

Genetic and biological characterization of selected Changuinola viruses (*Reoviridae*, *Orbivirus*) from Brazil

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The genus *Orbivirus* of the family *Reoviridae* comprises 22 virus species including the Changuinola virus (CGLV) serogroup. The complete genome sequences of 13 CGLV serotypes isolated between 1961 and 1988 from distinct geographical areas of the Brazilian Amazon region were obtained. All viral sequences were obtained from single-passaged CGLV strains grown in Vero cells. CGLVs are the only orbiviruses known to be transmitted by phlebotomine sandflies. Ultrastructure and molecular analysis by electron microscopy and gel electrophoresis, respectively, revealed viral particles with typical orbivirus size and morphology, as well as the presence of a segmented genome with 10 segments. Full-length nucleotide sequencing of each of the ten RNA segments of the 13 CGLV serotypes provided basic information regarding the genome organization, encoded proteins and genetic traits. Segment 2 (encoding VP2) of the CGLV is uncommonly larger in comparison to those found in other orbiviruses and shows varying sizes even among different CGLV serotypes. Phylogenetic analysis support previous serological findings, which indicate that CGLV constitutes a separate serogroup within the genus *Orbivirus*. In addition, six out of 13 analysed CGLV serotypes showed reassortment of their genome segments.

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The GenBank/EMBL/DDBJ accession numbers for the nucleotide sequences (S1 to S10) obtained for each of the 13 viral isolates are KF680168–KF680177, KF624614–KF624623, KF680178–KF680187, KF690591–KF690600, KF680188–KF680197, KF690601–KF690610, KF668547–KF668556, KF668527–KF668536, KF668557–KF668566, KF690611–KF690620, KF668537–KF668546, KF690581–KF690590 and KF690621–KF690630.

Four supplementary tables and 11 supplementary figures are available with the online version of this paper.

INTRODUCTION

The genus *Orbivirus* is one of the 15 distinct genera currently included in the family *Reoviridae*; the genus contains 22 distinct virus species recognized by the International Committee for the Taxonomy of Viruses (ICTV) (Attoui *et al.*, 2012). Viruses included in the genus *Orbivirus* are transmitted by culicoid midges, ticks, phlebotomine sandflies, as well as anopheline and culicine mosquitoes. Orbiviruses are icosahedral, non-enveloped and have a

genome consisting of 10 double-stranded RNA (dsRNA) segments encoding seven structural proteins (VP1–VP7), and at least three non-structural proteins (NS1–NS3) (Verwoerd *et al.*, 1972).

Insect-borne orbiviruses that have been reported to infect humans belong to the species *Changuinola virus* (CGLV), *Corriparta virus* (CORV), *Lebombo virus* and *Orungo virus* (Attoui *et al.*, 2005), while the human pathogenic tick-borne orbivirus species include Kemerovo virus (KEMV), Tribeč virus (TRBV) and Lipovnik virus (Dilcher *et al.*, 2012).

CGLV (BT-436) is the prototype of the CGLV serogroup and was originally isolated from a pool of phlebotomine sandflies (*Lutzomyia* sp.) collected in Panama in 1960. A second antigenically related agent, Irituia virus (BE AN 28873), was recovered from the serum of a rice rat (*Oryzomys goeldi*) collected in 1961 in the Brazilian Amazon Basin in the municipality of Ipixuna, state of Pará, Brazil (3° S 49° W). Subsequently, many additional isolates of Changuinola serogroup viruses have been obtained from sandflies and sloths in Panama, Colombia and other regions of Brazil (Seymour *et al.*, 1983; Tesh *et al.*, 1974; Travassos da Rosa *et al.*, 1984, 1998).

A single isolate of CGLV was also obtained from the blood of a febrile entomology field worker in Panama in 1966. To date, this is the only reported case of human illness associated with CGLV infection (Karabatsos, 1985; Peralta & Shelokov, 1966).

The Changuinola serogroup consists of a large number of antigenically related viruses that have been isolated from phlebotomine sandflies, mosquitoes and various species of wild mammals. To date, these viruses have been found only in Panama and tropical regions of South America, and they are presumed to be arthropod-borne (Travassos da Rosa *et al.*, 1984). By complement fixation (CF) test, the Changuinola group viruses are broadly cross-reactive and cannot be differentiated; consequently most of the available isolates have never been characterized beyond the serogroup level. Based on mouse neutralization tests, the Panamanian prototype BT-436 and 11 Brazilian isolates were shown to differ antigenically, and 12 distinct CGLV serotypes have now been recognized by the ICTV. These include Changuinola, Almeirim, Altamira, Caninde, Gurupi, Irituia, Jamanxi, Jari, Monte Dourado, Ourem, Purus and Saraca viruses (Attoui *et al.*, 2012). However, there are most likely more, since many of the existing isolates have never been examined by neutralization testing.

The first complete genome sequence of a CGLV serotype, Irituia virus (BE AN 28873), was published in 2013 (Silva *et al.*, 2013). The full genome sequence of a second serotype, Xaraira virus (BE AR 490492), was published in 2014 (Jaafar *et al.*, 2014). In the present study, we determined the full genome sequence of all 10 segments of Irituia, Caninde, Jari, Ourem, Saraca and Gurupi viruses

and seven additional CGLV serotypes isolated from wild animals and phlebotomine sandflies in the Brazilian Amazon region between 1961 and 1988. Analysis of their complete genomes and phylogenetic relationships indicate that the Changuinola group represents a genetically diverse species with multiple serotypes, many of which show signs of reassortment.

