Characterization of a New Potyvirus Infecting *Thevetia Ahouai*

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Research Article

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Abstract

A new potyvirus was found in *Thevetia ahouai* L. (Fam. *Apocynaceae*) plants exhibiting leaf white spots and fruit discoloration. The complete genome of two isolates of the virus, tentatively named thevetia white spot virus (ThWSV), was determined. The genome of ThWSV comprises 9,912 (isolate 1) and 9,904 (isolate 2) nucleotides (nt), encoding a polyprotein of 363 kDa. Sequence comparisons between the two isolates showed 80 and 87% identities at the nt and amino acid (aa) levels, respectively, whereas the overall identity between ThWSV and its closest relative is 69% and 71% at the nt and aa levels, respectively.

Full Text

Thevetia ahouai L. is an evergreen shrub with shiny, dark green, ovate leaves and bright, lobed red fruits with a milky sap. The shrub belongs to the *Apocynaceae* family, and is native from Brazil; although it can be found all the way to Mexico [3]. *T. ahouai* has been used in traditional medicine as treatment for hemorrhoids, toothache, and rheumatism, and has also shown to have anti-promastigote activity against *Leishmania* [6]. In recent years, the ornamental use of *T. ahouai* has expanded to private gardens and public places, such as urban parks or sidewalks, due to its colorful appearance.

In June of 2020, virus-like symptoms including leaf white spot and fruit discoloration were observed in *T. ahouai* plants at two different locations of Guayaquil, a coastal city of Ecuador. Symptomatic leaves from two selected shrubs, one located in an urban park (sample 1) and the second located in Prosperina, a tropical dry forest in the western side of the city (sample 2), were collected for virus identification.

Due to the lack of reports on viruses infecting *Thevetia sp.*, collected leaves were submitted to a partial virus purification protocol as described before [4]. Partially purified extracts were mounted on a carbon-coated formvar (1%) grid, and negatively stained with 2% PTA (phosphotungstic acid at pH 7.0). Grids were examined using a JEOL JEM-1400Plus transmission electron microscope hosted at the University of Minnesota Imaging Center. Flexuous filamentous virus-like particles of approximately 700 nm in length were identified in partially purified extracts from both samples (Online Resource 1).

High-throughput sequencing (HTS) was applied for virus identification using double-stranded RNA (dsRNA) for sample 1, or total RNA for sample 2, as initial template. The dsRNA was extracted from 15 g of fresh symptomatic leaf tissue following the protocol described by Morris and Dodds [7]. Total RNA was extracted from 100 mg of symptomatic leaf tissue using the RNeasy Plant Mini Kit (Qiagen, Germany) and subjected to plant ribosomal RNA (rRNA) depletion using Illumina's Ribo-Zero kit prior to the generation of a complementary DNA (cDNA) library using the TruSeq library prep kit. Sequencing was done on NovaSeq6000 Illumina platform as 150bp paired-end reads (Macrogen, South Korea).

A total of 22.3 and 21.2 million sequence reads were obtained for samples 1 and 2, respectively. Sequence data sets were analyzed using HTS-processing tools available from Geneious Prime® 2022.0.1. Raw sequences were trimmed for adapter removal and quality using the BBDuk plugin and *de novo* assembled using SPAdes. Several thousands of contigs were assembled from each sequence set. Blastx search identified a 9,528 nt long contig from sample 1, and a 9,542 nt long contig from sample 2, both showing sequence homology to several members of the potyvirus genus. A closer examination of each contig revealed that 15,844 reads (0.07%) were assembled into the potyvirus contig from sample 1; whereas 454,852 reads (2.15 %) were assembled into the potyvirus contig from sample 2. Blast searches of the remaining contigs revealed their host origin, which is expected as the RNA ribosomal depletion step does not subtract the whole host RNA. As for the difference in coverage observed between the viral contigs obtained from the two template types, dsRNA and total RNA, it is known that potyviruses yield low amounts of dsRNA compared to other plant viruses [12]. Given the low coverage of contig 1, a series of overlapping primers were designed to amplify and re-sequence contig 1 by cloning each RT-PCR fragment using a pGEM-T-easy Vector System (Promega, USA) followed by Sanger sequencing.

