Thladiantha Dubia Mosaic Virus: A Novel Potyvirus Infecting Manchurian Tubergourd (*Thladiantha dubia*) in Northeast China

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Abstract

A new virus with flexuous, filamentous particles approximately 650 nm long was discovered in Manchurian tubergourd (*Thladiantha dubia* Bunge) leaves exhibiting severe mosaic symptoms. The whole genome sequence of the virus was determined by reverse transcription-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE). The entire genome consisted of 10,112 nucleotides (nt) excluding the poly (A) tail, which shared the highest nucleotide sequence identity (73.8%) with that of papaya leaf distortion mosaic virus Hainan-DF isolate (PLDMV-Hainan-DF). A phylogenetic analysis showed that this virus clustered with PLDMV isolates in a subbranch within the potyviral clade. Of the 23 species of indicator plants tested,

only potato and its original host were systemically infected by the virus tested upon mechanical inoculation. A field survey showed that the virus was widely distributed on *T. dubia* and potatoes in Northeast China. Moreover, this virus displayed a high degree of genetic variation as evaluated by the sequences of the coat protein (CP) gene. Based on these results, the name *Thladiantha dubia* mosaic virus (ThDMV) is proposed for this new potyvirus.

Keywords: *Potyvirus*, *Thladiantha dubia*, *Thladiantha dubia* mosaic virus (ThDMV), biological characterization, complete nucleotide sequence, field survey

Manchurian tubergourd (*Thladiantha dubia* Bunge) is an herbaceous perennial climbing vine in the family Cucurbitaceae, and it is naturally distributed in Northeast Asia, ranging from Northeast China to the South Korean Peninsula, and Europe (Alegro et al. 2010; Nagao et al. 1990; Wang et al. 2011). This vine can establish large populations on the top of crop plants in the fields, and bushes or trees in the orchards and forests (Alegro et al. 2010). As a traditional Chinese medicine, it has been used to treat intractable diseases, such as mammary gland hyperplasia (Wang et al. 2011) and human cervical cancer (Jin et al. 2015; Shi et al. 2012; Zhou et al. 2016). The secondary metabolism products in the root or fruit have been shown to have anti-inflammatory and analgesic effects (Chen et al. 2012; Tong et al. 2012).

Studies on *T. dubia* have been mainly focused on its cultivation and medicinal activity (Wang 2016). Due to a lack of investigations, the pathogens that infect *T. dubia* have not been reported. Because *T. dubia* can survive the harsh winter in Northeast China as a tuber under the ground, it has the potential to serve as virus reservoir and thus may pose a potential risk to both cultivated crops and plants in the natural ecosystem. From 2014 to 2015, a survey of viral diseases in the region was carried out on plants used in traditional Chinese medicines, and *T. dubia* plants exhibiting severe mosaic symptoms

were discovered. Here, we report the identification and characterization of a novel potyvirus tentatively named *Thladiantha dubia* mosaic virus (ThDMV) that was collected from *T. dubia* plants showing severe mosaic symptoms.

Materials and Methods

Plant materials and virus inoculation. Symptomatic *T. dubia* leaves were collected in the experimental forest farm of the Northeast Forestry University in Heilongjiang Province, China, in July 2015. Samples were stored in a –80°C freezer.

For host range determination, a total number of 23 species of ThDMV-free plants were selected (Table 1). All plants were grown in pots in an insect-proof greenhouse under a natural photoperiod at 23°C. For mechanical inoculation, symptomatic *T. dubia* leaves were ground in 0.02 M phosphate buffer (pH 7.4), and the resulting sap was used as inoculum. Four-week-old seedlings were inoculated with sap from symptomatic *T. dubia* leaves or 0.02 M phosphate buffer (as the negative control) at the 3 to 5 true leaf stage. After inoculation, plants were observed in the greenhouse for symptom development. Symptoms were recorded for at least 4 weeks. Upper, noninoculated systemic leaves of all inoculated plants were analyzed by reverse transcription (RT)-polymerase chain reaction (PCR) to detect potential latent infection of ThDMV. All experiments were repeated three times for each treatment with three plants.

Viral particle purification and transmission electron microscopy (TEM). Viral particles were purified from symptomatic *T. dubia* leaves according to the protocol described previously with a few modifications (Chen 2010). In brief, the leaves were homogenized in 0.02 M phosphate buffer (pH 7.2) containing 1% Triton X-100 and 0.2% 2-hydroxy-1-ethanethiol. After filtering through four layers of gauze, the extract was clarified with 8% n-butanol and centrifuged at 6,000 × g for 20 min at 4°C. The aqueous supernatant was mixed with 4% (w/v) polyethylene glycol 6000 (PEG 6000) and 1.5% (w/v) sodium chloride and stirred overnight at 4°C. After centrifugation at 10,000 × g for 15 min at 4°C, the precipitate was resuspended in 0.02 M phosphate buffer (pH 7.2) and centrifuged at 10,000 × g for 15 min at 4°C. The aqueous supernatant was collected and centrifuged at 75,000 × g for 150 min at 4°C. The pellets containing purified virus particles were resuspended in 0.01 M phosphate buffer and stored at 4°C.

