# Characterization of a Novel *Tombusviridae* Species Isolated From *Paris polyphylla* var. *yunnanensis*

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# Abstract

A novel virus, Paris virus 2 (ParV2), was isolated from diseased *Paris polyphylla* var. *yunnanensis*, and its complete genome sequence was determined and analyzed. ParV2 is a positive-sense single-stranded RNA (+ssRNA) virus with a genome size of 4118 nucleotides. The ParV2 genome contains six putative open reading frames (ORFs) that encode proteins with predicted molecular weights of 40.14, 100.26, 7.31, 7.85, 26.09, and 8.77 kDa. The first ORF (ORF1) of ParV2 encodes a putative protein of 40.14 kDa (p40, nt: 20-1096), whiles the second ORF (ORF2, 888 aa) containing the GDD motif encodes the highly conserved RNA-dependent RNA polymerase protein (RdRP, nt:20-2683, p100, 100.26 kDa) of viruses in the family *Tombusviridae*. Multiple sequence alignments analysis showed that the complete genome sequence of ParV2 shares 31.7-55.5% nucleotide sequence identities with viruses in the family *Tombusviridae*. Ginger chlorotic fleck-associated tombusvirus (GCFaV-1,Accession No.QKE30557) had the highest sequence identity (55.5%) with ParV2, and also shares 59.2% RdRp and 34.9% CP amino acid sequence identity with GCFaV-1.Sequence comparisons and phylogenetic analysis of RdRp suggested that ParV2 is a novel member of the family *Tombusviridae*, and its closest known relative is GCFaV-1.


# Introduction

*Paris polyphylla* var. *yunnanensis* is a perennial herb that belongs to the genus *Paris* of the family *Trilliaceae*. It is widely distributed in Yunnan province, China.Due to its wide medicinal applications, it is used as one of the raw materials for various Chinese patent medicines.The scarcity of wild natural resources and large market demand of *Paris polyphylla* var. *yunnanensis* as a result of its medicinal importance have led to the rapid development of its artificial planting industry – a major cause of the continual emergence of new diseases. Reports indicate that Paris mosaic necrosis virus (PMNV), Paris polyphylla virus X (PPVX), Pepper mild mottle virus (PMMoV), Cowpea aphid borne mosaic virus (CABMV), and Paris virus 1 (ParV1) infect and cause viral diseases in *Paris polyphylla* var. *yunnanensis*, leading to significant losses in plant yield[1–6].

Viruses in the family *Tombusviridae* are positive-sense single-stranded RNA viruses that exhibit smooth or granular appearance with a diameter of 28–35 nm. The RNA of *Tombusviridae* is encapsulated in an icosahedral (T = 3) capsid that is composed of 180 identical protein subunits in three conformationally distinct states (A, B, C). With the exception of *Dianthoviruses* whose genome is bipartite, all members of *Tombusviridae* have a non-segmented (monopartite) linear genome of about 3.7–4.8 kb in size. The *Tombusviridae* consist of 16 genera that is divided into three subfamilies. All members of this family are readily transmitted by mechanical inoculation and through plant material used for propagation or grafting, and both the virion and the genetic material alone are infective. Most *Tombusviridae* species can be transmitted through the soil either dependent on, or independent of, a biological vector. Although, members of *Tombusviridae* can either infect monocotyledonous or dicotyledonous plants, no species has been found to infect both. The experimental host range is wide, but the natural host range of individual virus species is relatively narrow. Typical characteristics of viral diseases caused by the viruses of *Tombusviridae* include mottling, crinkling, necrosis and deformation of leaf, and some infections are symptomless in their natural hosts. Although variability exists in the number and location of genes within members of the family, they all have a conserved organizational feature.This unifying feature is a highly conserved polymerase that contains the canonical “GDD” motif.

We previously reported a novel potyviruses, Paris virus 1 (ParV1), isolated from diseased *Paris polyphylla* var. *yunnanensis* leaves exhibiting mosaic and mottle symptoms[6]. In the present study, we identified and characterized

the complete genome sequence of a novel virus (ParV2) infecting *Paris polyphylla* var. *yunnanensis* and confirmed its taxonomic and phylogenic relationship with other known viruses.

