

Aguacate virus, a new antigenic complex of the genus *Phlebovirus* (family *Bunyaviridae*)

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Genomic and antigenic characterization of Aguacate virus, a tentative species of the genus *Phlebovirus*, and three other unclassified viruses, Armero virus, Durania virus and Ixcanal virus, demonstrate a close relationship to one another. They are distinct from the other nine recognized species within the genus *Phlebovirus*. We propose to designate them as a new (tenth) serogroup or species (*Aguacate virus*) within the genus. The four viruses were all isolated from phlebotomine sandflies (*Lutzomyia* sp.) collected in Central and South America. Aguacate virus appears to be a natural reassortant and serves as one more example of the high frequency of reassortment in this genus.

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INTRODUCTION

The family *Bunyaviridae* currently comprises five genera that are differentiated by antigenic and molecular characteristics: *Hantavirus*, *Nairovirus*, *Orthobunyavirus*, *Phlebovirus* and *Tospovirus* (Nichol *et al.*, 2005) (<http://www.ictvonline.org/virusTaxonomy.asp?version=2008>). Viruses in the genus *Phlebovirus* are further subdivided into two groups: the sandfly fever and the Uukuniemi groups, primarily on the basis of the presence of the non-structural ORF in the medium (M) segment (Nichol *et al.*, 2005).

Because of the lack of biochemical characterization of most of the named phleboviruses, the International Committee on Taxonomy of Viruses (ICTV) defines species within the genus *Phlebovirus* by their serological relationships (Nichol *et al.*, 2005). Cross-complement fixation (CF) and plaque-reduction neutralization tests (PRNT) have generally been used for classification of the genus (Tesh *et al.*, 1976, 1982; Travassos da Rosa *et al.*, 1983). Using these criteria, 37 viruses have been assigned to nine *Phlebovirus* species. Sixteen other named viruses that show little serological

relationship to the nine recognized groups are classified as tentative species in the genus.

Here we describe the genetic and antigenic characterization of one of those tentative phleboviruses, Aguacate virus (AGUV) and of three other unclassified viruses: Armero virus (ARMV), Durania virus (DURV) and Ixcanal virus (IXCV). These four viruses are phylogenetically and antigenically related to each other and we propose that they comprise a new (tenth) species of the genus *Phlebovirus*, tentatively named *Aguacate virus*. The four viruses were all isolated from sandflies collected in Central or South America in the 1970s and 1980s. Although they are antigenically related to some other members of the phlebotomus fever serogroup by CF test, they are distinct from these other members and from each other by PRNT (Tesh *et al.*, 1974, 1982, 1989; Travassos da Rosa *et al.*, 1983). The present communication is the second report in our effort to develop a more precise classification system for the phleboviruses by sequencing most of the named viruses in the genus in order to clarify their phylogenetic relationships (Palacios *et al.*, 2011).

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The GenBank/EMBL/DDBJ accession numbers for the sequences of the three RNA segments making up the genomes of AGUV, ARMV, DURV and IXCV are HM566137–9, HM566140–2, HM566155–7, HM566161–3 and HQ661805–7.

Four supplementary figures are available with the online version of this paper.

RESULTS

Serological analysis

Results obtained in CF tests with AGUV, ARMV, DURV, IXCV and one representative from each of the eight