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Approximately 20 µl of purified virion solution was dropped on the top surface of a formvar film and incubated for 15 min at room temperature. The liquid on the formvar film was then absorbed with filter paper, and the sample was stained with 2% sodium phosphotungstate (pH 7.0) for 45 s. After drying, the formvar film containing viral particles was observed with a 200 kV JEM-2100 electron microscope (Jeol, Tokyo, Japan).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For the SDS-PAGE assay, approximately 10 µl of the purified virion solution was equally mixed with 2× SDS-PAGE loading buffer. After boiling at 100°C for 5 min, the sample was separated by 12% polyacrylamide gels at 120 V for approximately 1.5 h. The gel was stained by Coomassie Brilliant Blue G-250 (Amresco, Atlanta, GA).

Total RNA extraction, cDNA synthesis, and cloning. Total RNA was extracted using an E.Z.N.A. Plant RNA Kit (Omega Bio-Tek, Norcross, GA) following the manufacturer's instruction. First-strand cDNA was synthesized using a ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan) with random hexamers according to the manufacturer's instruction in a 20-µl volume system. PCR was performed in a 25-µl volume system containing 1 µl each forward and reverse primers (10 mM), 1 µl cDNA, and 12.5 µl 2× Phusion High-Fidelity DNA Polymerase Mix (New England Biolabs, Ipswich, MA). The thermal cycle consisted of 94°C initial denaturation for 4 min; 35 cycles of 94°C for 45 s, 45 to 60°C (depending on the T_m values of primers) for 45 s, and 72°C for 1 to 2 min (depending upon amplicon length); and then a final extension step at 72°C for 10 min. The PCR products were analyzed by electrophoresis in 1% agarose gel, stained with GelRed (Nachuan Biotech., Beijing, China) and visualized using a UV transilluminator (Bio-Rad, Hercules, CA). The amplicons were purified using an E.Z.N.A. Gel Extraction Kit (Omega Bio-Tek) and ligated into the pMD18-T vector (Kakara, Dalian, China). Plasmids containing correct inserts were sent to Kumei Comate Bioscience Co., Ltd. in Changchun, China, for sequencing. At least three independent clones of each amplicon were sequenced to avoid possible artificial mutations that might be introduced by PCR. All primers used in the present study are listed in Supplementary Table S1.

For virus detection, the specificity of the primers (CP-1 and CP-2) that can amplify the coding region of the viral coat protein (CP) was used. RT-PCR was performed as described above, and the amplicon

was ligated into the pMD18-T vector and sequenced. Plants infected by *Papaya leaf distortion mosaic virus* (PLDMV), *Potato virus Y* (PVY), and *Potato virus A* (PVA) were used as negative controls.

Rapid amplification of cDNA ends (RACE). The 3'- and 5'-end sequences were obtained using the SMARTer RACE 5'/3' Kit (Clontech, Mountain View, CA) according to the manufacturer's protocol using the primers listed in the supplementary material. The amplicons were ligated into the pMD18-T vector and sequenced with each amplicon of at least three independent plasmid clones.

Sequence analyses. The complete ThDMV nucleotide sequence was assembled by DNAMAN 6.0 (Lynnon Biosoft, San Ramon, CA). The BLAST program at the National Center for Biotechnology Information (NCBI) was used for a homology analysis (Johnson et al. 2008). Open reading frames (ORFs) and their deduced amino acid sequences were obtained using SnapGene software (GSL Biotech; available at snapgene.com). The phylogenetic tree was constructed using the maximum likelihood method with MEGA 6.0 software (Tamura et al. 2013).

ThDMV-specific antiserum and serological analysis. Polyclonal antiserum against ThDMV CP was prepared in a rabbit using prokaryotically expressed CP protein as the antigen (unpublished). Indirect enzyme-linked immunosorbent assay (ELISA) was performed as described (Clark and Adams 1977). In brief, approximately 0.1 g mixed leaf tissue was homogenized in 5 ml PBST buffer (10 mM potassium phosphate, 150 mM sodium chloride, 0.02% polyvinyl pyrrolidone, and 0.005% Tween-20, pH 7.4). The extracts were loaded onto polystyrene plate (100 µl per well), and incubated overnight at 4°C. After washing with PBST three times, the polyclonal antiserum that was diluted at 1:2,000 (v/v) in western primary antibody diluent (Bioss, Beijing, China) was added at 100 µl per well and the plates were incubated at 37°C for 2 h. After washing three times in PBST, the alkaline phosphatase-conjugated goat anti-rabbit IgG/AP (Bioss) that was diluted at 1:1,000 (v/v) in western secondary antibody diluent was added to each well at 100 µl per well. After 2 h incubation at 37°C, the plates were washed three times by PBST and dried by spin. The BCIP/NBT (Invitrogen, Carlsbad, CA) substrate was then added to at 100 µl per well, and the plates were kept in a lightproof humidity box for color development. The absorbance at 405 nm (A₄₀₅) was acquired with a microplate reader (Bio-Rad) at the desired time. Healthy leaves, PBST-treated, and original ThDMV infected leaves were used as negative, blank, and positive controls, respectively. Each sample was loaded in duplicates, and the mean A₄₀₅ value of the two duplicate wells was used. The threshold value for a positive sample was determined as its A₄₀₅ value when it was at least three times higher than that of the negative control.

