Complete Genome Sequence of Mulberry Vein Banding Associated Virus, a New Tospovirus Infecting Mulberry

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Abstract

Mulberry vein banding associated virus (MVBaV) that infects mulberry plants with typical vein banding symptoms had been identified as a tentative species of the genus Tospovirus based on the homology of N gene sequence to those of tospoviruses. In this study, the complete sequence of the tripartite RNA genome of MVBaV was determined and analyzed. The L RNA has 8905 nucleotides (nt) and encodes the putative RNA-dependent RNA polymerase (RdRp) of 2877 aa amino acids (aa) in the viral complementary (vc) strand. The RdRp of MVBaV shares the highest aa sequence identity (85.9%) with that of Watermelon silver mottle virus (WSMoV), and contains conserved motifs shared with those of the species of the genus Tospovirus. The M RNA contains 4731 nt and codes in ambisense arrangement for the NSm protein of 309 aa in the sense strand and the Gn/Gc glycoprotein precursor (GP) of 1,124 aa in the vc strand. The NSm and GP of MVBaV share the highest aa sequence identities with those of Capsicum chlorosis virus (CaCV) and Groundnut bud necrosis virus (GBNV) (83.2% and 84.3%, respectively). The S RNA is 3294 nt in length and contains two open reading frames (ORFs) in an ambisense coding strategy, encoding a 439-aa non-structural protein (NSs) and the 277-aa nucleocapsid protein (N), respectively. The NSs and N also share the highest aa sequence identity (71.1% and 74.4%, respectively) with those of CaCV. Phylogenetic analysis of the RdRp, NSm, GP, NSs, and N proteins showed that MVBaV is most closely related to CaCV and GBNV and that these proteins cluster with those of the WSMoV serogroup, and that MVBaV seems to be a species bridging the two subgroups within the WSMoV serogroup of tospoviruses in evolutionary aspect, suggesting that MVBaV represents a distinct tospovirus. Analysis of S RNA sequence uncovered the highly conserved 5’-3’-ends and the coding regions, and the variable region of IGR with divergent patterns among MVBaV isolates.

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Data Availability Statement: All relevant data are within the paper and all sequence data are available from the GenBank database with accession numbers KM819698-KM819709.

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Introduction

The mulberry (Morus spp.) is an economically important plant grown widely throughout Asia, for the cultivation of the silkworms (Bombyx mori Linn.) and for the sericulture industry. Diverse virus-like symptoms, including mosaic, vein banding, vein necrosis, chlorotic ringspots, and leaf deformation were frequently observed on mulberry. The viral diseases have been a major factor restricting yield and quality of mulberry. Two mulberry-infecting viruses, Mulberry latent virus (Genus Carlavirus) [1] and the Mulberry ringspot virus (Genus Nepovirus) [2], have been partly characterized in Japan. Recently, a mulberry-infecting Tospovirus, temporarily named Mulberry vein banding virus (MuVBV), was found in China and identified to be a new species of Tospovirus based on the homology of N protein sequence to other Tospovirus species [3].

Tospoviruses cause significant loss of yield and quality to vegetables, legumes, and ornamental crops worldwide and are transmitted by thrips in a circulative and propagative manner [4, 5]. Typical symptoms induced by members of the Tospovirus genus include foliar necrotic spots, necrotic stems, bronzing, wilting, and ring spots in leaves and fruits [4, 6]. Tospoviruses are characterized with enveloped quasi-spherical particles of approximately 80–120 nm in size and a tripartite negative and ambisense RNA genome, i.e. small RNA (S RNA), medium RNA (M RNA), and large RNA (L RNA) [4]. The 3’ termini of all RNA segments are highly conserved in the first 9 nucleotides and show inverted complementarity to the 5’ ends [7].

The L RNA is with a negative polarity and encodes a putative RNA-dependent RNA polymerase (RdRp), which is also called L protein, in the viral complementary (vc) strand for virus replication [4]. The other two genomic RNAs use an ambisense coding strategy. The M RNA encodes a cell-to-cell movement protein (NSm) and the envelope glycoprotein precursor (GP), whereas the S RNA encodes a nonstructural RNA-silencing suppressor protein (NSs) and the nucleocapsid protein (N) [7, 8]. The open reading frames (ORFs) in the M and S RNA segments are separated by large AU-rich intergenic regions (IGR), which forms a stable hairpin structure and is assumed to be involved in transcription termination [4, 9].

In recent years, new tospoviruses such as Pepper necrotic spot virus (PNSV) [10], Soybean vein necrosis associated virus (SVNaV) [11], Hippeastratum chlorotic ring virus (HCRV) [12–14], Bean necrotic mosaic virus (BeNMV) [15], Tomato necrotic spot virus (TNSV) [16], MuVBV [3], and Lisianthus necrotic ringspot virus (LNRV) [17] have been identified.

Tospoviruses are classified into four major serogroups, designated by their serological relatedness to the type viruses Watermelon silver mottle virus (WSMoV), Tomato spotted wilt virus (TSWV), Groundnut yellow spot virus (GYSV), and Iris yellow spot virus (IYSV) [18,19]. Impatiens necrotic spot virus (INSV) was classified together as a distinct serotype [18]. BeNMV and SVNaV, phylogenetically clustered into a new branch and exhibited low cross-reactivity with other species, may represent a new evolutionary lineage within the genus Tospovirus [15, 20]. As of to date, the full genome sequences were available for sixteen species in the genus Tospovirus [8, 11, 13, 21–24].