RESULTS

Electron microscopy and electrophoretic profile

Ultra structural analysis of ultrathin sections of Vero cells infected with Irituia virus (BE AN 28873) showed icosahedral particles in the cytoplasm, with a mean diameter of 75 nm. These particles were observed individually and aggregated (viroplasm) in the cytoplasm (Fig. 1). Analysis of the RNA electrophoretic profile of ten CGLVs, using agarose gel electrophoresis, revealed the presence of ten genome segments that were classified according to their molecular mass into three distinct groups: group 1, high molecular mass (S1–S3); group 2, intermediate molecular mass (S4–S6); and group 3, low molecular mass (S7–S10) (Fig. 2).

Genetic characterization

Full-length sequences were obtained for all 13 viral isolates listed in Table 1 for each of the ten dsRNA segments. The overall coverage of the genomes and the number and percentage of virus-specific reads are summarized in Table S1 (available in the online Supplementary Material). The analysis of similarity and homology with nucleotide and amino acid sequences available in the GenBank database (<http://blast.st-va.ncbi.nlm.nih.gov/Blast.cgi>) and in the InterProScan database (<http://www.ebi.ac.uk/Tools/pfa/iprscan/>) demonstrated that the nucleotide and amino

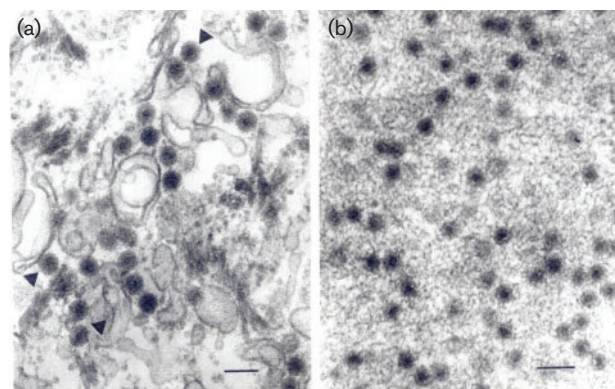


Fig. 1. Electron micrographs of serotype Irituia virus (BE AR 28873). (a) Virions 75 nm in diameter in the cytoplasm of a Vero cell (arrowheads). (b) A portion of a fibrillar aggregate (viroplasm) in the cytoplasm of a Vero cell with forming viruses. Bars, 100 nm.

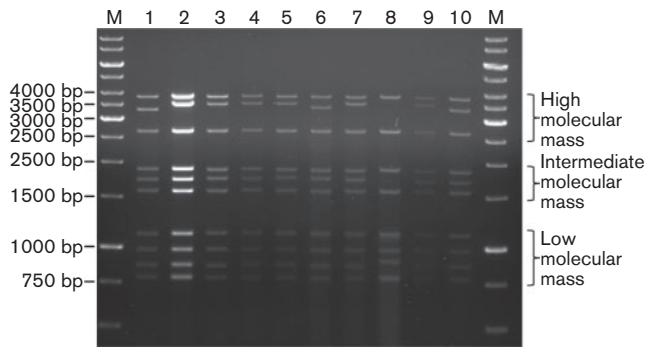


Fig. 2. Agarose gel electrophoretic profiles of selected CGLV serotypes. Lane M: molecular mass marker (size is shown in bp). BE AR 35646 (lane 1); BE AN 28873 (lane 2); BE AR 425269 (lane 3); BE AR 434080 (lane 4); BE AR 478620 (lane 5); BE AR 440489 (lane 6); BE AR 440541 (lane 7); BE AR 385278 (lane 8; in this lane, segments 2 and 5 had very low concentrations); BE AR 397956 (lane 9); BE AR 397374 (lane 10).

acid sequences of the CGLVs match with other known orbivirus genomes and proteins. The nucleotide sequences (S1 to S10) and respective proteins (VP1 to VP7 and NS1 to NS3) were deposited in GenBank (Table 1).

The predicted proteins ranged from 1309 aa (segment 1; VP1) to 231 aa (segment 10; NS3) in length (Table S2). Among the different CGLV serotypes, the length of some genome segments and the length of the encoded proteins varied significantly, whereas for other genome segments the length was rather constant (Table S2). The length of segments S1, S3, S4, S6 and S7 varied only by about 1 bp

among the different serotypes, leading to encoded proteins of identical length. In contrast, the length of segment 2 varied by about 245 nt, yielding VP2 proteins with size differences of about 78 aa. In addition, the length of segment 9 in BE AR 385278, with 940 nt, was significantly longer than that of segment 9 in the other CGLV isolates (see PAGE in Fig. 2), yielding a VP6 (Hel) protein that is 10 to 12 aa bigger than other CGLV VP6 (Hel) proteins. Size differences can also be seen in segment 5, where this segment is 52 nt shorter in BE AN 385199 than the longest segment 5 sequence. Regarding the non-coding regions (NCRs), the 5' termini were composed of short sequences ranging from 7 bp for segment 4 (VP4) to 34 bp for segment 5 (NS1). The 3' termini were longer, with NCRs ranging from 32 bp for segment 2 (VP2) to 108 bp for segment 5 (NS1).

