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Genetic characterization of the Wyeomyia group of orthobunyaviruses and their phylogenetic relationships

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Phylogenetic analyses can give new insights into the evolutionary history of viruses, especially of viruses with segmented genomes. However, sequence information for many viral families or genera is still limited and phylogenies based on single or short genome fragments can be misleading. We report the first genetic analysis of all three genome segments of Wyeomyia group viruses Wyeomyia, Taiassui, Macaua, Sororoca, Anhembi and Cachoeira Porteira (BeAr328208) in the genus *Orthobunyavirus* of the family *Bunyaviridae*. In addition, Tucunduba and Iaco viruses were identified as members of the Wyeomyia group. Features of Wyeomyia group members that distinguish them from other viruses in the Bunyamwera serogroup and from other orthobunyaviruses, including truncated NSs sequences that may not counteract the host's interferon response, were characterized. Our findings also suggest genome reassortment within the Wyeomyia group, identifying Macaua and Tucunduba viruses as M-segment reassortants that, in the case of Tucunduba virus, may have altered pathogenicity, stressing the need for whole-genome sequence information to facilitate characterization of orthobunyaviruses and their phylogenetic relationships.

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INTRODUCTION

Viruses with tripartite, negative-sense, ssRNA genomes are classified in the family *Bunyaviridae* (Fauquet *et al.*, 2005; Schmaljohn & Hooper, 2001). Among the five genera of the family, the genus *Orthobunyavirus* is the most complex, comprising 18 antigenic groups (Bishop *et al.*, 1980; Calisher, 1996) and 48 classified species (Fauquet *et al.*, 2005). Historically, orthobunyaviruses and many other arthropod-borne (arbo)viruses were classified into serogroups and serocomplexes based on cross-reactivity in complement fixation (CF) assays that reflect differences in nucleocapsid (N) protein epitopes, and haemagglutination-inhibition (HI) and neutralization (NT) assays that interrogate surface glycoprotein (G) determinants (Bishop, 1996; Casals & Whitman, 1960). However, in orthobunyaviruses, different genome segments encode these proteins. The smallest (S)

The GenBank/EMBL/DDBJ accession numbers for the L, M and S segments are JN572080–JN572082 for WYOV strain 'original', JN801033–JN801035 for WYOV strain TRVL8349, JN801036–JN801038 for WYOV strain Darien, JN572074–JN572076 for TAIAV, JN572068–JN572070 for MCAV, JN572071–JN572073 for SORV, JN572062–JN572064 for AMBV, JN968590–JN968591 for CPOV strain BeAr328208, JN572065–JN572067 for IACOV and JN572077–JN572079 for TUCV, respectively, and JN801039 for GROV L segment.

Five supplementary tables are available with the online version of this paper.

segment encodes N. In addition, the S segment of most sequenced viruses also encodes a non-structural protein NSs that is translated from a +1-shifted small ORF within the 5′ portion of the N sequence, whereas viruses in the Anopheles A, Anopheles B and Tete serogroups were found to lack the NSs ORF (Mohamed *et al.*, 2009). The medium-size (M) segment encodes the ORF for the polyprotein precursor that is cleaved co-translationally into two surface glycoproteins, Gn and Gc, and a small non-structural protein NSm. The largest (L) segment encodes the viral polymerase (POL). This segmented nature of the genome affords opportunities for reassortment, and examples of natural segment exchange in orthobunyaviruses that may have given rise to evolutionary shifts are increasingly recognized (Briese *et al.*, 2006, 2007; Gerrard *et al.*, 2004; Klimas *et al.*, 1981; Nunes *et al.*, 2005; Reese *et al.*, 2008; Yanase *et al.*, 2010). A major impediment to such analyses is the limited sequence information available. The majority of sequence information concerns the S segment, and only recently growing numbers of partial or complete sequences of M segments have become available; little information exists concerning L segments. The advent of unbiased high-throughput sequencing (UHTS) techniques affords new opportunities to sequence divergent genomes and obtain information that can provide better insight into virus evolution and phylogenetic relationships.

A group of South American viruses related serologically to the prototype Wyeomyia virus (WYOV) includes Taiassui (TAIAV), Macaua (MCAV), Sororoca (SORV), Anhembi (AMBV) and Cachoeira Porteira [CPOV; currently listed by its strain designation BeAr328208 (BAV)] viruses, which are all considered strains of the species *Wyeomyia virus* by the International Committee on Taxonomy of Viruses (Fauquet *et al.*, 2005). These viruses were all isolated in South America and are transmitted by various mosquito species, particularly including sylvan New World sabethine species (Table 1). The vertebrate host range has not been defined. A single isolation of MCAV from the rodent species *Proechimys guyannensis* and another of AMBV from *Proechimys iheringi* have been recorded (de Souza Lopes *et al.*, 1975; Travassos da Rosa *et al.*, 1998), whereas serological data may also indicate a bird reservoir (Aitken *et al.*, 1968; de Souza Lopes *et al.*, 1975). Aside from the original description of each virus, more frequent isolations were reported for WYOV from Colombia, Brazil, Panama and Trinidad (Aitken *et al.*, 1968; Srihongse & Johnson, 1965), although the specificity of the serological identification may not have been definitive in all cases. Their role in human disease remains uncertain, but antibodies to AMBV (de Souza Lopes *et al.*, 1975) and WYOV were reported from healthy people in the areas of virus isolation (Brazil for AMBV, Trinidad and Panama for WYOV), and WYOV has been isolated from a febrile patient (Aitken *et al.*, 1968; Srihongse & Johnson, 1965).

