BRIEF REPORT

Characterization of a new potyvirus infecting *Thevetia ahouai* in Ecuador

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Abstract

A new potyvirus was found in *Thevetia ahouai* L. (Fam. Apocynaceae) plants exhibiting white spots on leaves and fruit discoloration in Ecuador. The complete genome sequences of two isolates of this virus, tentatively named “thevetia white spot virus” (ThWSV), were determined and found to be 9,912 (isolate 1) and 9,904 (isolate 2) nucleotides (nt) in length, each encoding a polyprotein of 363 kDa. Sequence comparisons between the two isolates showed 80 and 87% identity at the nt and amino acid (aa) level, respectively, whereas the overall sequence identity between ThWSV and its closest relative was 69% and 71% at the nt and aa level, respectively.

*Thevetia ahouai* L. is an evergreen shrub with shiny, dark green, ovate leaves and bright, lobed red fruits with milky sap. The shrub belongs to the family Apocynaceae, and is native to South and Central America [1]. *T. ahouai* has been used in traditional medicine as treatment for hemorrhoids, toothache, and rheumatism and has also been shown to have anti-promastigote activity against *Leishmania* [2]. In recent years, there has been an increase in the use of *T. ahouai* as an ornamental in gardens and urban parks or along sidewalks.

In June of 2020, virus-like symptoms including white spots on leaves and fruit discoloration were observed in *T. ahouai* plants at two different locations in Guayaquil, a coastal city of Ecuador. Symptomatic leaves from two selected shrubs, one (sample 1) located in an urban park (GPS coordinates -2.24486, -79.89706) and the second (sample 2) located in Prosperina (GPS coordinates -2.15072, -79.95759), a tropical dry forest on the western side of the city, were collected for virus identification.

As there are no reports on viruses infecting *Thevetia* spp., attempts were made to purify virions from symptomatic leaves as described previously [3]. Partially purified extracts were mounted on carbon-coated formvar (1%) films and negatively stained with 2% phosphotungstic acid at pH 7.0. The films, mounted on grids, were examined using a JEOL JEM-1400plus transmission electron microscope at the University of Minnesota Imaging Center. Flexuous filamentous virion-like particles of approximately 700 nm in length were found in both samples (Online Resource 1).

High-throughput sequencing (HTS) was used to identify viruses in the plants, using double-stranded RNA (dsRNA) from sample 1 and total RNA from sample 2 as the initial templates. The dsRNA was extracted from 15 g of fresh symptomatic leaf tissue following the protocol described by Morris and Dodds [4]. Total RNA was extracted from 100 mg of symptomatic leaf tissue using an RNasey Plant Mini Kit (QIAGEN, Germany) and subjected to plant ribosomal RNA (rRNA) depletion using an Illumina Ribo-Zero Kit before generating the complementary DNA (cDNA) library using a TruSeq Library Prep Kit. Sequencing was done at Macrogen, South Korea, on a NovaSeq6000 Illumina platform with 150-bp paired-end reads.

A total of 22.3 and 21.2 million sequence reads were obtained from 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 assembled de novo using SPAdes. Several thousand contigs were assembled from each sequence set. A 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 similarity to several members of the genus *Potyvirus*. 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 failed to identify additional virus homologues; instead, they showed homology to several nuclear and chloroplast genes from *Thevetia peruviana*, *T. ahouai*, and *Asclepias* spp. among other plant species in the family Apocynaceae.

The difference in viral contig coverage by the two template types, dsRNA and total RNA, probably resulted from the fact that potyviruses tend to produce a small amount of dsRNA compared to other plant viruses [5]. 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 the 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 a 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 to the reverse transcription reaction. Five RT-PCR amplicons for each terminus were cloned as described above and sequenced in both directions.

The complete genome sequence, excluding the poly(A) tail, consisted of 9,912 nt (accession no. OM263475) and 9,904 nt (accession no. OM263476) for potyvirus sequence 1 and 2, respectively. The identity percentages between the two sequences (80% at the nt level and 86.7% at the amino acid level) suggest that both sequences are isolates of the same potyvirus (hereafter "isolate 1" and "isolate 2") [6].