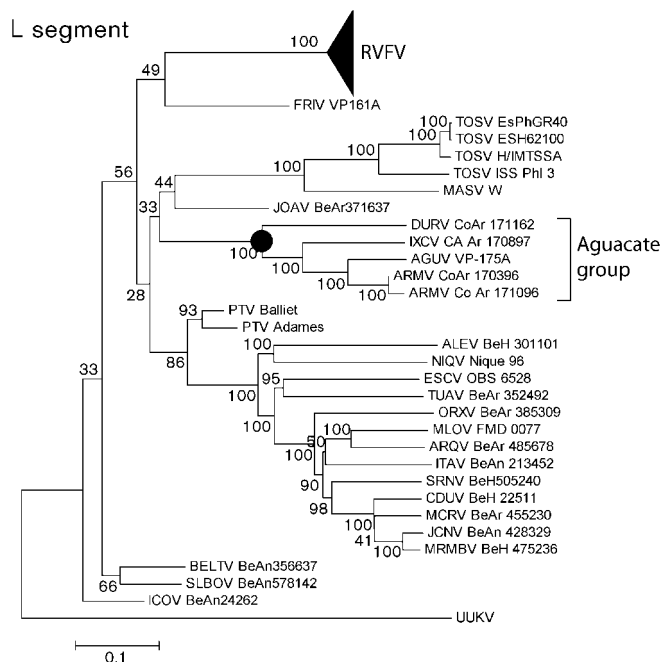


Fig. 1. Phylogenetic analysis of the available phlebovirus L ORF sequences. Neighbour-joining analysis at the amino acid level was performed because of the high variability of the underlying nucleotide sequences. The statistical significance of the tree topology was evaluated by bootstrap resampling of the sequences 1000 times. GenBank accession numbers used in this tree: RVFV (DQ375395, DQ375396, DQ375397, DQ375398, DQ375399, DQ375427, DQ375428, DQ375426, DQ375434, DQ375433, DQ375431, DQ375432, DQ375430, DQ375429, DQ375416, DQ375417, DQ375415, DQ375418, DQ375423, DQ375422, DQ375424, DQ375421, DQ375420, DQ375419, DQ375402, DQ375401, DQ375400, DQ375425, DQ375413, DQ375414, DQ375412, DQ375403, DQ37540, DQ375411, DQ375409, DQ375410, DQ375408, DQ375407, DQ375405, DQ375406, X56464), FRIV (EF076027), TOSV (FJ153280, FJ153279, FJ153281, X68414), MASV (EU725771), JOAV (EF076026), DURV (HM566155), IXCV (HM566161), AGUV (HM566137), ARMV (HQ661805, HM566140), PTV (DQ363409, DQ363408), ALEV (HM119401), NIQV (HM119425), ESCV (HM119410), TUAV (HM119431), ORXV (HM119434), MLOV (HM119413), ARQV (HM119404), ITAV (HM119416), SRNV (HM119428), CDUV (HM119407), MCRV (HM119419), JCNV (HM466934), MRMBV (HM119422), BELTV (EF076023), ICOV (EF076024), SLBOV (EF076025) and Uukuniemi virus (UUKV) (D10759).

Table 1. Results of complement-fixation tests with representative phleboviruses

Homologous antibody/antigen titres are shown in boldface type. CF titres are expressed as the highest antibody/highest antigen dilution. 0 = <8/Φ.

Antigen	Antibody											
	Aguacate (AGUV)	Armero (ARMV)	Durania (DURV)	Ixcanal (IXCV)	Bujaru (BUJV)	Chilibre (CHIV)	Candiru (CDUV)	Frijoles (FRIV)	Punta Toro (PTV)	Rift Valley (RVFV)	Salehabad (SALV)	Naples (SFNV)
Aguacate	128/8	128/2	128/2	256/2	32/2	16/2	0	0	0	0	0	0
Armero	128/6	≥ 32/2	128/2	128/2	–	–	0	0	0	0	0	0
Durania	128/3	≥ 32/2	128/2	128/2	–	–	0	0	0	0	0	0
Ixcanal	128/4	128/8	256/2	256/2	32/8	16/2	0	0	0	0	0	0
Bujaru	8/8	8/8	8/8	8/8	256/8	0	0	0	0	0	0	0
Chilibre	0	128/8	256/2	256/2	0	128/2	0	0	0	0	0	0
Candiru	0	0	0	0	0	0	≥ 512/8	0	0	0	0	0
Frijoles	0	0	0	0	0	0	0	≥ 256/8	0	0	0	0
Punta Toro	0	0	0	0	0	0	0	0	256/64	0	0	0
Rift Valley	–	–	–	–	–	0	0	0	0	64/32	0	0
Salehabad	0	0	0	0	0	0	0	0	0	0	512/128	0
Naples	0	0	0	0	0	0	0	0	0	0	0	32/32

