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Research paper

Molecular characterization of Capim and Enseada orthobunyaviruses

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ABSTRACT

Capim and Enseada viruses are members of the genus *Orthobunyavirus* isolated from mosquitoes and mammals in Brazil. Despite seroprevalence studies indicating human infections in Latin America, these viruses remain relatively unknown and unstudied. In order to better understand the genetic and evolutionary relationships among orthobunyaviruses, we sequenced the three genomic segments of Capim and Enseada orthobunyaviruses. Based on phylogenetic analysis, we demonstrated that these viruses depicted two new distinct clades, one represented by Enseada and another composed of Capim virus. In general, the genome organization and genetic traits of these viruses are similar to other orthobunyaviruses however, the open reading frame (ORF) of the putative nonstructural NSs protein of Enseada orthobunyavirus precedes the nucleocapsid ORF. Overall, our study provides details on the molecular characteristics of the prototype species of two groups within the *Orthobunyavirus* genus, revealing novel features into the genetic diversity and evolution of this genus.

1. Introduction

Orthobunyavirus is one of the five well-characterized genera of the *Bunyaviridae*. These enveloped negative-sense RNA viruses are transmitted to their vertebrate hosts by hematophagous arthropods, mostly from the *Ceratopogonidae* or *Culicidae* insect families (Elliott, 2014; Mohamed et al., 2009). Similar to other members of the family *Bunyaviridae*, the orthobunyaviruses genomes comprises three RNA segments: a large segment (L) encoding the viral RNA dependent RNA polymerase (RdRp); a medium segment (M) encoding two envelope glycoproteins (Gn and Gc) and a non-structural protein (NSm) that are translated as a polyprotein and processed in the endoplasmic reticulum and Golgi of the host cell; and a small segment (S) encoding the viral nucleocapsid protein (NP) and in most of the virus species a small non-structural protein (NSs) that acts as a modulator of the innate immune response (Elliot and Schmaljohn, 2013; Elliott, 2014).

Currently, there are more than 40 species of orthobunyaviruses officially recognized, which are widely distributed around the world (Plyusnin et al., 2012). These viruses have detrimental effects on human and animal health, although little is known about the burden of orthobunyavirus disease (Elliott, 2014). In humans, infections caused by orthobunyaviruses are responsible for the occurrence of several disease syndromes, including acute self-limiting febrile illness (Oropouche fever), hemorrhagic fever (Ngari hemorrhagic fever) and encephalitis (California encephalitis) (Briese et al., 2006; Gerrard et al., 2004; Martins Vdo et al., 2014; Rogstad et al., 2015; Vasconcelos et al., 2011). In animals, some orthobunyaviruses, such as Akabane, Schmallenberg and Cache Valley viruses can cause a relatively mild disease, teratogenic effects, congenital malformations and abortions in ruminants (Chung et al., 1990; Lievaart-Peterson et al., 2015). There are no specific therapeutic interventions or vaccines available to treat or prevent orthobunyavirus infection.

There are more than 170 named viruses in the *Orthobunyavirus* genus, which are grouped into 18 serogroups based on serological relatedness, as determined by complement fixation, neutralization and hemagglutination inhibition assays (Calisher, 1996; Elliott, 2014). However,

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few of these viruses have been studied at the molecular level and knowledge of orthobunyavirus evolution using full coding sequence is restricted to 10 out of the 18 recognized orthobunyavirus serogroups. Therefore, as more information becomes available for different viruses, it is important to clarify the diversity, distribution, and also the evolutionary and genetic aspects of these viruses. Also, it is clear that there is an enormous diversity among orthobunyaviruses, and it is highly likely that there will be exceptions to the generalizations that are outlined.

In this study, we describe the nearly complete sequences and molecular characterization of two orthobunyaviruses, Capim virus (CAPV) and Enseada virus (ENSV), both isolated in Brazil. The CAPV strain BeAn 8582 was originally isolated from a sample from a Bare-tailed woolly opossum (*Caluromys philander*) captured on February 1958 in Utinga watershed forest, Para State, Brazil (Zarate et al., 1968). The ENSV strain 76V-25880 was isolated from mosquitoes (*Culex epanastasis*) in August 1976 in Cananéia town, Sao Paulo State, in the Southeast Coastal area of Brazil (Calisher et al., 1983) and remains as an unclassified orthobunyavirus. Both viruses have not been related to human disease and their epidemiology and biology are poorly understood.

