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Additional Information

## Identification and genomic characterization of a novel tobamovirus from prickly pear cactus

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### Abstract

In this work, we describe the complete sequence and genome organization of a novel tobamovirus detected in the prickly pear plant (*Opuntia sp.*) by High-throughput sequence (HTS), tentatively named “Opuntia virus 2”. The full genome of Opuntia virus 2 is 6,453 nucleotides in length and contains four open reading frames (ORFs) coding for the two subunits of the RNA polymerase, the movement protein and the coat protein, respectively. Phylogenetic analysis using the complete nucleotide sequence, revealed that the virus is included in the genus *Tobamovirus* (family *Virgaviridae*), showing the highest nucleotide identity percentage of 49.8% with cactus mild mottle virus (CMMoV), being grouped in the subgroup Cactaceae of this genus.

The prickly pear plant (*Opuntia sp.*) is an important crop in Mexican culture and gastronomy [1]. The main viruses affecting this crop are the potexvirus opuntia virus X and schlumbergera virus X and the tobamovirus rattail cactus necrosis-associated virus, which are associated with chlorotic ring spots [2,3,4]. In cacti, two species of tobamovirus have been identified to date: cactus mild mottle virus (CMMoV) isolated from *Gymnocalycium mihanovichii* [5], and rattail cactus necrosis-associated virus (RCNaV) isolated from *Aporocactus flagelliformis* [6]. Viruses assigned to the tobamovirus genus, are economically important in agriculture affecting plants belonging to the Solanaceae and Cucurbitaceae families [7,8]. In 2017, prickly pear fruit and vegetable crops (*O. albicarpa* and *O. ficus-indica*) showing chlorotic annular spots (Fig. 1a) and located in farms from municipality of Otumba, state of Mexico, were subjected to HTS analysis. To do that, total messenger RNA extracted from a mixed sample of symptomatic cladodes of the two species (*O. albicarpa* and *O. ficus-indica*) were sequenced, yielding approximately 30 million sequences of 75 pb each. All sequences were filtered and trimmed, using the Geneious 9.0 software (Biomatters Ltd, Auckland, New Zealand) and the *de novo* assembly was performed with the Trinity software that uses Bruijn graphs [9]. Contigs were analyzed with BLASTx [10] and the identified ORFs were aligned using Geneious 9.0. A contig of 6456 nt was identified from these mixed sample of prickly pear plants, showing the highest identity percentages with CMMoV and RCNaV (49.8%) (Supplementary Table 1). The 5' and 3' termini of the new virus were determined by RACE analysis (Roche Diagnostics, Mannheim Germany) using specific primers (VP3374AsTobamo-5' and VP3373sTobamo-3'; Supplementary Table 2) designed from the ensembled RNA-seq sequence and total RNA extracted from an *O. ficus-indica* symptomatic plant collected in farms from Otumba, state of Mexico. In the next step, the full genome of the new virus was amplified by RT-PCR using the Superscript III One-step High Fidelity (Thermo Fisher Scientific, Carlsbad, USA) kit, total RNA extracted from the same sample used in RACE analysis and the VP3379sTobamo\_genome and VP3380sTobamo\_genome primers (Supplementary Table 1), targeting the 5' and 3' ends, respectively. The obtained amplicon of 6.5 kb was cloned using the CloneJET PCR Cloning Kit (Thermo Fisher Scientific, Carlsbad, USA) and the