In our previous survey, a new tospovirus tentatively named Mulberry vein banding virus (MuVBV) with highest N protein homology of 74.4% to Capsicum chlorosis virus (CaCV) was identified from mulberry with vein-banding symptom in Guangxi Province of China by RT-PCR and sequencing of a fragment of the S RNA of the viral genome [3]. Since this virus has not been fully characterized, particularly due to the lack of the vector information, we rename this virus Mulberry vein banding associated virus (MVBaV), to more precisely reflect its current taxonomic status. To better understand the nature of this virus, we cloned and sequenced the whole genome of MVBaV, and characterized its histopathology and serology properties. Our results show that MVBaV is a distinct member of Tospovirus belonging to the WSMoV serogroup.
Material and Methods

Ethics Statement

This work was supported by the government of Guangxi province and the sampling activities were conducted with the permission of the agricultural authority of local counties or authorities in charge. The mulberry orchards where the plant samples were collected were in public domain and in regular agricultural farm lands where no endangered or protected species were involved. The sampling locations of the isolates of MVBaV in this study and the authorities who issued the permission were listed in S1 Table.

Virus sources, electron microscopy and indirect enzyme-linked immunosorbent assay (ELISA)

During 2011 to 2012, ten isolates of MVBaV designated XCSY-3, XCBY-1, XZDL-1, SL-3, HX-2, NN-5, NN-10, NN-16, YZ-3, YZ-4, were collected from mulberry plants showing characteristic symptoms including vein banding, mosaic, chlorotic ringspots, necrotic ringspots or vein necrosis on leaves in mulberry orchards around Guangxi province, China. Due to difficulty of sap transmission, the virus was maintained on mulberry by grafting to the virus-free mulberry seedlings. The leaf samples were stored at −80°C until total RNA was extracted. Ultrathin sections of diseased tissues were prepared as described [25] and examined with the H-7650 transmission electron microscope (Hitachi High-Technologies, Tokyo, Japan).

Mixed antisera of WSMoV/Groundnut bud necrosis virus (GBNV) for WSMoV serogroup, Groundnut ringspot virus (GRSV)/Tomato chlorotic spot virus (TCSV) for TSWV serotype, and antisera against TSWV for TSWV serogroup, IYSV for IYSV serogroup, and INSV for the distinct serotype, were purchased from Agdia Inc. (Elkhart, IN, USA). Indirect ELISA was performed as described Chen et al. (2010) with minor modifications [18]. Crude extracts from infected and healthy mulberry plants were diluted 1:30 and used as the antigen source and negative control, respectively.

RNA extraction and rapid amplification of cDNA ends (RACE)

Total RNA was extracted from the infected mulberry leaves using RNAplant plus reagent (TIANGEN, Beijing, China) according to the manufacturer’s instruction and the quality of the purified RNA was evaluated by gel electrophoresis on 0.8% (w/v) agarose gels. The 5′ and 3′-terminal sequences of the viral genomic RNA were determined by using the RACE cDNA Amplification Kit (BD Biosciences, Franklin Lakes, NJ, USA) coupling with Sanger sequencing. Primers were designed according to the available sequences obtained through viral small RNA deep sequencing. The RACE products were purified using DNA gel recovery kit (Sangon, Shanghai, China) and cloned into the pCR2.1-TOPO vector (Thermo Fisher Scientific, Waltham, MA, USA) before sequencing.

Amplification and cloning of viral genomic cDNAs

MVBaV-specific primer pairs were designed based on the sequence information obtained by 5′ and 3′ RACE, and used for the amplification of complete fragments of the MVBaV genome. The primers LRNAF1/LRNAR1 and LRNAF2/LRNAR2 were used for the PCR amplification of MVBaV L RNA, MRNAF/MRNAR for MVBaV M RNA, and SRNAF/SRMAR for S RNA, respectively. The reverse transcription reaction was carried out using the Reverse Transcriptase Kit (Thermo Fisher Scientific) and the PCR was carried out in a total reaction volume of 25 μl. After denaturizing at 95°C for 2 min, the PCR were performed with the following parameters: 35 cycles with each cycle at 94°C for 30 s, 55–58°C for 30 s (the temperature varied with the
length of template fragments), 72°C for 45 s, and final extension at 72°C for 10 min. The PCR products were analyzed by electrophoresis on a 0.8% (w/v) agarose gel. The PCR products of interest were recovered from the gel and cloned into pCR2.1-TOPO vector plasmid. The primers used for RACE and amplifying the MVBaV Genome are shown in Table 1.

The cloned PCR products were sequenced with sequencer ABI 3700. At least three independent clones were sequenced for a target PCR product. The assembled full-length sequence data of MVBaV genome were deposited in the GenBank with accession numbers KM819698-KM819709.

### Analysis of viral RNA sequences

Sequence analysis was carried out using programs within the software VECTOR NTI V11.0 (Thermo Fisher Scientific). Phylogenetic trees were constructed by the neighbor-joining method with 1000 bootstrap replications using a program included in MEGA 6.06 [26]. The putative peptide cleavage sites and the N- and O-linked glycosylation sites of the GP of MVBaV were predicted using the software programs SignalP 4.1, NetNGlyc 1.0, and NetOGlyc 3.1, respectively. For transmembrane domain prediction, the TMHMM Server 2.0 program was used. The sequences of Tospoviruses were down loaded from the GenBank and listed in Table 2.