Analysis of the 5' and 3' NCRs demonstrated that all of the segments share five conserved nucleotides at their 5' and 3' ends (5'-GUAAA----CUUAC-3'). Segment 2, which encodes the outer capsid protein VP2, showed the highest genetic variability among the 13 studied CGLV serotypes, with only 19.2% identity (712 nt). The amino acid alignment showed 17.1% identity. The size of segment 2 is rather variable, ranging from 3450 to 3695 nt in the different CGLV serotypes. Furthermore, the G + C content of CGLV serotypes showed values ranging between 33.1 and 42.1 mol%.

All CGLV serotypes encode the helicase protein on segment 9 (VP6, ORF +2); however, an additional putative protein was also found to be encoded on the same segment but in a different reading frame (ORF +3). This putative protein ranged from 87 aa (ORF of 261 nt) to 89 aa (ORF of 267 nt) in length with a mean identity of 59.4% (from

Table 1. CGLV serotypes isolated from 1961 to 1988 in the Amazon region of Brazil

Prototype number	Serotype	GenBank accession numbers	Isolation information		
			Host	Date	Material (place*)
BE AR 35646	Gurupi virus	KF680168–KF680177	<i>Phlebotominae</i> spp.	1961	Pool (BR 14, km 94)
BE AN 28873	Irituia virus	KF624614–KF624623	<i>Oryzomys goeldi</i>	1961	Blood (BR 14, km 94)
BE AR 41067	Ourem virus	KF680178–KF680187	<i>Phlebotominae</i> spp.	1962	Pool (BR 14, km 94)
BE AR 54342	Caninde virus	KF690591–KF690600	<i>Phlebotominae</i> spp.	1963	Pool (BR 14, km 94)
BE AN 385199	Jari virus	KF680188–KF680197	<i>Choloepus didactylus</i>	1980	Viscera (Jari, Monte Dourado)
BE AR 385278	Saraca virus	KF690601–KF690610	<i>Phlebotominae</i> spp.	1980	Pool (Porto de Trombetas, Oriximiná)
BE AR 397956	Tumucumaque virus	KF668547–KF668556	<i>Lutzomyia umbratilis</i>	1981	Pool (Jari, Monte Dourado)
BE AR 397374	Jutai virus	KF668527–KF668536	<i>Lutzomyia umbratilis</i>	1981	Pool (Jari, Monte Dourado)
BE AR 425269	Aracai virus	KF668557–KF668566	<i>Phlebotominae</i> spp.	1984	Pool (Tucuruí)
BE AR 434080	Tapirope virus	KF690611–KF690620	<i>Phlebotominae</i> spp.	1984	Pool (Serra Norte, Marabá)
BE AR 440489	Jandia virus	KF668537–KF668546	<i>Phlebotominae</i> spp.	1985	Pool (Tucuruí)
BE AR 440541	Timbozal virus	KF690581–KF690590	<i>Phlebotominae</i> spp.	1985	Pool (Tucuruí)
BE AR 478620	Balbina virus	KF690621–KF690630	<i>Phlebotominae</i> spp.	1988	Pool (Balbina)

*All virus isolates were obtained in the state of Pará, except for Balbina virus, which was recovered at the Balbina hydro-electric dam in Balbina, Amazonas state. Kilometre markers are indicated for locations on Brazil road BR 14.

33.7% to 97%). The most divergent strain was the Saraca virus (BE AR 385278), showing a mean identity of 36.15%.

Phylogenetic analysis

Phylogenetic reconstruction based on multiple alignments of VP1 amino acid sequences generated trees with similar topologies regardless of the method used. In this case, the maximum likelihood (ML) tree was selected as the best phylogenetic tree to represent the genetic relationship for CGLVs, as it was the one that best represented the known taxonomy. The analysis using the VP1 protein (segment 1, viral polymerase) confirmed that the CGLVs belong to the genus *Orbivirus* and are more closely related to Bluetongue virus (BTV), Epizootic hemorrhagic disease virus (EHDV), Pata virus (PATV), Eubenberg virus (EUBV) and Tilligerry virus (TILV), which are all *Culicoides*-borne orbiviruses (Fig. 3).

The phylogenetic relationship based on the amino acid sequences of orbivirus T2 proteins (segment 3 for *Culicoides*-borne viruses and segment 2 for mosquito-borne and tick-borne orbiviruses) demonstrated that the CGLV serotypes studied belong to a new group near the *Culicoides*-borne group (Fig. 4). Figs S1 to S8 summarize the genetic relationships for the proteins encoded by the other genome segments.

Genetic reassortment

The analysis of genetic reassortment demonstrated that six out of 13 CGLVs showed strong phylogenetic permutation signs (>90%) involving at least one reassorted RNA segment: Ourem virus (BE AR 41067), Tapirope virus (BE AR 434080), Jandia virus (BE AR 440489), Tumucumaque virus (BE AR 397956), Jari virus (BE AN 385199) and Jutai virus (BE AR 397374) (Figs 5, S9 and S10). Reassortment events mainly involved the segments S2, S4, S5, S8, S9 and S10 (encoding VP2, VP4, NS1, NS2, VP6 and NS3, respectively). No reassortment event was observed for S1, S3, S6 or S7 (encoding VP1, VP3, VP5 and VP7 proteins, respectively).