1 sequence of an individual colony was determined using specific primers (Supplementary Table 2). The complete genome  
2 sequence of the selected individual clone has 6543 nt (Accession number MF434821) and showed a nucleotide identity  
3 percentage of 98.4% with the ensembled RNA-seq sequence. The complete genome of the new virus, that we tentatively  
4 named ‘Opuntia virus 2’ (OV2), has the typical genomic organization of other members of the tobamovirus genus: the 5’ and  
5 3’ non-coding regions of 57 nt and 286 nt, respectively; two 5’ proximal ORFs (ORF 1 and ORF 2) of 3303 nt and 4797 nt  
6 coding for the two subunits of the RNA polymerase of 128 kDa and 187 kDa, respectively; an ORF of 765 nt coding for the  
7 movement protein (MP) of 27.8 kDa and finally the 3’ proximal ORF of 540 nt coding for the capsid protein (CP) of 19.2  
8 kDa (Fig.1b). Phylogenetic analysis of the complete genome was performed in MEGA 7 [11] and a Neighbour-joining tree  
9 was obtained with Jukes-Cantor substitution model and 1500 bootstrap pseudoreplicates. A dendrogram was obtained by  
10 aligning the complete genome of OV2 and 34 tobamovirus species (39 sequences) available at the GenBank database. Other  
11 species assigned to different genus of the *Virgaviridae* family and two potexvirus were included as outgroups. The genus  
12 Tobamovirus has been historically divided into three subgroups based on natural host range, genomic organization and  
13 phylogenetic clustering. Taking into account the plant families to which tobamoviruses infect they have been divided into  
14 five (Brassicaceae, Cactaceae, Cucurbitaceae, Malvaceae, and Solanaceae/Orchidaceae) [5], or six subgroups (the previous  
15 five plus members infecting members of the family Passifloraceae) [12]. The obtained tree included OV2 in the Cactaceae  
16 subgroup, closely related to other species isolated from cacti (CMMoV and RCNaV), with a bootstrap value of 100% (Fig.2).  
17 More recently, Gibbs et al. [13] have shown that, with the exception of three of the twenty-nine species, all tobamoviruses  
18 fall into three clusters that have either asteroid or rosid or caryophyllid hosts (i.e. the major subdivisions of eudicotyledonous  
19 plants). OV2 clustered in the Caryophyllid group, as expected (Fig. 2) reinforcing the hypothesis that tobamoviruses and their  
20 hosts have probably co-diverge [13].

21 The prevalence of OV2 in cultivars of prickly pear plants and other wild species of *Opuntia*, collected from Puebla  
22 and Ciudad de Mexico, was analyzed by RT-PCR (Superscript III One-step, Thermo Fisher Scientific) using specific primers  
23 (Tobamo\_2nopF and Tobamo\_2nopR) targeting a conserved region of the capsid protein gene. Total RNA extracted from *O.*  
24 *albicarpa* germinated plants was used as negative control. For the analysis, we selected twenty symptomatic wild (*O. pilifera*:  
25 3 plants; *O. depressa*: 2 plants; *O. tomentosa*: 2 plants; *O. robusta*: 2 plants; *O. streptacantha*: 1 plant) and commercial (*O.*  
26 *albicarpa*: 7 plants; *O. ficus-indica*: 3 plants) plants. The expected amplicon of 890 nt (located in CP gene to 3’end) was  
27 obtained in all analyzed samples, indicating an association of symptoms with the OV2 infection. No amplification was  
28 observed in the negative control. To confirm the presence of the new virus, the amplicons obtained from *O. streptacantha*, *O.*  
29 *pilifera*, *O. depressa*, *O. robusta* and *O. ficus-indica* samples were extracted from gel, using the Wizard DNA Clean-up  
30 System kit (Promega) and directly sequenced. All amplicons corresponded to the new OV2 virus with identity percentages  
31 that ranged between 94,2% and 96,6%.

32 Accordantly to the results presented herein, we propose that Opuntia virus 2 should be placed in the genus *Tobamovirus*,  
33 family *Virgaviridae*. Based on species demarcation criteria proposed by the International Committee of Taxonomy of Virus  
34 [14], the Opuntia virus 2 should be considered a new species into genus *Tobamovirus* due to the low level of identity  
35 percentage (less than 90%) of the complete nucleotide sequence with the rest of tobamovirus species. Further work is needed  
36 to evaluate more precisely the incidence of OV2 in other prickly pear plants grown in commercial plots or wild *Opuntia*  
37 species throughout the country, where the specific primers designed herein could be an attractive tool.

38

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#### 7 **Compliance with ethical standards**

8 **Ethical approval:** This article does not contain any studies performed on human participants or animals by any of the authors.