### Results

#### Symptomatology and virion morphology

The symptoms of MVBaV-infected leaves varied widely, depending on the growing stage of mulberry and the environmental conditions. Initial symptoms of vein banding, mosaic, or chlorotic ringspots (Fig 1A–1C) were observed in early April and leaf deformation could develop later (Fig 1D). In the autumn, necrotic ringspots and vein necrosis on leaves were common (Fig 1E). The diseased mulberry leaf samples were further examined by electron microscopy. Enveloped particles with a quasi-spherical shape of 80–100 nm in diameter were

| Primer | Sequence (5' to 3') | Location and orientation |
|--------|---------------------|--------------------------|
| Tos-UPA | GACCACGCGTATGAGTGCAGACAGCAATCAGGGTATTAATT | 3' termination of S, M and L RNA |
| SRNA 5–1 | GCTGGGAACCTGGCTCAGAAAGGC | S 670-695(vc) |
| SRNA 5–2 | ATGTCTCTCTCTCAGAATGACGTTG | S 610-634(vc) |
| S RNA 3 | ATGTCTCTGCTCAGAATGACGTTG | S 2547-2570(v) |
| S RNAR | AGAGCAATCAGGGTATTAATT | S 1-21(v) |
| S RNA 5–1 | AGAGCAATCAGGGTATTAATT | S 3268-3294(vc) |
| MRNA 5–1 | CACTTCTAAGATGCTCCCATATC | M 961-984(vc) |
| MRNA 5–2 | CTAATACATGCTACATCACAATC | M 937-961(vc) |
| M RNA 3 | GGTATGCTTTGATGATGATGAGAAAGTG | M3378-3406(v) |
| MRNAF | AGAGCAATCAGGGTATTAATT | M 1-30(v) |
| M RNAR | AGAGCAATCAGGGTATTAATT | M4706-4731(vc) |
| LRNA 5–1 | GCCCTGGAATCAAGAATGACGAG | L1402-1425(vc) |
| LRNA 5–2 | TCCTCTAGTGAATAATGACGTTG | L1241-1264(vc) |
| LRNA 3 | TCAGTTATAGCATCTGTAAGTCG | L 7380-7405(v) |
| LRNAR1 | AGAGCAATCAGGGTATTAATT | L 1-27(v) |
| LRNAR2 | AGAGCAATCAGGGTATTAATT | L 4328-4349(vc) |
| LRNAR2 | AGAGCAATCAGGGTATTAATT | L 4328-4349(vc) |
| LRNAR2 | AGAGCAATCAGGGTATTAATT | L 4328-4349(vc) |
| LRNAR2 | AGAGCAATCAGGGTATTAATT | L 4328-4349(vc) |
| LRNAR2 | AGAGCAATCAGGGTATTAATT | L 4328-4349(vc) |
observed to accumulate in the endoplasmic reticulum (Fig 2A) or dispersed in the cytoplasm as single particles (Fig 2B) in leaf cells. These particles had morphological features typical of tospoviruses. The tospovirus-like particles were also present in the symptom-showing leaves of plants infected by grafting inoculation (data not shown), suggesting that the symptoms were associated with MVBaV.

Serological characterization of MVBaV

The diseased samples reacted strongly with WSMoV/GBNV mixed antisera, but not with antisera to TSWV, INSV, IYSV, and GRSV/TCSV in ELISA assays (data not shown), indicating that MVBaV is serologically related to the members of the WSMoV serogroup.

| Virus                                      | Abbreviation | S RNA       | M RNA        | L RNA       |
|--------------------------------------------|--------------|-------------|--------------|-------------|
| Mulberry vein banding associated virus MVBaV | KM819701.1   | KM819699.2  | KM819698.1   |             |
| Alstromeria necrotic streak virus ANSV     | GQ4768668.1(N)| -           | -            | -           |
| Bean necrotic mosaic virus BeNMV           | JN587269.1   | JN587269.1  | JF417980.1   |             |
| Calla lily chlorotic spot virus CCSV       | AY967502.1   | FJ829961.1  | FJ829962.1   |             |
| Capsicum chlorosis virus AIT isolate CaCV-AIT | NC_008301.1 | NC_008303.1 | NC_008302.1 |             |
| Capsicum chlorosis virus HT-1 isolate (synonyms: Gloxinia tospovirus) CaCV-HT-1 | AF059578 (N) AF059577 (NSs) | AF023172.1 | -           |
| Chrysanthemum stem necrosis virus CSNV     | KM114548     | KM114547    | KM114546     |             |
| Groundnut bud necrosis virus GBNV          | AY871098.2   | U42555.1    | AF025538.1   |             |
| Groundnut chlorotic fan-spot virus GCFSV  | AFO80526.1   | -           | -            | -           |
| Groundnut ringspot virus (South African isolate) GRSV-SA | AF487516.1 for N, JN571117.1 for NSs | AY574055.2 for GPs, AF213673.1 for NSm | -           |
| GRSV (south Florida isolate from the United State) GRSV-US | HQ644414.1 | HQ644414.1 | HQ644414.2  |             |
| Groundnut yellow spot virus GYSV           | AFO13994.1   | -           | -            | -           |
| Hippeastrum chlorotic ringspot virus HCRV  | JX833564     | JX833565    | HG763961     |             |
| Impatiens necrotic spot virus INSV         | X66972.1     | DQ425095.1  | DQ425094.1   |             |
| Iris yellow spot virus IYSV                | AFO01387.1   | FJ361359.1  | FJ623474.1   |             |
| Lisianthus necrotic ringspot virus LNRV    | AB852525     | -           | -            | -           |
| Melon severe mosaic virus MMSV             | EU275149.1   | -           | -            | -           |
| Melon yellow spot virus MYSV               | AB038343.1   | AB061773.1  | AB061774.1   |             |
| Pepper necrotic spot virus PNSV            | HE584762.1   | -           | -            | -           |
| Physalis silver mottle virus PhySMV        | AFO67151     | -           | -            | -           |
| Polygonum ringspot virus PolRSV            | KJ541744     | KJ541745    | KJ541746     |             |
| Soybean vein necrosis associated virus SVNaV | HQ728387.1  | HQ728386.1  | HQ728385.1   |             |
| Tomato chlorotic spot virus TCSV           | AF282982.1 (N) | AY574054.2 for GPs, AF213674.1 for NSm | HQ700667.1  |
| Tomato necrosis virus TNeV                 | AY647437.1 (N) | -           | -            | -           |
| Tomato necrotic ringspot virus TNRV        | FJ489600.2   | FJ947152.1  | -            | -           |
| Tomato necrotic spot virus TNSV            | KM355773     | -           | -            | -           |
| Tomato spotted wilt virus TSWV             | AF020660.1   | JN664253.1  | NC_002052.1  |             |
| Tomato yellow ring virus TYRV              | AY686718.1   | JN560177    | JN560178     |             |
| Tomato zonate spot virus TZZV              | EF552433.1   | EF552434.1  | EF552435.1   |             |
| Watermelon bud necrosis virus WBNV         | GU584184.1   | GU584185.1  | GU735408.1   |             |
| Watermelon silver mottle virus WSMoV       | ABO42650.1   | DQ157768.1  | AY863200.1   |             |
| Zucchini lethal chlorosis virus ZLCV       | AF067069.1(N) | ABO74207.1 for GPs, AF213674.1 for NSm | -           |

