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Characterization of the Uukuniemi Virus Group (*Phlebovirus*: *Bunyaviridae*): Evidence for Seven Distinct Species

Gustavo Palacios,[a] Nazir Savji,[b]* Amelia Travassos da Rosa,[c] Hilda Guzman,[c] Xuejie Yu,[c] Aaloki Desai,[b] Gail Emilia Rosen,[b] Stephen Hutchison,[d] W. Ian Lipkin,[b] Robert Tesh[c]

Center for Genomic Sciences, United States Army Medical Research Institute for Infectious Diseases, Frederick, Maryland, USA[a]; Center for Infection and Immunity, Mailman School of Public Health, Columbia University, New York, New York, USA[b]; Center for Biodefense and Emerging Infectious Diseases, Department of Pathology, University of Texas Medical Branch, Galveston, Texas, USA[c]; 454 Roche Life Sciences, Branford, Connecticut, USA[d]

Evolutionary insights into the phleboviruses are limited because of an imprecise classification scheme based on partial nucleotide sequences and scattered antigenic relationships. In this report, the serologic and phylogenetic relationships of the Uukuniemi group viruses and their relationships with other recently characterized tick-borne phleboviruses are described using full-length genome sequences. We propose that the viruses currently included in the *Uukuniemi virus* group be assigned to five different species as follows: *Uukuniemi virus*, *EgAn 1825-61 virus*, *Fin V707 virus*, *Chizé virus*, and *Zaliv Terpenia virus* would be classified into the *Uukuniemi* species; *Murre virus*, *RML-105-105355 virus*, and *Sunday Canyon virus* would be classified into a *Murre virus* species; and *Grand Arbaud virus*, *Precarious Point virus*, and *Manawa virus* would each be given individual species status. Although limited sequence similarity was detected between current members of the *Uukuniemi* group and *Severe fever with thrombocytopenia syndrome virus* (SFTSV) and *Heartland virus*, a clear serological reaction was observed between some of them, indicating that SFTSV and *Heartland virus* should be considered part of the *Uukuniemi virus* group. Moreover, based on the genomic diversity of the phleboviruses and given the low correlation observed between complement fixation titers and genetic distance, we propose a system for classification of the *Bunyaviridae* based on genetic as well as serological data. Finally, the recent descriptions of SFTSV and Heartland virus also indicate that the public health importance of the Uukuniemi group viruses must be reevaluated.

The family *Bunyaviridae* currently includes more than 350 RNA viruses assigned to five genera: *Orthobunyavirus*, *Nairovirus*, *Hantavirus*, *Phlebovirus*, and *Tospovirus* (1). Three unique molecules of negative or ambisense single-stranded RNA (ssRNA), designated L (large), M (medium), and S (small), make up bunyavirus genomes, totaling 11 to 19 kb. Within each genus, viruses share similar segment and structural protein sizes and characteristic terminal sequences at the 3′ and 5′ ends of each segment. Genetic reassortment among related bunyaviruses has been demonstrated both *in vitro* and *in vivo* (2–5).

Except for the hantaviruses, bunyaviruses in the other four genera are transmitted by arthropods (mosquitoes, culicoid midges, phlebotomine sandflies, or ticks to vertebrates and thrips to plants). At present, the genus *Phlebovirus* is comprised of approximately 70 named viruses that are classified, based on their antigenic, genomic, and vector relationships, into two groups: the Sandfly fever group and the Uukuniemi group (1). Viruses in the Sandfly fever group are transmitted by phlebotomine sandflies and mosquitoes; Uukuniemi viruses (UUKV) are transmitted by ticks.

Uukuniemi virus strain S23, the prototype of the latter group, was originally isolated in 1960 from a pool of *Ixodes ricinus* ticks collected in southern Finland (6). Subsequent isolations of UUKV have been reported from a number of other countries in Scandinavia and central and eastern Europe and from Azerbaijan in central Asia (CDC Arbovirus catalog [wwwn.cdc.gov/arbocat/]). As other related viruses were discovered in Europe and elsewhere in the world, it was proposed that UUKV and other antigenically related tick-borne viruses should be assigned to a new genus, *Uukuvirus*, within the family *Bunyaviridae* (7). Later, because the phleboviruses and uukuviruses are more closely related to each other than they are to viruses of other genera in this family, it was decided to integrate them into a single genus, *Phlebovirus* (8).

Fourteen named viruses are currently included within the *Uukuniemi virus* group: *Catch-me-cave virus* (CMCV); *EgAN 1825-61 virus* (EGAV), *Fin V707 virus* (FINV), *Grand Arbaud virus* (GAV), *Manawa virus* (MWAV), *Murre virus* (MURV), *Oceanside virus* (OCV), *Ponteves virus* (PTVV), *Precarious Point virus* (PPV), *RML-105355 virus* (RMLV), *St. Abbs Head virus* (SAHV), *Tunis virus* (TUNV), *Uukuniemi-S23 virus* (UUKV), and *Zaliv Terpeniya virus* (ZTV) (1, 9). A provisional 15th member of the group, designated *Chizé virus* (CHZV), was isolated from nymphs of *Ixodes frontalis* collected from a dead bird in western France (10). A 16th provisional member, designated *Sunday Canyon virus* (SCAV), is currently classified as an ungrouped *Bunyavirus* and was isolated from *Argas cooleyi* ticks collected in swallow nests in southern Texas (11). Although most Uukuniemi group viruses are antigenically related (10, 12), only UUKV has biochemical and structural analysis available, and none exists for the group as a

