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Short communication

A diverse assemblage of RNA and DNA viruses found in mosquitoes collected in southern Portugal

Manuel Silva[a], Paulo Morais[a,b], Carla Maia[b,c], Carolina Bruno de Sousa[b,d], António Paulo Gouveia de Almeida[b,c,e], Ricardo Parreira[a,b,*]

[a] Unidade de Microbiologia Médica, Instituto de Higiene e Medicina Tropical (IHTM)/Universidade Nova de Lisboa (NOVA), Lisboa, Portugal
[b] Global Health and Tropical Medicine (GHTM) Research Centre, Lisboa, Portugal
[c] Unidade de Parasitologia Médica, (IHMT/NOVA), Faro, Portugal
[d] Centro de Ciências do Mar, Universidade do Algarve, Faro, Portugal
[e] Centre for Viral Zoonoses, Faculty of Health Sciences, University of Pretoria, South Africa

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ABSTRACT

This work describes the detection and partial characterization of mosquito-borne virus genomic sequences, based on the analysis of mosquitoes collected from the Spring to Fall of 2018 in the Algarve (southern Portugal). The viral survey that was carried out using multiple primer sets disclosed the presence of both RNA and DNA viral sequences in these mosquitoes, which were subsequently analysed using maximum likelihood and Bayesian phylogenetic reconstruction methods. The obtained results brought to light three lineages of insect-specific flaviviruses, a monophyletic cluster of bunyaviruses from an unassigned group within the *Phenuiviridae* family, as well as brevidensoviruses (*Parvoviridae, Densovirinae*:). The latter two groups of viruses were here described for the first time in mosquitoes from Portugal. Results relating to the tentative isolation of the putative viruses identified in C6/36 cells are also shown, and the serendipitous, although not unexpected, isolation a Negev-like *Nelorpivirus* from *Culex laticinctcus* mosquitoes is reported.

Among invertebrates, mosquitoes are frequently the focus of viral surveys because they may serve as vectors for many pathogenic agents with (re)emerging potential, including viruses (Gould et al., 2017). Despite their potential to transmit viral agents which may affect human health, mosquitoes have also been shown to harbor many others that seem to display restricted replication capacity in vertebrate cells. These viruses are regarded as insect-specific (Calisher and Higgs, 2018; Junglen and Drosten, 2013), are genetically diverse, and have been tentatively placed in a multitude of viral *taxa* (Abudurexiti et al., 2019; Bolling et al., 2015).

This report describes the results of a survey that was carried out aiming at the detection of a selection of both RNA and DNA viruses, including flaviviruses, phleboviruses, and densoviruses. We based our analysis on mosquitoes recently collected in the Algarve, the southernmost region of the country. This region is climatically influenced by its proximity to the Mediterranean sea, is a hotspot for tourism, and a temporary haven for migratory birds as they fly to/from Africa/northern Europe. Furthermore, the Algarve displays a combination of ecological and climatic conditions that support the development of multiple species of mosquitoes to high densities, some of which may serve as vectors for arboviruses (Almeida et al., 2008).

The mosquitoes that were analysed in this work were collected between April and November of 2018 in the district of Faro and corresponded to a convenience sample obtained using CDC-light traps that were not baited with CO_2. Due to logistic constraints, the collected mosquitoes were maintained at −20 °C until they were brought to IHMT in Lisbon, where their morphological identification was carried out on ice-bricks using appropriate identification keys (Becker et al., 2010; Ribeiro and Ramos, 1999). These mosquitoes were grouped according to species, sex, geographic origin, and blood-feeding status, and divided into pools with a minimum of 5, and a maximum of 60 specimens. The detection of viral genomes was carried out exclusively using female mosquitoes.

The preparation of mosquito homogenates, nucleic acids extraction, cDNA synthesis, PCR amplification, and DNA cloning was performed as previously described (Carapeta et al., 2015; Pimentel et al., 2019). Detection of *Flavivirus ns5* sequences was carried out using previously described primers and reaction conditions (Vázquez et al., 2012).

* Corresponding author at: Unidade de Microbiologia Médica (Grupo de Virologia), Instituto de Higiene e Medicina Tropical (IHMT)/Universidade Nova de Lisboa (NOVA), Global Health and Tropical Medicine (GHTM). Rua da Junqueira 100, 1349-008, Lisboa, Portugal.
E-mail address: ricardo@ihmt.unl.pt (R. Parreira).