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Genome organization of the MVBaV

In our previous deep sequencing of small RNAs from MVBaV-infected samples, three incomplete RNA segments of MVBaV responding to the L, M, and S RNA had been assembled (our unpublished data). The complete genome sequence of the MVBaV isolate XCSY-3 was obtained by combining the deep sequencing data, RT-PCR and RACE data (Fig 3, Table 1).

**L RNA.** Two overlapping PCR fragments generated by RT-PCR constitute the complete MVBaV L RNA of 8905 nt in length (GenBank accession number KM819698). There is a single open reading frame (ORF) in the viral complementary strand (vc-strand) that encodes a deduced RdRp (L protein) of 2877 aa with a molecular mass of 331.6 kDa. The 5' untranslatable regions (UTRs) and 3'-UTRs of the L RNA were 241 and 30 nt in length, respectively. The 17-nt termini of the L RNA formed a panhandle structure with a mismatch at the 11th nucleotide.

The RdRp protein of MVBaV has a conserved region similar to those of other tospoviruses. The five conserved motifs, motif A (DXXKW), motif B (QGXXXXXSS), motif C (SDD), motif D (K), and motif E (EXXS), were all found within the RdRp of MVBaV. Furthermore, three motifs, Motif F1 (TDF), Motif F2 (KxQRTK) and Motif F3 (DREIY), which were found in the
RdRp of CaCV, IYSV, TYRV, WBNV, GBNV, WSMoV, TZSV, and CCSV [21], were also present in the MVBaV RdRp (Fig 4).

**M RNA.** The complete nucleotide sequence of the MVBaV M RNA is 4731 nt in length and contains two ORFs encoding NSm and GP, in an ambisense coding strategy and separated by an IGR of 322 nts (GenBank accession number KM819699). The 5' and 3' UTRs of

![image of electron microscopy of MVBaV infected plant leaves](image)

**Fig 2.** Electron micrograph of a MVBaV-infected mulberry plant leaves showing typical tospovirus-like particle morphology. Typical spherical, enveloped virions are shown accumulating in the endoplasmic reticulum (A) or dispersing in the cytoplasm as single particles in leaf cells (B). The scale bar represents 200 nm.

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![image of cloning strategies for L, M, and S RNAs](image)

**Fig 3.** Cloning strategies for L, M, and S RNAs. Arrows indicate the annealing positions of each primer. To determine the 5'-terminal sequences, total RNAs were denatured by heating at 65°C for 5 min and then mixed with the first primer, LRNA 5'–1 for L RNA, MRNA 5'–1 for M RNA, and SRNA 5'–1 for S RNA, respectively. After removal of template RNAs by RNaseH digestion, PCR amplification of the 5'-cDNAs was performed with Ex Taq DNA polymerase (Takara Bio, Dalian, China) using UPM primer (provided with the kits) and a nested primer, LRNA 5'–2 for L RNA, MRNA 5'–2 for M RNA, and SRNA 5'–2 for S RNA, respectively. To determine the 3'-terminal sequence, first strand cDNA was synthesized by using the primer Tos-UPA. PCR amplification of the 3'-cDNAs was performed with Ex Taq DNA polymerase using the primers Tos-UPA and the LRNA 3 primer for L RNA, MRNA 3 for M RNA, and SRNA 3 for S RNA, respectively. The primers used for RACE and amplifying the MVBaV Genome are shown in Table 1.

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MVBaV M RNA contain 57 and 47 nts, respectively, forming a panhandle structure with 21 base pairs and 3 mismatches at the termini. The ORF on the viral strand codes for the NSm protein of 309 aa with a molecular mass of 34.3 kDa. The ORF on the vc-strand was 3,375 nt in length and codes for a 1,124 aa GP with a molecular mass of 128.1 kDa.

The NSm protein, probably involved in cell-to-cell movement, belongs to the 30 K movement protein superfamily [27, 28]. The conserved motifs of the 30 K movement protein superfamily, such as the D-motif and G-residue, were found in the NSm protein of MVBaV, while the P/D-L-X motif and the phospholipase A2 catalytic site (PLA2-motif) present in NSm proteins of TSWV, GRSV, CSNV, and TCSV [28, 29], was absent from the MVBaV (Fig 5).

In topology of MVBaV GP protein, four N-glycosylation sites (N294, N549, N930, and N1101) and five transmembrane domains (aa positions 4–26, 297–316, 321–343, 414–436, and 1051–1073) were predicted and two potential signal peptides with predicted cleavage sites at aa 24 (VYL-LN) and aa 436 (SIA-LQ) to yield the glycoproteins Gn (47.8 kDa) and Gc (77.7 kDa) were present, whereas Arg-Gly-Asp (RGD) motif was not found.

S RNA. The complete nucleotide sequence of the MVBaV S RNA is 3,294 nt in length (GenBank accession code KM819701). The S RNA contains two ORFs in an ambisense coding strategy separated by an AU-rich IGR of 1008 nt in length spun from coordinates 1386–2393.

