Insights into the RNA virome of the corn leafhopper *Dalbulus maidis*, a major emergent threat of Maize in Latin America

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Abstract

The maize leafhopper (*Dalbulus maidis*) is a significant threat to maize crops in tropical and subtropical regions, causing extensive economic losses. While its ecological interactions and control strategies are well-studied, its associated viral diversity remains largely unexplored. Here, we employ high-throughput sequencing data mining to comprehensively characterize the *D. maidis* RNA virome, revealing novel and diverse RNA viruses. We characterized six new viral members belonging to distinct families, with evolutionary cues of beny-like viruses (*Benyviridae*), bunya-like viruses (*Bunyaviridae*) iflaviruses (*Iflaviridae*), orthomyxo-like viruses (*Orthomyxoviridae*), and rhabdoviruses (*Rhabdoviridae*). Phylogenetic analysis of the iflaviruses places them within the genus *Iflavirus* in affinity with other leafhoppers-associated iflaviruses. The five-segmented and highly divergent orthomyxo-like virus showed a relationship with other insect associated orthomyxo-like viruses. The rhabdo virus is related with a leafhopper associated rhabdo-like virus. Furthermore, the beny-like virus belonged to a cluster of insect-associated beny-like viruses, while the bi-segmented bunya-like virus was related with other bi-segmented insect-associated bunya-like viruses. These results highlight the existence of a complex virome linked to *D. maidis* and paves the way for future studies investigating the ecological roles, evolutionary dynamics, and potential biocontrol applications of these viruses on the *D. maidis*—maize pathosystem.

Key words: RNA virome; *Dalbulus maidis*; maize leafhopper; data mining

1. Introduction

The maize leafhopper *Dalbulus maidis* (DeLong & Wolcott) (Hemiptera: Auchenorrhyncha) is a significant threat to maize crops causing extensive economic losses [1]. This insect pest is widely distributed in the Americas, from the southern United States to northern Argentina [2]. In addition to the direct damage caused by its feeding, this insect also serves as the vector for the mollicute *Spiroplasma kunkelii* Whitcomb, the causal agent of Corn Stunt disease, and the maize bushy stunt (MBS) phytoplasma [1]. *D. maidis* is also the vector of two viruses: maize rayado fino virus (MRFV) [1], and maize striate mosaic virus (MSV) [3]. While its ecological interactions [4, 5] and control strategies, such as germplasm resistance, insecticide sprays and some cultural practices are well-studied [5, 6], its associated viral diversity remains largely unexplored.

Viruses are the most abundant microbes on the planet [7-9] and RNA viruses dominate the eukaryotic virome [10, 11]. Hundreds of viruses have been identified in recent years in arthropods [10-15], illustrating that invertebrates usually have a greater and more diverse virome than vertebrates [16]. Nevertheless, over the last years there was a rapid increase of vertebrate-associated virus entries in the NCBI-GenBank, while low numbers of invertebrate-associated virus were identified and uploaded into public repositories [16]. The availability of a great amount of RNA-seq data (metatrancriptomics) in public databases has revolutionized the field of virus discovery, fostering the identification of novel viruses even without a previous knowledge of their genome sequence [7, 17, 18]. The comprehensive analysis of the RNA virosphere led to the identification of a great number of novel viruses thus shedding light not only on the huge diversity of RNA viruses in arthropods, but also on their evolutionary histories as well as on their genomic organization complexity [11, 12, 15, 16, 19, 20]. Furthermore, this advances on the knowledge of the virosphere are leading to opportunities and challenge on virus discovery [21] and to develop a new, genome-based virus taxonomy [22-26].

This paper reports the analysis of 23 publicly available *D. maidis* transcriptome datasets from USA, Brazil and Argentina, that resulted in the discovery of six novel RNA viruses associated to this insect. To our knowledge, this is the first report of insect viruses associated to this economically important insect, revealing that the *D. maidis* RNA virome is rich and diverse.

2. Material and Methods

2.1 Exploration of the D. maidis virome from public RNA-seq datasets, sequence assembly and virus identification

We analyzed all *D. maidis* Sequence Read Archives (SRA) deposited in the NCBI (https://www.ncbi.nlm.nih.gov/sra). The raw nucleotide sequence reads from each SRA experiment were downloaded from their associated NCBI BioProjects (Table S1). The datasets were pre-processed by trimming and filtering with the Trimmomatic v0.40 tool as implemented in http://www.usadellab.org/cms/?page=trimmomatic with standard parameters. The resulting reads were assembled *de novo* with rnaSPAdes using standard parameters on the Galaxy server (https://usegalaxy.org/). The transcripts obtained from *de novo* transcriptome assembly were subjected to bulk local BLASTX searches (E-value < $1e^{-5}$) against a curated database of the publicly available reference viral proteins. The resulting viral sequence hits of each dataset were explored in detail. Tentative virus-like contigs were curated (extended and/or confirmed) by iterative mapping of each SRA library's filtered reads. This strategy was used to extract a subset of reads related to the query contig, used the retrieved reads from each mapping to extend the contig and then repeat the process iteratively using as query the extended sequence [27]. The extended and polished transcripts were reassembled using Geneious v8.1.9 (Biomatters Ltd.) alignment tool with high sensitivity parameters.

2.2 Bioinformatics tools and analyses

2.2.1 Sequence analyses

Open reading frames (ORFs) were predicted with ORFfinder (minimal ORF length 120 nt, genetic code 1, https://www.ncbi.nlm.nih.gov/orffinder/), functional domains and architecture of translated gene products were determined using InterPro (https://www.ebi.ac.uk/interpro/search/sequence-search) and the NCBI Conserved domain database - CDD v3.20 (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) with e-value = 0.1. Further, HHPred and HHBlits as implemented in https://toolkit.tuebingen.mpg.de/#/tools/ were used to complement annotation of divergent predicted proteins by hidden Markov models. Transmembrane domains were predicted using the TMHMM version 2.0 tool (http://www.cbs.dtu.dk/services/TMHMM/) and signal peptides were predicted using the SignalP version 6.0 tool (https://services.healthtech.dtu.dk/services/SignalP-6.0/), while nuclear localization signals (NLS) were predicted using cNLS Mapper tool (https://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi). The presence of gene junction sequences flanking ORFs was also included as a criterion to determine the potential coding

sequences. The predicted proteins were then subjected to NCBI-BLASTP searches against the non-redundant protein sequences (nr) database.

2.2.2 Phylogenetic analysis

Phylogenetic analysis based on the RdRp proteins, was done using MAFFT 7.505 with multiple aa sequence alignments using FFT-NS-i as the best-fit model. The aligned aa sequences were used as the input in Mega11 software [28] to generate phylogenetic trees by the maximum-likelihood method using the best-fit substitution model. Local support values were computed using bootstraps with 1,000 replicates.

