available upon request. Reverse transcription (RT) reactions were performed using Expand reverse transcriptase (Roche Applied Science) and gene-specific primers or random hexamers according to the manufacturer's instructions. The PCRs were performed using *Taq* DNA polymerase (Fermentas) and *Taq* Extender (Promega) using standard PCR protocols, with extension times varying from 1 to 3 min depending on the amplicon size. The RT-PCR products were cleaved from agarose gels when necessary and either sequenced directly using gene-specific forward and reverse primers or first cloned into pGEM-T vector (Promega) for sequencing.

Sequence alignments and phylogenetic analysis. The nucleotide sequences were translated into amino acid sequences by using ExPASy translate tool (available online at http://ca.expasy.org/tools/dna.html). The LAMV nucleotide sequences were aligned with other flavivirus sequences from the GenBank (see the supplemental table [http://www.hi.helsinki.fi/zoonoosivirukset/files/SupplementaryTableJVI.pdf]) using CLUSTAL W2 (http://www.ebi.ac.uk/Tools/clustalw2). The phylogenetic trees were estimated with the neighbor-joining method by using the Kimura two-parameter analysis on MEGA3 software version 3.1 (27).

Bioinformatic tools used in analysis of LAMV proteins. The LAMV polyprotein signalase cleavage sites were predicted by using SignalIP 3.0 (http://www.cbs.dtu.dk/services/SignalP/). Polyprotein amino acid hydropathy plots were estimated by using the Kyte and Doolittle method (http://www.expasy.org/cgi-bin/protscale.pl). The NetNGlyc 1.0 server was used in predicting potential N-glycosylation sites on LAMV glycoproteins (http://www.cbs.dtu.dk/services/NetNGlyc/).

Mosquito species identification using PCR. The virus isolation positive mosquito homogenate pool was derived from 24 homogenates of individual mosquitoes. Total nucleic acids were extracted from the remaining individual homogenates with EZ-Biorobot by using the EZ1 virus minikit (Qiagen) and studied for the presence of viral RNA by using a real-time one step RT-PCR method (35). For identifying the mosquito species, the mosquito mitochondrial cytochrome *c* oxidase subunit I gene sequences were amplified by using the primer pairs UEA3 (34)/FLY10 (37) and LCO/HCO2198 (16) according to standard PCR protocols and directly sequenced.

RESULTS

Virus isolation and viral antigen screening by IFA. Approximately 3,900 mosquitoes collected in 2004 at two locations in Finland were studied for the presence of arboviruses by virus isolation procedures using mosquito C6/36 and mammalian Vero E6 cells. When we screened the infected cells using IFA with a panel of antibodies to arboviruses, one of the C6/36 cell cultures was determined to be positive for flavivirus antigen using an IgG-positive human serum. Flavivirus genus E-protein specific monoclonal antibodies were also positive in IFA, confirming that the isolate was a member of the genus *Flavivirus*. The isolate was designated Lammi virus according to the mosquito collection site in Southern Finland. None of the virus

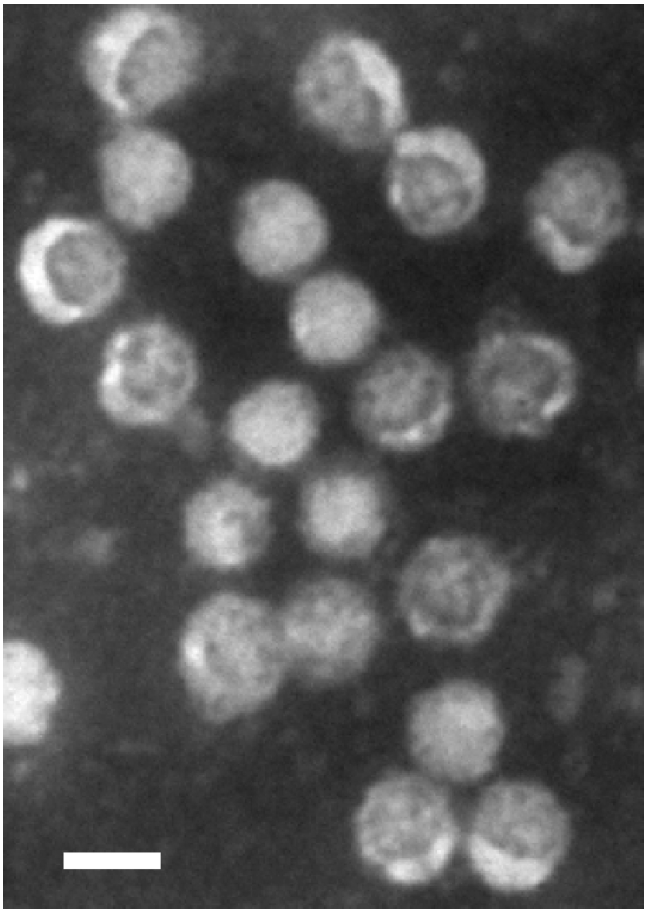


FIG. 1. Electron microscopic image of LAMV virions concentrated by ultracentrifugation. Scale bar, 50 nm.

isolation trials in the present study were positive for alpha- or orthobunyavirus antigen in the IFA.

HI. Tween/ether-inactivated extracts from the supernatant medium of infected C6/36 cells agglutinated goose erythrocytes at pH 6.0, and the prepared LAMV HA antigen was positive in HI tests with human sera from other flavivirus infections (DENV, WNV, and TBEV) demonstrating antigenic cross-reactivity.