2. Material and methods

2.1. Viruses

The CAPV strain BeAn 8582 was originally isolated from the liver and spleen of a *C. philander* captured in 1958 in Utinga forest, Belem, Para State, Northern of Brazil (Zarate et al., 1968), while ENSV strain 76V-25880 was isolated from *C. epanastasis* mosquitoes collected in 1976 in Cananéia town, Sao Paulo State in coastal of Southeast Brazil (Supplementary Fig. 1) (Calisher et al., 1983; Zarate et al., 1968).

2.2. Viral culture, RNA purification

The viruses were propagated in C6/36 *Aedes albopictus* cell cultures and maintained in Leibovitz's-15 (L-15) medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 50 mg/ml of gentamicin and 2 mg/ml of amphotericin B (Vitrocell, Brazil). The infected cells were cultured for six days at 28 °C until visualization of cytopathogenic effect.

The viral culture supernatants were clarified by centrifugation and precipitated by adding 40% polyethylene glycol (PEG) 8000 and 30% NaCl solution and then stirred the solution overnight at 4 °C. The precipitated viruses were pelleted by centrifugation at 14,000 *g* for 60 min at 4 °C. Then, supernatants were decanted, and viral pellets resuspended in a 1 ml of Hank's balanced salt solution (HBSS) (Gibco, USA). Viral suspensions were applied to sterile 30% sucrose gradients and ultracentrifuged for 3 h at 180,000 *g*. Viral supernatants were discarded, and then viral pellets were resuspended in 200 μl of HBSS. To remove the naked DNA and RNA, the resuspended pellets were digested in a cocktail of 0.1 mg/ml of RNase A (Life Technologies, USA), 20 U of Turbo DNase (Ambion, USA) and 25 U of Benzonase (Novagen, USA) at 37 °C for 1 h. The RNA was extracted using the QIAamp viral RNA extraction kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol.

2.3. Genome sequencing and assembling

Total RNA from CAPV and ENSV orthobunyaviruses were converted to double-stranded cDNA using the Superscript III cDNA synthesis kit (Invitrogen, USA) according to the manufacturer's instructions. The cDNAs were prepared for high-throughput sequencing using *RAPID* module with the TruSeq Universal adapter (Illumina, USA) protocols and standard multiplex adaptors. A paired-end, 150-base-read protocol in *RAPID* module was used for sequencing on an Illumina HiSeq 2500 instrument as recommended by the manufacturer's protocol. Sequencing was performed in Life Sciences Core Facility (LaCTAD) from State University of Campinas (UNICAMP), Brazil.

To assemble viral genomes, reads were first quality-filtered (Quality score ≥ Q30) using the program FastQC v0.11.3 and any adapter sequences were removed using Trimmomatic-0.33 software (Bolger et al., 2014). Reads were assembled by the *de novo* strategy into contigs using IDBA UD-1.1.1 (Peng et al., 2012). The largest contigs were submitted to BLAST-based searches to identify the viruses. Subsequently, the annotations of putative ORF genes were predicted using the Geneious v.8.0.3 (Biomatters, New Zealand). Details are available upon request.

2.4. Molecular characterization of Capim and Enseada viruses

Deduced amino acid sequences were predicted in Geneious 8.0.3 (Biomatters, New Zealand) and submitted to TMHMM Server v. 2.0 (Krogh et al., 2001), SignalP 4.1 Server (Petersen et al., 2011) and NetNglyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/) for identification of transmembrane regions, signal peptide and glycosylation sites, respectively. The protein molecular weight for each identified viral proteinwas predicted using the Protein Molecular Weight Calculator tool (http://www.sciencegateway.org/tools/proteinmw.htm).

2.5. Phylogenetic analysis

Phylogenetic trees were reconstructed using our CAPV and ENSV sequences and additional 71 orthobunyaviruses complete coding sequences (S, M and L) available in the GenBank database (http://www.ncbi.nlm.nih.gov/) composing a final dataset of 73 viruses (Supplementary Table 1). The viral sequences were aligned using the MAFFT v7.158b (Katoh and Standley, 2013).