BLASTn searches revealed that both isolates share up to ~73% nt sequence identity (86% coverage) with their closest relative, a metagenomic potyvirus sequence (UPHV-3) from unidentified weeds in a papaya orchard of Chiapas, Mexico [7] (accession no. MN203192). When the complete genome sequence of this potyvirus was compared to both isolates of the thevetia potyvirus, the identity was 69%. According to the species demarcation criteria for potyviruses [6], at this genomic identity level, it can be inferred that the thevetia virus described here represents a new species in the genus *Potyvirus*.

Symptomatic leaves infected with each virus isolate were used for mechanical inoculation of *T. ahouai* virus-free seedlings (n = 15 for each isolate) as described [8]. White spots were observed on non-inoculated young leaves (n = 7 for isolate 1 and n = 8 for isolate 2) at an average of 15 days post-inoculation, with no differences between the symptoms induced by the two isolates. The presence of the virus in the inoculated plants was confirmed by RT-PCR and Sanger sequencing using the primers Det_F (5’-TCAGGAACGGTCTCGGTTC-3’) and Det_R (5’-CCATCATCACCCAAAA
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CTCCAT-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. The original symptoms, including white spots on leaves and fruit discoloration, were reproduced in the inoculated plants, and no other virus-like sequences were 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

**Fig. 2** Genome organization and phylogeny of thevetia white spot virus (ThWSV)*. (A) The 5’ and 3’ untranslated regions (UTRs) are represented by a solid line, and the open reading frame (ORF) is represented by an open box with a 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 phylogenetic tree (500 bootstrap replicates) based on the putative polyprotein sequences of ThWSV and 34 representative members of the genus *Potyvirus*. The tree was constructed in MEGA X using the LG+G+F+I model. The clade containing the two ThWSV isolates and their closest relatives in the tobacco etch lineage is indicated. Cucumber vein yellowing virus, a member of the genus *Ipomovirus*, family *Potyviridae*, was used as an outgroup. NCBI accession numbers are indicated for each virus sequence used in the analysis.
Table 1  Conserved motifs in the polyprotein of thevetia white spot virus isolates (ThWSV 1 and ThWSV 2) with respect to those of representative potyvirids. The amino acid positions for each motif are shown. An ‘x’ represents any amino acid in that position. Shaded residues indicate a difference with respect to the reference conserved motif. Footnote: * Potyvirus consensus motifs obtained from Worrall et al. 2019

