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.

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)
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 itheringi have been recorded (de Souza Lopes et al., 1975; Travassos da Rosa et al., 1975), 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>
<thead>
<tr>
<th>Virus/strain</th>
<th>Source</th>
<th>Country</th>
<th>Year</th>
<th>Reference</th>
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<tr>
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<td>Roca-Garcia (1944)</td>
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<td>1955</td>
<td>Aitken et al. (1968)</td>
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<tr>
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<td>Panama</td>
<td>1963</td>
<td>Srihongse &amp; Johnson (1965)</td>
<td>Febrile illness</td>
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<td>Anhembi</td>
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<tr>
<td>SPAr2984</td>
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*http://www.cdc.gov/nczved/divisions/dvbid/arbovirus.html

Table 1. Wyeomyia group viruses studied
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)

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<th>Virus</th>
<th>Nucleotide sequence (nt)*</th>
<th>Amino acid sequence (aa)</th>
<th>S segment</th>
<th>M segment</th>
<th>L segment</th>
<th>N</th>
<th>NSs</th>
<th>Gn</th>
<th>NSm</th>
<th>Gc</th>
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<td>6922</td>
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<td>26+</td>
<td>286</td>
<td>144</td>
<td>946</td>
<td>2236</td>
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<td>6923</td>
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<td>286</td>
<td>143</td>
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<td>92</td>
<td>281</td>
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<td>968</td>
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</table>

*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.
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
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 (N60/61NS/T; the second possible glycosylation site, N247/248KS, 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 (N616DI/T in GROV (Briese et al., 2004) and the fourth (N615/616/617D/ST, except for WYOV-Darien and -TRVL, and N1153/1154/1155K/RT, respectively; Fig. 1b) predicted sites were mainly conserved. An additional potential glycosylation site conserved throughout Wyeomyia group Gc sequences was N656/657/658K/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 N615/616/617D/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 G1053/1054/1055EGC 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 S1163DD (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 by Wyeomyia and other orthobunyaviruses.
within the group: TUCV being related to WYOV and TAI AV 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, TAI AV 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 TAI AV/WYOV-Darien/TAI AV genotype for TUCV. The high sequence identity between TAI AV 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 TAI AV, 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 TAI AV/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>
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</table>

*Non-infected mouse brain extract.

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.
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 KRV, 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 KRV) 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 ≥ 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 (Geneviz).

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 EBI/OMU 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, to parse the results for all comparisons. Topology and targeting for amino acid alignments:EMBOSS (Rice et al., 2000) and WYOV, S-segment genomic 3’ terminal sequences were amplified by using the conserved 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.

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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