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Gouléako Virus Isolated from West African Mosquitoes Constitutes a Proposed Novel Genus in the Family *Bunyaviridae*▽

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The family *Bunyaviridae* is the most diversified family of RNA viruses. We describe a novel prototypic bunyavirus, tentatively named Gouléako virus, isolated from various mosquito species trapped in Côte d'Ivoire. The S segment comprised 1,087 nucleotides (nt), the M segment 3,188 nt, and the L segment 6,358 nt, constituting the shortest bunyavirus genome known so far. The virus had shorter genome termini than phleboviruses and showed no evidence of encoded NSs and NSm proteins. An uncharacterized 105-amino-acid (aa) putative open reading frame (ORF) was detected in the S segment. Genetic equidistance to other bunyaviruses (74 to 88% aa identity) and absence of serological cross-reactivity with phleboviruses suggested a proposed novel *Bunyaviridae* genus.

The family *Bunyaviridae* comprises the five genera *Hantavirus*, *Nairovirus*, *Orthobunyavirus*, *Phlebovirus*, and *Tospovirus* (26). Classification was originally based on serological relationships but has been extended to include virion morphology, genome organization, and phylogenetic relationships. Congeneric members have further features in common, such as conserved genome termini, identical coding strategies, and encoded proteins (26). The segmented, negative-stranded RNA genome codes for a nucleocapsid (N) protein, two glycoproteins (Gn and Gc), and an RNA-dependent RNA polymerase (RdRp) on the S, M, and L segments, respectively. S and M segments of the genera *Orthobunyavirus*, *Phlebovirus*, and *Tospovirus* encode two additional nonstructural proteins, NSs and NSm. However, these proteins are not consistently represented throughout those genera (8, 22). More recently identified novel bunyaviruses, as well as recently sequenced bunyaviruses from archived material, consistently belonged to any of the five known genera (6, 7, 18–20, 23, 37, 38, 39, 40).

During an arbovirus surveillance study in Côte d'Ivoire, an RdRp fragment of a novel bunyavirus was identified (14). The virus was detected with a relatively high prevalence of 6.5% in different species of *Anopheles*, *Culex*, and *Uranotaenia* mosquitoes in a diverse range of habitat types, indicating a widespread virus that is promiscuous regarding arthropod vectors. The virus was tentatively termed Gouléako virus (GOUV), after the village from which the first isolate originated. Here we determined the complete genome sequence and investigated criteria to formally classify GOUV.

Virus isolation from 432 pools of 4,839 female mosquitoes was done in *Aedes albopictus* (C6/36) cells as described before (13). All positive pools induced similar cytopathic effects (CPE) after 3 to 7 days postinfection (dpi), and maximum genome copies were reached after 5 dpi (Fig. 1a, b, and d). Polymorphic, enveloped virions with a bunyavirus-like morphology were detected by electron microscopy in infected cell culture supernatants (Fig. 1c) (13, 28).

To investigate the GOUV cell tropism, infectious cell culture supernatant of one isolate (A5/CI/2004) was used to infect various insect, reptile, bird, and mammalian cells with multiplicities of infection (MOIs) of 10, 1, 0.5, and 0.1 (measured by 50% tissue culture infective dose [$TCID_{50}$]), and cells were incubated at 33°C and 37°C (Table 1). Cell culture supernatants were passaged in fresh cells every 7 days in 1/10 dilutions for five consecutive passages and tested by real-time reverse transcription-PCR (RT-PCR) (14). GOUV replicated well on U4.4 cells, but on all other cell lines tested, no CPE was observed, and no virus growth was detected.

Furthermore, 269 pools of 1,716 adult male mosquitoes were tested for GOUV by real-time RT-PCR, yielding two positive pools of *Culex* spp. and *Anopheles* spp.

The third passage in C6/36 cells of isolate A5/CI/2004 was completely sequenced. Initial sequences were obtained by adaptor-based random amplification (Fig. 1e to g) (13, 32). Two hundred eighty-one clones with inserts between 500 and 1,500 nucleotides (nt) were sequenced and compared to GenBank sequences, showing distant relationships on the amino acid (aa) level with members of the genus *Phlebovirus*. Larger contiguous sequence fragments of 5,275 nt (corresponding to the L gene), 1,049 nt (S gene), and 762 nt (M gene, two fragments) were assembled. M gene fragments were combined into a 1,776-nt fragment. Lateral parts of genome segments were amplified with contig-specific primers and oligonucleotides