Field survey. Three to five plants exhibiting viral-like symptoms (mosaic, shrinking, curling, and yellowing) were randomly collected in each field, and each plant had three to five leaves. Three to five plants without viral-like symptoms were also collected in some fields where no plants with viral-like symptoms were available. Samples were stored in a -80°C freezer until use. All samples were tested two times by RT-PCR with the ThDMV-specific primers CP-1 and CP-2 and indirect ELISA using antiserum raised against ThDMV CP.

Results

Symptomatology and disease incidence. From 2014 to 2015, a survey of viral diseases of traditional Chinese medicinal plants was carried out in Northeast China. *T. dubia* plants with severe mosaic symptoms on leaves were observed in many locations in Northeast China, including Heilongjiang, Jilin, and Liaoning provinces (Fig. 1A). A preliminary survey showed that more than 60% of *T. dubia* population contain symptomatic *T. dubia* plants. Because it is an herbaceous climbing vine, the precise disease incidence per population cannot be determined. A symptomatic *T. dubia* plant was collected in the experimental forest farm of the Northeast Forestry University in Heilongjiang province in July 2015 and used as an inoculum for mechanical inoculation of healthy-looking *T. dubia* seedlings. Approximately 4 weeks postinoculation, all *T. dubia* seedlings inoculated with the sap from the symptomatic *T. dubia* plant developed similar

Table 1. *Thladiantha dubia* mosaic virus (ThDMV) host range analysis

Plant species	Varieties	Symptoms	PCR
<i>Brassica oleracea</i> L.	Jingfeng 1	None	–
<i>Brassica pekinensis</i> (Lour.) Rupr.	9812	None	–
<i>Capsicum annuum</i> L.	Longjiao 8	None	–
<i>Chenopodium amaranticolor</i>		None	–
<i>Chenopodium quinoa</i> Willd.		None	–
<i>Cucumis sativus</i> L.	Longyuanxiuchun	None	–
<i>Cucurbita moschata</i> (Duch. ex Lam.) Duch. ex Poiret	Landrace	None	–
<i>Cucurbita pepo</i> L.	Zaoqingyidai	None	–
<i>Datura innoxia</i> Mill.		None	–
<i>Gomphrena globosa</i> L.		None	–
<i>Luffa cylindrica</i> (L.) Roem.	Landrace	None	–
<i>Lycopersicon esculentum</i> Mill.	Dongnong 725	None	–
<i>Momordica charantia</i> L.	Landrace	None	–
<i>Nicotiana benthamiana</i>		None	–
<i>Nicotiana glutinosa</i>		None	–
<i>Petunia hybrid</i> Vilm.		None	–
<i>Physalis alkekengi</i> L.		None	–
<i>Pisum sativum</i> L.	Landrace	None	–
<i>Solanum nigrum</i> L.		None	–
<i>Solanum tuberosum</i> L.	Dongnong 310	None	+
<i>Thladiantha dubia</i> Bunge		Mosaic	+
<i>Vigna unguiculata</i> (L.) Walp.	Shenxin 888	None	–
<i>Zea mays</i> L.	Kennian 1	None	–

mosaic symptoms in upper, noninoculated leaves, whereas those inoculated with buffer remained symptomless (Fig. 1B and C). These data suggested that the mosaic symptoms of *T. dubia* plants is likely caused by a viral agent or agents.

Spectroscopy, TEM, and protein composition. To obtain evidence of a viral infection in the symptomatic *T. dubia* plants, a purified viral preparation was examined using spectroscopy and TEM. A typical nucleoprotein ultraviolet absorption profile was observed

Fig. 1. Symptom and morphology of Thladiantha dubia mosaic virus (ThDMV). Healthy (A) or diseased (B) *T. dubia* leaves. (C) *T. dubia* plant mechanically inoculated with sap from diseased *T. dubia* leaves at 4 weeks postinoculation. (D) Morphology of ThDMV under transmission electron microscopy. (E) SDS-PAGE analysis of purified virion of (ThDMV). Lane 1, protein molecular mass marker; lane 2, purified virion preparation.