Table 1. Wyeomyia group viruses studied

Virus/strain	Source	Country	Year	Reference	Human infection
Wyeomyia					
Original	*Wyeomyia melanocephala*	Colombia	1940	Roca-Garcia (1944)	
TRVL8349	*Psorophora albipes*	Trinidad	1955	Aitken *et al.* (1968)	
Darien	Human	Panama	1963	Srihongse & Johnson (1965)	Febrile illness
Anhembi					
SPAr2984	*Phoniomyia pilicauda*	Brazil	1965	de Souza Lopes *et al.* (1975)	Human seropositivity
Macaua					
BeAr306329	*Sabethes soperi*	Brazil	1976	International Catalogue of Arboviruses*	Human seropositivity
Iaco					
BeAr314206	*Wyeomyia* spp.	Brazil	1976	International Catalogue of Arboviruses*	
Sororoca					
BeAr32149	*Sabethini* spp.	Brazil	1961	International Catalogue of Arboviruses*	
Tucunduba					
BeAr278	*Wyeomyia* spp.	Brazil	1955		Febrile illness with encephalitic symptoms
Taiassui					
BeAr671	*Sabethini* spp.	Brazil	1955		
Cachoeira Porteira					
BeAr328208	*Sabethes glaucadaemon*	Brazil	1977		

*http://www.cdc.gov/nczved/divisions/dvbid/arbovirus.html

Here we report the nearly complete sequence for all three genome segments of these viruses and present phylogenetic analyses that show a relationship to Guaroa virus (GROV) for their S segments, but a closer link to other Bunyamwera serogroup viruses for their M and L segments, and identify two viruses as intra-group reassortants. Furthermore, we characterize distinguishing genetic features and identify two additional members of the group.

RESULTS AND DISCUSSION

Genomic sequence information for orthobunyaviruses is sparse. For some groups and species, sequence information is missing or represents only small portions of one or two of the genome segments. One such group comprises South American viruses related serologically to WYOV, the prototype of the group initially isolated from *Wyeomyia melanocephala* mosquitoes trapped in Colombia in 1940 (Roca-Garcia, 1944) (Table 1). In order to discern genome characteristics of the Wyeomyia group viruses and assess their phylogenetic relationships to other members of the genus, we determined the nearly complete sequence of S, M and L segments of different isolates of WYOV, as well as TAIAV, MCAV, SORV, AMBV and CPOV (GenBank accession numbers JN801033–JN801038, JN572062–JN572064, JN572068–JN572076, JN572080–JN572082 and JN968590–JN968591, respectively). Employing consensus primers that were designed during the course of the work, additional orthobunyaviruses were screened and amplification products with high similarity to Wyeomyia group viruses were recovered for Tucunduba (TUCV) and Iaco (IACOV) viruses (Table 1), two other South American viruses currently attributed to the species *Bunyamwera virus* of the genus *Orthobunyavirus* (Fauquet *et al.*, 2005). Subsequent genomic sequencing identified these two viruses as additional members of the Wyeomyia virus group (GenBank accession numbers JN572077–JN572079 and JN572065–JN572067, respectively).

Wyeomyia virus group S segments contain A-rich runs in the 3′-UTR of the antigenomic RNA that result in S-segment sizes of >1000 nt (Table 2). These stretches separate the termination codon of the N ORF from a conserved TGGG-TGGG-TGGT motif that was described as part of a universal primer-binding site in California encephalitis (CE) group viruses (Bowen *et al.*, 1995; Campbell & Huang, 1996), but is also found in Bunyamwera group members (Dunn *et al.*, 1994). Overall nucleotide conservation was highest with respect to GROV (range 42 %/CPOV to 39 %/WYOV-TRVL) and Kairi virus (KRIV; 43 %/AMBV to 40 %/SORV) (Table S1, available in JGV Online); at the amino acid level, the identity values ranged from 72 % (IACOV/WYOV-Darien) to 67 % (MCAV), and from 72 % (TUCV/TAIAV/WYOV) to 66 % (IACOV/AMBV), respectively. Motifs characteristic of orthobunyavirus N were recognized, and deduced amino acid sequences were aligned with 'bunyavirus N protein' (PFAM accession

Table 2. Lengths of Wyeomyia group S, M and L nucleotide and amino acid sequences in comparison to those of Bunyamwera virus (BUNV) and La Crosse virus (LACV)

Virus	Nucleotide sequence (nt)*			Amino acid sequence (aa)					
	S segment	M segment	L segment	N	NSs	Gn	NSm	Gc	L
CPOV	1046	4680	6922	233	26†	286	144	946	2236
SORV	>1033	4649	6923	233	26†	286	143	946	2236
IACOV	>1013	4641	7149	233	27†	286	144	946	2236
AMBV	1049	4612	7009	233	26†	286	144	945	2236
MCAV	1050	4583	6881	233	5†	286	144	946	2236
WYOV-Darien	1113	4563	6868	233	32†	286	143	946	2236
WYOV-TRVL	1146	4623	6867	233	32†	286	143	946	2236
TUCV	1082	4545	6869	233	17†	286	143	946	2236
TAIAV	1082	4554	6869	233	17†	286	142	946	2236
WYOV	1082	4554	6869	233	17†	286	142	946	2236
GROV‡	954	>4452	6824	233	83	287	142	939	2231
BUNV‡	961	4458	6875	233	101	286	146	956	2238
LACV‡	984	4527	6980	235	92	281	145	968	2263

*Nucleotide sequence length for Wyeomyia group viruses includes conserved terminal primer sequences used for PCR amplification, except for SORV and IACOV S segments, where genomic 5′-terminal sequence was not obtained. UHTS data and RACE yielded authentic genomic termini for AMBV (S and M, 3′), MCAV (S and M, 3′), TUCV (L, 3′), and WYOV (S, 5′; M, 3′/5′; L, 5′) that matched in all cases the known conserved terminal orthobunyavirus sequences used for PCR priming.

†Truncated ORF that may not be expressed.

‡GenBank accessions used: LACV: S, NC_004110; M, NC_004109; L, NC_004108; BUNV: S, NC_001927; M, NC_001926; L, NC_001925; GROV: S, X73466; M, AY380581.