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Address correspondence to Gustavo Palacios, gustavo.f.palacios.ctr@us.army.mil.
* Present address: Nazir Savji, School of Medicine, New York University, New York, New York, USA.
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TABLE 1 Names, abbreviations, strain numbers, sources, dates and locality of isolation, and accession numbers of the viruses used in this study

Virus name	Abbreviation	Strain	Yr of isolation	Source of isolate	Geographical location	Accession no(s).
Uukuniemi virus	UUKV	S 23	1960	*Ixodes ricinus*	Uukuniemi, Finland	NC005214, NC005220-1
EgAN 1825-61 virus	EGAV	EgAn 1825-61	1961	*Phylloscopus trochilus* (bird)	Nile Delta, Egypt	HM566158-60
Manawa virus	MWAV	Argas T-461	1964	*Argas abdussalami*	Lahore, Pakistan	JQ924565-7
Grand Arbaud virus	GAV	Argas 2	1966	*Argas reflexus*	Southern France	JF838327-9
Sunday Canyon virus	SCAV	RML 52301-11	1969	*Argas cooleyi*	Randal County, TX, USA	
Zaliv Terpeniya virus	ZTV	LEIV-271Ka	1970	*Ixodes (Ceratixodes) putus*	Bering Island, Kamchatka, Russia	HM566191-93
Murre virus	MURV	Murre H	1973	*Uria aalge* (bird)	Alaska, USA	JF838330-2
FinV707 virus	FINV	FinV707	1975	*Ixodes (Ceratixodes) uriae*	Rost Islands, Norway	JQ924562-4
Precarious Point virus	PPV	MI 19334	1975	*Ixodes (Ceratixodes) uriae*	Southern Ocean, Australia	HM566179-81
RML-105355 virus	RMLV	RML-105355	1977	*Ixodes (Ceratixodes) uriae*	Alaska, USA	JF838333-5
Chizé virus	CHZV	Brest Ar/T2913	1993	*Ixodes (Trichotoixodes) frontalis*	Chizé Forest, western France	JF838324-6
Severe fever thrombocytopenia syndrome virus	SFTSV	HB29	2009	Human (blood)	Henan Province, China	NC018136-8
Heartland virus	HRTV	MO-4	2009	Human (blood)	Northwestern Missouri, USA	JX005842-47

whole. No structural or biochemical analyses of the group have been reported.

In general, the Uukuniemi group viruses have not been considered to be of public health significance, although antibodies to some of its members have been detected in humans in serologic surveys (13–15). Three cases of acute UUKV infection were reported from southern Russia; these patients had an acute self-limited illness characterized by fever, headache, muscle and joint pains, facial hyperemia, and body rash (13). No human illness has yet been reported with the other 16 Uukuniemi group viruses noted above.

Recently, a novel bunyavirus has been isolated from patients in eastern and central China, presenting with fever, thrombocytopenia, leukocytopenia, and multiorgan dysfunction (16, 17). The new virus has been designated *Severe fever with thrombocytopenia syndrome virus* (SFTSV) (16), or *Huaiyangshan virus* (HYSV) (18). SFTSV RNA has been detected in *Haemaphysalis longicornis* and *Rhipicephalus microplus* ticks collected in the region of endemicity (16, 18). Published phylogenetic studies of the sequences of multiple SFTSV isolates (16, 17) have suggested that the new virus probably represents a third distinct lineage (group) within the genus *Phlebovirus*. In addition, in 2012, another phlebovirus named *Heartland virus* was described and associated with two human cases of severe febrile illness with thrombocytopenia in Missouri (19). *Heartland virus* is closely related to SFTSV, and the human illness was associated with a tick bite. Finally, the genomes of Bhanja and Palma viruses, two tick-borne phleboviruses isolated in India in 1954 and in Portugal in 1994, respectively, were recently characterized by pyrosequencing and reported to be related to UUKV and SFTSV (20). However, these analyses were impaired by the paucity of genomic information available for other members of the Uukuniemi group; until now, only the prototype S23 UUKV sequence was available for comparison.

In an effort to develop a more precise taxonomic system for phleboviruses, we have attempted to sequence all of the available named viruses in the genus to clarify their phylogenetic relationships. This is the third in a series of publications describing this work (21, 22). The present communication describes the antigenic and phylogenetic relationships among Uukuniemi group viruses and with selected members of the Sandfly fever group, SFTSV, and HRTV.

MATERIALS AND METHODS

Viruses. Viruses used in this study were obtained from the World Reference Center for Emerging Viruses and Arboviruses at the University of Texas Medical Branch (UTMB). Table 1 provides the names, strain numbers, sources, and dates and localities of isolation, as well as GenBank accession numbers. UUKV, SFTSV, and HRTV were not sequenced, as their full genome sequences were already available; but these three viruses were used to prepare mouse brain antigen and immune sera (see below). Catch-me-cave, Oceanside, Ponteves, and St. Abbs Head viruses were not available to us for analysis; and our sequence analysis of Tunis virus demonstrated that it does not belong to the *Phlebovirus* genus (data not shown). Consequently, the last five viruses were not sequenced or included in our serologic tests.