Maximum likelihood (ML) phylogenies for all segments were inferred using the PhyML 3.0 software, employing SPR branch-swapping and a general time-reversible (GTR) model of nucleotide substitution with among site rate heterogeneity parameter (γ) and a proportion of invariant sites (I), as determined by jModelTest version 2.1.7 program (Darriba et al., 2012; Guindon et al., 2010). The phylogeny for the amino acid partial sequence was inferred using the LG model (Le and Gascuel, 2008) substitution models in PhyML 3.0. Statistical support for individual nodes was estimated using the approximate likelihood ratio test (aLRT) available in PhyML and by bootstrap analysis (1000 replicates, nearest neighbor interchange branch swapping) in PhyML.

2.6. Reassortment analysis

To identify potential reassortment events, the data were mined for evidence of distinct phylogenetic topologies based on the depicted trees at the nucleotide level, as described above. Also, we concatenated all genes in a single sequence and performed a multiple alignment using the program MAFFT v7.158b (Katoh and Standley, 2013). Potential reassortment events were then analyzed using the Bootscan, geneconv, Chimaera and MaxChi methods implemented in RDP4 (Martin et al., 2015). Common program settings for all methods were used to perceive sequences as linear, to require phylogenetic evidence, to refine breakpoints and to check alignment consistency. The highest acceptable P value was set at 0.05, after considering Bonferroni correction for multiple comparisons. All method-specific program settings remained at their default values.

2.7. Genetic distance and prediction of conserved motifs

The genetic distances among clades were calculated using the p-distance values. Standard error estimations were calculated by

bootstrapping method (1000 replicates) using the MEGA v.6 program (Tamura et al., 2013). The results were presented in box and whisker plot. The presence of potential motifs characteristic for *Orthobunyavirus* was identified using Geneious v.8.0.3 (Biomatters, New Zealand).

2.8. Nucleotide sequence

The genome sequences determined in this study were deposited in GenBank under the following accession numbers; CAPV, S segment (GenBank Accession KU178982); M segment (GenBank Accession

