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SHORT REPORT

Characterization of a new bunyavirus and its derived small RNAs in the brown citrus aphid, *Aphis citricidus*

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Abstract

High-throughput sequencing is widely used for virus discovery, and many RNA viruses have been discovered and identified. A new negative-sense single-stranded RNA virus was identified in the brown citrus aphid and named *Aphis citricidus bunyavirus*. The genome consists of large (7037 nt), medium (3462 nt), and small (1163 nt) segments. Phylogenetic analysis and amino acid sequences identities of this virus with other bunyaviruses suggest that it is a new species belonging to the family *Phenuiviridae*. The small interfering RNA pathway could be involved against the infection of this virus in brown citrus aphid as supported by the viral derived small RNAs. The discovery of this virus illustrates the diversity of RNA viruses and contributes to the classification of bunyaviruses.

Keywords Bunyaviruses · Aphids · RNAi · Small RNA

Bunyavirus are enveloped viruses with a helical and oval or spherical capsid ranging from 80–120 nm of diam [1]. The genome consists of single-stranded RNA of negative polarity divided into three segments: large (L), medium (M), and small (S). The large segment encodes a single protein RNA-dependent RNA polymerase (RdRp). This protein is an enzyme that catalyzes the replication of RNA from an RNA template and plays an essential role in viral replication. The M segment encodes two envelope glycoproteins, Gn and Gc, which are involved in viral attachment to the cell and the fusion of the viral and cellular membrane. The nucleocapsid protein is encoded by the S segment, which is involved in viral RNA replication [2]. This group of viruses has a wide range of hosts, including vertebrates, invertebrates, and plants [3]. The order *Bunyavirales* currently contains 12 families comprised of 46 genera recognized by the International Committee on Taxonomy of Viruses (https://talk.ictvonline.org/taxonomy/).

The brown citrus aphid, *Aphis citricidus*, was formerly in the genus *Toxoptera* which has now been placed in the genus *Aphis* [4]. *A. citricidus* is the main vector of *citrus tristeza virus* worldwide [5]. *Citrus tristeza virus* is one of the most widely distributed and destructive diseases of citrus [6]. The development of high-throughput sequencing technologies have enabled a large number of insect viruses to be identified [7]. Aphids host diversified viruses, such as *Aphid lethal paralysis virus* [8], *Aphis glycines virus 2* [9], *Aphis glycines virus 3* [10], *Rosy apple aphid virus* [11], and *Brevicoryne brassicae virus* [12]. However, no studies have reported viruses harbored in brown citrus aphid. In this study, we discovered a new bunyavirus in the brown citrus aphid tentatively named "*Aphis citricidus bunyavirus*".

Stock colonies of brown citrus aphid were originally collected from a citrus greenhouse at Southwest University, Chongqing, China, in 2012, and maintained at a constant temperature at 25 °C, relative humidity of 65 ± 5%, and a 14 h:10 h (L:D) photoperiod [13]. Total RNA was isolated from 20 adults and 20 nymphs randomly selected

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from the colonies using TRIzol reagent (Invitrogen, Carlsbad, CA). The RNA was then used to construct two libraries. First, an RNA-seq library was used for exploring the potential sequences of RNA viruses. Briefly, the rRNA was removed from the total RNA using a Ribo-Zero Magnetic Kit (Epicentre, Madison, WI); then, an RNA-seq library was constructed using a TruSeq Total RNA Sample Prep Kit (Illumina, San Diego, CA). Then, RNA sequencing was performed on a HiSeq 2500 platform with PE150 bp (Illumina), which generated ~ 8 Gbp raw data (NCBI-SRR6981553). Second, a small RNA library was used for analyzing the potential viral-derived small RNAs. This library was established from an RNA-depleted sample using a small RNA Sample Pre Kit (Illumina, San Diego, CA) and sequenced by Illumina HiSeq 2500 platform to generate ~ 10 Mbp raw data (NCBI-SRR6981554). Raw reads were cleaned by removing adaptors, low-quality sequences, and reads with more than 20% low quality bases. The sRNA and RNA-seq data were assembled with Kmer values (17 for sRNA and for RNA-seq) using Velvet, CLC, and IDBA. The unique sequences were obtained by removal of redundant contigs. After assembling the RNA-seq data, a comparative analysis of Blastx in NCBI nucleotide and non-redundant protein sequence databases demonstrated that these sequences might belong to an uncharacterized virus that in the order *Bunyavirales*. The genome of the virus consisted of three segments: L (7037 nt), M (3462 nt), and S (1163 nt) (Fig. 1a). To confirm these segments by RT-PCR, we sequenced the L segment through five parts with a small overlap of about 100–200 nt, M segment by two parts, and S segment by one part (Fig. S1, Table S1). Viral ORFs were predicted using Gene Finding in Viral Genomes (http://linux1.softberry.com/berry.phtml?topic=virus0&group=programs&subgroup=gfindv). Identification of conserved and functional domains of the predicted proteins in *Aphis citricidus bunyavirus* was made using the Conserved Domain Database in NCBI (https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) and the SMART tool (http://smart.embl-heidelberg.de/smart/set_mode.cgi?GENOMIC=1). Molecular weight and the isoelectric point were predicted by the online tool (https://web.expasy.org/compute_pi/).

