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Virome of the vegetable prickly pear cactus in the central zone of Mexico

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ABSTRACT

Objective / Background. In this study, the ability of high-throughput sequencing (HTS) to detect viruses in vegetable prickly pear cactus was exploited.

Materials and Methods. Samples from State of Mexico (EDMX), Hidalgo, and Morelos, as well as Mexico City (CDMX), were analyzed.

Results. In the sample from EDMX, the genomes of *Opuntia virus 2* (OV2, genus *Tobamovirus*) and *Cactus carlavirus 1* (CCV-1, genus *Carlavirus*) were detected and recovered. In the sample from CDMX, in addition to OV2 and CCV-1, a new viroid and potexvirus were detected. The former has a circular RNA genome with a length of 412 nt for which the name "*Opuntia viroid I*" (OVd-I) is proposed. The primary structure of this viroid showed a nucleotide sequence identity of less than 80% with any of the currently known viroids and a phylogenetic relationship with the genus *Apscaviroid* (Family *Pospiviroidae*) with which it shares conserved structural motifs.

Conclusion. The new potexvirus was named *Opuntia potexvirus A* (OPV-A), whose viral replicase sequence has a 77.7 % amino acid identity with *Schlumbergera virus X*. Finally, CCV-1 was detected in 93 (72 %) of 129 vegetable prickly pear cactus samples collected in the four entities.

Keywords: Cactaceae, HTS, Viroid, *Potexvirus*, *Tobamovirus*, *Carlavirus*.

Opuntia ficus-indica stands as the most economically significant cactus globally, with cultivation spanning across the continents of America, Africa, Asia, Europe,

and Oceania (Barbera *et al*., 1992). Originating in Mexico, this species underwent domestication (Griffith, 2004), a process that has yielded the greatest diversity of cultivars within the region (Bravo, 1978). The fruits and cladodes of *Opuntia ficus-indica* are utilized, contingent upon the specific cultivar. In Mexico, an estimated 12,618 hectares are allocated to the cultivation of "vegetable prickly pear cactus" for human consumption (SIAP, 2022). Notably, the primary producing regions for vegetable prickly pear cactus are Morelos and Mexico City, jointly constituting 50% of the total cultivated area. Diverse products and by-products are derived from the cladodes, serving as raw materials for the production of various anti-diabetic preparations. Additionally, the flowers are employed in the preparation of diuretic beverages, while the fruits find application in the creation of juices, jellies, honey, preserves, and pastes, among other products. The seeds of *Opuntia ficus-indica* are a source of oil. Furthermore, this cactus is cultivated as a host for the "cochineal scale" (*Dactylopius coccus*), an insect utilized in the production of carminic acid dye (Barbera *et al*., 1992).

Globally, various viruses have been identified in cacti, the majority of which possess a single-stranded RNA genome (Sastry *et al*., 2019). Furthermore, two caulimoviruses that infect *Epiphyllum* spp. have been documented (Lan *et al*., 2019; Zheng *et al*., 2020), along with three geminiviruses found in several cactus species (Fontenele *et al.,* 2020; 2021).

In Mexico, specifically in the municipalities of Axapusco, Otumba, and San Martín de las Pirámides (State of Mexico), *Rattail cactus necrosis-associated virus* (RCNaV), a tobamovirus, was reported in prickly pear cactus plants exhibiting small spots and yellow rings on the cladodes (De La Torre-Almaraz *et al.,* 2016a). In the same region, the presence of a potexvirus, *Schlumbergera virus X* (SchVX), was also identified in prickly pear cactus plants showing irregular spots and deformation of cladodes (De La Torre-Almaraz *et al.,* 2016a). Additionally, in cladodes of prickly pear cactus displaying symptoms of chlorotic halos and irregular spots, both a tobamovirus and a potexvirus were detected in the localities of San Martín de las Pirámides (State of Mexico) and Cuautepec de Hinojosa (Hidalgo), respectively. However, the species could not be conclusively determined (De La Torre-Almaraz *et al.,*2007; Alonso-Barrera *et al*., 2015). Lastly, a tobamovirus named *Opuntia virus 2* (OV2) was identified through high-throughput sequencing (HTS) in species of the *Opuntia* genus in Mexico (Salgado-Ortíz *et al*., 2020).

Other significant plant pathogens include viroids, small circular single-stranded RNA molecules of approximately 400 nucleotides (nt) in length that can replicate autonomously. In contrast to viruses, there is no evidence that viroids encode any proteins, and their genomes are not protected by a capsid (Di Serio *et al*., 2014). Taxonomically, viroids are classified into two families: a) *Pospiviroidae*, comprising five genera (*Apscaviroid*, *Cocadviroid*, *Coleviroid*, *Hostuviroid*, and *Pospiviroid*),

and b) *Avsunviroidae*, consisting of three genera (*Avsunviroid*, *Pelamoviroid*, and *Elaviroid*) (Di Serio *et al*., 2018; 2021). Some viroids can severely impact the production of various crops, while others replicate without inducing symptoms in susceptible plants (Di Serio *et al*., 2014).

Advances in sequencing technologies have made High-Throughput Sequencing (HTS) more accessible to a broader range of users (Vučurović *et al*., 2021). The application of HTS has revolutionized plant virology, expediting the discovery of new virus species in ecosystems and enhancing our understanding of the diversity of these phytopathogens (Fontenele *et al*., 2020; Vučurović *et al*., 2021). Consequently, the objective of this study was to explore the virome of vegetable prickly pear cactus and determine the distribution of *Cactus carlavirus 1* (CCV-1) in four vegetable prickly pear cactus-producing states in Mexico.

Materials and Methods

High Throughput Sequencing (HTS). Two samples displaying putative virus symptoms, referred to as CDMX-1 and EM-1, underwent HTS. CDMX-1 comprised cladodes (n=4) exhibiting irregular yellow patterns, yellow rings, and mosaic, from the Milpa Alta borough in Mexico City (Figure 1A-D). The EM-1 sample (n=1) was a plant from Oxtotipac, in the Otumba Municipality, showing chlorotic spots around the thorns, locally termed "pinto" by producers (Figure 1E). In both cases, RNA was extracted using the SV Total RNA Isolation System kit (Promega, USA), following the manufacturer's instructions. The EM-1 sample was sent to Macrogen Inc. (Korea), where the library was prepared using TruSeq stranded total RNA, pre-treated with Ribo-Zero Plant. Sequencing was performed on an Illumina NovaSeq6000 instrument with 150-bp PE. The CDMX-1 sample was sent to BGI Genomics (China), where the Eukaryotic Strand-specific mRNA library was constructed, and sequencing took place on the DNBSEQ platform with 150-bp PE.

Bioinformatics analysis. Adapters and low-quality sequences from the data obtained from Macrogen were removed using Fastp (Chen *et al*., 2018). On the other hand, sequences from BGI were received clean and without adapters, and they were verified using FastQC (Andrews, 2010). Clean reads were *de novo* assembled using the SPAdes software (Prjibelski *et al*., 2020). The obtained contigs underwent BLASTx and BLASTn searches against a local database of viruses/viroids. Subsequently, the narrowed list of viral hits was compared to the complete non-redundant (nr) GenBank database using BLASTx and BLASTn (Vučurović *et al*., 2021).

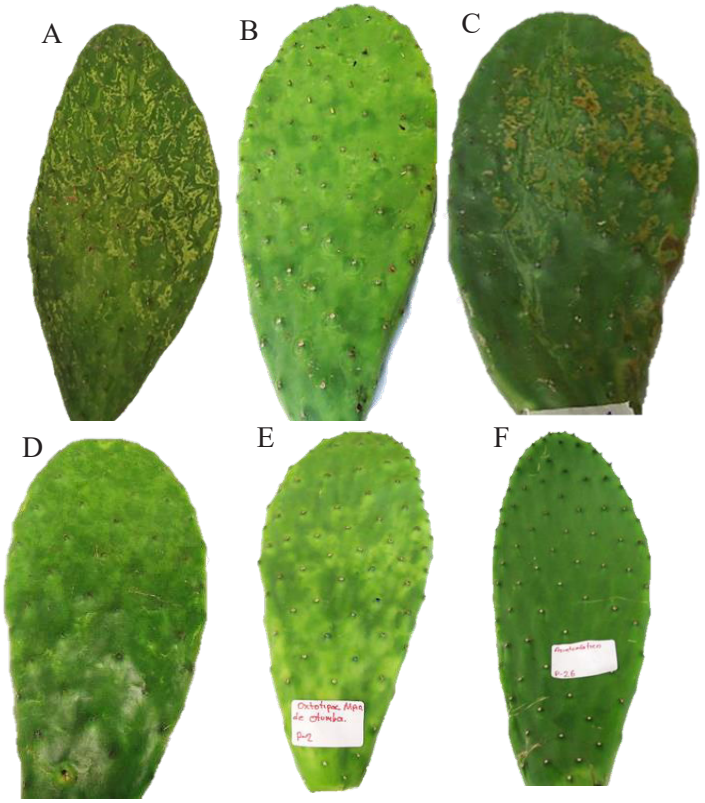

Figure 1. Symptoms associated with virosis in vegetable prickly pear cactus cladodes. (A-D) Samples of prickly pear cactus collected in Mexico City, Milpa Alta Borough, (E) Prickly pear cactus sample collected in the State of Mexico, Otumba Municipality, (F) Healthy prickly pear cactus sample from the State of Mexico.