In vitro culture of LAMV. LAMV infection caused a CPE in infected C6/36 cells 3 days postinfection. The C6/36 mosquito-cell cultured LAMV was not found to infect any of the tested mammalian cells or chicken embryo fibroblasts, as demonstrated by the failure of LAMV to induce CPE or to produce viral antigen when tested with flavivirus antibodies by IFA.

Virus amplification in mice. The intracerebrally administered LAMV produced no signs of disease, and LAMV RNA could not be detected in the brains of newborn mice, suggesting that no virus replication occurred in the inoculated animals.

EM. The sedimented LAMV appeared under EM as flaviviruslike spherical enveloped virions ~50 nm in diameter (Fig. 1).

Sequence analysis. The complete coding sequence of LAMV was determined and found to contain a single open reading frame of 10,302 nucleotides in length (GenBank accession no. FJ606789) encoding a polyprotein of 3,434 amino acids. Conserved domains were detected for the three flavivirus structural proteins and seven nonstructural proteins, including the functional domains NTPase/helicase, serine protease, and RNA-dependent RNA polymerase. The genomic sequence of LAMV was found to be divergent from any of the previously known flaviviruses (Table 1), sharing only ca. 50 to 55% homologies with other flaviviruses in both nucleotide and amino acid sequences. The highest homology percentages were observed with members of the *Culex* sp.-associated JEV group

TABLE 1. Percent identity comparisons of LAMV protein sequences and other flaviviruses

Protein	LAMV protein size (aa)[a]	% Identity[b]										
		Mosquito-borne (*Culex* spp.)			Mosquito-borne (*Aedes* spp.)			NKV	Tick-borne	Unclassified		
		SLEV	JEV	BSQV	YFV	DENV4	ENTV	APOIV	LGTV	CFAV	TABV	NOUV
C (virion)	104	28.5	27.6	24.7	17.9	24.7	16.6	12.5	12.3	9.9	12.9	28.5
CTHD	18											
pr	93											
prM	169	40.8	37.8	38.5	31.9	39	39.5	26.7	28.4	13.9	13.0	40.2
E	497	49.1	48.5	47	41	41.5	36.7	30.6	35.6	12.4	20.1	42.2
NS1	355	45.9	45.9	49	48.4	44.5	48.7	32.8	41.1	21.0	8.9	49.1
NS2A	227	37.7	35.9	34.1	29.8	23.7	18.6	12	17.5	7.5	6.7	33.1
NS2B	131	32.8	35.8	43.5	32.8	37.4	27.4	12.9	16	12.2	9.9	30.5
NS3	621	57.8	58.4	59.4	50.2	54.3	46.8	45	44.2	26.8	22.1	51.9
NS4A	125	43.2	41.6	34.1	32.8	33.3	38.4	24	31.7	13.3	13.3	36
2K	24											
NS4B	255	40.5	38.6	37.8	37.2	38.8	31.7	23.7	26.0	7.2	5.3	33.4
NS5	908	62.9	61.3	64.0	59.6	59.9	57.4	51.5	55.3	41.5	24.9	58
Polyprotein sequence	3,434	50.5	49.6	50.4	45.4	46.4	42.7	35.5	38.9	23	17	46.1
Complete ORF nucleotides	10,302 (bp)	54.3	53.9	53.4	50.4	52	49.2	44	46.6	35.8	33.8	54.7

[a] aa, amino acids. All values are given in number of amino acids except as noted.
[b] NKV, no known vector. ENTV has currently no known arthropod vector. Abbreviations: BSQV, Bussuquara virus; APOIV, Apoi virus; LGTV, Langat virus; TABV, Tamana bat virus.

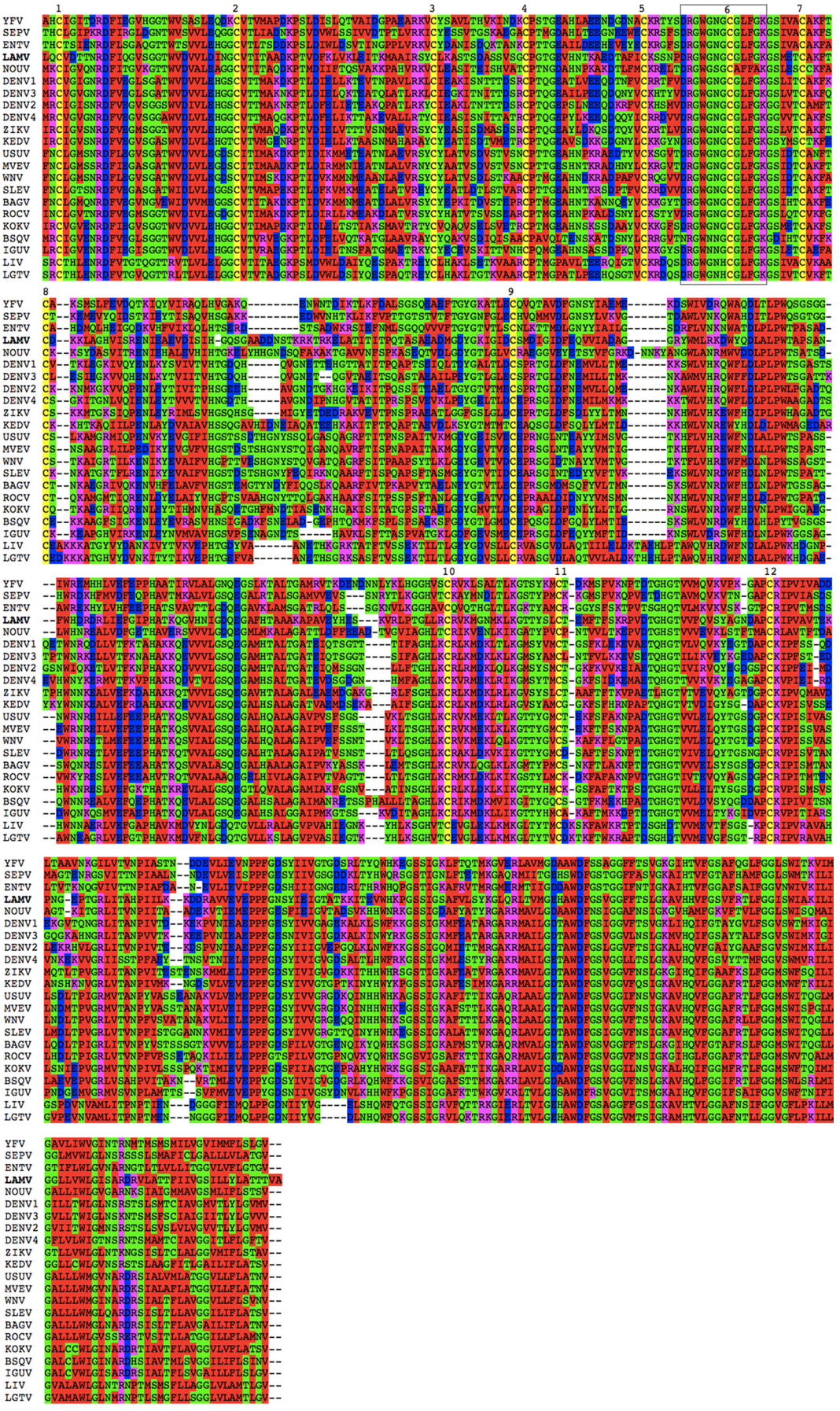
TABLE 2. Putative polyprotein cleavage sites of LAMV polyprotein and other flavivirus polyproteins for comparison[a]

