Full-length sequencing and genetic characterization of Breu Branco virus (Reoviridae, Orbivirus) and two related strains isolated from Anopheles mosquitoes

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Breu Branco virus (BE AR 492347) was isolated in 1988 from Anopheles (Nyssorhynchus) triannulatus mosquitoes captured in Tucurui, Pará State, northern Brazil. No cross-reactivity by complement-fixation tests was observed between Breu Branco virus and other known arboviruses. Results of electron microscopy and physicochemical tests suggested that Breu Branco virus may be a member of the family Reoviridae. In order to elucidate its taxonomic status, a comprehensive genetic characterization was conducted for Breu Branco virus and related strains (BE AR 494475 and BE AR 486204) that were also isolated from Anopheles mosquitoes in the same area. This included full-length genome sequencing, determination of genetic traits and phylogenetic analysis. Breu Branco virus showed a similar genome organization to members of the genus Orbivirus, family Reoviridae. Genetically, Breu Branco virus was indistinguishable from strains BE AR 494475 and BE AR 486204. Phylogenetic analysis suggested that Breu Branco virus BE AR 492347 and its related strains constitute a novel species of the genus Orbivirus. Breu Branco virus is the first Brazilian orbivirus and the fifth orbivirus in the world to be sequenced fully.

INTRODUCTION

Breu Branco virus (BE AR 492347) was isolated in 1988 from a pool of Anopheles (Nyssorhynchus) triannulatus mosquitoes collected in the municipality of Tucurui, Pará State, northern Brazil (03° 46‘ S 49° 40‘ W). The virus was initially tested by the complement-fixation (CF) technique, and no cross-reactivity was observed against immune sera of 195 other arboviruses previously isolated in the Brazilian Amazon. By electron microscopy, Breu Branco virus was revealed to be a non-enveloped, spherical particle with icosahedral symmetry and a diameter of approximately 70 nm. The absence of an envelope structure was confirmed by the persistence of virus infectivity after treatment with sodium deoxycholate, as described elsewhere (Theiler, 1957). Two other virus strains, BE AR 494775 and BE AR 486204, were also isolated in the same area in 1984 and 1987 from a mixed pool of Anopheles (Nys.) oswaldoi/Anopheles (Nys.) nuneztovari, and from a pool of A. (Nys.) triannulatus, respectively. CF and neutralization tests showed that these two isolates were indistinguishable from Breu Branco virus, and they are therefore classified as strains of this virus.

The family Reoviridae contains the genus Orbivirus as well as other well-established genera (including Orthoreovirus, Rotavirus, Coltivirus, Aquareovirus, Cypovirus, Fijivirus, Phytoreovirus, Oryzavirus, Seadornavirus, Mycoreovirus and Idnoreovirus). There are currently 22 recognized
species in the genus *Orbivirus* that are vectored by different insects, including mosquitoes (Anophelinae and Culicinae), Ceratopogonidae (*Culicoides* midges), ticks and Phlebotominae flies. Orbiviruses have a genome characterized by a ten-segmented, double-stranded (ds) RNA (Mertens *et al.*, 2005).

The most well-known species of the genus *Orbivirus* is *Bluetongue virus* (BTV). This virus has been found in different countries and continents, such as Australia, USA, Africa, the Middle East, Asia and Europe, where it is considered an important veterinary problem due to serious disease in livestock (sheep, goats, cattle and deer). Its occurrence is seasonal in affected Mediterranean countries (Purse *et al.*, 2005). BTV, African horse sickness virus (AHSV) and epizootic hemorrhagic disease virus (EHDV) are vectored by *Culicoides* midges. These viruses are among the most economically important orbiviruses that infect vertebrates (Mertens *et al.*, 2005).