DISCUSSION

Between 1960 and 1990, a large number of arthropod-borne viruses were isolated in the Brazilian Amazon region and described as new members of the Changuinola serogroup (Travassos da Rosa *et al.*, 1998). In the past, arbovirus classification was based on several criteria, which included virion morphology and size, the nature of the RNA (segmented, non-segmented, ssRNA or dsRNA) and antigenic relationships. More recently, the analysis of genetic relationships within a given group of viruses, using a specific signature gene (e.g. polymerase gene) has been used (Attoui *et al.*, 2005).

Based on these criteria, 12 different serotypes are currently recognized within the Changuinola species complex (Attoui

et al., 2012). This classification is supported by the icosahedral ultrastructure of the virions (Fig. 1), electrophoretic profiles demonstrating 10 dsRNA segments (Fig. 2), and their genetic relationship with other members of the genus *Orbivirus* on the basis of phylogenetic reconstructions using the amino acid sequences of the polymerase and T2 proteins (Figs 3 and 4). These molecular and structural data are in agreement with antigenic, biological, and chemical properties described previously for members of the Changuinola serogroup (Jaafar *et al.*, 2014; Silva *et al.*, 2013; Travassos da Rosa *et al.*, 1984).

The VP2 protein is the most variable protein found in orbiviruses (Attoui *et al.*, 2012). This outer shell protein is associated with virus antigen specificity, as well as with virus–host cell attachment, virulence, and virus entry into host cells (Hwang & Li, 1993; Kahlon *et al.*, 1983). High genetic divergence and size heterogeneity have been observed among VP2 proteins from mosquito, *Culicoides* and tick-associated orbiviruses. In fact the VP2 proteins of tick-transmitted orbiviruses are half the size of those of mosquito- and *Culicoides*-associated orbiviruses (Schoehn *et al.*, 1997).

In the case of the Brazilian CGLV isolates, the VP2 proteins are the largest VP2 proteins of all known orbiviruses. Considerable segment 2 size heterogeneity was even found among different CGLV serotypes (Table S2) reflected in proteins of different sizes. For BE AR 35646, BE AR 385278, BE AR 54342, BE AR 397374 and BE AR 440489 respective segment 2 sizes of 3450 nt, 3450 nt, 3503 nt, 3536 nt and 3548 nt are 113–245 nt smaller than in the segments 2 of the remaining Changuinola serotypes (3661–3695 nt). These differences could be related to viral protein selection and evolutionary adaptation to a given host. Although many conserved motifs were found among CGLV isolates, unique sequences or motifs were observed in the region of VP2 comprising amino acid positions 210–380 aa (Fig. S11). Furthermore, three isolates BE AR 35646 (Gurupi virus), BE AR 54342 (Caninde virus) and BE AR 385278 (Saraca virus) had a deletion of 70 aa in their VP2. It is important to assess how this deletion might affect VP2 structure and function.

Cysteine residues are unique among coding amino acids because they contain a reactive sulfhydryl group. Therefore, two cysteine residues may form a cystine (disulfide link) between various parts of the same protein or between two separate polypeptide chains (Miseta & Csutora, 2000). Several cysteine residues were found to be conserved among the CGLV VP2 proteins (Fig. S9). This suggests that the basic structure of the VP2 protein, and consequently its function, remains unchanged for the Brazilian CGLV serotypes. However, further studies are necessary to assess and validate the antigenic sites of the CGLV group members.

In addition to the viral helicase VP6 (reading frame +2), segment 9 of CGLV seems to code for an additional putative protein, since all CGLV strains contain a 261 to