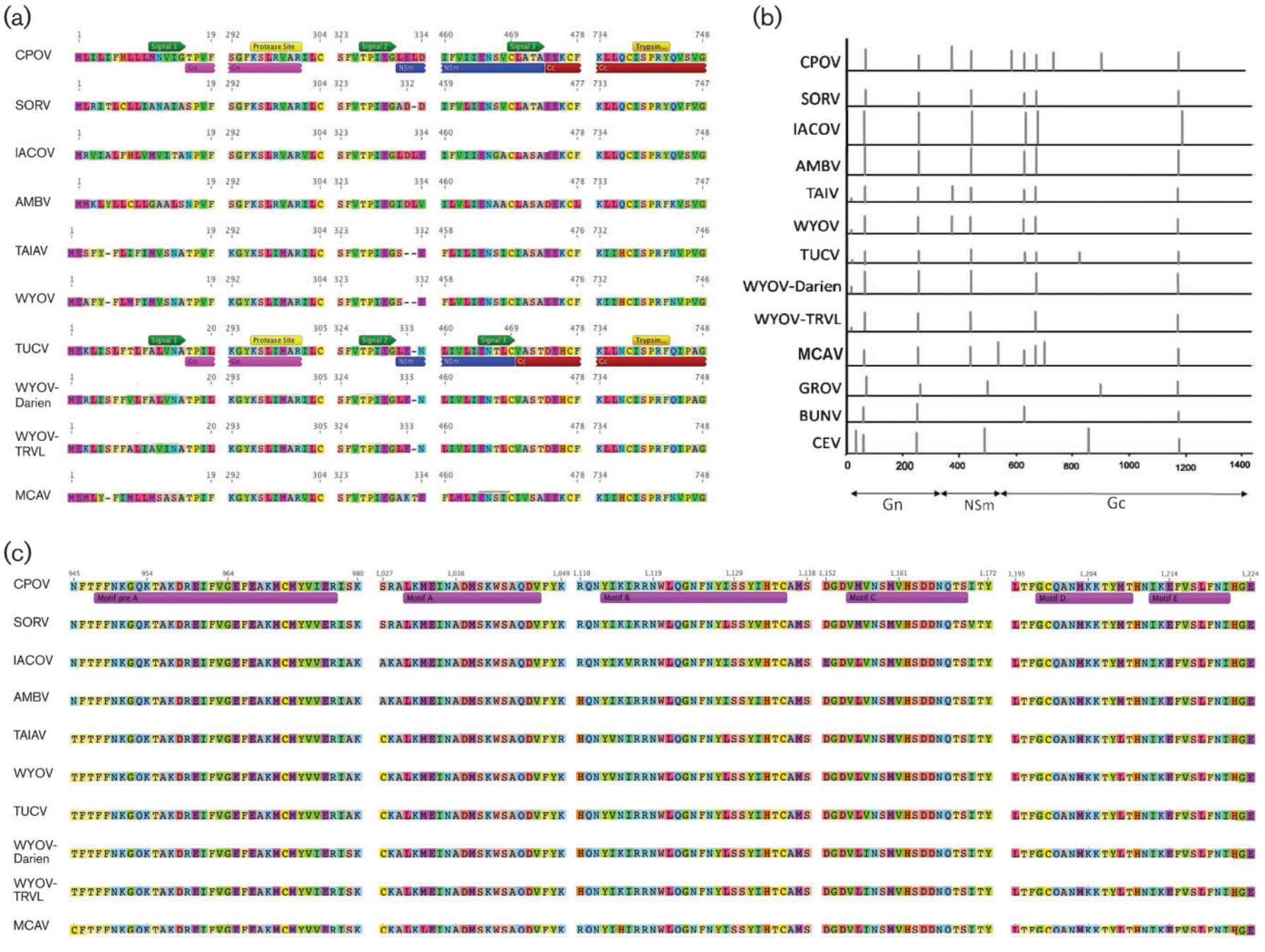
(a)
CPOV
SORV
IACOV
AMBV
TAIAV
WYOV
TUCV
WYOV-Darien
WYOV-TRVL
MCAV
Signal 1
Protease Site
Signal 2
Signal 3
Trypsin...
Gn
NSm
Gc
(b)
CPOV
SORV
IACOV
AMBV
TAIV
WYOV
TUCV
WYOV-Darien
WYOV-TRVL
MCAV
GROV
BUNV
CEV
0
200
400
600
800
1000
1200
1400
Gn
NSm
Gc
(c)
CPOV
SORV
IACOV
AMBV
TAIAV
WYOV
TUCV
WYOV-Darien
WYOV-TRVL
MCAV
Motif pre A
Motif A
Motif B
Motif C
Motif D
Motif E

Fig. 1. Analysis of Wyeomyia group M- and L-segment ORFs. (a) Deduced M-segment polyprotein sequences of Wyeomyia group viruses were analysed for predicted processing sites for the generation of mature proteins Gn, NSm and Gc. In addition to the conserved protease motif (protease site) and trypsin-like site (trypsin), potential signal-peptide cleavage sites were analysed using algorithms implemented in Geneious and SignalP-NN/SignalP-HMM; three sites were predicted (signal 1, 2 and 3). Different signal 2 sites out of the two highest-scoring motifs (AT/SA-E/DE or T/SL/IC-V/IA/V) were favoured for different viruses by the algorithms. (b) Deduced M-segment polyprotein sequences of Wyeomyia group viruses were analysed for predicted *N*-glycosylation sites. The likelihood of glycosylation at a given site is indicated by the height of the line in the diagram. (c) RNA-dependent RNA polymerase motifs pre-A, A, B, C, D and E were identified in the deduced L-segment amino acid sequence of Wyeomyia group viruses.

