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A novel rhabdovirus persistently infects lepidopteran cell lines

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ABSTRACT

A novel rhabdovirus was identified in BmN-4 (BmN) cells derived from *Bombyx mori* and Sf9 cells derived from *Spodoptera frugiperda*. Genome sequence homology revealed that this novel virus is distinct from the previously reported Spodoptera frugiperda rhabdovirus (SfRV). Given the high similarity of the *large protein* sequence of this virus to that of the Taarstrup virus, we named it Bombyx mori taarstrup virus (BmTV). When BmTV-negative *B. mori*-derived cultured cells were inoculated with BmTV, an increase in virus RNA was observed, but no impact on host cell proliferation occurred, suggesting that BmTV exhibits latent infection in insect cells. On the other hand, oral and hemocoel inoculation in *B. mori* larvae did not cause significant increase in BmTV replication. After subcutaneous inoculation, no difference in adult metamorphosis was observed between BmTV- and mock-inoculated larvae. No virus RNA was detected in eggs laid by adults that had been inoculated with BmTV at larval stage. These findings demonstrate that BmTV is a novel rhabdovirus that persistently infects several lepidopteran cultured cells.

1. Introduction

Rhabdoviruses are taxonomically classified into thirteen genera within the family *Rhabdoviridae* and the order Mononegavirales. These viruses are infectious for vertebrates, invertebrates, plants, fungi, and protozoans. Rhabdoviruses are naturally transmitted through various routes, including horizontal transmission via insect vectors, sap, aerosols, animal bites, and immersion in contaminated water (reviewed in Kuzmin et al., 2009). The viral particles are mostly enveloped, exhibiting a characteristic bullet-like or rod shape, measuring 100 to 430 nm in length and 45 to 100 nm in diameter. They contain non-segmented, negative-sense, single-stranded RNA genomes ranging from 11 to 16 kb in length. All rhabdovirus genomes encode, in order from the 3′ end, nucleoprotein (nucleocapsid protein, N), phosphoprotein (P), matrix protein (M), glycoprotein (G), large protein (L, RNA-dependent RNA polymerase), and may also encode accessory proteins (reviewed in Dietzgen et al., 2017).

Baculoviruses are enveloped, insect-specific viruses with double-stranded circular DNA genomes ranging from 80 to 180 kb. The baculovirus expression vector system (BEVS) is a powerful eukaryotic method for recombinant protein expression. Due to the extremely strong activity of the *polyhedrin* promoter and the accurate post-translational modifications performed in virus-infected cells, recombinant proteins produced via BEVS exhibits high yields with proper biological activity and function. As a result, BEVS has been utilized for developing next-generation vaccines, vectors for gene therapy, and other complex biopharmaceutical proteins (reviewed in Felberbaum, 2015). Autographa californica multicapsid nucleopolyhedrovirus (AcMNPV) and Bombyx mori nucleopolyhedrovirus (BmNPV), both classified under group I alphabaculovirus, have been commonly used as BEVS in cultured cells and larvae (reviewed in Rohrmann, 2019).

Recombination of foreign genes into the baculovirus genome is performed using *Spodoptera frugiperda*-derived Sf9 cells and *B. mori*-derived BmN-4 (BmN) cells, which are permissive for the replication of AcMNPV and BmNPV, respectively. However, insect cultured cells have been reported to be contaminated with persistently infectious RNA viruses (Friesen et al., 1980; Katsuma et al., 2005; Li et al., 2007; Ma et al., 2014). *B. mori*-derived BmN cells, for example, are contaminated with Bombyx mori latent virus (BmLV), an unclassified member of the family *Tymoviridae* (Katsuma et al., 2005). To address the safety concerns of

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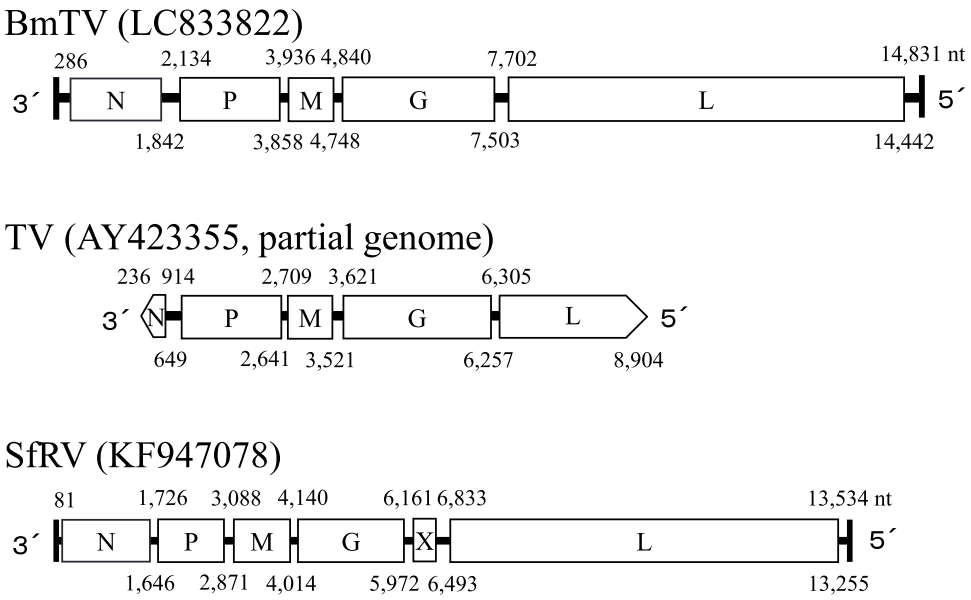

Fig. 1. Structure of full-length cDNA of BmTV. The five putative rhabdoviral genes, nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and large protein (L, RNA-dependent RNA polymerase), are shown for both BmTV and SfRV. Additionally, SfRV may encodes an accessory gene (*X*).

