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Identification and molecular characterization of a novel *alphanucleorhabdovirus* associated with cacti in Mexico

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Abstract

High-throughput sequencing revealed a putative novel alphanucleorhabdovirus associated with *Opuntia pilifera*, *O. depressa*, and *Cephalocereus tetetzo* collected in Mexico. Three viral genomes (12,946–13,122 nt) exhibited the typical nucleorhabdovirus gene organization (N–P–P3–M–G–L) and shared 86–92% nucleotide sequence identity. Phylogenetic analysis of the RNA-dependent RNA polymerase placed the isolates in a distinct clade closely related to *Agave tequilana virus 1*. Viral RNA was detected by RT-PCR in field-collected cacti, and rhabdovirus-like particles were observed by transmission electron microscopy. These findings support the identification of a putative novel alphanucleorhabdovirus, tentatively named *Opuntia nucleorhabdovirus A*.

Prickly pear (*Opuntia* spp.) is an important crop in Mexican culture and gastronomy [1]. Viral pathogens infecting *Opuntia* spp. include potexviruses and tobamoviruses, some of which have been associated with chlorotic ringspot symptoms [2]. Recently, *Opuntia virus 2* (OV2), a tobamovirus, was reported in wild *O. pilifera* and *O. depressa* plants in Mexico [3]. Members of the genus *Alphanucleorhabdovirus* (family *Rhabdoviridae*) have negative-sense, single-stranded, unsegmented RNA genomes that typically encode six proteins: nucleoprotein (N), phosphoprotein (P), movement protein (P3), matrix protein (M), glycoprotein (G), and RNA-dependent RNA polymerase (L). In recent years, numerous novel plant

rhabdoviruses have been identified using high-throughput sequencing (HTS) [4]. However, the diversity of rhabdoviruses infecting members of the family Cactaceae remains largely unexplored, and no alphanucleorhabdovirus has been reported in cactus species to date. In this study, we report the identification and molecular characterization of a putative novel *alphanucleorhabdovirus* associated with three cactus species in Mexico.

In 2016, *Opuntia pilifera*, *O. depressa* and *Cephalocereus tetetzo* plants showing virus-like symptoms were collected in Zapotitlan, Puebla, within the Tehuacán-Cuicatlán Biosphere Reserve, Mexico. Total RNA extracted from *O. pilifera*, *O. depressa* and *C. tetetzo* was used to prepare stranded mRNA libraries with the TruSeq kit (Illumina, USA). Sequencing was performed on an Illumina NextSeq 500 platform, generating approximately 10 million 75-nt paired-end reads per library. Raw reads were quality-trimmed using Trimmomatic v0.41, and duplicate reads were removed using CD-HIT-DUP v4.8.1. Host-derived reads were filtered by mapping the processed reads against a previously curated *Opuntia ficus-indica* reference genome using BBMap v40.02. Unmapped reads were subsequently assembled de novo using Trinity v2.15.2, and the resulting contigs were analyzed using BLASTn and BLASTx searches against the NCBI databases to identify sequences of viral origin. BLASTx searches of the assembled contigs against the NCBI non-redundant protein database identified three viral contigs: a 13,101-nt contig from *O. pilifera* (GenBank accession QB134307), a 13,122-nt contig from *C. tetetzo* (QB134308), and a 12,946-nt contig from *O. depressa* (QB134309). Multiple sequence alignment of the three viral contigs was performed using Geneious v9.0 (Biomatters), revealing pairwise nucleotide sequence identities ranging from 86% to 92%. The viral sequences identified in this study showed approximately 43% whole-genome nucleotide sequence identity to their closest known relative, *Agave tequilana virus 1* (NC_076970.1), as determined by whole-genome sequence alignment using Geneious v9.0. An average of 41,245 reads per sample mapped to the viral contigs, corresponding to an estimated mean sequencing depth of approximately 237× (197× for *O. pilifera*, 302× for *C. tetetzo*, and 212× for *O. depressa*). The

