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Discovery of two novel totiviruses from *Culex tritaeniorhynchus* classifiable in a distinct clade with arthropod-infecting viruses within the family *Totiviridae*

Yong Huang[1] · Xiaofang Guo[2] · Shuyan Zhang[3] · Qiumin Zhao[1] · Qiang Sun[1] · Hongnin Zhou[2] · Jiusong Zhang[1] · Yigang Tong[1]

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Abstract

Two double-stranded RNA viruses, named Culex tritaeniorhynchus totivirus NJ2 (CTV_NJ2) and NJ3 (CTV_NJ3), were discovered from wild-captured *Culex tritaeniorhynchus* mosquitoes. The complete genomes (7,624 and 7,612 bp in length) were obtained using RNA sequencing. Both CTV_NJ2 and CTV_NJ3 encode a putative capsid protein and an RNA-dependent RNA polymerase. The most similar strain to CTV_NJ2/3 is Omono River virus strain AK4 (ORV-AK4). The CP and RdRp identities of AK4 are different to CTV_NJ2 (84% and 87%) and CTV_NJ3 (47% and 62%). Phylogenetic analysis showed that taxonomically speaking CTV_NJ2/3 grouped within the unclassified *Totiviridae* and formed a distinct clade with other arthropod-infecting viruses.

Viruses in the family *Totiviridae* are now been classified into five genera: *Totivirus*, *Victorivirus*, *Trichomonasvirus*, *Giardiavirus* and *Leishmaniavirus* [4]. However, many toti-like viruses have not yet officially been assigned to the family *Totiviridae*. To date, only 28 viruses have been assigned to one of the genera, according to the 10th ICTV report. Totiviruses are double-stranded RNA viruses with a genome consisting of two open reading frames (ORF) encoding a capsid protein (CP) and an RNA-dependent RNA polymerase (RdRp). The natural hosts of the classified totiviruses are protozoa and fungi. However, the unassigned toti-like viruses have a complicated and wider range of hosts including protozoa, fungi, plants, fish [2, 6, 7] and arthropods [5, 8, 10, 12]. To date, three reported toti-like viruses have been isolated from mosquitoes: Armigeres subalbatus virus SaX06-AK20 (AsTV) isolated from *Armigeres subalbatus* [12] and Omono River viruses ORV-AK4 and ORV-Y61 isolated from *Culex inatomii* [3]. In this paper, we report another two toti-like viruses isolated from Culex tritaeniorhynchus, whose genomes and coding proteins are distinct from previously reported toti-like viruses.

Mosquitoes were sampled during the active mosquito season (from June to September) in Yunnan Province, southwest China, from 2007 to 2010 using light traps baited with CO2. Collected mosquitoes were grouped into separate pools based on collection site and mosquito classification. Each mosquito pool included approximately 50–100 mosquitoes. Each pool of samples were milled and cultured with Aedes albopictus C6/36 cells. The pools that caused cytopathic effect (CPE) were selected to extract virus particles and inoculate new cell lines. C6/36 cell culture supernatants inoculated with virus for 3d were used for virus purification. Viruses from other virus families were found in some of

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Yong Huang, Shuyan Zhang and Xiaofang Guo contributed equally to this work.

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✉ Hongnin Zhou
zhouhn66@163.com

✉ Jiusong Zhang
zhang_jiusong113@163.com

✉ Yigang Tong
tong62035@gmail.com

[1] Beijing Institute of Microbiology and Epidemiology, 20 Dong-Da Street, Fengtai District, Beijing 100071, China

[2] Yunnan Provincial Key Laboratory of Vector-borne Diseases Control and Research, Yunnan Institute of Parasitic Diseases, Pu'er, Yunnan, China