Fig. 2. Schematic representation of the genomic organization of Thladiantha dubia mosaic virus (ThDMV). The numbers below the diagram indicate the starting position (nt) for each gene. The position and dipeptide motif of the cleavage sites are indicated. Relative position of the primers and the strategy to amplify and sequence the ThDMV genome are also indicated below the diagram.

with an A_{260}/A_{280} ratio of 1.33. The concentration of the virion was estimated to be approximately 0.354 mg per ml, which was equal to ~1.416 mg of virus per 100 g of infected tissue (Choi et al. 1977). Under TEM, flexuous filamentous particles of about 650 nm in length were observed, which resembled the particles of a potyvirus (Fig. 1D). No other shapes were observed under the TEM examination. A protein of about 35 kilodalton (kDa) was observed by the SDS-PAGE analysis of the protein obtained from the purified virion, consistent with the size of a potyviral CP (Fig. 1E). Together, these results not only confirmed the presence of a virus in the symptomatic *T. dubia* plants, but also suggested that the virus might belong to the family *Potyviridae*.

Viral genome determination. Three sets of degenerate primers for potyviruses were synthesized to amplify the partial coding regions of this virus. Three amplicons with desired sizes (680, 683, and 340 bp, respectively) were obtained by the three pairs of primers (Fig. 2). The remaining parts of the genome were then amplified as five fragments and the 5'- and 3'-ends were obtained by RACE as shown in Figure 2. The complete genome sequence of this virus (GenBank accession no. MF420353) consists of 10,112 nucleotides (nt) excluding the terminal poly (A) tail. The genome has a 134-nt 5'-untranslated region (UTR), a single large ORF (135–9,914 nt), and a 135-nt 3'-UTR. The ORF encodes a 3,260 amino-acid (aa) polyprotein, which has a predicted molecular mass of 371.2 kDa. Based on

Fig. 3. Phylogenetic tree of *Thladiantha dubia* mosaic virus (ThDMV) and representative members of nine genera in the family *Potyviridae*. The tree was constructed based on the complete genome sequences using the maximum likelihood method with the general time reversible (+G+I) substitution model and 1,000 bootstrap replicates in MEGA 6.0 software. Viruses in the genus *Bymovirus* were not included in the analysis because they have a bipartite genome. The numbers on the branches indicate the bootstrap supporting values. The full name and GenBank accession number of each virus are shown, and the location of ThDMV is indicated by a black arrow.

the conserved cleavage sites of viruses in the family *Potyviridae* (Adams et al. 2005), the putative cleavage sites and 10 mature proteins were predicted (Fig. 2): P1 (1–469 aa), HC-Pro (470–927 aa), P3 (928–1,275 aa), 6K1 (1,276–1,327 aa), CI (1,328–1,961 aa), 6K2 (1,962–2,014 aa), NIa-VPg (2,015–2,201 aa), NIa-Pro (2,202–2,444 aa), NIb (2,445–2,964 aa), and CP (2,965–3,260 aa). Moreover, a conserved potyviral polymerase spillage motif (GAAAAAA) was found in the P3 cistron between 3,381 and 3,387 nt. A 225-nt ORF (3,381–3,610 nt) encoding a 74-aa PIPO, which is +2 frame relative to the large ORF, was found flanking the 3'-end of the potyviral polymerase spillage motif (Chung et al. 2008).

Sequence comparison and phylogeny. A BlastN search showed that the complete genome sequence of this virus was most closely related to the genome of a Hainan isolate of PLDMV-Hainan-DF (GenBank accession no. JX9745551). To gain further insights into the

phylogenetic relationship between this virus and members in the family *Potyviridae*, the full genome of the most closely related potyviruses and typical members of each genus in the family *Potyviridae* were downloaded from the NCBI database. Multiple alignment showed that the genome and polyprotein of this virus shares 27.1–73.8% nt and 14.3–79.7% aa sequence identities with that of selected viruses in the family *Potyviridae* (Supplementary Table S2). A phylogenetic tree based on the complete nucleotide sequences of this virus and representative members of nine genera in the family *Potyviridae* was constructed. The results showed that the virus clustered with PLDMV isolates in the same subbranch within the *Potyvirus* genus (Fig. 3), which further confirmed that this virus is phylogenetically related to PLDMV. We also compared the nt and aa identities of each protein between this virus and all available PLDMV isolates (Table 2). The results showed that P1 is the least conserved protein among the 11 viral proteins, whereas the CP was the most conserved.