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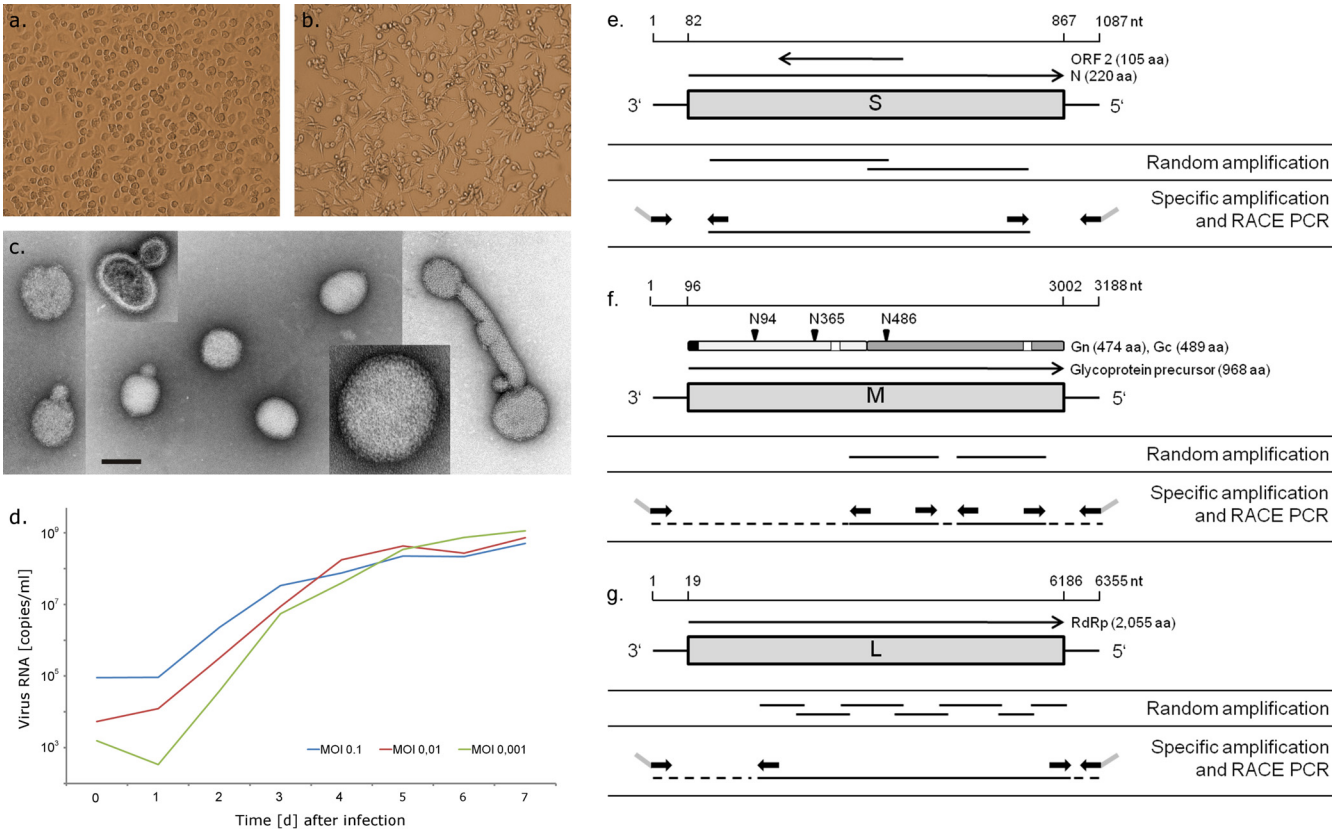


FIG. 1. GOUV growth on insect cells, morphology, and genome characteristics. (a) Uninfected C6/36 cells. (b) C6/36 cells 4 days after infection with GOUV. (c) Negative staining electron microscopy of purified GOUV particles. Bar = 100 nm. (d) Numbers of GOUV genome copies per ml in cell culture supernatant of C6/36 cells infected with GOUV at MOIs of 0.1, 0.01, and 0.001 were measured by RT-PCR for 7 days. (e to g) Strategies used for full genome sequencing. The top panel shows the genome segments S (e), M (f), and L (g). Boxes represent open reading frames (ORFs) flanked by noncoding regions (NCR), which are indicated by lines. Coding directions are indicated as arrows. Glycoprotein precursor properties were identified by signalP-NN, TMHMM, and NetNGlyc 1.0 and are marked as follows: signal peptide, black box; Gn, light-gray box; Gc, dark-gray box; transmembrane domains (TMD), white boxes; and glycosylation sites, black triangles. Bars in the middle panel indicate genome fragments generated in initial random amplification reactions. The bottom panel shows specific PCRs used for genome walking. Oligonucleotide orientations and positions are marked by arrowheads.