3. Results and Discussion

RNA viruses associated with *D. maidis*

Insects are the most abundant group of animals on earth [29]. These animals have a rich and diverse virome which is being unraveled in the later years using high-throughput sequencing [10, 11, 14, 15, 30]; however, only a small portion of the insect viromes diversity has been described so far [31]. Several viruses were identified in some leafhoppers, mainly iflaviruses, [14]. In addition, a rhabdovirus [32], an orthomyxo-like virus [12], a chuvirus [33], a reovirus [34], a nido-like virus [35], and nine RNA viruses, belonging to seven viral clades (Picorna-Calici, Permutotetra, Bunya-Arena, Reo, Partiti-Picobirna, Luteo-Sobemo and Toti-Chryso) [36] were also identified, which illustrates how rich and diverse could be the virome associated to leafhoppers. There are more than 20,000 described species of leafhoppers (*Cicadellidae*) which illustrate that the handful of viruses linked to this invertebrate is scarce, poorly miss-represents the diversity of these invertebrates, andeven important agronomic pests as the maize leafhopper virome are still unexplored. Thus, here we analyzed transcriptome datasets of maize leafhopper collected in Argentina, USA and Brazil. The *de novo* assembling of those 23 libraries followed by similarity analyses resulted in the robust identification of six novel highly divergent RNA viruses, including a beny-like virus, a bunya-like virus, two iflaviruses, a orthomyxo-like virus and a rhabdovirus, which were tentatively named Chicharrita del maiz beny-like virus (ChMBLV), Chicharrita del maiz bunya-like virus (ChMBV), Chicharrita del maiz iflavirus 1 (ChMIfV1), Chicharrita del maiz iflavirus 2 (ChMIfV2), Chicharrita del maiz orthomixo-like virus (ChMOMLV) and Chicharrita del maiz rhabdoirus (ChMRV), respectively. This first glimpse of the virus repertoire reveals how rich and diverse is the virome associated to the maize leafhopper. We assembled the full-length coding region sequence for all viruses, but the rhabdovirus, which its nearly complete coding region was able to be assembled. Revealing the virus diversity in this

organism is crucial to further exploit the identified viruses as specific and targeted tools for the biological control of insect populations.

Molecular and phylogenetic characterization of the newly identified maize leafhopper Beny-like viruses

The *Benyviridae* is a family with members that have positive-sense, single stranded RNA viruses with only one genus, the *Benyvirus*, recognized by the ICTV [37]. Benyviruses are plant-infecting viruses with a multipartite genome [37]. Nevertheless, metagenomic studies as well as the metatrancriptomic analysis of publicly data deposited in NCBI resulted in the discovery of many benyviruses related sequences, that resemble to newly unassigned *Benyviridae* members, that likely infect a wide range of non-plant hosts, such as insects and fungi [38-41]. Moreover, the insect-associated beny-like viruses genomes are monopartite which is contrasting to the multipartite genomes displayed by the plant-associated counterparts. Here, a novel beny-like virus was identified and named as Chicharrita del maiz beny-like virus (ChMBLV), which was only detected in one transcriptome datasets of maize leafhopper, that was collected in USA (Table S1). The positive-sense, single stranded RNA ChMBLV sequence (GenBank accession number BK068250) is 6,887 nucleotides (nt) long and has two ORFs encoding a non-structural polyprotein of 2,003 amino acids (aa) and a putative structural protein of 224aa (Fig. 1A) (Table 1). This genomic organization is similar to that reported for the beetle associated viruses Diabrotica undecimpunctata virus 2 (DuV2) [42] and Guiyang benyvirus 1 (GuBV1) (Feng, unpublished). ChMBLV non-structural polyprotein showed the highest BlastP similarity with that one encoded by DuV2, with 46.61% identity (Table 1); therefore, based on genetic distance ChMBLV is a new virus distantly related to other NCBI database entries of insect viruses. Two conserved domains, the Viral_helicase1, and the ps-ssRNAv_RdRp-like, were identified in the polyprotein at aa positions 617-848 and 1,673-1,857, respectively. Similar conserved domains were found in those non-structural polyproteins encoded by related beny-like viruses [38, 39, 41, 42] ChMBLV structural protein showed the highest BlastP similarity with that one encoded by DuV2, with 29.75% identity (Table 1). This protein encoded by the ChMBLV ORF2 is likely a coat protein, since the TMV_coat super family conserved domain was identified at aa position 108-180. A similar conserved domain was also identified in the ORF2 encoded proteins of DuV2 [42], and GuBV1 (Feng, unpublished).

Phylogenetically analysis of the non-structural polyprotein amino acid sequence placed ChMBLV in a clade containing several insect-associated beny-like viruses, where ChMBlV was clustered together with the beetle Lepidiota negatoria beny-like virus within the insect clade of beny-like viruses, which is separated from the clades composed of fungi-

associated beny-like viruses and plant-associated ones (Fig. 1B). Thus, we propose to create a new genus, to be named as "*Insebenyvirus*" within the family *Benyviridae*, to accommodate the insect-associated beny-like viruses.

To our knowledge, ChMBLV is the first reported beny-like virus associated to leafhoppers.

Molecular and phylogenetic characterization of the newly identified maize leafhopper Bunya-like virus

Bunyavirales is a viral order including several families of negative-sense single-stranded RNA (-ssRNA) viruses with a multi segmented genome [43]. Bunyavirids are highly diverse and have a wide host range and a considerable genome complexity. Many proposed members of this family have been discovered in several insect species [12]. Here, a novel bunya-like virus was identified and named as Chicharrita del maiz bunyan-like virus (ChMBV), which was detected in the three transcriptome datasets of maize leafhopper from Brazil (Table S1). The full-length ChMBV coding sequence ((GenBank accession numbers BK068254 and BK068255) comprised two segments (L and S) of single-stranded, negative-sense RNA of 6,651nt and 1,281nt, respectively. Segment L encodes a putative RdRp protein of 2,166 aa, and the segment S encodes a putative nucleoprotein (NP) of 327 aa (Fig. 2A) (Table 1).

As expected, the structural NP encoding segment was the most abundant one in the three libraries from Brazil, whereas the segment encoding the RdRp was the one that accumulated less on those datasets where ChMBV was identified (Table S1). ChMBV present low homology to other bunyavirids (Table 1). The RdRp protein shows the highest BlastP similarity with the leafhopper associated Hubei insect virus 1 (HbIV1) RdRp, with 49.84% identity (Table 1), and a Bunya_RdRp super family conserved domain was identified in its sequence at aa positions 603-1,303. The NP protein shows the highest BlastP similarity with the HbIV1 NP, with 38.01% identity (Table 1), and a Tenui_N super family conserved domain has been identified in its sequence at aa positions 40-191.

The phylogenetic tree based on ChMBV RdRp encoded protein, showed that this virus is evolutionary related with leafhopper associated bunya-like viruses. ChMBV clustered with HbIV1, Hemipteran phenui-related virus OKIAV285 and the rice green leafhopper associated virus Hangzhou nephotettix cincticeps phenuivirus 1, and this cluster is linked with the planthopper-associated Sanya nilaparvata lugens phenuivirus 1 (Fig.2B). Bunyavirids have different number of genome segments; however, those viruses phylogenetically related with ChMBV, are all bisegmented RNA viruses [11]. Thus, these leafhopper-associated bunya-like viruses appear to be a new cluster of bisegmented RNA viruses that could be classified into a new genus within the *Bunyavirales* order.

Molecular and phylogenetic characterization of the newly identified maize leafhopper Iflaviruses

Iflaviridae is a family composed of only one genus, the *Iflavirus,* of positive-sense single-stranded RNA (-ssRNA) viruses with a monopartite genome, that contains an ORF encoding a single polyprotein of approximately 3,000 aa. All reported iflaviruses are associated to arthropod hosts, mainly insects [44, 45], and are among the most common viruses identified in different species of arthropods [46]. Here, two iflaviruses were identified and named as Chicharrita del maiz iflavirus virus 1 (ChMIfV1) and Chicharrita del maiz iflavirus 2 (ChMIfV2). ChMIfV1 was detected in five transcriptome datasets of the maize leafhopper (one from Argentina, another from USA and three from Brazil), while ChMIfV2 was detected in 18 transcriptome datasets, all from USA (Table S1). The iflaviruses were the most prevalent and abundant among the six viruses identified in this study (Table S1).