Genetic studies have been conducted for some of the insect-borne orbiviruses, including the recent descriptions of the full-length sequences of Yunnan orbivirus (YOV) – a novel orbivirus isolated from *Culex tritaeniorynchus* mosquitoes in China (Attoui *et al.*, 2005), Middle Point orbivirus, isolated from sentinel cattle in Australia (Cowled *et al.*, 2007), Stretch Lagoon orbivirus, isolated from *Culex* and *Aedes* mosquitoes (Cowled *et al.*, 2009), and Toggenburg orbivirus (Chaingnat *et al.*, 2009). In contrast, little attention has been paid to tick-borne orbiviruses. There is currently a lack of genetic data available for orbiviruses in terms of entire genome sequencing, which is mainly represented by nucleotide sequences obtained for BTV serotype 17 (Mertens *et al.*, 2005), Broadhaven virus (BRDV; Moss *et al.*, 1992) and St Croix River virus (SCRV; Attoui *et al.*, 2001).

In this study, we have determined the full-length sequence of Breu Branco orbivirus and related strains were amplified by using the single-primer amplification technique (SPAT) as described by Attoui *et al.* (2000a, b).

**Methods**

**Virus strains.** Breu Branco virus (BE AR 492347) and related strains (BE AR 494475 and BE AR 486204) used in this study are listed in Table 1 and represent relatively low-passage isolates obtained from the WHO Collaborating Centre for Research, Diagnostic Reference and Training in Arbovirus, Arbovirus Section and Hemorrhagic Fevers, Evandro Chagas Institute, Ananindeua, Pará State, Brazil.

**Virus culture and RNA extraction.** Viruses were grown in monolayer cultures of *Aedes albopictus* (C6/36) cells. Cells were infected with the viruses and observed daily for detection of cytopathic effect (CPE). When cells displayed 75% CPE, the supernatants of infected cell cultures were collected, centrifuged at 3000 r.p.m. at 4°C for cell-debris precipitation, aliquotted and either stored at −70°C or used immediately for RNA extraction. This was performed as follows: 6 ml infected supernatant was transferred to a 50 ml polypropylene tube containing a solution of 50% polyethylene glycol (PEG) 8000 (Invitrogen) and 23% NaCl for virus precipitation. Subsequently, supernatants were removed by centrifugation at 3000 r.p.m. at 4°C for 10 min and the virus pellets were eluted into 250 μl HPLC RNase-free water (Invitrogen). RNA extraction was carried out by using TRIzol LS reagent (Invitrogen) or by using a commercial kit (QIAamp Viral RNA mini kit; Qiagen), following the manufacturer’s instructions.

**Electrophoretic profile.** For evaluation of the RNA electrophoretic profiles of Breu Branco virus and related strains, PAGE was performed. A 10% PAGE gel (2 M Bis acrylamide, pH 8.8; 10% ammonium persulfate; TEMED) was run as described elsewhere (Shapiro *et al.*, 1967). The RNA was run with Irituia virus (IRIV), an Amazonian orbivirus isolated in 1961 from Oryzomys sp. on the Belem–Brasilia highway km-92 (Travassos da Rosa *et al.*, 1984), which was used as a positive control. RNA extracted from uninfected C6/36 cells was used as a negative control.

**Isolation and purification of virus RNA.** Polysegmented Breu Branco virus RNA extracted from supernatant of infected C6/36 cells was segregated by conventional electrophoresis in 1.2% agarose gel stained with ethidium bromide (0.5 μg ml⁻¹). After that, each RNA segment was removed from the gel by using a sterile stylus and purified by using a commercial Qiagquick Gel Extraction kit (Qiagen) according to the manufacturer’s instructions.

**Genome amplification.** The RNA segments of Breu Branco virus and related strains were amplified by using the single-primer amplification technique (SPAT) as described by Attoui *et al.* (2000a, b).

**Sequencing.** The cDNA products generated by SPAT were partially sequenced by the dyeoxide chain-termination method using an ABI PRISM Dye Terminator kit (Applied Biosystems), and analysed in an ABI 377 DNA sequencer. The complementary primer (B: 5′-GGTGACGGGTCTACGAGACCT-3′) to the anchor 3′-amino-blocked primer A (5′-PO4-AGGTCTCGTAGACCGTGCACC-NH2-3′) (Attoui *et al.*, 2000b) was used for cDNA sequencing in both directions. Specific primers were designed for each segment by using the overlapping strategy as far as the sequences were obtained (see Supplementary Table S1, available in JGV Online).