BEVS in BmN cells, it was determined that BmLV does not replicate in mammalian-derived cultured cells (Innami et al., 2016), and physical methods for inactivating BmLV were developed (Uchiyama et al., 2016). Additionally, BmLV-negative cultured cells were established from *B. mori* embryos, leading to the development of a BmLV-negative BmNPV-based BEVS (Iwanaga et al., 2012, 2014). On the other hand, *S. frugiperda*-derived Sf9 cells are persistently infected with Spodoptera frugiperda rhabdovirus (SfRV), a member of the family *Rhabdoviridae* (Ma et al., 2014). For safety aspects, SfRV-negative SfRVN cells have been developed and are used for AcMNPV-based BEVS (Maghodia et al., 2016). Recently, it was reported that SfRV replicates not only in *S. frugiperda*-derived Sf9 cells but also in *B. mori*-derived BmN cells, *Heliothis sebflexa*-derived HsAM cells, and *S. frugiperda* larvae (Schroeder et al., 2019). Interestingly, vertical transmission of SfRV was suggested in field-collected *S. frugiperda* larvae (Schroeder et al., 2019).

In this study, we focused on a cDNA identified through transcriptome analysis of *B. mori*-derived cells, which showed high similarity to the Taarstrup virus (TV) isolated from the leafhopper *Psammotettix alienus* (Bock et al., 2004). We have termed this virus Bombyx mori taarstrup virus (BmTV). RT-PCR analysis revealed that several cultured cell lines derived from both *B. mori*- and *S. frugiperda-derived* were already contaminated with BmTV.

2. Materials and methods

2.1. Cells and larvae

B. mori-derived BmN, BmVF, BmVF-MLV, *B. mandarina*-derived BoMa-529b, and *S. frugiperda*-derived Sf9 cells were maintained in the appropriate medium, as previously described (Iwanaga et al., 2004, 2009, 2012). *Trichoplusia ni*-derived High Five cells were maintained in Express Five medium (Thermo Fisher Scientific). *B. mori* larvae (F1 hybrid N124 × C124) were reared on artificial diets, as described by Tsukui et al. (2019).

2.2. Identification of full-length cDNA sequence

The BmTV contig was assembled by reanalyzing the RNA-seq data of zs00291 (Katsuma et al., 2018) using the Trinity RNA-seq assembly and analysis package (ver. 2.0.6) (Grabherr et al., 2011). The cDNA sequence of the BmTV genome was verified through Sanger sequencing using the primers listed in Table S1. To determine the terminal sequences of the BmTV genome, viral particles were precipitated by centrifuging 2000 ml of conditioned medium collected from BmTV-positive BmN cells at 20,000 × g for 30 mins. Viral RNA was extracted from these particles using RNAisoPlus (Takara Bio) and subjected to RACE analysis. The 3' RACE was performed using the 5' RACE kit (Takara Bio) with the primers listed in Table S1. The 3′-terminal cDNA of BmTV was concatemerized and amplified by PCR, followed by nested PCR. The 5' RACE was carried out using the First Choice RLM-RACE Kit (Thermo Fisher Scientific) with the primers shown in Table S1, and the 5′-terminal cDNA of BmTV was amplified by PCR, followed by nested PCR.

2.3. PCR analysis

Genomic DNAs was extracted from *B. mori*-derived BmN and BmVF cells, as well as from fat body tissue, using the standard phenol: chloroform: isoamyl alcohol method and then subjected to genomic PCR. Total RNA was isolated from cultured cells derived from *B. mori, S. frugiperda, T. ni*, and *B. mandarina* using RNAisoPlus. To verify whether virus-positive cells released BmTV or SfRV particles extracellularly, 30 ml of conditioned medium from Sf9 and VF-MLV cells was concentrated with Amicon Ultra-15 Centrifugal filter 100 K (Millipore) and used for RNA preparation. For RT-PCR, cDNAs was reverse-transcribed from total RNAs using ReverTra Ace (Toyobo) and then subjected to PCR with Quick Taq HS DyeMix (Toyobo). For qRT-PCR, cDNA was reverse-transcribed using the PrimeScript RT Reagent Kit (Takara Bio) and subjected to qPCR with SYBR Premix Ex Taq II (Takara Bio) using the Light Cycler 96 System (Roche), as described previously (Innami et al., 2016). The primers used for PCR are listed in Table S1.

2.4. Sequence analysis

The DNA products from RT-PCR and RACE were cloned into the T-vector pMD19 (Takara Bio) and sequenced using the BigDye terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, USA) with a 3500 Genetic Analyzer (Applied Biosystems). A phylogram was constructed using Molecular Evolutionary Genetics Analysis version 10 (MEGA10) with the Maximum Likelihood method and a bootstrap of 1000 replicates (Kumar et al., 2018).

2.5. Infection experiment

The BmTV solution was prepared as follows: conditioned medium from BmTV-positive BmVF-MLV and Sf9 cells was centrifuged at 1000 × g for 10 mins at 4 °C. After centrifugation, the supernatant was filtered using a 0.22 μm pore-size filter. Around 2×10^5 BmTV-negative BmVF cells were inoculated with 1 ml of the conditioned medium and harvested at appropriate time points for qRT-PCR analysis. For oral infection experiments, newly hatched *B. mori* larvae were reared on an artificial diet (2 cm × 2 cm × 5 mm) containing 100 μl of BmTV solution until the end of the third instar, after which they returned to an artificial diet without BmTV. On day 5 of the fifth instar, larvae were dissected, and total RNAs was subjected to RT-PCR. For hemocoel infection experiments, day 1, fifth instar larvae were starved for three hours to prevent vomiting and anesthetization with ice. They were then injected with 100 μl of BmTV solution and returned to an artificial diet. At appropriate time points, larvae were dissected, and total RNA was subjected to RT-PCR. For vertical transmission experiments, larvae inoculated with BmTV in the hemocoel were allowed to mate after eclosion. Eggs laid by the adult females were collected, and total RNA was subjected to RT-PCR.