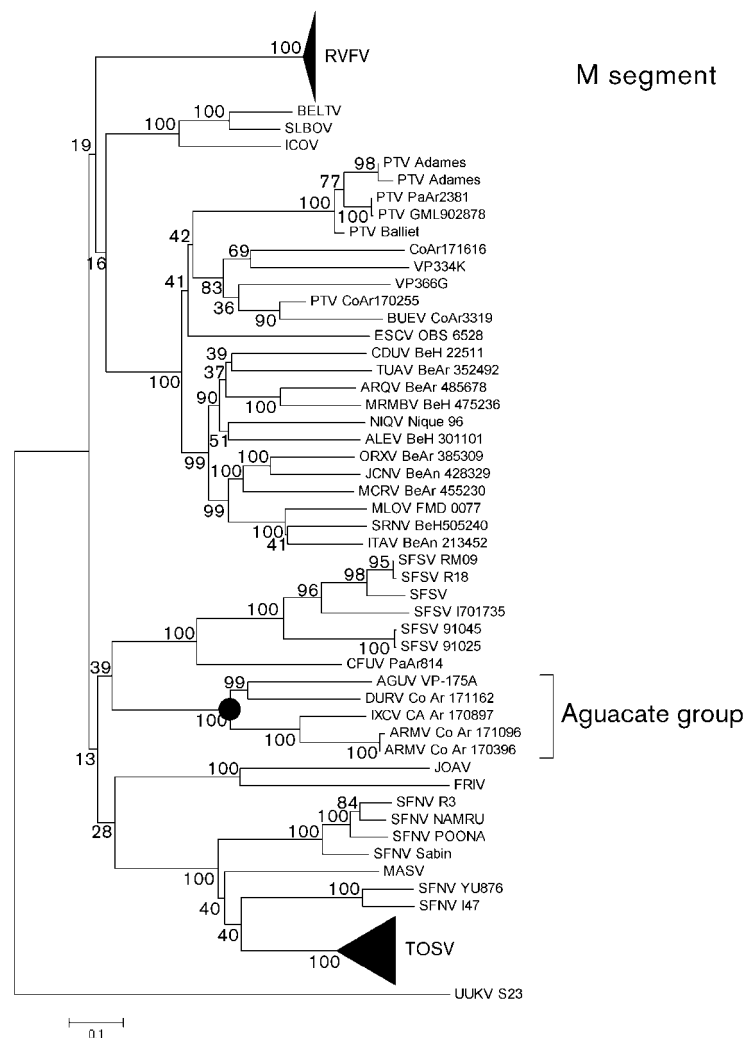


Fig. 2. Phylogenetic analysis of the available phlebovirus M ORF sequences. Neighbour-joining analysis at the amino acid level was performed because of the high variability of the underlying nucleotide sequences. The statistical significance of the tree topology was evaluated by bootstrap resampling of the sequences 1000 times. GenBank accession numbers used in this tree: RVFV (M11157, DQ380199, DQ380202, DQ380201, DQ380200, DQ380207, DQ380206, M25276, DQ380208, DQ380204, DQ380203, DQ380205, DQ380210, DQ380209, DQ380221, DQ380198, DQ380197, DQ380196, DQ380214, DQ380211, DQ380213, DQ380212, DQ380219, DQ380218, DQ380220, DQ380217, DQ380215, DQ380216, DQ380195, DQ380222, DQ380187, DQ380186, DQ380183, DQ380185, DQ380184, DQ380194, DQ380193, DQ380192, DQ380191, DQ380190, DQ380189, DQ380188), BELTV (EF076018), SLBOV (EF076020), ICOV (EF076019), PTV (DQ363407, AY129745, AY129751, AY129747, M11156, AY129746, AY129752, AY129748, AY129750), BUEV (AY129749), ESCV (HM119411), CDUV (HM119408), TUAV (HM119432), ARQV (HM119405), MRMBV (HM119423), NIQV (HM119426), ALEV (HM119402), ORXV (HM119435), JCNV (HM466935), MCRV (HM119420), MLOV (HM119414), SRNV (HM119429), ITAV (HM119417), SFSV (AY129741, AY129742, U30500, AY129743, AY129740, AY129739), CFUV (AY129744), AGUV (HM566138), DURV (HM566156), IXC (HM566162), ARMV (HM566141), ARMV (HQ661806), JOAV (EF076021), FRIV (EF076022), SFNV (AY129736, AY129738, AY129734, AY129733, AY129735, AY129732), MASV (EU725772), UUKV (M17417) and TOSV (AY129737, FJ153284, FJ153283, FJ153282, DQ479891, DQ479903, DQ479906, DQ479890, DQ479914, DQ479912, DQ479898, DQ479910, DQ479907, DQ479909, DQ479916, DQ479913, DQ479915, DQ479892, DQ479893, DQ479900, DQ479902, DQ479899, DQ479908, X89628, DQ479911, DQ479904, DQ479896, DQ479895, DQ479905, DQ479894, DQ479897, DQ479901).

recognized species in the sandfly fever group of phleboviruses are summarized in Table 1. All antisera were reactive with their homologous antigens. Antisera to

AGUV, ARMV, DURV and IXC were markedly cross-reactive with each other in a pattern consistent with a serocomplex comprising these four viruses. Chilibre virus