**Assembly of overlapping sequences.** Partial nucleotide sequences obtained for Breu Branco virus and related strains were first inspected for quality and then assembled by using the SeqMan II program v. 5.03 (Lasergene DNAStar software package) in order to produce contiguous full-length sequences. This software was also used to predict the entire open reading frame (ORF) for the polyprotein, as well as for both 5′ and 3′ non-coding regions (NCRs).

<table>
<thead>
<tr>
<th>Virus/strain</th>
<th>Mosquito species</th>
<th>Place of isolation</th>
<th>Date of isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breu Branco virus (BE AR 492347)</td>
<td><em>Anopheles</em> (Nys.) <em>trianulatus</em></td>
<td>Tucuruí/PA, Base 4</td>
<td>15 August 1988</td>
</tr>
<tr>
<td>BE AR 494475</td>
<td><em>Anopheles</em> (Nys.) <em>oswaldoi/nunezovari</em></td>
<td>Tucuruí/PA, Arumateua</td>
<td>20 October 1984</td>
</tr>
<tr>
<td>BE AR 486204</td>
<td><em>Anopheles</em> (Nys.) <em>trianulatus</em></td>
<td>Tucuruí/PA, Base 4</td>
<td>10 October 1987</td>
</tr>
</tbody>
</table>
Scanning of Breu Branco virus ORFs and identification of related proteins. For the identification of related Breu Branco virus proteins, the full-length predicted ORFs for each of the ten segments (S1–S10) of Breu Branco virus and related strains were scanned against other orbivirus amino acid sequences available in GenBank by the protein–protein BLAST program, available on the NCBI homepage (http://www.ncbi.nlm.nih.gov/Blast.cgi), using standard default parameters. Sequences represented by the lowest E value and highest P score were selected to be used in multiple sequencing alignments (gene by gene) using CLUSTAL W software (Thompson et al., 1997). The percentage identity for nucleotide and amino acid sequences, as well as the mutations along the entire genome, were assessed with the MegAlign software (LaserGene DNASTAR package).

Phylogeny. For phylogenetic analysis of the sequences obtained for Breu Branco virus and the related strains BE AR 494475 and BE AR 486204, amino acid sequences corresponding to the viral polymerase (VP1, S1), T2 protein [which is encoded by S2 (VP2) or S3 (VP3)] and T13 protein (S7, VP7) were compared with sequences of representative members belonging to different genera within the family Reoviridae (Orthoreovirus, Aquareovirus, Phytovirus, Cypovirus, Rotavirus, Seadornavirus, Coltivirus, Oryzavirus and Fijiivirus) (see Supplementary Table S2, available in JGV Online). Neighbour-joining (NJ) (Saitou & Nei, 1987) and maximum-parsimony (MP) (Swofford, 1999) methods implemented in the MEGA 3.0 software (Kumar et al., 2004) and PAUP 4.0 beta version (Swofford, 1999) were performed. For NJ analysis, the distance matrix was calculated from the aligned sequences by using the Kimura two-parameter formula (Kimura, 1980), and a weight of four transitions versus one transversion was selected. In order to obtain a tree with MP, the heuristic algorithm was used in MP analysis. For determination of the reliability of the tree topology, bootstrap analysis (Felsenstein, 1985) was carried out on 2000 replicates. The bootstrap-resampling technique (Efron, 1982) was then used to further evaluate the reliability of bootstrap analysis with a confidence value of 95%. Furthermore, 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.

RESULTS

Electrophoretic profile

Analysis of the RNA electrophoretic profile of Breu Branco virus by using PAGE revealed the presence of a ten-segmented genome, which was classified according to its 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. 1).