## Materials And Methods

Diseased leaf samples exhibiting leaf mosaic and chlorotic symptoms, and suspected of viral infection (Fig. 1A) were sent to Oebiotech Co. Ltd (Shanghai, China) for high throughput sequencing (HTS) on the Illumina HiSeq 2500 platform. Total RNA was extracted from diseased leaf samples using OMEGA® Plant RNA Kit (TaKaRa Bioengineering, Dalian) according to manufacturer's instructions. To confirm the sequencing results, total RNA isolated from virus-infected leaves was reverse transcribed into cDNA using the ABM® 5X All-In-One RT MasterMix (Macklin Biochemical Co. Ltd, Shanghai). Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was performed using 2×Taq PCR MasterMix (Biomed Gene Technology Co. Ltd., Beijing, China) and the specific primers ParV2:1–13 F/R designed based on the sequence of the assembled contigs to confirm the presence of the virus, and also amplify the whole genome sequence. The 5' and 3' terminals of the viral genome were amplified using 5' RACE and 3' RACE kit (Sangon Biotech, Shanghai; order No. B605102 and B605101) and specific reverse transcription primers 2R, R1, R2, F1 and F2. The sequences of all primers used in this study have been listed in Supplementary Tables S1 and S2. To confirm the classification status of the virus and determine its relationship with other viruses, ORFs in the viral genome sequence and protein molecular masses were predicted by NCBI ORF finder (www.ncbi.nlm.nih.gov/projects/gorf). The nucleotide (nt) and protein amino acid (aa) sequences of related viruses were retrieved from NCBI database and used for sequence alignments. Sequence identities were calculated using the ClustalW algorithm in BioEdit 7.0 program.Phylogenetic relationships between the novel virus and related viruses were determined using the Neighbor-Joining method (NJ) within the MEGA7.0 program with replicas bootstrapped to 1000.

## Results

Raw reads were processed to remove adaptor and low-quality reads. The transcript sequences were *de novo* assembled into contigs using paired-end splicing method in the Trinity program. BLASTx analysis of assembled contigs revealed that one contig consisting of 4118 nt was most similar to the genome sequences of viruses of the family *Tombusviridae*. This result suggests that a *Tombusviridae* species could be responsible for the virus-disease-like symptoms in diseased *Paris polyphylla* var. *yunnanensis* plants. Sequencing of RT-PCR products of the total RNA isolated from virus-infected *Paris polyphylla* var. *yunnanensis* leaves produced a sequence of 4118 nt that includes the 5' and 3' RACE data for the isolated virus which was deposited in the GenBank database under the accession number MW423699.

The complete RNA genome of ParV2 (MW423699) consists of 4,118 nt, and contains six ORFs, 5'-UTR (19 nt) and 3'-UTRs (359 nt) (Fig. 1B). The first ORF of the ParV2 putatively encodes a P40 protein (nt:20-1096) of 40.14 kDa. If the amber codon UAG (nt:1094–1096) is readthrough, the ORF2 (nt:20-2683, 888 aa) predicted to encode a 100.26 kDa RNA-dependent RNA polymerase (RdRp, P100) protein is continuously translated. This ParV2 RdRp is similar to the highly conserved RdRp of members of the family *Tombusviridae* that contains a conserved $^{684}$GDD$^{686}$ motif [7–9]. The ParV2 sequence 5'AAA UAG GGG 3' (nt:1091–1099) surrounding the amber stop codon is consistent with the proposed relative efficiency of readthrough sequences (A/C/U) (A/U) AUAG (G/C) (G/A). This amber stop codon surrounded sequence of ParV2 is 100% identical to the corresponding sequence present in the members of *Tombusviridae*[10–12].ORF3 (nt:2641–2844,68aa,7.31kDa) encodes the putative movement protein 1 (MP1, P7), that may be involved in virus movement, contains the FNF motif (at the C-terminus) that is conserved within the putative

movement proteins of Carmoviruses, Machlomoviruses and Panicoviruses[13, 14], an indication that ParV2 is a member of the *Tombusviridae*. As seen in some animal and plant viruses whose initiation codons for mRNA translation are non-AUG start codons CUG, ACG and GUG[13, 15, 16],the ORF4 (nt:2844–3062,72aa,7.85kDa,P8) encoding the putative movement protein 2 (MP2) of ParV2 has the non-canonical start codon $^{2844}$ACG$^{2846}$. The last adenine base of ORF3 stop codon is the first base of ORF4 start codon. Similarly, the MP2 of panicoviruses such as Cocksfoot mild mosaic virus (CMMV), Panicum mosaic virus (PMV), Thin paspalum asymptomatic virus (TPAV) and Bermuda grass latent virus (BGLV) initiates with CUG, GUG, CUG and CUG start codons respectively[15, 17, 18]. ORF5 (nt:3031–3759, 243 aa) encodes the 26.09 kDa coat protein (CP, P26). In addition to pre-readthrough and readthrough proteins (RdRp), MP1, MP2, CP, ParV2 also contains ORF6 (nt:3207–3434,75aa) that is embedded within the CP gene, and encodes the P9 (accessory protein) of 8.77kDa. The existence of *Tombusviridae* motifs and other *Tombusviridae*-specific features in ParV2 genome suggest that ParV2 is evolutionally close to viruses in this family, and that ParV2 is a member of the family *Tombusviridae*.