priming conserved panhandle elements of phleboviruses ligated to an anchor sequence. Genome termini were determined by rapid amplification of cDNA ends-PCR (RACE-PCR). The complete genome was resequenced for confirmation on both strands by primer walking techniques. S-segment size was confirmed by RACE-PCR with virus obtained from cells infected with an MOI of 0.001 and harvested at 3 dpi to avoid detection of defective interfering (DI) RNAs. The GOUV genome was

TABLE 1. Cell lines inoculated with GOUV

Cell line	Host	Tissue	Comment	GOUV growth
C6/36	*Aedes albopictus*	Larvae	RNAi deficient[a]	Positive
CEF	*Gallus gallus domesticus*	Embryo	Primary cells	Negative
BHK-21	*Mesocricetus auratus*	Kidney		Negative
BHK-J	*Mesocricetus auratus*	Kidney		Negative
EiNi/41	*Eidolon helvum*	Kidney		Negative
ICR-2A	*Rana pipiens*	Embryo		Negative
L929	*Mus musculus*	Fibroblasts		Negative
MEF MDA5$^{-/-}$	*Mus musculus*	Fibroblasts	MDA5 knockout	Negative
MEF RIG-I$^{-/-}$	*Mus musculus*	Fibroblasts	RIG-I knockout	Negative
PSEK	*Sus scrofa domestica*	Kidney		Negative
RoNi/7-NPro.1	*Rousettus aegyptiacus*	Kidney		Negative
S2	*Drosophila melanogaster*	Larvae		Negative
U4.4	*Aedes albopictus*	Larvae		Positive
Vero B4	*Cercopithecus aethiops*	Kidney		Negative
Vero E6/7	*Cercopithecus aethiops*	Kidney		Negative
VH2	*Daboia russelii*	Heart		Negative

[a] RNAi, RNA interference.

TABLE 2. Terminal nucleotide sequences and segment sizes of GOUV compared to those of members of other genera in the family *Bunyaviridae*

Genus/virus	Consensus terminal nucleotides[a]	Segment size, in nucleotides (GenBank accession no.)		
		S	M	L
Hantavirus/	3′ AUCAUCAUCUG–	1,696	3,616	6,533
Hantaan virus	5′ UAGUAGUAUGC–	(M14626)	(M14627)	(X55901)
Orthobunyavirus/	3′ UCAUCACAUG–	961	4,458	6,875
Bunyamwera virus	5′ AGUAGUGUGC–	(D00353)	(M11852)	(X14383)
Nairovirus/	3′ AGAGUUUCU–	1,712	4,888	12,255
Dugbe virus	5′ UCUCAAAGA–	(M25150)	(M94133)	(U15018)
Tospovirus/	3′ UCUCGUUA–	2,916	4,821	8,897
tomato spotted wilt virus	5′ AGAGCAAU–	(D00645)	(S48091)	(D10066)
Phlebovirus/	3′ **UGUGUUUC**–	1,690	3,885	6,404
Rift Valley fever virus	5′ **ACACAAAG**–	(X53771)	(M11157)	(X56464)
Phlebovirus/Uukuniemi virus	3′ **UGUGUUUC**–	1,720	3,229	6,423
	5′ **ACACAAAG**–	(M33551)	(M17417)	(D10759)
Phlebovirus/SFTSV	S segment, 3′ **UGUGUUUC**–	1,744	3,378	6,368
	5′ **ACACAAAG**–	(HM745930)	(HM745931)	(HM745932)
	M segment, 3′ **UGUGU**UUC–			
	5′ **ACACA**GAG–			
	L segment, 3′ **UGUGU**UUC–			
	5′ **ACACA**GAG–			
Unassigned/Gouléako virus	S segment, 3′ **UGUGU**UUC–	1,087	3,188	6,358
	5′ **ACACA**GUG–	(HQ541736)	(HQ541737)	(HQ541738)
	M segment, 3′ **UGUGU**UUC–			
	5′ **ACACA**GUG–			
	L segment, 3′ **UGUGUUUC**–			
	5′ **ACACAAAG**–			

[a] Boldface type indicates consensus terminal nucleotides between phleboviruses, SFTSV, and Gouléako virus.

shorter than that of any known member of the *Bunyaviridae* and most similar to those of members of the genus *Phlebovirus* (Table 2) (29). The genome termini of GOUV were most similar to those in the genus *Phlebovirus*, albeit S and M genome termini were shorter, with a length of only 5 instead of 8 nt (Table 2). Notably, a novel phlebovirus, severe fever with thrombocytopenia syndrome virus (SFTSV), was recently identified in patients in China (39). SFTSV also had shorter genome termini, of only 5 instead of 8 nt, in its M and L segments (Table 2). The genome termini are generally conserved within but invariably different between bunyavirus genera (26).