Virus	Sequence (5′–3′)			
	VirC/CTHD (VSP)	VirC-CTHD/preM (HS)	Pr/M (furin)	M/E (HS)
BAGV	RGKKKR/GGTTVG	LGVAQA/IKIGSL	ARRSRR/SITVHH	IAPAYS/FNCLGM
KEDV	INKRKR/SPVNWF	FGVLTA/VKIGDY	PRRSRR/SVSLPP	VAPAYS/IRCIGV
ZIKV	KERKRR/GADTSI	LTTAMA/AEITRR	ARRSRR/AVTLPS	IAPAYS/IRCIGV
SLEV	PSKKR/GGTRS	GLASS/LQLST	RRSRR/SISVQ	APAYS/FNCLG
RBV	KKQRR/GGTES	TGLMA/MQVSQ	HRLKR/SLSIT	APSYS/TQCVN
APOIV	KGGRR/GGKSV	PIALS/AVVMN	TRTRR/DVTIQ	APAYA/STCVS
MODV	KTKQR/SAGWT	GTILS/IEVVK	NRVRR/AVNIA	LPSFA/TNCVT
CxFV	ALEAKR/SAKNA	MMVLGA/VVIDM	KRERR/VASTN	RTTVKG/EFVEP
CFAV	ALESRR/TTGNP	TVLCGC/VVIDM	KKREKR/SREPP	WTTVKG/EFVEP
KRV	RLEKQR/SGPNL	LGLCYG/EMLRY	QVRRRR/APQPQ	WNVVKA/SSIEP
TABV	QKRQK/SSGGY	MVIFC/GYQSG	HRTRR/SVTET	ILVIA/QFYLAD
NOUV	VSKRR/GSASL	GVASA/VTFTT	QRSRR/SVGIS	IPAYS/MKCIG
LAMV	KKNGKR/STEIS	FGAAMA/ASMFT	RRGKR/SVALAP	LIGPAYS/LQCVD
	E/NS1 (HS)	NS1/NS2A (unknown, HS?)	NS2A/NS2B (VSP)	NS2B/NS3 (VSP)
BAGV	ATNVHA/DTGCAV	KSRVTA/YDGAGM	KPSNRR/GWPVSE	THSPKR/SGAIWD
KEDV	ATSVNG/DQGCAM	KARVSA/GSGSGV	TGEKRR/SWPPTE	DKKAKR/SGALWD
ZIKV	STAVSA/DVGCSV	RSMVTA/GSTDHM	TRSGKR/SWPPSE	VKTGKR/SGALWD
SLEV	TSVQA/DSGCA	SRVTA/GVAGG	PNGKR/SWPAS	KHSKR/GGALD
RBV	TGVMG/DHGCA	VYAGS/MTALE	HRGQR/ATDYT	DATQR/SIIVG
APOIV	TGVVG/EIGCM	VMAFD/EEPVW	RSGQR/SVDPI	RSIQK/SNTSS
MODV	TGVMG/DHGCV	GLVMA/SDGEK	PRHIR/GVDYV	GKEQR/SLIVY
CxFV	FVYTKA/DVGCG	EVTIDA/DGEDM	LRASRR/SLVAG	CWWNKR/ARVAT
CFAV	FYYVRA/DLGCG	YGKANA/QSDFR	WAAEKA/HQPTV	LTASNR/SDDLL
KRV	VRSVSA/DVGCG	YGKAHA/CSDFR	WAAERA/QQPTI	LSEQNR/SDDLL
TABV	EVVAA/DKYVL	NVVKA/SKMNK	Not known	NLRDK/SKGLI
NOUV	TSVSA/ELGCS	LGVLA/MTMMF	KTTKR/SVPQS	ENRKR/SNDTP
LAMV	ATTTVA/LSEVGCS	RSKVSA/GTFQIDD	YTSGKR/SWPAGE	YVKSGRR/GTVLWD
	NS3/NS4A (VSP)	NS4A/2K (VSP)	2K/NS4B (HS)	NS4B/NS5 (VSP)
BAGV	FACGKR/SAIGVS	EPERQR/SQTDSH	VGTVAS/NEMGWL	NGSMRR/GGKGR
KEDV	FAAGRR/GATAGV	EPERQR/SVQDNY	LGLVAA/NEAGLL	VSSTKR/SNRGLG
ZIKV	FAAGKR/GAALGV	EPEKQR/SPQDNQ	LGLITA/NELGWL	GLVKRR/GGGTGE
SLEV	AAGKR/SALGM	EPEKQ/RSQTD	GVVAA/NEMGL	PKGKR/GGGKG
RBV	AQMRR/SGVLL	EEGMQ/RTQID	VTVVA/NEMRL	RSDRR/GIVTS
APOIV	AKGKR/SGMTI	EEGMQ/RTQVD	AAVVA/NEMGF	SENRR/GVSSS
MODV	AEMRR/SSVWL	EGQQR/TQIDT	GLVIA/NELRW	TSNRR/GICSS
CxFV	NELDKR/SKIML	Not known	VNGVVA/WEMDL	SRMALR/SLVKT
CFAV	EWETRK/VSIDF	Not known	ICGVLA/WEMRM	FNQFRA/LEKST
KRV	EWDTRK/LSIEF	Not known	VCGVLA/WEMRL	FNQFRA/LEKST
TABV	PLVQR/VFSGI	GITQR/EKSTG	YYILA/DGEIL	KTTQR/FRSSI
NOUV	AGGKR/SAVDL	EGKQR/SMVDN	GAVAA/NEYGM	AYKKR/GIWEV
LAMV	EFAEGRR/SYVPL	EPGSQ/RSVQDNT	AALIAA/NETGLL	KGVPRR/GMTIC