no. PF00952). Commonly conserved motifs were present around invariant amino acids T_{91}/R_{94} and G_{147}/PL_{160}, a motif proposed to participate in N multimerization was recognizable as F_{17}NPDV/N (Leonard *et al.*, 2005), and usually conserved individual amino acids were mostly maintained (Table S2) (Eifan & Elliott, 2009). Interestingly, the amino acid change E128/A, consistently observed in Wyeomyia group viruses, has been found to be associated with a small-plaque/high-titre phenotype in BUNV (Eifan & Elliott, 2009). Other unique motifs characteristic of Wyeomyia group members included K_{55}RSEES/T, D_{87}E/DAM/L, and A_{204}L/VA/L/VVV. An NSs ORF comparable to those of other sequenced orthobunyaviruses was not present. Indeed, comparison of the 10 S segments shows appropriately spaced AUG codons in a reading frame overlapping that of N. In WYOV-TRVL and IACOV, this includes a tandem AUG initiation codon, a property described for Bunyamwera group virus NSs ORFs (Dunn *et al.*, 1994) and later for the CE group viruses (Bowen *et al.*, 1995; Huang *et al.*, 1996) and several Oropouche virus strains (Saeed *et al.*, 2000), but not for other Simbu serogroup members (Akashi *et al.*, 1984, 1997; Saeed *et al.*, 2001a, b) or group C viruses (Nunes *et al.*, 2005). In Wyeomyia group viruses, the approximately 100 residues following the initiation codon show recognizable conservation with respect to NSs amino acid sequences of other orthobunyaviruses (particularly AMBV to KRIV/GROV and WYOV-TRVL to KRIV). However, these amino acids are not present in a continuous ORF. The sequences are interrupted by multiple termination codons, such that potential NSs proteins expressed from the initiation codons are much shorter (5–32 aa) than those described in other orthobunyaviruses (Table 2). The observed sequence conservation may thus reflect the close relatedness of the overlapping N ORFs, instead of functional selection.

The organization of the M segments follows common patterns by encoding a polyprotein that is predicted to be cleaved into mature proteins Gn, NSm and Gc. Overall sequence conservation in comparison to other orthobunyaviruses was recognized (Table S3), with significant matches to 'bunyavirus glycoprotein G2' (PFAM accession no. PF03563) and 'bunyavirus glycoprotein G1' (PFAM accession no. PF03557). As for S segments, the antigenomic 3′-UTRs were characterized by A-rich regions that resulted in large M-segment sizes (Table 2). Signal-peptide sequences preceding predicted mature protein sequences and protease-cleavage sites identified in other orthobunyaviruses were recognized, although some variation was evident in the proposed cytoplasmic cleavage site preceding NSm and the trypsin-like motif in Gc (Fig. 1a) (Fazakerley *et al.*, 1988). Whereas the N-terminal (lumenal) portion of NSm appears more variable among Wyeomyia group viruses than among other orthobunyaviruses, the C-terminal (cytoplasmic) portion, which includes conserved dual zinc-finger motifs presumably involved in viral RNA binding, was highly conserved (Estrada & De Guzman, 2011). The overall structures of Gn and Gc appear well-preserved, as cysteine