Antigens and antisera. Antisera for serologic tests were prepared in adult mice, using 10% crude homogenates of infected newborn mouse brain in phosphate-buffered saline as the immunogens (21). The immunization schedule consisted of four intraperitoneal injections of antigen mixed with Freund's adjuvant, given at weekly intervals. After the final immunization, mice were inoculated with sarcoma 180 cells, and the resulting immune ascitic fluids were collected. All animal work was done at UTMB under an institutional review board (IRB)-approved animal use protocol.

Serologic tests. Complement fixation (CF) tests were performed by the microtiter technique (23), using 2 units of guinea pig complement and overnight incubation of the antigens and antibodies at 4°C. Antigens used in the CF tests were prepared from infected newborn mouse brain by the sucrose acetone extraction method (24) and were inactivated with 0.05% β-propriolactone (Sigma, St. Louis, MO) or by gamma irradiation. CF titers were recorded as the highest dilutions giving 3+ or 4+ fixation of complement on a scale of 0 to 4+.

Genome sequencing. Viral stocks were extracted using TRIzol LS (Invitrogen, Carlsbad, CA). Total RNA extracts were treated with DNase I (DNA-Free; Ambion, Austin, TX). cDNA was generated using the Superscript II system (Invitrogen) employing random hexamers linked to an arbitrary 17-mer primer sequence (25). Resulting cDNA was treated with RNase H and then randomly amplified by PCR with a 9:1 mixture of primer corresponding to the 17-mer sequence and the random hexamer-linked 17-mer primer (25). Products greater than 70 bp were selected by column chromatography (MinElute; Qiagen, Hilden, Germany) and ligated to specific adapters for sequencing on the 454 Genome Sequencer FLX (454 Life Sciences, Branford, CT) without fragmentation (26–28). Software programs accessible through the analysis applications at the GreenePortal website (http://tako.cpmc.columbia.edu/Tools/) were used for removal of primer sequences, redundancy filtering, and sequence assembly. Sequence gaps were completed by PCR using primers based on

TABLE 2 Results of complement fixation tests with SFTSV and 11 Uukuniemi group viruses

	CF test result[b] with antibody from:											
	Uukuniemi complex					Murre complex			Others			
Antigen[a]	UUKV	EGAV	CHZV	ZTV	FINV	MURV	RMLV	SCAV	SFTSV	GAV	PPV	MWAV
UUKV	**256/32**	**16/≥8**	**16/32**	**64/≥8**	**16/≥8**	0[c]	0	0	0	0	0	0
EGAV	**32/≥8**	**256/≥512**	**512/≥8**	**128/≥8**	**16/≥8**	0	**64/≥8**	0	**8/≥8**	**8/8**	0	0
CHZV	**32/128**	**256/≥8**	**512/128**	**128/128**	**16/≥8**	0	0	0	**8/≥8**	**16/8**	0	0
ZTV	**32/32**	**64/≥8**	**64/32**	**1,024/32**	**256/≥8**	0	0	0	0	0	0	0
FINV	**32/≥2**	**32/≥2**	**32/≥2**	**512/≥2**	**256/≥16**	0	0	0	0	0	0	0
MURV	**16/≥8**	0	0	0	0	**512/128**	**1,024/128**	**64/128**	0	0	0	0
RMLV	**16/≥8**	0	0	0	0	**512/128**	**1,024 /28**	**64/128**	0	0	0	0
SCAV	**16/≥8**	0	0	0	0	**512/128**	**1,024/128**	**64/128**	0	0	0	0
SFTSV	0	0	0	**64/32**	0	0	0	0	**512/128**	0	0	0
GAV	**16/16**	**8/≥8**	**32/32**	**8/32**	0	0	**32/≥8**	0	0	**512/128**	0	0
PPV	0	0	0	0	0	0	**8/≥8**	0	0	**8/8**	**32/32**	0
MWAV	0	0	0	0	0	0	0	0	0	0	0	**16/32**

[a] UUKV, Uukuniemi virus; EGAV, EgAn 1825-61 virus; ZTV, Zaliv Terpeniya virus; CHZV, Chizé virus; SFTSV, Severe fever with thrombocytopenia syndrome virus; FINV, FINV 707 virus; MURV, Murre virus; RMLV, RML 105355 virus; SCAV, Sunday Canyon virus; GAV, Grand Arbaud virus; PPV, Precarious Point virus; MWAV, Manawa virus. The significance of shaded areas is discussed in the text.

[b] CF titers are expressed as the ratio of highest antibody dilution to highest antigen dilution.

[c] A value of "0" indicates that the titers are <8/<8.

pyrosequencing data. Amplification products were size fractionated on 1% agarose gels, purified (MiniElute; Qiagen), and directly sequenced in both directions with ABI Prism BigDye Terminator 1.1 Cycle Sequencing kits on ABI Prism 3700 DNA Analyzers (Perkin-Elmer Applied Biosystems, Foster City, CA). For the termini of each segment, a primer with the 8-nucleotide (nt) conserved sequence was used for a specific reverse transcription with additional arbitrary nucleotides on the 5′ end (5′-AAGCAGTGGTATCAACGCAGAGTAC**ACACAAAG**-3′), where the bold portion represents the conserved nucleotides. This primer is designed to bind to the 3′ end of the genomic RNA; however, it was also useful in sequencing the 5′ end of the genome as well, suggesting it likely binds to the 3′ end of the mRNA. Sequences of the genomes were verified by classical dideoxy sequencing using primers designed from the draft sequence to create products of 1,000 bp with 500-bp overlaps. The assembled data revealed seven phlebovirus genomes.