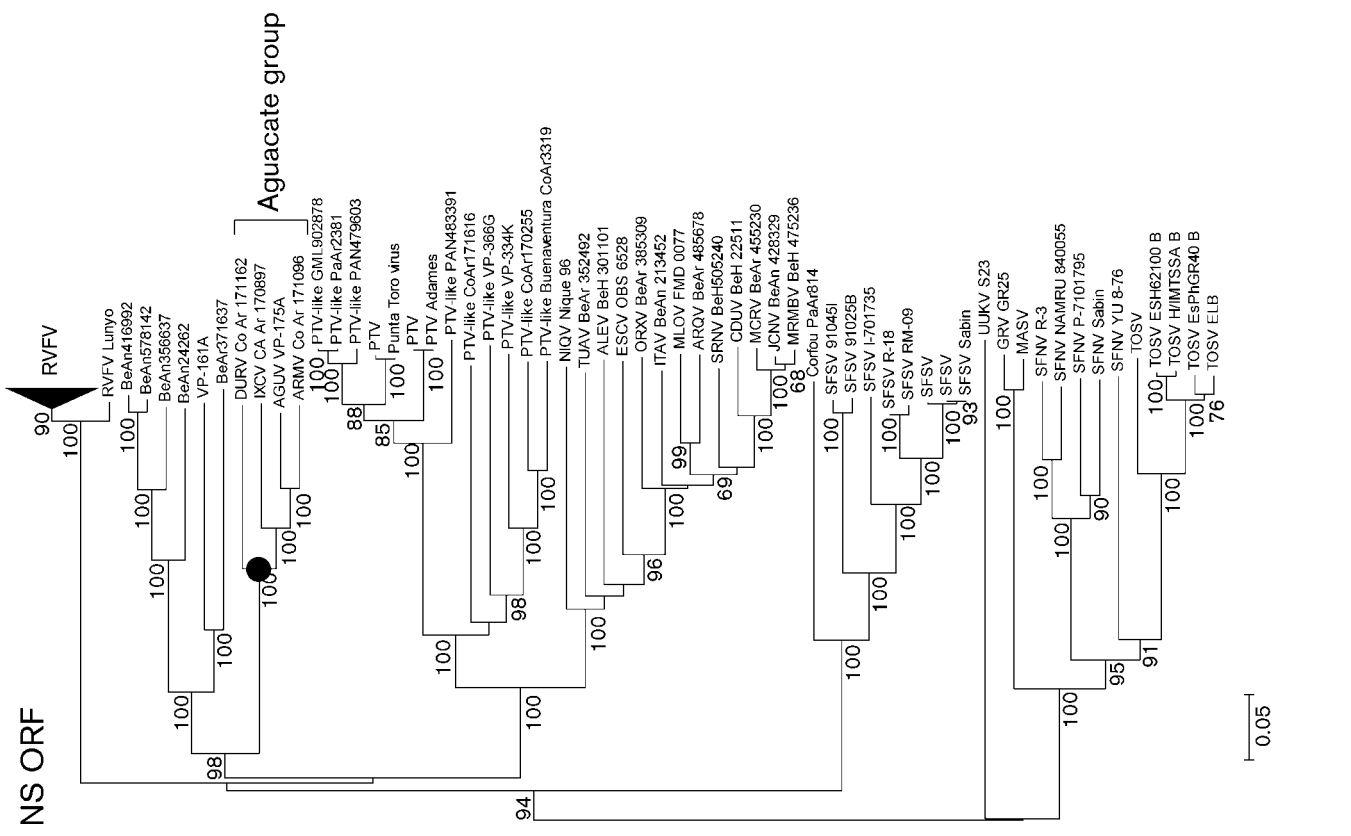
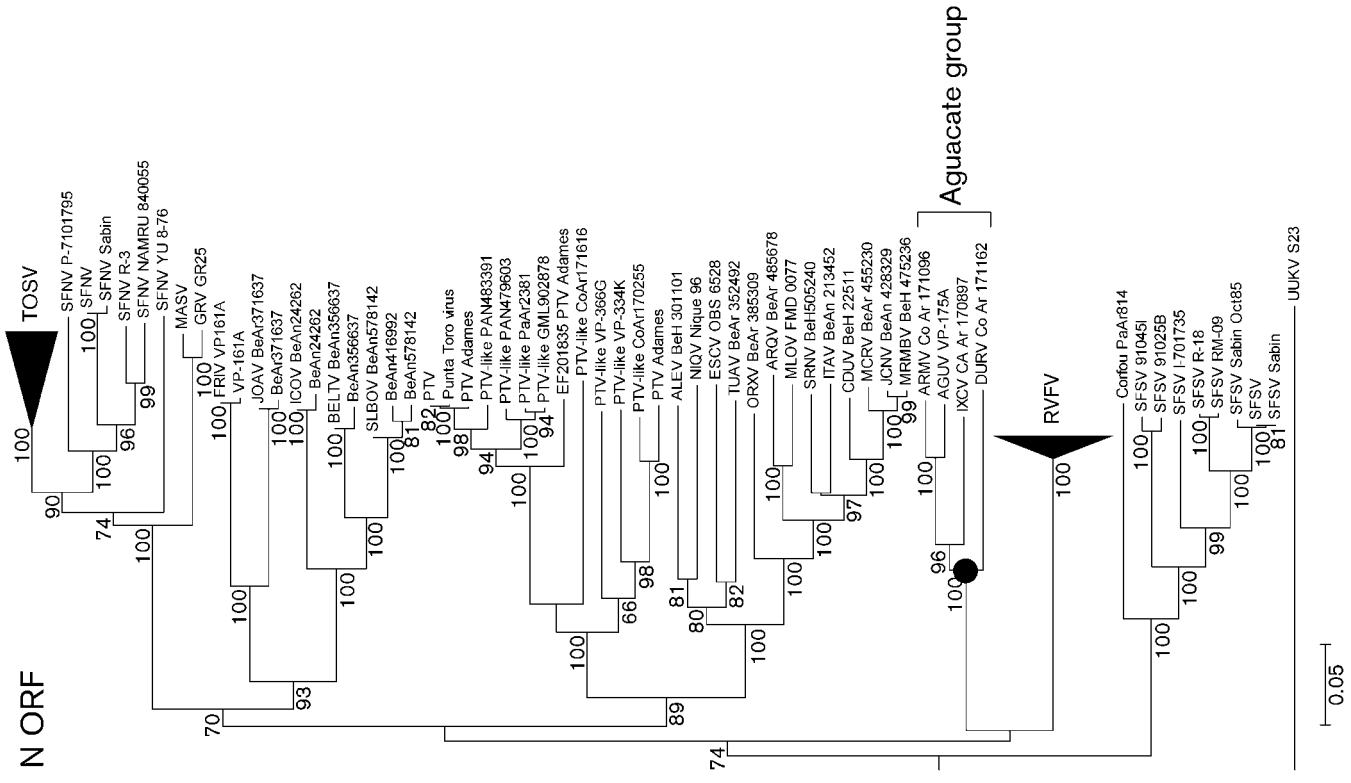