The original template was used to obtain the 5' and 3' terminal sequences using the 5´/3´ RACE Kit, 2nd Generation (Roche, Germany), according to manufacturer instructions. For the dsRNA, an additional denaturation step (96 C for 10 min) was used prior the reverse-transcription reaction. RT-PCR amplicons for each terminus (n =5) were cloned as described above and sequenced in both directions.

The complete genomic sequence, excluding the poly (A) tail, consisted of 9,912 nt (acc. numb. OM263475) and 9,904 nt (acc. numb. OM263476), for potyvirus sequence 1 and 2, respectively. The identity percentages between the two sequences (80% nt level and 86.7% amino acid level) suggest that both sequences belong to isolates of the same potyvirus (hereafter isolate 1 and isolate 2) [15].

Blastn searches revealed that both isolates share up to ~ 73% nt identity (86% coverage) with their closest relative, a potyvirus sequence from weeds in a papaya orchard of Chiapas, Mexico (access. numb. MN203192). When the complete genome of this potyvirus was compared to both isolates of the thevetia potyvirus, the identity was 69%. According to the species demarcation criteria for potyviruses [15], at this genomic identity level, it can be inferred that the thevetia virus described in this study represents a new member of the potyvirus genus.

Symptomatic leaves infected with each virus isolate were used for mechanical inoculations onto *T. ahouai* virus-free seedlings as described [9]. Leaf white spots were observed at an average of 15 days post-inoculation, with no differences between the symptoms induced by each isolate. The presence of the virus in the inoculated plants was confirmed by RT-PCR and Sanger sequencing using the primers: Det_F: 5'-

TCAGGAACGGTCTCGGTTCC-3' and Det_R: 5'-CCATCATCACCCAAACTCCAT-3', which amplify a 292 bp fragment of the virus coat protein (CP) gene. Inoculated plants were maintained under controlled conditions and symptoms were monitored for one year. Original symptoms including leaf white spots and fruit discoloration were reproduced in the inoculated plants, while no other virus-like sequence was found in the HTS data sets. Taken together, these findings suggest that the new potyvirus is the causal agent of the described symptomatology (Fig 1). Hence, the name thevetia white spot virus (ThWSV) is proposed and will be used hereafter.

In order to test seed-transmission, as reported for other potyviruses [11], five symptomatic fruits from a single virus-inoculated plant, were collected and all the twenty seeds (each fruit has 4 seeds) were potted in sterile germination medium. The third true leaf of each seedling was tested for the virus by RT-PCR as described above. None of the plants tested positive for the virus and no symptoms were observed during the study.

The genome organization of ThWSV is identical to those of recognized potyviruses, containing a long open reading frame (ORF) at nt positions 123-9,671 for isolate 1, and 118-9,663 for isolate 2. The hypothetical polyprotein precursor from isolate 1 has 3,183 amino acids with a predicted molecular mass of 363.5 kDa; while the polyprotein from isolate 2 has 3,182 aa (363.6 kDa), having a single lysine deletion (derived from codon AAA) at the N-terminus of the coat protein (CP) with respect to isolate 1.

Analyses of the hypothetical polyprotein of ThWSV identified the nine conserved proteolytic cleavage sites previously described for potyviruses [1], resulting in ten mature putative proteins (Fig 2A). In addition, typical potyvirus conserved motifs [14] were identified in the proteins of ThWSV, with slight differences in a few motifs between the two isolates (Table 1).

The putative small ORF termed PIPO was also identified in both isolates (Fig 2A), overlapping with the P3 coding region through the presence of the highly conserved motif $G_{1-2}A_{6-7}$ at the beginning of the PIPO ORF (isolate 1: ^{3324}GAAAAAT^{3330}; isolate 2: ^{3315}GAAAAAT^{3325}). In both isolates, the PIPO ORF is found in a non-frame fashion, suggesting its expression through a -1 ribosomal frameshifting from the P3 coding region, which would result in a fused protein (P3N-PIPO) as previously described [13].