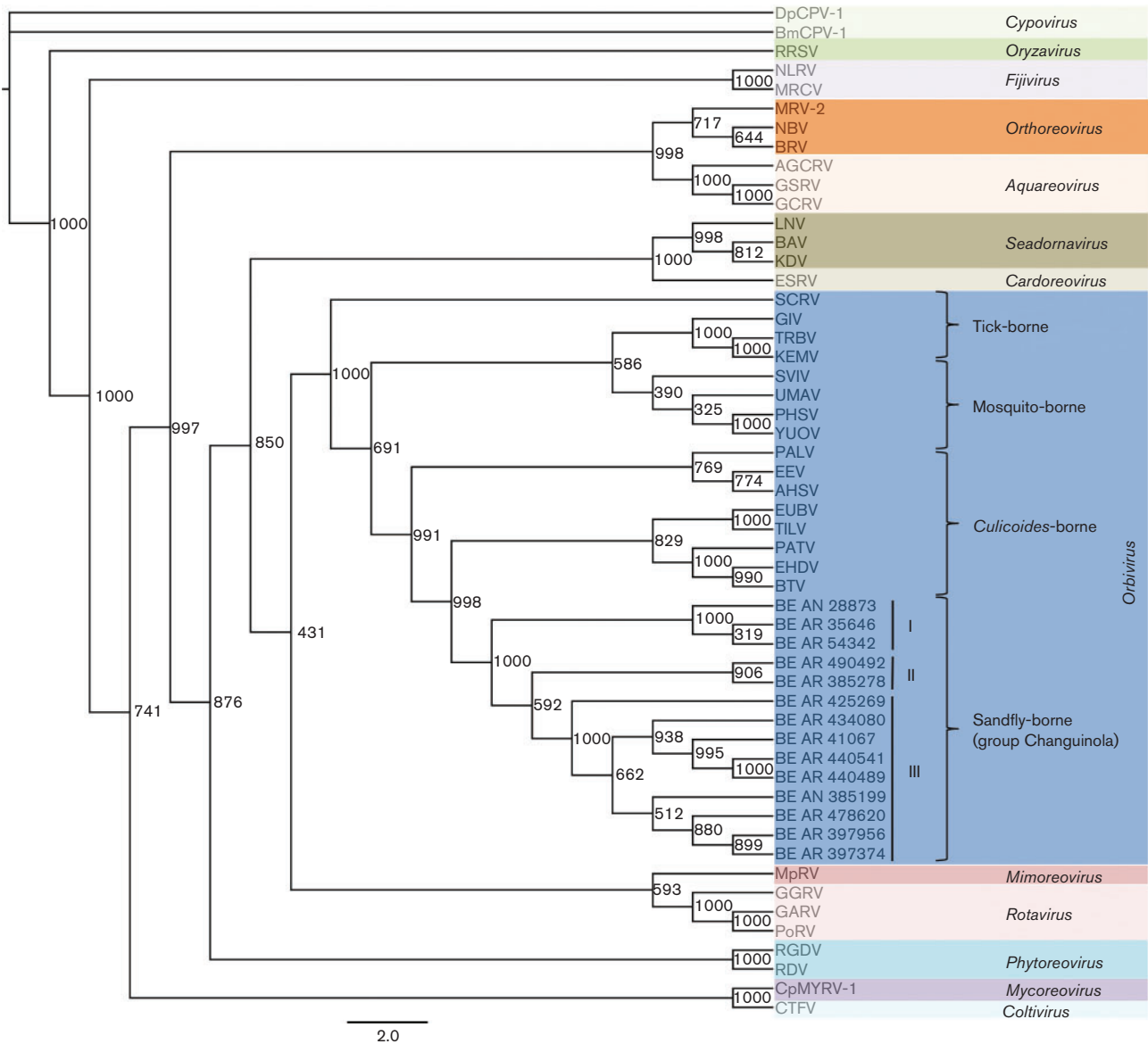


Fig. 3. Phylogenetic analysis using the ML method of different genera within the family *Reoviridae*. Based on the complete amino acid sequences of the viral RNA polymerase (VP1). Different phylogenetic groups are labelled in different coloured boxes. The genus *Orbivirus* is represented as a separate group (dark blue) within the tree. Numbers at each main node of the tree correspond to bootstrap values (1000 replicates). Viral abbreviations and GenBank accession numbers are available in Table S3. The scale bar corresponds to 2% of genetic divergence among amino acid sequences.

267 nt long ORF (reading frame +3) overlapping VP6 at the same position. VP6 overlapping ORF's in different reading frames have been discovered in several other orbivirus genomes (Belhouchet *et al.*, 2010; Dilcher *et al.*, 2012; Firth, 2008). For *Culicoides*-borne Bluetongue virus (BTV) and tick-borne Great Island virus (GIV) it has been shown that this VP6 overlapping ORF encodes a fourth non-structural protein, NS4, which can also be detected in infected mammalian cells, where it seems to counteract the antiviral response of the host. In addition it has been shown that it protects DNA from degradation by DNase,

indicating an ability to bind dsDNA (Belhouchet *et al.*, 2011; Ratnien *et al.*, 2011).

The genetic relationship of the genus *Orbivirus* in the *Reoviridae* was previously analysed using basic information on both polymerase (VP1) and T2 proteins (Attoui *et al.*, 2009). The viral polymerase protein has the highest degree of genetic conservation among the orbiviruses, and has been used for taxonomic classification (Kapoor *et al.*, 2013; Vieira *et al.*, 2009). The phylogenetic analysis of the VP1 proteins performed in this study, in comparison to other reoviruses, groups the Brazilian CGLV isolates as members