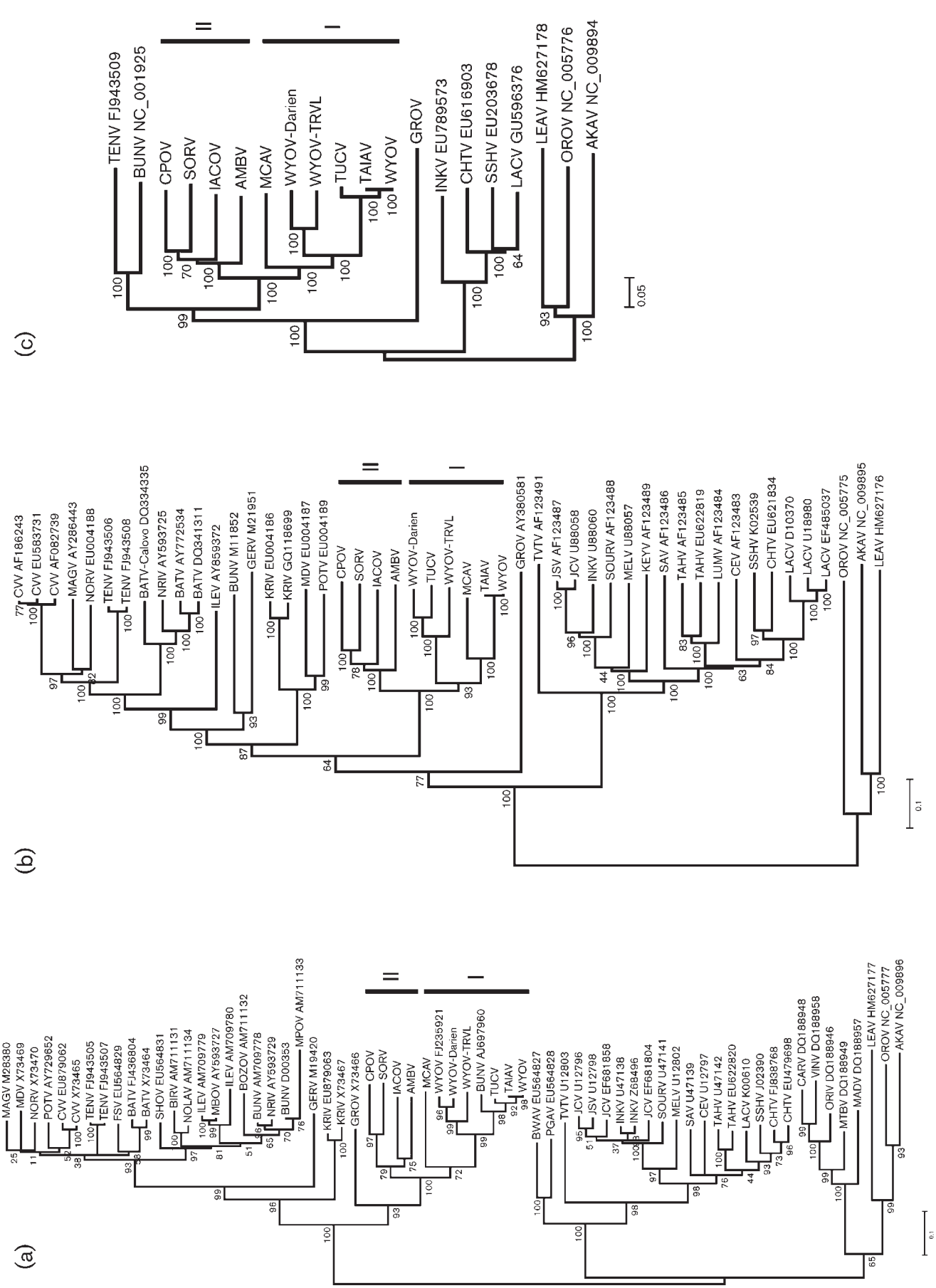
(a)
MAGV M28380
MDV X73469
NORV X73470
POTV AY729852
CVV EU879062
CVV X73465
TENV FJ943505
TENV FJ943507
FSV EU564829
BATV FJ436804
BATV X73464
SHOV EU564831
BIRV AM711131
NOLAV AM711134
ILEV AM709779
MBOV AY593727
ILEV AM709780
BOZOV AM711132
BUNV AM709778
NRIV AY593729
BUNV D00353
MPOV AM711133
GERV M19420
KRIV EU879063
KRIV X73467
GROV X73466
CPOV
SORV
IACOV
AMBV
MCAV
WYOV FJ235921
WYOV-Darien
WYOV-TRVL
BUNV AJ697960
TUCV
TAIAV
WYOV
BWAV EU564827
PGAV EU564828
TVTV U12803
JCV U12796
JSV U12798
JCV EF681858
INKV U47138
INKV Z68496
JCV EF681804
SOURV U47141
MELV U12802
SAV U47139
CEV U12797
TAHV U47142
TAHV EU622820
LACV K00610
SSHV J02390
CHTV FJ838768
CHTV EU479698
CARV DQ188948
VINV DQ188958
ORIV DQ188946
MTBV DQ188949
MADV DQ188957
LEAV HM627177
OROV NC_005777
AKAV NC_009896
0.1
(b)
CVV AF186243
CVV EU583731
CVV AF082739
MAGV AY286443
NORV EU004188
TENV FJ943506
TENV FJ943508
BATV-Calovo DQ334335
NRIV AY593725
BATV AY772534
BATV DQ341311
ILEV AY859372
BUNV M11852
GERV M21951
KRIV EU004186
KRIV GQ118699
MDV EU004187
POTV EU004189
CPOV
SORV
IACOV
AMBV
WYOV-Darien
TUCV
WYOV-TRVL
MCAV
TAIAV
WYOV
GROV AY380581
TVTV AF123491
JSV AF123487
JCV U88058
INKV U88060
SOURV AF123488
MELV U88057
KEYV AF123489
SAV AF123486
TAHV AF123485
TAHV EU622819
LUMV AF123484
CEV AF123483
SSHV K02539
CHTV EU621834
LACV D10370
LACV U18980
LACV EF485037
OROV NC_005775
AKAV NC_009895
LEAV HM627176
0.1
(c)
TENV FJ943509
BUNV NC_001925
CPOV
SORV
IACOV
AMBV
MCAV
WYOV-Darien
WYOV-TRVL
TUCV
TAIAV
WYOV
GROV
INKV EU789573
CHTV EU616903
SSHV EU203678
LACV GU596376
LEAV HM627178
OROV NC_005776
AKAV NC_009894
0.05

Fig. 2. Phylogenetic relationships of Wyeomyia group viruses amongst themselves and to other representative orthobunyaviruses. Deduced amino acid sequences of the S (N ORF) (a), M (Gn, NSm, GC polyprotein ORF) (b) and L (POL ORF) (c) segments of Wyeomyia group viruses were aligned with those of representative orthobunyaviruses, and phylogenetic trees were reconstructed with the maximum-likelihood method as implemented in MEGA5, performing 1000 pseudoreplicates. The resulting bootstrap values are indicated at the respective nodes. Bars, 0.1 substitutions per site (a, b); 0.05 substitutions per site (c). GenBank accession numbers are given next to virus abbreviations: MAGV, Maguari virus; MDV, Main Drain virus; NORV, Northway virus; POTV, Potosi virus; CVV, Cache Valley virus; TENV, Tensaw virus; FSV, Fort Sherman virus; BATV, Batai virus; SHOV, Shokwe virus; BIRV, Birao virus; NOLAV, Nola virus; ILEV, Ilesha virus; MBOV, Mboke virus; BOZOV, Bozo virus; BUNV, Bunyamwera virus; NRIV, Ngari virus; MPOV, M'Poko virus; GERV, Germiston virus; KRIV, Kairi virus; GROV, Guaroa virus; BWAV, Bwamba virus; PGAV, Pongola virus; TVTV, Trivittatus virus; CHTV, Chatanga virus; SSHV, snowshoe hare virus; LACV, La Crosse virus; TAHV, Tahyna virus; CEV, California encephalitis virus; SAV, San Angelo virus; MELV, Melao virus; SOURV, South River virus; JCV, Jamestown Canyon virus; JSV, Jerry Slough virus; CARV, Caraparu virus; VINV, Vinces virus; ORIV, Oriboca virus; MTBV, Marituba virus; MADV, Madrid virus; LEAV, Leanyer virus; OROV, Oropouche virus; AKAV, Akabane virus. GenBank accession AJ697960 is reported as representing members of an uncharacterized orthobunyavirus group from Peru (Mores *et al.*, 2009), possibly related to TUCV when comparing the reported partial Gn sequence to the sequences presented in this paper.