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Bunyavirus L-coding sequences were targeted for amplification using *Phlebovirus* and *Orthobunyavirus* primers and reaction conditions either previously described (Matsuno et al., 2015; Pereira et al., 2017), or defined in the course of this work. Densovirus sequences were amplified using primers targeting the viral NS1 encoding gene. All the primers, as well as the thermal profiles used for PCR, are listed in Supplementary Table 1. Virus isolation in cell culture was carried out using the *Aedes albopictus* C6/36 cell line, as described before (Carapeta et al., 2015).

Multiple alignments of nucleotide (nt) or amino acid (aa) sequences were performed using the iterative G-INS-I method, as implemented in MAFFT vs. 7. Also, phylogenetic analyses using the maximum likelihood optimization criterium or following a Bayesian approach were carried out essentially as described in previous reports (Pereira et al., 2017; Pimentel et al., 2019).

For molecular confirmation of the morphological identification of mosquitoes, partial mitochondrial cytochrome c oxidase subunit I (COI) sequences were obtained and analysed as previously described (Parreira et al., 2012). However, this analysis was only performed on the pools of mosquitoes where molecular screenings suggested the presence of a viral genome (see below). In all cases, it confirmed the morphological identifications that had been performed. All the nt sequences obtained in the course of this study were deposited in the public sequence databases (GenBank/ENA/DDBJ consortium) under accession numbers LC480777-LC480779 (*ns5*-flaviviruses), LC480766-LC480776 (*L*-bunyaviruses), LC483875 (*ORF1*-Negev-like virus), LC486533 and LC486534 (*NS1*-brevidensoviruses), and LC480766-LC480779, and LC484858 (COI).

The mosquitoes analysed in this work totaled 2837 specimens (Supplementary Table 2). Most were female (80%, 2276/2837), and the majority were unfed, with only 14.9% (340/2276) evidencing a blood-meal. They were classified into 6 genera (*Anopheles*, *Culex*, *Culiseta*, *Aedes*, *Coquillettidia*, and *Uranotaenia*), and 16 species. The genus *Culex* encompassed the largest number of specimens distributed into 5 species [*Culex pipiens* s. l. (Linnaeus, 1758), *Cx. theileri* (Theobald, 1903), *Cx. laticinctus* (Edwards, 1913), *Cx. univittatus* (Theobald, 1901), *Cx. hortensis* (Ficalbi, 1889)].

Among the 2276 female mosquitoes that were collected, 79.1% (n = 1801) were associated into 50 pools, all of which were subsequently processed for viral screening using a combination of different PCR/RT-PCR protocols (Supplementary Table 1). *Flavivirus* and *Phlebovirus*-like genomes were detected in multiple pools of *Aedes*, *Anopheles*, *Culiseta*, and *Culex* mosquitoes, as described in Supplementary Table 2. While the presence of *Orthobunyavirus* sequences could not be unambiguously confirmed in any of the pools analysed, the use of *Brevidensovirus*-specific primers allowed the observation of the expected amplification products when cDNA extracts prepared from *Cx. laticinctus* (n = 1) and *Cs. longiareolata* (Macquart, 1838) (n = 1) macerates were used.

Flavivirus-specific amplicons were obtained from four species of mosquitoes indicating the presence of *Flavivirus* genomes in *Cx. laticinctus*, *Cs. annulata* (Schrank 1776), *Ae. caspius*, and *An. petragnani* (Del Vecchio 1939). However, attempts to obtain a high-quality sequence from *An. petragnani* (Del Vecchio 1939) systematically failed, even when recombinant plasmid clones carrying the *Flavivirus*-specific amplicon were used as template for cycle-sequencing, probably due to very low plasmid copy number. On the other hand, analysis of the obtained sequence data (Supplementary Fig. 1) clearly showed that they clustered among the so-called classical insect-specific flaviviruses (cISF), but segregated in three genetically distinct lineages. Two viral sequences detected in pools of *Cx. laticinctus* and *Ae. caspius* were associated with previously identified genetic clusters of viruses circulating in the Iberian Peninsula (Ferreira et al., 2013; Parreira et al., 2012; Vázquez et al., 2012). In addition, a viral sequence obtained from *Cs. annulata* showed high identity (> 98% by BLASTn), and shared common ancestry with another one (KU958176) recently obtained from *Cs. annulata* mosquitoes from Turkey (Ergünay et al., 2017). Curiously, high sequence identity also extended to two other viral sequences (> 96% identity with JF707859-JF707860 using BLASTn) previously found to be integrated within the genomes of *Ae. vexans* (Meigen, 1830) mosquitoes from Spain (Vázquez et al., 2012).