Fig. 3. Phylogenetic analysis of the available phlebovirus N and NS ORF sequences. Neighbour-joining analysis at the amino acid level was performed because of the high variability of the underlying nucleotide sequences. The statistical significance of the tree topology was evaluated by bootstrap resampling of the sequences 1000 times. GenBank accession numbers used in this tree: TOSV (EF120631, AY705941, FJ153285, EF120631, AY705940, AY705935, EF120628, AY705938, EF120632, AY705936, AY705943, AY705942, EF201833, FJ153286, EF120630, AY705934, EF120629, AY705933, AY705939, AY705937, AY766034), SFNV (EF201830, AY705944, EF201829, EF201832, EF201828, EF201831), MASV (EU725773), GRV (GU135608), FRIV (EF076017, EF201819), JOAV (EF076016, EF201820), ICOV (EF076014, EF201818), BELTV (EF076013, EF201817), SLBOV (EF076015, EF201816, EF201815), PTV (K02736, EF201834, DQ363406, EF201844, EF201843, EF201841, EF201837, EF201835, EF201836, EF201838, EF201842, EF201840), ALEV (HM119403), NIQV (HM119427), ESCV (HM119412), TUAV (HM119433), ORXV (HM119436), ARQV (HM119406), MLOV (HM119415), SRNV (HM119430), ITAV (HM119418), CDUV (HM119409), MCRV (HM119421), JCNV (HM466936), MRMBV (HM119424), ARMV (HM566142), AGUV (HM566139), IXCV (HM566163), DURV (HM566157), CFUV (EF201821), SFSV (EF201824, EF201823, EF201827, EF201826, EF201825, AJ811547, J04418, EF201822), UUKV (M33551) and RVFV (DQ380177, DQ380178, DQ380179, DQ380180, DQ380181, DQ380173, DQ380158, DQ380176, DQ380174, EF530207, DQ380175, DQ380159, AF134532, DQ380157, DQ380156, DQ380155, DQ924959, DQ380171, DQ380169, DQ380170, DQ380165, DQ380166, DQ380167, DQ380168, DQ380161, DQ380160, DQ380164, AF134531, DQ380182, DQ380162, DQ380163, DQ380172, DQ380144, DQ380143, DQ380150, DQ380149, DQ380148, DQ380147, DQ380153, DQ380145, DQ380146, AF134535, AF134533, DQ380152, AF134534, DQ380151, X53771, AF134530, X53771, DQ380154).

(CHIV) antigen was strongly reactive with ARMV, DURV and IXCV antibodies, and Bujaru virus (BUJV) antigen was slightly reactive to AGUV, ARMV, DURV and IXCV antibodies. Likewise, CHIV and BUJV antibodies reacted slightly with AGUV and IXCV antigens, but the titres were significantly lower than with the homologous antibodies.

Genomic characterization

AGUV, ARMV, DURV and IXCV all have the genomic organization characteristic of phleboviruses: three RNA segments that include a large (L) segment encoding the RNA polymerase, a medium segment encoding a polyprotein that includes the non-structural protein (NSm) and both glycoproteins (Gn and Gc), as well as a small (S) segment encoding the nucleocapsid (N) protein (NP) and, in ambisense orientation, a non-structural protein (NSs) (GenBank accession nos HM566137–9, HM566140–2, HM566155–7, HM566161–3, HQ661805–7).