[a] Abbreviations: BAGV, Bagaza virus; KEDV, Kedougou virus; ZIKV, Zika virus; RBV, Rio Bravo virus; APOIV, Apoi virus; MODV, Modoc virus; CxFV, *Culex* flavivirus; KRV, Kamiti river virus; TABV, Tamana bat virus; SEPV, Sepik virus; YOKV, Yokose virus; VSP, viral serine protease; HS, host signalase; VirC, viral capsid protein, CTHD, C-terminal hydrophobic domain.

viruses and some members of *Aedes* sp.-associated viruses. The most conserved LAMV proteins were NS3 and NS5 sharing over 50% identity with other mosquito-borne flaviviruses. In the compared sequences, the highest polyprotein sequence identities were observed between LAMV and St. Louis encephalitis virus (SLEV) (50.5%) and in nucleotide sequences of the coding region between LAMV and NOUV (54.7%), followed by LAMV and SLEV (54.3%).

Despite the low sequence homology, the hydropathy profile (data not shown) and the putative polyprotein cleavage sites (Table 2) (2, 10, 13, 23, 29, 30, 33) of the LAMV polyprotein sequence were found to resemble those of other mosquito-borne flaviviruses. The putative cleavage sites in virC/CTHD, preM/M, NS2A/NS2B, NS2B/NS3, NS3/NS4A, and NS4B/NS5 followed the rule of occurring after two basic residues (R or K) and being preceded by short side chain residue (G or S) that are probably cleaved by viral serine protease and furin. However, the predicted cleavage site between NS4A/2K (EPGSQ/RSVQD) did not fit this rule but was similar to that of SLEV (EPEKQ/RSQTD). The predicted host signalase cleavage sites resembled those reported for other mosquito-borne flaviviruses, especially in the cleavage site of M/E, where the preceding residues (PAYS) were identical in LAMV to those of other mosquito-borne flaviviruses. The flaviviral conserved cysteine residues—6 in the preM, 12 in the NS1, and 12 in the E protein (Fig. 2) (15)—were also present in LAMV proteins. Two potential N-glycosylation sites were found in the LAMV preM protein, interestingly resembling those of the YFV group



viruses. A single N-glycosylation site was predicted in LAMV E protein, at a conserved position (N153), similar to many of the JEV group viruses. The integrin-binding motif "RGD" present in several JEV group viruses was not conserved in LAMV and was instead "TAT." Flaviviruses have two to three potential N-glycosylation sites in the NS1 protein, with the exception of KRV and CFAV that have four conserved sites (10). A total of four N-glycosylation sites were predicted in the LAMV NS1 protein (N96, N109, N193, and N293) that differed from those of CFAV and KRV.

The phylogenetic analysis of complete open reading frame nucleotide sequences of LAMV and other flaviviruses (see the supplemental table [http://www.hi.helsinki.fi/zoonoosivirukset/files/SupplementaryTableJVI.pdf]) showed that LAMV represents a distinct lineage positioned among the *Aedes* spp. transmitted mosquito-borne viruses. The conservation in the nucleotide sequence was highest in the NS3 and NS5 genes, and the analyses showed a similar result for the position of LAMV in the phylogenetic trees (data not shown). When the newly discovered NOUV was included to the analysis, it was shown to form a sister lineage with LAMV. The results of the sequence analysis of the complete coding sequence (Fig. 3A) and NS3 gene (Fig. 3B) suggest that YFV group diverged before the NOUV and LAMV lineages that preceded the rest of the mosquito-borne viruses including the dengue, Ntaya, and Kokobera groups and all of the *Culex* sp.-associated viruses.