Amplicons with a size compatible with the presence of a *Phlebovirus* L segment were detected in some of the pools analysed corresponding to five species of mosquitoes (Supplementary Table 2). Surprisingly, high sequence-identity *Phlebovirus* homologs could not be found in the databases when the obtained sequences were analysed with BLASTn/x. This suggested they had been amplified from *Phlebovirus*-like viruses, but not legitimate phleboviruses. Furthermore, phylogenetic reconstruction using nt alignments placed all of them (n = 11) outside the *Phlebovirus*, *Banyangvirus*, *Bandavirus*, and *Goukovirus* genera (not shown). Since these sequences diverged from any of the *taxa* mentioned above, their identity was investigated using phylogenetic analysis performed on aligned datasets of amino acid sequences of the viral-encoded RNA polymerases (L protein) from the viral groups that compose the Order *Bunyavirales*. Regardless of the method/parameters used for phylogenetic reconstruction, the obtained trees displayed congruent topologies that placed the viral sequences obtained in this study within the *Phenuiviridae* family (Fig. 1). Within this radiation of bunyabiruses, they formed a strongly supported monophyletic cluster that also included viral sequences previously detected using metagenomics/NGS (Chandler et al., 2015; Li et al., 2015; Sadeghi et al., 2018). This viral lineage remains unnamed, as it has not been yet assigned any official designation by the International Committee on Taxonomy of Viruses. Although some of the reference sequences within this cluster had been previously appointed as members of the *Peribunyaviridae* family (Sadeghi et al., 2018), the analysis shown here contradicts that statement.

The analysis of the obtained densovirus NS1 sequences placed them within the *Brevidensovirus* genus (Fig. 2A), while the analysis of a *Brevidensovirus*-only nt sequence dataset (Fig. 2B) revealed that the sequences here described from *Cx. laticinctus* and *Cs. longiareolata* shared a common ancestor with those from brevidensoviruses previously identified in mosquitoes from Russia and Brazil (accession numbers M37899 and GU452799, respectively).

Seven macerates from six species of mosquitoes (*Ae. berlandi*, *Ae. caspius*, *An. petragnani*, *Cs annulata*, *Cs. longiareolata*, and *Cx. laticinctus*) were selected for viral isolation in C6/36 cells. After two weeks of culture, and when compared with the negative controls, C6/36 exposed to a *Cx. laticinctus* macerate revealed evident CPE. This was characterized by cell growth arrest, cell rounding and detachment from the solid surface (Supplementary Fig. 2A). Somewhat surprizingly, when screened by PCR/RT-PCR using the same primers employed for viral genome screening, none of the culture supernatants revealed the presence of any of the targeted virus-groups. However, the observed CPE recalled previous virus isolation attempts carried out in our laboratory, and suggested the presence of a nelorpivirus in the culture supernatant. This was confirmed using Negev-like virus-specific primers combined with a phylogenetic analysis of the obtained partial ORF1 sequence (Supplementary Fig. 2C). The low success rate of isolation of viruses using C6/36 cells must take into account the fact that only one blind passage was performed. Although this strategy may have conditioned the possibility of obtaining high titer viral suspensions, the success of viral isolation may have been more seriously compromised by the fact that the mosquitoes were maintained at −20 °C from the day of their collection up to the point when they were identified and macerated. While this does not seem to have affected the infectivity of Negev-like viruses, it may have influenced that of the other viruses detected in the mosquitoes that were analysed.

To conclude, this report brought to evidence the circulation of a diverse array of viruses in mosquitoes collected in southern Portugal. While *bona fide* arboviruses were not identified, three lineages of cISF were described in as many different species of *Culex*, *Aedes* and *Culiseta* mosquitoes. Two of these lineages had been described previously