In the phylogenetic analysis, major nodes that represented viruses belonging to the same species or antigenic complex were clearly distinct and confirmed previously reported topologies (Charrel *et al.*, 2009; Collao *et al.*, 2009). The four viruses reported here clustered together, defining an *Aguacate virus* antigenic-complex node (Figs 1, 2 and 3). Partial sequencing data for BUJV and CHIV demonstrate that the viruses reported here do not belong to the BUJV antigenic complex (data not shown). Similar topology was observed in phylogenetic trees at the nucleotide level (data not shown). Branching inconsistencies observed inside this node suggested the possibility of reassortment. Therefore, we searched for evidence of reassortment using concatenated full genomes. These analyses indicate that AGUV is a reassortant virus that has obtained its M segment from an as-yet unknown virus, probably of the same antigenic complex (Fig. 4).

ORFs

RNA-dependent RNA polymerase (L). The four members of the *Aguacate virus* antigenic complex show high conservation with previously conserved functional domains described in phlebovirus sequences (Supplementary Figs S1 and S2, available in JGV Online) (Aquino *et al.*, 2003; Palacios *et al.*, 2011; Poch *et al.*, 1989; Xiong & Eickbush, 1990).

Polyprotein (M). The polyprotein is cotranslationally cleaved into three protein products: NSm, Gn and Gc. The topology of the polyprotein of the *Aguacate virus* antigenic complex is similar to the predicted topology for TOSV, PTV, RVFV and CDUV (Gerrard & Nichol, 2002; Grò *et al.*, 1997; Ihara *et al.*, 1985; Matsuoka *et al.*, 1996; Valentini *et al.*, 2008). Signal sequences, transmembrane domains and predicted cleavage sites for the protein products are conserved (Supplementary Fig. S3, available in JGV Online).

NP. Similarly, all the functional domains described in the NP are conserved (Gerrard & Nichol, 2002; Le May *et al.*,