Phylogenetic analysis. A set of phlebovirus sequences (103 for the L segment, 147 for the M segment, 171 for the N gene, and 103 for the NS gene) comprising all nucleotide sequences from GenBank available on 1 August 2012 were aligned using the CLUSTAL algorithm (as implemented in the MEGA package version 3) at the amino acid level, with additional manual editing to ensure the highest possible quality of alignment. Neighbor-joining (NJ) analysis at the amino acid level was performed due to the observed high variability of the underlying nucleotide sequences. The robustness of the phylogenetic calculations was evaluated by bootstrap resampling of the sequences 1,000 times. Phylogenetic analyses were performed by using MEGA software (29).

Detection of recombination events. Systematic screening for the presence of recombination patterns was pursued by using the nucleotide alignments and the Recombination Detection Program (RDP [30]), Bootscan (31), MaxChi (32), Chimaera (33), LARD (34), and Phylip Plot (35).

Sequence analysis. Geneious 4.8.3 (Biomatters Inc.) was used for sequence assembly and analysis. Topology and targeting predictions were generated by employing SignalP, NetNGlyc, TMHMM (http://www.cbs.dtu.dk/services), the web-based version of TopPred2 (http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html), and integrated predictions in Geneious (36–40).

RESULTS

Antigenic characteristics. The results of CF tests with 11 Uukuniemi group viruses and SFTSV are shown in Table 2. Most viruses demonstrated some antigenic relatedness with others in the group. Two antigenic complexes are seen. One group includes UUKV, EGAV, CHZV, ZTV, and FINV. A second group includes MURV, RMLV, and SCAV; by CF test, these three viruses were indistinguishable.

SFTSV, GAV, and PPV show weak antigenic relationships to some members of the aforementioned UUKV and MURV complexes, but they are antigenically distinct. Likewise, MWAV showed no serologic relationship with any of the other 11 viruses; however, the CF titers of both MWAV antigen and antibody were weak compared to the reagents for most of the other viruses.

Genomic characterization. Consistent with the genomic organization of other members of the genus *Phlebovirus*, viruses in the Uukuniemi antigenic group are comprised of three RNA segments, including a large (L) segment encoding the RNA polymerase, a medium (M) segment encoding both glycoproteins (Gn and Gc), and a small (S) segment encoding the nucleocapsid protein (NP) and, in an ambisense orientation, a nonstructural protein (NSs). However, the members of the Uukuniemi group do not encode a small nonstructural protein in the M segment, the NSm. Interestingly, Heartland virus and SFTSV do not encode an NSm protein either. Tunis virus, a virus isolated from *Argas reflexus hermanni* ticks in Tunisia (18), previously listed as a member of the UUKV group, appears to be a nairovirus (data not shown) and was not included in our data set. Phylogenetic analyses of the L, M, and S gene segment sequences of the other 13 viruses were consistent with earlier reports, confirming that viruses belonging to the same *Phlebovirus* group cluster together (41, 42). As anticipated, based on their cross-reactivity in CF tests (12), members of the UUKV serocomplex generally clustered together (Fig. 1, 2, and 3). Based on L, M, and S segment sequences, UUKV, EGAV, CHZV, ZTV, and FINV were closely related. Likewise, MURV, RMLV, and SCAV were closely related; in fact, RMLV and MURV appear to be a single virus, as do EGAV and CHZV. Clearly, SFTSV and Heartland, Bhanja, and Palma viruses show little relationship with the other members of the UUKV virus group. MWAV, a virus isolated from *Argas abdussalami* ticks collected in vulture roosting