Phylogenetic inferences were done using the complete polyprotein amino acid sequences of 36 representative potyvirus species and a member of the *Ipomovirus* genus (*Potyviridae*), which was used as outgroup. Multiple sequence alignment was done using MUSCLE [8] and the best fitted protein model (LG+G+F+I) was obtained. The phylogenetic tree was generated using the maximum-likelihood method with 500 bootstrap replicates in MEGAX [10]. The topology of the tree was consistent with previous Blast results, showing a most recent common ancestor for ThWSV, a potyvirus sequenced from an unknown weed in Mexico and asclepias virus A, a perennial herb in the same family (*Apocynaceae*) as *T. ahouai*. Other closely related potyviruses include pokeweed mosaic virus, tobacco vein mottling, potato virus A and potato virus B (Fig 2B). Amino acid identities among most closely related species ranged from 51% to 72%.

To the best of our knowledge, this is the first report of a virus infecting *T. ahouai.* ThWSV induced a range of symptoms including white spots on the leaves, darkening and black ringspots on the stems, and fruit discoloration (Fig 1). Further studies should be conducted to investigate the host range and natural vector of this new virus, especially due to its increased use as ornamental, which might pose a threat to cultivated plants. Based on the presence of aphid-transmission related motifs, such as KITC (KIAC in isolate 2) and PTK, in the helper component protein (HC-Pro), and DAG in the CP (Table 1), it is reasonable to speculate that natural transmission of ThWSV is mediated by aphids. However, epidemiology studies should focus on identifying those aphid species that are more common in *T. ahouai* and closely related species such as *Catharanthus roseus* (L.), which is ubiquitously found as ornamental in tropical regions. Sequence comparisons at nucleotide and amino acid level indicate that ThWSV is most closely related to a potyvirus found in an unknown weed growing within papaya orchards in Mexico [2] and asclepias virus A [5], isolated from *Asclepias syriaca*, a perennial herb in the same family (*Apocynaceae*) as *T. ahouai*.

Declarations

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Compliance with ethical standards

Ethics approval

This article does not contain any studies with human participants or animals performed by any of the authors. Plant samples were collected under *Genetic Resource Access Permit #* MAE–DNB–CM–2018–0098 granted by the Department of Biodiversity of the Ecuadorean Ministry of the Environment.

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Conflict of interest declaration

The authors declare no conflict of interest.

Author contributions

Study conception and design: Diego F. Quito-Avila. Material preparation, data collection and analysis were performed by Maria G. Cañada-Bautista, Edison G. Reyes-Proaño, Juan F. Cornejo-Franco, Robert A. Alvarez-Quinto and Dimitre Mollov. The first draft of the manuscript was written by Maria G. Cañada-Bautista and Diego F. Quito-Avila; all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability

The genomic sequences of the two virus isolates reported here have been deposited in the GenBank under accession numbers: OM263475 and OM263476.

Online Resource 1 Transmission electron micrograph of partially purified extracts from symptomatic leaves of *Thevetia ahouai.* A representative single flexuous filamentous virus-like particle is portrayed. Scale bar equals 100 nm. Magnification 60,000 X.