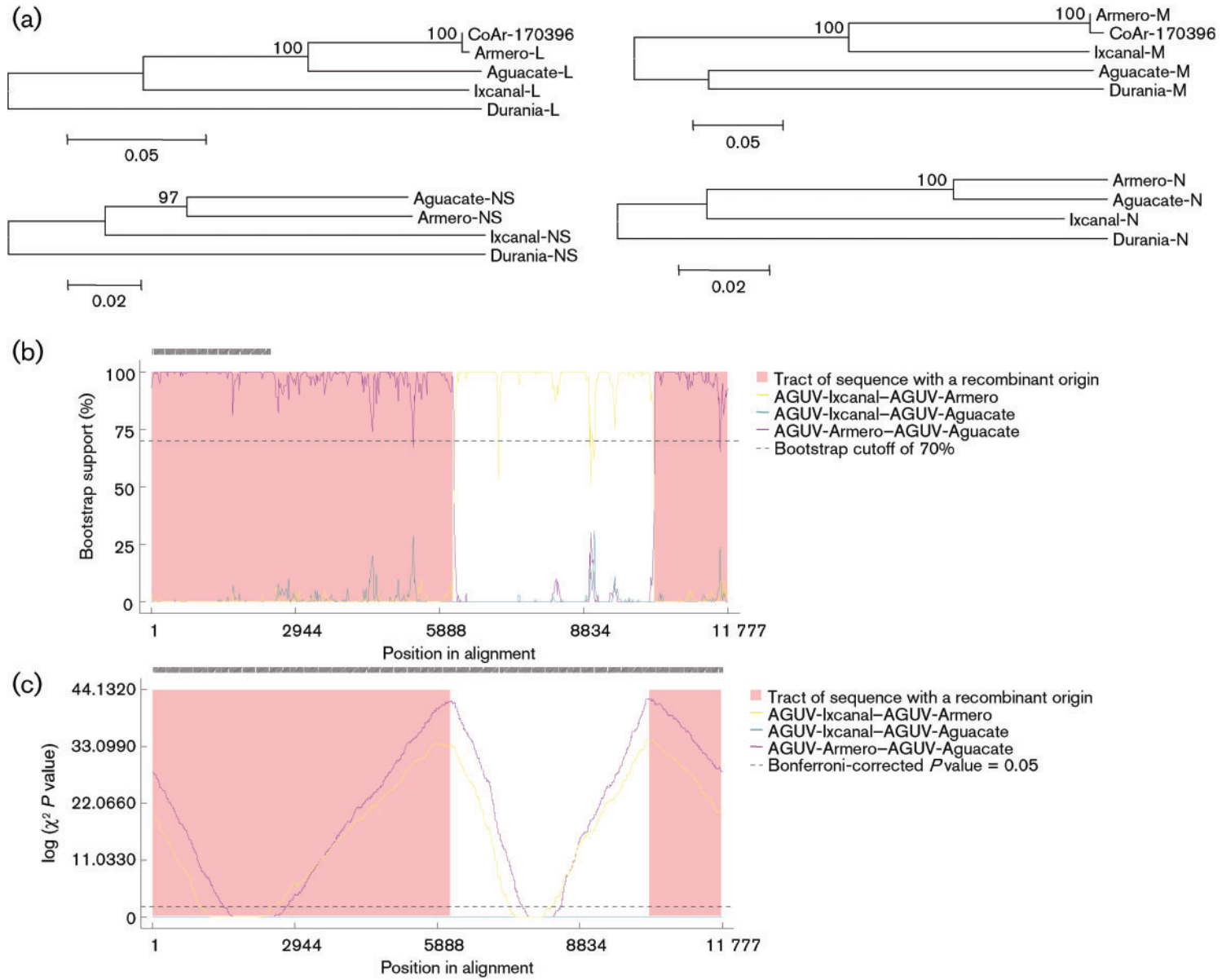


Fig. 4. Evidence supporting AGUV as a reassortant. (a) Phylogenetic, (b) BOOTSCAN and (c) CHIMERA analysis all demonstrate AGUV as being a reassortant.

2005; Palacios *et al.*, 2011) (Supplementary Fig. S4, available in JGV Online).

DISCUSSION

Despite the clinical importance of phleboviruses as pathogens of both humans and livestock, we have only limited insight into their phylogenetic diversity. Until now, speciation of phleboviruses has been largely driven by antigenic studies. However, it is no longer feasible to test newly isolated phleboviruses by serology against all other known members of the genus because of their abundance and diversity, the frequency of recombination events (Collao *et al.*, 2010; Palacios *et al.*, 2011) and the fact that some of these viruses do not produce readable plaques in cell culture or produce illness in newborn mice. Thus, high-throughput sequencing offers another option for their characterization and classification. We are presently sequencing the genomes of the known phleboviruses to determine their taxonomic relationships; this is one of several planned publications on our findings.

The results of this study demonstrate that AGUV, ARMV, DURV and IXCv are closely related to one another in terms of sequence and serology and that they are more distantly related to members of the other nine recognized species within the genus *Phlebovirus*. We propose that this warrants their designation as a new (tenth) species (*Aguacate virus*) within the genus. Interestingly, the prototype Aguacate virus strain (VP-175A) appears to be a natural reassortant among the members of the serological complex. Reassortment among RNA segments of related viruses in the family *Bunyaviridae* (including phleboviruses) has been reported before (Beatty *et al.*, 1985; Briese *et al.*, 2006, 2007; Collao *et al.*, 2010; Dunn *et al.*, 1994; Nunes *et al.*, 2005; Palacios *et al.*, 2011; Saeed *et al.*, 2001); it is thought to be a mechanism which permits rapid evolution of viruses in this taxon. Although Bird *et al.* (2008) characterized the diversity of RVFV as being low, reassortment may explain the abundance and diversity observed between different phleboviruses circulating in nature and the difficulty in their precise identification and taxonomic classification.

METHODS

Viruses

The phlebovirus strains used in this study were Aguacate (AGUV) strain VP-175A, Armero (ARMV) strains Co Ar 171096 and Co Ar 170396, Durania (DURV) strain Co Ar 171162, Ixcanal (IXCV) strain CA Ar 170897, Bujaru (BUJV) Be An 47693, Candiru (CDUV) Be H 22511, Chilibre (CHIV) strain VP-118D, Frijoles (FRIV) strain VP-161A, Punta Toro (PTV) strain D 4021A, Rift Valley fever (RVFV) strain MP-12, Salehabad (SALV) strain I-81 and sandfly fever Naples (SFNV) Sabin strain. All virus stocks were obtained from the World Reference Center for Emerging Viruses and Arboviruses at the University of Texas Medical Branch.

AGUV. Multiple isolates of AGUV were made from separate pools of male and female sandflies (*Lutzomyia* sp.) collected in secondary

tropical forest at two localities (El Aguacate, central Panama, and Limbo, in the Panama Canal Zone) in Panama between 1969 and 1971 (Tesh *et al.*, 1974). The prototype strain (VP-175A) was isolated in Vero cell culture. Initially, it did not produce illness or death in newborn mice, but it was subsequently adapted by serial intracerebral (ic) blind passage (Tesh *et al.*, 1974).

ARMV. Strains Co Ar 171096 and Co Ar 170396 were isolated in Vero cells from pools of female sand flies (*Lutzomyia* sp.) collected in a secondary forest in the municipality of Mariquita, Department of Tolima, Colombia, in 1986 (Tesh *et al.*, 1989). It produced illness in newborn mice approximately 9 days after ic inoculation.