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**Fig 4.** The RdRp conserved motifs in viruses of the genus *Tospovirus*. The consensus amino acid residues in each RdRp motif of the family *Bunyaviridae* are shown in bold and underlined. Identical amino acid (aa) residues are indicated with dots and deficient aa residues with hyphens. The positions of the conserved motifs in RdRp are indicated. Abbreviations and accession numbers of the analyzed sequences of tospoviruses are listed in Table 2.

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**Fig 5.** The NSm conserved motifs in viruses of the genus *Tospovirus*. The positions of the conserved motifs in NSm are indicated. Abbreviations and accession numbers of the analyzed sequences of tospoviruses are listed in Table 2.

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in the v-strand. The 65- and 67-nt untranslated regions in the 5'- and 3'-UTRs can form a panhandle structure of 22 bp with 2 mismatches. The ORF on the v-strand codes for a 439 aa NSs protein with a molecular mass of 49.1 kDa and the ORF on the vc-strand encodes a 277 aa nucleoprotein (N protein) of 30.69 kDa. Three highly conserved motifs, Walker A (GxxxxGKT), Walker B (DEXX), and YL, were present in the NSs proteins of some tospoviruses [30, 31]. Walker A and YL were present (aa positions 175–182 and 416–417), while the Walker B motif was not found in the NSs protein of MVBaV (Fig 6).

Evolutionary relationship of the MVBaV to other tospoviruses

MVBaV RdRp shares the highest sequence homology to that of WSMoV (85.8%) and high sequence homology to GBNV, WBNV, and CaCV (83.9–84.9%). To the rest members of the WSMoV serogroup (MYSV, TZSV, CCSV), the homology were 76.1–77.8%. Intriguingly, the RdRp homology between MVBaV and topoviruses of different serotypes was all less than 70%, with members of TSWV and the distinct serotypes being the lowest (42.3–45.2%) (Table 3). An inspection of the proteins encoded by the M RNA and S RNA of different tospoviruses revealed that MVBaV was closest to WSMoV serogroup (M RNA, 64.6–83.2% homology; S RNA, 45.4–74.4% homology) and most distant from TSWV, GYSV, and Distinct (M RNA, 34.1–39.2% homology; S RNA, 15.9–29.9% homology), with IYSV serogroup in between (M RNA, 66.2–68.5% homology; S RNA, 41.9–49.4% homology).

Fig 6. The NSs conserved motifs in viruses of the genus Tospovirus. The positions of the conserved motifs in NSs are indicated. Abbreviations and accession numbers of the analyzed sequences of tospoviruses are listed in Table 2.

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Table 3. Comparison of the RNAs and deduced proteins of MVBaV with those of other Tospoviruses.

| M RNA | Full length nt | 5'UTR nt | NSm nt | aa | Identity (%) | IGR nt | GPs nt | aa | Identity % | 3'UTR nt |
|-------|----------------|----------|--------|----|--------------|--------|--------|----|------------|---------|
| WSMoV serogroup | | | | | | | | | | |
| MVBaV | 8905 | 241 | 8634 | 2877 | 30 | 85.8 | 32 | |
| WSMoV | 8917 | 248 | 8637 | 2878 | 32 | 84.9 | 32 | |
| GBNV | 8911 | 245 | 8634 | 2877 | 31 | 84.6 | 32 | |
| WBNV | 8916 | 247 | 8637 | 2878 | 32 | 84.5 | 31 | |
| CaCV-AIT | 8912 | 247 | 8634 | 2877 | 32 | 84.4 | 32 | |
| CCSV | 8911 | 230 | 8652 | 2883 | 29 | 77.8 | 28 | |
| TZSV | 8919 | 233 | 8658 | 2885 | 28 | 76.9 | 28 | |
| MYSV | 8918 | 273 | 8613 | 2870 | 27 | 76.1 | 32 | |
| IYSV serogroup | | | | | | | | | | |
| HCRV | 8908 | 250 | 8622 | 2873 | 33 | 69.0 | 32 | |
| PolRSV | 8893 | 230 | 8631 | 2876 | 32 | 68.8 | 32 | |
| TYRV | 8877 | 223 | 8622 | 2873 | 32 | 68.5 | 32 | |
| IYSV | 8880 | 225 | 8622 | 2873 | 33 | 67.8 | 32 | |
| TSWV serogroup | | | | | | | | | | |
| CSNV | 8955 | 397 | 8625 | 2874 | 33 | 45.2 | 32 | |
| GRSV-US | 8876 | 217 | 8625 | 2874 | 34 | 45.2 | 32 | |
| TCSV | 8868 | 215 | 8622 | 2873 | 31 | 44.8 | 31 | |
| TSWV | 8897 | 236 | 8628 | 2875 | 33 | 43.1 | 33 | |
| Other serotype | | | | | | | | | | |
| INSV | 8780 | 147 | 8598 | 2865 | 35 | 44.9 | 32 | |
| SVNaV | 9010 | 184 | 8796 | 2931 | 30 | 42.5 | 30 | |
| BeNMV | 9040 | 220 | 8799 | 2932 | 21 | 42.3 | 21 | |
| WSMoV serogroup | | | | | | | | | | |
| MVBaV | 4731 | 57 | 930 | 309 | — | 322 | 3375 | 1124 | — | 47 |
| CaCV-AIT | 4823 | 56 | 927 | 308 | 82.6 | 427 | 3366 | 1121 | 80.9 | 47 |
| CaCV-HT-1 | 4780 | 29 | 927 | 308 | 82.3 | 436 | 3369 | 1122 | 78.9 | 19 |
| GBNV | 4801 | 56 | 924 | 307 | 81.9 | 408 | 3366 | 1121 | 84.3 | 47 |
| WBNV | 4794 | 55 | 924 | 307 | 79.9 | 402 | 3366 | 1121 | 81.6 | 47 |
| TZSV | 4945 | 54 | 930 | 309 | 77.3 | 546 | 3369 | 1122 | 73.2 | 46 |
| WSMoV | 4877 | 55 | 939 | 312 | 75.2 | 470 | 3366 | 1121 | 80.7 | 47 |
| CCSV | 4704 | 54 | 930 | 309 | 75.1 | 503 | 3372 | 1123 | 73.6 | 45 |
| TNRV | 4716 | 59 | 933 | 310 | 73.1 | 307 | 3369 | 1122 | 65.3 | 48 |
| MYSV | 4815 | 58 | 927 | 308 | 64.6 | 398 | 3384 | 1127 | 63.4 | 48 |
| IYSV serogroup | | | | | | | | | | |
| TYRV | 4766 | 62 | 927 | 308 | 68.5 | 354 | 3393 | 1130 | 62.9 | 50 |
| IYSV | 4821 | 63 | 936 | 311 | 68.1 | 394 | 3411 | 1136 | 60.3 | 17 |
| PolRSV | 4710 | 62 | 927 | 308 | 68.2 | 263 | 3408 | 1135 | 60.0 | 50 |
| HCRV | 4741 | 47 | 927 | 308 | 65.9 | 330 | 3390 | 1129 | 61.9 | 47 |
| TSWV serogroup | | | | | | | | | | |
| GRSV-SA | — | — | 912 | 303 | 39.2 | — | 3402 | 1133 | 33.4 | — |
| GRSV-US | 4848 | 99 | 912 | 303 | 38.6 | 348 | 3405 | 1334 | 34.1 | 84 |
| CSNV | 4830 | 101 | 912 | 303 | 38.6 | 326 | 3408 | 1135 | 34.5 | 83 |
| TCSV | — | — | 912 | 303 | 38.6 | — | 3405 | 1134 | 34.1 | — |