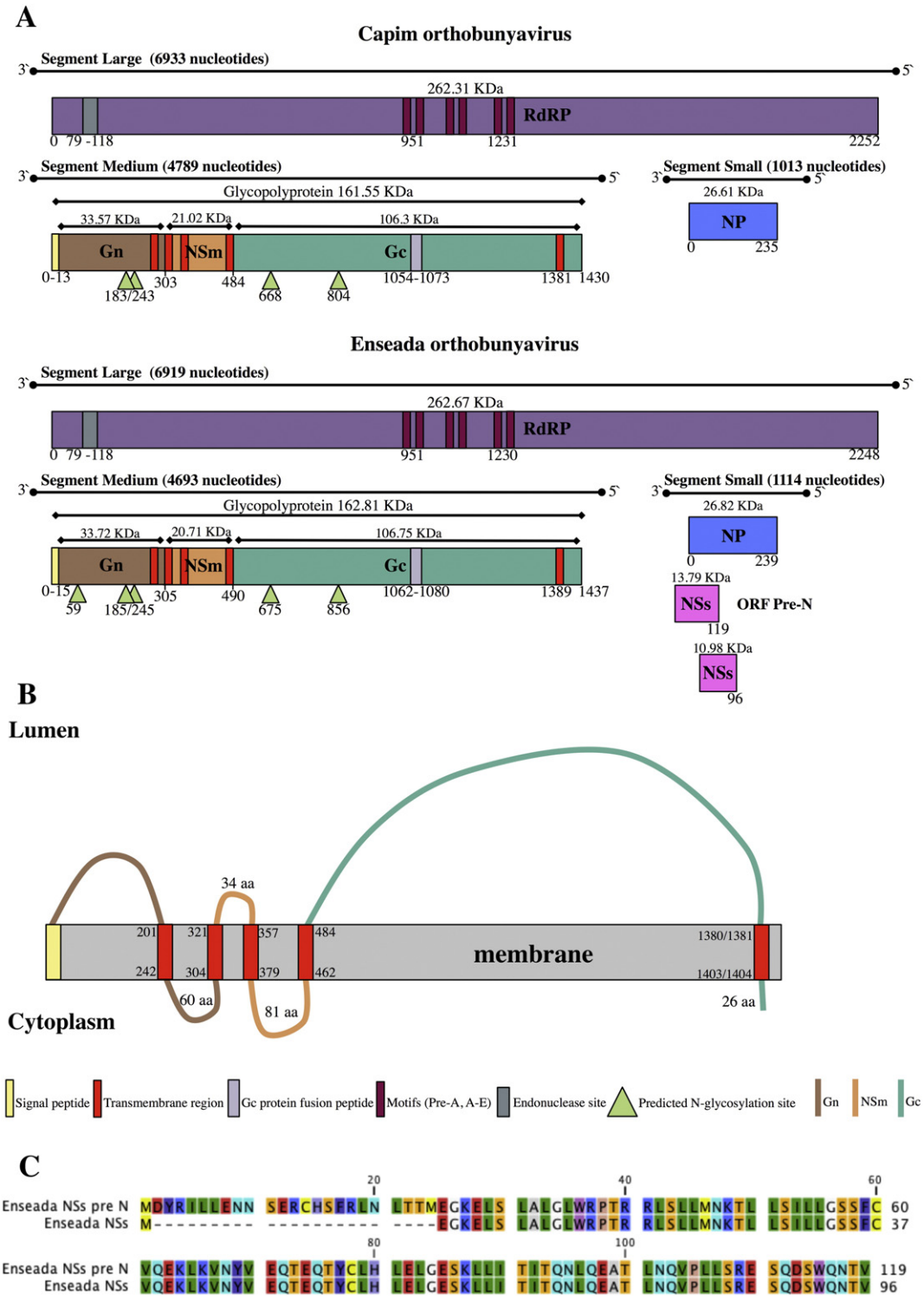

Fig. 1. Virus genome structures. A) Schematic view of the genome organization of Capim and Enseada viruses. B) The predicted topology of glycoprotein of Capim and Enseada viruses. C) Alignment of the two putative NSs ORFs from Enseada virus. Legend: Signal peptide (yellow rectangle); Transmembrane region (red rectangle); Gc protein fusion peptide (gray rectangle); Motifs — Pre A, A to E (dark red rectangle); Endonuclease site (dark gray rectangle); Predicted N glycosylation site (green triangle); Gn (brown line), NSm (orange line), Gc (green line).

KU178981) and L segment (GenBank Accession KU178980) and ENSV, S segment (GenBank Accession KU178985); M segment (GenBank Accession KU178984) and L segment (GenBank Accession KU178983).

3. Results

3.1. Molecular characterization of Capim and Enseada orthobunyaviruses

The genome of both CAPV strain BeAn 8582 and ENSV strain 76V25880 comprises three negative sense RNAs with sizes ranging from 1013 (S) to 6933 (L) and deduced amino acid length similar to other orthobunyaviruses (Fig. 1A). The L segment of CAPV codes for a RNA dependent RNA polymerase (RdRp) of 2.252 amino acids, with a predicted molecular weight of 262.31 kDa, while ENSV codes for a RdRp of 2.248 amino acids with a molecular weight of 262.67 kDa. Both proteins contain the predicted N-terminal endonuclease motifs H, PD, and DxK (Supplementary Fig. 2A), and the conserved polymerase activity domains consisting of Pre-Motif A and Motifs A through E (amino acids 951–1231), highly conserved in negative sense RNA viral polymerases and common to all other *Orthobunyaviruses* characterized so far (Supplementary Fig. 2B) (Reguera et al., 2010). The M polyprotein coded by the M segment is 1430 aa in length for CAPV and 1437 aa for ENSV and contains the characteristic N-terminal signal peptide that is common to all members of the genus. The M polyprotein is cleaved into two different structural proteins, Gn and Gc, and a non-structural protein named NSm (Savji et al., 2011; Shi et al., 2007). The topology of the glycoproteins is highlighted in Fig. 1B and Supplementary Fig. 3. The glycoproteins are predicted to contain transmembrane regions (TMDs): a single TMD in Gn (close to the C-terminus); three TMD domains in NSm; and a single one close to the C-terminus of Gc. Both TMDs in Gn and Gc are responsible for anchoring the glycoproteins in the viral lipid bilayer and it has been shown that the C-terminus of Gn is responsible for interacting with the nucleocapsid protein during viral assembly (Shi et al., 2007). The Gc protein of these viruses also has a fusion peptide that is important during viral uncoating within cellular endosome (regions 1054 to 1073 in CAPV and 1062 to 1080 in ENSV). The asparagine sites predicted to be N-glycosylated in the M polyprotein are also observed, with two sites in Gn and Gc for CAPV (Fig. 1A and Supplementary Fig. 3). ENSV presented the same sites in similar positions, but contains an extra site at the N-terminal region of the Gn protein. These N-glycosylation sites are located at the portion of the polyprotein that is facing the lumen of the endoplasmic reticulum and Golgi apparatus of the predicted protein topology, indicating that they could be glycosylated. However, further investigation on biological data needs to be done in order to address whether they are actually being glycosylated *in vivo*.