The positive-sense single-stranded RNA ChMIfV1 genome genome (GenBank accession number BK068251) is 11,104 nt in length, while ChMIfV2 genome (GenBank accession number BK068252) is 10,318 nt in length (Fig. 3A) (Table 1) and are A/U rich, similar to other iflaviruses [47]. Both viruses have a single ORF which encodes a polyprotein of 3,306aa and 3,102aa, respectively (Fig. 3A) (Table 1).

ChMIfV1 and ChMuIfV2 polyproteins share only a 26.3% aa identity between them, and ChMIfV1 polyprotein showed the highest BlastP similarity with that one encoded by the rice green leafhopper associated virus Nephotettix cincticeps positive-stranded RNA virus 1 (NcPSRV1), with 64.03% identity (Table 1); while ChMIfV2 polyprotein showed the highest BlastP similarity with that one encoded by the American grapevine leafhopper associated virus Scaphoideus titanus iflavirus 1 (StIfV1) with 53.04% identity (Table 1). The prediction of the conserved domains present in both ChMIfV1 and ChMuIfV2 polyproteins, showed that both viruses have the typical iflavirus gene organization. The structural domains are located at the N-terminal region of the polyprotein, while the non-structural domains are located downstream in the C-terminal region [44]. Two rhinovirus-like capsid domains (Rhv-like, aa 338-518 and aa 597-785 in ChMIfV1, aa 327-508 and aa 602-786 in ChMIfV2) and a cricket paralysis virus–like capsid domain (CRPV_capsid super family, aa 997-1,148 im ChMIfV1 and aa 1,057-1,209 in ChMIfV2) were identified in the N-terminal region of the ChMIfV1 and ChMIfV2 polyproteins, while an helicase (RNA_Helicase, aa 1,550-1,656 in ChMIfV1 and aa 1,552-1,658 in ChMIfV2), a Picornavirales 3C/3C-like protease (3C-Pro, aa 2,529-2,741 in ChMIfV1 and aa 2,301-2,530 in ChMIfV2) and RNA-directed RNA polymerase (RdRp, aa 2,952-3,256 in ChMIfV1 and aa 2,738-3,055 in ChMIfV2) conserved domains were identified in the C-terminal region of the ChMIfV1 and ChMuIfV2 polyproteins (Fig. 3A).

Phylogenetic analysis based on the RdRp conserved region shows that ChMIfV1 and ChMIfV2 clustered within the *Iflaviridae* members with other leafhopper-associated iflaviruses; where both viruses were placed in different clades as predicted by the low identity observed between them (Fig. 3B). ChMIfV1clustered with NcPSRV1 and the rice pest "zig-zag leafhopper" associated virus Hangzhou recilia dorsalis iflavirus 1 (Fig. 3B); while ChMIfV2 clustered with the American grapevine leafhopper StIfV1 and StIfV2, Hangzhou recilia dorsalis iflavirus 2 and the rice green leafhopper associated virus Congyang nephotettix cincticeps iflavirus 1 (Fig. 3B). All in all, the branching of both viruses withing the group of Iflavirus members indicates that both viruses should be included in the family *Iflaviridae* (Fig.3B).

Iflavirus infections are usually asymptomatic; however, some ifllaviruses have been reported to be harmful to the host insects leading to morphological deformities, behavioral change, and death [48-50]. Moreover, recently the the armyworm Spodoptera exigua iflavirus 1 (SeIV1) was shown to enhance the susceptibility of the armyworm to the disease caused by Spodoptera exigua multiple nucleopolyhedrovirus (SeMNPV) [51], as well as its susceptibility to Bacillus thuringiensis-based insecticide formulations and the parasitism by the wasp Hyposoter didymator [52]. Moreover, iflaviruses have been proposed for VIGS to regulate the expression of insect metabolic genes [53]. Thus, it would be critical to further explore if the iflaviruses identified in this study are pathogenic to its insect host for their potential deployment as tools for biocontrol agents.

Molecular and phylogenetic characterization of the newly identified maize leafhopper Orthomyxo-like virus

Orthomyxoviridae is a family of negative-sense single-stranded RNA (-ssRNA) viruses with a multi segmented genome [54] . Members belonging to the *Orthomyxoviridae* family are highly diverse, with a wide host range and complex genomes. Many members of this family have been discovered in various insect species [12]. Here, a novel orthomyxo-like virus was identified and named as Chicharrita del maiz orthomyxo -like virus (ChMOMLV), which was detected in three transcriptome datasets of maize leafhopper, one from USA and two from Brazil. The library from USA showed the highest abundance of virus reads, and among segments, as expected, the ones encoding for the structural nucleoprotein (NP) and hemagglutinin protein (HA) showed the highest RNA titers in all ChMOMLV positive libraries (Table S1). The ChMOMLV genome sequence (GenBank accession numbers BK068245-BK068250) comprised 5 segments of single-stranded, negative-sense RNAs of 2,557nt, 2,460nt, 2,348nt, 1,817nt and 1,681nt (Fig. 4A). RNA1 and RNA2 encode polymerase PB1 and PB2 proteins of 794 and 777 aa, respectively, and RNA3 encodes a putative polymerase

PA of 739aa. RNA4 encodes a putative NP of 572aa, while RNA5 encodes a putative HA of 503 aa (Fig. 4A) (Table 1). ChMOMLV presents low homology to other orthomixo-like virus and members belonging to the *Orthomyxoviridae* family (Table 1). PB1 protein shows the highest BlastP similarity with the brown marmorated stink bug associated virus Halyomorpha halys orthomyxo-like virus 1 (HhOlV1) PB1, with 49.41% identity, and a Flu_PB1 super family conserved domain has been identified in its sequence at aa positions 41-747. PB2 protein shows the highest BlastP similarity with HhOlV1-PB2, with 26.32% identity and a Flu_PB2 super family conserved domain has been identified in its sequence at aa positions 15-409. The PA protein shows the highest BlastP similarity with the Bat faecal associated orthomyxo-like virus 1 PA, with 34.59% identity and a Flu_PA super family conserved domain was detected in its sequence at aa positions 126-731. The putative NP protein shows the highest BlastP similarity with the Hemipteran orthomyxo-related virus OKIAV183 NP, with 29.87% identity with a Flu_NP super family conserved domain in its sequence at aa positions 340-553. The HA protein shows the highest BlastP similarity with the parasitoid wasp associated virus Cotesiavirus orthomyxo HA, with 24.56% identity and an envelope glycoprotein conserved domain at aa positions 295-495.

The phylogenetic tree based on ChMOMLV PB1 encoded protein, showed that this virus is clustered with the insect-associated orthomyxo-like viruses HhOlV1 and Hemipteran orthomyxo-related virus OKIAV191 and this cluster its related with a clade formed by putative quaranjavirus (Fig.4B). Orthomixovirids have different number of genome segments; however, those viruses phylogenetically related with ChMOMLV also have 5 RNA segments [12, 55]. Viruses belonging to the genus *Quaranjavirus* are known as argasid tick-borne viruses and have genomes with 5 to 6 RNA segments [56]. Many quaranji-like viruses were recently described in several insects; therefore, the diversity of quaranjaviruses might be greatly underestimated in arthropods other than the previously described hematophagous insects [39]. Thus, future studies should asses whether this group of unclassified quaranja-like viruses including ChMOMLV could be classified within the genus *Quaranjavirus*, or if would be more appropriate to encompass these fifth segmented viruses in a novel genus within the family *Orthomyxoviridae*.

To our knowledge, ChMOMLV is the first reported orthomyxo-like virus associated to leafhoppers.