(Continued)
|        | mRNA | Full length nt | 5'UTR nt | NSm nt | aa | Identity (%) | IGR nt | GPs nt | aa | Identity % | 3'UTR nt |
|--------|------|----------------|----------|--------|----|--------------|--------|--------|----|------------|----------|
| TSWV   |      | 4767           | 100      | 909    | 302| 37.5         | 266    | 3408   | 1135| 34.1       | 84       |
| ZLCV   |      |                |          | 912    | 303| 34.3         |        | 3408   | 1135| 34.5       |          |
| Other serotype |     |                |          |        |    |              |        |        |    |            |          |
| BeNMV  |      | 4886           | 64       | 954    | 317| 37.6         | 299    | 3486   | 1161| 33.1       | 83       |
| INSV   |      | 4962           | 85       | 912    | 303| 36.9         | 465    | 3333   | 1110| 32.9       | 86       |
| SVNaV  |      | 4955           | 57       | 951    | 316| 34.1         | 267    | 3588   | 1195| 30.1       | 92       |
| S RNA  |      |                |          |        |    |              |        |        |    |            |          |
| WSMoV serogroup |   |                |          |        |    |              |        |        |    |            |          |
| MVaBaV |      | 3294           | 65       | 1320   | 439| 71.3         | 1199   | 828    | 275 | 74.4       | 67       |
| CaCV-AIT |     | 3477           | 66       | 1320   | 439| 70.6         | 773    | 831    | 276 | 71.5       | 67       |
| CaCV-HT-1 |    | 3401           | 66       | 1320   | 439| 70.4         | 1120   | 828    | 275 | 71.8       | 67       |
| TNeV   |      |                |          |        |    |              |        |        |    |            |          |
| WSMoV  |      | 3558           | 67       | 1320   | 439| 70.8         | 1278   | 828    | 275 | 70.0       | 65       |
| GBNV   |      | 3057           | 66       | 1320   | 439| 70.6         | 773    | 831    | 276 | 71.5       | 67       |
| WBNV   |      | 3401           | 66       | 1320   | 439| 70.4         | 1120   | 828    | 275 | 71.8       | 67       |
| TNSV   |      | 3012           | 66       | 1380   | 459| 61.7         | 664    | 834    | 277 | 61.5       | 68       |
| TZSV   |      | 3279           | 64       | 1380   | 459| 60.6         | 934    | 837    | 278 | 59.4       | 64       |
| CCSV   |      | 3172           | 66       | 1383   | 460| 59.6         | 825    | 834    | 277 | 60.4       | 64       |
| TNRV   |      | 3023           | 65       | 1356   | 451| 47.3         | 690    | 846    | 281 | 56.2       | 66       |
| MYSV   |      | 3232           | 68       | 1410   | 469| 45.4         | 847    | 840    | 279 | 58.4       | 67       |
| PhySMV |      | 3257           | 68       | 1410   | 469| 45.0         | 872    | 840    | 279 | 58.4       | 67       |
| IYSV serogroup | |                |          |        |    |              |        |        |    |            |          |
| IYSV   |      | 3105           | 70       | 1332   | 443| 49.4         | 811    | 822    | 273 | 44.8       | 70       |
| TYRV   |      | 3061           | 71       | 1332   | 443| 49.4         | 762    | 825    | 274 | 44.1       | 71       |
| HCRV   |      | 2744           | 73       | 1338   | 445| 48.1         | 437    | 825    | 274 | 42.7       | 71       |
| PoiRSV |      | 2485           | 73       | 1332   | 443| 46.7         | 183    | 825    | 274 | 41.9       | 72       |
| TSWV serogroup | |                |          |        |    |              |        |        |    |            |          |
| MeSMV  |      | 3283           | 80       | 1368   | 455| 20.2         | 887    | 789    | 262 | 28.5       | 159      |
| TSWV   |      | 2955           | 88       | 1404   | 467| 17.7         | 535    | 777    | 258 | 29.9       | 151      |
| GRSV-SA |    |                |          |        |    |              |        |        |    |            |          |
| GRSV-US |     | 3049           | 87       | 1404   | 467| 17.7         | 630    | 777    | 258 | 29.2       | 151      |
| CSNV   |      | 2947           | 79       | 1404   | 467| 17.3         | 529    | 783    | 260 | 27.0       | 152      |
| PNSV   |      | 2949           | 87       | 1404   | 467| 17.0         | 529    | 777    | 258 | 28.5       | 153      |
| ZLCV   |      |                |          |        |    |              |        |        |    |            |          |
| TCSV   |      |                |          |        |    |              |        |        |    |            |          |
| ANSV   |      |                |          |        |    |              |        |        |    |            |          |
| GYSV serotype |   |                |          |        |    |              |        |        |    |            |          |
| GCFSV  |      | 2833           | 67       | 1419   | 472| 16.3         | 455    | 813    | 270 | 20.8       | 79       |
| GYSV   |      | 2970           | 57       | 1443   | 480| 15.9         | 653    | 741    | 246 | 22.9       | 76       |
| Other serotype | |                |          |        |    |              |        |        |    |            |          |
| INSV   |      | 2992           | 62       | 1350   | 449| 19.9         | 642    | 789    | 262 | 24.3       | 149      |
| BeNMV  |      | 2584           | 60       | 1320   | 439| 19.1         | 315    | 813    | 270 | 27.3       | 76       |
| SVNaV  |      | 2603           | 58       | 1323   | 440| 18.8         | 318    | 834    | 277 | 29.8       | 70       |
| LNRV   |      | 2768           | 85       | 1326   | 441| 17.7         | 385    | 807    | 268 | 19.9       | 159      |