In deduced amino acid sequences, the highest L-segment similarity was identified with Uukuniemi virus (UUKV) (28%), SFTSV (27%), and Rift Valley fever virus (RVFV) (27%). The conserved motif III of the RdRp gene was most similar to that of members of the genus *Phlebovirus* (Fig. 2a) (1, 24). However, there were clear differences discriminating GOUV from all other bunyaviruses. Notably, between the two invariant residues KW in the tentative RdRp motif A, GOUV showed a V insertion. This insertion was unique among RdRp of negative-strand viruses (27). Valine is an uncharged residue and performs due to its dipolar compounds as zwitterion. An insertion of cysteine or tyrosine, also zwitterions, into polymerase active sites has been reported in retroviruses and retrotransposons (27).

The M segment was distantly related to the glycoproteins of SFTSV (24%), UUKV (21%), and Punta Toro virus (PTV) (21%). Three in-frame translation initiation codons (AUG) at genomic positions 96, 111, and 141 were found. The first AUG seemed to be in best Kozak context for initiation of translation (16, 17). The mosquito-borne RVFV and the sandfly-borne PTV have 5 and 13 in-frame AUG codons, respectively, while the tick-borne UUKV has only one (9, 15, 21, 29) and SFTSV has two. As GOUV is in basal phylogenetic position to a clade formed by the *Phlebovirus* main group (sandfly fever group [SFG]), UUKV, and SFTSV, functional start codons may have been acquired convergently in SFG and GOUV or lost in UUKV and SFTSV. Translation of different proteins from alternative AUG codons has been reported for RVFV (15). However, the function of multiple AUG codons is still unclear.

The most likely cleavage site of the Golgi retention and targeting signal was found between aa 21 and 22 (CYS-QV) (Fig. 1f). Conserved domains of the phleboviral G1 superfamily (pfam07243) and phleboviral G2 superfamily (pfam07245) were detected by alignment to the pfam database, suggesting Gn to be encoded from aa 1 to 474 and Gc from aa 479 to 968. These coding regions could be confirmed by aligning the GOUV glycoprotein precursor sequence with those from representative phleboviruses. The putative cleavage site between Gn and Gc was identified at aa 479 (CSSRA/TP-CSTSVV, with amino acids conserved among GOUV and phleboviruses underlined) (Fig. 1f and Fig. 2b). It should be mentioned that SFTSV does not contain the conserved CS motif. Determination of the hydropathy profile predicted two transmembrane domains at aa 370 to 392 and aa 932 to 954, suggesting type I transmembrane topologies for Gn and Gc (Fig. 1f). N-linked glycosylation sites are conserved within bunyavirus genera except for the genus *Phlebovirus*, where UUKV represents an exception. Members of the SFG contain one N-linked glycosylation site in NSm, one in Gn, and four in Gc, whereas UUKV contains four sites in both glycoproteins (15, 29) and SFTSV contains two sites in Gn. The distinct predicted glycosylation pattern in GOUV is another criterion of its distinction from phleboviruses (Fig. 1f).