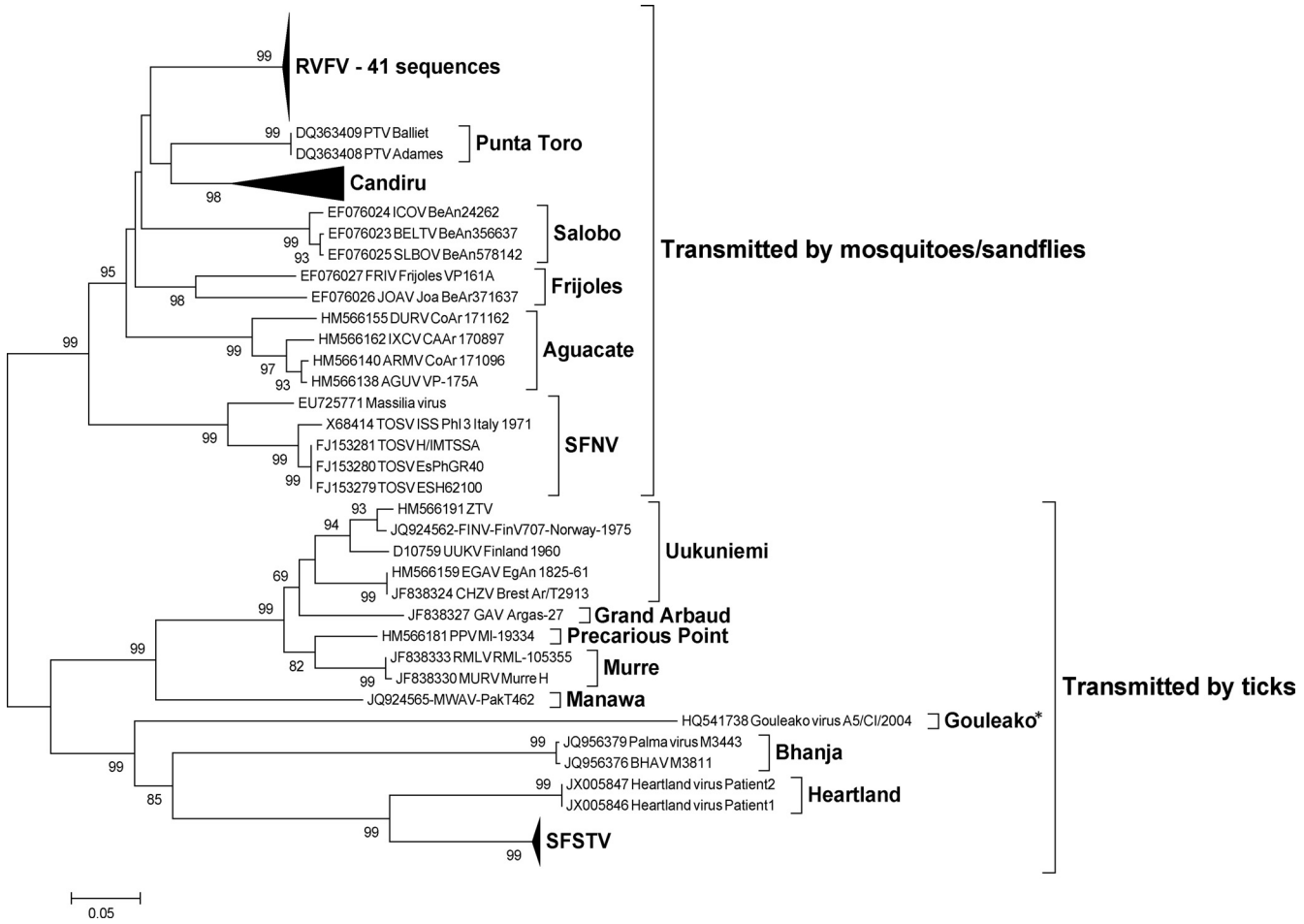

FIG 1 Phylogenetic analysis of the available sequences of phlebovirus L ORF. Neighbor-joining (NJ) analysis at the amino acid level was performed due to the observed high variability of the underlying nucleotide sequences. The statistical significance of the tree topology was evaluated by bootstrap resampling of the sequences 1,000 times. Phylogenetic analyses were performed using MEGA software (29). *, Gouleako virus was isolated from mosquitoes (69).

sites along the Indus River in Pakistan, is the most remote member of this clade. Based on the intragroup and intergroup differences among other members of the genus *Phlebovirus*, MWAV should be considered a distinct species in the *Uukuvirus* serogroup.

The topology of the phylogenetic trees within the UUKV complex node did not reveal branching inconsistencies suggesting reassortment events. Searching for reassortment using RDP, Bootscan, MaxChi, LARD, and Phylip Plot confirmed the absence of reassortment (data not shown).

Open reading frames (ORFs). (i) L protein (RNA-dependent RNA polymerase). Several regions of the RNA-dependent RNA polymerase overlap conserved regions found in all available phlebovirus sequences, confirming an association with function. Region I is located in the amino terminus (amino acids [aa] 107 to 131) and is centered on the amino acid $P_{114}D$; region II (aa 673 to 731), also located in the amino terminus, is centered around the amino acid $R_{696}Y$. Regions I and II are conserved in all bunyaviruses and arenaviruses (43). Region III (aa 936 to 1223), located in the center of the protein, contains the polymerase motifs (pre-A [aa 936 to 970], A [aa 1008 to 1026], B [aa 1107 to 1129], C [aa 1152 to 1168], and D and E [aa 1197 to 1223]) found in all RNA-dependent polymerases from positive, negative-, and double-stranded RNA viruses (see Fig. S1 in the supplemental material) (44, 45). Region IV (1225 to 1324), identified by Aquino et al. (46), and two additional regions of high conservation (region V, aa 1420 to 1458, and region VI, aa 1551 to 1646) are shown in Fig. S2 in the supplemental material.

(ii) M protein. Among the viruses in the UUKV complex, the length of the M protein varies in length from 1,007 aa in EGAV, RMLV, and Chizé virus to 1,010 aa in PPV. The M ORF of Sandfly fever group viruses has lengths of 1,299 to 1,338 aa, while the M ORF of SFTSV has a length of 1,074 aa. This reflects the fact that the M polyprotein of Sandfly fever group viruses is cotranslationally cleaved into three products, NSm, Gn, and Gc, while the polyprotein of Uukuniemi virus group or SFTSV appeared to be cleaved only in Gn and Gc. Signal sequences, transmembrane domains, cleavage sites for the cellular signal signalase protease, and Golgi retention signals for Gn and Gc are conserved in the majority of the viruses in the Uukuniemi virus group; there is also little variation in the patterns of predicted glycosylation sites (see Fig. S3 in the supplemental material). However, SFTSV has fewer and different glycosylation sites from those of the Uukuniemi virus group members.