Mosquito identification using PCR. From the geographical origin of the LAMV isolate mosquitoes, altogether 1,200 individuals were collected and studied. By studying separately the 24 individual mosquito homogenates comprising the virus isolation positive pool for the presence of viral RNA, the source of LAMV was identified as a single infected mosquito. The identification of the mosquito species was not obtained before the homogenization took place. The genetic identification of mosquito species based on mitochondrial cytochrome *c* oxidase 1 (CO1) DNA sequences have proven suitable and a useful method for studying the vectors of arboviruses (8, 12). For identification of the mosquito species carrying LAMV, a partial CO1 sequence was amplified and compared to available sequences in GenBank and the Barcode of Life Data Systems (BOLD) CO1-based species identification database (available at www.barcodinglife.org). Unfortunately, no sequences from Northern European mosquitoes were available for comparison. However, highly similar sequences were found, suggesting that the mosquito species was *Aedes cinereus*. The obtained CO1 sequence of 1,324 bp (FJ805239) was longer than the sequences yielding the highest identity percentage. The sequence was found 99% identical in the alignable region of (652 bp) to the single available *A. cinereus* CO1 sequence in the GenBank (AF253027), originating from France. In addition, from an alignable region of 568 bp, it was found to be 94.75 to 95.57% identical to the four available *A. cinereus* sequences from Canada in the BOLD database. The data available on Finnish mosquitoes lists 38 species, including 25 *Aedes* species. Except for the northernmost regions, *A. cinereus* has been detected throughout Finland and is known to be an anthropophilic species (41).

DISCUSSION

We report here the first mosquito-borne member of the genus *Flavivirus* from Northern Europe, LAMV, and show that this novel flavivirus is genetically divergent from other flaviviruses and yet related to the human pathogenic mosquito-borne flaviviruses of the tropics. LAMV was isolated from a mosquito, which was shown to be most likely *A. cinereus*, a known anthropophilic mosquito species. In Finland, the previously known endemic mosquito-borne arboviruses include members of the virus genera *Alphavirus* (Sindbis virus) (32) and *Orthobunyavirus* (Inkoo virus) (3). Like LAMV, these viruses are genetically related to viruses found from other continents, since Inkoo virus was found to be related to orthobunyaviruses in North America (42), and the Finnish Sindbis virus isolates were found to be related to the African strains (32). The previously known mosquito-borne flaviviruses in Europe, WNV and Usutu virus, originated in tropical Africa but, unlike LAMV, have been associated with ornithophilic mosquito species and migratory birds.

The phylogenetic analysis has shown the grouping of the mosquito-borne flaviviruses to separate the mainly *Aedes*-transmitted YFV group from the rest of the mosquito-borne flaviviruses, which include *Aedes*-transmitted viruses related to DENV and the large group of *Culex*-transmitted viruses. The recently described novel flavivirus NOUV from Africa along with LAMV from Northern Europe are phylogenetically positioned between the YFV group and the rest of the recognized mosquito-borne flaviviruses. LAMV and NOUV are highly divergent from each other in their genomic sequences, with less than 50% homology in the protein sequence and less than 55% in the nucleotide sequences. Based on the phylogenetic analysis, they form long branches, suggesting that they represent separate virus species or lineages. However, NOUV and LAMV seem to share the property of infecting only or at least primarily mosquito cells. In the present study, LAMV was not found to infect any of the tested mammalian cells, chicken embryo fibroblasts, or newborn mice. The flavivirus in vitro replication properties correlate well with their host range (28), and the restricted ability to replicate only in insect cells is a recognized property of the tentatively classified insect flaviviruses. The determination of in vitro and in vivo culture prop-

FIG. 2. Amino acid alignment of envelope proteins of different flaviviruses. Amino acid alignment of envelope proteins of different flaviviruses. Color coding on amino acids: red = A, V, F, P, M, I, L, and W; blue = D and E; magenta = R and K; green = S, T, Y, H, C, N, G, and Q. Exceptions are conserved cysteines that are shown on yellow. The fusion loop area is boxed. Representative species of the mosquito-borne flavivirus groups: YFV group, YFV and SEPV; DENV group, DENV1 to DENV4 and KEDV; Spondweni virus group, ZIKV; JEV group, USUV, MVEV, WNV, and SLEV; Ntaya virus group, BAGV and ROCV; Kokobera virus group, KOKV; Aroa virus group, Bussuquara virus and IGUV. Representative species of the no-known-vector flavivirus, the ENTV group and the mammalian tick-borne virus group (Langat virus and LIV) were also included.