Molecular and phylogenetic characterization of the newly identified maize leafhopper Rhabdovirus

Rhabdoviridae is a family of negative-sense single-stranded RNA (-ssRNA) viruses with a monopartite genome as a hallmark of almost all rhabdoviruses. However, plant-associated

rhabdoviruses with bi-segmented and tri-segmented genomes have been described [27, 57] . Members belonging to the *Rhabdoviridae* family are highly diverse and have a wide host range and a great genome complexity and many members of this family have been discovered in many insect species, some of which may serve as unique hosts or may act as biological vectors for transmission to other animals or plants [12, 57, 58]. Here, a novel rhabdo-like virus was identified and named as Chicharrita del maiz rhabdovirus (ChMRV), which was only detected in the transcriptome dataset of maize leafhopper collected in Argentina (Table S1). The partial negative-sense single-stranded RNA ChMRV genome (GenBank accession number BK068253) is 12,271nt in length (Table 1, Fig. 5A). ChMRV genome has the five canonical rhabdoviral structural protein genes nucleocapsid (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and the polymerase (L), as is the typical architecture within the family *Rhabdoviridae* [57, 58]. Two additional genes, named as U1 and U2, were detected between the G and L genes. Thus, the genomic organization of ChMRV is 3'-N-P-M-G-U1-U2-L-5´ (Fig. 5A), which is similar to the one reported for a few insect-associated rhabdoviruses, such as the mosquito associated Xiangyun mono-chu-like virus 11 [57] and the leafhopper associated viruses Guiyang nephotettix cincticeps rhabdovirus 1 (GuNCRV1) (Feng, unpublished).

The ORF1, which is 1,365nt in length, encodes a putative N protein of 454aa (Table 1). One nuclear localization signal (NLS) was identified at aa positions 405–440. A NLS was also identified in the N protein encoded by the leafhopper associated Nephotettix cincticeps negative-stranded RNA virus-1 (NcNSRV1) [32]. The ORF2, which is 936nt in length, encodes a putative P protein of 311aa (Table 1). One NLS was identified at aa positions 103–132. A NLS was also identified in the P protein encoded by NcNSRV1 and Spodoptera frugiperda rhabdovirus [32, 59]. The ORF3, which is 729nt in length, encodes a putative M protein of 242aa (Table 1). One NLS was identified at aa positions 232–241. A NLS was also identified in the M protein encoded by Vesicular stomatitis virus [60]. The ORF4, which is 1,989nt in length, encodes a putative G protein of 662aa (Table 1). A signal peptide was predicted in the N-terminus of this protein at aa positions 21-22, while a transmembrane domain was predicted in the C-terminus at aa positions 611-633. Signal peptides and transmembrane domains are present in every rhabdovirus glycoproteins, due to its membrane-associated function in the rhabdoviral life cycle [61].

The partial ORF7, encodes the putative L protein (Table 1). Two NLS were identified at aa positions 159–170 and 723-751. A NLS was also identified in the L protein encoded by NcNSRV1 and GuNCRV1 [32]. As stated above, two accessory ORFs were predicted between the G and L genes, which was not surprising, since the G-L gene junction is the

most commonly occupied site of accessory genes within rhabdoviruses [62]. Moreover, the presence of two additional genes in both ChMRV and GuNCRV1 could be an evidence of gene duplication, which has been mentioned as a mechanism involved in the evolutionary process in other rhabdoviruses [63]. The ORF5, which is 273nt in length, encodes a putative U1 protein of 90aa (Table 1). A transmembrane protein was predicted at aa positions 54-76. This protein has the structural characteristics of a class I viroporin, that has been speculated to display membrane-associated functions [64, 65]. The ORF6, which is 159nt in length, encodes a putative U1 protein of 52aa (Table 1). A transmembrane protein was predicted at aa positions 20-42. Thus, like U1, this protein also should have membrane-associated functions. As expected, transmembrane domains were also identified in the U1 and U2 proteins encoded by GuNCRV1.

BlastP analysis of each protein encoded by ChMRV genome showed that the highest identities of the N, M, G and L proteins was with the GuNCRV1 homologous proteins, with low sequence identities (Table 1). On the other hand, both P and P5 proteins have no hits with any protein present in the NCBI database.

The consensus gene junction 3´-UUAUUUUUUGGUAA-5´ was identified in the non-coding regions of ChMRV genome. This sequence is highly similar to that reported for GuNCRV1 and was used to identify the accessory genes as bona fide predictions.

To explore the relationships of ChMRV with members belonging to the family *Rhabdoviridae*, phylogenetic analysis based on the L protein sequence were carried out, which showed that ChMRV is clustered with the leafhopper associated GuNCRV1, and the froghopper associated hemipteran rhabdo-related virus OKIAV30 (Fig. 5B). These viruses should be classified into a novel genus within the subfamily *Deltarhabdovirinae* in the family *Rhabdoviridae*.

Concluding remarks

Analysis of publicly available RNA-seq data led to the identification of several novel viruses which have expanded the knowledge about the virosphere, shedding light on the diversity and phylogenetic relationships of viruses infecting a wide number of host including insects [10-15]. The six viruses associated with the maize leafhopper were related with other leafhopper-associated viruses, thus supporting the host assignments of those viruses identified in this study. Knowledge of the virome (insect viral community) of insect pests is essential to develop bio-insecticides based on viruses; therefore, the identification of the maize leafhopper virome would be useful to develop novel and sustainable approaches based on virocontrol to manage this agronomically important pest.

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FIGURE LEGENDS

Figure 1. (A) Schematic representation of the genome organization of Chicharrita del maiz beny-like virus. (B) Maximum likelihood phylogenetic trees reconstructed using the RdRp protein sequence of Chicharrita del maiz beny-like virus and of representative beny-like viruses. Bootstrap values above 50% are shown (1,000 replicates). Chicharrita del maiz beny-like virus is indicated with a green square. The scale bar the substitution per site.

Figure 2. (A) Schematic representation of the genome organization of Chicharrita del maiz bunyan-like virus. (B) Maximum likelihood phylogenetic trees reconstructed using the RdRp protein sequence of Chicharrita del maiz bunyan-like virus and of representative bunyan-

like viruses. Bootstrap values above 50% are shown (1,000 replicates). Chicharrita del maiz bunyan-like virus is indicated with a green square. The scale bar the substitution per site.

Figure 3. (A) Schematic representation of the genome organization of Chicharrita del maiz iflavirus 1 and Chicharrita del maiz iflavirus 2. (B) Maximum likelihood phylogenetic trees reconstructed using the RdRp protein sequence of Chicharrita del maiz iflavirus 1 and Chicharrita del maiz iflavirus 2 and of representative iflaviruses. Bootstrap values above 50% are shown (1,000 replicates). Chicharrita del maiz iflavirus 1 and Chicharrita del maiz iflavirus 2 are indicated with green squares. The scale bar the substitution per site.