| Hypothetical protein | Conserved potyvirus motifs | Putative function* | Motifs in the polyprotein of each isolate of ThWSV, showing amino acid positions |
|----------------------|-----------------------------|-------------------|--------------------------------------------------------------------------------|
|                      |                             |                   | ThWSV 1                                                                           | ThWSV 2                                                                           |
| P1                   | IxFG                        | Protease activity | 5IMFG8                                                                           | 5IMFG8                                                                           |
|                      | Hx5Dx12SGx22RG              |                   | 355Hx5Dx12SGx22RG114                                                             | 355Hx5Dx12SGx22RG114                                                             |
|                      | GxSG                        |                   | 397GMSG400                                                                       | 397GMSG400                                                                       |
|                      | FIVRG                       |                   | 410ILVRG414                                                                      | 410ILVRG414                                                                      |
|                      | Cx6C21C22C23                 | Zinc finger       | 456Cx6C21C22C23496                                                               | 456Cx6C21C22C23496                                                               |
|                      | IGN                         | Cell-to-cell and long-distance movement | 688IGN460                                                                      | 688IGN460                                                                      |
| KITC                 |                             | Aphid transmission | 490KITC493                                                                      | 490KIAC493                                                                      |
| PTK                  |                             |                   | 748PTK750                                                                        | 748PTK750                                                                        |
| FRNKx12CDNQLD        |                             | Cell-to-cell movement | 619FRNKx12CDNQLD640                                                             | 619FRNKx12CDNQLD640                                                             |
| HAKRFF               |                             |                   | 653HAKRFF568                                                                    | 653HAKRFF568                                                                    |
| CCCVT                |                             | Long-distance movement | 730CCCVT734                                                                     | 730CCCVT734                                                                     |
| GYCY                 |                             |                   | 780GFCY783                                                                       | 780GFCY783                                                                       |
| NIFLAML              |                             | Protease activity | 785NIFLAML791                                                                   | 785NIFLAML791                                                                   |
| AELPRLVDH            |                             |                   | 840AELPRLVDH849                                                                  | 840AELPRLVDH849                                                                  |
| Cx22H                |                             |                   | 842C22H55                                                                        | 842C22H55                                                                        |
| P3                   | GAVGSGKST                   | NTP binding       | 928QPYx7SPx2LxAx2NxNx2Ex5W958                                                     | 928QPYx7SPx2LxAx2NxNx2Ex5W958                                                     |
|                      | EPYx7SPx7LxAx2NxNEx2Ex5W     | Protease activity | 1372VLLLETRP11401                                                                 | 1372VLLLETRP11401                                                                 |
|                      |                             |                   | 1460VLLLETRP11401                                                                 | 1460VLLLETRP11401                                                                 |
|                      |                             |                   | 1488VLLLETRP11401                                                                 | 1488VLLLETRP11401                                                                 |
|                      |                             |                   | 1539LIY1542                                                                      | 1539LIY1542                                                                      |
|                      |                             |                   | 1590VATIVENGT1601                                                                  | 1590VATIVENGT1601                                                                  |
|                      |                             |                   | 1631GERIQLRGVR1645                                                                  | 1631GERIQLRGVR1645                                                                  |
|                      |                             |                   | 2206Hx5Dx12GxCGx4H2327                                                           | 2206Hx5Dx12GxCGx4H2327                                                           |
|                      |                             |                   | 2572SLKAEL2577                                                                   | 2572SLKAEL2577                                                                   |
|                      |                             |                   | 2647CDADGS2652                                                                   | 2647CDADGS2652                                                                   |
|                      |                             |                   | 2709GNNSQPSTVVDN-TIMV27425                                                       | 2709GNNSQPSTVVDN-TIMV27425                                                       |
|                      |                             |                   | 2752GDD2755                                                                       | 2752GDD2755                                                                       |
|                      |                             |                   | 2714QSTVVDN2721                                                                   | 2714QSTVVDN2721                                                                   |
|                      |                             |                   | 2605CVDFD2610                                                                      | 2605CVDFD2610                                                                      |
|                      |                             |                   | 2594FTAAPID2597                                                                    | 2594FTAAPID2597                                                                    |
|                      |                             |                   | 2839AMIESWG2845                                                                   | 2839AMIESWG2845                                                                   |
|                      |                             |                   | 2922DAG2929                                                                       | 2922DAG2929                                                                       |
|                      |                             |                   | 3114AFDF3117                                                                      | 3114AFDF3117                                                                      |
|                      |                             |                   | 3132QMKAAB3139                                                                      | 3132QMKAAB3139                                                                      |
|                      |                             |                   | 3033MVWCIENTG53041                                                                 | 3033MVWCIENTG53041                                                                 |
|                      |                             |                   | 3047WMMDGE3053                                                                      | 3047WMMDGE3053                                                                      |
|                      |                             |                   | 3099PYMPRYG3099                                                                    | 3099PYMPRYG3099                                                                    |
|                      |                             |                   | 3159EDTERH3164                                                                     | 3159EDTERH3164                                                                     |
| Nia-Pro, Nib         | Hx5Dx12GxCGx4H              | Proteolytic activity | 3030MVWCIENTG53041                                                                 | 3030MVWCIENTG53041                                                                 |
|                      | SLKAEEL                    | RNA-dependent RNA polymerase activity | 3132QMKAAB3139                                                                      | 3132QMKAAB3139                                                                      |
|                      | CHADGS                     |                   | 3033MVWCIENTG53041                                                                 | 3033MVWCIENTG53041                                                                 |
|                      | GNNSQPSTVVDN-TIMV27425     |                   | 3047WMMDGE3053                                                                      | 3047WMMDGE3053                                                                      |
|                      |                             |                   | 2752GDD2755                                                                       | 2752GDD2755                                                                       |
|                      |                             |                   | 2714QSTVVDN2721                                                                   | 2714QSTVVDN2721                                                                   |
|                      |                             |                   | 2605CVDFD2610                                                                      | 2605CVDFD2610                                                                      |
|                      |                             |                   | 2594FTAAPID2597                                                                    | 2594FTAAPID2597                                                                    |
|                      |                             |                   | 2839AMIESWG2845                                                                   | 2839AMIESWG2845                                                                   |
|                      |                             |                   | 2922DAG2929                                                                       | 2922DAG2929                                                                       |
|                      |                             |                   | 3114AFDF3117                                                                      | 3114AFDF3117                                                                      |
|                      |                             |                   | 3132QMKAAB3139                                                                      | 3132QMKAAB3139                                                                      |
|                      |                             |                   | 3033MVWCIENTG53041                                                                 | 3033MVWCIENTG53041                                                                 |
|                      |                             |                   | 3047WMMDGE3053                                                                      | 3047WMMDGE3053                                                                      |
|                      |                             |                   | 3099PYMPRYG3099                                                                    | 3099PYMPRYG3099                                                                    |
|                      |                             |                   | 3159EDTERH3164                                                                     | 3159EDTERH3164                                                                     |
"thevetia white spot virus" (ThWSV) is proposed and will be used hereafter in this report.