viral sequences are presented in the 5′–3′ antigenomic orientation. The *O. pilifera* viral sequence (QB134307) contained six predicted ORFs (N, nt 210–1574; P, nt 1610–2560; P3, nt 2706–3557; M, nt 3700–4434; G, nt 4638–6452; and L, nt 6650–12481) (Fig.1). The other two viral sequences showed the same genome organization. Multiple sequence alignment of the three viral sequences revealed a highly conserved 5′-ATTAANAAAAACCCA-3′ motif in the antigenomic orientation at the end of each predicted ORF, corresponding to the putative transcription termination/polyadenylation signal. Total RNA was analyzed by one-step RT-PCR using the SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (Invitrogen) and the specific primers NucleoRhab_506-F (5′-GACAAAACCTTTTTTGAAC-3′) and NucleoRhab_1103-R (5′-TTGATAATTGTTGCGATTGT-3′), which amplify a 598-bp fragment of the N gene. Reverse transcription was performed at 50 °C for 30 min, followed by initial denaturation at 95 °C for 2 min and 30 cycles of 95 °C for 15 s, 55 °C for 15 s, and 68 °C for 30 s, with a final extension at 68 °C for 5 min. The assay was performed on ten symptomatic *Opuntia* plants (five *O. pilifera* and five *O. depressa*), four *C. tetetzo* plants, and two *Myrtillocactus geometrizans* plants, all collected from Zapotitlan, Puebla. Seedlings of *O. albicarpa* grown under greenhouse conditions were included as negative controls. The expected 598 bp amplicon was obtained from all field-collected plants tested, whereas no amplification was observed in the *O. albicarpa* negative controls. Amplicons obtained from *O. pilifera* and *O. depressa* were Sanger sequenced, and the resulting sequences were deposited in GenBank under accession numbers QB136256 and QB136257. BLASTn analysis showed 95–96% nucleotide sequence identity with the *O. pilifera* viral contig obtained by HTS. Phylogenetic analysis was performed using the complete amino acid sequences of the RNA-dependent RNA polymerase (L) protein from the three cactus-associated viral isolates and representative members of the genus *Alphanucleorhabdovirus*. A maximum likelihood phylogenetic tree was constructed using RAxML under the Jones–Taylor–Thornton (JTT) amino acid substitution model with 1,000 bootstrap replicates. *Maize fine streak virus* (genus *Gammanucleorhabdovirus*) was included as an outgroup. The

three cactus-associated isolates formed a well-supported monophyletic clade (100% bootstrap support), closely related to *Agave tequilana virus 1*. Within this clade, the isolates from *O. pilifera* and *C. tetetzo* clustered together (100% bootstrap support), whereas the *O. depressa* isolate formed a distinct sister lineage (Fig. 2). Transmission electron microscopy of partially purified extracts from *O. pilifera*, negatively stained with uranyl acetate, revealed bacilliform to bullet-shaped particles with morphology consistent with that of plant rhabdoviruses (Fig. 3). Virions of members of the genus *Alphanucleorhabdovirus* are typically bacilliform and approximately 130–300 nm long and 45–100 nm in diameter, supporting the morphological compatibility of the observed particles with members of this genus. According to the species demarcation criteria established by the ICTV for the genus *Alphanucleorhabdovirus*, a whole-genome nucleotide sequence identity below 75% is one of the characteristics distinguishing viruses assigned to different species [5]. The viral sequences identified in this study showed approximately 43% whole-genome nucleotide sequence identity to their closest known relative, *Agave tequilana virus 1*. Together with their distinct phylogenetic placement within the genus *Alphanucleorhabdovirus*, these results support the classification of the virus as a putative novel species. We propose the tentative name *Opuntia nucleorhabdovirus A* (OpNV-A). To our knowledge, this is the first report of an alphanucleorhabdovirus associated with members of the family Cactaceae [6]. Further studies are needed to determine its geographic distribution, host range, and incidence in cultivated prickly pear in Mexico. The primers developed in this study may provide a useful tool for its detection in future epidemiological surveys.

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Statements and Declarations

Author Contributions

H. Salgado-Ortiz: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Visualization, Writing – original draft, Project administration. **R. De La Torre-Almaraz:** Project administration, Supervision, Resources, Writing – review & editing. **J. E. Campos-Contreras:** Resources, Supervision, Writing – review & editing. **M. L. Rojas-Morales:** Investigation, Methodology, Formal analysis (transmission electron microscopy), Writing – review & editing.

Competing Interests

The authors declare that they have no competing interests.

Data Availability

The viral genome sequences generated in this study have been deposited in GenBank under accession numbers QB134307 (*Opuntia pilifera*), QB134308 (*Cephalocereus tetetzo*), and QB134309 (*Opuntia depressa*). The Sanger-derived sequences have been deposited under accession numbers QB136256 and QB136257. The raw high-throughput sequencing data are available in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1538445.