The S segment of these viruses presents some peculiarities. The nucleocapsid (N) protein is 235 amino acids and 26.61 kDa for CAPV and 239 amino acids and 26.82 kDa for ENSV. The S segment of CAPV does not present an open reading frame (ORF) for a non-structural protein (NSs), a protein that is common in most *Orthobunyaviruses* that have been isolated from vertebrates. On the other hand, ENSV presents two putative ORFs that could code for a NSs protein. One of the ORFs starts at an ATG codon +31 nucleotides upstream from the N ORF start codon, which potentially encodes for a NSs of 96 amino acids (10.98 kDa), a molecular size that is close to other *Orthobunyaviruses* (Fig. 1A). The second NSs ORF starts at an ATG codon −38 downstream from the N ORF, a distinct feature that was also observed in Brazoran virus (Lanciotti et al., 2013). This second putative NSs ORF codes for a 119 amino acid protein with 13.79 kDa that has the exact amino acid sequence of the other NSs, but with an extra N-terminal portion of 23 amino acids (Fig. 1A and C).

3.2. Phylogenetic relationship of Capim and Enseada viruses within the Orthobunyavirus genus

The 73 orthobunyaviruses clustered into 14 well-supported monophyletic groups; however, many of the deeper nodes were unresolved throughout the phylogeny. Thirteen of the well-supported clades corresponded to already established serogroups: Capim, Tete, Group C, Gamboa, Nyando, Bwamba, California Encephalitis, Guaroa, Bunyamwera, Wyeomyia, Mapputta and Simbu Clades A and B. Furthermore, we assigned a divergent clade as a proposed new "group", containing the ENSV prototype species (Fig. 2).

The ML trees based on nucleotide or amino acid sequences produced the same clustering pattern of groups, but with different topologies (Fig. 2 and Supplementary Fig. 3). In the S segment tree, all known orthobunyaviruses could be positioned into four well supported "phylogroups", which represent the most recent common ancestor for each of these groups, and their corresponding clades (groups), as described above. The first phylogroup comprised the Gamboa and Alajuela viruses isolated in mosquitoes (*Aedeomyia squamipennis*) collected in