Genome analyses

The complete genome sequences of Breu Branco virus and strains BE AR 494475 and BE AR 486204 were obtained for all ten RNA segments by assembling nucleotide sequences obtained for each segment. The segment sizes ranged from 4965 nt for S1 to 773 nt for S10. The predicted proteins ranged from 1630 aa (Pol, viral polymerase; S1) to 237 aa (NS3a, S10) in length. Regarding NCRs, the 5’ termini were composed of short sequences ranging from 8 nt for S4 to 30 nt for S6. The 3’ terminus was longer, with NCRs ranging from 33 nt (S6) to 106 nt (S7). Conserved sequences were observed in both 5’ and 3’ termini of all genomic segments and corresponded to GUUAA and UAC, respectively. It is important to note that size variability in the 3’ NCR region was also observed in S10 of Breu Branco virus and its related strains (Table 2), due to an insertion or deletion of the nucleotide sequences CGCGCGGTT/TTCGCGCGGTTCGCGCGGTT.

The genome scanning implemented in the protein–protein BLAST using the entire ORFs of each RNA segment of Breu Branco virus and its related strains revealed protein identity with other members of the genus Orbivirus (E values=0.001; P scores ranging from 1226 to 4071). The highest identity was observed with AHSV S1 (P score=4071; 49.1%). On the other hand, the lowest identity was observed with S6 of SCRV (P score=1226; 19.1%). As shown in Table 3, the function of each RNA segment is referred to in comparison with homologous segments of BTV, AHSV and SCRV.

Multiple sequence alignment carried out between ORFs of Breu Branco virus and its related strains BE AR 49475 and BE AR 486204 revealed a high degree of nucleotide sequence identity, ranging from 91.4% for S9 to 99.6% for S6. For amino acid sequences, the identities were estimated to be in the range 95% (S9) to 100% (except for...
Mutations were observed along all RNA segments when sequences were compared among Breu Branco, BE AR 494475 and BE AR 486204 viruses. However, S9 was the most divergent, showing 51 mutations, 45 of which were identified as transitions and six as transversions. Of 51 mutations, four (7.8 %), six (11.7 %) and 41 (80.5 %) were observed in the first, second and third codons, respectively. Thirteen of these mutations were characterized as non-synonymous mutations leading to amino acid changes. Furthermore, four mutations modified the biochemical properties of the amino acid involved (see Supplementary Table S4, available in JGV Online).

**Phylogeny**

The phylogenetic analysis based on the amino acid sequences (S1, S2 and S3) obtained for Breu Branco, BE

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**Table 2.** Genome organization of Breu Branco virus and strains BE AR 494475 and BE AR 486204 according to RNA segment, size, ORFs, protein and 5' and 3' NCRs

<table>
<thead>
<tr>
<th>RNA segment</th>
<th>Size (nt)</th>
<th>ORF (nucleotide position)</th>
<th>Predicted protein size (aa)</th>
<th>5' NCR Size (nt)</th>
<th>Terminal sequence</th>
<th>3' NCR Terminal sequence</th>
<th>Size (nt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>4965</td>
<td>14–4903</td>
<td>1630</td>
<td>13</td>
<td>GUUAAAU----</td>
<td>----UUAC</td>
<td>62</td>
</tr>
<tr>
<td>S2</td>
<td>3221</td>
<td>16–3186</td>
<td>1057</td>
<td>15</td>
<td>GUUAAAU----</td>
<td>----UUAC</td>
<td>35</td>
</tr>
<tr>
<td>S3</td>
<td>2789</td>
<td>21–2738</td>
<td>906</td>
<td>20</td>
<td>GUUAAAU----</td>
<td>----UUAC</td>
<td>51</td>
</tr>
<tr>
<td>S4</td>
<td>1999</td>
<td>9–1952</td>
<td>648</td>
<td>8</td>
<td>GUUAAUA----</td>
<td>----UUAC</td>
<td>47</td>
</tr>
<tr>
<td>S5</td>
<td>1707</td>
<td>36–1649</td>
<td>538</td>
<td>35</td>
<td>GUUAAAA----</td>
<td>----AUAC</td>
<td>58</td>
</tr>
<tr>
<td>S6</td>
<td>1670</td>
<td>39–1637</td>
<td>533</td>
<td>38</td>
<td>GUUAAAA----</td>
<td>----UUAC</td>
<td>33</td>
</tr>
<tr>
<td>S7</td>
<td>1281</td>
<td>18–1175</td>
<td>386</td>
<td>17</td>
<td>GUUAAAA----</td>
<td>----UUAC</td>
<td>106</td>
</tr>
<tr>
<td>S8</td>
<td>1125</td>
<td>20–1078</td>
<td>353</td>
<td>19</td>
<td>GUUAAAA----</td>
<td>----UUAC</td>
<td>47</td>
</tr>
<tr>
<td>S9</td>
<td>920</td>
<td>22–855</td>
<td>278</td>
<td>22</td>
<td>GUUAAAA----</td>
<td>----AUAC</td>
<td>65</td>
</tr>
<tr>
<td>S10</td>
<td>773/762/782</td>
<td>21–740</td>
<td>240</td>
<td>20</td>
<td>GUUAAAA----</td>
<td>----UUAC 33/22/42</td>
<td></td>
</tr>
</tbody>
</table>