9 **Conflict of Interest:** The authors declare that they have no conflict of interest.

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5  
6

7 **Fig.1 a.** *O. albicarpa* plants showing chlorotic annular spots (left) and plants showing no symptoms (right), **b.** Genomic  
8 organization of opuntia virus 2 isolated from nopal verdura. Numbers indicate the initial and last nucleotide of each ORF and  
9 the complete genome. The arrows indicated the location of Tobamo\_2nopF and Tobamo\_2nopR primers used in the diagnostic  
10 RT-PCR assay.

11 **Fig. 2** Phylogenetic analysis of the genus *Tobamovirus* using the complete nucleotide sequence of 34 species available at the  
12 GenBank database plus the new virus opuntia virus 2 identified in prickly pear cactus (MF434821), which is indicated by a  
13 diamond (◆). Tobamovirus clustering based on the phylogenetic relationships of original host is conserved from different  
14 classification approaches: A. Historical classification based on natural host range, genomic organization and site of assembly  
15 origin. B. Classification in host families based on Song et al. 2006 [12]. C. Division on three groups of eudicotyledonous host  
16 plant lineages, asterids, rosids, and caryophyllids based on Gibbs et al. 2015 [13]. Numbers indicate the bootstrap percentage  
17 value (1500 pseudoreplicates) for each node. Branches with bootstrap values less than 50% were collapsed. Potexvirus potato  
18 virus X (KF575174) and schlumbergera virus X (KU854929) are used as outgroups. ‘Other genera’ is referred to other viral  
19 species assigned to different genus of the family *Virgaviridae*. The scale bar shows the number of substitutions per site.  
20