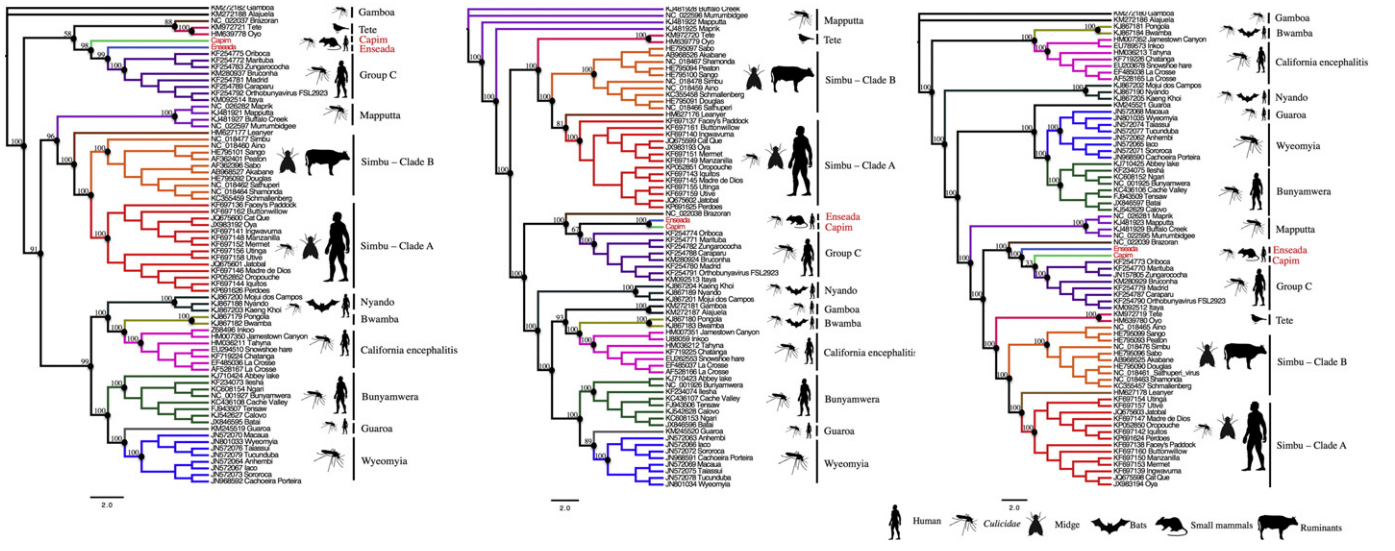

Fig. 2. ML phylogenetic trees of the *Orthobunyavirus* genus. The S segment (left), M segment (center) and L segment (right). Branches are colour-coded according to group, while the principal animal host species (where known) or vector are shown by indicated symbols. Horizontal branch lengths are drawn to a scale of nucleotide substitutions/site, and all bootstrap proportion (BSP) values. Newly proposed groups are indicated with a red color.

Central America, as described previously (Nunes et al., 2014). Notably, this phylogroup occupied a basal position in comparison to the remaining viruses both in the S and L segment topologies, but not for the M segment tree (Fig. 2). Phylogroup II was comprised by CAPV and ENSV, which are firstly described in this study and that are closely related to the Group C and Tete groups and the Brazoran virus (bootstrap < 58). The Phylogroup III contained six clades, including Nyando, Bwamba, California Encephalitis, Guaroa, Bunyamwera and Wyeomyia groups (bootstrap < 99). Finally, the fourth phylogroup included the Simbu and Mapputta groups, but this clade did not form three clearly distinct monophyletic groups in all segments. Importantly, the topologies of the ML trees estimated using amino acid sequences of all structural proteins were consistent with those of the trees based on the nucleotide sequences, indicating that site saturation has not adversely affected our phylogenetic inference (Fig. 2 and Supplementary Fig. 4).

The phylogenetic analysis reveals that all segments of CAPV and ENSV have the same evolutionary origin of the Group C group, and interestingly form a monophyletic subclade in the M segment, sharing the same common ancestor for this segment (Fig. 2).

Pairwise amino acid sequence distance analysis of the nucleocapsid, M polyprotein and RdRp proteins of CAPV and ENSV together with selected orthobunyaviruses was conducted and the results of an interclade analysis are depicted in Fig. 3. The genetic distances between CAPV and ENSV were estimated to be 52%, 39% and 32% for the N, M and RdRp, respectively. Capim and Enseada groups exhibit a high distance for the N protein and M polyprotein, with a distribution of 40 to 70% between other serogroups within the *Orthobunyavirus* genus, while the distance rates for the RdRp ranged between 30 to 52%, the lowest among the three segments (Fig. 3). The amino acid distances that these viruses share with the viruses of other groups are comparable to other inter-group correlations within the genus *Orthobunyavirus* (Fig. 3). Interestingly, the same patterns observed in ML trees are noted in the evolutionary distance, especially when comparing CAPV and ENSV with other members of the Group C serogroup (Figs. 2 and 3).

In order to identify reassortment events between CAPV and ENSV within the complete sequences from other orthobunyaviruses we inspected the nucleotide ML phylogenies for discordances in clade clustering between the S, M and L trees (Fig. 2), together with Sliding-window RDP4 reassortment analyses based on the concatenated amino acid sequences of the three segments of all 73 viruses included here. We found no reassortment events, indicating that these viruses are not potentially reassortant candidates, however future studies may help clarify this point.

4. Discussion

Here we present the complete coding sequences and molecular characteristics of CAPV and ENSV, two prototype species of different groups within the genus *Orthobunyavirus*, and their genetic and evolutionary relatedness to other orthobunyaviruses. Currently, CAPV and ENSV are recognized as individual species by the International Committee on Taxonomy of Viruses (Plyusnin et al., 2012), with CAPV virus considered the prototype species of its serogroup. Our phylogenetic analyses, together with previously serologic studies that have shown the absence of antigenic relationship, suggest that the previously unclassified ENSV virus is distinct from the Capim serogroup and most likely forms a new potential serogroup within the *Orthobunyavirus* genus (Calisher et al., 1983). However, future studies are necessary to establish accurate serogroup classification of Enseada orthobunyavirus, as well as the description of new strains within this new virus group.