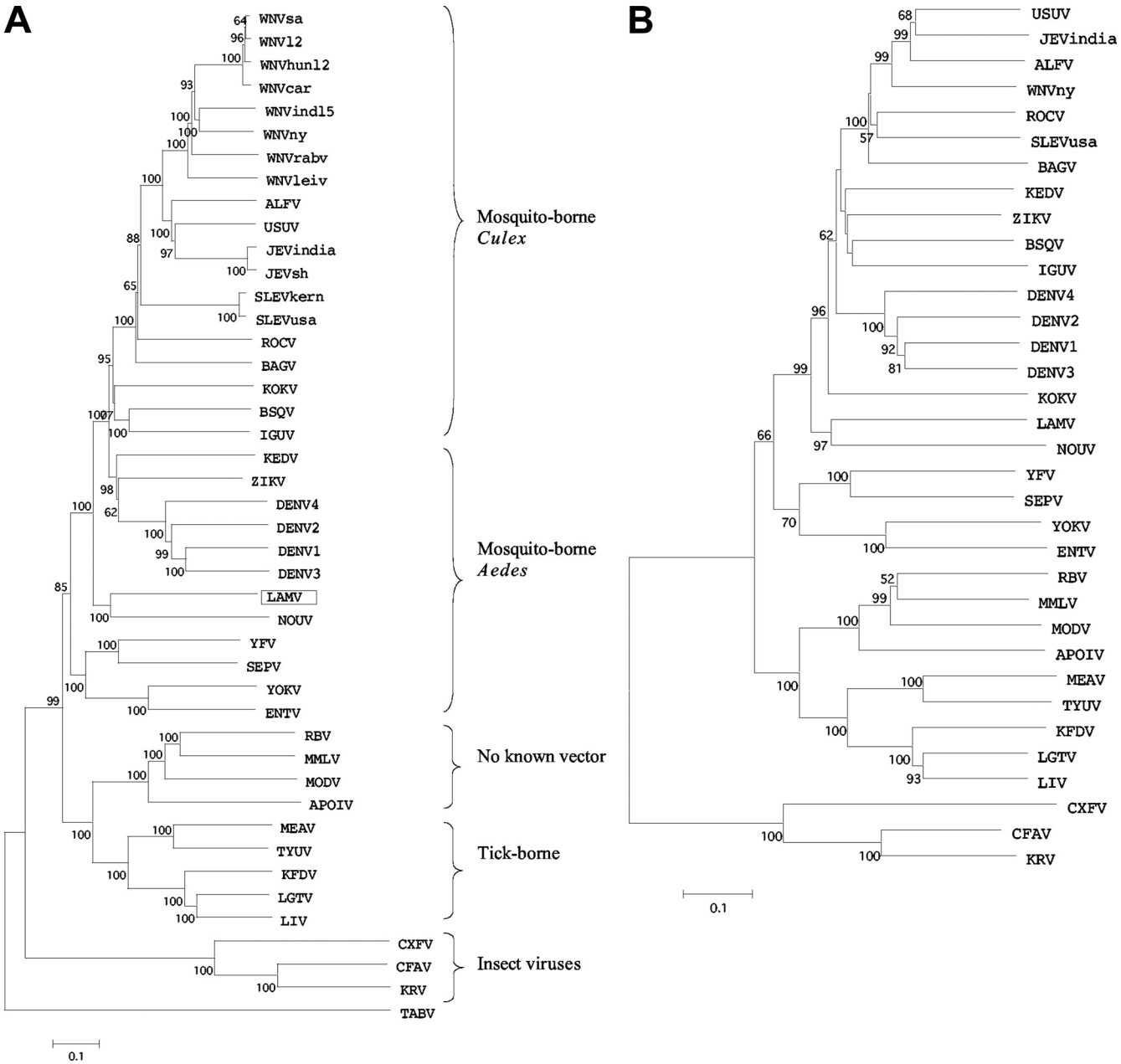


FIG. 3. (A) Neighbor-joining phylogenetic tree of complete open reading frame nucleotide sequences. The bar represents nucleotide substitutions/site. (B) Neighbor-joining phylogenetic tree of NS3 gene.

erties of LAMV merits further experiments as most antigenically cross-reactive flaviviruses can be cultured in mouse brain and/or a variety of vertebrate cells.

The observed lack of ability of LAMV to infect vertebrate cells appears to be contradictory versus its phylogenetic position among the mosquito-borne viruses that are generally associated with vertebrate hosts. A similar contradiction also appears to apply for the recently characterized NOUV (23). The observed biological properties and phylogenetic position differ also in the no-known-vector flaviviruses Entebbe bat virus (ENTV), Sokuluk virus (SOKV), and Yokose virus (YOKV), since they have no currently known arthropod vectors but group phylogenetically with the mosquito-borne viruses (31). This has been explained in evolutionary terms by secondary loss of transmission through vectors (9). Using a similar argument, LAMV could have lost the capacity to replicate in vertebrate cells. However, more exhaustive studies will be required before the ability of LAMV to infect vertebrate cells can be ruled out. The infectivity and possible human or veterinary disease associations of LAMV also remain to be investigated.

Currently, studies for evidence of possible vertebrate hosts of LAMV using human and wildlife sera are ongoing. In addition, mosquitoes collected at various sites are being studied

to determine the distribution of LAMV in Finland. In Northern Europe, prior to our report, the only flaviviruses known to be endemic were TBEV and louping ill viruses (24), and although no mosquito-borne flaviviruses have previously been isolated, serological evidence of WNV and Usutu virus circulation, e.g., in the United Kingdom, has been reported (4).

During the past few years our knowledge of the spectrum of flaviviruses has widened as new species in the genus *Flavivirus* have been isolated and characterized. These findings, together with this report characterizing the first mosquito-borne flavivirus from Northern Europe, show that much still remains to be learned of the variety and evolutionary history of flaviviruses.