For genome reconstruction, Bowtie2 (Langmead and Salzberg, 2012), Samtools (Li *et al.*, 2009), and Integrative Genomic Viewer (IGV) version 2.3 (Thorvaldsdóttir *et al*., 2013) were employed. The assembly characteristics of the obtained genomes were determined using Qualimap (Okonechnikov *et al*., 2016), where the average coverage per nucleotide for each genome was calculated. Finally, the obtained genomes were annotated using ORFinder from the National Center for Biotechnology Information (NCBI) and deposited in GenBank.

Phylogenetic analysis. The obtained genomes were aligned with representative species of the *Tobamovirus* genus from the NCBI database. The GTR+F+R5 nucleotide substitution model was used. Additionally, a phylogenetic tree was constructed by aligning complete genome sequences of representative members of the *Carlavirus* genus (family*: Betaflexiviridae*), obtained from the NCBI, along with a virus isolate obtained in this study. The estimated evolutionary model was

GTR+F+R5. For new viruses, two phylogenetic trees were constructed by aligning amino acid sequences of viral replicase and capsid protein from different species in the corresponding family obtained from the International Committee on Taxonomy of Viruses (ICTV) (Kreuze *et al*., 2020), including the sequence obtained in this study. The evolutionary model for the viral replicase was LG+F+R7, and for the capsid protein, it was LG+F+R5. Regarding the phylogenetic analysis of the new viroid, sequences of representative species from both families of these pathogens obtained from the NCBI were used, and the evolutionary model applied was HKY+F+G4.

In all cases, trees were generated using the Maximum Likelihood method with IQTREE 2 (Minh *et al*., 2020), multiple sequence alignment with MAFFT (Katoh *et al*., 2019), and the evolutionary model determined by the Bayesian Information Criterion implemented in ModelFinder (Kalyaanamoorthy *et al*., 2017). Nodal support was assessed with ultrafast bootstrap (UFBoot) by running a total of 1000 replicates. Finally, tree visualization and editing were carried out using iTOL (Letunic and Bork, 2021).

Pairwise identity analysis. To strengthen the classification of the new species, pairwise identity was estimated using SDT v1.2 (Muhire *et al*., 2014), comparing the replicase and capsid protein of the closest viruses, as well as different genomes from the *Pospiviroidae* family in the case of the viroid.

Secondary structure prediction of the new viroid. It was obtained using the UNAFold web server (www.mfold.org, accessed on November 18, 2022) (Zuker, 2003).

Validation of the detection of the new viruses and viroid. A portion of the RNA extracted from symptomatic cladodes that was submitted for HTS underwent RT-PCR to confirm the presence of the viroid and viruses identified. Various primers were designed (Table 1) based on the obtained sequences. Additionally, RT-PCR analyses were conducted to detect one of the viruses in four independent samples collected in different locations: Mexico City (n=1, asymptomatic), State of Mexico (n=2), Hidalgo (n=2), and Morelos (n=2).

In all cases, cDNA synthesis was carried out using M-MLV Reverse Transcriptase (200 U μL^{-1}) (Promega, USA) from 1 µL of total RNA and 1 µL (10 mM) of the reverse primer, following the manufacturer's instructions. The obtained cDNA served as a template for PCR, and the reaction mixture consisted of 2 µL of reaction buffer (5X Green GoTaq®), 0.6 µL of MgCl2 (25 mM), 0.2 µL of dNTPs Mix (10 mM), 0.6 µL (10 µM) of each primer (forward and reverse), 4.9 µL of ultrapure water, 0.1 µL of GoTaq® DNA Polymerase (5 U μL^{-1}) (Promega, USA), and 1 µL of

Table 1. Primers used in RT-PCR assays for the validation of viruses/viroids detected in the HTS data from two vegetable prickly pear cactus libraries.

Primers name	Virus/Viroide (Region)	Sequence (5'->3')	Amplicon (bp)	Alignment temperature (°C)
OV2-F	*Opuntia virus* 2 (Movement protein)	CGG GTC CTT TTC CTT TTG TCT GTC	667	55
OV2-R		GAGAGCGAGTAGAAACTCCAACG		
CCV1-F	*Cactus carlavirus* 1 (Viral replicase)	GAAGCGCATGGGCTGTATTG	459	55
CCV1-R		GTCTGCGCAGCTTTAGCATC		
Opuntia_Potex-F(RdRp)	*Opuntia potexvirus* A (Viral replicase)	ACCCACTAGCCATTGAAGCC	580	55
Opuntia_Potex-R(RdRp)		AGGTGGTTGGCACCTTTTGA		
Caulimovirus_F	Caulimovirus (Retro transcriptase)	TCCTCACACTAGTCCTGCCT	846	55
Caulimovirus_R		TTGAGGACGGCACTCCAATG		
Alphavirus-F(RdRp)	Alphavirus (Coat protein)	CACTGGCGTGTTTTAGCCAC	579	55
Alphavirus-R(RdRp)		TCAGCCTCCAGCCACAAAAT		
Tymovirus-F	Timovirus (Triple gene block protein)	CTGGAGCGCCTTGGAACTAA	745	55
Tymovirus-R		TTTGCCAACCCTCACCATGA		
Vityvirus-F	Vityvirus (Movement protein)	GAACTGCTTGATATGCGCCG	229	55
Vityvirus-R		CCTGACGGACCACACTCATC		
Banmivirus_Opun-F	Banmivirus (Viral replicase)	CAGAGCGCCAATTAACCTGC	707	55
Banmivirus_Opun-R		CGCTCTTGAAGGTTAGCCCA		
OPV-I-1F	*Opuntia viroid I* (Part of the genome)	TTGACCTAGGTTGGCTCTCG	362	55
OPV-I-1R		GCAGGAGAAGGCCAGGAAG		
OPV-I-2F	*Opuntia viroid I* (Part of the genome)	GGGATCAACCTTGTGGTTCCT	351	55
OPV-I-2R		GAAAGAAAAGCACCTGTGGGC		

cDNA in a final volume of 10 µL. PCR conditions included an initial incubation at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 40 s, and a final extension cycle at 72 °C for 10 min. PCR products were sequenced using the Sanger method at Macrogen Inc.

CCV-1 Detection. A total of 129 samples collected from cladodes in the State of Mexico, Hidalgo, Morelos, and Mexico City were analyzed using RT-PCR for the detection of CCV-1 with the CCV1-F/R primers, aiming to determine their presence or absence.

Transmission electron microscopy. In order to ascertain the presence of virions from the viruses identified in the HTS results, symptomatic vegetable prickly pear cactus plants were utilized to prepare 300-mesh copper grids coated with Formvar for observation. The "leaf dip" technique was employed, and 2% phosphotungstic acid served as the contrast agent. The grids were examined using a FEI Tecnai G2 Spirit TWIN transmission electron microscope at the Electron Microscopy Unit of the College of Postgraduates.

Results

Virus Detection by HTS. A total of 32,921,232 reads were obtained for sample EM-1, and 48,292,940 reads for sample CDMX-1. The raw reads have been deposited in NCBI under the BioProject number PRJNA922236. Analysis of these reads revealed the presence of sequences from various viruses and a viroid in the vegetable prickly pear cactus (Table 2), which are described below:

Table 2. Comparison of the complete or partial genome of the viruses identified through HTS in the two vegetable prickly pear cactus libraries with the most closely related reference sequence from GenBank.

Sample	Virus/Viroid isolation detected	GenBank accession number	Part of the genome	Reference sequence accession number	Percent nucleotide similarity
EM-1	CCV-1-Edo-MeX	OQ240442	Complete	KU854930.4	87.71
	OV2-Edo-Mex	OQ240440	Complete	NC_040685.2	97.67
CDMX-1	CCV-1-CD-MX	OQ240444	Partial	KU854930.4	97.5
	OV2-CDMX	OQ240441	Complete	NC_040685.2	97.61
	Opuntia potevirus A	OQ240443	Complete	LC654699.2	74.42
	Opuntia viroid I	OQ240445	Complete	X95292.1	88.52*

*Query coverage 14%.