DURV. Strain Co Ar 171162 was isolated in Vero cells from a pool of female sand flies (*Lutzomyia* sp.) collected in 1986 from a coffee plantation 8 km from the town of Durania, Norte de Santander Department, Colombia (Tesh *et al.*, 1989). On first passage in infant mice, it produced hind-limb paralysis and death within 13 days of ic inoculation (Tesh *et al.*, 1989).

IXCV. Strain CA Ar 170897 was isolated in Vero cells from a pool of male sand flies (*Lutzomyia* sp.) collected in 1982 in the vicinity of two small villages (Aldeas Ixcanal and Puerto Progreso) in the riparian habitat of the Cato river valley in Guatemala. IXCv did not initially produce disease in newborn mice, but it was subsequently adapted by serial passage (Tesh *et al.*, 1989).

Antigens and immune reagents. Methods used to prepare antigens for the CF tests and immune ascitic fluids have been described previously (Beatty *et al.*, 1989; Travassos da Rosa *et al.*, 1983; Xu *et al.*, 2007). Antigens and antibodies were both prepared in mice.

CF tests. CF tests were performed by using the microtitre technique (Beatty *et al.*, 1989; Xu *et al.*, 2007), using 2 U of guinea pig complement and overnight incubation of the antigen and antibody at 4 °C. CF titres were recorded as the highest dilutions giving 3+ or 4+ fixation of complement (0–25 % haemolysis).

Genome sequencing. Viral stocks were extracted using TRIzol LS (Invitrogen). Total RNA extracts were treated with DNase I (DNA free; Ambion). cDNA was generated using a Superscript II system (Invitrogen) employing random hexamers linked to an arbitrary 17-mer primer sequence (Palacios *et al.*, 2007). The 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 (Palacios *et al.*, 2007). Products >70 bp were selected by column chromatography (MiniElute; Qiagen) and ligated to specific adapters for sequencing on a 454 Genome Sequencer FLX (454 Life Sciences) without fragmentation (Cox-Foster *et al.*, 2007; Margulies *et al.*, 2005; Palacios *et al.*, 2008). 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 by using primers based on pyrosequencing data. Amplification products were size-fractionated on 1% agarose gels, purified (MiniElute; Qiagen) and directly sequenced in both directions with ABI Prism Big Dye Terminator 1.1 Cycle Sequencing kits on ABI Prism 3700 DNA Analyzers (Perkin-Elmer Applied Biosystems). For the termini of each segment, a primer with the 8 nt conserved sequence was used for a specific reverse transcription reaction with additional arbitrary nucleotides on the 5' end (5'-AAGCAGTGGTATCAACGAGAGTACACA-CAAAG-3'; the boldface portion indicates the conserved nucleotides). This primer is designed to bind to the 3' end of the genomic RNA and the 3' end of the mRNA. The sequences of the genomes were verified by classical dideoxy sequencing by using primers designed from the draft sequence to create products of 1000 bp with 500 bp overlap.

Phylogenetic analysis A set of phlebovirus sequences (70 for the L segment, 122 for the M segment, 131 for the N gene and 98 for the NS gene), comprising all sequences available from GenBank (June 2010), was used to determine the phylogenetic relationships of AGUV, ARMV, DURV and IXC. All sequences were aligned using the CLUSTAL algorithm (as implemented in MEGA version 3) at the amino acid level, with additional manual editing to ensure the highest possible quality of alignment. Neighbour-joining analysis at the amino acid level was performed because of the observed high variability of the underlying nucleotide sequences. Nucleotide trees were also produced using neighbour-joining analysis and the Kimura two-parameter model. The statistical significance of tree topology was evaluated by bootstrap resampling of the sequences 1000 times. Phylogenetic analyses were performed using MEGA software (Kumar *et al.*, 2004).

Detection of recombination events. Systematic screening for the presence of recombination patterns was achieved by using the nucleotide alignments and the recombination detection program (RDP) (Martin & Rybicki, 2000). The algorithms BOOTSCAN (Salminen *et al.*, 1995), MaxChi (Smith, 1992), CHIMAERA (Posada & Crandall, 2001), LARD (Holmes, 1998) and PHYLIP Plot (Felsenstein, 1989) were also used for the detection of recombination.

Sequence analysis. GENEIOUS 4.8.3 (Biomatters) was used for sequence assembly and analysis. Topology and targeting predictions were generated by employing SignalP, NetNGlyc, TMHMM (<http://www.cbs.dtu.dk/services>), TopPred2 (<http://mobyle.pasteur.fr/cgi-bin/portal.py?#forms:toppred>), and integrated predictions in Geneious (Bendtsen *et al.*, 2004; Claros & von Heijne, 1994; Kahsay *et al.*, 2005; Käll *et al.*, 2004; Krogh *et al.*, 2001).

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