2.6. Cell proliferation assay

BmVF cells (1.5×10^6) were inoculated with a BmTV solution prepared from the conditioned medium of Sf9 cells that were BmLV-

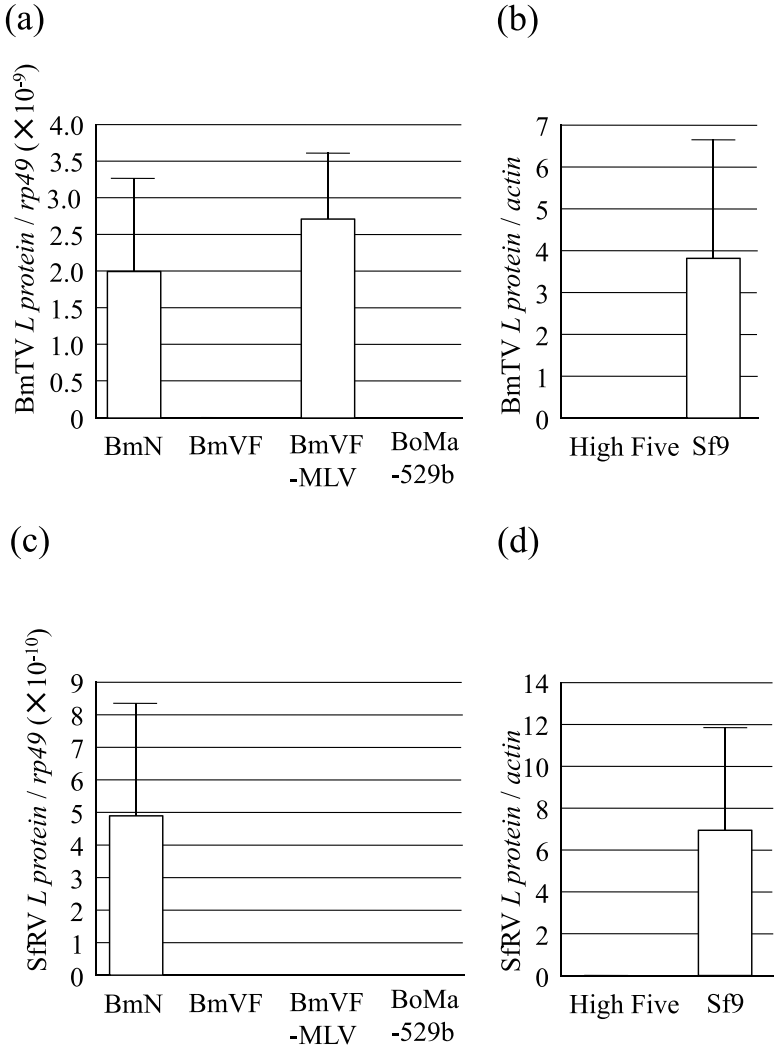

Fig. 2. Replication of BmTV. (A) Replication of BmTV (ab) and SfRV (cd) in lepidopteran cultured cells. RNA was extracted from *B. mori*-derived BmN, BmVF, and BmVF-MLV cells; *B. mandarina*-derived BoMa529b cells; *T. ni*-derived High Five cells; and *S. frugiperda*-derived Sf9 cells. The RNA samples were then subjected to qRT-PCR for the L *protein* of BmTV and SfRV. Results represent the average of three independent experiments, with error bars indicating standard deviation. (B) Genomic PCR analysis of *B. mori*-derived cultured cells and fat bodies. DNA was extracted from *B. mori*-derived BmN and BmVF cells as well as from fat bodies and subjected to PCR for the N *protein* of BmTV and SfRV. *B. mori* actin was used as a control. (C) RT-PCR analysis of conditioned media from Sf9 and VF-MLV cells. RNA was extracted from the conditioned medium and subjected to RT-PCR for the L *protein* of BmTV and SfRV. (D) BmTV infection experiment. Conditioned medium from BmTV-positive BmVF-MLV cells was inoculated into BmTV-negative BmVF cells. After inoculation, RNA was extracted at designated times points and subjected to qRT-PCR analysis of the BmTV L *protein*. Results represent the average of three independent experiments, with error bars indicating standard deviation. (E) BmTV infection experiments. Conditioned medium from BmTV-positive Sf9 cells was inoculated into BmVF cells. After inoculation, RNA was extracted at 60 and 120 hpi and subjected to qRT-PCR analysis of the BmTV L *protein*. Results represent the average of three independent experiments, with error bars indicating standard deviations. (F) Effect of BmTV inoculation on host cell proliferation. BmVF cells were inoculated with the conditioned medium from BmTV-positive Sf9 cells and harvested at the indicated time post-infection, then counted with a hemocytometer. Results represent the average of three independent experiments, with standard deviations indicated. Means not sharing any letter are significantly different by the Tukey-test at the 5 % level of significance. (G) Photographs of mock- or BmTV-infected BmVF cells. BmVF cells were inoculated with the conditioned medium from BmTV-positive Sf9 cells and harvested at 120 hpi. The scale bar is 50 μm.