(iii) N protein. The bunyavirus NP is the most abundant pro-

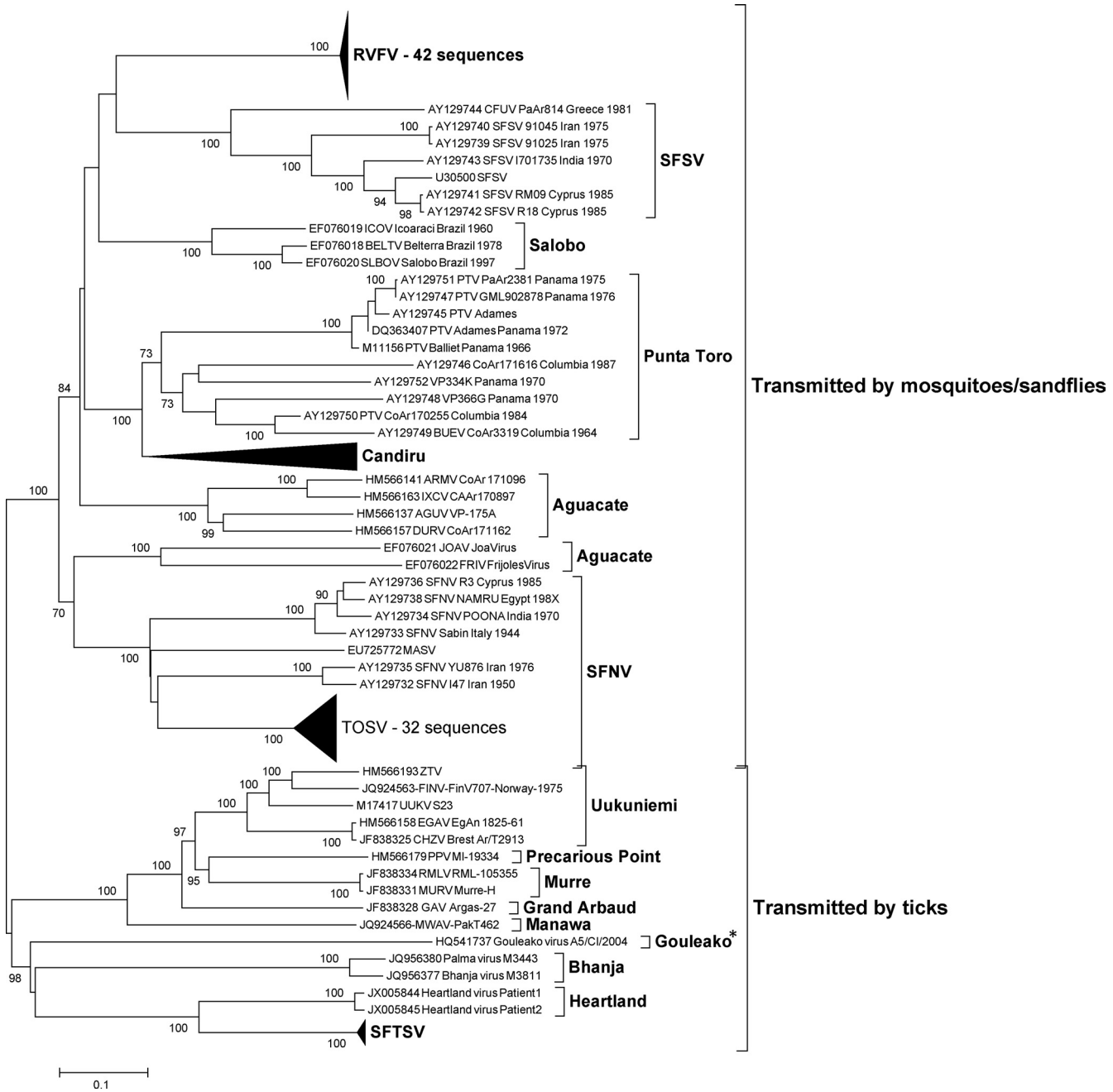

FIG 2 Phylogenetic analysis of the available sequences of phlebovirus M ORF. A set of 133 M phlebovirus sequences comprising all partial or complete sequences from GenBank were analyzed under conditions identical to those of Fig. 1. *, Gouleako virus was isolated from mosquitoes (69).

tein in infected cells and is posited to regulate the transition between primary transcription and replication (47). The NP forms homodimers at their amino termini. In Rift Valley fever virus (RVFV), the interacting domain is located in the N-terminal 71 residues; the amino acids Tyr_4, Phe_{11}, Asp_{17}, and Trp_{24} are conserved in all Sandfly fever group viruses. Although the alpha helices crucial for this interaction are conserved, none of those amino acid sites are conserved among Uukuniemi group viruses or SFTSV (see Fig. S4 in the supplemental material) (48). The NP contains several clusters of conserved domains separated by around 40 to 50 aa; Uukuniemi and Sandfly fever group viruses maintain this conservation; SFTSV is significantly different, and the conserved domain cannot be located (see Fig. S4 in the supplemental material).