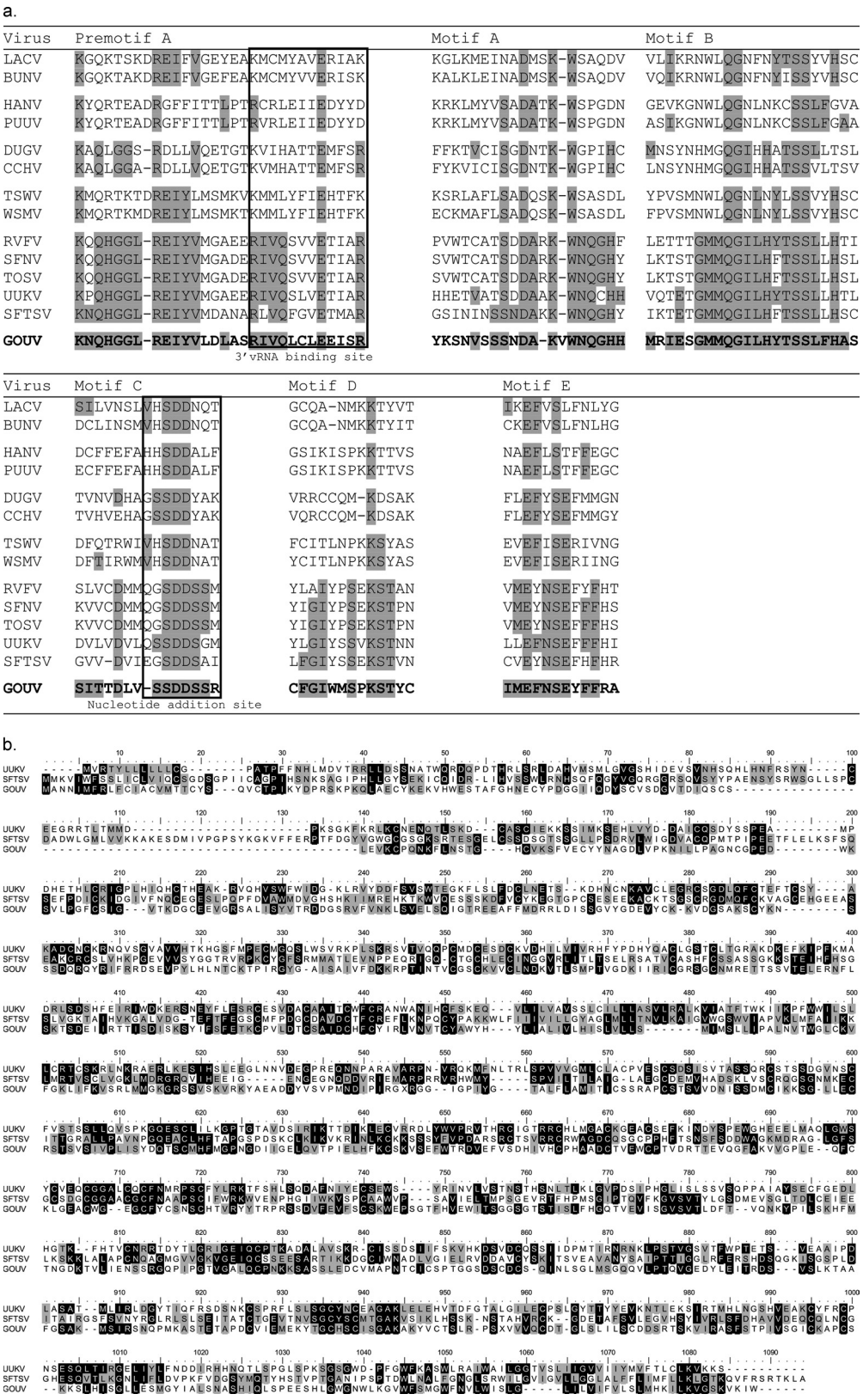


FIG. 2. Multiple sequence alignments of putative GOUV RNA-dependent RNA polymerase and glycoprotein precursor genes. (a) Alignment of GOUV and RdRp genes, third conserved motif. Premotif A and motifs A, B, C, D, and E are indicated. Amino acids conserved between GOUV and other bunyaviruses are marked in gray. Active sites corresponding to the PB1 protein of influenza virus (1) are highlighted by boxes. (b) Alignment of putative GOUV, UUKV, and SFTSV glycoprotein precursor proteins. Highly conserved amino residues are marked in black and conserved residues in gray. Abbreviations: BUNV, Bunyamwera virus; CCHV, Crimean-Congo hemorrhagic fever virus; DUGV, Dugbe virus; GOUV, Gouléako virus; HANV, Hantaan virus; LACV, La Crosse virus; PUUV, Puumala virus; RVFV, Rift Valley fever virus; SFNV, sandfly fever Naples virus; SFTSV, severe fever with thrombocytopenia syndrome virus; TOSV, Toscana virus; TSWV, tomato spotted wilt virus; UUKV, Uukuniemi virus; WSMV, watermelon silver mottle virus.