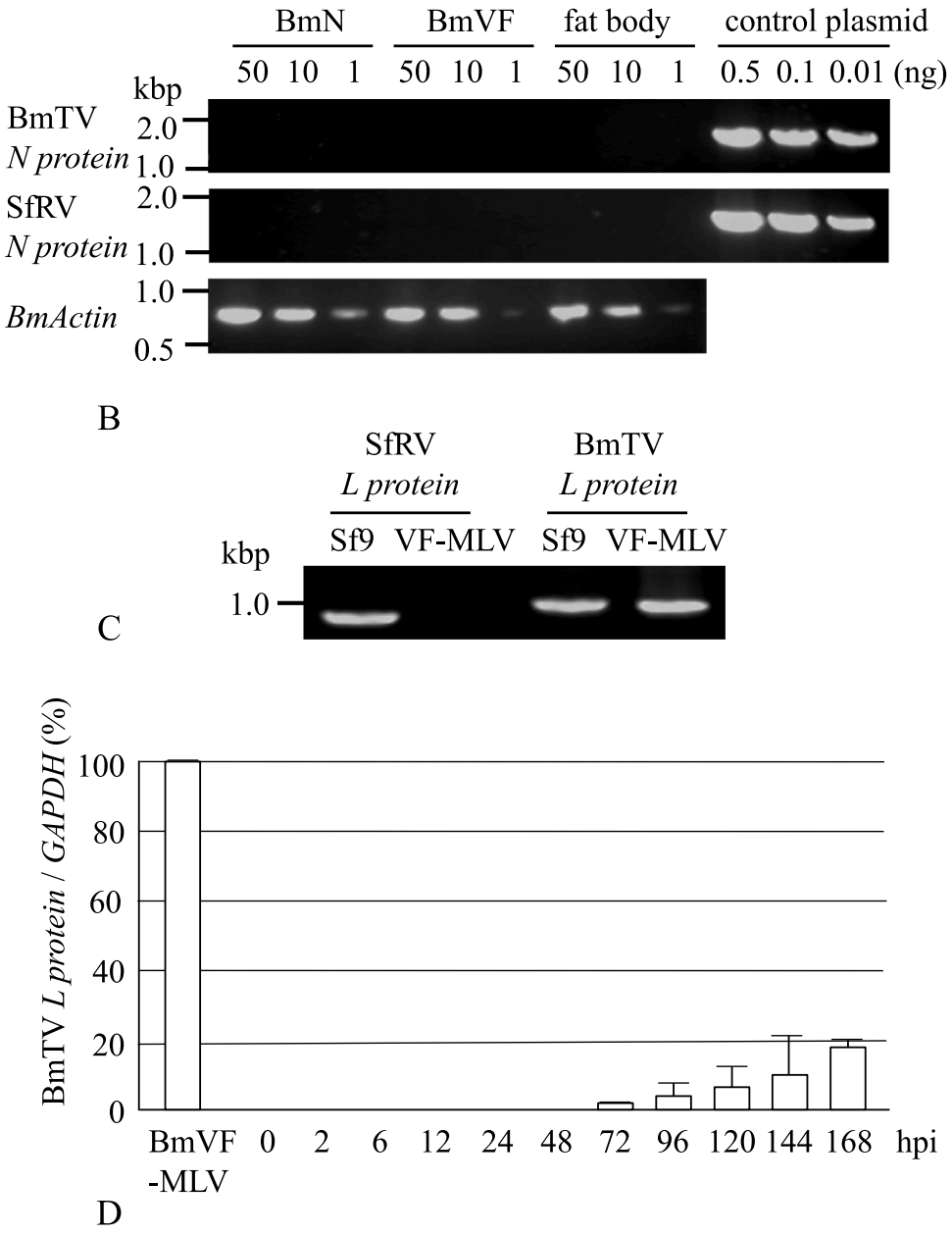

Fig. 2. (*continued*).