References

1. Adams MJ, Antoniw JF, Beaudoin F (2005) Overview and analysis of the polyprotein cleavage sites in the family Potyviridae. Mol Plant Pathol 6:471–487
2. Alcalá-Briseño RI, Casarrubias-Castillo K, López-Ley D, Garrett KA, Silva-Rosales L (2020) Network Analysis of the Papaya Orchard Virome from Two Agroecological Regions of Chiapas, Mexico. mSystems 5
3. Alvarado-Cárdenas LO, Ochoterena H (2007) A Phylogenetic Analysis of the Cascabela-Thevetia Species Complex (Plumerieae, Apocynaceae) Based on Morphology. Ann Mo Bot Gard 94:298–323
4. Alvarez-Quinto RA, Serrano J, Olszewski N, Grinstead S, Mollov D, Lockhart BEL (2022) Complete genome sequences of two isolates of spiraea yellow leafspot virus (genus Badnavirus) from Spiraea x bumalda 'Anthony Waterer'. Arch Virol
5. Baumann MM, Kiambi RG, Gomez T, Alvarez-Quinto RA, Lockhart BEL (2021) Asclepias yellow vein virus: a proposed new potyvirus in milkweed in the USA. New Disease Reports 44:e12025
6. Calderón Ál, Vázquez Y, Solís PN, Caballero-George C, Zacchino S, Gimenez A, Pinzón R, Cáceres A, Tamayo G, Correa M, Gupta MP (2006) Screening of Latin American Plants for Cytotoxic Activity. Pharm Biol 44:130–140
7. Dodds JA, Morris TJ, Jordan RL (1984) Plant Viral Double-Stranded RNA. Annu Rev Phytopathol 22:151–168
8. Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32:1792–1797
9. Hull R (2009) Mechanical Inoculation of Plant Viruses.Current Protocols in Microbiology13:16B.16.11-16B.16.14
10. Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. Mol Biol Evol 35:1547–1549
11. Simmons HE, Holmes EC, Gildow FE, Bothe-Goralczyk MA, Stephenson AG (2011) Experimental Verification of Seed Transmission of Zucchini yellow mosaic virus. Plant Dis 95:751–754
12. Valverde RA, Dodds JA, Heick JA (1986) Double stranded RNAs from plants infected with viruses having elongated particles and undivided genomes. Phytopathology:7
13. Vijayapalani P, Maeshima M, Nagasaki-Takekuchi N, Miller WA (2012) Interaction of the Trans-Frame Potyvirus Protein P3N-PIPO with Host Protein PCaP1 Facilitates Potyvirus Movement. PLoS Pathog 8:e1002639
14. Worrall EA, Hayward AC, Fletcher SJ, Mitter N (2019) Molecular characterization and analysis of conserved potyviral motifs in bean common mosaic virus (BCMV) for RNAi-mediated protection. Arch Virol 164:181–194
15. Wylie SJ, Adams M, Chalam C, Kreuze J, López-Moya JJ, Ohshima K, Praveen S, Rabenstein F, Stenger D, Wang A, Zerbini FM, Ictv Report C (2017) ICTV Virus Taxonomy Profile: Potyviridae. J Gen Virol 98:352–354

Tables

Table 1. Conservation of motifs in the polyprotein of thevetia white spot virus isolates (ThWSV 1 and ThWSV 2) with respect to those of representative members of the potyvirus genus. The amino acid (aa) positions for each motif are shown shown. The ‘x’ denotes any amino acid in that position. Shaded residues indicate differences with respect to the reference conserved motif. Footnote: * Potyvirus consensus motifs taken from Worrall et al. 2019.

Hypothetical protein	Conserved potyvirus motifs	Putative function*	Motifs in the polyprotein derived from both isolates of ThWSV showing amino acid positions	
			ThWSV 1	ThWSV 2
P1	IxFG	Protease activity	^{5}IMFG^{8}	^{5}IMFG^{8}
	$Hx_8Dx_{32}SGx_{22}RG$	Protease activity	$^{355}Hx_9Dx_{33}SGx_{12}RG^{414}$	$^{355}Hx_9Dx_{33}SGx_{12}RG^{414}$
	GxSG	Protease activity	^{397}GMSG^{400}	^{397}GMSG^{400}
	FIVRG	Protease activity	^{410}ILVRG^{414}	^{410}TLVRG^{414}
HC-Pro	$Cx_8Cx_{18}Cx_2C$	Zinc finger	$^{465}Cx_8Cx_{18}Cx_2C^{496}$	$^{465}Cx_8Cx_{18}Cx_2C^{496}$
	IGN	Cell to cell and long-distance movement	^{688}IGN^{690}	^{688}IGN^{690}
	KITC	Aphid transmission	^{490}KITC^{493}	^{490}KIAC^{493}
	PTK	Aphid transmission	^{748}PTK^{750}	^{748}PTK^{750}
	$FRNKx_{12}CDNQLD$	Symptomatology	$^{619}FRNKx_{12}CDNQLD^{640}$	$^{619}FRNKx_{12}CDNQLD^{640}$
	HAKRFF	Cell to cell movement	^{653}HAKRFF^{658}	^{653}HAKRFF^{658}
	CCCVT	Long-distance movement	^{730}CCCVT^{734}	^{730}CCCVT^{734}
	GYCY	Cysteine proteinase	^{780}GFCY^{783}	^{780}GFCY^{783}
	NIFLAML	Protease activity	^{785}NIFLAML^{791}	^{785}NIFLAML^{791}
	AELPRILVDH	Protease activity	^{840}AELPKILVDH^{849}	^{840}AELPKILVDH^{849}
	$Cx_{72}H$	Protease activity	$^{782}Cx_{72}H^{855}$	$^{782}Cx_{72}H^{855}$
P3	GAVGSGKST	NTP binding	^{1372}GAVGSGKST^{1380}	^{1372}GAVGSGKST^{1380}
	$EPYx_7SPx_2LxAx_2NxGx_2Ex_5N$	Protease activity	$^{928}QPYx_7SPx_2LxAx_2NxNx_2Ex_5W^{958}$	$^{928}QPYx_7SPx_2LxAx_2NxNx_2Ex_5W^{958}$
CI	VLLLEPTRPL	Helicase activity	^{1392}VLLLEPTRPL^{1401}	^{1392}VLLLEPTRPL^{1401}
	DExH	Helicase activity	^{1461}DECH^{1464}	^{1461}DECH^{1464}
	KVSATPP	Helicase activity	^{1488}KVSATPP^{1494}	^{1488}KVSATPP^{1494}
	LVYV	Helicase activity	^{1539}LIYV^{1542}	^{1539}LVYV^{1542}
	VATNIIENGVTL	Helicase activity	^{1590}VATNIVENGVTL^{1601}	^{1590}VATNIVENGVTL^{1601}
	GERIQRLGRVGR	Helicase activity	^{1634}GERIQRLGRVGR^{1645}	^{1634}GERIQRLGRVGR^{1645}
Nia-Pro	$Hx_{34}Dx_{67}GxCGx_{14}H$	Proteolytic activity	$^{2206}Hx_{34}Dx_{67}GxCGx_{14}H^{2327}$	$^{2206}Hx_{34}Dx_{67}GxCGx_{14}H^{2327}$
Nib	SLKAEL	RNA polymerase activity	^{2572}SLKAEL^{2577}	^{2572}SLKAEL^{2577}