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REFERENCES

1. **Aranda, C., M. P. Sanchez-Seco, F. Caceres, R. Escosa, J. C. Galvez, M. Masia, E. Marques, S. Ruiz, A. Alba, N. Busquets, A. Vazquez, J. Castella, and A. Tenorio.** 2009. Detection and monitoring of mosquito flaviviruses in Spain between 2001 and 2005. Vector Borne Zoonotic Dis. **9:**171–178.
2. **Billoir, F., R. de Chesse, H. Tolou, P. de Micco, E. A. Gould, and X. de Lamballerie.** 2000. Phylogeny of the genus flavivirus using complete coding sequences of arthropod-borne viruses and viruses with no known vector. J. Gen. Virol. **81:**2339.
3. **Brummer-Korvenkontio, M., P. Saikku, P. Korhonen, I. Ulmanen, T. Reunala, and J. Karvonen.** 1973. Arboviruses in Finland. IV. Isolation and characterization of Inkoo virus, a Finnish representative of the California group. Am. J. Trop. Med. Hyg. **22:**404–413.
4. **Buckley, A., A. Dawson, and E. A. Gould.** 2006. Detection of seroconversion to West Nile virus, Usutu virus, and Sindbis virus in UK sentinel chickens. Virol. J. **3:**71.
5. **Cammisa-Parks, H., L. A. Cisar, A. Kane, and V. Stollar.** 1992. The complete nucleotide sequence of cell fusing agent (CFA): homology between the nonstructural proteins encoded by CFA and the nonstructural proteins encoded by arthropod-borne flaviviruses. Virology **189:**511–524.
6. **Clarke, D. H., and J. Casals.** 1958. Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. Am. J. Trop. Med. Hyg. **7:**561–573.
7. **Cook, S., S. N. Bennett, E. C. Holmes, R. De Chesse, G. Moureau, and X. de Lamballerie.** 2006. Isolation of a new strain of the flavivirus cell fusing agent virus in a natural mosquito population from Puerto Rico. J. Gen. Virol. **87:**735–748.
8. **Cook, S., M. Diallo, A. A. Sall, A. Cooper, and E. C. Holmes.** 2005. Mitochondrial markers for molecular identification of Aedes mosquitoes (Diptera: Culicidae) involved in transmission of arboviral disease in West Africa. J. Med. Entomol. **42:**19–28.
9. **Cook, S., and E. C. Holmes.** 2006. A multigene analysis of the phylogenetic relationships among the flaviviruses (family: *Flaviviridae*) and the evolution of vector transmission. Arch. Virol. **151:**309–325.
10. **Crabtree, M. B., R. C. Sang, V. Stollar, L. M. Dunster, and B. R. Miller.** 2003. Genetic and phenotypic characterization of the newly described insect flavivirus, Kamiti River virus. Arch. Virol. **148:**1095–1118.
11. **Crochu, S., S. Cook, H. Attoui, R. N. Charrel, R. De Chesse, M. Belhouchet, J. J. Lemasson, P. de Micco, and X. de Lamballerie.** 2004. Sequences of flavivirus-related RNA viruses persist in DNA form integrated in the genome of Aedes spp. mosquitoes. J. Gen. Virol. **85:**1971–1980.
12. **Cywinska, A., F. F. Hunter, and P. D. Hebert.** 2006. Identifying Canadian mosquito species through DNA barcodes. Med. Vet. Entomol. **20:**413–424.
13. **de Lamballerie, X., S. Crochu, F. Billoir, J. Neyts, P. de Micco, E. C. Holmes, and E. A. Gould.** 2002. Genome sequence analysis of Tamana bat virus and its relationship with the genus *Flavivirus*. J. Gen. Virol. **83:**2443–2454.
14. **Farfan-Ale, J. A., M. A. Lorono-Pino, J. E. Garcia-Rejon, E. Hovav, A. M. Powers, M. Lin, K. S. Dorman, K. B. Platt, L. C. Bartholomay, V. Soto, B. J. Beaty, R. S. Lanciotti, and B. J. Blitvich.** 2009. Detection of RNA from a novel West Nile-like virus and high prevalence of an insect-specific flavivirus in mosquitoes in the Yucatan Peninsula of Mexico. Am. J. Trop. Med. Hyg. **80:**85–95.
15. **Fauquet, C. M., M. A. Mayo, J. Maniloff, U. Desselberger, and L. A. Ball (ed.).** 2005. Virus Taxonomy Eight Report of the International Committee on the Taxonomy of Viruses. Elsevier Academic Press, Amsterdam, The Netherlands.
16. **Folmer, O., M. Black, W. Hoeh, R. Lutz, and R. Vrijenhoek.** 1994. DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. Mol. Mar. Biol. Biotechnol. **3:**294–299.
17. **Gaunt, M. W., and E. A. Gould.** 2005. Rapid subgroup identification of the flaviviruses using degenerate primer E-gene RT-PCR and site specific restriction enzyme analysis. J. Virol. Methods **128:**113–127.
18. **Gaunt, M. W., A. A. Sall, X. de Lamballerie, A. K. Falconar, T. I. Dzhivanian, and E. A. Gould.** 2001. Phylogenetic relationships of flaviviruses correlate with their epidemiology, disease association and biogeography. J. Gen. Virol. **82:**1867–1876.
19. **Gould, E. A., A. Buckley, N. Cammack, A. D. Barrett, J. C. Clegg, R. Ishak, and M. G. Varma.** 1985. Examination of the immunological relationships between flaviviruses using yellow fever virus monoclonal antibodies. J. Gen. Virol. **66:**1369–1382.
20. **Grard, G., J. J. Lemasson, M. Sylla, A. Dubot, S. Cook, J. F. Molez, X. Pourrut, R. Charrel, J. P. Gonzalez, U. Munderloh, E. C. Holmes, and X. de Lamballerie.** 2006. Ngoye virus: a novel evolutionary lineage within the genus *Flavivirus*. J. Gen. Virol. **87:**3273–3277.
21. **Henchal, E. A., M. K. Gentry, J. M. McCown, and W. E. Brandt.** 1982. Dengue virus-specific and flavivirus group determinants identified with monoclonal antibodies by indirect immunofluorescence. Am. J. Trop. Med. Hyg. **31:**830–836.
22. **Hoshino, K., H. Isawa, Y. Tsuda, K. Yano, T. Sasaki, M. Yuda, T. Takasaki, M. Kobayashi, and K. Sawabe.** 2007. Genetic characterization of a new insect flavivirus isolated from *Culex pipiens* mosquito in Japan. Virology **359:**405–414.
23. **Junglen, S., A. Kopp, A. Kurth, G. Pauli, H. Ellerbrok, and F. H. Leendertz.** 2009. A new flavivirus and a new vector: characterization of a novel flavivirus isolated from *Uranotaenia* mosquitoes from a tropical rainforest. J. Virol. **83:**4462–4468.
24. **Kallio-Kokko, H., N. Uzcategui, O. Vapalahti, and A. Vaheri.** 2005. Viral zoonoses in Europe. FEMS Microbiol. Rev. **29:**1051–1077.
25. **Karabatsos, N.** 1995. International catalogue of arboviruses including certain other viruses of vertebrates. American Society of Tropical Medicine and Hygiene, Deerfield, IL.
26. **Kim, D. Y., H. Guzman, R. Bueno, Jr., J. A. Dennett, A. J. Auguste, C. V. Carrington, V. L. Popov, S. C. Weaver, D. W. Beasley, and R. B. Tesh.** 2009. Characterization of culex flavivirus (*Flaviviridae*) strains isolated from mosquitoes in the United States and Trinidad. Virology **386:**154–159.
27. **Kumar, S., K. Tamura, and M. Nei.** 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform. **5:**150–163.
28. **Kuno, G.** 2007. Host range specificity of flaviviruses: correlation with in vitro replication. J. Med. Entomol. **44:**93–101.
29. **Kuno, G., and G. J. Chang.** 2007. Full-length sequencing and genomic characterization of Bagaza, Kedougou, and Zika viruses. Arch. Virol. **152:**687–696.
30. **Kuno, G., and G. J. Chang.** 2006. Characterization of Sepik and Entebbe bat viruses closely related to yellow fever virus. Am. J. Trop. Med. Hyg. **75:**1165–1170.
31. **Kuno, G., G. J. Chang, K. R. Tsuchiya, N. Karabatsos, and C. B. Cropp.** 1998. Phylogeny of the genus *Flavivirus*. J. Virol. **72:**73–83.
32. **Kurkela, S., T. Manni, A. Vaheri, and O. Vapalahti.** 2004. Causative agent of Pogosta disease isolated from blood and skin lesions. Emerg. Infect. Dis. **10:**889–894.
33. **Leyssen, P., N. Charlier, P. Lemey, F. Billoir, A. M. Vandamme, E. De Clercq, X. de Lamballerie, and J. Neyts.** 2002. Complete genome sequence, taxonomic assignment, and comparative analysis of the untranslated regions of the Modoc virus, a flavivirus with no known vector. Virology **293:**125–140.
34. **Lunt, D. H., D. X. Zhang, J. M. Szymura, and G. M. Hewitt.** 1996. The insect cytochrome oxidase I gene: evolutionary patterns and conserved primers for phylogenetic studies. Insect Mol. Biol. **5:**153–165.
35. **Moureau, G., S. Temmam, J. P. Gonzalez, R. N. Charrel, G. Grard, and X. de Lamballerie.** 2007. A real-time RT-PCR method for the universal detection and identification of flaviviruses. Vector Borne Zoonotic Dis. **7:**467–477.
36. **Saikku, P., and M. Brummer-Korvenkontio.** 1975. Tick-borne viruses in Finland. Med. Biol. **53:**317–320.
37. **Sallum, M. A. M., T. R. Schultz, P. G. Foster, K. Aronstein, R. A. Wirtz, and R. C. Wilkerson.** 2002. Phylogeny of *Anophelinae* (Diptera: Culicidae) based