DISCUSSION

During the past 10 years, the field of virus taxonomy has been moving toward a sequence-based taxonomic classification of viruses. In this classification system, virus clusters are delineated objectively by establishing a family-wide genetic divergence threshold for each level of classification. In some cases, as in the *Bunyaviridae* family, a genetically based system was intended to

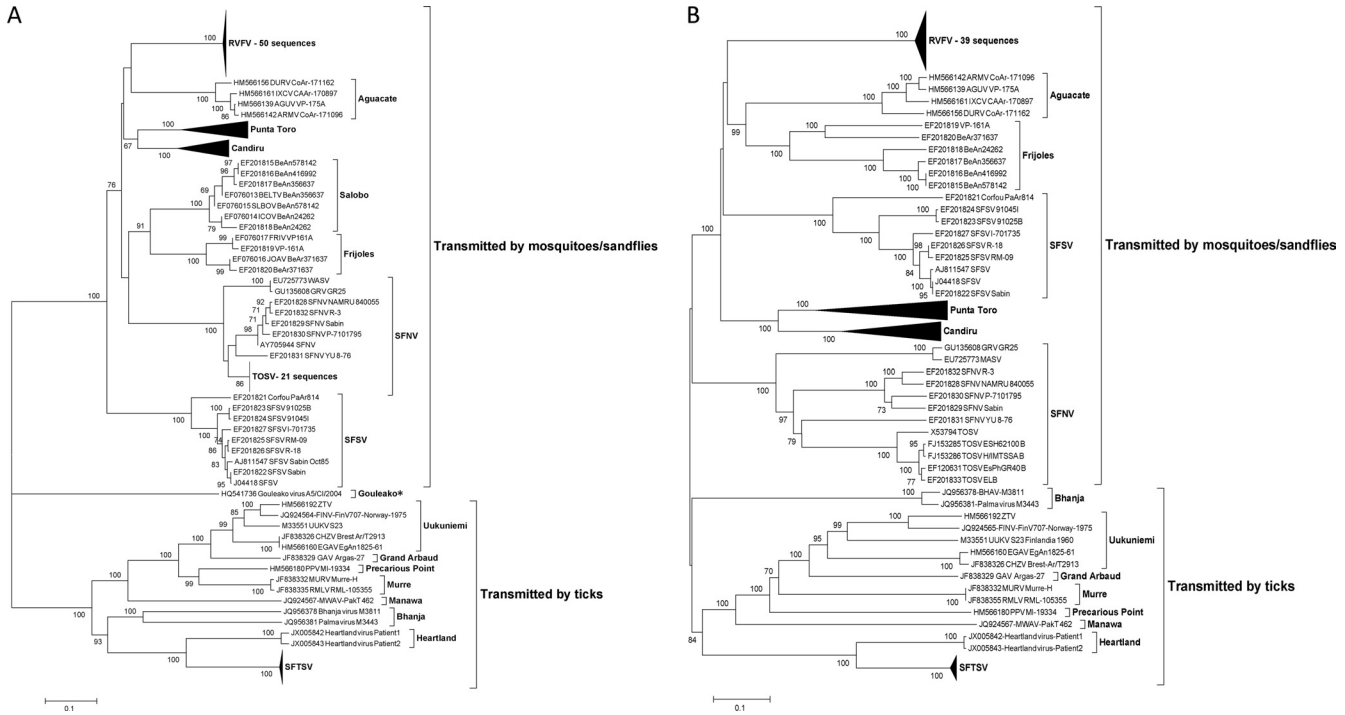

FIG 3 Phylogenetic analysis of the available sequences of phlebovirus S ORF. (A) N ORF. A set of 154 N phlebovirus sequences comprising all partial or complete sequences from GenBank were analyzed under conditions identical to those of Fig. 1. (B) NS ORF. A set of 115 NS phlebovirus sequences comprising all partial or complete sequences from GenBank were analyzed under conditions identical to those of Fig. 1. *, Gouleako was isolated from mosquitoes.

replace the traditional serologically based classification. Our results put that vision in perspective. Although no sequence similarity was detected between current members of the Uukuniemi group SFTSV and HRTV (Table 2), a clear serological relationship was observed between some members of the UUKV group and SFTSV. CF tests are believed to correlate with reactivity against the viral NP. While an analysis of the N protein identified several conserved domains between SFTSV and UUKV, this similarity is not higher than that of other phleboviruses that show no cross-reactivity. Our analyses also demonstrate that RMLV, MURV, and SCAV are closely related (Table 2). Although these viruses were isolated from different geographical locations in North America, their close relation with ticks and migratory birds might explain their similarities. For the above reasons and based on the serologic and phylogenetic evidence, we propose that MURV, RMLV, and SCAV be considered a single entity. MURV and RMLV were collected at the same locality in Alaska; MURV was isolated from the blood of a common murre (*Uria aalge*), an aquatic seabird, and RMLV was isolated from ticks (*Ixodes uriae*) collected in nests of the same bird species. SCAV was isolated from another tick species (*Argas cooleyi*) collected from nests of cliff swallows (*Petrochelidon pyrrhonota*) in Texas.

Five other tick-associated viruses (UUKV, EGAV, CHZV, ZTV, and FINV) are closely related by CF test (Table 2) and form a second clade. In contrast, GAV, PPV, and MWAV are more distantly related to each other and to other members of the Uukuniemi and Murre complexes. In fact, each of the last three viruses probably represents a unique clade. SFTSV and Heartland virus are more distantly related but appear to represent another clade.

Segment reassortment in bunyaviruses has been reported with increasing frequency, especially in the genus *Orthobunyavirus* (49–59). Previously, we demonstrated that the extent of reassortment in the Candiru complex (5 of 13 named viruses) was unprecedented (21). Nonetheless, our analysis of the members of the Uukuniemi virus group does not indicate any reassortment events.