Seed transmission of potyviruses is not uncommon, but the rate at which it occurs can vary significantly depending on the virus. For instance, papaya ringspot potyvirus has a seed transmission rate of 0.15% in papaya, whereas in *Robinia pseudocacia*, an unusual host, seed transmission rates of up to 50% have been observed [9]. Likewise, seed transmission of soybean mosaic potyvirus has been shown to vary from 0 to 64% depending on virus and host genotypes [10]. In order to investigate seed transmission of ThWSV, five symptomatic fruits from a single virus-inoculated plant (one with each virus isolate) were collected, and all 20 seeds (each fruit had 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 during the study, suggesting that, under our experimental conditions, the rate of seed transmission of ThWSV is no higher than 5%.

The genome organization of ThWSV is identical to that of other 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), with a single lysine deletion (deletion of an AAA codon) at the N-terminus of the coat protein (CP) compared to isolate 1. Analysis of the hypothetical polyprotein of ThWSV showed that it contains the nine conserved proteolytic cleavage sites described previously for other potyviruses [11], resulting in 10 mature putative proteins (Fig. 2A). In addition, typical potyvirus-conserved motifs [12] 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_1–2A_6–7 at the beginning of the PIPO ORF (isolate 1, 3324GAAAAAT_3330; isolate 2, 3315GAAAAAT_3325). In both isolates, the PIPO ORF is out of frame from P3, suggesting that it is expressed through a -1 ribosomal frameshifting mechanism from the P3 coding region, which would result in a fused protein (P3N-PIPO) as described previously [13].

The phylogeny was inferred from the complete polyprotein amino acid sequences of 36 representative potyviruses and a member of the genus *Ipomovirus* (family *Potyviridae*), which was used as an outgroup. The sequences were aligned using MUSCLE [14], and the best-fitting protein substitution model (LG+G+F+I) was chosen. A phylogenetic tree was generated using the maximum-likelihood method with 500 bootstrap replicates in MEGA X [15]. The topology of the tree was consistent with previous BLAST results, showing the most recent common ancestors of ThWSV to be a potyvirus sequenced from an unidentified weed in Mexico [7] and asclepias virus A, from a perennial herb in the same family (*Apocynaceae*) as *T. ahouai*. Other closely related potyviruses include pokeweed mosaic virus, tobacco vein mottling virus, potato virus A, potato virus B, and other members of the tobacco etch virus lineage [16], most of which were first found in the Americas (Fig. 2B). Amino acid identity values among the most closely related viruses 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 an ornamental. 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, which are found in most potyviruses (Table 1), it is reasonable to speculate that aphids transmit ThWSV in nature. However, epidemiological studies should focus on identifying the aphid species associated with *T. ahouai* and closely related species, such as *Catharanthus roseus* (L.), which is widely used as an ornamental in tropical regions, as transmission of ThWSV is likely to be non-persistent.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s00705-022-05450-6.

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**Author contributions** Study conception and design: DFQ-A. Material preparation, data collection, and analysis were performed by MGC-B, EGR-P, JFC-F, RAA-Q, and DM. The first draft of the manuscript was written by MGC-B and DFQ-A. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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

**Declarations**

**Conflict of interest** The authors declare no conflict of interest.

**Ethical 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-1465
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