	CHADGS	RNA-dependant polymerase activity	^{2647}CDADGS^{2652}	^{2647}CDADGS^{2652}
	GNNSGQPSTVVDNTLMV	RNA-dependant polymerase activity	^{2709}GNNSGQPSTVVDNTIMV^{27425}	^{2709}GNNSGQPSTVVDNTIMV^{27425}
	GDD	RNA-dependant polymerase activity	^{2753}GDD^{2755}	^{2753}GDD^{2755}
	QPSTVVDN	RNA-dependant polymerase activity	^{2714}QPSTVVDN^{2721}	^{2714}QPSTVVDN^{2721}
	CVDDFN	RNA-dependant polymerase activity	^{2605}CVDDFN^{2610}	^{2605}CVDDFN^{2610}
	FTAAP[L/I][D/E]	RNA-dependent polymerase activity	^{2591}FTAAPID^{2597}	^{2591}FTAAPID^{2597}
	[A/S]M[I/V]E[S/A]WG	RNA polymerase activity	^{2839}AMIESWG^{2845}	^{2839}AMIESWG^{2845}
CP	DAG	Aphid transmission	^{2927}DAG^{2929}	^{2927}DAG^{2929}
	AFDF	unknown	^{3114}AFDF^{3117}	^{3113}AFDF^{3116}
	QMKAAA	Aphid transmission	^{3134}QMKAAA^{3139}	^{3133}QMKAAA^{3138}
	MVWCI[E/D]NGTSP	Aphid transmission	^{3031}MVWCIENGTSP^{3041}	^{3030}MVWCIENGTSP^{3040}
	W[V/T]MMDG[D/E/N]	unknown	^{3047}WVMMDGE^{3053}	^{3046}WVMMDGE^{3052}
	[P/R/A]YMPRYG	unknown	^{3094}PYMPRYG^{3100}	^{3093}PYMPRYG^{3099}
	E[N/D]TERH	unknown	^{3159}EDTERH^{3164}	^{3158}EDTERH^{3163}