[3] Military General Hospital of Beijing PLA, Beijing, China

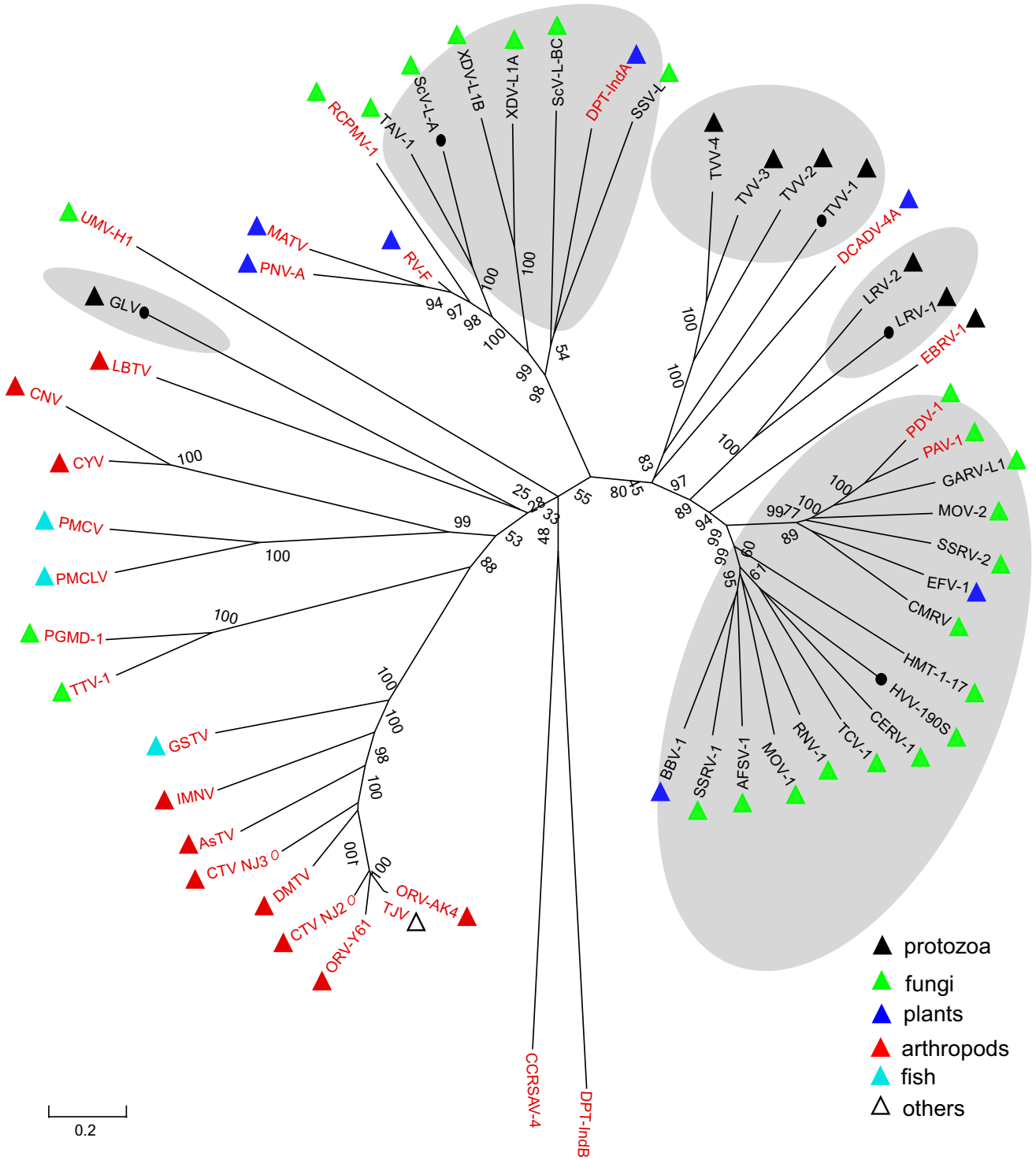

Fig. 1 A phylogenetic tree comparing 50 viruses classifiable within the family *Totiviridae* (based on the RdRp protein). The 28 officially assigned viruses are in black font while the 23 unassigned toti-like viruses are in red. The grey area represents the current classification of the five genera, while the black circles indicate the representative strains. CTV_NJ2/3 are marked with hollow red rings. Viral hosts are distinguished using different colored triangles near the strain name: black for protozoa, green for fungi, blue for plants, light blue for fish and red for arthropods. The white triangle represent other hosts, such as bat feces. The phylogenetic tree was constructed with the maximum likelihood method, with a 1000-replicate bootstrap test

the pools, published previously [9, 13]. Two novel viruses were obtained from another two collection pools of *Culex tritaeniorhynchus*.

Viral RNA was extracted using the QiagenViral RNA Mini Kit (Qiagen, Valencia, CA, USA) and used to construct a cDNA library according to the manufacturers' protocols using the Ion Total RNA-Seq Kit V2 (Life Technologies). The library was subsequently sequenced with the Ion Torrent™ sequencing platform. A total of 567,338 and 372,958 raw reads were generated and assembled into CTV_NJ2 and CTV_NJ3 using Newbler (version 2.8). The average reads length of CTV_NJ2 and CTV_NJ3 were 154 bp and 163 bp, respectively. The ORFs were detected using Glimmer [1]. CTV_NJ2 is 7,624 nucleotides in length and encodes two ORFs, a 1689 aa putative CP and a 876 aa RdRp. CTV_NJ3 is 7,612 nucleotides in length and encodes a 1686 aa CP and 842 aa RdRp protein. Both of them have a putative RdRp motif (RDRP-4). The blastp analysis of the amino acid sequences revealed that the CP of CTV_NJ2 and CTV_NJ3 shared the highest identities to that of strain ORV-AK4 (84% and 47%, respectively) and 46% between themselves. The blastp results for the RdRp were similar (CTV_NJ2, 87% to ORV-AK4; CTV_NJ3, 62% to ORV-AK4; and 60% between themselves). The complete genomes were deposited in GenBank with the accession numbers KX456218 (CTV_NJ2) and KX456219 (CTV_NJ3).

To explore the relationships of CTV_NJ2/3 with other classifiable members of the family *Totiviridae*, all 28 assigned viruses and 23 toti-like unclassified viruses (supplemental table) were selected to generate a tree based on the RdRp gene (Figure 1). The natural hosts of the 51 viruses are indicated with colored triangles. The 28 assigned strains grouped into five main clades, which were consistent with the current classification (grey area). However, viruses that infect arthropods were grouped into a large clade. The strains isolated from mosquitoes (AsTV, ORV-AK4, ORV-Y61, CTV_NJ2, CTV_NJ3) were closest in terms of their phylogenetic placement. Interestingly, TJV was isolated from bat feces [11], and GSTV was isolated from golden shiner [6], which are both insectivorous animals feeding on insects, especially mosquitoes. The phylogenetic analysis showed a distinctive feature: all the arthropod-infecting toti-like viruses grouped in a clade. This may indicate a novel genus should be assigned to family *Totiviridae*, as proposed by Yougang Zhai et al[12].

With the increasing number of toti-like viruses being identified, the current classification is no longer sufficient to represent all the viruses classifiable within family *Totiviridae*. CTV_NJ2 and CTV_NJ3 formed a distinct clade with other arthropod-infecting toti-like viruses. CTV_NJ2/3 were isolated from asymptomatic adult mosquitoes and are genetically distinct from any previously reported totiviruses. Characterization of their virulence and their host range warrants further investigation.

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Compliance with ethical standards

Conflict of interest There is no conflict of interest for all the authors.

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