***Opuntia virus 2*.** The presence of the virus was detected and confirmed in the two samples subjected to HTS, resulting in two complete genomes named OV2-Edo-Mex and OV2-CDMX. The average coverage per nucleotide was 634,104.1X and 500.5X, respectively (Figure 2A, 3A). The 6,453 nt OV2-Edo-Mex genome exhibits the typical organization of tobamoviruses (Figure 2B), including a 5' untranslated region (UTR) (nt 1-57), an open reading frame (ORF) encoding the 183 kDa viral replicase (RdRp) (nt 58-4,854), a 128 kDa methyltransferase/helicase (nt 67-3,360), a movement protein (MP) (nt 4,844-5,608), a coat protein (CP) (nt 5,514-6,167), and a 3' UTR (nt 6,168-6,453). The OV2-Edo-Mex genome shares a nucleotide identity of 97.6% with the NC_040685.2 OV2 isolate (Table 2).

The genome of the OV2-CDMX isolate had a length of 6,453 nt, displaying a typical tobamovirus organization (Figure 3B) that includes a 5' untranslated region (UTR) (nt 1-61), an open reading frame (ORF) encoding the 183 kDa RdRp (nt 62-4,858), a 128 kDa methyltransferase/helicase (nt 62-3,364), MP (nt 4,848-5,612), CP (nt 5,632-6,171), and a 3' UTR (nt 6,172-6,453). The genome sequence of this isolate shared a nucleotide identity of 97.61% with the NC_040685.2 OV2 isolate (Table 2). Phylogenetic analysis placed both OV2 isolates obtained in this study within the subgroup of tobamoviruses that infect cacti (Figure 4).

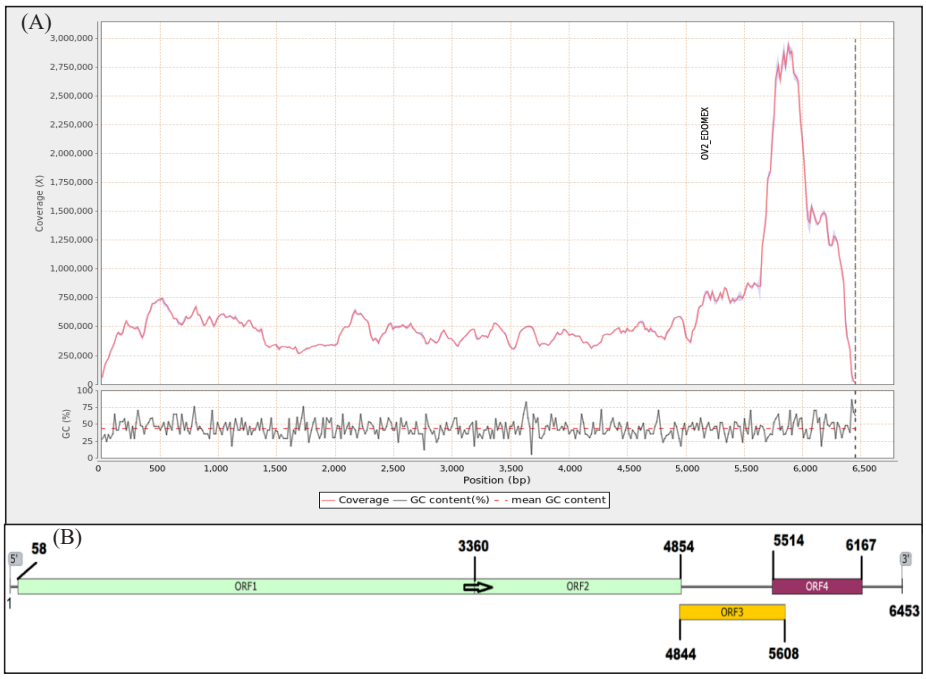

Figure 2. (A) Read coverage across the genome of the OV2-Edo-Mex isolate and the percentage of guanine-cytosine content, (B) Genomic map of OV2-Edo-Mex. The arrow indicates the termination codon site of the complete 128 kDa protein, with readthroughs.

***Cactus carlavirus 1* (CCV-1).** The virus was detected in both samples subjected to HTS. However, the complete genome was only recovered from the State of Mexico sample, supported by an average coverage per nucleotide of 15,516X (Figure 5A). The CCV-1 genome organization is similar to other known carlaviruses, featuring six ORFs (Figure 5B): ORF1 (nt 61-5,880) encodes a polyprotein (220 kDa) involved in viral replication; ORF2 (TGB1) (nt 5,910-6,599), ORF3 (TGB2) (nt 6,577-6,897), and ORF4 (TGB3) (nt 6,879-7,067) encode three proteins of 26 kDa, 11 kDa, and 7 kDa, respectively, representing the movement-associated triple gene block (TGB) proteins associated with 'potex-like' cell-to-cell movement (Morozov and Solovyev, 2003); ORF5 (nt 6,866-8,011) encodes a protein with a carlavirus-typical coat protein (CP) domain and a Flexi_CP domain; ORF6 (nt 8,004-8,324) encodes a 12 kDa nucleic acid binding (NB) protein; and finally, the 5' UTR (nt 1-60) and 3' UTR (8,325-8,395) regions. Phylogenetic analysis grouped CCV-1-Edo-Mex with the two available genomes of this virus in GenBank (Figure 6) with a nucleotide similarity of 87.86 and 90.41%.

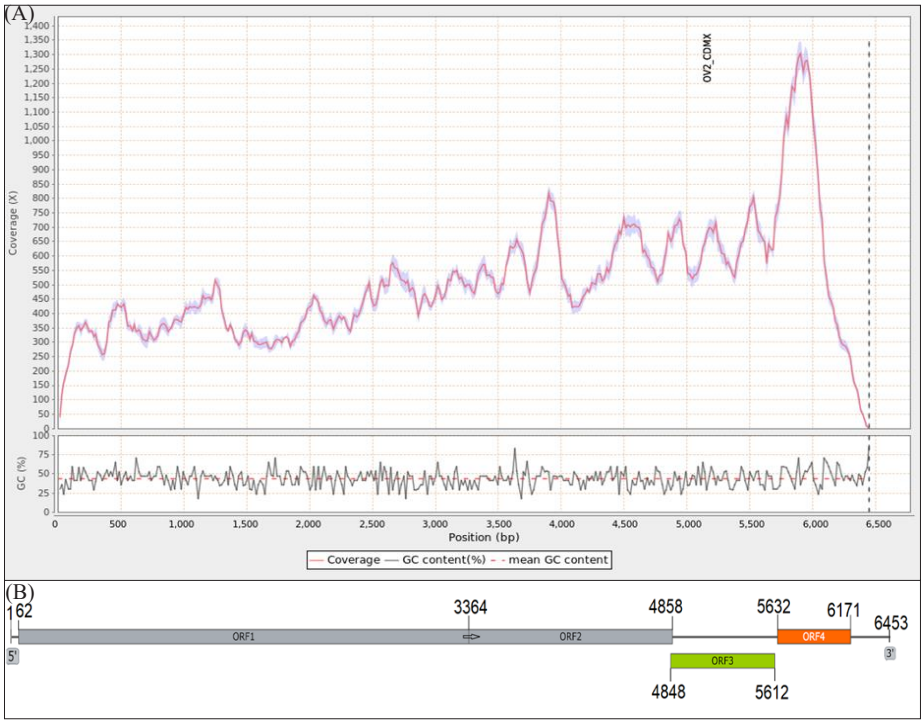

Figure 3. (A) Read coverage across the genome of the OV2-CDMX isolate and the percentage of guanine-cytosine content. (B) Genomic map of OV2-CDMX. The arrow indicates the termination codon site of the complete 128 kDa protein, with readthroughs.

***Opuntia potexvirus A* (OPV-A).** In the composite sample from Mexico City, a 6,671 nt contig was detected, which in the BLASTx search showed 77.5% identity with *Schlumbergera virus X* (SchVX). Additional inspection of the viral genome transcription and curation through interactive mapping of filtered reads resulted in a consolidated viral sequence of 6,644 nt supported by 10,536,601 reads with an average coverage per nucleotide of 238,206.9X (Figure 7A). The genome is organized into five ORFs (Figure 7B). Like other potexviruses, ORF1 (nt 85–4737) encodes the viral replicase consisting of 1,550 amino acids (aa). It also includes a set of three partially overlapping ORFs: ORF2 (4,737–5,426 nt), ORF3 (5,389–5,721 nt), and ORF4 (5,651–5,842 nt), displaying the typical configuration of the three-gene block (TGB) of potexviruses. ORF5 (5,854–6,531 nt) encodes the coat protein (CP). Finally, UTR regions of 84 and 113 nt were detected at the 5' and 3' ends, respectively.