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Fig. 1. Schematic representation of the genomic organization of Opuntia nucleorhabdovirus A (OpNV-A) identified in Opuntia pilifera (GenBank accession no. QB134307). The 13,101-nt sequence is shown in the 5′–3′ antigenomic orientation. Colored arrows represent the six predicted open reading frames encoding the nucleoprotein (N), phosphoprotein (P), movement protein (P3), matrix protein (M), glycoprotein (G), and RNA-dependent RNA polymerase (L). Nucleotide positions are indicated above the diagram.

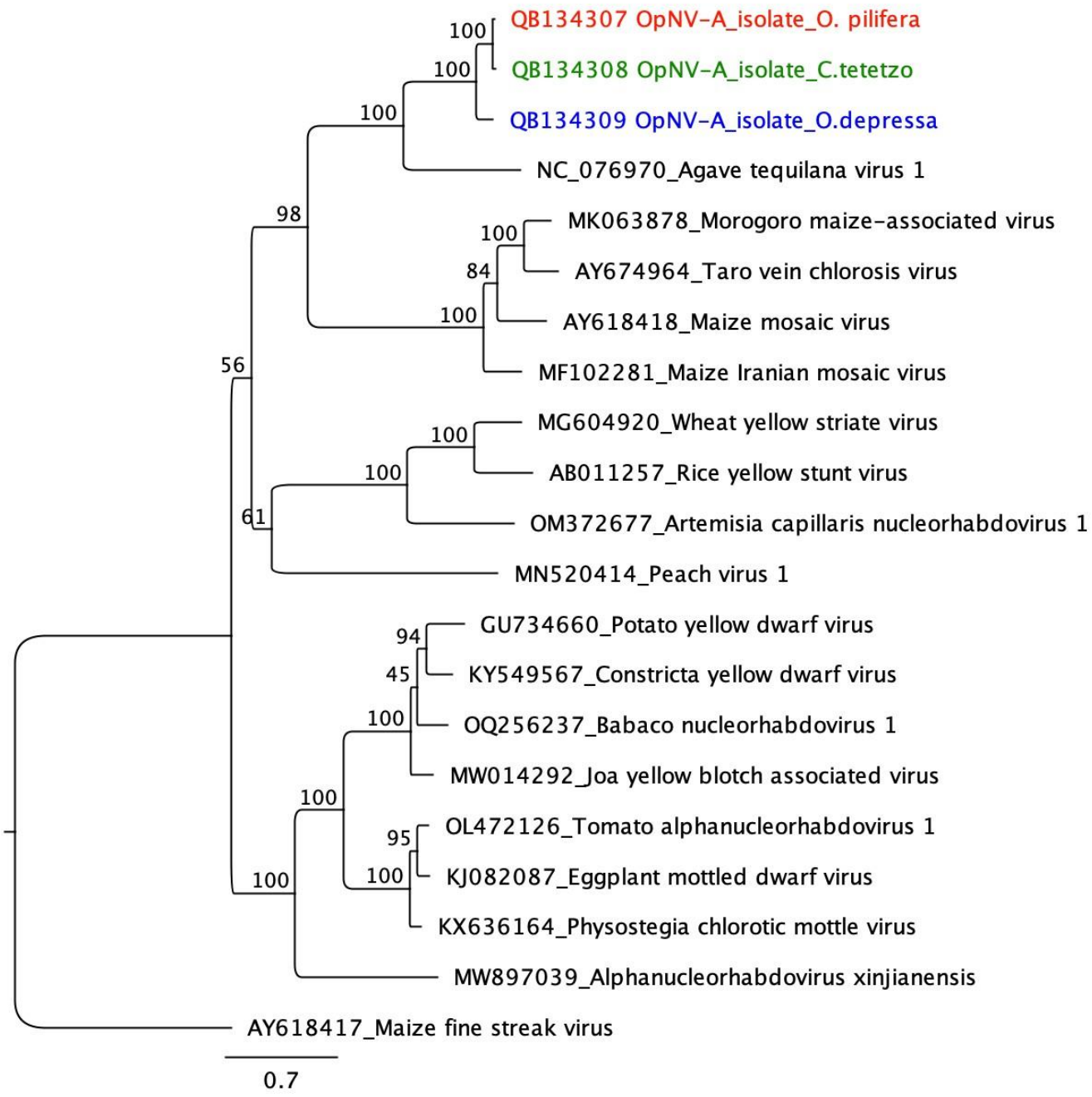


Fig. 2. Maximum likelihood phylogenetic tree based on the complete amino acid sequences of the RNA-dependent RNA polymerase (L) protein from three Opuntia nucleorhabdovirus A (OpNV-A) isolates and representative members of the genus Alphanucleorhabdovirus. Phylogenetic inference was performed using RAxML under the Jones–Taylor–Thornton (JTT) amino acid substitution model with 1,000 bootstrap replicates. Bootstrap support values (%) are indicated at the nodes. The three cactus-associated isolates are highlighted in color. Maize fine streak virus (genus Gammanucleorhabdovirus; GenBank accession no. AY618417) was used as the outgroup**.** Branch lengths are proportional to the estimated number of amino acid substitutions per site. The scale bar indicates 0.7 substitutions per site.