Table 1
Nucleotide (nt) and amino acid (aa) sequence identities of ParV2 and other members of a few genus within *Tombusviridae*.

| Genus of *Tombusviridae* | Virus Name | Accession number | Viral genome sizes(nt) | Sequence identity (%) complete genomes(nt) | Sequence identity (%)for encoded proteins | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | RdRP | MP1 | MP2 | CP |
| Unclassified | Ginger chlorotic fleck-associated tombusvirus | MN581046 | 4143 | 55.5 | 59.2 | 42.8 | 47.2 | 34.9 |
| Panicovirus | Bermuda grass latent virus | NC-032405 | 4044 | 43.7 | 40.2 | 23.2 | 18.6 | 19.8 |
| | Thin paspalum asymptomatic virus | JX848616 | 4193 | 43.5 | 38.5 | 19.1 | 24.0 | 21.2 |
| | Thin paspalum asymptomatic virus | NC-021705 | 4195 | 41.9 | 38.5 | 20.5 | 24.0 | 18.1 |
| | Panicum mosaic virus | MH885652 | 4324 | 40.6 | 36.3 | 17.8 | 25.3 | 23.6 |
| | Cocksfoot mild mosaic virus | NC-011108 | 4198 | 42.4 | 38.6 | 24.3 | 16.0 | 24.2 |
| Pelarspovirus | Jasmine mosaic-associated virus 2 | MG958506 | 3885 | 31.7 | 31.1 | 12.8 | 13.0 | 7.7 |
| | Jasmine mosaic-associated virus 2 | MF991300 | 3975 | 32.5 | 30.2 | 11.4 | 11.9 | 8.0 |
| | Jasmine virus H | MH231176 | 3867 | 33.4 | 30.4 | 11.4 | 10.8 | 8.0 |
| | Jasmine virus H | MH231180 | 3867 | 33.4 | 30.2 | 12.8 | 11.9 | 8.3 |
| | Jasmine mosaic-associated virus 1 | MG958505 | 3867 | 32.5 | 29.8 | 11.4 | 10.8 | 8.6 |
| Machlomovirus | Maize chlorotic mottle virus | MF510225 | 4439 | 38.9 | 38.9 | 25.7 | 14.8 | 17.0 |
| | Maize chlorotic mottle virus | MF510235 | 4439 | 39.1 | 38.9 | 25.7 | 14.8 | 17.0 |

| Genus of *Tombusviridae* | Virus Name | Accession number | Viral genome sizes(nt) | Sequence identity (%) complete genomes(nt) | Sequence identity (%)for encoded proteins | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | RdRP | MP1 | MP2 | CP |
| Betacarmovirus | Turnip crinkle virus | MK301398 | 4061 | 33.8 | 30.6 | 15.0 | 7.6 | 10.1 |
| | Japanese iris necrotic ring virus | NC-002187 | 4041 | 37.8 | 26.3 | 10.5 | 13.6 | 10.1 |
| Gammacarmovirus | Melon necrotic spot virus | AY122286 | 4271 | 32.2 | 29.3 | 15.7 | 23.6 | 9.3 |
| | Pea stem necrosis virus | NC-004995 | 4048 | 32.1 | 30.7 | 15.7 | 18.0 | 8.1 |
| | Cowpea mottle virus | NC-003535 | 4029 | 37.3 | 29.0 | 18.3 | 7.8 | 8.7 |
| Alphacarmovirus | Angelonia flower break virus | NC-007733 | 3962 | 31.7 | 29.9 | 17.6 | 13.4 | 9.3 |
| | Pelargonium flower break virus | NC-005286 | 3923 | 32.2 | 29.4 | 18.0 | 11.1 | 8.5 |
| Gallantivirus | Galinsoga mosaic virus | NC-001818 | 3803 | 34.4 | 29.3 | 16.9 | 14.8 | 9.7 |

Further pairwise comparison of the complete genome nucleotide sequence and ORFs of ParV2 indicated that ParV2 share 31.7–55.5% complete genome nt, 26.3–59.2% RdRp, 10.5–42.8% MP1, 7.6–47.2% MP2, and 7.7–34.9% CP aa identities with 21 virus isolates of the family *Tombusviridae* (Table 1). The highest identity (55.5%) was seen with GCFaV-1 (QKE30557) followed by members of the genus *Panicovirus* (40.6–43.7%).Again, the highest aa identities of the individual proteins of ParV2 were seen with GCFaV-1, followed by *Machlomovirus* (RdRp and MP1) or *Panicovirus* (MP2 and CP). These results indicate that ParV2 is a member of the *Tombusviridae*, although it may not have all the characteristics of specific genera within the family *Tombusviridae*.