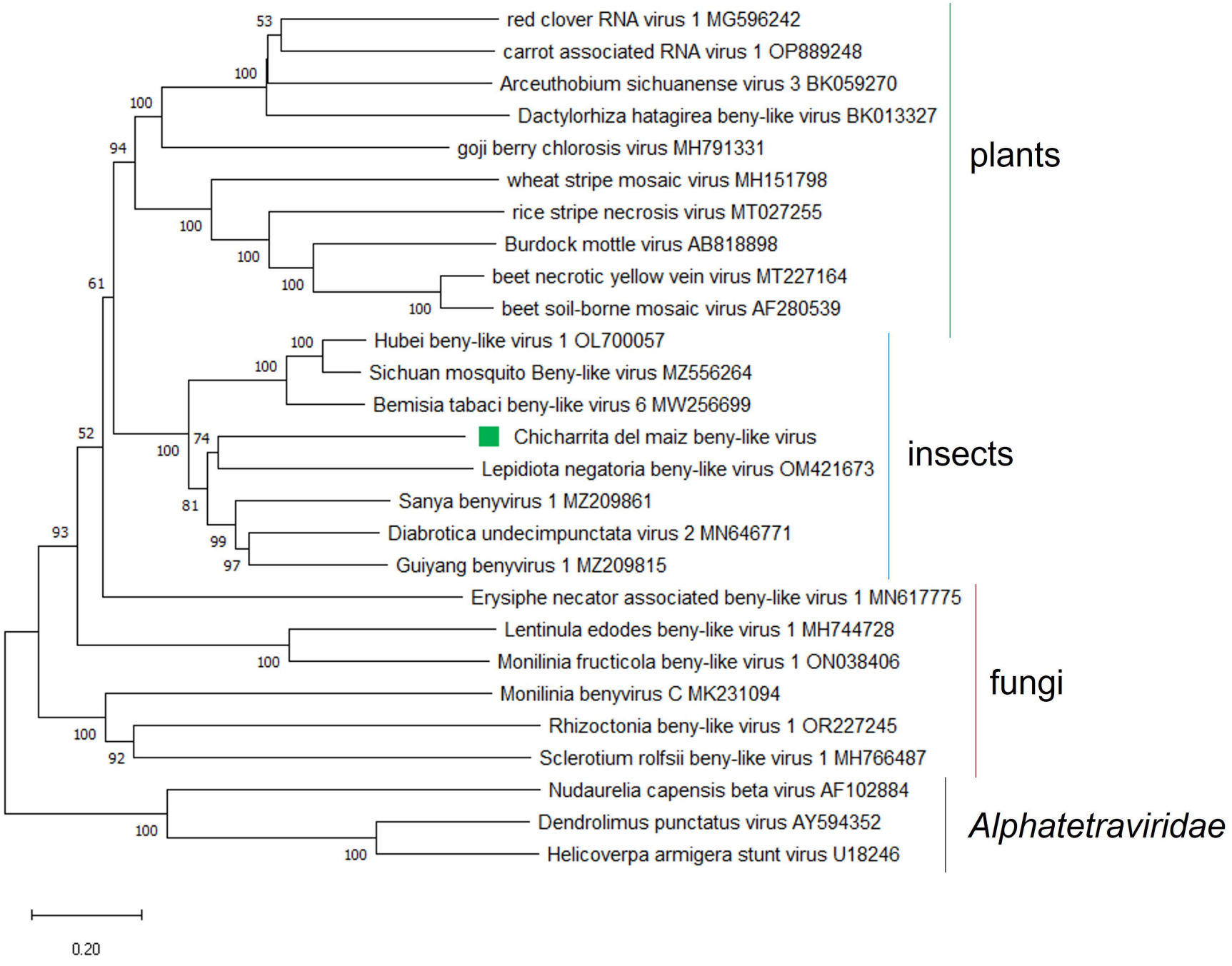
Figure 4. (A) Schematic representation of the genome organization of Chicharrita del maiz orthomyxo-like virus. (B) Maximum likelihood phylogenetic trees reconstructed using the PB1 proteins sequence of Chicharrita del maiz orthomyxo -like virus and of representative orthomyxo -like viruses. Bootstrap values above 50% are shown (1,000 replicates). Chicharrita del maiz orthomyxo -like virus is indicated with a green square. The scale bar the substitution per site.

Figure 5. (A) Schematic representation of the genome organization of Chicharrita del maiz rhabdovirus. (B) Maximum likelihood phylogenetic trees reconstructed using the L protein sequence of Chicharrita del maiz rhabdovirus and of representative rhabdoviruses. Bootstrap values above 50% are shown (1,000 replicates). Chicharrita del maiz rhabdovirus is indicated with a green square. The scale bar the substitution per site.