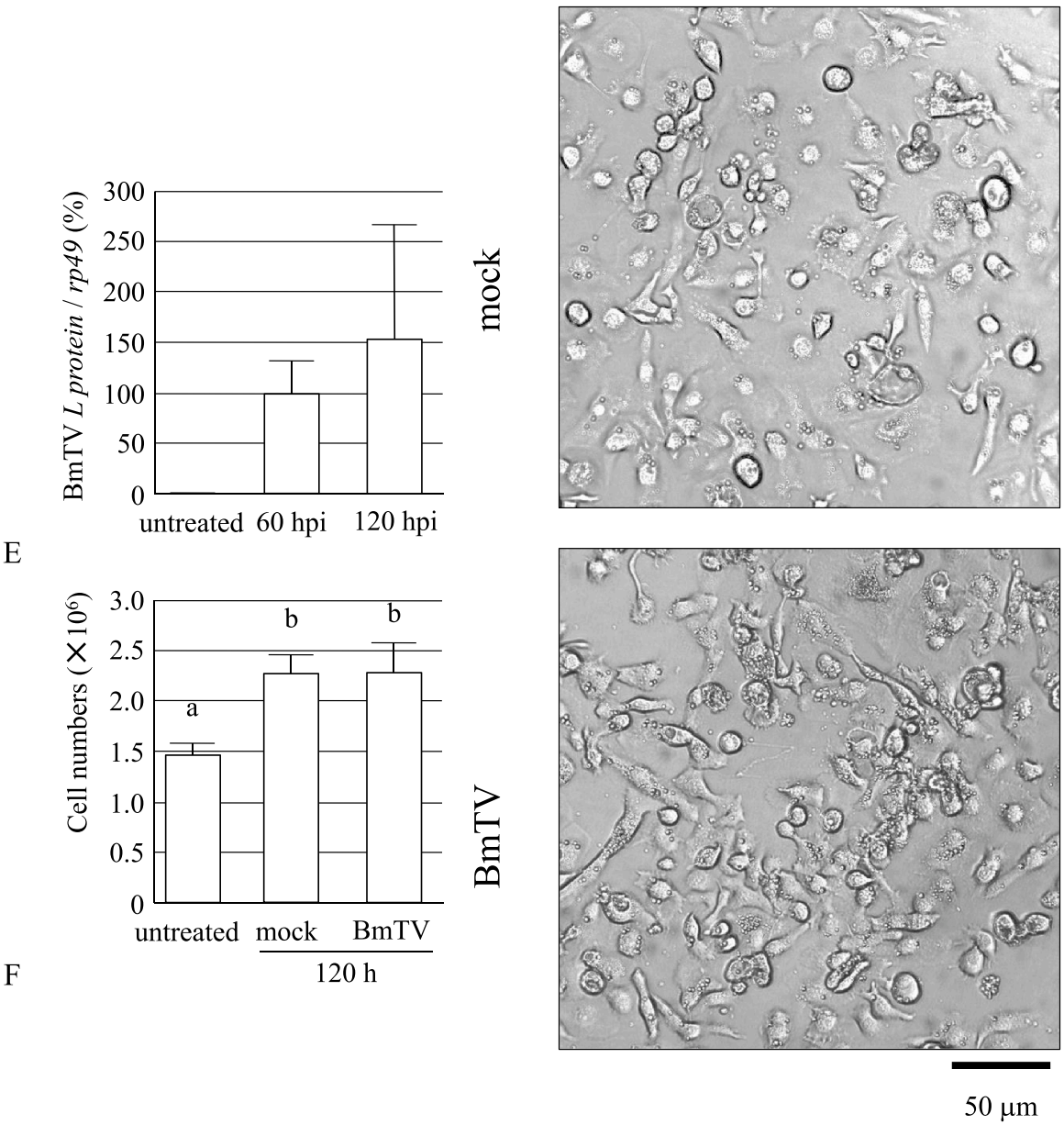

Fig. 2. (*continued*).

negative and BmTV-positive. Cell numbers were counted by a hemocytometer.

3. Results

3.1. Identification of Bombyx mori taastrup virus

A cDNA sequence exhibiting high similarity to rhabdovirus L *protein* genes was obtained from the transcriptome data of BmLV-inoculated BmVF cells (Katsuma et al., 2018). This cDNA showed 78 % and 49 % amino acid identity with the L protein of TV and SfRV, respectively. The upstream region of this cDNA was further investigated by assembling sequences from the transcriptome data. Consequently, a contig of 14, 709 bases, resembling the rhabdovirus genome, was discovered. This contig sequence was verified as Sanger sequencing of cDNAs obtained through RT-PCR, and the full-length cDNA, comprising 14,831 bases, was determined using RACE. As shown in Fig. 1, five putative rhabdoviral genes, nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and large protein (L, a subunit of the viral RNA-dependent RNA polymerase), were identified from this full-length cDNA. Since the L *protein* gene of this cDNA showed the highest sequence identity to that of TV, we designated this virus as BmTV. Although the genome structure of BmTV was similar to that of SfRV, the *X* gene present in the SfRV genome was not found in the BmTV genome.

Next, we conducted qRT-PCR analysis to determine whether BmTV RNA could be detected in various insect-derived cells. As shown in Fig. 2A-ab, BmTV RNA was present in both BmN and Sf9 cells. BmTV RNA was also detected in BmVF-MLV cells, which were established by inoculating BmTV-negative BmVF cells with conditioned medium from BmTV-positive BmN cells (Fig. 2A-a). Additionally, SfRV RNA was found in both BmN and Sf9 cells, but not in BmVF and BmVF-MLV cells (Fig. 2A-cd). Neither BmTV nor SfRV RNA was detected in *T. ni*-derived High Five cells and *B. mandarina*-derived BoMa-529b cells. To determine whether the detected viral RNA was transcribed from the host genome,