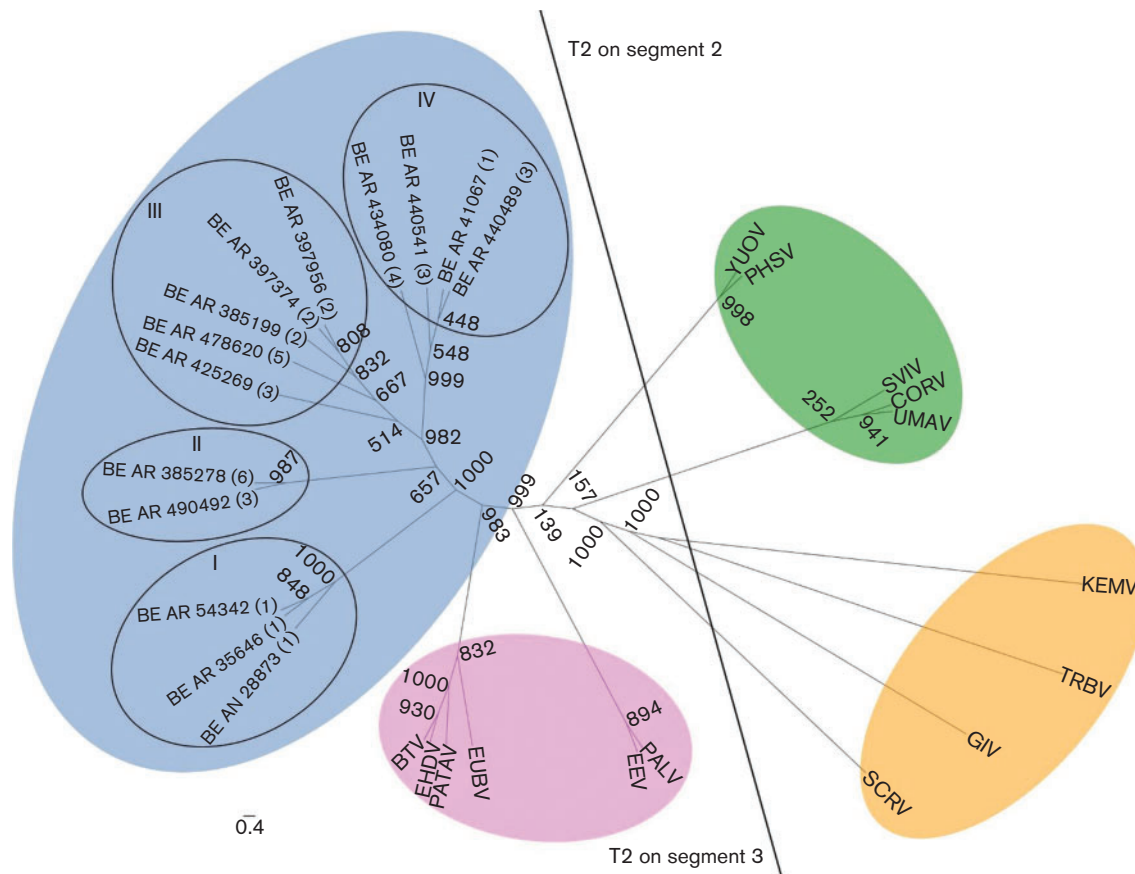


Fig. 4. Relationships between complete amino acid sequences of T2 proteins of different orbiviruses. Maximum-likelihood unrooted tree showing distinct phylogenetic groups based on the T2 protein. Group 'T2 on segment 3' includes viruses in which the T2 protein [in this case VP3 (T2)] is encoded on segment 3: *Culicoides*-borne (pink) and sandfly-borne (blue) viruses composed of different serotypes of the Changuinola group. Group 'T2 on segment 2' includes viruses in which the T2 protein [in this case VP2 (T2) or VP3 (T2)] is encoded on segment 2: mosquito-borne (green) and tick-borne (orange). Bootstrap values are placed at each main node of the tree. Roman numerals (I to IV) represent the phylogenetic lineages. Arabic numerals (1 to 5) correspond to different geographical locations from where the viruses were isolated. (1) Belém–Brasília highway km 94, Pará state; (2) Monte Dourado/Jari, Pará state; (3) base 4/Tucuruí, Pará state; (4) Serra Norte/Carajás, Pará state; (5) Balbina/Pte. Figueiredo, Amazonas state; (6) Porto Trombetas, Pará state. Full names of virus isolates and accession numbers of T2 protein sequences used for comparative analysis are listed in Table S4. The scale bar represents 0.4% genetic divergence.

of the genus *Orbivirus*. These results confirm previous serological grouping (Travassos da Rosa *et al.*, 1984). Within the clade corresponding to orbiviruses, the closest relative group to the Brazilian CGLV subclade are the orbiviruses transmitted by *Culicoides*. Similar results were also observed by others, using partial CGLV VP1 nt sequences (132 nt) (Palacios *et al.*, 2011). The T2 protein is largely used to assess the evolutionary relationship of orbiviruses and their natural hosts (Attoui *et al.*, 2001, 2005, 2009; Belaganahalli *et al.*, 2012; Dilcher *et al.*, 2012; Jaafar *et al.*, 2014; Kapoor *et al.*, 2013; Vieira *et al.*, 2009). The analysis of the T2 protein of the 13 Brazilian CGLV isolates also confirmed their closest genetic association with *Culicoides* orbiviruses (Fig. 4). These findings suggest a possible ecological, epidemiological and evolutionary relationship between the CGLV clade transmitted by

phlebotomine sandflies and orbiviruses transmitted by *Culicoides* midges (Fig. 4).

The evolutionary process of segmented RNA viruses occurs basically by three mechanisms (mutation, genetic reassortment and genetic recombination) (Bishop & Beaty, 1988; Bolker *et al.*, 2009; Talbi *et al.*, 2010). Although several studies have been performed, the effective contribution of these three mechanisms for biogenesis of segmented viruses is not well understood. However, in the specific case of Brazilian orbiviruses, genetic reassortment appears to be directly associated with virus biodiversity since 6 out of 13 Brazilian CGLV serotypes demonstrated reassortment events (Fig. 5).