Conserved amino acid motifs QDGAML, HQQAKDE, and TFDANTE were identified in the C-terminal region of the viral replicase (Van der Vlugt and Berendsen, 2002). Additionally, a catalytic replicase domain containing the characteristic

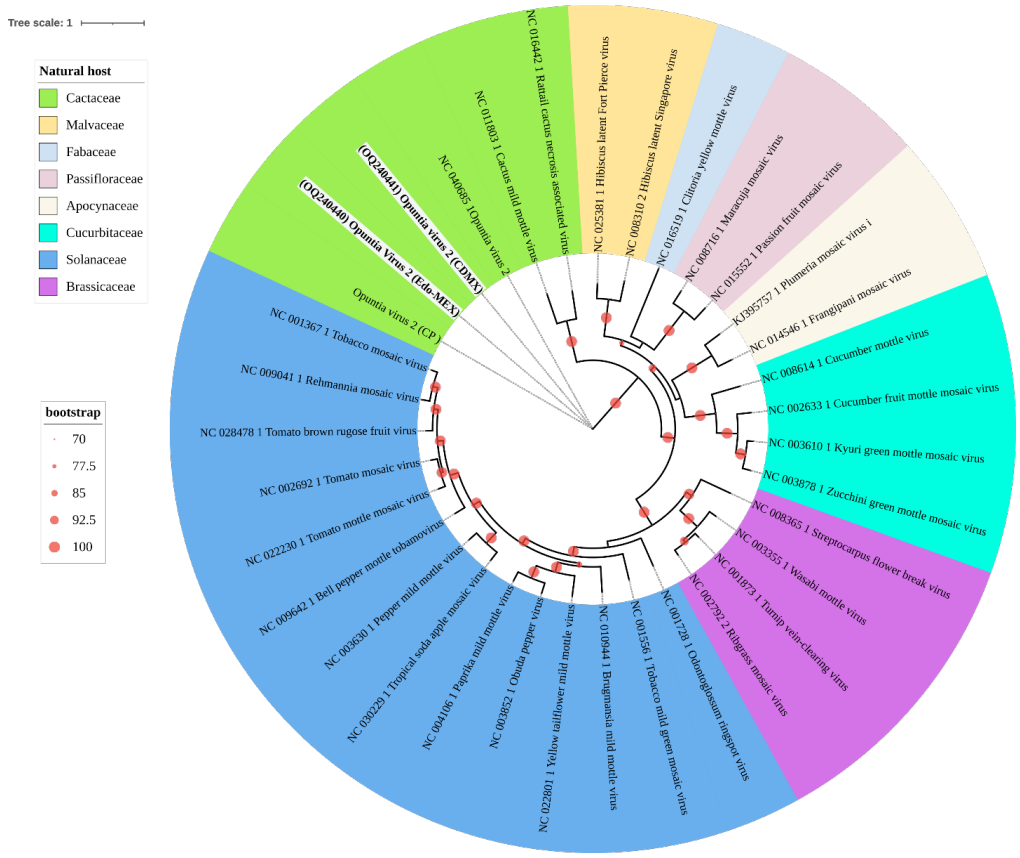

Figure 4. Maximum Likelihood phylogenetic tree of the Tobamovirus genus showing the relationships between genomes obtained in this study (in bold) for OV2 and those of previously reported isolates. Different colors represent botanical families as natural hosts where each virus has been reported. Circles on branches indicate UFBoot support values > 70%.

central consensus motif TGX3TX3NTX22GDD found in potexviruses (Martelli et al., 2007) was present near the C-terminal end of the replicase. Furthermore, the hexanucleotide motif 5'-GGAAAA-3' was found at the 5'-UTR, which is observed in some potexviruses (Chen *et al*., 2005), along with a putative polyadenylation signal at the 3'-UTR.

Pairwise comparisons revealed amino acid identities ranging from 59.8 to 82.2% for the CP and from 53.6 to 77.7% for the viral replicase (Figure 8a, b). The species demarcation criterion for potexviruses states that distinct species have <72% nt or <80% aa identity for the replicase or coat protein genes, respectively (Adams *et al*., 2004). Therefore, the virus detected in the vegetable prickly pear cactus is considered a new species of the genus *Potexvirus*, proposed to be named "*Opuntia potexvirus A*" (OPV-A). The genome sequence of OPV-A has been deposited in GenBank under the accession number OQ240443.

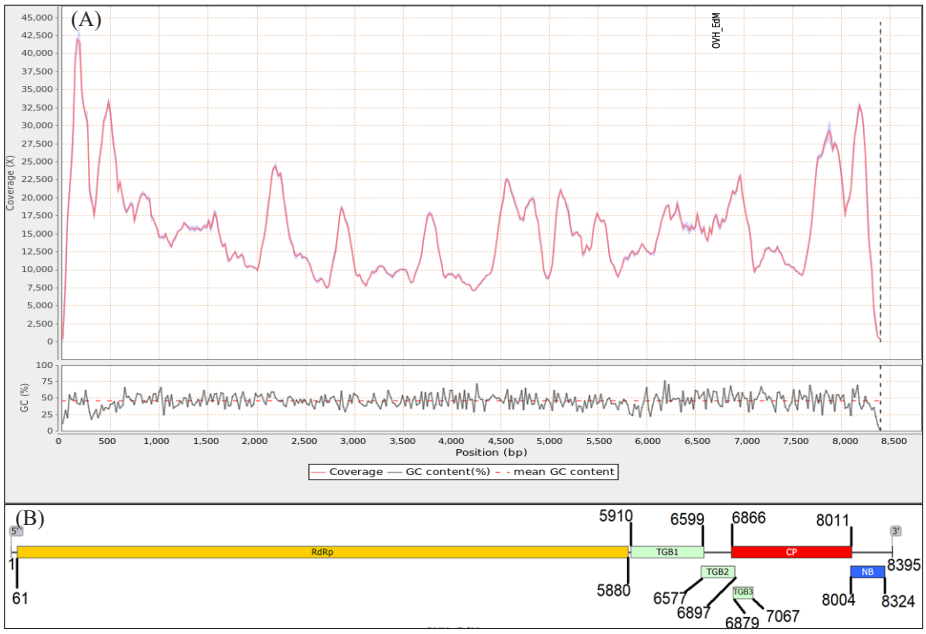

Figure 5. (A) Read coverage across the genome of the CCV-1-Edo-Mex isolate and the percentage of guanine-cytosine content. (B) Genome organization of CCV1-Edo-Mex, depicting six open reading frames and their corresponding products. RNA-dependent RNA polymerase (RdRp); coat protein (CP); nucleic acid-binding protein (NB); triple gene block (TGB).

The phylogenetic trees based on amino acid sequences of the viral replicase and coat protein of viruses belonging to the *Alfaflexiviridae* family show that OPV-A groups with potexviruses: *Pitaya virus X* (PiVX), *Zygocactus virus X* (ZyVX), *Cactus virus X* (CVX), SchVX, and *Opuntia virus X* (OpVX), in a highly supported clade (Figures 9 and 10).

Opuntia viroid I. In sample CDMX-1, a contig similar to that of viroids in the family Pospiviroidae was identified and designated as "*Opuntia viroid I*" (OVd-I). BLASTn analysis results indicated similarity to Columnea latent viroid-B, with a 14% query coverage and an E value of 4e-08. To determine the genus of OVd-I, manual inspection and sequence curation were performed. A final contig of 412 bp was obtained, and mapping onto HTS data confirmed its presence with an average coverage per nucleotide of 1,492X. Confirmation of OVd-I was achieved through RT-PCR and Sanger sequencing.