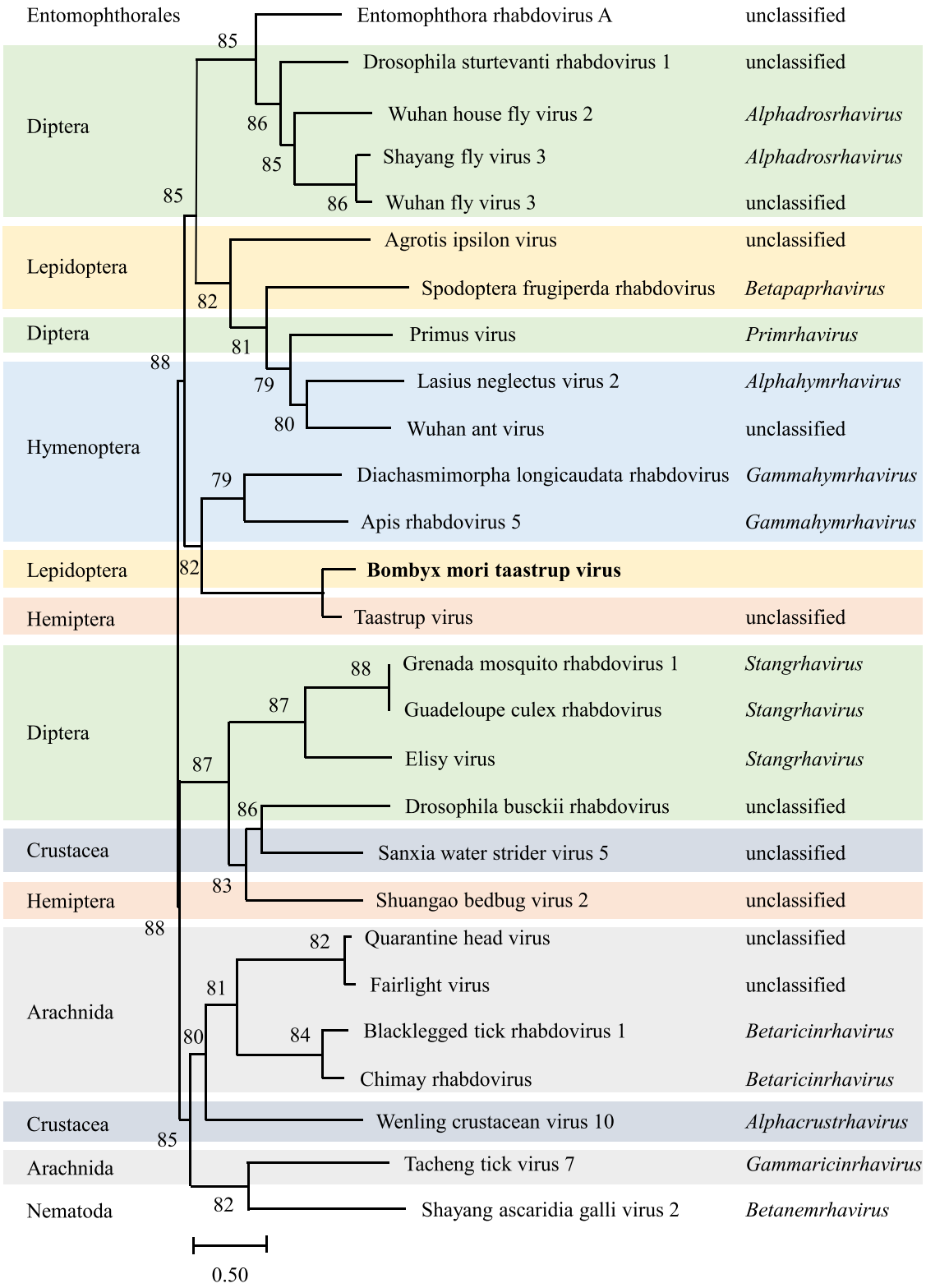

Fig. 3. Phylogenetic analysis of L *protein* genes from 27 viruses; Agrotis ipsilon virus (QKY64636.1), Apis rhabdovirus 5 (UCR92551.1), Blacklegged tick rhabdovirus 1 (YP_010797554.1), Chimay rhabdovirus (YP_010797586.1), Diachasmimorpha longicaudata rhabdovirus (YP_009259650.1), Drosophila busckii rhabdovirus (AMK09240.1), Drosophila sturtevanti rhabdovirus 1 (AMK09264.1), Elisy virus (QRW41819.1), Entomophthora rhabdovirus A (QED42948.1), Fairlight virus (AYP67531.1), Grenada mosquito rhabdovirus 1 (AVP26977.1), Guadeloupe culex rhabdovirus (QEM39085.1), Lasius neglectus virus 2 (YP_010797908.1), Primus virus (QIS62334.1), Quarantine head virus (AYP67539.1), Sanxia water strider virus 5 (YP_009289352.1), Shayang ascaridia galli virus 2 (YP_010797278.1), Shayang fly virus 3 (YP_009300674.1), Shuangao bedbug virus 2 (YP_009300677.1), Spodoptera frugiperda rhabdovirus (YP_009094312.1), Taastrup virus (AY423355.1), Tacheng tick virus 7 (YP_009304476.1), Wenling crustacean virus 10 (YP_009336512.1), Wuhan ant virus (YP_009304559.1), Wuhan fly virus 3 (AJG39162.1), Wuhan house fly virus 2 (YP_009304985.1). L *protein* genes homologous to the BmTV L *protein* were analyzed using the neighbor-joining method in MEGAX software. Bootstrap values (1000 replicates) are shown next to the branches. The host organism orders and the virus genus names are indicated on the left and right. The scale bar represents 0.50 substitutions per nucleotide position.

Table 1
Comparison of nucleotide sequence identity between BmTV (LC833822), TV (AY423355) and SfRV (KF947078) ORFs.

ORF	BmTV/TV identity(%)	BmTV/SfRV identity(%)
Nucleoprotein	22.5	49.4
Phosphoprotein	53.9	42.3
Matrix protein	65.0	47.1
Glycoprotein	63.6	44.8
Large protein	71.9	48.2

Table 2
Comparison of nucleotide sequence identity of BmTV and SfRV N protein genes (100–700 nt) in BmN and Sf9 cells.

Host	BmTV/SfRV identity(%)	Virus	BmN/Sf9 identity(%)
BmN	38.8	BmTV	99.0
Sf9	38.0	SfRV	97.8

genomic PCR analysis was performed. As shown in Fig. 2B, BmTV-derived fragments were not amplified from the genomes of BmTV-positive BmN cells, BmTV-negative BmVF cells, and *B. mori*. Similarly, SfRV-derived fragments were not amplified from these genomic DNA samples. To verify whether virus-positive cells released BmTV and SfRV extracellularly, total RNA was extracted from conditioned media from Sf9 and BmVF-MLV cells and subjected to RT-PCR. As shown in Fig. 2C, both BmTV- and SfRV-derived fragments were amplified from the conditioned medium from BmTV- and SfRV-positive Sf9 cells, whereas only SfRV-derived fragments were amplified from that from BmTV-positive BmVF-MLV cells. To confirm whether BmTV is an infectious virus, virus-negative BmVF cells were inoculated with conditioned medium from virus-positive BmVF-MLV cells. As shown in Fig. 2D, BmTV RNA gradually increased, reaching approximately 18 % of the level found in BmVF-MLV cells a week after inoculation. Also, since BmVF cells are BmLV-negative, when condition medium from BmLV-positive BmVF-MLV cells was used as an inoculum for BmTV, the cell proliferation of BmVF could be affected by BmLV infection (Iwanaga et al., 2012). Therefore, BmVF cells were inoculated with BmTV prepared in the conditioned medium of BmLV-negative, BmTV-positive Sf9 cells. As shown in Fig. 2E, F, G, BmTV RNA gradually increased, and there was no significant difference in cell proliferation and morphological change between virus- and mock-inoculated BmVF cells. Combining the results of these qRT-PCR, genomic PCR, and infection experiments, we concluded that BmTV contaminating *B. mori*- and *S. frugiperda*-derived cultured cells is an infectious virus and not embedded in the host genome.