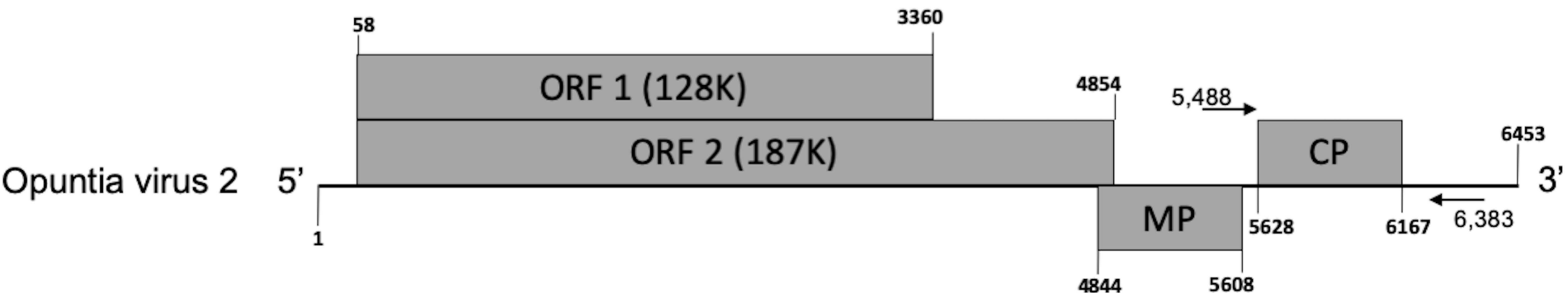
a

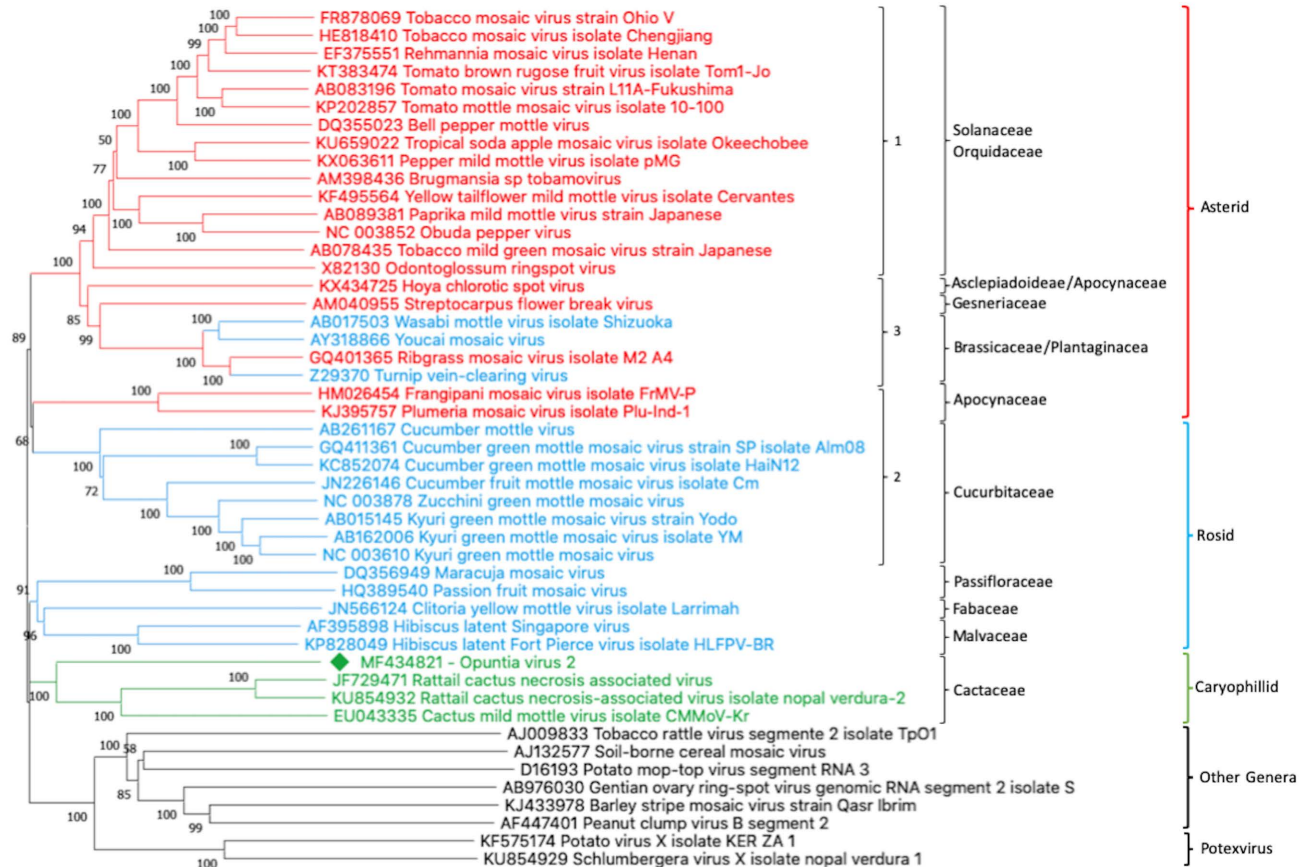


Symptomatic

non-Symptomatic

b





**Supp. Table 1.** Percentage identities among the complete genome (nt) of the opuntia virus 2 and different tobamovirus from the Genbank database.