The secondary structure prediction of OVd-I revealed a rod-shaped molecule with a conserved central region (CCR) (Figure 11), a key criterion for classifying

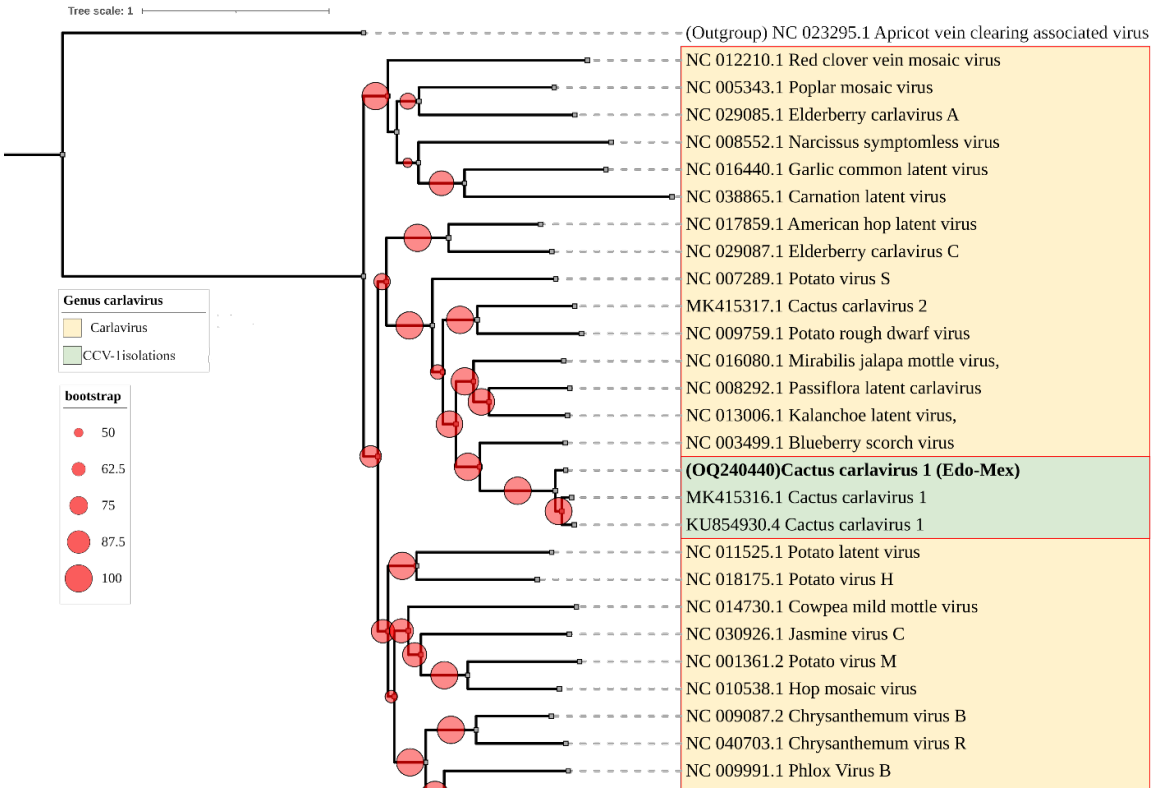

Figure 6. Maximum Likelihood phylogenetic tree of complete nucleotide sequences of CCV-1 genomes from this study (in bold), along with other isolates, and various species within the Carlavirus genus. Apricot vein clearing associated virus, Prunevirus genus, was used as an outgroup. Circles on branches indicate UFBoot support values > 50%.

a viroid as a new member of the family *Pospiviroidae*. The presence of CCR and a conserved terminal region (TCR) was considered to place OVd-I within the genus *Apscaviroid* (Di Serio *et al*., 2014). No changes were detected in the TCR; however, in the upper CCR, five changes (T/G, T/C, T/G, A/G, G/C) were observed, and in the lower CCR, six changes (G/T, T/C, G/C, C/A, G/C, A/C) were detected, likely due to the divergence of OVd-I from the *Apscaviroid* genus.

The results of the Maximum Likelihood phylogenetic analysis (Figure 12) placed OVd-I in the clade of the *Apscaviroid* genus within the *Pospiviroidae* family. The SDT analysis, based on pairwise comparisons, shows that none of the analyzed viroid sequences exhibit more than 80% similarity with the genome of OVd-I (Figure 13). Finally, OVd-I was detected in all four independent samples from Mexico City and in a sample from Morelos.

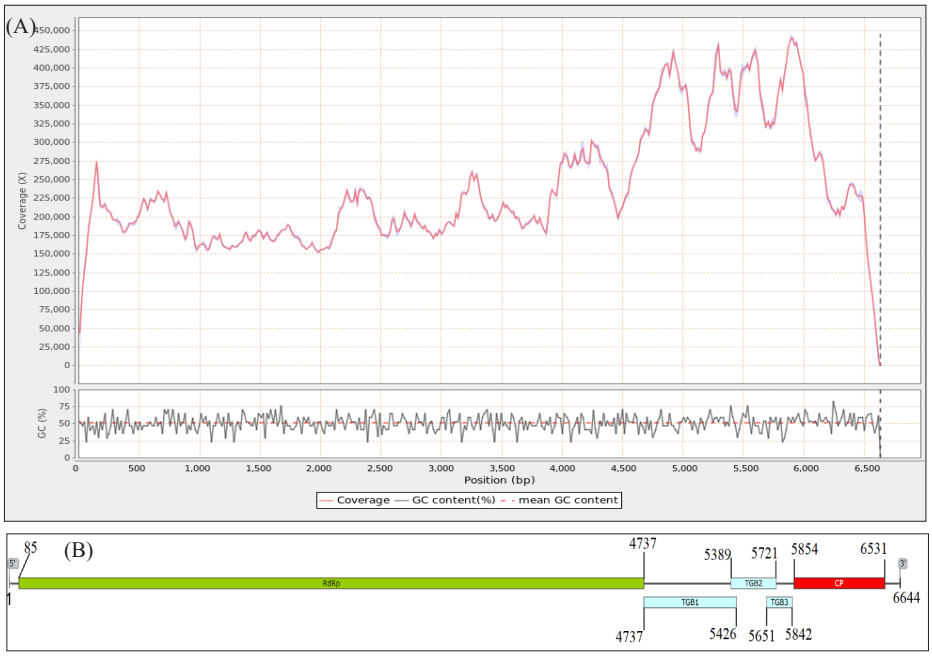

Figure 7. (A) Read coverage across the genome of *Opuntia potexvirus A* (OPV-A) and guanine/cytosine percentage. (B) Genome organization of OPV-A, displaying five open reading frames and their corresponding products: RdRp, viral replicase; TGB, triple gene block; CP, coat protein.

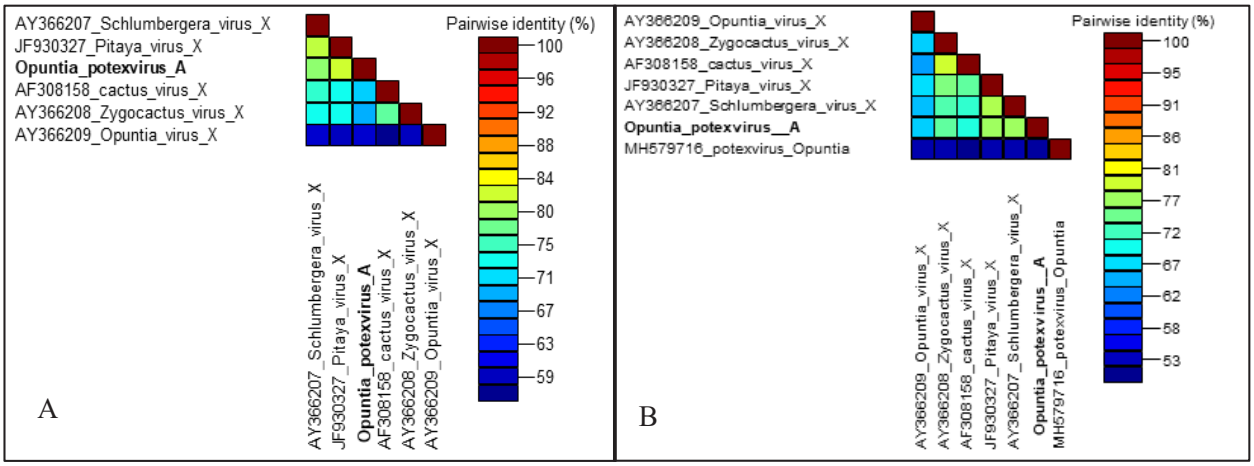

Figure 8. Matrix of amino acid identity percentage for the coat protein (A) and viral replicase (B) of species within the Potexvirus genus closest to *Opuntia potexvirus A* (in bold).