The L segment of *Aphis citricidus bunyavirus* encoded one ORF (ORF1) starting at nucleotide position 289 nt and ending at 6829 nt. ORF1 encoded an RNA-dependent RNA polymerase (RdRp) of 2180 amino acids (aa) and a molecular weight of approximately 253.1 kDa. The domain of RdRp consisted of L_protein_N, DUF3770 and the core of RdRp. L protein N: endonuclease domain at the N-terminus of bunyavirus L proteins [14]. DUF3770: protein of unknown function (DUF3770). This domain family is found in viruses and is approximately 250 aa long. M segment encoded a protein of 1003 aa with a molecular weight of 117.1 kDa, spanning nucleotide position 412-3423. Members of the *Bunyaviridae* acquire an envelope by budding through the lipid bilayer of the Golgi complex (i.e., Phlebovirus_G1 and Phlebovirus_G2). For the S segment, the encoded ORF started at nucleotide position 1251, including a nucleoprotein (NP) of 279 aa with a molecular weight of 33.6 kDa (Fig. 1a). Based on the Conserved Domain Database and SMART analyses, S segment encoded proteins had the conserved domains, Tenui_N super family (Accession cl05345). This is a typical domain in nucleocapsid protein of viruses in the genera *Tenuivirus* and *Phlebovirus*, with a Pfame-value of 5.4D-16 that is different from those in other genera of *Bunyavirales*. In addition, phylogenetic analysis using NP proteins of representative viruses in the order *Bunyavirales* revealed that *Phasivirus* was separated from the cluster of *Phleobovirus* and *Tenuivirus*.

To further study the classification and evolution of *Aphis citricidus bunyavirus* in the *Bunyavirales*, sequence alignments of the core motif in RdRp from selected species were made (Fig. S2). The alignment of RdRp of *Aphis citricidus bunyavirus* with other (-ss) RNA viruses suggests that their RdRp have six conserved motifs (motif A-E and premotif A), which are highly conserved regions in the RdRp of members of the *Bunyavirales*. Bunyavirus RdRp contain motifs including premotif A, motif A, motif B, motif C, and motif D. Premotif A has five conserved aa ($KxQx_5Rx_9K/Rx6E$), motif A exhibits a conserved Dx_2KW sequence, motif B contains the conserved QGx_5SS residues, motif C exhibits conserved SDD residues, motif D contains a conserved lysine, and motif E has conserved Ex_2S residues [15]. Based on the phylogenetic tree constructed by the aa sequences of the putative RdRp using MEGA6 [16], this new virus clustered with viruses from *Wenrivirus* in the *Phenuiviridae* (Fig. 1b). We also conducted a phylogenetic analysis using the aa sequences of GP and NP. In consistence with the phylogenic analysis of RdRp, *Aphis citricidus bunyavirus* was also placed in the *Phenuiviridae* (Fig. S3). However, it was more closely clustered to *Phlebovirus* in the phylogenic tree of GP (Fig. S3A) and more closely clustered to *Phasivirus* in the phylogenic tree of NP (Fig. S3B). To further analyze the evolutionary status of this virus, aa sequences identities between the novel virus and other known bunyaviruses were conducted using the DNAstar MEGalign tool. The domains of RdRp and NP were closely related to *Phlebovirus*, but the GP domain was closely related to *Hartmanivirus* in the *Arenaviridae* (Tables S2; S3). RdRp shared 75.3% similarity with *Uukuniemi virus* (genus *Phlebovirus*). In addition, the highest similarity (82.4%) for the GP were found with *Haartman Institute snake virus* (genus *Hartmanivirus*). Moreover, the NP domain also showed a high aa sequence identity (71.5%) to that of *Uukuniemi virus* (Table S2). Therefore, we postulated that *Aphis citricidus bunyavirus* belongs to a new genus. *Aphis citricidus bunyavirus* was searched for in the citrus leaves being infested by brown