3.2. Difference between BmTV and SfRV genome

To explore the relationship between BmTV and SfRV, we conducted a phylogenetic analysis using the amino acid sequences of the L protein. As shown in Fig. 3, the sequence most closely related to BmTV was TV, which was discovered in stink bugs. Both BmTV and TV belong to the same phylogenetic clade as Diachasmimorpha longicaudata rhabdovirus and Apis rhabdovirus 5, both of which were discovered from Hymenoptera and classified in the genus *Gammahymrhavirus*. In contrast, SfRV, classified in the genus *Betapaprhavirus* was grouped separately from BmTV (Fig. 3).

To further characterize BmTV, we compared the five putative genes of BmTV (LC833822) with those of SfRV (KF947078) and TV (AY423355). As shown in Table 1, the highest sequence identity between the BmTV and SfRV genes was observed in the N *protein* at 49.4 %, while the lowest was in the *P protein* at 42.3 %. On the other hand, the highest sequence identity between the BmTV and TV genes was observed in the L *protein* at 71.9 %, and the *P protein*, M *protein*, and G *protein* showed >50 % identity. Additionally, we compared the partial

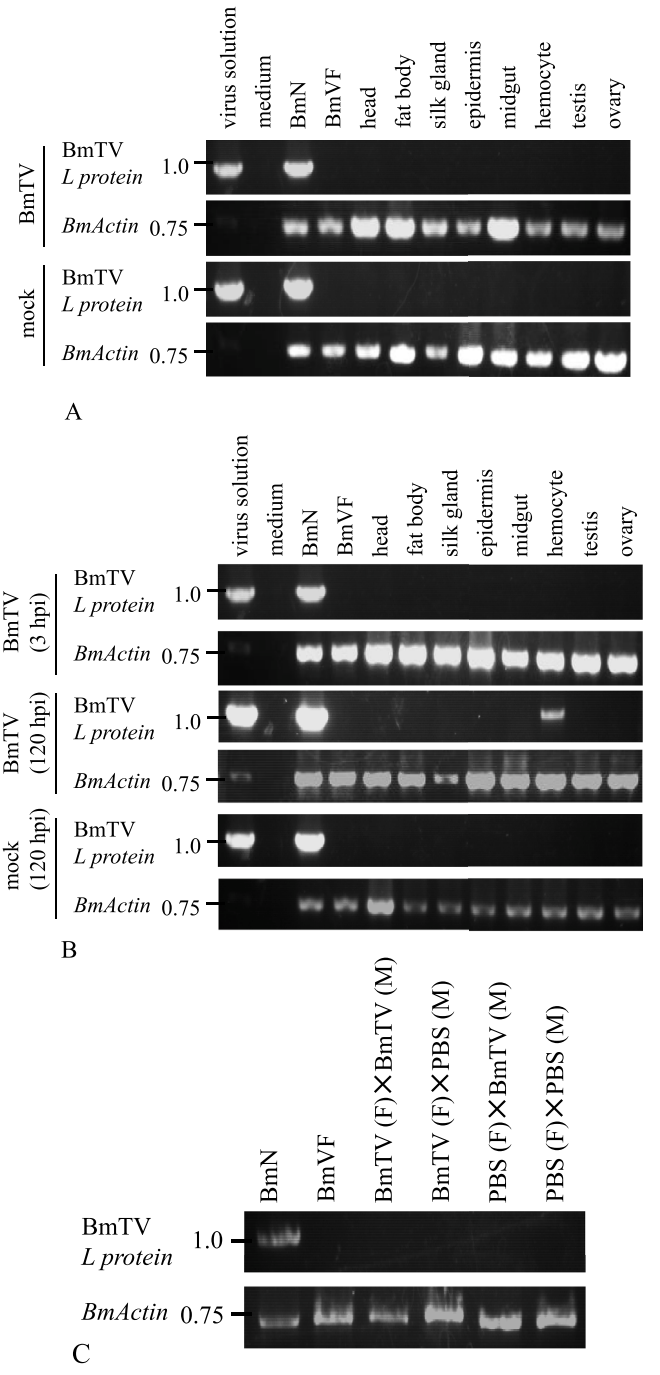

Fig. 4. BmTV inoculation into *B. mori* larvae. (A) Oral inoculation of BmTV in *B. mori* larvae. After hatching, larvae were fed an artificial diet containing BmTV until the third instar, after which they were switched to a normal diet. On the fifth day of the fifth instar, RNA was extracted from the excised heads and organs and analyzed by RT-PCR. RNA from BmN and BmVF cells served as positive and negative controls, respectively. (B) Hemocoel inoculation of BmTV in *B. mori* larvae. On the first day of the fifth instar, larvae were inoculated with BmTV via the hemocoel. RNA was extracted from the excited head and organs at 3 and 120 hpi and analyzed by RT-PCR. (C) Mating of adults following BmTV inoculation. Larvae on the first day of the fifth instar were inoculated with BmTV and raised on a normal diet. After eclosion, adults were mated in the combinations indicated at the top. F and M denote females and males, respectively.

nucleotide sequences of the N *protein* genes of BmTV and SfRV cloned from BmN and Sf9 cells maintained our laboratory. As shown in Table 2, the N *protein* gene similarity between these viruses in BmN and Sf9 cells was 38.8 % and 38.0 %, respectively. However, the identity between the BmTV N *protein* genes was 99.0 %, while the sequence identity between the SfRV N *protein* genes was 97.8 %. These results clearly demonstrate that BmTV and SfRV are distinct viruses, and BmN and Sf9 cells are persistently infected with both viruses.