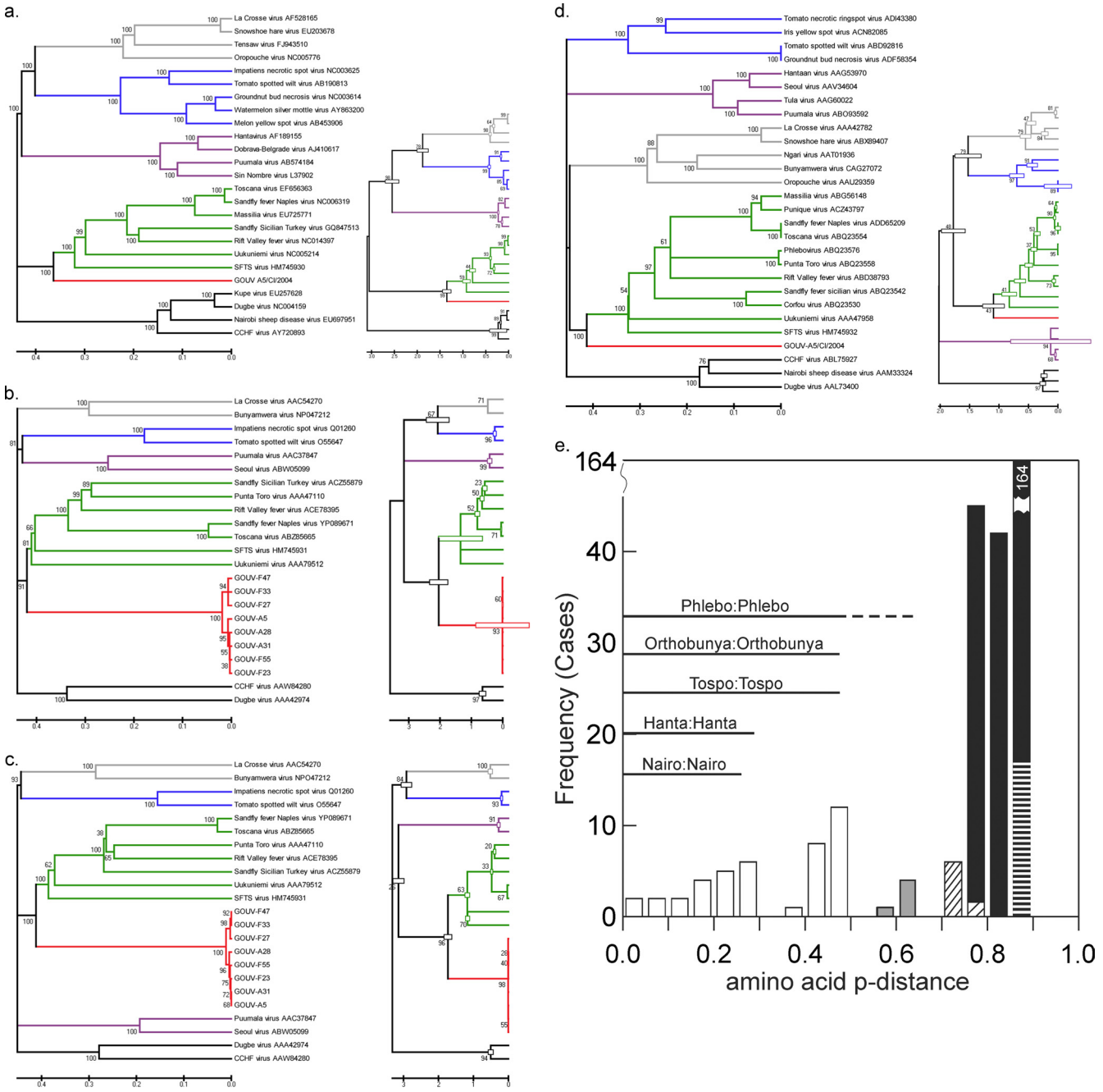
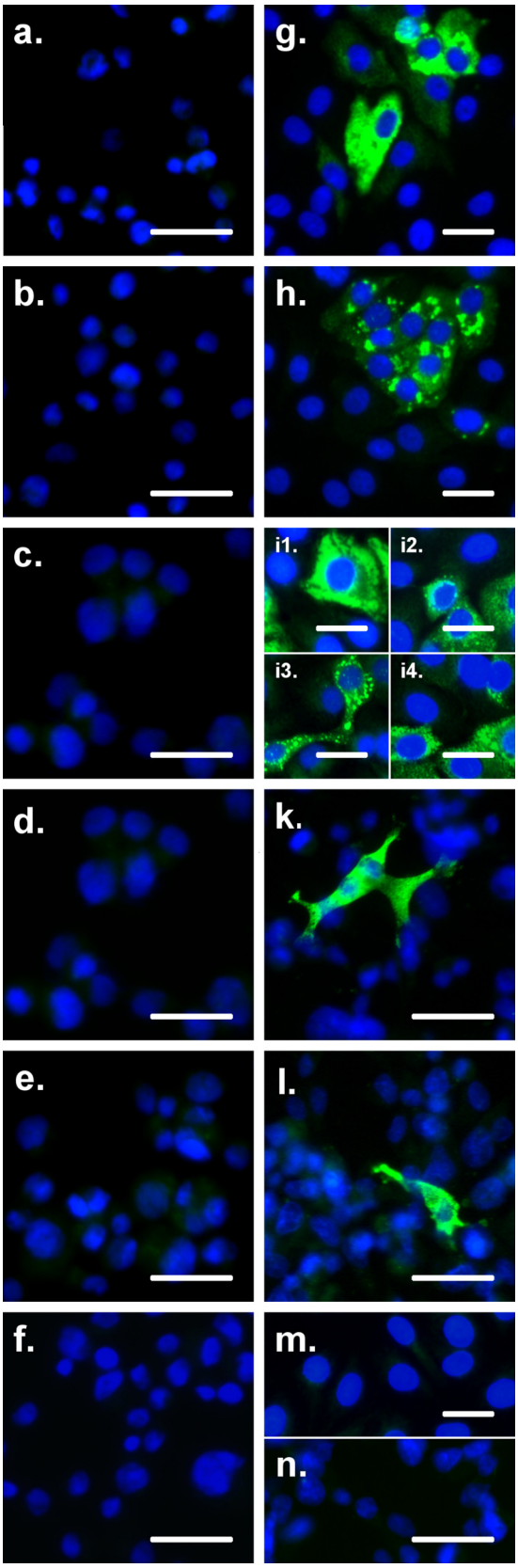