The results of our phylogenetic analysis, using the polymerase and T2 proteins, demonstrated that the

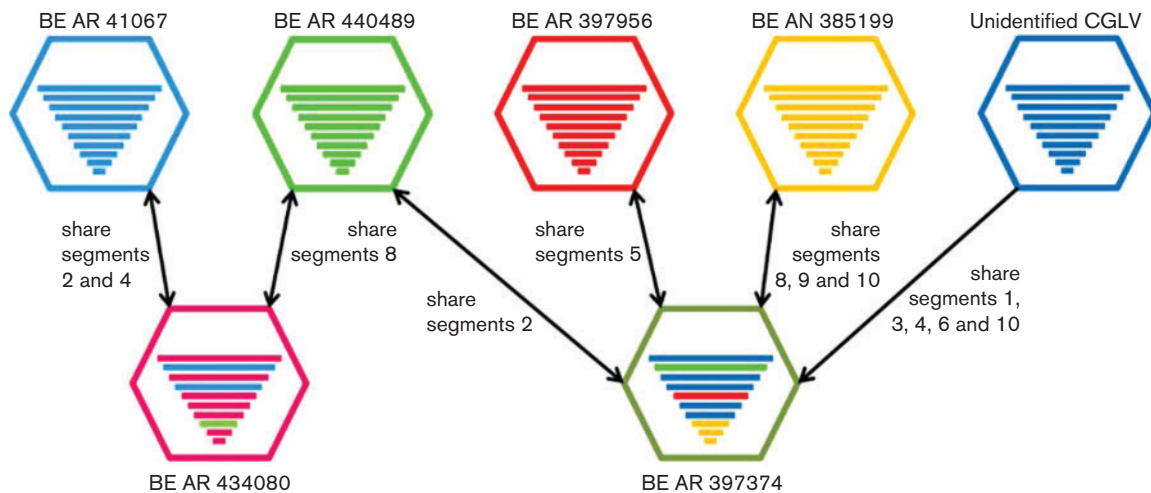


Fig. 5. Scheme of genetic reassortment between CGLV serotypes. Each hexagon represents a different CGLV serotype. The 10 internal lines represent all genome segments. The arrows indicate shared segments. The sample Tapirope virus (BE AR 434080) presents a reassortment event with at least two different CGLVs. Jutai virus (BE AR 397374) appears to harbour segments which originate from four different viruses.

Brazilian CGLVs isolated in distant geographical locations of the Brazilian Amazon (e.g. Pará and Amazonas states) were generally genetically diverse (Figs 3 and 4), suggesting that the geographical isolation of a group of viruses can lead to genetic modifications and in specific cases to a virus speciation over a period of time (Bolker *et al.*, 2009; Talbi *et al.*, 2010).

The combined analysis and the results using phylogenetic topologies, percentage of permutation trees in Simplot analysis, and geographical information, suggested that genetic reassortment and geographical location play an important role in viral biodiversity among Changuinola serogroup members. However, further studies including complete sequencing of other CGLV serotypes isolated in Brazil and in other geographical regions are needed for a better understanding of the factors involved in viral diversity.

METHODS

Virus strains. Thirteen CGLV serotypes recovered from distinct geographical areas of the Brazilian Amazon region and isolated between 1961 and 1988 were used in this study. The 13 CGLV serotypes were initially isolated by intracerebral inoculation of newborn mice; viruses were single-passaged in Vero cells to obtain viral RNA for sequencing. The studied strains were obtained from the World Health Organization/Pan American Health Organization Collaborating Center for Arbovirus Reference Research at the Department of Arbovirology and Hemorrhagic Fevers, Evandro Chagas Institute, Brazilian Ministry of Health, Ananindeua, Brazil. A summary of information about the 13 viral isolates (serotype, designation, geographical area, source and year of isolation) is presented in Table 1.

Transmission electron microscopy. Vero cells infected with Irituia virus (BE AN 28873) were used for ultrastructural analysis. Ultrathin

sections of infected cells were first fixed for at least 1 h in a mixture of 2.5% formaldehyde prepared from paraformaldehyde powder, and 0.1% glutaraldehyde in 0.05 M cacodylate buffer pH 7.3 to which 0.03% picric acid and 0.03% CaCl₂ were added. The monolayers were washed in 0.1 M cacodylate buffer; cells were scraped off and processed further as a pellet. The pellets were post-fixed in 1% OsO₄ in 0.1 M cacodylate buffer pH 7.3 for 1 h, washed with distilled water and stained en bloc with 2% aqueous uranyl acetate for 20 min at 60 °C. The pellets were dehydrated in ethanol, processed through propylene oxide and embedded in Poly/Bed 812 (Polysciences). Ultrathin sections were cut on a Leica EM UC7 ultramicrotome (Leica Microsystems), stained with lead citrate and examined in a Philips 201 transmission electron microscope at 60 kV.

Treatment to remove contaminant host RNA. Approximately 20 ml of cell culture supernatant from infected Vero cells, showing 80–90% cytopathic effect (CPE) was collected between 3 and 5 days post-infection; this material was first centrifuged for 10 min at 2000 r.p.m. (700 g) and then for 5 min at 4000 r.p.m. (2800 g) and passed through 0.2 µm filters, followed by PEG-precipitation to enrich for virus particles (Dilcher *et al.*, 2012). Subsequently, the virus pellets were resuspended in 250 µl PBS, and 25 µl RNase A (final concentration, 2 mg ml⁻¹) was added to digest cellular RNAs. The samples were kept at 37 °C for 30 min after which 225 µl PBS was added.