The Capim serogroup is serologically constituted by Guajara, Acara, Benevides, Moriche, Bushbush and Benfica viruses (Zarate et al., 1968). CAPV has not yet been associated with febrile illness in humans but has also been isolated from a sample from a Bare-tailed woolly opossum (*C. philander*) and detected in Guyenne Spiny-rat (*Proechimys quyannensis*) and mosquitoes from the *Culex* genus (Travassos da Rosa,

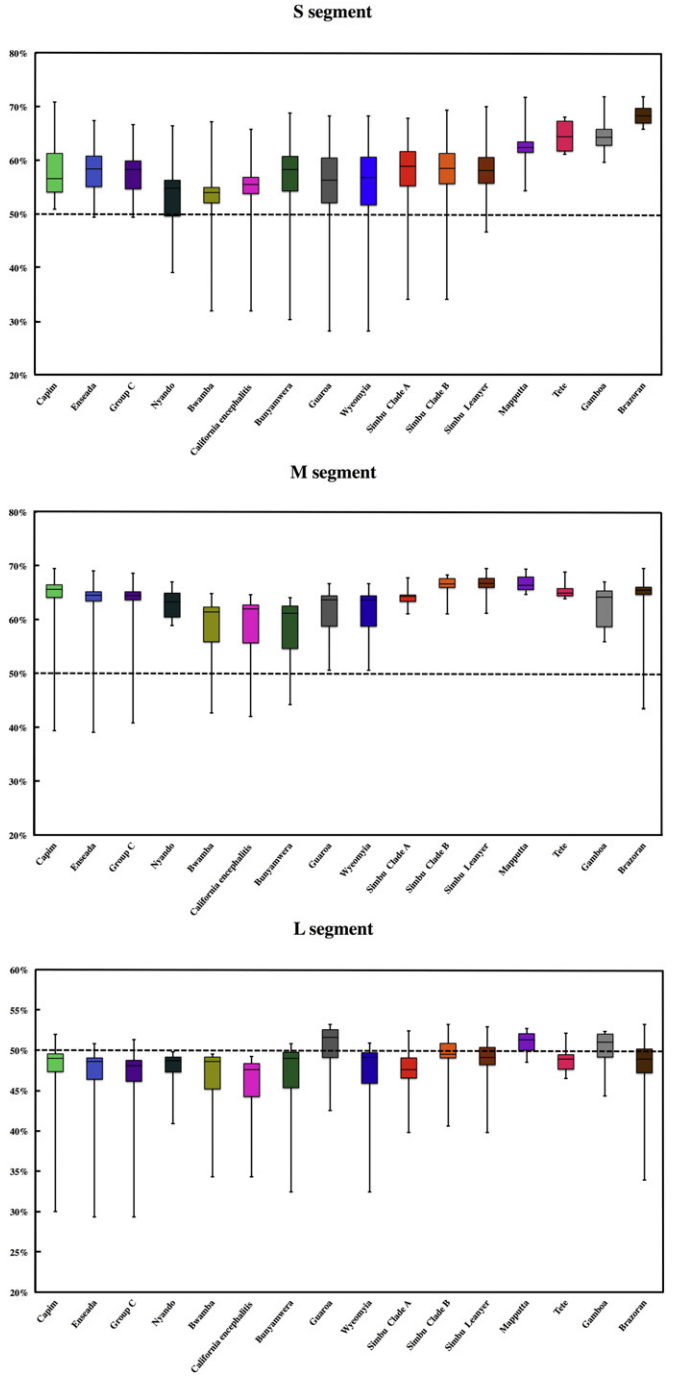

Fig. 3. Pairwise genetic similarities (amino acid p-distance) among groups of orthobunyaviruses based on the S segment (top), M segment (middle) and L segment (bottom).