on nuclear ribosomal and mitochondrial DNA sequences. Syst. Entomol. **27:**361–382.
38. **Sang, R. C., A. Gichogo, J. Gachoya, M. D. Dunster, V. Ofula, A. R. Hunt, M. B. Crabtree, B. R. Miller, and L. M. Dunster.** 2003. Isolation of a new flavivirus related to cell fusing agent virus (CFAV) from field-collected flood-water Aedes mosquitoes sampled from a dambo in central Kenya. Arch. Virol. **148:**1085–1093.
39. **Scaramozzino, N., J. M. Crance, A. Jouan, D. A. DeBriel, F. Stoll, and D. Garin.** 2001. Comparison of flavivirus universal primer pairs and development of a rapid, highly sensitive heminested reverse transcription-PCR assay for detection of flaviviruses targeted to a conserved region of the NS5 gene sequences. J. Clin. Microbiol. **39:**1922–1927.
40. **Stollar, V., and V. L. Thomas.** 1975. An agent in the *Aedes aegypti* cell line (Peleg) which causes fusion of *Aedes albopictus* cells. Virology **64:**367–377.
41. **Utrio, P.** 1979. Geographic distribution of mosquitoes (Diptera, Culicidae) in eastern Fennoscandia. Notulae Entomologicae **59:**105–123.
42. **Vapalahti, O., A. Plyusnin, Y. Cheng, T. Manni, M. Brummer-Korvenkontio, and A. Vaheri.** 1996. Inkoo and Tahyna, the European California serogroup bunyaviruses: sequence and phylogeny of the S RNA segment. J. Gen. Virol. **77:**1769–1774.