FIG. 3. Relationship of GOUV to other bunyaviruses. Phylogenetic analyses including representative members of all *Bunyaviridae* genera were performed on a gap-free amino acid alignment guided by the BLOSUM62 substitution matrix, using the neighbor-joining (NJ) algorithm with a uniform-rates substitution model and confidence testing by 1,000 bootstrap replicates in MEGA version 5.0 (33). Maximum-likelihood (ML) analyses were performed with the Dayhoff substitution model and are shown in smaller scale on the right. Phylogenies were investigated for the RdRp (a), Gn (b), Gc (c), and N (d) protein genes. Bars indicate evolutionary substitutions per position in the alignment. (e) Distribution of pairwise amino acid sequence distances between putative RdRp proteins in the family *Bunyaviridae*. A distance matrix of pairwise identity values was calculated with MEGA 5.0 (33) for 28 L-segment sequences. For each range of identity values (*x* axis), the incidence in the matrix is plotted on the *y* axis. White bars indicate pairwise distances between viruses of same genera (intragenus), and black bars indicate pairwise distances between viruses of different genera (intergenus). Pairwise distances between Uukuniemi virus and main-group phleboviruses (sandfly fever group) are shaded in gray. Pairwise distances between Gouléako virus and phleboviruses are hatched, and ranges of pairwise distances between GOUV and orthobunya-, hanta-, nairo-, and tospoviruses are marked by horizontal bars. Horizontal lines indicate ranges of pairwise sequence distances within each of the five established genera of the family *Bunyaviridae*. CCHF virus, Crimean-Congo hemorrhagic fever virus.



Contrary to the SFG but in agreement with UUKV and SFTSV, no NSm protein was identified for GOUV, based on sequence alignments and homology searches (8, 29, 39) (Fig. 2b).

Pairwise comparison of the S segment revealed equally low maximal amino acid similarities with the N protein genes of sandfly fever Sicilian virus (27%), RVFV (27%), and SFTSV (25%). Four open reading frames (ORFs) in reverse orientation overlapped the putative N ORF; three seemed too small to encode relevant proteins (all were <70 aa), but one ORF might encode a putative uncharacterized protein of 11.6 kDa (Fig. 1e), a predicted molecular mass similar to that of the NSs protein of orthobunyaviruses. However, in orthobunyaviruses NSs is encoded within the N ORF in the same coding direction. In phlebo- and tospoviruses, NSs is between 29 and 52 kDa and is encoded in ambisense in a nonoverlapping ORF separated from N by an RNA hairpin fold (10, 31). Downstream of N, GOUV contained a small ORF of 38 aa (3.9 kDa) in ambisense orientation with a putative intergenic region of 61 nt and 63.9% A+T content, comparable to that of UUKV (74 nt, 62% A+T content) and SFTSV (55 nt, 67% A+T content) (31). By use of mfold, hairpin structures were predicted for the GOUV region downstream of N up to the 5′ terminus, suggesting a function in the regulation of transcription as assumed for viruses using an ambisense coding strategy (30, 41). No putative NSs ORF using a coding strategy similar to that for other bunyaviruses could be identified. However, whether ORF2 is expressed and might serve functions similar to those of bunyaviral NSs proteins remain to be determined.