Consensus sequence: GUUAAww---- ----wUAC

*Breu Branco virus, 773 nt; BE AR 494475, 762 nt; BE AR 486204, 782 nt.
†Breu Branco virus, 33 nt; BE AR 494475, 22 nt; BE AR 486204, 42 nt.
§w=A or U.

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**Table 3.** Genome comparison and related functions of Breu Branco virus proteins in comparison with selected members of the genus *Orbivirus*

<table>
<thead>
<tr>
<th>Putative protein function</th>
<th>Genomic segment</th>
<th>BTV</th>
<th>AHSV</th>
<th>SCRV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viral polymerase, Pol</td>
<td>S1</td>
<td>48.3</td>
<td>49.1</td>
<td>38.7</td>
</tr>
<tr>
<td>Outer shell protein, VP2</td>
<td>S2</td>
<td>29.0</td>
<td>33.8</td>
<td>29.9</td>
</tr>
<tr>
<td>Major subcore protein, VP3 (T2)</td>
<td>S3</td>
<td>28.2</td>
<td>31.1</td>
<td>20.4</td>
</tr>
<tr>
<td>Minor core and capping enzyme, VP4</td>
<td>S4</td>
<td>31.7</td>
<td>30.5</td>
<td>34.2</td>
</tr>
<tr>
<td>Tubules, NS1</td>
<td>S5</td>
<td>39.6</td>
<td>29.0</td>
<td>36.8</td>
</tr>
<tr>
<td>Outer capsid protein, VP5</td>
<td>S6</td>
<td>28.1</td>
<td>30.3</td>
<td>19.1</td>
</tr>
<tr>
<td>Major core surface protein, VP7 (T13)</td>
<td>S7</td>
<td>35.4</td>
<td>38.2</td>
<td>19.4</td>
</tr>
<tr>
<td>Viral inclusion body, NS2</td>
<td>S8</td>
<td>22.2</td>
<td>23.5</td>
<td>21.7</td>
</tr>
<tr>
<td>Minor core protein, helicase, VP6</td>
<td>S9</td>
<td>27.6</td>
<td>25.2</td>
<td>23.7</td>
</tr>
<tr>
<td>Virus release, NS3</td>
<td>S10</td>
<td>24.8</td>
<td>19.7</td>
<td>22.1</td>
</tr>
</tbody>
</table>
AR 494475 and BE AR 486204 viruses and other representative members of the family Reoviridae using the MP and NJ methods resulted in trees with similar topologies, although bootstrap values in the MP consensus tree were slightly lower than those in the NJ tree (data not shown). Accordingly, the NJ tree was used to represent the phylogeny of the selected viruses. By using reliable bootstrap (>85%) and confidence (S1, ≤28% ± 2SD; S2 or S3, ≤13% ± 2SD) values as criteria for group inclusion, the phylogram using the entire polymerase (S1) amino acid sequences revealed the genetic relationship of Breu Branco, BE AR 494475, and BE AR 486204 viruses to other members of the genus Orbivirus (Fig. 2). The phylogeny for the T2 protein (S2 or S3) depicted the segregation of two major groups, hereafter designated the Culicoides- and Culicidae-borne group (Cul) and the tick-borne group. In the Cul group, 15 clades (I–XV) were formed and the Breu Branco virus and related strains constituted a single phylogenetic subclade (I), related more closely to subclade II (Kasba virus and D’Aguilar virus). Furthermore, the Breu Branco viruses were also related to orbiviruses in which the T2 protein is encoded on S3 (VP3) (Fig. 3).