Table 2. Nucleotide and amino acid sequence identities between Thladiantha dubia mosaic virus (ThDMV) and Papaya leaf distortion mosaic virus (PLDMV) isolates^a

PLDMV isolates	P1 ^a	HC-Pro	P3	PIPO	6K1	CI	6K2	VPg	NIa	NIb	CP
PLDMV P	62.5/57.7	80.9/89.5	68.4/65.2	80.1/75.0	74.4/92.3	74.5/84.7	73.7/79.3	75.0/85.6	77.8/86.8	75.7/84.8	81.6/77.4
PLDMV Taiwan-KS	62.5/56.8	81.5/90.4	68.1/65.2	80.3/71.4	74.4/94.2	74.9/84.4	75.6/81.1	74.3/84.0	76.5/87.2	75.7/85.8	81.6/77.3
PLDMV LM	63.1/57.8	81.4/91.3	67.8/64.9	80.1/72.4	74.4/92.3	75.0/84.5	75.0/77.4	74.7/84.0	77.8/87.2	75.7/85.0	82.3/79.1
PLDMV Hainan-DF	63.3/58.3	81.4/91.3	67.9/64.7	79.7/72.4	74.4/92.3	74.9/84.4	75.0/77.4	74.9/84.0	77.9/87.2	75.3/85.0	82.1/79.4
PLDMV Taiwan-CZ	63.3/58.3	81.4/91.3	67.9/64.7	79.7/72.4	74.4/92.3	74.9/84.4	75.0/77.4	74.9/84.0	77.9/87.2	75.3/85.0	82.1/79.4
PLDMV XX7	62.5/56.5	77.2/88.0	70.8/70.7	79.2/71.1	80.1/92.3	74.8/84.9	70.5/77.4	76.3/87.7	78.2/86.0	76.3/86.7	82.0/81.5
PLDMV J69P	–	–	–	–	–	–	–	–	–	–	80.0/79.1
PLDMV J179P	–	–	–	–	–	–	–	–	–	–	80.3/78.7
PLDMV J199C	–	–	–	–	–	–	–	–	–	–	80.1/81.4
PLDMV P56	–	–	–	–	–	–	–	–	–	–	80.3/79.4
PLDMV TW-P-WZ	–	–	–	–	–	–	–	–	–	–	80.5/79.1
PLDMV TW-P-ZS	–	–	–	–	–	–	–	–	–	–	80.4/79.1
PLDMV TW-P-TD	–	–	–	–	–	–	–	–	–	–	80.6/79.7

^a Data are presented as nt/aa identities.

Table 3. Detection of Thladiantha dubia mosaic virus (ThDMV) on different field plants^a

Family	Plant species	Dalian, Liaoning			Majiaogou, Harbin			Horticulture branch, Heilongjiang			Panshi, Jilin		
		Symptoms	RT-PCR	ELISA	Symptom	RT-PCR	ELISA	Symptom	RT-PCR	ELISA	Symptom	RT-PCR	ELISA
Amaranthaceae	<i>Amaranthus retroflexus</i> L.	N	–	–	N	–	–	N	–	–	N	–	–
Chenopodiaceae	<i>Chenopodium hybridum</i> L.	N	–	–	N	–	–	N	–	–	N	–	–
Cucurbitaceae	<i>Citrullus lanatus</i> (Thunb.) Matsum. et Nakai	M	–	–		NT	NT	M	–	–		NT	NT
	<i>Cucumis melo</i> L.	M	–	–		NT	NT		NT	NT		NT	NT
	<i>Cucumis sativus</i> L.	M	–	–	M	–	–	M	–	–	M	–	–
	<i>Cucurbita moschata</i> (Duch. ex Lam.) Duch.ex Poir.	M	–	–		NT	NT	M	–	–		NT	NT
	<i>Cucurbita pepo</i> L.	M	–	–		NT	NT	M	–	–	M	–	–
	<i>Lagenaria siceraria</i> (Molina) Standl.	N	–	–		NT	NT	M	–	–		NT	NT
	<i>Momordica charantia</i> L.	N	–	–		NT	NT	N	–	–		NT	NT
	<i>Thladiantha dubia</i> Bunge	M	+	+	M	+	+	M	+	+	M	+	+
	<i>Glycine max</i> (L.) Merr.	M	–	–	M	–	–	M	–	–	M	–	–
Leguminosae	<i>Phaseolus vulgaris</i> L.	M	–	–		NT	NT		NT	NT		NT	NT
	<i>Vigna umbellata</i> (Thunb.) Ohwi et Ohashi		NT	NT	M	–	–		NT	NT		NT	NT
	<i>Vigna unguiculata</i> (L.) Walp.	N	–	–	M	–	–	N	–	–	N	–	–
Solanaceae	<i>Capsicum annuum</i> L.	N	–	–	N	–	–	N	–	–	N	–	–
	<i>Lycopersicon esculentum</i> Mill.	N	–	–	N	–	–	N	–	–	N	–	–
	<i>Solanum melongena</i> L.	N	–	–	N	–	–	N	–	–	N	–	–
	<i>Solanum tuberosum</i> L.		NT	NT	SC	–	–	M	+	+	SC	+	+

^a N, no symptom; M, mosaic; SC, shrinking and curling; NT, not tested; –, ThDMV was not detected; +, ThDMV was detected.