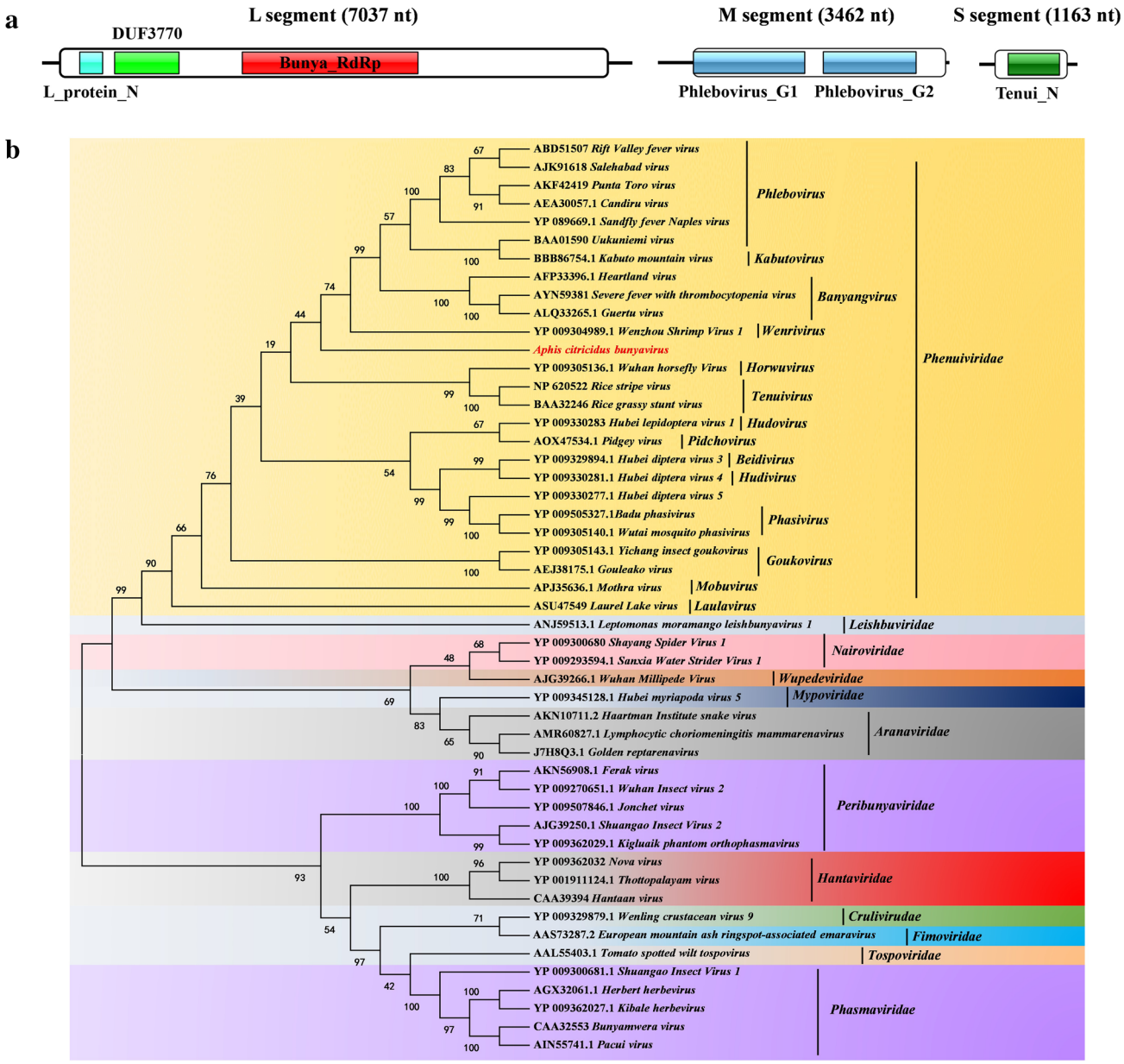

Fig. 1 **a** Schematic representation of *Aphis citricidus bunyavirus* genome organizations. L_protein_N: endonuclease domain at the N-terminus of bunyavirus L proteins. DUF3770: Protein of unknown function (DUF3770). This domain family is found in viruses and is approximately 250 amino acids in length. Bunya_RdRp: RNA-dependent RNA polymerase of bunyaviruses. Phlebovirus_G1: *Phlebovirus* glycoprotein G1 sequences. Phlebovirus_G2: *Phlebovirus* glycoprotein G2 sequences. Tenui_N: nucleocapsids of *tenuiviruses*. **b** Phylogenetic tree of *Aphis citricidus bunyavirus* with the amino acid sequences of RdRp, and the corresponding proteins of representative other bunyaviruses. The tree was conducted with 1000 replicates using Muscle alignment and the neighbor-joining method

citrus aphid, but the virus was not found in this host plants (Fig. S4).