3.3. Infection study of bmtv to B. mori larvae

To determine whether BmTV replicates in *B. mori* larvae, we conducted infection studies. As shown in Fig. 4A, five days after oral inoculation with BmTV, no viral RNA was detected in any tissues and organs. Additionally, five days after hemocoel inoculation with BmTV, viral RNA was detected only in hemocytes and was absent from all other tissues or organs (Fig. 4B). Furthermore, to investigate the possibility of vertical transmission of BmTV, we mated adult moths emerged from larvae with hemocoel inoculation. RNAs were extracted from the laid eggs and analyzed by RT-PCR. As shown in Fig 4C, no viral RNA was detected in eggs laid by adults inoculated via the hemocoel with BmTV. These results suggest that BmTV does not actively replicate in *B. mori*.

4. Discussion

SfRV not only persistently infects Sf9 cells but has also been detected in wild populations of *S. frugiperda* (Schroeder et al., 2019). It is believed that SfRV was introduced into the cultured cells during the establishment of *S. frugiperda*-derived cell lines. In the present study, since BmTV replication was observed only in *B. mori* hemocytes (Fig. 4B), it remains possible that BmTV was similarly introduced into the cultured cells when cultured *B. mori* cells were established. Interestingly, both BmTV and SfRV were detected in *B. mori*-derived BmN cells and *S. frugiperda*-derived Sf9 cells (Fig. 2A). However, as shown in Tables 1 and 2, the sequences of BmTV and SfRV are distinctly different throughout the genome, suggesting that BmTV is unrelated to SfRV and likely has a different origin. It is possible that Sf9 and BmN cells were contaminated with BmTV and SfRV, respectively, during the subculture process.

BmTV RNA was not detected in any tissue or organ other than hemocytes, according to RT-PCR analysis of virus-inoculated *B. mori* larvae (Fig. 4B). Although BmTV chronically infects several *B. mori*-derived cell lines (Fig. 2A), its propagation in these cultured cells was very slow, with viral RNA levels not reaching 20 % of virus-positive cells even 168 hpi (Fig. 2C). Therefore, to determine whether BmTV is vertically transmitted in *B. mori*, it is necessary to investigate multiple generations of virus-inoculated *B. mori*.

BmN cells, the most widely used cultured cells derived from *B. mori*, are contaminated with three viruses: BmLV, SfRV, and BmTV. Interestingly, in BmVF-MLV cells, which were generated by inoculating BmVF cells with conditioned medium derived from BmN cells, BmTV RNA was detected, while SfRV was not (Fig. 2A, C). The reason why SfRV replicates in BmN cells but not in BmVF cells remains unclear. Future studies should determine whether SfRV is unable to enter BmVF cells or if its replication is blocked by host defenses after entry. Additionally, BmVF-MLV cells proliferate more slowly than VF cells, a delay previously attributed to the persistent infection of BmLV (Iwanaga et al., 2012). However, given that the transcript levels of BmLV and BmTV in BmVF-MLV cells were 22,294.27 (Katsuma et al., 2018) and 1112.49 TPM (this study), respectively, it is possible that the persistent infection of BmTV also contribute to this slowed cell growth.

Several *B. mori*-derived cell lines and *S. frugiperda*-derived Sf9 cells are chronically infected with BmTV. Since these cell lines are often used for BmNPV- and AcMNPV-based BEVS, it is crucial to establish BmTV-negative cultured cells. Previous studies have shown that BmLV can be inactivated by UV-C, gamma rays, and heat treatment (Uchiyama, 2016), indicating the need to develop similar physical inactivation methods for BmTV. Additionally, it has also been reported that BmLV does not replicate in most mammalian cells, whereas SfRV may persistently infect some mammalian cell lines (Innami et al., 2016; Menghini et al., 2023). In the future, it will be important to determine whether BmTV can replicate in mammalian cells to ensure the safety of recombinant proteins produced in BmN and Sf9 cells.

5. Conclusions

In this study, we identified a novel rhabdovirus in *B. mori*-derived cells and named it BmTV based on phylogenetic analysis of the L *protein* gene. BmTV was detected not only in multiple *B. mori*-derived cell lines but also in *S. frugiperda*-derived Sf9 cells. Although BmTV was observed to replicate in *B. mori*-derived BmVF cells, its proliferation rate was notably slow. Additionally, BmTV was found to multiply in the hemocytes of *B. mori* larvae. Since *B. mori*- and *S. frugiperda*-derived cell lines are commonly used in BEVS, future research should focus on detailed analysis of BmTV replication, inactivation methods, and safety evaluation.

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CRediT authorship contribution statement

Takeo Fukushima: Investigation. **Shohei Takamura:** Investigation. **Keisuke Shoji:** Formal analysis, Data curation. **Tomoyo Touguchi:** Investigation. **Susumu Katsuma:** Writing – review & editing. **Masashi Iwanaga:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2025.199548.

Data availability

Data will be made available on request. The complete sequence of a full-length cDNA for BmTV has been submitted to the DDBJ, EMBL, and GenBank data banks under accession number LC833822.

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