Interestingly, none of the Uukuniemi group viruses or SFTSV or HRTV encoded an NSm-like protein in their M segment. This observation was previously described when sequencing the complete genome of UUKV and is one of the criteria for differentiating the Sandfly fever and Uukuniemi groups (1). Our work confirms the lack of NSm as a feature of the Uukuniemi group and suggests that it might be related to its ecological niche. Both Rift Valley fever virus nonstructural genes (NSs and NSm) were found to function as virus virulence factors and determinants of mammalian host pathogenesis (60–62). NSm functions as a virus virulence factor by suppressing the host cell apoptotic pathway following infection (62); and NSm was found to be indispensable for efficient RVFV growth in both interferon-competent and interferon-deficient cell cultures (63, 64). In fact, a recombinant RVFV lacking the entire NSm coding region was attenuated (65). These findings were used as foundations to create a rationally designed highly attenuated RVFV vaccine lacking NSm and NSs genes (66).

Given the low correlation observed between complement fixation titers and genetic distance, we propose a system for classification of the *Bunyaviridae* based on genetic as well as serological data. The current taxonomic system, which is based exclusively on antigenic classification, can be misleading given the phenomenon of segment reassortment. But, as the current data demonstrate, a system based exclusively on sequence identification can be equally misleading. In most instances, the two measures will correlate; but

in some, only serology or genetic relatedness will identify a similarity of novel viruses to previously characterized ones. In some instances, the results of the two methods may clash; in such cases, we propose that other biological properties also be used for classification.

Although the 9th report of the International Committee on Taxonomy of Viruses (ICTV) does define species demarcation based on a 4-fold difference in two-way neutralization (67), the main reason for this definition is the lack of biochemical or sequence data. This standard of classification of phleboviruses is no longer feasible, as we previously discussed (21, 22). First, there are more than 75 named viruses classified as members of the genus *Phlebovirus*. This number continues to grow. Several of these viruses do not produce cytopathic effect or plaques in vertebrate cells. Others fail to kill mice, making it difficult to produce a "clean" antiserum or mouse immune ascitic fluid. Obviously, if the virus does not grow well or produce plaques in vertebrate cells, it is not feasible to utilize plaque reduction and neutralization assays (PRNTs) as a routine characterization method. This is not a problem restricted to the phleboviruses; a similar problem arises when working with hantaviruses or nairoviruses. In particular, many UUKV group viruses do not produce hemagglutinins, making hemagglutination inhibition (HI) tests difficult or impossible. In addition, several novel phleboviruses have been recently described based solely on partial genome sequences, for which no actual viral isolate exists, making serological analysis impossible. Producing recombinant antigens or infectious clones on such viruses for the purpose of 2-way neutralization tests is not realistic. In addition, current shipping restrictions on infectious viruses make it incredibly difficult for a single lab to acquire all >75 viruses.

After our study was completed and while the current report was in preparation, a new publication appeared (20) demonstrating that *Bhanja virus* (BHAV) and the closely related *Palma virus* (PALV) were also members of the genus *Phlebovirus*, based on their full-genome sequences. BHAV was initially isolated from *Haemaphysalis intermedia* ticks collected from a paralyzed goat in India. It has subsequently been isolated in Europe and Africa from several other tick species, domestic animals, and humans (13, 68; CDC Arbovirus catalog). BHAV has been associated with disease in domestic animals (sheep, goats, and cows) and with febrile illness and meningoencephalitis in humans (13, 68). PALV was isolated from *Haemaphysalis punctata* collected off cattle in Portugal, but it has not yet been associated with human or animal disease. Genetically, BHAV and PALV form another clade distinct from those of SFTSV/HRTV and UUKV; pending further genetic and antigenic studies, they probably should also be considered tentative members of the UUKV group. Likewise, the veterinary and public health importance of the Uukuniemi group viruses must be reevaluated.

Until now, viruses included in the Uukuniemi serogroup were considered to be members of a single species (*Uukuniemi virus*) within the genus *Phlebovirus* (1, 67). But based on the aforementioned data, that no longer seems tenable. Consequently, we propose that the 15 Uukuniemi group viruses examined in this study, including SFTSV, HRTV, BHAV, and PALV, be assigned to seven different species. In this new classification scheme, UUKV, EGAV, CHZV, ZTV, and FINV would be members of a *Uukuniemi virus* species; MURV, RMLV, and SCAV would be assigned to a Murre species; and SFTSV/HRTV, GAV, MWAV, and BHAV/PALV would each be given individual species status. PPV also shows moderate sequence similarity with the other members of the Uukuniemi species, but it does not show any CF cross-reactivity with them. In this situation, it would be desirable to obtain PRNT or HI data to verify that the observed difference is not a limitation of the CF tests. Unfortunately, PPV does not grow well or produce plaques in vertebrate cells and does not produce a hemagglutinin, thus making further testing impossible. Based on the available evidence, we believe PPV should also be considered a distinct species. The status of CMCV, OCV, PTVV, and SAHV remains uncertain pending further study. However, all of these viruses would remain in the *Uukuniemi virus* group of the *Phlebovirus* genus, based on the absence of the NSm protein on their M segment, serologic relationships, and common arthropod vector (ticks).

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