A

5´
Non-structural polyprotein
Structural protein
3´

B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wheat stripe mosaic virus MH151798
rice stripe necrosis virus MT027255
Burdock mottle virus AB818898
beet necrotic yellow vein virus MT227164
beet soil-borne mosaic virus AF280539
Hubei beny-like virus 1 OL700057
Sichuan mosquito Beny-like virus MZ556264
Bemisia tabaci beny-like virus 6 MW256699
Chicharrita del maiz beny-like virus
Lepidiota negatoria beny-like virus OM421673
Sanya benyvirus 1 MZ209861
Diabrotica undecimpunctata virus 2 MN646771
Guiyang benyvirus 1 MZ209815
Erysiphe necator associated beny-like virus 1 MN617775
Lentinula edodes beny-like virus 1 MH744728
Monilinia fructicola beny-like virus 1 ON038406
Monilinia benyvirus C MK231094
Rhizoctonia beny-like virus 1 OR227245
Sclerotium rolfsii beny-like virus 1 MH766487
Nudaurelia capensis beta virus AF102884
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insects
fungi
Alphatetraviridae
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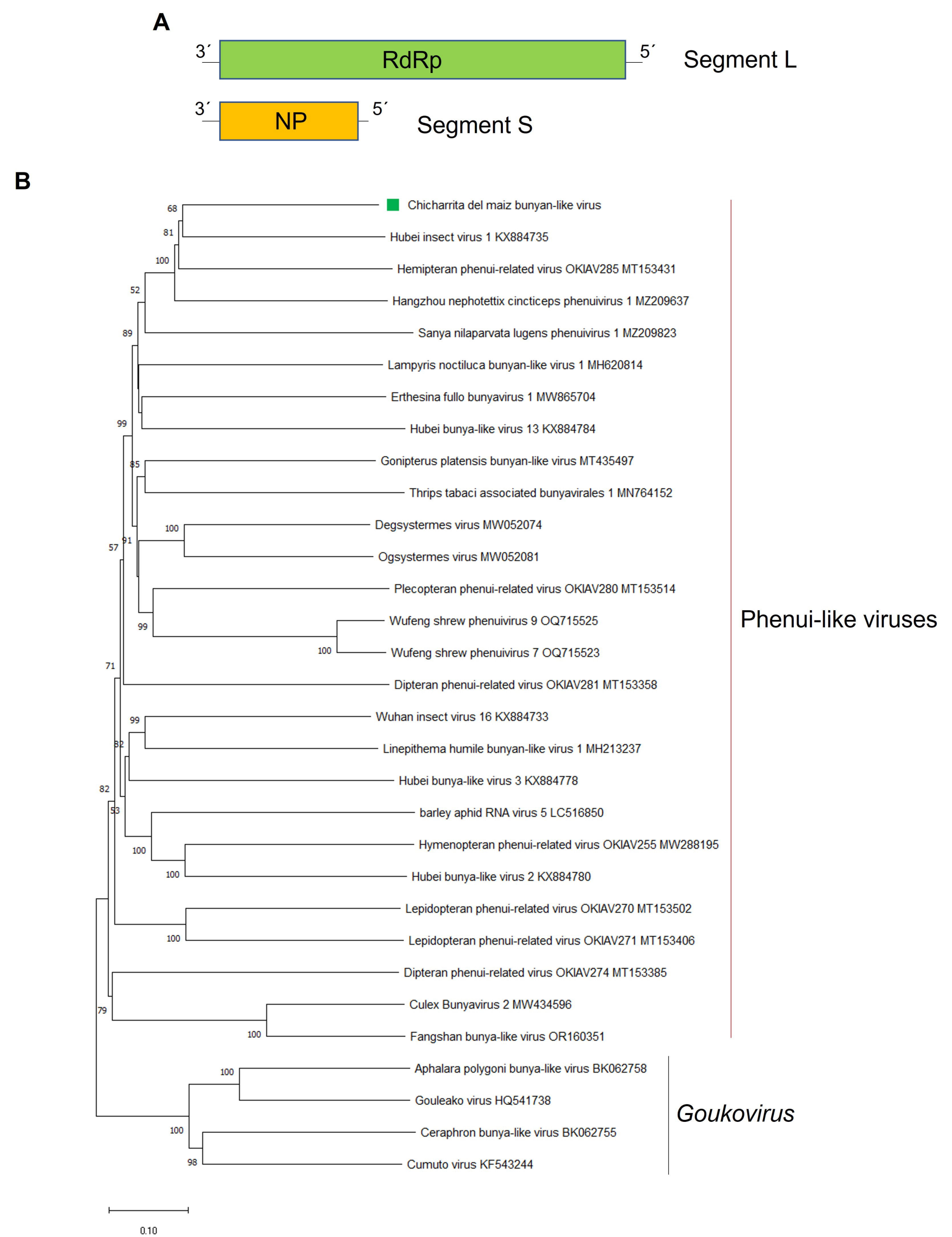
A
3´
RdRp
5´
Segment L
3´
NP
5´
Segment S
B
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Hemipteran phenui-related virus OKIAV285 MT153431
Hangzhou nephotettix cincticeps phenuivirus 1 MZ209637
Sanya nilaparvata lugens phenuivirus 1 MZ209823
Lampyris noctiluca bunyan-like virus 1 MH620814
Erthesina fullo bunyavirus 1 MW865704
Hubei bunya-like virus 13 KX884784
Gonipterus platensis bunyan-like virus MT435497
Thrips tabaci associated bunyavirales 1 MN764152
Degsystermes virus MW052074
Ogsystermes virus MW052081
Plecopteran phenui-related virus OKIAV280 MT153514
Wufeng shrew phenuivirus 9 OQ715525
Wufeng shrew phenuivirus 7 OQ715523
Dipteran phenui-related virus OKIAV281 MT153358
Wuhan insect virus 16 KX884733
Linepithema humile bunyan-like virus 1 MH213237
Hubei bunya-like virus 3 KX884778
barley aphid RNA virus 5 LC516850
Hymenopteran phenui-related virus OKIAV255 MW288195
Hubei bunya-like virus 2 KX884780
Lepidopteran phenui-related virus OKIAV270 MT153502
Lepidopteran phenui-related virus OKIAV271 MT153406
Dipteran phenui-related virus OKIAV274 MT153385
Culex Bunyavirus 2 MW434596
Fangshan bunya-like virus OR160351
Aphalara polygoni bunya-like virus BK062758
Gouleako virus HQ541738
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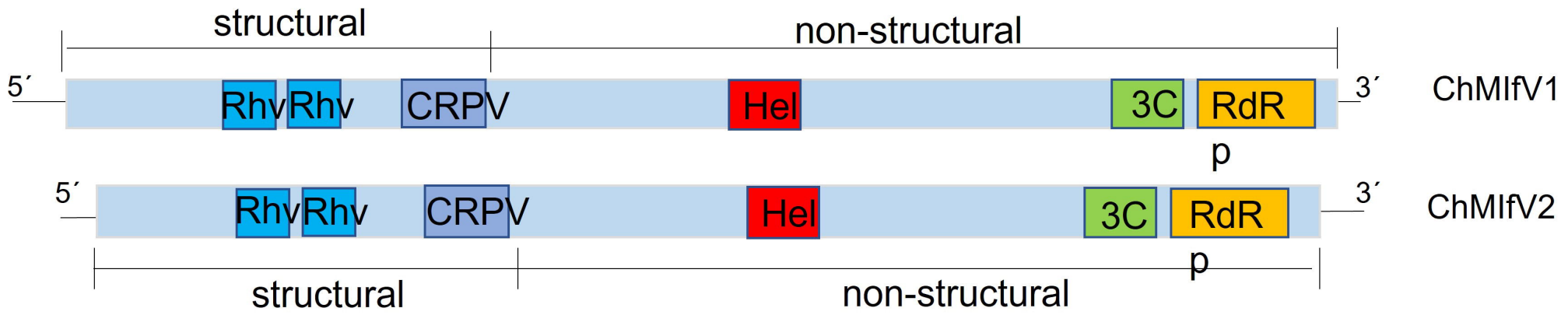
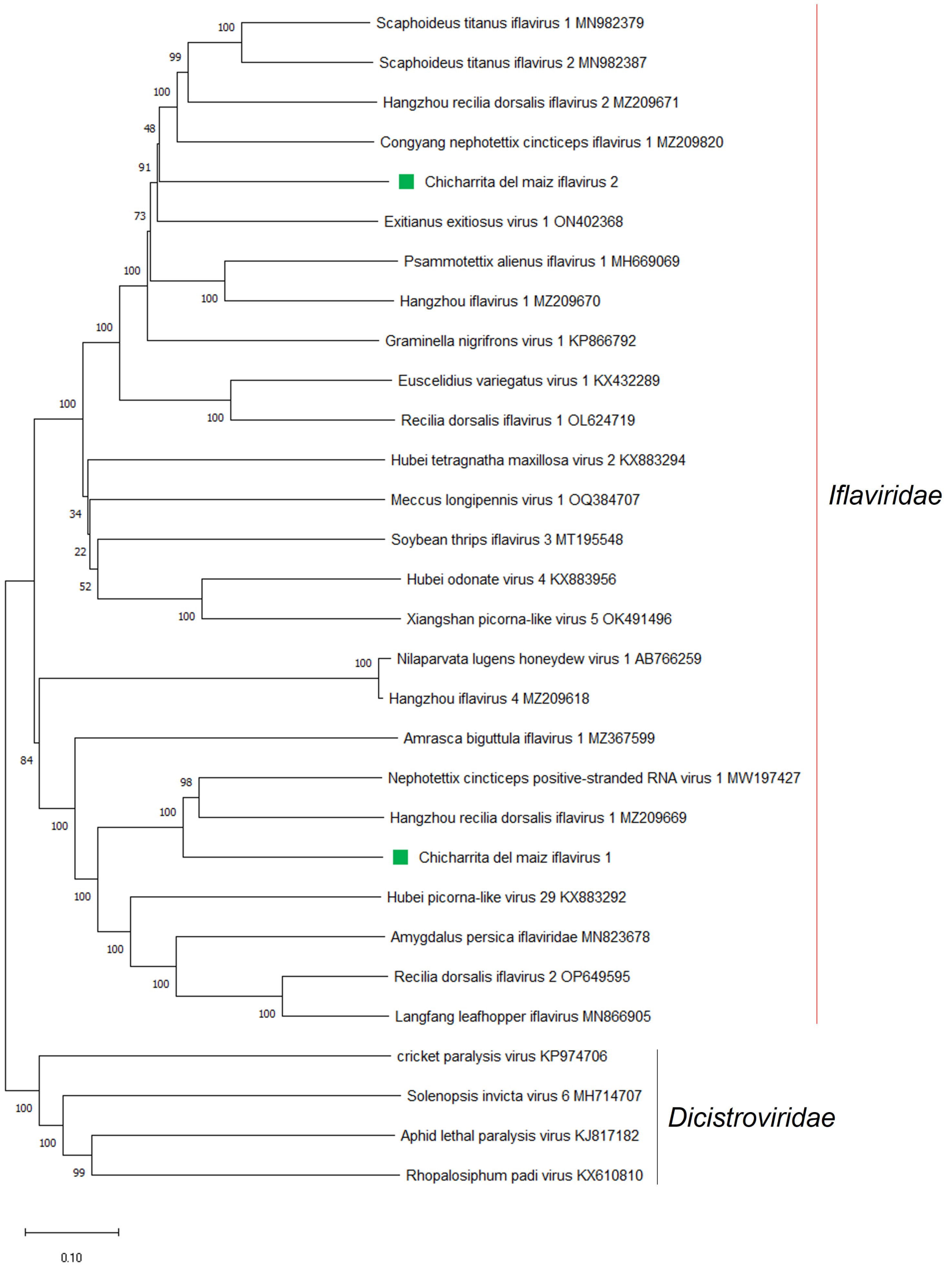
Cumuto virus KF543244
Phenui-like viruses
Goukovirus
0.10