Virus-related dsRNAs such as viral dsRNA replication intermediates, the viral genome itself, and viral transcripts, can trigger an antiviral response from the small interfering RNA (one of the key pathways of RNAi) [17]. Thus, the small RNA reads derived from *Aphis citricidus bunyavirus* genome could provide evidence for infection activity of this virus in *A. citricidus* and also activation of the host antiviral immunity. We found that the length distribution of vsiRNAs of this virus exhibited a high peak at the 22 nt-long vsiRNAs in all segments (Fig. 2a). In particular, the 22 nt-long vsiRNAs derived from the virus genome and antigenome (Fig. 2b) may represent the slicing activity of Dicer-2 to virus-related dsRNAs [18].

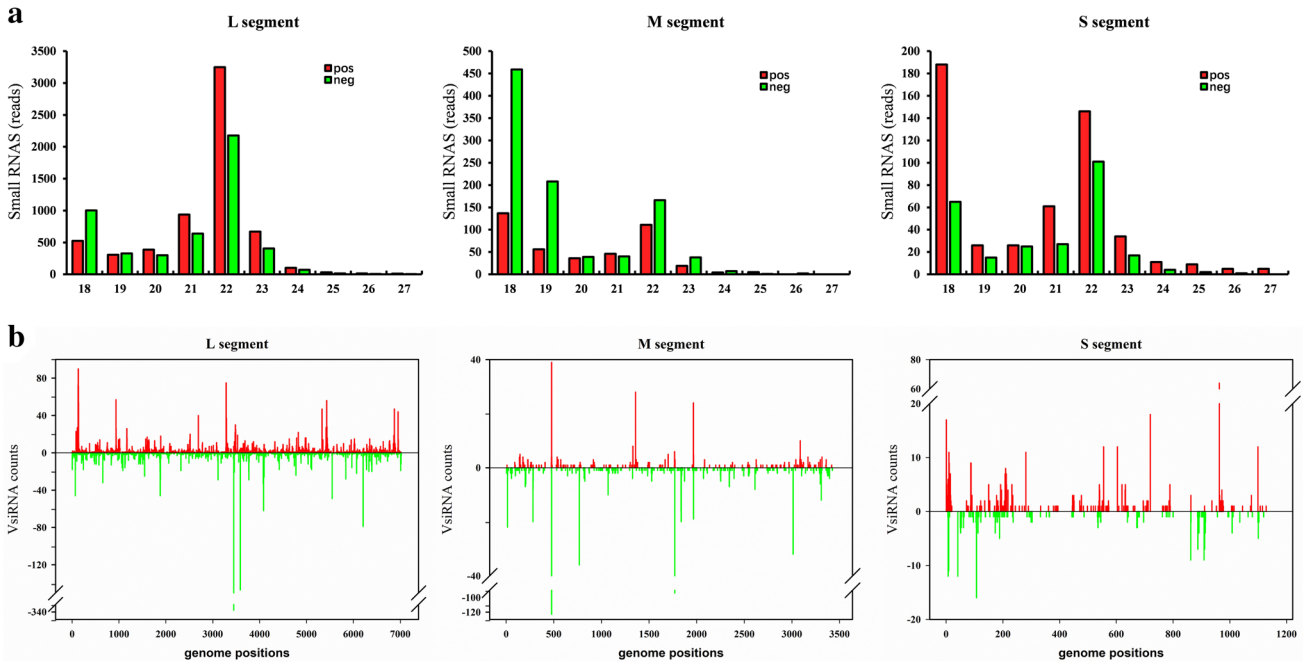

Fig. 2 Distribution of *Aphis citricidus bunyavirus*-derived small RNAs **a** The length distribution of *Aphis citricidus bunyavirus*-derived small RNAs, including three segments. **b** The distribution of ~ 22 nt-long small RNAs (vsiRNA) on regions of *Aphis citricidus bunyavirus* genome and antigenome. The red bars represent the counts of visRNAs which comes from *Aphis citricidus bunyavirus* genome, and the green bars are derived from antigenome

In conclusion, based on the genomic structure, amino acid sequence identities, and phylogenetic relationships, we believe that *Aphis citricidus bunyavirus* is a new species in the family *Phenuiviridae*. The small interfering RNA pathway could be involved in against the infection of this virus in brown citrus aphid. Further studies on the interactions of this new virus and its hosts will be required to determine the potential of RNAi and viruses for pest control [19].

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Author contributions WZ, JN and JW designed the study. The experiments are performed by WZ, TW, TC, LY, YT and CY. WZ, TW and TC contributed to sequencing data and PCR analysis. TC, LY, YT, MG performed the alignment and phylogenetic tree, MG contributed to test *Aphis citricidus bunyavirus* in citrus. WZ wrote the initial draft. JN and JW edited the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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