Figures

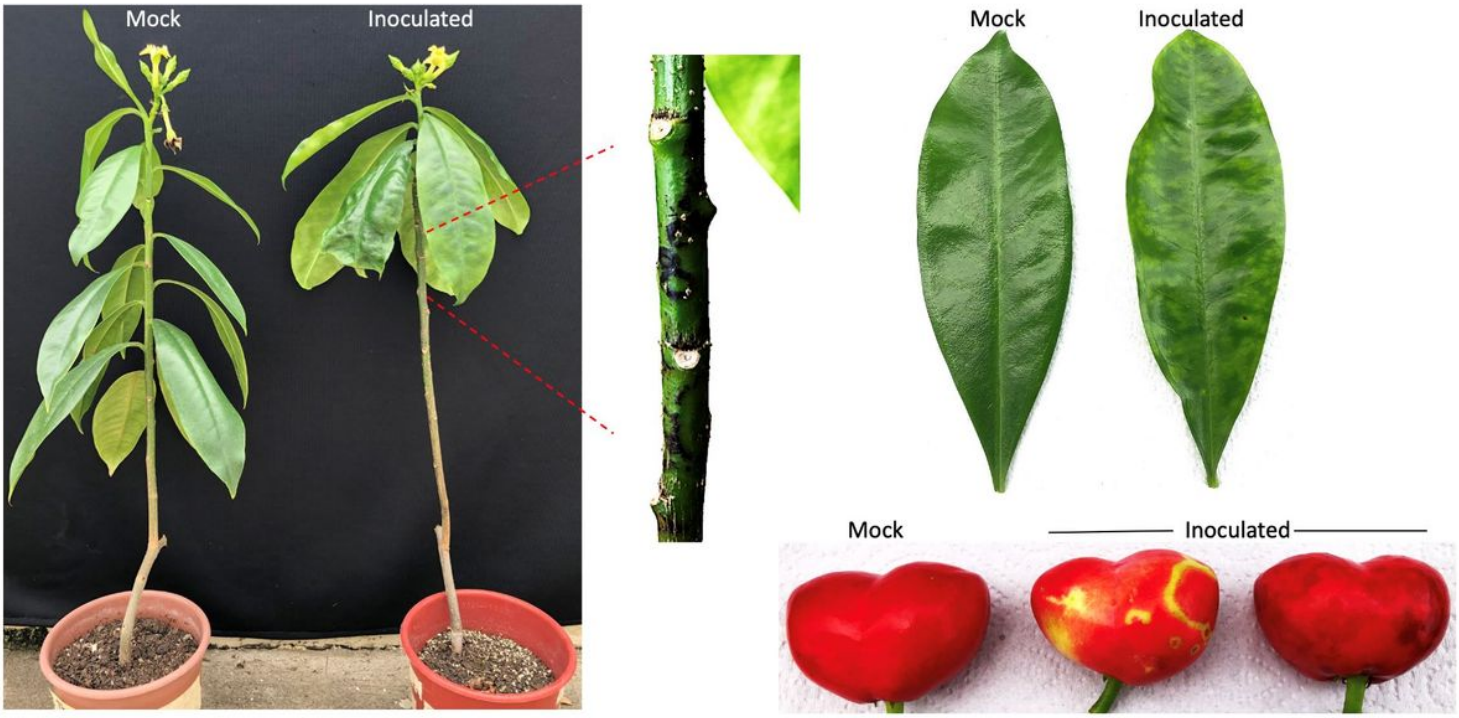

Figure 1

Symptoms observed on *Thevetia ahouai* inoculated with thevetia white spot virus (ThWSV). From the left: the overall appearance of the mock inoculated versus the virus inoculated plant. Note (enlarged image) the darkening and black ringspotting on the stem of the virus-inoculated plant. At the right: leaf white spotting (top) and fruit discoloration (including white rings) on the fruits (bottom) from plants inoculated with ThWSV.