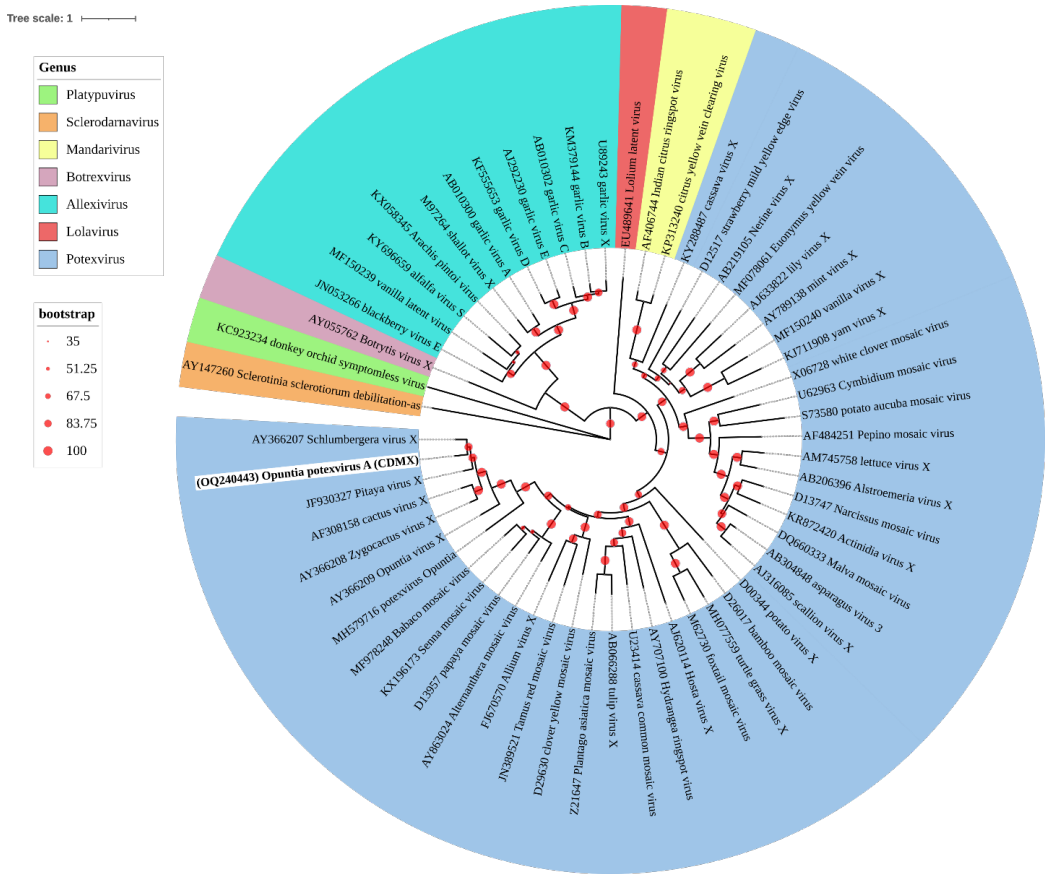

Figure 9. Maximum Likelihood phylogenetic tree of the Alphaflexiviridae family based on viral replicase amino acid sequences; the *Opuntia potexvirus A* sequence is highlighted in bold. Tree colors represent different genera within this family. Circles on branches indicate UFBoot support values >70%.

Other Viruses Detected. Additionally, in sample CDMX-1, additional viral contigs were detected (Table 3). However, only one caulimovirus and one vityvirus were confirmed by RT-PCR. Therefore, it is necessary to proceed with additional efforts to recover the complete genomes of these potential new virus species.

CCV-1 Detection. CCV-1 was detected in all four entities under investigation in 72% of the analyzed samples (Table 4); this marks the initial documentation of CCV-1 presence in commercial vegetable prickly pear cactus plantations in Mexico.

Electron microscopy. In the analyzed samples, virions of various sizes and shapes were observed (Figure 14). Rigid rod-shaped particles, approximately 300 nm in length and 18 nm in diameter characteristic of tobamoviruses were identified.

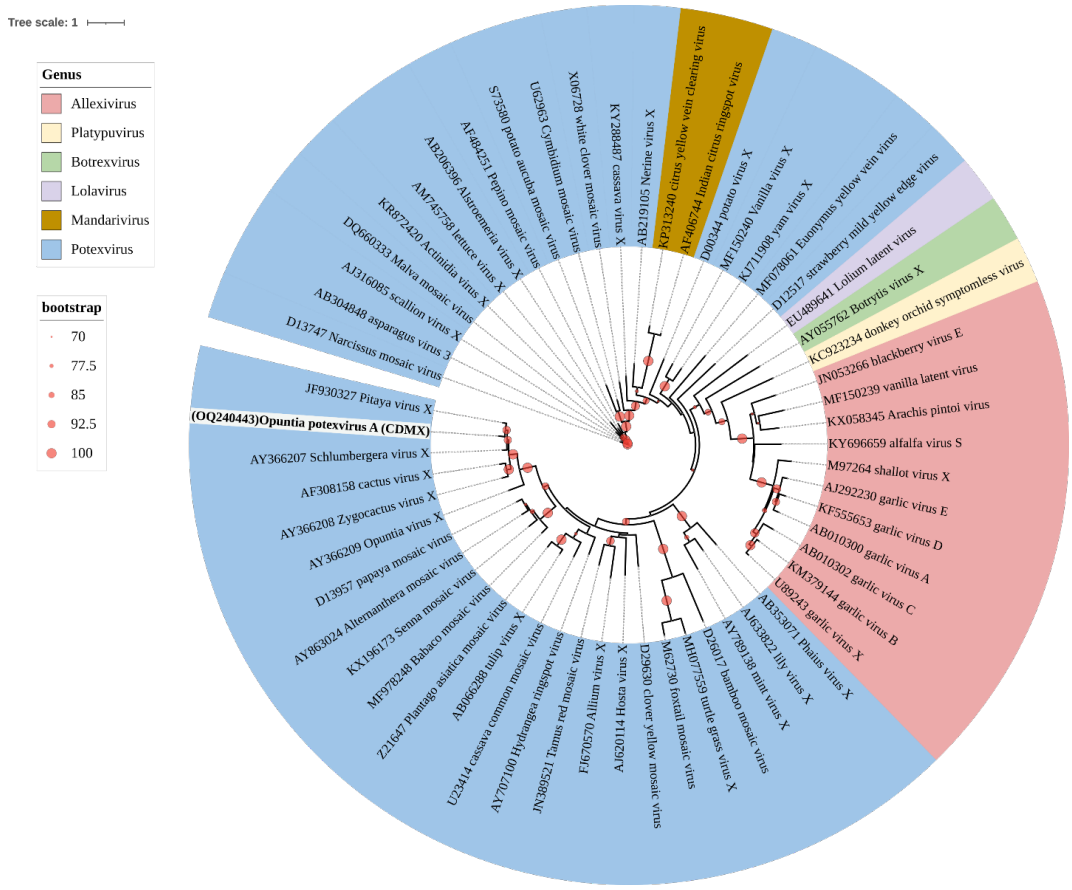

Figure 10. Maximum Likelihood phylogenetic tree of the Alphaflexiviridae family based on amino acid sequences of the coat protein; the *Opuntia potexvirus A* sequence is highlighted in bold. Tree colors represent different genera within this family. Circles on branches indicate UFBoot support values >70%.

Additionally, flexible rod-shaped virions, with an average length of 700 nm and a diameter of 15 nm, were found, possibly associated with CCV-1. Furthermore, flexible viral particles of approximately 500 nm in length were also observed.

Discussion

In recent years, high-throughput sequencing (HTS) has facilitated the discovery of new viral species (Maliogka *et al*., 2018; Villamor *et al*., 2019). This has, in turn, revealed the intricacies of viromes, highlighting their complexity (Al Rwahnih *et al*., 2011; Vučurović *et al*., 2021).

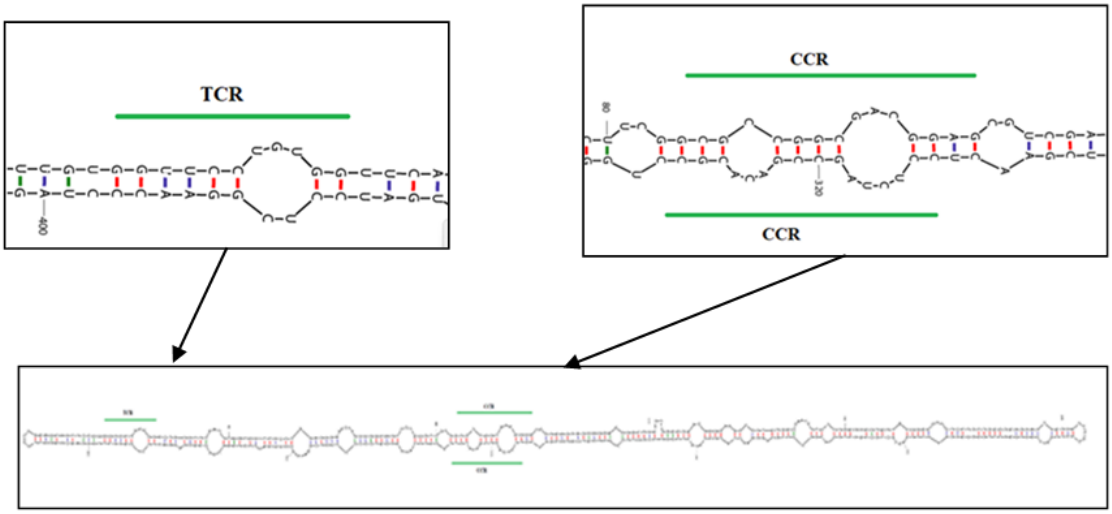

Figure 11. Predicted secondary structure using the UNAFold web server (UNAFold tool available online: www.mfold.org) for OVd-I. Highlighted are the sites forming the terminal conserved region (TCR) at positions 16 to 26 and the central conserved region (CCR) at positions 84 to 100 and 313 to 329.