In the phlebovirus RVFV, the NSs and NSm proteins are

FIG. 4. Indirect immunofluorescence assay (IIFA) with GOUV and phleboviruses. GOUV-infected C6/36 cells were used to prepare slides for immunofluorescence assays. GOUV infection was confirmed by determination of infectious particles (5.0×10^4 $TCID_{50}$/ml) and by measurement of virus RNA copies/ml (5.27×10^{11}/ml). GOUV-infected cells were tested with mouse anti-RVFV serum (a), mouse anti-RVFV nucleocapsid serum (b), human anti-sandfly fever virus serum (Euroimmun AG, Lübeck, Germany) (c), mouse anti-UUKV serum (9b) (d), and mouse anti-UUKV serum (8b) (e). Reactivity of all sera was confirmed on IFA slides spotted with EU14 cells infected with each respective virus. These slides were taken from the commercially available "Sandfly Fever Virus Mosaic 1" and "Phlebovirus Mosaic 1" detection kits (Euroimmun AG, Lübeck, Germany). These positive controls are shown as follows: mouse anti-RVFV serum (g), mouse anti-RVFV nucleocapsid serum (h), human anti-sandfly fever Cyprus virus (SFCV) serum (i1), human anti-sandfly fever Naples virus (SFNV) serum (i2), human anti-TOSV serum (i3), and human anti-SFSV serum (i4). Additional control experiments were done by incubation of 2 different mouse anti-UUKV sera (designated 8b and 9b) on IFA slides spotted with UUKV-infected BHK-21 cells, as shown in panels k and l. Experiments with negative controls were performed using uninfected C6/36 cells incubated with human anti-sandfly fever virus serum (f), uninfected EU14 cells incubated with human anti-sandfly fever virus serum (m), and uninfected BHK-21 cells incubated with mouse anti-UUKV serum (9b) (n). IFA detection of human and murine sera, respectively, was performed with an anti-human IgG conjugate (Euroimmun AG, Lübeck, Germany) and with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse serum (Sifin, Berlin, Germany). Cells were stained with DAPI (4′,6-diamidino-2-phenylindole). Bars, 20 μm (C6/36 cells) and 50 μm (EU14 and BHK-21 cells). All photographs were taken at equivalent exposure settings.

dispensable for replication in cell culture but play a major role in viral pathogenesis (9, 12, 25, 36). Orthobunyaviruses lacking NSs proteins are likely nonpathogenic for humans (22). The NSs proteins of phlebo- and orthobunyaviruses (RVFV, Bunyamwera virus, La Crosse virus) efficiently inhibit type I interferon synthesis and are relevant for infection of mammals (2, 4, 5, 11, 35). NSs and NSm proteins might thus have been acquired convergently by bunyaviruses during adaptation to vertebrate hosts. This matches our observations that GOUV could not be passaged to vertebrate cells, suggesting that the virus might depend entirely on insects rather than vertebrates for maintenance in nature (34). Indeed, our finding of GOUV in two pools of male mosquitoes suggests transovarial or transvenereal transmission. This idea is supported by the existence of an NSs protein in SFTSV that is phylogenetically placed between GOUV and phleboviruses and that can infect vertebrates (39).

To provide an estimate of genetic diversity within GOUV viruses, the coding regions for Gn and Gc proteins were sequenced from eight randomly chosen isolates. The isolates were clearly diversified, with a maximal distance of 4.1% at the amino acid level (Fig. 3b and c). Phylogenetic analysis yielded five major clades reflecting the established *Bunyaviridae* genera and GOUV as an additional clade (Fig. 3a to d). GOUV was placed in a basal phylogenetic relationship to the *Phlebovirus* genus and was more distant from the SFG than UUKV and SFTSV were, which already constitute outliers within the genus *Phlebovirus* (3).

To characterize the amino acid distance pattern within the family *Bunyaviridae*, a distance matrix using the complete RdRP ORFs was calculated (it should be noted that this analysis excluded SFTSV, due to its unclassified status). Viruses within genera were found to be up to 47% distant from each other, except for UUKV, which showed between 57 and 61% distances from the SFG of phleboviruses (Fig. 3e). Intergenus pairwise distances ranged between 77 and 90%. GOUV was approximately equidistant from all bunyaviruses, with distances ranging between 74 and 88%.

To examine the antigenic distinction of GOUV from the genus *Phlebovirus*, immunofluorescence assays were done on GOUV-infected cells, using antisera against a broad panel of prototypic phleboviruses, including SFV Toscana, Sicilia, and Naples strains and RVFV, as well as UUKV. No cross-reactivity was detected, while all controls showed reaction patterns as expected (Fig. 4).

In summary, we have identified a prototypic mosquito-associated bunyavirus that differs from the established bunyavirus genera in all taxonomically relevant genetic features and that is antigenically distinct. We assume that GOUV defines a novel genus within the family *Bunyaviridae*.

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