A


structural
non-structural
5´
Rhv
Rhv
CRPV
Hel
3C
RdR
p
3´
ChMIfV1
5´
Rhv
Rhv
CRPV
Hel
3C
RdR
p
3´
ChMIfV2
structural
non-structural

B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Congyang nephotettix cincticeps iflavirus 1 MZ209820
Chicharrita del maiz iflavirus 2
Exitianus exitiosus virus 1 ON402368
Psammotettix alienus iflavirus 1 MH669069
Hangzhou iflavirus 1 MZ209670
Graminella nigrifrons virus 1 KP866792
Euscelidius variegatus virus 1 KX432289
Recilia dorsalis iflavirus 1 OL624719
Hubei tetragnatha maxillosa virus 2 KX883294
Meccus longipennis virus 1 OQ384707
Soybean thrips iflavirus 3 MT195548
Hubei odonate virus 4 KX883956
Xiangshan picorna-like virus 5 OK491496
Nilaparvata lugens honeydew virus 1 AB766259
Hangzhou iflavirus 4 MZ209618
Amrasca biguttula iflavirus 1 MZ367599
Nephotettix cincticeps positive-stranded RNA virus 1 MW197427
Hangzhou recilia dorsalis iflavirus 1 MZ209669
Chicharrita del maiz iflavirus 1
Hubei picorna-like virus 29 KX883292
Amygdalus persica iflaviridae MN823678
Recilia dorsalis iflavirus 2 OP649595
Langfang leafhopper iflavirus MN866905
Iflaviridae
cricket paralysis virus KP974706
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Aphid lethal paralysis virus KJ817182
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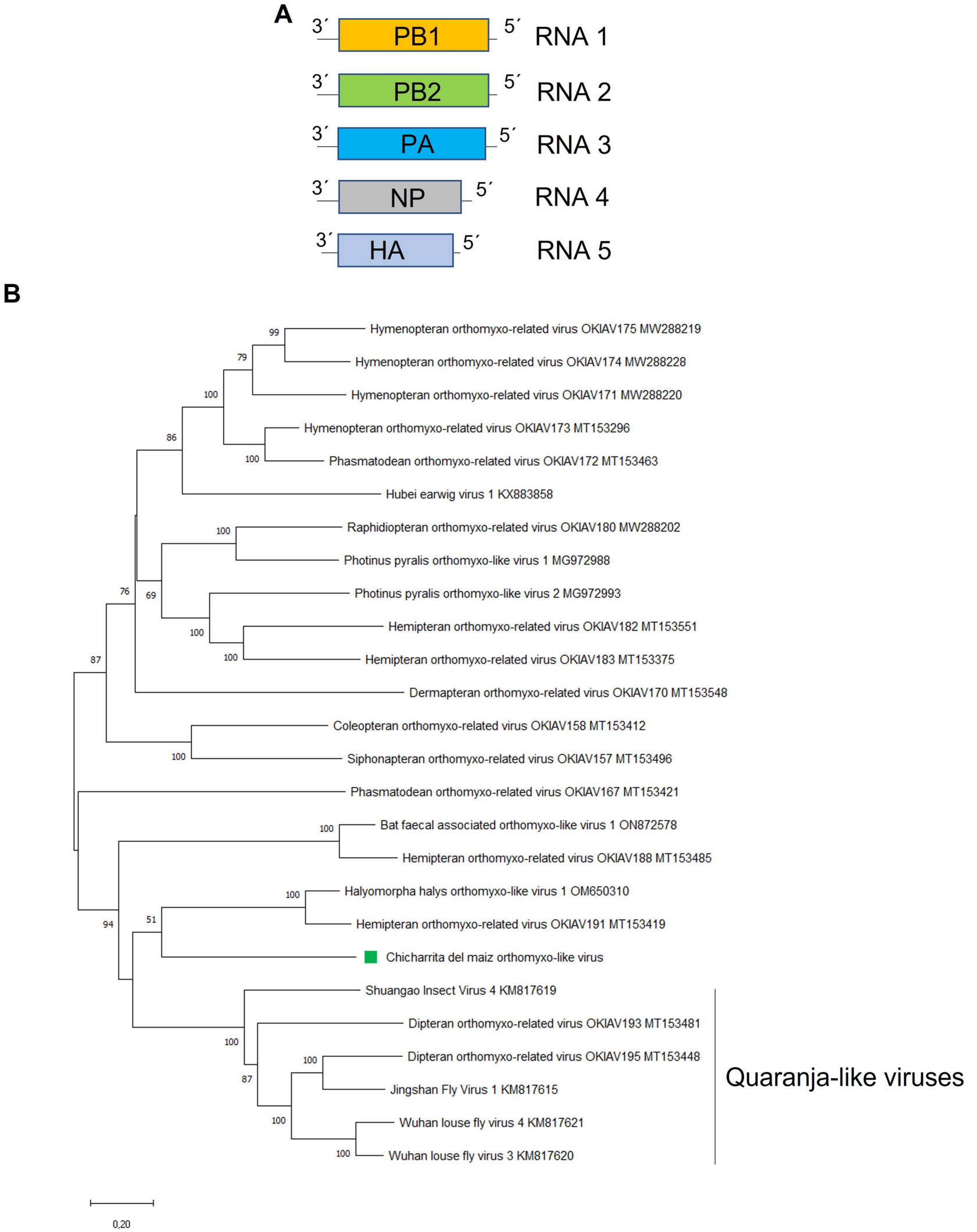
Dicistroviridae
0.10