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Of particular interest is the envelop glycoproteins (GPs). Although the aa homology between MVBaV and most members of WSMoV serogroup is greater than 70%, two members, TNRV and MYSV, share only 63.4% and 65.3% homology, compatible to the 62.9% of TYRV, a member belonged to IYSV serogroup.

The phylogenetic trees constructed with RdRps, NSm, GnG, NSs, and N all clearly showed that MVBaV was a distinct member of WSMoV serogroup, more closely related to a subgroup composed of CaCV, WBNV, WSMoV, and GBNV (Fig 7).
S RNA sequence diversity

To assess the genome sequence diversity of MVBaV, the S RNA of nine additional MVBaV isolates were cloned, sequenced, and compared with that of the type isolate XCSY-3. As shown in Table 3, the lengths of the S RNA of these isolates range from 3273 to 3324 nt with six length patterns and the IGRs from 987 to 1038 nt with 7 patterns among a total of 10 isolates. However, the 5’-UTR and 3’-UTR were completely conserved at length of 65 and 67 nt, respectively. The N and NSs proteins were highly conserved with only one aa substitution in the 277-aa N protein in two isolates and 2–6 aa substitutions in the 439-aa NSs proteins in eight isolates (Table 4). Therefore, the rich sequence diversity of the S RNA was reflected mainly in the length of the RNA fragment and the IGS.

Discussion

The N protein sequence, in combination with biological characters such as the thrips vector species and host range, represents the main classification criteria for the establishment of a new tospovirus species. In term of molecular differentiation, N protein sequence identity of 90% has been set as the threshold to establish a new species of tospovirus. As of to date, nine definitive species and fourteen tentative species of tospoviruses have been recognized by the International Committee on Taxonomy of Viruses [7, 32]. Among the tentative species, Iris yellow spot virus and Watermelon bud necrosis virus are pending for final approval as definitive species [32]. The recently reported vain-banding syndrome on mulberry (Fig 1) added a new plant to the host list of tospovirus [3]. Data of genome analysis presented in this study clearly demonstrates that MVBaV is indeed a new member of Tospovirus.

The genome of MVBaV is composed of L, M, and S RNAs. The genome organization strategy and the size as well as the proteins encoded are typical of the genus Tospovirus, particularly the WSMoV serogroup (Table 3). Phylogenetic analyses with five functional proteins encoded in L, M, and S RNAs all show that MVBaV is a distinct member of the WSMoV serogroup (Fig 7). This conclusion was also supported by the serological investigation. RdRp is responsible for the genome replication and may associates with host factors to fulfill its function [4]. Alignment of RdRp aa sequences revealed that all six conserved motifs for tospoviruses were present in the RdRp of MVBaV (Fig 4). The highest homology of viral proteins between MVBaV and other tospoviruses was found in RdRp, 83.9–85.8% to that of WSMoV, GBNV, WBNV, and CaCV. These five species form a subgroup within the WSMoV serogroup, while the rest members in the same serogroup share homology of 76.1–77.8% with MVBaV, compared with 67.8–
69% for viruses from the IYSV serogroup and 42.5–45.2% for viruses from TSWV and distinct serotypes (Table 3). High homology in RdRp may suggest a conserved functional mechanism for this protein.

The NSm protein encoded by M RNA is thought to be probably involved in cell-to-cell movement and belongs to the 30 K movement protein superfamily [27, 28]. Two of the four feature motifs, P/D-L-X motif and PLA2-motif, characteristic in TSWV serotype viruses did not find their counterparts in viruses of other serogroups. For P/D-L-X motif, rather than DLF, all other tospoviruses except BeNMV and SVNaV, have a motif of DSL; for PLA2-motif, instead of CCQHLHMC, most WSMoV serogroup viruses contain CMQNLNTS, following the motif pattern CXXLNLTTS. Of particular note is that MYSV contains a sequence of CTQLIFTS in this motif positions, very different from all other members in the WSMoV serogroup (Fig 5).