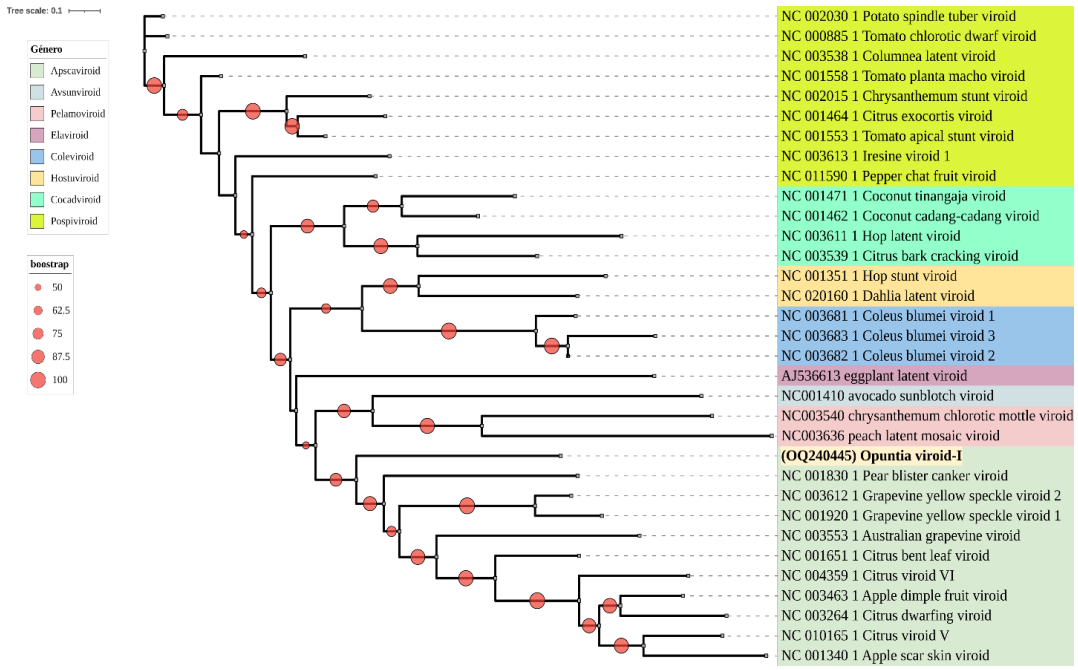

Figure 12. Maximum Likelihood phylogenetic tree of the Pospiviroidae and Avsunviroidae families based on nucleotide sequences of complete genomes from representative species of different genera. The sequence obtained from OVd-I is highlighted in bold. Various colors represent viroid genera. Circles on branches indicate UFBoot support values >50%.

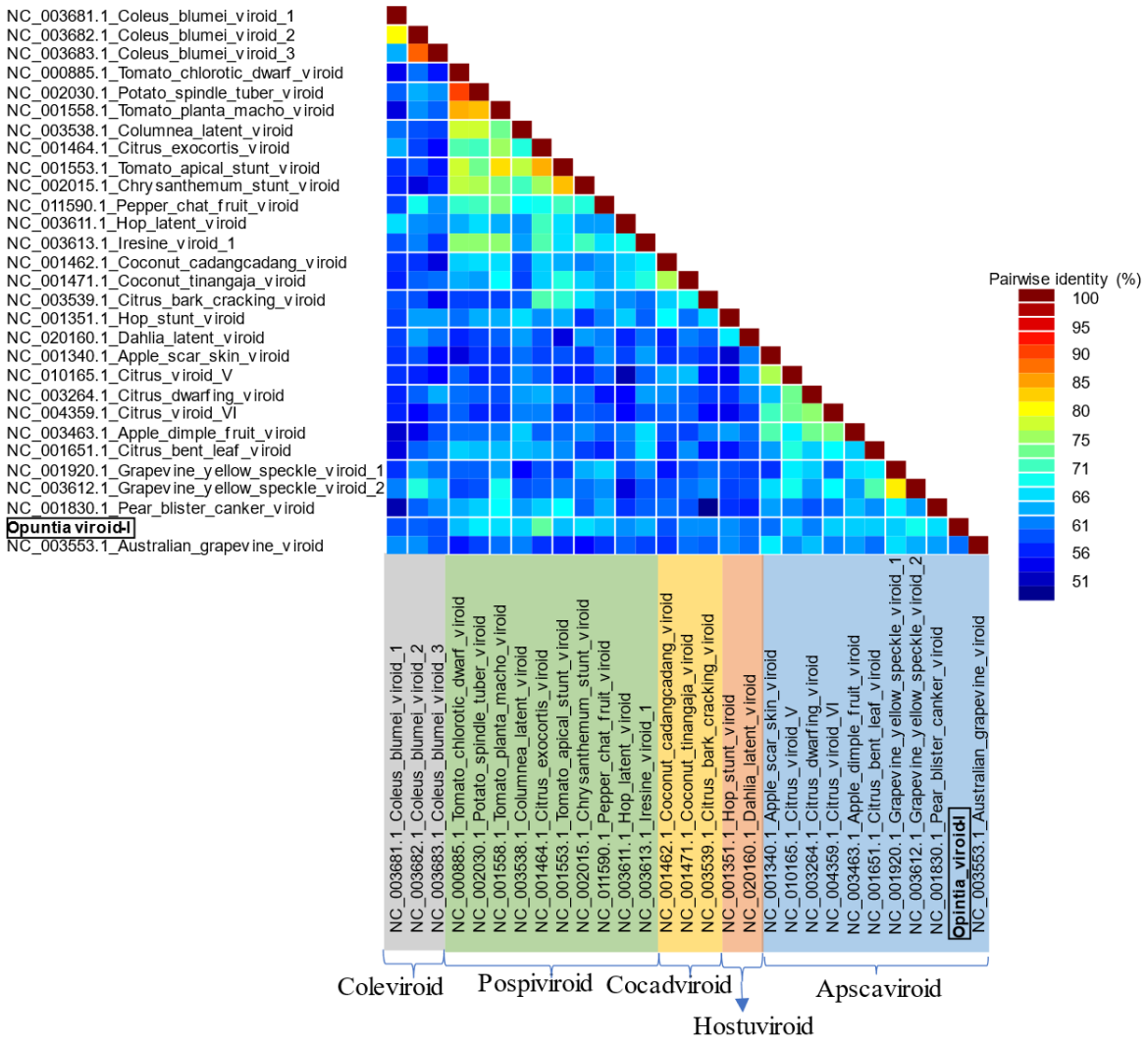

Figure 13. Pairwise identity frequency distribution obtained using the Sequence Demarcation Tool (SDT) (Muhire *et al*., 2014) from complete genome sequences of *Pospiviroidae* family species to support the demarcation of *Opuntia viroid I* as a new species.

In Mexico, research has been conducted to identify viruses associated with vegetable prickly pear cactus showing putative symptoms of virosis. However, conventional techniques such as RT-PCR, ELISA, transmission electron microscopy, and biological indexing have, in several studies, faced challenges in determining the associated virus or viruses due to their inherent limitations (De La Torre-Almaraz *et al*., 2007; De La Torre-Almaraz *et al*., 2016a; De La Torre-Almaráz *et al*., 2016b; Alonso-Barrera *et al*., 2015). The use of HTS allows for the identification of all viruses (known or unknown) present in a sample, a capability

Table 3. Result of Blastx analysis for potentially present viruses in the CDMX-1 library, confirmed through RT-PCR and sequencing.

Virus	Contig length (nucleotide)	Reference sequence accession number	Identity percentage (query coverage)	Part of the genome	Confirmation by RT-PCR	Confirmation by Sanger sequencing
Caulimovirus	3168	QYR69113.1	47.14 (51)	Putative viral replicase	+	+
Vityvirus	1242	AVL25840.1	52.21 (64)	Movement protein	+	+
Alphavirus	2932	QYD13417.1	75 (27)	Coat protein	-	-
Bammivirus	2110	UUL90866.1	39.75 (99)	Viral replicase	-	-
Betaflexiviridae (Tymovirales)	1096	AZM69108.1	36.36 (52)	Three-gene block protein 1	-	-

+= Positive; -= Negative

Table 4. Detection of CCV-1 in four vegetable prickly pear cactus producing states in Mexico.

State	Samples analyzed	CCV-1 positive samples (%)
Estado de México	24	22 (91)
Morelos	26	16 (64)
Hidalgo	24	10 (41)
CDMX	55	45 (81)
Total	129	93 (72)

not shared by other techniques. For instance, this approach successfully detected a new tobamovirus (OV2) in a sample composed of RNA extracted from *O. ficus-indica* and *O. albicarpa* collected in the State of Mexico (Salgado-Ortíz *et al*., 2020).