To determine the phylogenetic status of ParV2 and the evolutionary relationship between ParV2 and other viruses of the family *Tombusviridae*, phylogenetic tree was constructed using the aa sequences of RdRp protein (a reliable indicator for virus taxonomy).It can be seen from the tree that ParV2 clustered with the unassigned/unclassified GCFaV-1, and machlomoviruses and panicoviruses of the subfamily *Procedovirinae.* GCFaV-1 is the closest known relative of ParV2 (Fig. 2). It can be inferred from the tree that ParV2 shares a recent common ancestor with viruses of the genera *Machlomovirus* and *Panicovirus*. ParV2 and GCFaV-1 diverged from the branch that gave rise to the genera *Machlomovirus* and *Panicovirus* (Fig. 2). It can be concluded that Parv2 is a member of the subfamily *Procedovirinae* of the family *Tombusviridae*, although it couldn't be allocated to any *Procedovirinae*-specific genera. These results confirm our pairwise comparison, and genome analyses results which indicated that ParV2 is a novel member of the family *Tombusviridae*.

# Discussion

In this study, we identified and characterized a novel plant virus that belongs to the family *Tombusviridae*, and is tentatively named Paris virus 2. ParV2 has a typical *Tombusviridae* genome organization[19–22]. Multiple sequence alignments analysis showed that ParV2 share 31.7–55.5% nt sequence identities with members of the family *Tombusviridae.* Although, ParV2 clustered with viruses in the genera *Machlomovirus* and *Panicovirus*, the closest known relative of ParV2 is the unclassified/unassigned GCFaV-1, which showed the highest sequence similarity with ParV2. Similar to machlomoviruses and panicoviruses genomes that encode a 7–8 kDa MP1 and 6–9 kDa MP2, ParV2 genome also encode an MP1 and MP2 of 7.31kDa and 7.85kDa respectively. In contrast, genomes of tombusviruses and aureusviruses encode a conserved 22–27 kDa MP, whiles that of dianthoviruses encode another type of MP of about 34 kDa. These results indicate the relationship of ParV2 with viruses of the genera *Machlomovirus* and *Panicovirus*.

One of two distinct groups of CP subunit always exist in the capsids of *Tombusviridae* species. In one group of genera (*Machlomovirus*, *Necrovirus* and *Panicovirus*) that have structured CP lacking P domain, the CP subunit ranges in size of 25–30 kDa, whiles in the other group of genera with a CP-containing P domain, the CP subunit ranges in size of 37–48 kDa. Our comparative sequence analysis results revealed that CP of ParV2 has a size of 26.09 kDa, that is similar to the structured CP of the genera *Machlomovirus*, *Necrovirus* and *Panicovirus*. The genomic RNA of *Machlomovirus* and *Panicovirus* are 4.4 kb (encoding four ORFs) and 4.3 kb (encoding five ORFs) respectively. In contrast, ParV2 and GCFaV-1 have genomic RNA of 4.1kb that encode six ORFs. These data suggest that although ParV2 and GCFaV-1 share various characteristics with *Machlomovirus* and *Panicovirus* species, ParV2 and GCFaV-1 belong to a distinct genus. These results also confirm our phylogenetic data that indicate that GCFaV-1 is the closest known relative of ParV2, and that ParV2 and GCFaV-1 share a recent common ancestor with the genera *Machlomovirus* and *Panicovirus.*

Taken together, ParV2 should be considered a new species of the family *Tombusviridae*, and its taxonomic status should be further studied. We therefore propose that ParV2 and GCFaV-1 should be assigned to a new genus within the subfamily *Procedovirinae* in the family *Tombusviridae.* In addition to the viral-symptoms, there were obvious insect bites on the infected leaves of *Paris polyphylla* var. *yunnanensis*. It is possible that the virus infected the plants through insect transmission. This is the first report of a virus of *Tombusviridae* infecting *Paris polyphylla* var. *yunnanensis*. As a new virus, further studies are required to ascertain its method of transmission and possible vector.

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