Based on these results and the current sequence-based species demarcation criteria for *Potyviridae* (Wylie et al. 2017), this virus should be classified as a distinct member of the genus *Potyvirus*, and the name *Thladiantha dubia* mosaic virus (ThDMV) is tentatively proposed.

Host range studies. A total of 23 species of plants were mechanically inoculated with the sap of ThDMV-infected *T. dubia* leaf tissue (Table 1). The plants were kept in the greenhouse for symptom development for at least 4 weeks. However, none of the plants developed visible systemic symptoms except for the original *T. dubia* host plants, which were used as a positive control. RT-PCR showed that no amplicon was obtained from healthy *T. dubia* or plants infected by PLDMV, PVY, or PVA, demonstrating a high level of specificity of the primers. An amplicon with the desired size was amplified from potato and *T. dubia* but not from the other 21 indicator plants. The subsequent sequencing confirmed the presence of ThDMV in the upper, noninoculated leaves of potato (variety Dongnong310) and *T. dubia* (Table 1), indicating a productive systemic infection in these two hosts. Taken together, these data suggested that potato may serve as an alternative host of ThDMV.

Distribution of ThDMV in nature. From 2016 to 2018, a total of 17 plant species (three to five plants per species) from five families (Solanaceae, Cucurbitaceae, Chenopodiaceae, Amaranthaceae, and Leguminosae) were collected from four locations in Northeast China (Table 3). Most of these samples exhibited viral-like symptoms (mosaic, shrinking, and curling). The presence of ThDMV was evaluated by RT-PCR using specific primers (CP-1 and CP-2) and by indirect ELISA with the polyclonal antiserum against ThDMV CP. The results showed that all samples of *T. dubia* that had mosaic symptoms were positive for ThDMV via both RT-PCR and ELISA. Interestingly, the potato varieties Youjin-885 from the Horticulture Branch

of Heilongjiang Province and Huiyin 1 from Panshi, Jilin Province, were also positive for ThDMV via both RT-PCR and ELISA (Table 3). These results not only confirmed that potato is susceptible to ThDMV infection but also suggested that it is a host under natural conditions.

We further surveyed the presence of ThDMV on different potato varieties in the major potato-producing areas in Heilongjiang Province. Samples of nine potato varieties (Fujin, Helan 7, Kexin 13, Luyin 1, Zaodabai, Youjin-885, Xingjia 2, Yanshu 9, and 806) from five locations, namely Keshan, Suihua, Hulan, Mudanjiang, and Jagedaqi, were collected and detected by RT-PCR and ELISA (Table 4). The results showed that three potato cultivars, namely Youjin-855, Kexin 13, and 806, were infected by ThDMV and ThDMV was detected in all five locations surveyed (Table 4). These results suggested that ThDMV is widely distributed in the potato growing areas in Heilongjiang Province.

Genetic variation of ThDMV CP. To further analyze the genetic variation of ThDMV, the full-length CP gene of ThDMV was amplified from six field-collected ThDMV-infected potato samples and sequenced. Six sequence variants were obtained (GenBank accession nos. MK752525 to MK752530). A sequence comparison showed that the CP gene of the six ThDMV isolates shared 90.8 to 91.1% nt and 93.9 to 94.6% aa identities with that of the *T. dubia* ThDMV isolate (Table 5). These results suggested that ThDMV has a considerable level of genetic variation.

Discussion

In this study, we described a tentative new potyvirus species from *T. dubia* plants with severe mosaic symptoms found in Northeast China. This virus has flexuous, filamentous particles of approximately 650 nm containing a single CP of 35 kDa. The genome shared the highest nt sequence identity (73.8%) with PLDMV isolate Hainan-DF; whereas the large ORF had the highest aa identity with that of PLDMV isolate XX7 (79.7%). In the phylogenetic tree constructed based on full genomic sequences of selected viruses in the family *Potyviridae*, this virus clustered with PLDMV in a subbranch within the potyviral clade (Fig. 3). Moreover, the P1 and CP of this virus had the highest aa identities (58.3 and 81.5%) with PLDMV isolates (Table 2). Based on the species demarcation criteria in the 10th report of the International Committee on Taxonomy of Viruses (ICTV) (Wylie et al. 2017), the virus should be recognized as a new member of the genus *Potyvirus* in the family *Potyviridae*, and the name ThDMV is tentatively proposed.