This study reveals, for the first time, the virome of commercially grown vegetable prickly pear cactus in the central region of Mexico, comprising three viruses (two of which are new, belonging to the genera *Potexvirus* and *Carlavirus*) and one viroid (genus *Apscaviroid*).

Opuntia potexvirus A (OPV-A) is proposed as a new member of the family *Alphaflexiviridae*, genus *Potexvirus*, based on the species demarcation criteria for this genus (Jan *et al*., 2020). Potexviruses have elongated particles approximately 700 nm in length, infect herbaceous hosts, lack known vectors, and are primarily transmitted mechanically (Jan *et al*., 2020). Phylogenetically, OPV-A is closely related to PiVX and SchVX, which naturally infect cacti (Figures 9 and 10).

Recently, potexviruses CVX, OpVX, PiVX, SchVX, and ZyVX were reported in ornamental cacti, either as single infections or mixed infections with up to four

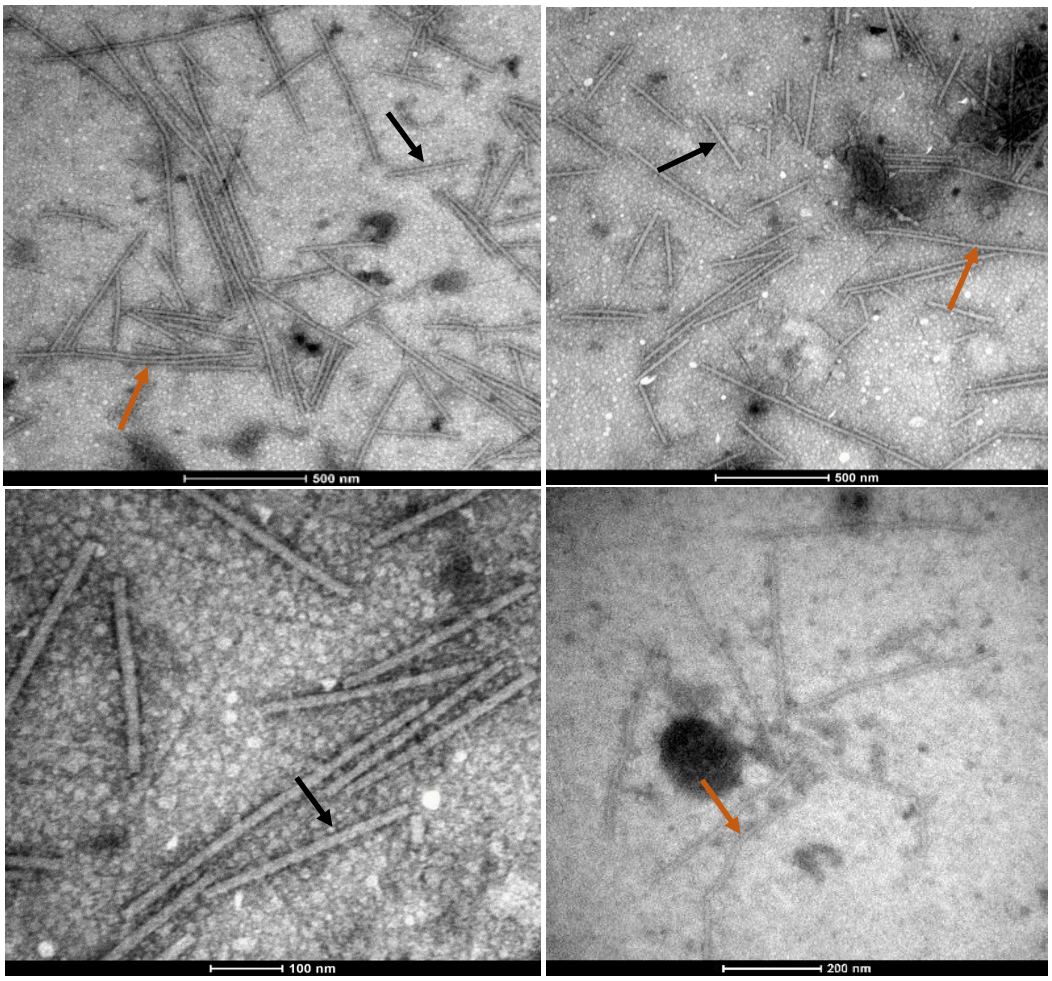

Figure 14. Viral particles in the form of rigid rods (➔) and flexible filaments (➔) of varying lengths were observed through transmission electron microscopy in vegetable prickly pear cactus exhibiting irregular yellow patterns, yellow rings, mosaic patterns, and chlorotic spots around the spines, employing the "leaf dip" technique.

of them (CVX, PiVX, SchVX, and ZyVX) in a single plant (Park *et al*., 2021). Additionally, it was noted that the cactus *Notocactus leninghausii* f. cristatus can be co-infected with up to six viruses, including four potexviruses (CVX, PiVX, SchVX, and ZyVX) and two tobamoviruses (CMMoV and RCNaV) (Park *et al.,* 2018). These reports highlight cacti as natural hosts for both potexviruses and tobamoviruses. However, the symptoms caused by these viruses individually have not been clearly described, and their host range is not well-known. Therefore, future studies will need to address the biological characterization of the potexvirus detected in this study to determine, among other things, whether it poses a risk to prickly pear cactus production in Mexico.

In the HTS analysis, CCV-1 was detected in samples from both Mexico City and the State of Mexico. However, the complete genome was only recovered from the cladodes collected in the latter. The partial genome detection in the Mexico City sample is likely attributed to the suppression of CCV-1 replication by OPV-A and OV2. Consequently, the number of sequences obtained for CCV-1 did not cover the entire genome. The CCV-1 isolated from the State of Mexico grouped in the same clade as the MK415316.1 isolate reported in the United States and a sequence from Mexico (KU854930.4) but in a distinct branch. This virus was detected in all four sampled entities (Table 4), both in plants exhibiting virosis symptoms and asymptomatic plants, the latter case only in the states of Hidalgo, Morelos, and Mexico City. Carlaviruses are known to be transmitted by non-persistent aphids or whiteflies, predominantly infect herbaceous plants, and many cause latent or asymptomatic infections, explaining their detection in asymptomatic prickly pear cactus without visible alterations (Kreuze *et al.*, 2012). CCV-1 and cactus carlavirus 2 (CC-2) were first detected in an asymptomatic 'Professor Ebert' hybrid of *Epiphyllum* spp. (Peng *et al*., 2019). Therefore, subsequent studies should investigate the biological behavior of both viruses to determine if, under certain environmental conditions, they induce symptoms in vegetable prickly pear cactus and other economically significant plants. Additionally, it needs to be established whether they pose a risk to vegetable prickly pear cactus or other crops in production.

OV2 was first reported in Mexico, infecting various cultivated (*O. ficus-indica* and *O. albicarpa*) and uncultivated (*O. pilifera*, *O. depressa*, *O. tomentosa*, *O. robusta*, and *O. streptacantha*) cactus species in the states of Mexico, Puebla, and Mexico City (Salgado-Ortíz *et al*., 2020). The *Tobamovirus* genus is categorized into seven groups based on their natural hosts, including the families Solanaceae, Brassicaceae, Cucurbitaceae, Malvaceae, Cactaceae, Passifloraceae, Apocynaceae, and Leguminosae (Li *et al*., 2017; Song *et al*., 2006). The phylogenetic analysis is consistent with this classification, placing OV2 in the clade of tobamoviruses that infect cacti (Figure 4). Another tobamovirus reported in Mexico that infects prickly pear cactus is the *Rattail cactus necrosis-associated virus* (RCNaV). RCNaV was previously documented in the Axapusco, Otumba, and San Martín de las Pirámides regions of the State of Mexico in prickly pear cactus displaying small yellow rings (De La Torre-Almaráz *et al*., 2016a). However, RCNaV was not detected in any of the samples analyzed by HTS in this study.

This research reports, for the first time worldwide, the presence of a viroid in vegetable prickly pear cactus (OVd-I). However, since it was found in samples with mixed infections involving OV2, CCV-1, and OPV-A, it remains unknown whether it induces symptoms individually.

Currently, there is no regulatory framework in Mexico governing the movement of prickly pear cactus propagative material. Consequently, there is a risk of

introducing the viruses and viroid identified in this study to new production areas of this species.

Conclusions

The virome of commercially grown vegetable prickly pear cactus in four central Mexico production zones consisted of *Opuntia virus 2* (genus *Tobamovirus*), *Cactus carlavirus 1* (genus *Carlavirus*), a new virus proposed to be named *Opuntia potexvirus A* (genus *Potexvirus*), and a new viroid suggested to be named *Opuntia viroid I* (genus *Apscaviroid*). *Cactus carlavirus 1* was detected in all four sampled states known for vegetable prickly pear cactus production.

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