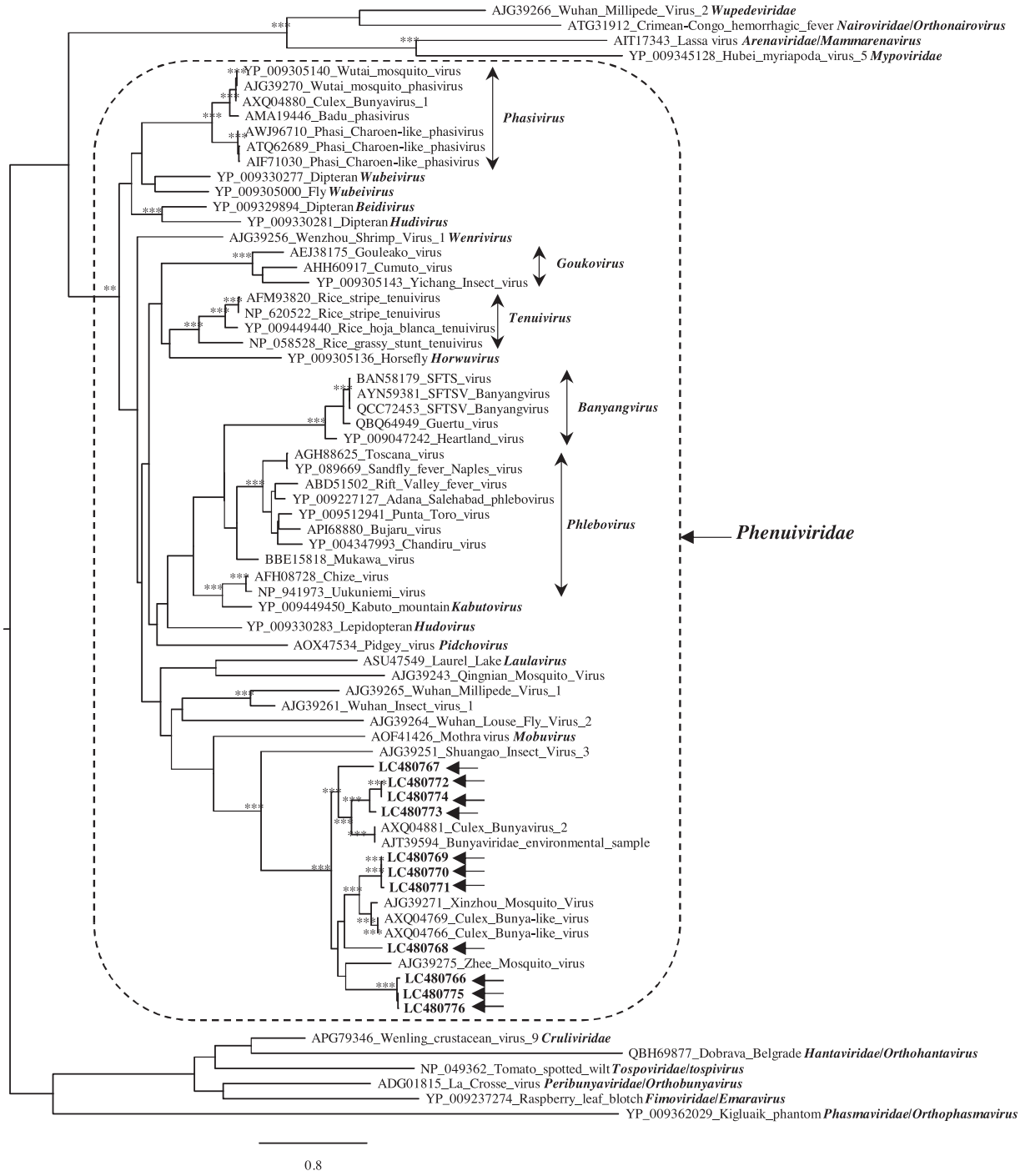

Fig. 1. Phylogenetic analysis of partial amino acid sequences of the viral-ended RNA polymerase of viruses within the Order *Bunyavirales*. At specific branches the number of "*" indicates the support revealed by the different phylogenetic reconstructions methods used, assuming as relevant bootstrap values ≥75% and posterior probability values ≥0.80. Some of the taxonomic groups (viral families and genera) are indicated in boldface and by vertical arrows. The sequences are indicated by their accession number_virus name. The sequences described in this work are indicated by their accession numbers, highlighted in boldface, and signaled by the horizontal arrows. The size bar indicates the number of amino acid substitutions per site.

(Ferreira et al., 2013; Parreira et al., 2012; Vázquez et al., 2012), but another one associated with *Culiseta* specimens had not been described before in the Iberian Peninsula. In addition, the presence of genus-unassigned phenuiviruses (*Buniavirales*) and brevidensoviruses (*Parvoviridae*, *Densovirinae, Brevidensovirus*) were here described for the first time. Some, or even all, of these viruses, may correspond to viral mutualistic symbionts that are part of the mosquito microbiota, as previously described (Roossinck, 2011).

Author contributions

MS was involved in mosquito identification and processing, as well as molecular screening and phylogenetic analysis; PM was responsible for screening of densoviruses and the analyais of their sequences; CM and CBS were involved in mosquito collection; APGA supervised mosquito identification and processing and manuscript writing; RP supervised all the laboratory tasks involved in this work, including phylogenetic analysis, and wrote the manuscript.