The host range study showed that only potato and *T. dubia* were susceptible to ThDMV infection among the 23 indicator plants tested (Table 1). These results suggested that ThDMV might have a relatively narrow host range. Nevertheless, the actual natural host range of ThDMV is yet to be investigated. On cultivated crops, ThDMV was only found in three out of the nine field potato cultivars surveyed (Table 3 and 4). Therefore, further experiments are needed to confirm whether the other five potato cultivars are also susceptible to ThDMV infection. Noticeably, our results suggested that ThDMV might be widely distributed on potato in Heilongjiang Province because it was found at all five locations surveyed. In addition, we found that all ThDMV-positive potato samples had the mosaic

Table 4. Detection of *Thladiantha dubia* mosaic virus (ThDMV) on different potato varieties in five regions of Heilongjiang Province

Sampling location	Potato varieties	Symptoms ^a	PCR/ELISA ^b
Keshan	Youjin-885	N	+/+
	Zaodabai	N	–/–
Suihua	Youjin-885	M	–/–
	Helan 7	M	–/–
	Yanshu 9	N	–/–
	Fujin	Y	–/–
	806	M	+/+
Hulan	Youjin-885	N	+/+
Mudanjiang	Kexin 13	N	+/+
	Youjin-885	N	+/+
Jagedaqi	Youjin-885	N	+/+
	Fujin	N	–/–
	Luyin 1	N	–/–
	Zaodabai	N	–/–
	Xingjia 2	N	–/–
	Kexin 13	N	–/–

^a N, no symptom; M, mosaic; Y, yellowing.

^b + and – indicate ThDMV was and was not detected in the samples, respectively.

Table 5. Percent CP nucleotide (888 nt, above diagonal) and amino acid (296 aa, below diagonal) sequence identity of the CP-encoding sequences of *Thladiantha dubia* mosaic virus (ThDMV) isolates

SN	Isolate	Origin ^a	Host	SN						
				1	2	3	4	5	6	7
1	ThDMV	NEFU	<i>T. dubia</i>	–	91.1	91.1	90.9	90.8	91.0	91.1
2	Youjin-885	Keshan	Potato	94.6	–	100	99.8	99.7	99.7	99.8
3	Kexin 13	Mudanjiang	Potato	94.6	100	–	99.8	99.7	99.7	99.8
4	Youjin-885	Mudanjiang	Potato	93.9	99.3	99.3	–	99.4	99.4	99.6
5	806	Suihua	Potato	93.6	99.0	99.0	98.3	–	99.3	99.4
6	Youjin-885	Hulan	Potato	94.6	100	100	99.3	99.0	–	99.4
7	Youjin-885	Jagedaqi	Potato	94.2	99.7	99.7	99.0	98.6	99.7	–

^a NEFU: Northeast Forestry University; all sites are in Heilongjiang, China.

symptoms; however, the relationship between ThDMV infection and mosaic symptoms still needs to be verified in the future.

Viruses in the genus *Potyvirus* are transmitted mainly by aphids in a nonpersistent manner in nature (Boquel et al. 2013; Ng and Perry 2004). However, they can be transmitted by a large number of aphid species, including both colonizing and noncolonizing species (Pelletier et al. 2012). Although we noticed that diseased *T. dubia* and potato plants are always associated with large populations of green peach aphid (*Myzus persicae*), the aphid species that is able to transmit ThDMV still needs to be verified in the future. *T. dubia* is an herbaceous climbing vine plant that always forms a complex population on the top of other plants. Therefore, ThDMV could be transmitted by a direct connection between diseased and healthy plants in the same population. Nevertheless, further investigations are needed to verify these possibilities.

Recent studies have highlighted the importance of wild plants in outbreaks of viral diseases on crops, the evolution of plant viruses, and the subsistence of viruses in nature (Kaliczak and Syller 2009; Malmstrom et al. 2005; Shargil et al. 2017; Webster et al. 2007). In the present study, we confirmed that *T. dubia* is the natural reservoir for ThDMV, a potential threat to potato production. Therefore, special attention should be paid to the damage that it may cause to potato in Northeast China.

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Literature Cited

Adams, M. J., Antoniw, J. F., and Beaudoin, F. 2005. Overview and analysis of the polyprotein cleavage sites in the family *Potyviridae*. *Mol. Plant Pathol.* 6: 471-487.

Alegro, A., Bogdanović, S., Rešetnik, I., and Boršić, I. 2010. *Thladiantha dubia* Bunge (*Cucurbitaceae*), new alien species in croatian flora. *Natura Croat.* 19: 281-286.

Boquel, S., Giguère, M. A., Clark, C., Nanayakkara, U., Zhang, J., and Pelletier, Y. 2013. Effect of mineral oil on *Potato virus Y* acquisition by *Rhopalosiphum padi*. *Entomol. Exp. Appl.* 148:48-55.

Chen, J. S. 2010. Page 260 in: *Experimental Plant Virology*. Zhejiang University Press, Hangzhou, Zhejiang, China.

Chen, J. S., Zhang, Y. L., Zhao, B., Tong, J. M., Mao, X. X., and Liu, Y. P. 2012. Anti-inflammatory effect of the total saponins from *Thladiantha dubia* roots. *Chin. J. Exp. Tradit. Med. Formul.* 18:163-166.