A
3´
PB1
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RNA 1
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PB2
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RNA 2
3´
PA
5´
RNA 3
3´
NP
5´
RNA 4
3´
HA
5´
RNA 5
B
Hymenopteran orthomyxo-related virus OKIAV175 MW288219
Hymenopteran orthomyxo-related virus OKIAV174 MW288228
Hymenopteran orthomyxo-related virus OKIAV171 MW288220
Hymenopteran orthomyxo-related virus OKIAV173 MT153296
Phasmatodean orthomyxo-related virus OKIAV172 MT153463
Hubei earwig virus 1 KX883858
Raphidiopteran orthomyxo-related virus OKIAV180 MW288202
Photinus pyralis orthomyxo-like virus 1 MG972988
Photinus pyralis orthomyxo-like virus 2 MG972993
Hemipteran orthomyxo-related virus OKIAV182 MT153551
Hemipteran orthomyxo-related virus OKIAV183 MT153375
Dermapteran orthomyxo-related virus OKIAV170 MT153548
Coleopteran orthomyxo-related virus OKIAV158 MT153412
Siphonapteran orthomyxo-related virus OKIAV157 MT153496
Phasmatodean orthomyxo-related virus OKIAV167 MT153421
Bat faecal associated orthomyxo-like virus 1 ON872578
Hemipteran orthomyxo-related virus OKIAV188 MT153485
Halyomorpha halys orthomyxo-like virus 1 OM650310
Hemipteran orthomyxo-related virus OKIAV191 MT153419
Chicharrita del maiz orthomyxo-like virus
Shuangao Insect Virus 4 KM817619
Dipteran orthomyxo-related virus OKIAV193 MT153481
Dipteran orthomyxo-related virus OKIAV195 MT153448
Jingshan Fly Virus 1 KM817615
Wuhan louse fly virus 4 KM817621
Wuhan louse fly virus 3 KM817620
Quaranja-like viruses
99
79
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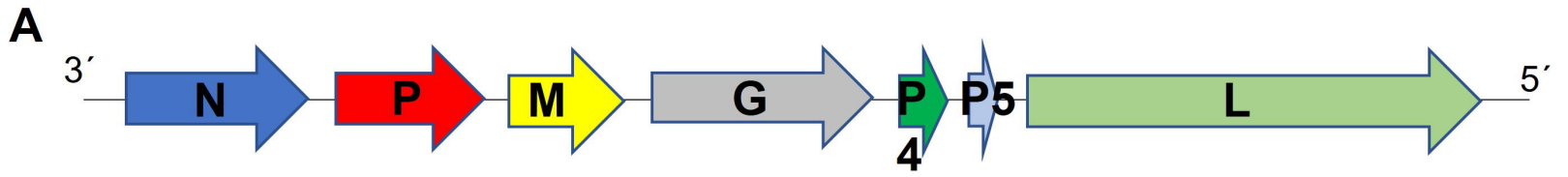
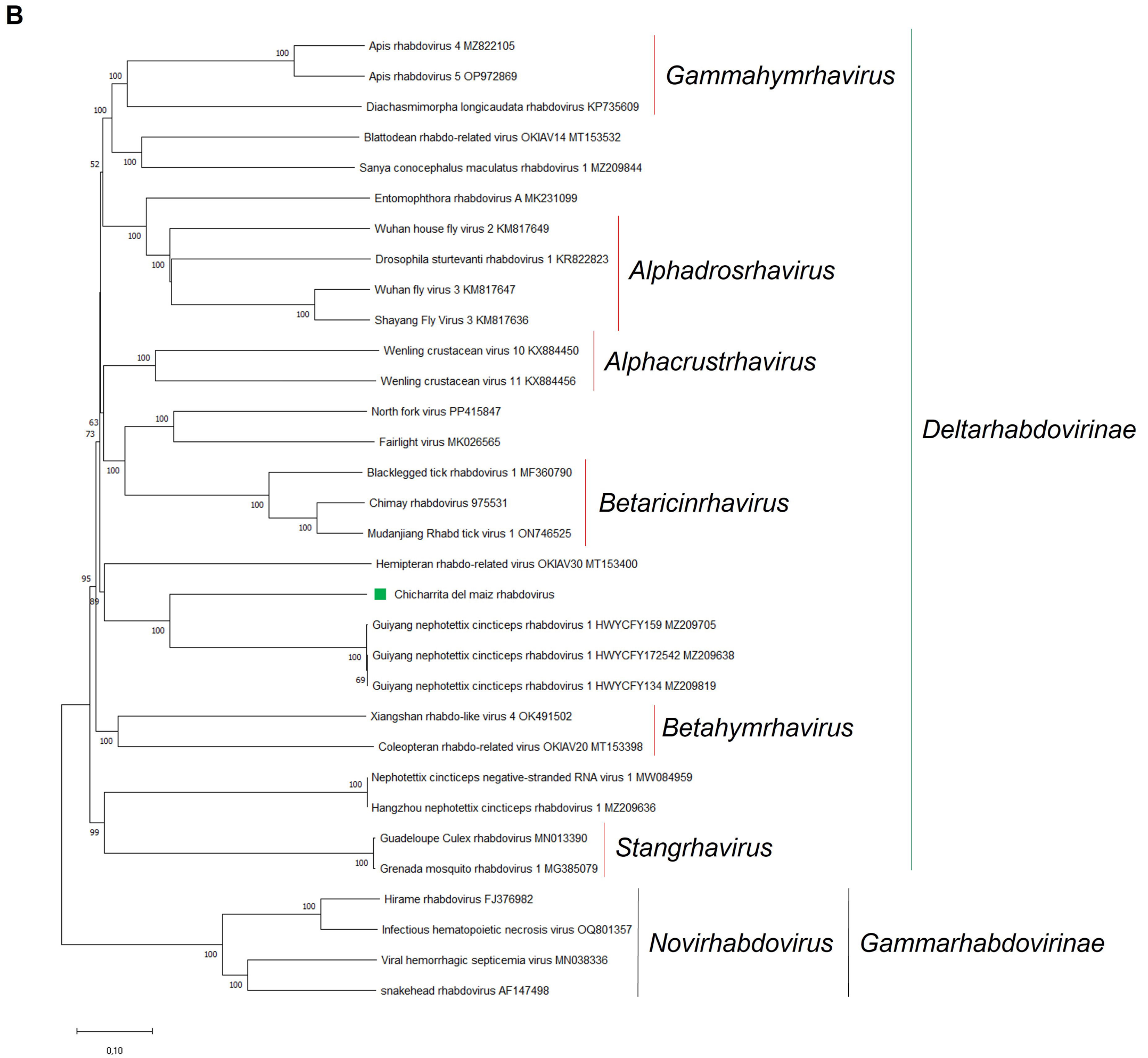
87
100
100
0,20


A
3´
N
P
M
G
P4
P5
L
5´


B
Apis rhabdovirus 4 MZ822105
Apis rhabdovirus 5 OP972869
Diachasmimorpha longicaudata rhabdovirus KP735609
Gammahymrhavirus
Blattodean rhabdo-related virus OKIAV14 MT153532
Sanya conocephalus maculatus rhabdovirus 1 MZ209844
Entomophthora rhabdovirus A MK231099
Wuhan house fly virus 2 KM817649
Drosophila sturtevanti rhabdovirus 1 KR822823
Wuhan fly virus 3 KM817647
Shayang Fly Virus 3 KM817636
Alphadrosrhavirus
Wenling crustacean virus 10 KX884450
Wenling crustacean virus 11 KX884456
Alphacrustrhavirus
North fork virus PP415847
Fairlight virus MK026565
Blacklegged tick rhabdovirus 1 MF360790
Chimay rhabdovirus 975531
Mudanjiang Rhabd tick virus 1 ON746525
Betaricinrhavirus
Deltarhabdovirinae
Hemipteran rhabdo-related virus OKIAV30 MT153400
Chicharrita del maiz rhabdovirus
Guiyang nephotettix cincticeps rhabdovirus 1 HWYCFY159 MZ209705
Guiyang nephotettix cincticeps rhabdovirus 1 HWYCFY172542 MZ209638
Guiyang nephotettix cincticeps rhabdovirus 1 HWYCFY134 MZ209819
Xiangshan rhabdo-like virus 4 OK491502
Coleopteran rhabdo-related virus OKIAV20 MT153398
Betahymrhavirus
Nephotettix cincticeps negative-stranded RNA virus 1 MW084959
Hangzhou nephotettix cincticeps rhabdovirus 1 MZ209636
Guadeloupe Culex rhabdovirus MN013390
Grenada mosquito rhabdovirus 1 MG385079
Stangrhavirus
Hirame rhabdovirus FJ376982
Infectious hematopoietic necrosis virus OQ801357
Viral hemorrhagic septicemia virus MN038336
snakehead rhabdovirus AF147498
Novirhabdovirus
Gammarhabdovirinae
0,10