Tospoviruses are transmitted by insects belonging to the order Thysanoptera [33]. Glycoproteins encoded by the M RNA had been shown to be involved in vector transmission in TSWV virus [34, 35]. The glycoproteins (GPs) of MVBaV are 84.3% homologous to GBMV and 78.7–81.6% to CaCV, WSMoV, and WBNV (Table 3). Similarity in glycoproteins may imply a similar insect vector. Members of WSMoV serogroup all have apparent origin in Asia and are predominantly transmitted by only two species of the genus *Thrips* that have been proposed as WSMoV-Thrips-Asian type [4]. *Thrips palmi*, the major vector of the several tospoviruses in the WSMoV serogroup, was found abundantly in agriculture fields in China [36, 37]. Since *Pseudodendrothrips mori* is widely distributed in the mulberry field in China [38], it may well be the vector for MVBaV. Confirmation of the disease transmissibility by this thrips is currently under investigation.

N proteins encoded by the S RNA contain antigenic determinants that form the bases for the serotype differentiation in tospoviruses [18, 19]. The N protein of MVBaV shares the highest homology of 74.4% with that of CaCV, 70.0–71.8% with those of WSMoV, GBNV, and WBNV, and 56.2–61.5% to the rest members of the WSMoV serotype (Table 3). It was proposed that tospovirus species sharing N homology identity above 51.8% were serologically related [39].

The NSs protein encoded in S RNA has been shown to participate in viral movement, replication, and suppression of the host defense mechanism in TSWV and GBNV [30, 40, 41]. The NSs protein of MVBaV shares 70.2–71.3% homology with CaCV, WSMoV, GBNV, and WBNV, 54.4–61.7% with the rest members of the WSMoV serogroup. This protein also shares homology of 46.7–49.6% with members of the IYSV serogroup, but only 17.7% homology with TSWV (Table 3). Alignment of NSs proteins showed that WSMoV has the typical Walker A and YL motifs, but not the Walker B motif (Fig 6).

Phylogenetic analysis and homology calculation of the viral proteins RdRp, NSm, GP, NSs, and N of tospoviruses places MVBaV between the CaCV subgroup composed of CaCV, GBNV, WSMoV, and the CSSV subgroup composed of CSSV, TZSV, TRNV, and MYSV within the WSMoV serogroup of *Tospovirus* (Fig 7, Table 3). Thus MVBaV may represent a species that bridges the lineage within the WSMoV serogroup of *Tospovirus*.

Genetic diversity of MVBaV was investigated by sequencing the S RNA of 10 MVBaV isolates collected from mulberry orchards in different locations in this study. As shown in Table 4, the sizes of the RNA range from 3273 to 3324 nt, showing a disperse pattern of 3288 (2 isolates), 3293 (2 isolates), 3294 (2 isolates), 3273 (1 isolate), 3276 (1 isolate), 3286 (1 isolate), and 3324 nt (1 isolate). However, the 5’ UTR (65 nt), 3’ UTR (67 nt), the NSs (1320 nt, 439 aa), and N (834 nt, 277 aa) are completely conserved in length among all isolates. Minor substitutions in NSs (2–6 in 439 aa, 9 isolates) and N (1 in 277 aa, 2 isolates) were observed. Variations in size of the S RNA were caused by the varied length in the IGR, which ranges from 987 to 1038 nt. Intriguingly, the IGR length pattern was perfectly matched with that of S RNA size pattern,
i.e., S RNA size of 3288 nt with IGR of 1002 nt (isolates NN-5 and NN-10); S RNA size of 3293 nt with IGR of 1007 nt (isolates YZ-3 and XZDL-1); S RNA size of 3294 nt with IGR of 1008 nt (isolates XCSY-3 and HX-2). Thus, it is suggested that IGR of S RNA can be used as a genetic marker for MVBaV population analysis.

Genome reassortment and recombination play a role in the evolution of RNA virus. In this regard, genome reassortment and recombination have been observed to take place in several species of Tospovirus [42–43]. It was noted that the proteins GPs and NSm encoded by the M RNA of GRSV-US, a strain from the south Florida of the United State [43], do not group with the South African isolate (GRSV-SA), but with those of TCSV (Fig 7), demonstrating a genome fragment reassortment. Indeed, a large survey of the GRSV in vegetables in Florida and the Southeastern United States showed that all GRSV contained a reassorted genome with M RNA from TCSV [44]. Although no genome reassortment event has been observed for any of the ten Tospovirus members reported in mainland China [6, 12, 13, 45, 46], detailed survey by screening the genome components is required to evaluate the epidemic potential of these viruses, as genome reassortment may change the virus-vector or virus—host specificity.

We did not succeed in transmitting MVBaV by sap inoculation to any plants including mulberry. However, the virus could be transmitted by grafting (data not shown), establishing a positive correlation of MVBaV to the disease. Of important note, 32 out of 48 (66.7%) mulberry plants with viral disease-like symptoms were found to be infected with MVBaV in our preliminary survey, demonstrating the dominant nature of MVBaV in the field [3]. The high incidence of MVBaV in the mulberry orchards and the vast importance of tospovirus to crops in general imply that this virus may represent a substantial threat to the silkworm industry in China [3]. Future studies should focus on the biology of MVBaV, including the plant-virus interaction and vector-virus interaction, disease epidemiology, and practical measures for the disease control. In these regards, elucidation of the genome organization of MVBaV lays a solid foundation for future studies.

Supporting Information

S1 Table. Information of virus sampling, virus-induced symptom and permission for collection of the samples of Mulberry vein banding associated virus (MVBaV).

(DOC)

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Author Contributions

Conceived and designed the experiments: BC JM. Performed the experiments: PL LZ. Analyzed the data: JM CZ. Contributed reagents/materials/analysis tools: JL. Wrote the paper: JM BC.

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