Choi, J. K., Maeda, T., and Wakimoto, S. 1977. An improved method for purification of *Turnip mosaic virus*. *Jpn. J. Phytopathol.* 43:440-448.

Chung, B. Y., Miller, W. A., Atkins, J. F., and Firth, A. E. 2008. An overlapping essential gene in the *Potyviridae*. *Proc. Natl. Acad. Sci. USA* 105:5897-5902.

Clark, M. F., and Adams, A. N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *J. Gen. Virol.* 34:475-483.

Jin, J. H., Wang, A. L., Feng, T. Y., He, H. B., Qin, H. L., Wang, J. Z., and Zhang, Y. F. 2015. Studies of the inhibition effects from *Thladiantha nudiflora* Hemsl on Caski cells and survivin gene expression and Bcl-2 protein family relationships. *Pharmacol. Clin. Chin. Mater. Med.* 31:150-154.

Johnson, M., Zaretskaya, I., Raytselis, Y., Merezhuik, Y., McGinnis, S., and Madden, T. L. 2008. NCBI BLAST: a better web interface. *Nucleic Acids Res.* 36:W5-W9.

Kaliczak, A., and Syller, J. 2009. New hosts of *Potato virus Y* (PVY) among common wild plants in Europe. *Eur. J. Plant Pathol.* 124:707-713.

Malmstrom, C. M., Hughes, C. C., Newton, L. A., and Stoner, C. J. 2005. Virus infection in remnant native bunchgrasses from invaded California grasslands. *New Phytol.* 168:217-230.

Nagao, T., Tannaka, R., Okabe, H., and Yamauchi, T. 1990. Studies on the constituents of *Thladiantha dubia* Bunge. II. Structures of dubiosides D, E and F, neutral saponins of quillaic acid isolated from the tuber. *Chem. Pharm. Bull. (Tokyo)* 38:378-381.

Ng, J. C. K., and Perry, K. L. 2004. Transmission of plant viruses by aphid vectors. *Mol. Plant Pathol.* 5:505-511.

Pelletier, Y., Nie, X., Giguère, M. A., Nanayakkara, U., Maw, E., and Footitt, R. 2012. A new approach for the identification of aphid vectors (*Hemiptera: Aphididae*) of *Potato virus Y*. *J. Econ. Entomol.* 105:1909-1914.

Shargil, D., Smith, E., Lachman, O., Reingold, V., Darzi, E., Tam, Y., and Dombrovsky, A. 2017. New weed hosts for *Cucumber green mottle mosaic virus* in wild Mediterranean vegetation. *Eur. J. Plant Pathol.* 148:473-480.

Shi, M. Q., Liu, X., Zhou, J. G., Yang, W. Y., Luo, T., and Xiong, X. J. 2012. Apoptosis of cervix cancer HeLa cells induced by *Thladiantha nudiflora* Hemsl extract. *J. Third Mil. Med. Univ.* 34:1844-1848.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 30: 2725-2729.

Tong, J. M., Liu, Y. L., Liu, Y. P., and Mao, X. X. 2012. Mechanism of analgesic effect of total saponin from the root of *Thladiantha dubia* Bunge. *Lishizhen Med. Mater. Med. Res.* 23:1085-1087.

Wang, L. S., Zhao, D. Q., Di, L., Cheng, D. Y., Zhou, X. F., Yang, X. W., and Liu, Y. H. 2011. The anti-hyperplasia of mammary gland effect of *Thladiantha dubia* root ethanol extract in rats reduced by estrogen and progesterone. *J. Ethnopharmacol.* 134:136-140.

Wang, Y. F. 2016. Cultivation of medicinal plant *Thladiantha dubia* Bunge. *Chin. Wild Plant Resour.* 35:74-75.

Webster, C. G., Coutts, B. A., Jones, R. A. C., Jones, M. G. K., and Wylie, S. J. 2007. Virus impact at the interface of an ancient ecosystem and a recent agroecosystem: studies on three legume-infecting potyviruses in the southwest Australian floristic region. *Plant Pathol.* 56:729-742.

Wylie, S. J., Adams, M., Chalam, C., Kreuze, J., Lopez-Moya, J. J., Ohshima, K., Praveen, S., Rabenstein, F., Stenger, D., Wang, A., Zerbini, F. M., and ICTV Report Consortium. 2017. ICTV Virus Taxonomy Profile: *Potyviridae*. *J. Gen. Virol.* 98:352-354.

Zhou, Q., Yang, X. J., Shi, M. Q., Liu, Y., Qin, H. L., Feng, T. Y., Zhang, Y. F., and Yu, X. Q. 2016. Alcohol extraction from *Thladiantha nudiflora* Hemsl promotes apoptosis of human cervix cancer Caski cells through PI3K/Akt/mTOR pathway. *Chin. J. Clin. Pharmacol. Ther.* 21:42-49.