residues common to sequenced Bunyamwera and CE group virus M-segment sequences are conserved. Features similar to GROV M-segment sequences include conservation of a single glycosylation site in Gn ($N_{60/61}$NS/T; the second possible glycosylation site, $N_{247/248}$KS, is predicted to have a cytoplasmic localization). The conservation of glycosylation sites in Gc was variable; sites equivalent to the first and third site of GROV were mostly absent, whereas the second [N_{616}DI/T in GROV (Briese *et al.*, 2004)] and the fourth ($N_{615/616/617}$D/ST, except for WYOV-Darien and -TRVL, and $N_{1153/1154/1155}$K/RT, respectively; Fig. 1b) predicted sites were mainly conserved. An additional potential glycosylation site conserved throughout Wyeomyia group Gc sequences was $N_{656/657/658}$K/ST, which is also found in CE group viruses of the Melao clade except for Keystone virus (KEYV), and in Bunyamwera group viruses except for BUNV, Germiston virus (GERV) and Ilesha virus (ILEV). Additional glycosylation sites were predicted for individual viruses (Fig. 1b). The $N_{615/616/617}$D/ST site is located in, and N-terminally flanked by, putative antigenic domains (Brockus & Grimstad, 2001). At the C terminus, the site is followed by a trypsin-like motif that is surrounded by a highly divergent orthobunyaviral Gc sequence region (Briese *et al.*, 2007). In Wyeomyia group viruses, this region is similarly characterized by indels between different viruses, and might also represent serologically relevant epitopes. Conservation is observed for the proposed fusion-peptide domain (Plassmeyer *et al.*, 2007), which displays a $G_{1053/1054/1055}$EGC motif, instead of the N/ST/D/EGC motif of Bunyamwera and CE group viruses.

L-segment sequences were well-conserved, and deduced amino acid sequences showed significant similarity to 'bunyavirus RNA-dependent RNA polymerase (RdRp)' (PFAM accession no. PF04196). Sequence motifs readily recognized in L segments of Wyeomyia group viruses included the POL block III motifs pre-A to E (Fig. 1c) and the proposed active-site domain S_{1163}DD (Jin & Elliott, 1992; Müller *et al.*, 1994; Poch *et al.*, 1989). L segments were generally of comparable length to those of other orthobunyaviruses (Table 2), with AMBV and IACOV including longer AT-rich 3′-UTRs that, however, differed in composition from those seen in S and M segments. Only short AT-rich patches were present in the 3′-UTRs of the other Wyeomyia group virus L segments. Given the observed relationships between S and M segments of the Wyeomyia group viruses and GROV, it was also of interest to compare their L segments. Hence, we determined the GROV L-segment sequence (GenBank accession no. JN801039). At the nucleotide level, Wyeomyia group sequences were related most closely to GROV (range 62 %/WYOV-TRVL to 59 %/IACOV), whereas at the amino acid level, higher similarity existed to Bunyamwera group viruses BUNV (68 %/IACOV and WYOV-Darien to 66 %/SORV) and TENV (68 %/IACOV to 66 %/SORV) (Table S4).

Phylogenetic analyses confirmed the inclusion of TUCV and IACOV in the Wyeomyia group, with each of the viruses matching with one of the major clades formed

within the group: TUCV being related to WYOV and TAIAV in clade I, and IACOV being related to AMBV, CPOV and SORV, forming clade II (Fig. 2). Limited serological cross-reactivity between IACOV and some Wyeomyia group viruses (AMBV, CPOV, SORV) was detected previously (International Catalogue of Arboviruses; http://www.cdc.gov/nczved/divisions/dvbid/arbovirus.html; Calisher & Karabatsos, 1988), which resulted in the inclusion of all these viruses in the Bunyamwera serogroup, but exclusion of IACOV from the species *Wyeomyia virus*. At the translated amino acid sequence level, the IACOV and AMBV S segments (N-ORF) show a similarly close relationship to each other as is seen between SORV and CPOV across all genome-segment sequences. However, for the M and L segments, the AMBV sequence appears ancestral to IACOV and the other clade II viruses. TUCV, TAIAV and WYOV are not distinguished from each other in CF tests (Table 3), but are differentiated in NT tests (A. Travassos da Rosa, unpublished data), a result that is in line with the observed closer sequence relationship between their S segments (Fig. 2a) than between their M segments (Fig. 2b). This finding, combined with the observed L-segment sequence relationships (Fig. 2c), is compatible with M-segment reassortment, suggesting a possible TAIAV/WYOV-Darien/TAIAV genotype for TUCV. The high sequence identity between TAIAV and WYOV strain 'original' throughout all genome segments, including the UTRs, is remarkable (Fig. 2; Tables S1, S3 and S4). The relationships found for WYOV strain 'original' were consistent when compared with GenBank accessions EU004150 [partial WYOV S-segment sequence (Mores *et al.*, 2009)], AY593741 and AY593740 [partial M-segment sequences for WYOV and TAIAV, respectively (Gerrard *et al.*, 2004)]. In contrast, analyses of strains TRVL8349 and Darien, which were characterized as isolates of WYOV by CF (Aitken *et al.*, 1968) or CF and NT (Srihongse & Johnson, 1965) tests, respectively, indicated a closer match of their S segments with the WYOV sequence in GenBank accession FJ235921 (Lambert & Lanciotti, 2009), which differs from GenBank accession EU004150. MCAV represents the most ancestral clade I S- and L-segment sequences and is probably another reassortant virus, as its M segment matches those of TAIAV/WYOV more closely (Fig. 2). Although a distant relationship between Wyeomyia group members and Birao virus may be deduced from analyses of short M-segment sequences (Gerrard *et al.*, 2004), a similar relationship was not supported when we analysed the full M-segment sequence of Birao virus (data not shown).

The Wyeomyia group viruses share a common ancestor with GROV for their S-segment sequences. However, for their M- and L-segment sequences, the last common ancestor appears to be with other Bunyamwera serogroup viruses rather than with GROV. Although the bootstrap support for this branching is low for the M-sequence tree (64/77 %), this assignment is supported by nucleotide and amino acid sequence identity scores that were higher for

Table 3. Reactivity of Wyeomyia group viruses in CF assay

CF titres are expressed as the highest antibody dilution/highest antigen dilution; 0=<8/<8.