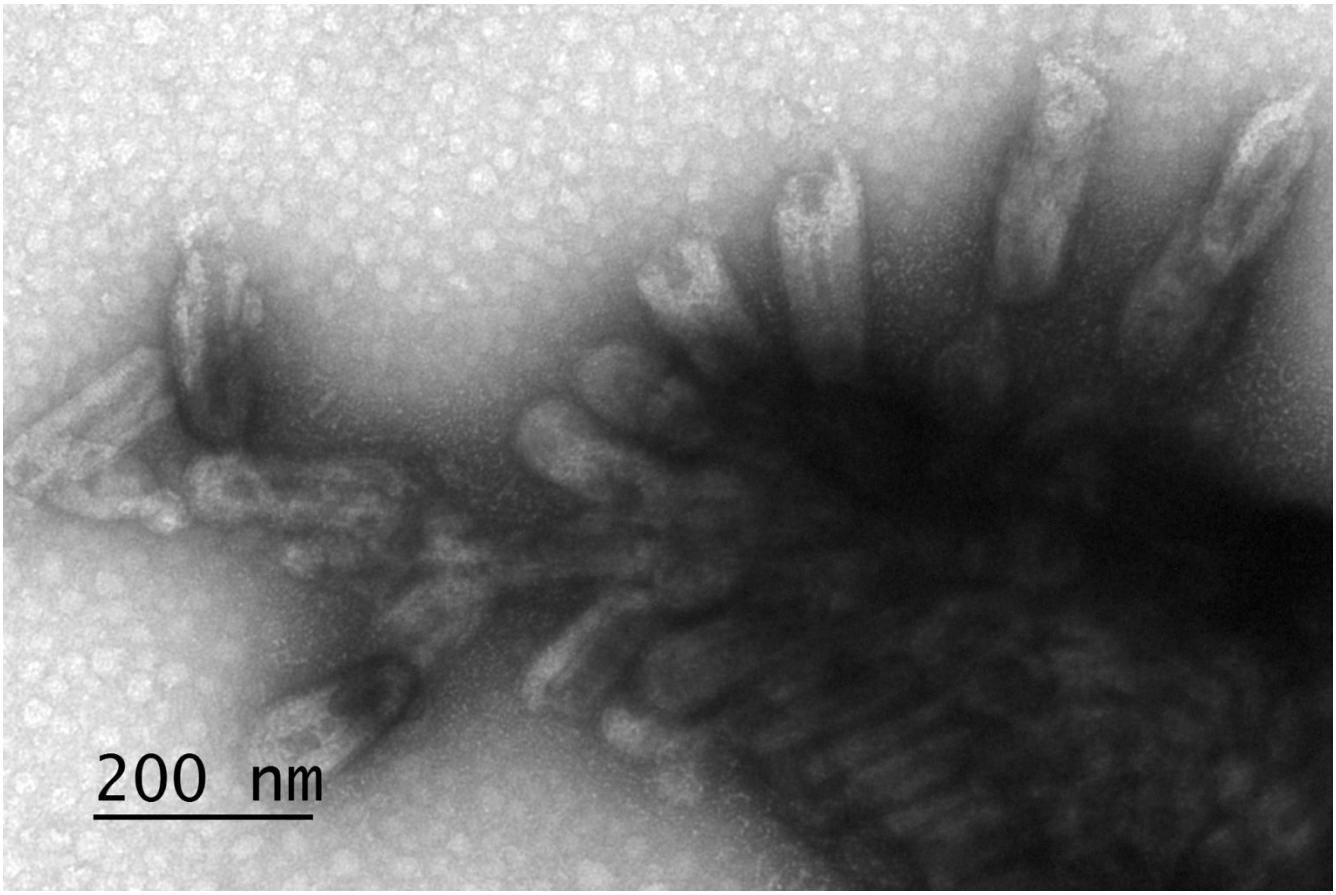


Fig. 3. Transmission electron micrograph of negatively stained, partially purified extracts from *Opuntia pilifera*, showing elongated, bacilliform particles with morphology consistent with that of plant rhabdoviruses. Samples were negatively stained with uranyl acetate. Scale bar = 200 nm.