Nucleic acid isolation and gel electrophoresis. The dsRNA was extracted from virus pellets using a guanidinium isothiocyanate (Trizol) procedure according to the manufacturer's instructions (peqGOLDTrifast FL; Peqlab). To improve the RNA precipitation, 2 µl glycogen (35 µg µl⁻¹) was added together with one volume of 2-propanol per sample and kept overnight at -20 °C. Samples were then centrifuged for 15 min at 12 000 g and 4 °C and washed twice with 75% ethanol. After drying, each RNA pellet was resuspended in 25 µl RNase-free diethyl pyrocarbonate (DEPC)-treated water and incubated for 5 min at 57 °C in a heating block. After DNA digestion via the Turbo-DNA-free kit (Ambion) and Pellet-Paint precipitation of the purified RNAs (Pellet Paint NF Co-Precipitant; Novagen), cellular ssRNAs that might still have been present were precipitated overnight in 2 M LiCl at 4 °C (Attoui *et al.*, 2000). The viral dsRNA

was precipitated from the supernatant by addition of 0.25 volume 7.5 M ammonium acetate and 1 volume (RNA + ammonium acetate) 2-propanol for 2 h at -20°C . The RNA pellet was washed twice with 70% ethanol and once with 100% ethanol and resuspended in 18 μl DEPC-treated water after drying. The RNA concentration was measured using a Nanodrop 2000 device (Peqlab). Electrophoresis was carried out in long 1% TAE agarose gels at 4°C in a cold room. A total of 1–2 μg of purified dsRNA was used per lane and subsequently run for 2 h at 90 V, then for 4 h at 100–120 V (total 6 h) at 4°C . The RNA segments were visualized via staining with ethidium bromide. The sizes of the RNA bands were estimated using the GeneRuler 1 kb DNA Ladder (Thermo Scientific).

Genome sequencing. Complete genomes were obtained using the pyrosequencing approach (Shendure & Ji, 2008). In order to recover the 5' and 3' ends of the dsRNA segments, the same anchor primer sequence used in the FLAC-method (full-length amplification of cDNAs) was ligated to the 3' ends of the viral RNAs prior to pyrosequencing as previously described (Maan *et al.*, 2007; Potgieter *et al.*, 2009). 500 ng of purified viral dsRNA was ligated to 500 ng of FLAC-anchor primer. The concentration of the adaptor-ligated dsRNA was determined using a NanoDrop 2000 (Peqlab). Then, 60 ng of adaptor-ligated viral RNA was amplified and converted to cDNA using a TransPlex Whole Transcriptome Amplification kit (WTA2; Sigma-Aldrich).

After purification with the QIAquick PCR Purification kit (Qiagen), an additional size exclusion step via Ampure-XP beads (Agencourt) in a ratio of 1 volume WTA2 product to 0.7 volume Ampure-XP beads was used to remove fragments shorter than 300 bp. Furthermore, 300 ng of the whole genome amplified cDNA were used for titanium shotgun library preparation and pyrosequencing as described in the FLX Titanium Rapid Library Preparation Protocol starting with the end-repair step (Roche Applied Sciences) (Margulies *et al.*, 2005). In this case, the DNA fragmentation by nebulization step was skipped. In the RL adaptor ligation step, RL-MID adapters were used to allow pooling of several samples.

A pyrosequencing library was prepared and used for sequencing on a GS FLX pyrosequencer (Roche, 454 Life Sciences) at the Department of Virology, University Medical Center, Göttingen, Germany. In parallel, the same libraries were sequenced on a GS FLX pyrosequencer (Roche, 454 Life Sciences) in the Center for Technological Innovation at the Evandro Chagas Institute, Ministry of Health, Brazil.

Genome assembly and phylogenetic analysis. The assembly of the obtained sequences was carried out using the GS De Novo Assembler program (Newbler v. 2.6). For further analysis, the Geneious v. 6.1.4 software and a set of programs (SeqMan Pro, SeqBuilder, Protean) available in the DNASTAR package (Lasergene) were used. The dataset used to reconstruct the phylogenetic trees consisted of amino acid sequences obtained for each genome segment.

The alignment was made using the Mafft v. 7 program (Katoh & Standley, 2013). The reconstruction of phylogenetic trees was made using the ML method (Myung, 2003), implemented in the PhyML v. 3.0 (<https://github.com/stephanguindon/phyml-downloads/releases/download/stable/phyml-20120412.tar.gz>) computational program. For determination of the reliability of the tree topology, bootstrap analysis (Felsenstein, 1985) was carried out on 1000 replicates. Confidence values used as criteria for group inclusion or exclusion were calculated based on the mean of the amino acid sequence identities within and among selected members of the different genera of the family *Reoviridae*.

Evaluation of genetic reassortment. In order to evaluate the possibility of natural genetic reassortment among the 13 CGLV serotypes analysed, concatenate sequences of the 10 dsRNA segments

of each virus were used. Multiple sequence alignments were carried out using Mafft v. 7 (Katoh & Standley, 2013) and Simplot v. 3.5.1 (Lole *et al.*, 1999) for evaluation of RNA segment shifting. Values of permutation trees were assigned as percentages. Genetic reassortment was considered when the percentage of permutation trees was more than 90% across a given entire genomic segment.

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