Antigen	Antibody								
	TUCV BeAr278	TAIAV BeAr671	WYOV TRVL8349	IACOV BeAr314206	MCAV BeAr306329	SORV BeAr321149	CPOV BeAr328208	AMBV SPAr2984
Tucunduba (TUCV)	64/⩾8	32/⩾8	32/⩾8	16/⩾8	16/⩾8	0	16/⩾8	32/⩾8
Taiassui (TAIAV)	64/⩾8	32/⩾8	32/⩾8	16/⩾8	16/⩾8	0	8/⩾8	16/⩾8
Wyeomyia (WYOV)	64/⩾8	32/⩾8	64/⩾8	16/⩾8	32/⩾8	0	16/⩾8	32/⩾8
Iaco (IACOV)	0	0	0	64/⩾8	16/⩾8	16/⩾8	8/⩾8	32/⩾8
Macaua (MCAV)	0	0	0	16/⩾8	256/⩾8	0	8/⩾8	8/⩾8
Sororoca (SORV)	8/⩾8	0	8/⩾8	32/⩾8	8/⩾8	16/⩾8	64/⩾8	32/⩾8
C. Porteira (CPOV)	8/⩾8	0	8/⩾8	32/⩾8	16/⩾8	8–16/⩾8	64/⩾8	64/⩾8
Control*	0	0	0	0	0	0	0	0

*Non-infected mouse brain extract.

other Bunyamwera serogroup members than for GROV (Table S4). Reports on the presence in South America of Bunyamwera group viruses such as Maguari virus (MAGV), Cache Valley virus (CVV) and KRIV, and the widespread distribution of GROV attest to a potential for geographical overlap and mixed infections (Aguilar *et al.*, 2010; Aitken & Spence, 1963; Anderson *et al.*, 1960; Calisher *et al.*, 1988; Causey *et al.*, 1961; Downs *et al.*, 1961; Forshey *et al.*, 2010; Vasconcelos *et al.*, 1998). Although homologous segment reassortment between GROV mutants is readily achieved experimentally (Iroegbu & Pringle, 1981), heterologous reassortment between contemporary GROV and Bunyamwera group viruses BUNV, MAGV and BATV has not been observed *in vitro* (Iroegbu & Pringle, 1981). This restriction may not apply for other Bunyamwera group viruses (e.g. CVV or KRIV) or ancestors of contemporary viruses. The divergent genetic distances observed between GROV, Bunyamwera and Wyeomyia group viruses may potentially reflect rather ancient events where long evolutionary histories obscure recognizable sequence conservation.

The finding that TUCV is a potential reassortant between WYOV-Darien and TAIAV/WYOV is intriguing. The WYOV-Darien isolate was obtained from a febrile illness case in Darien Province, Panama (Srihongse & Johnson, 1965). Similarly, TUCV has been linked to human disease when the virus was isolated from a child with transient fever, diarrhoea, meningismus and flaccid paraparesis (Pinheiro *et al.*, 1994; Vasconcelos *et al.*, 1992). Thus, the reassorted M segment of these viruses may include conserved markers that potentially relate to human infection and enhanced pathogenicity.

In this context, the presence of truncated NSs ORFs in the Wyeomyia group viruses is of note. NSs proteins of orthobunyaviruses are considered non-essential for basic virus replication, but counteract the host interferon response by inhibiting mRNA and protein synthesis in mammalian cells, and thus act as a virulence factor (Blakqori *et al.*, 2007; Bridgen *et al.*, 2001; Weber *et al.*, 2002). The deletion of NSs in a recombinant BUNV construct resulted in smaller plaque size, lower titre, impaired inhibition of cellular mRNA and protein synthesis, and the induction of interferon (Bridgen *et al.*, 2001). In experimental mouse infections, these deficiencies lead to slower dissemination of virus and delayed disease in comparison with wild-type virus. Similarly, the NSs-lacking Anopheles A, Anopheles B and Tete group viruses were found to induce interferon (Mohamed *et al.*, 2009). These viruses are also not known to infect humans, with the exception of Tacaiuma virus, which is linked to human disease and was found to suppress interferon production, albeit through an NSs-independent mechanism. Phylogenetically, the NSs-lacking viruses map in a distinct clade that is very distant from the Wyeomyia group viruses (data not shown), and a potential insect-only life cycle has been proposed for these viruses (Mohamed *et al.*, 2009). The Wyeomyia group viruses may therefore also be considered of diminished pathogenicity for mammals, as it seems unlikely that they express NSs proteins that counteract the interferon system. It has been shown that the action of NSs includes interaction of its C-terminal portion with Mediator protein MED8 (Léonard *et al.*, 2006), but that the N-terminal part is also crucial for interferon suppression (van Knippenberg *et al.*, 2010). Thus, it does not appear likely that the short peptides possibly expressed by Wyeomyia group viruses are active in suppressing the interferon response. This may explain the comparable behaviour of WYOV and Anopheles A virus in mouse infection experiments described during their initial isolation (Aitken *et al.*, 1968). Nevertheless, Wyeomyia group viruses have also been linked to human disease. However, although seropositivity appears not to be rare in exposed populations, only two isolated cases of transient disease have been reported, i.e. mild febrile illness in an adult and febrile illness with neurological symptoms in a young child (Aitken *et al.*, 1968; Srihongse & Johnson, 1965; de Souza Lopes *et al.*, 1975). Further studies will be required to determine the pathogenic potential of the Wyeomyia group viruses.