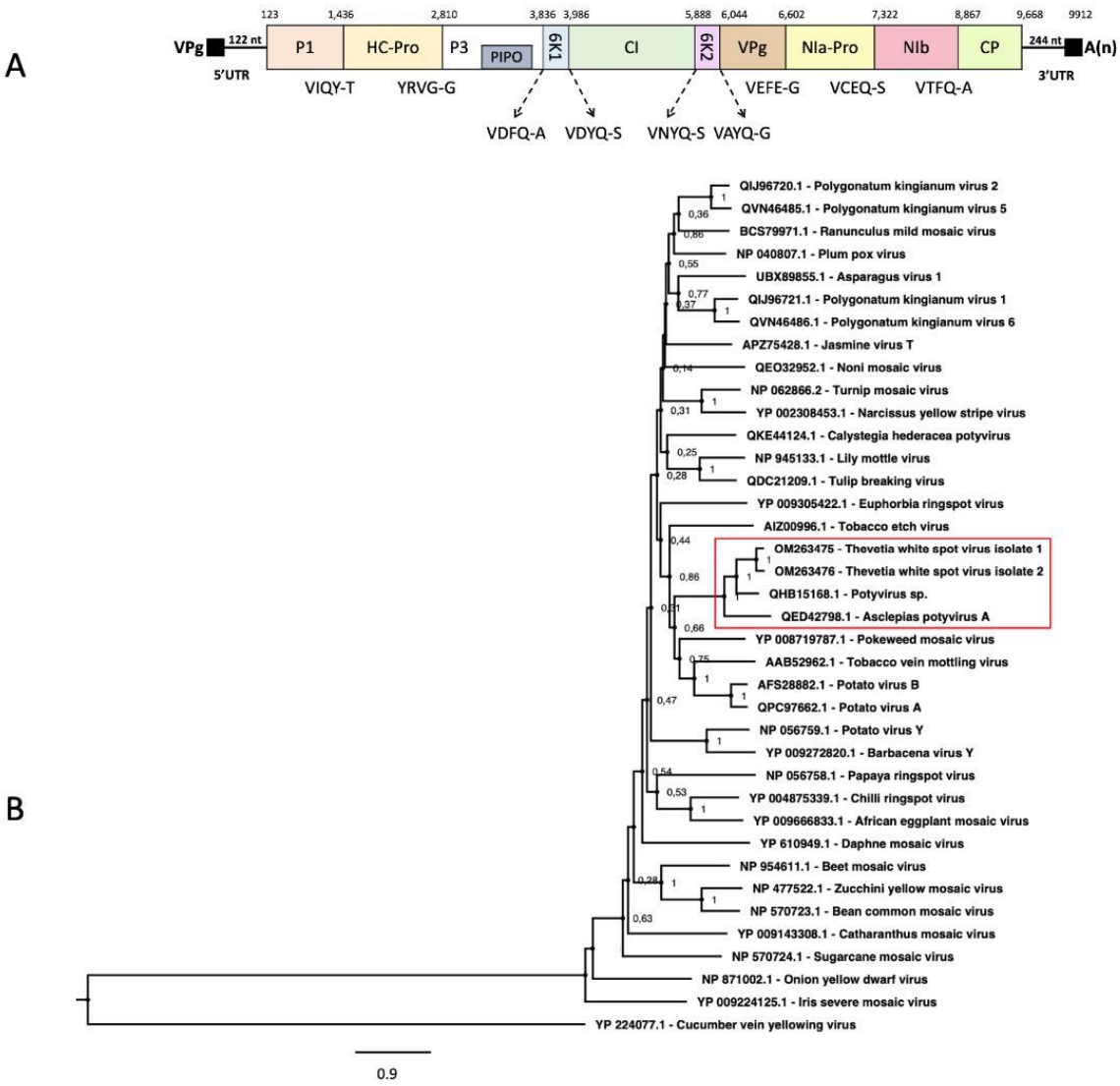

Figure 2

Genome organization and phylogeny of thevetia white spot virus (ThWSV)*. A) The 5'- and 3'- untranslated regions (UTR) are represented by a solid line, and the open reading frame (ORF) is represented by an open box with solid line. Nucleotide positions and putative cleavage sites for each protein are shown. (* genome coordinates and amino acid sites are those corresponding to ThWSV -isolate 1). B) Maximum-likelihood tree analysis (500 bootstrap replicates) based on the putative polyprotein sequence of ThWSV and 34 representative members of genus *Potyvirus*. Phylogenetic tree was constructed with MEGAX using LG+G+F+I model. The clade clustering the two ThWSV isolates and their closest relatives is indicated. Cucumber vein yellowing virus, a member of genus *Ipomovirus (Potyviridae)*, was used as an outgroup. NCBI accession numbers are indicated for each virus sequence used in this analysis.

Supplementary Files

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- Onlineresource.jpg