**DISCUSSION**

Due to the isolation of Breu Branco virus and related strains from mosquitoes of the genus Anopheles, it was expected that these viruses would be grouped in the Anopheles A serogroup of the family Bunyaviridae, genus Orthobunyavirus, where several other viruses isolated from these mosquitoes are grouped (Travassos da Rosa et al., 1992). However, the results obtained by the CF test did not show cross-reactivity between Breu Branco virus and any other Anopheles A member, or with other arboviruses belonging to different antigenic groups (group A, B, C, Bunyamwera, Simbu and California) that commonly circulate in the Brazilian Amazon region. Breu Branco virus reacted only with its homologous serum and with the immune sera prepared for BE AR 494475 and BE AR

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**Fig. 2.** Phylogenetic analysis of the viral polymerase S1 of Breu Branco virus and strains BE AR 494475 and BE AR 486204 in comparison with other members of the family Reoviridae, using the NJ method. Bootstrap values and percentages of divergence (in parentheses) are given above each main node of the tree. *Amino acid divergence of 0.5%. Bar, 50% amino acid sequence divergence.
486204 viruses (CF titres in reciprocal serum/antigen reaction: \(\geq 64/\geq 64 \pm 2\)-fold dilutions). Thus, further analyses were necessary to assess the classification status of this viral agent.

As recently observed for YOV (Attoui et al., 2005), the classification of Breu Branco virus and strains BE AR 494475 and BE AR 486204 as members of the family Reoviridae (more specifically, as a novel virus species within the genus Orbivirus) has also been supported by data including the size of virus particles, morphology, physicochemical properties (sodium deoxycholate sensitivity), electrophoretic profile (10 RNA segments; Fig. 1) and the genetic relationship with other members of the genus (Figs 2, 3).

Genetic characterization using the full-length sequences obtained for Breu Branco virus and related strains was helpful to better understand the genetic relationship among these viruses and with other, previously completely sequenced orbiviruses. The analysis of the 5’ and 3’ NCRs revealed highly conserved sequences that are similar to those identified in other known orbiviruses (Table 2) (Mertens et al., 2005).

The genetic diversity of Breu Branco, BE AR 494475 and BE AR 486204 viruses was most evident for S9, which encodes the VP6 protein – a minor core protein with helicase, NTPase and single-stranded RNA/dsRNA-binding functions. The analysis of mutations revealed significant

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**Fig. 3.** Phylogenetic tree of the amino acid sequences of the T2 proteins of Breu Branco virus, strains BE AR 494475 and BE AR 486204 and other orbivirus species, using the NJ method (analysis was based on partial amino acid sequences between positions 393 and 548 relative to the BTV sequence). Bootstrap values are given above each main node of the tree. The value in parentheses represents the amino acid divergence between Breu Branco virus and related strains. GenBank accession numbers are available in Supplementary Table S2 (in JGV Online). Bar, 10% amino acid sequence divergence.
T2 protein is an important evolutionary marker for orbiviruses (Figs 2, 3), reinforcing the hypothesis that the demonstration of the segregation between T2-VP2 and T2-VP3 value (other orbiviruses was significantly lower than the threshold AR 486204 and BE AR 494475 viruses (28.2–29.9 %) with acid sequence identity of the T2 protein of Breu Branco, BE strains BE AR 486204 and BE AR 494475, associated with a distinct species in the genus Orbivirus, isolated from mosquito species of the genus Anopheles.

Breu Branco virus is the first Brazilian orbivirus and the fifth orbivirus in the world to be sequenced fully. The availability of its full-length sequence should be a helpful tool for the development of new PCR-detection strategies, as well as for a better understanding of the molecular epidemiology of this novel orbivirus isolated in the Brazilian Amazon region. Finally, we propose the abbreviation BBV for Breu Branco virus.

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