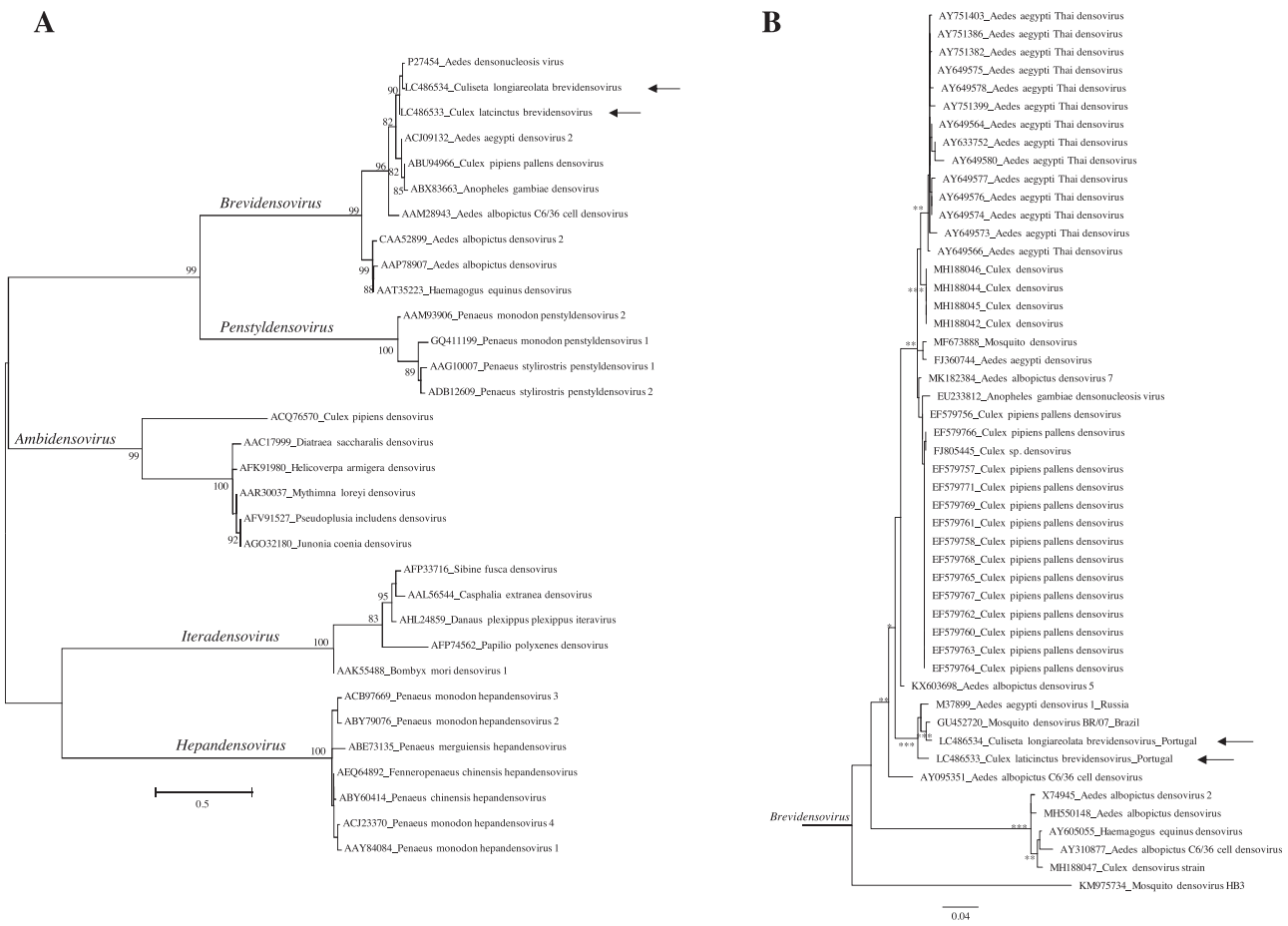

Fig. 2. Phylogenetic analysis of partial *NS1* amino acid (A) or nucleotide (B) sequences of viruses in the sub-family *Densovirinae* (family *Parvoviridae*)(A) or the *Brevidensovirus* genus (B). In (A) only a maximum likelihood tree is shown, with bootstrap values (≥75%) indicated at specific branches. In (B), at specific branches the number of "*" indicates the support revealed by the different phylogenetic reconstructions methods used, assuming as relevant bootstrap values ≥75% and posterior probability values ≥0.80. The sequences obtained in this work are highlighted in boldface and indicated by the horizontal arrows. The size bar indicates the number of amino acid (A) or nucleotide (B) substitutions per site.

Research involving human and animal participants

This article did not contain any study with human participants or animals.

Declaration of Competing Interest

All authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2019.197769.

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