1998). Recently, in parallel to our study, the complete coding sequences for all three genome segments of 15 orthobunyaviruses, including the Capim orthobunyavirus were reported (Shchetinin et al., 2015). Thus, as previously reported, our phylogenetic analysis suggests that CAPV shares the same origin of Group C and Enseada groups, including a common evolutionary origin for the M segment. This fact is highlighted in the amino acid distance analysis, showing the same pattern of evolutionary distance (Figs. 2 and 3). The molecular characterization of the putative ORFs coded by the S segment reveals that this virus does not code for a non-structural NSs protein, which is interesting, since it is one of the few orthobunyaviruses described so far that has been associated with human infection that does not contain a putative ORF for this protein

(Chowdhary et al., 2012; Mohamed et al., 2009). It has been demonstrated that orthobunyaviruses isolated from vertebrates associated with human diseases contain an ORF for a NSs protein that is involved in the virulence and pathogenesis of these pathogens (Leonard et al., 2006; van Knippenberg et al., 2010; Verbruggen et al., 2011). Apparently, viruses isolated from mosquitoes that have not been associated with human diseases do not contain this ORF (Chowdhary et al., 2012; Mohamed et al., 2009), and it was reported that Tacaiuma virus, a member of the Anopheles A serogroup, which also does not contain the NSs ORF, is able to inhibit IFN production, suggesting that other viral proteins control IFN production in infected cells (Mohamed et al., 2009). More full coding sequences of other species from this serogroup are necessary to clarify the evolutionary processes driving the lack of NSs and if this is a common feature among them. This will ultimately lead to the understanding of the importance of this virulence gene in human infections.

The ENSV strain 76V-25880 was isolated from mosquitoes, but remains an unclassified orthobunyavirus. In our analysis of the complete coding sequences of the three genomic segments and the molecular characterization of this virus shows that ENSV forms a unique clade, which is probably a new serogroup within the *Orthobunyavirus* genus. ENSV presents the same phylogenetic origin from viruses in Group C and Capim groups. However, although ENSV has not been associated with human infections, the close phylogenetic relationship between this virus and the Capim and Group C group members could suggest that an association of this virus and human diseases cannot be excluded. It is also important to notice that ENSV presents two putative ORFs for the NSs protein: a short sequence with 96 amino acids, after the start codon of the N protein, and a second larger protein of 119 amino acids, which have a pre-N start codon, as previously described for Brazoran virus (Lanciotti et al., 2013). Both proteins share the exact amino acid sequence, but the larger NSs contains 23 extra amino acids in its N-terminus. Whether both proteins are translated and have diverse functions during viral replication remains to be determined. It has already been demonstrated for Bunyamwera virus that when the start codon of the NSs ORF is removed in a reverse genetics generated virus, the virus is still capable of producing an NSs protein from a downstream start codon (van Knippenberg et al., 2010), which suggests that the host translation machinery could possibly "slip" and recognize both Met codons (ATGs) and translate both ENSV NSs proteins.

Pairwise amino acid sequence distance analysis of the nucleocapsid, M polyprotein and RdRp proteins of CAPV and ENSV demonstrated a divergence of 52%, 39% and 32% for the N, M and RdRp proteins, respectively, and when these viruses are compared to the amino acid distance from other groups we can conclude that the degree of evolutionary divergence between these two clades is equivalent with the levels of divergence that can be observed among the other orthobunyavirus groups, and that they could be considered two separate clades with possible biological significance. Both viruses exhibit a higher amino acid distance within the *Orthobunyavirus* genus for the M polyprotein, while the distance rates for the nucleocapsid protein were lower. It is also important to note that the amino acid distance values for the RdRp were the lowest in our analysis, which means that these viruses are under the same selective pressure, with a more conserved polymerase protein and more plastic glycoproteins. This is confirmed by an alignment of the RdRp amino acid sequence, which reveals a high degree of conserved residues in the protein domains that are involved in the polymerase activity (Motifs Pre-A, and A through E, Supplementary Fig. 2B).

The current study provides better understanding of the genetic and evolutionary relationships of CAPV and ENSV among the *Orthobunyavirus* genus. In particular, we show the molecular and phylogenetic analyses of two prototype species from two groups, Capim and Enseada orthobunyaviruses. Our analysis reveals novel features among orthobunyaviruses, since ENSV virus contains two putative ORFs for the NSs protein. Our analysis regarding genomic reassortment events based on complete coding sequences has not suggested any relevant aspect that could include these viruses as reassortant species, but the potential of co-infection in nature does not exclude this possibility and it should be further investigated in future studies (Aguilar et al., 2011).

Our study provides insight into the genetic diversity and evolution of *Orthobunyaviruses*. In the future, the availability of more complete genetic information of other orthobunyaviruses, together with an understanding of their genetic relationships, will aid into better defining the evolutionary and molecular characteristics, as well as the distribution, epidemiology and public health impact of these viruses.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.meegid.2016.02.024.

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