Conclusions

Our genetic analysis of all three genome segments of Wyeomyia group viruses identified distinctive 3′-UTRs of this group. However, review of proximal sequence confirms their grouping with the Bunyamwera serogroup viruses, although distinguishing them by the absence of an NSs ORF comparable to most other orthobunyaviruses. Together, these findings are consistent with a classification of WYOV, TAIAV, MCAV, SORV, AMBV, CPOV, TUCV and IACOV as members of the species *Wyeomyia virus* in the genus *Orthobunyavirus*. Phylogenetic reconstruction suggests intra-group genome-segment reassortment as a driving force in the evolution of the group that contributed, together with genetic drift, to diversification.

METHODS

Viruses. Virus stocks were obtained from the World Reference Center for Emerging Viruses and Arboviruses collection at the University of Texas Medical Branch at Galveston, TX, USA, and the WHO Collaborating Center for Arbovirus Reference and Research at the Instituto Evandro Chagas, Ananindeua, Pará, Brazil (Table 1). Total RNA was extracted from 250 µl virus stock with Tri-Reagent (MRC), suspended in 35 µl nuclease-free water and stored at −80 °C.

CF assay. Viral antigens were prepared by sucrose/acetone extraction of newborn mouse brains (Clarke & Casals, 1958) infected with the respective viruses. Mouse hyperimmune ascites fluids served as antibody preparations. Four intraperitoneal injections of antigen mixed with Freund's complete adjuvant were given at weekly intervals; thereafter, mice were inoculated with sarcoma cells and immune ascitic fluid was collected. CF tests were performed in a microtitre-plate format by incubation at 4 °C overnight in the presence of 2 U guinea pig complement (Beaty *et al.*, 1989, 1995). On a scale of 0 (complete haemolysis) to 4+ (no haemolysis), CF titres were scored as the highest antibody/antigen dilutions that gave a 3+/4+ fixation of complement; titres $\geqslant$1 : 8 were rated positive.

UHTS and RT-PCR. Genomic sequences were generated by applying a combination of consensus RT-PCR and UHTS. For UHTS, aliquots (0.5 μg) of total RNA extracts were treated with DNase I (DNA-free; Ambion) prior to reverse transcription by SuperScript II (Invitrogen) with random octamer primers linked to an arbitrary, defined 17-mer primer sequence. The cDNA was RNase H-treated and randomly amplified by PCR with AmpliTaq (Applied Biosystems) and a primer mix, including the octamer-linked 17-mer sequence primer and the defined 17-mer sequence primer in a 1 : 9 ratio (Quan *et al.*, 2007). Amplification products >70 bp were purified (MinElute; Qiagen) and ligated to linkers for sequencing on a GS-FLX Sequencer (454 Life Sciences) (Margulies *et al.*, 2005). Sequence reads were stripped of primer sequences and highly repetitive elements, then clustered and assembled into contiguous fragments (contigs) for comparison by BLAST search (Altschul *et al.*, 1990) against GenBank at the nucleotide (BLASTN) and deduced amino acid (BLASTX) levels.

Based on the sequences obtained through UHTS, multiple primer sets were designed and used to validate the draft genome sequences by sequencing overlapping PCR products that covered the entire genome. Terminal sequences were amplified by using the conserved 14–15 terminal bases of each segment for PCR priming. In some cases, authentic termini were obtained from the UHTS data (AMBV and WYOV, S-segment genomic 3′ terminus) or in other cases by RACE kits (Invitrogen) (Table 2). With increasing sequence information, consensus primers (Table S5) were designed and used to amplify and sequence additional viruses. Gaps between the amplification products generated by consensus PCR were filled through additional specific PCR amplifications, and the draft genome sequences were subsequently resequenced by overlapping PCR products along the genome. The consensus and the specific PCRs routinely used 1 μl random hexamer-primed cDNA (SuperScript II; Invitrogen), primers at 0.2 mM concentration and Platinum *Taq* DNA polymerase (Invitrogen). Amplification products were purified (QIAquick PCR purification kit; Qiagen) and directly dideoxy-sequenced on both strands (Genewiz).

Bioinformatic analyses. Sequence assembly and analysis were performed with programs of the Wisconsin GCG Package (version 10.3; Accelrys, Inc.), MEGA 5 (Tamura *et al.*, 2011), Geneious 5.5 (Drummond *et al.*, 2011) and NewblerAssembler 2.4 (454 Life Sciences). Amino acid sequence identity and similarity were calculated with the Needleman–Wunsch algorithm, applying an EBLOSUM62 substitution matrix [gap open/extension penalties of 10/2 for amino acid alignments; EMBOSS (Rice *et al.*, 2000)] and a Perl script to parse the results for all comparisons. Topology and targeting predictions were obtained by employing SignalP-NN/SignalP-HMM, NetNGlyc and TMHMM at the Center for Biological Sequence Analysis (http://www.cbs.dtu.dk/services), the web-based version of TopPred2 (http://www.sbc.su.se/~erikw/toppred2/) and Phobius (http://www.ebi.ac.uk/Tools/phobius/) (Claros & von Heijne, 1994; Käll *et al.*, 2004; Krogh *et al.*, 2001; Nielsen & Krogh, 1998; Nielsen *et al.*, 1997). Functional and protein family domain predictions were obtained by comparison with the PFAM database (http://pfam.sanger.ac.uk/). Multiple sequence alignments were generated using CLUSTAL 2.0.12 (Chenna *et al.*, 2003), and programs implemented in MEGA and Geneious software were applied for phylogenetic analyses. Trees were generated with the maximum-likelihood method as implemented in MEGA5.

NOTE ADDED IN PROOF

Recently, the CDC, Fort Collins, CO, USA (Barbara Johnson and Brandy Russel) generously provided the WYOV isolate from which the S segment sequence (GenBank accession no. FJ235921) was obtained for additional sequence analyses. Phylogenetic analyses of its M and L segments (GenBank accession nos JQ743065 and JQ743066, respectively) indicated, in both cases, WYOV Darien as the closest relative (identities at the nucleotide/amino acid levels of 94/98 % for S, 97/98 % for M, and 98/99 % for L sequence), resulting in a branching comparable to that seen for the S segment (Fig. 2).

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