|   | 1*          | 2    | 3    | 4    | 5    | 6    | 7    | 8    | 9    | 10   | 11   | 12   | 13   | 14   | 15   | 16   | 17   | 18   | 19   | 20   | 21   | 22   | 23   |
|---|-------------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|
| 1 MF434821 Opuntia virus 2                            |             | 49.8 | 49.8 | 47.4 | 44.9 | 43.9 | 43.2 | 44.1 | 44.6 | 45.5 | 45.4 | 44.7 | 45.2 | 45.9 | 45.9 | 45.3 | 45.3 | 44.7 | 45.2 | 45.6 | 45.1 | 45.4 | 44.6 |
| 2 EU043335_1_Cactus_mild_mottle_virus                 | <b>49.8</b> |      | 62.9 | 47.6 | 45   | 42.3 | 42.8 | 43   | 44   | 44.4 | 44.2 | 43.7 | 43.7 | 44.6 | 44.8 | 44.4 | 44.1 | 43.5 | 44.9 | 44.2 | 44.1 | 44.9 | 43.7 |
| 3 KU854932_1_Rattail_cactus_necrosis-associated_virus | <b>49.8</b> | 62.9 |      | 47.5 | 45.2 | 42.6 | 42.5 | 43.5 | 44.8 | 45.1 | 45   | 44.8 | 44.9 | 44.6 | 45   | 44   | 44.5 | 43.9 | 43.8 | 43.5 | 43.7 | 44.8 | 43.6 |
| 4 KP828049_1_Hibiscus_latent_Fort_Pierce_virus        | 47.4        | 47.6 | 47.5 |      | 50.6 | 46.5 | 47.4 | 46.8 | 47.9 | 46.9 | 46.4 | 46.9 | 47.1 | 46.6 | 47.4 | 47.2 | 46.8 | 46.9 | 47.2 | 47.1 | 46.8 | 46.9 | 46.1 |
| 5 JN566124_1_Clotria_yellow_mottle_virus_             | 44.9        | 45   | 45.2 | 50.6 |      | 44.7 | 43.8 | 43.6 | 44.8 | 44.7 | 44.7 | 44.8 | 45.1 | 44.5 | 45   | 44.8 | 44.2 | 44.1 | 44.9 | 44.9 | 45   | 44.6 | 44.1 |
| 6 HQ389540_1_Passion_fruit_mosaic_virus               | 43.9        | 42.3 | 42.6 | 46.5 | 44.7 |      | 43.3 | 43   | 43.8 | 45.2 | 44.4 | 44.4 | 44.6 | 44.2 | 44   | 43.9 | 43.7 | 43.4 | 43.5 | 43.8 | 43.5 | 43.4 | 42.8 |
| 7 NC_003610_1_Kyuri_green_mottle_mosaic_virus         | 43.2        | 42.8 | 42.5 | 47.4 | 43.8 | 43.3 |      | 61.1 | 46.5 | 45.6 | 45.6 | 45.9 | 45.6 | 45.1 | 45.8 | 45.6 | 46.2 | 45.9 | 46   | 46.5 | 45.5 | 46.2 | 45.4 |
| 8 AB261167_1_Cucumber_mottle_virus                    | 44.1        | 43   | 43.5 | 46.8 | 43.6 | 43   | 61.1 |      | 47.3 | 45.3 | 45.4 | 45.5 | 45.2 | 45   | 47.2 | 45.8 | 45.7 | 45.5 | 46   | 46.3 | 45.8 | 46.6 | 45   |
| 9 HM026454_1_Frangipani_mosaic_virus                  | 44.6        | 44   | 44.8 | 47.9 | 44.8 | 43.8 | 46.5 | 47.3 |      | 46.9 | 47.1 | 46.4 | 46.6 | 47.2 | 47.3 | 47.2 | 47.9 | 46.6 | 47.1 | 47.6 | 46.1 | 46.9 | 46.4 |
| 10 AB017503_1_Wasabi_mottle_virus                     | 45.5        | 44.4 | 45.1 | 46.9 | 44.7 | 45.2 | 45.6 | 45.3 | 46.9 |      | 84.7 | 82.1 | 82.2 | 58.8 | 57.8 | 56.7 | 56.4 | 56.3 | 56.5 | 56.5 | 55.5 | 56   | 57.9 |
| 11 AY318866_1_Youcai_mosaic_virus                     | 45.4        | 44.2 | 45   | 46.4 | 44.7 | 44.4 | 45.6 | 45.4 | 47.1 | 84.7 |      | 81.1 | 81.7 | 58.9 | 57.6 | 56.7 | 56.4 | 56.6 | 56.3 | 57   | 55.5 | 56.5 | 58.5 |
| 12 GQ401365_1_Ribgrass_mosaic_virus                   | 44.7        | 43.7 | 44.8 | 46.9 | 44.8 | 44.4 | 45.9 | 45.5 | 46.4 | 82.1 | 81.1 |      | 86.9 | 59.2 | 57.8 | 57.2 | 56.6 | 57.2 | 56.3 | 56.6 | 55.7 | 56.3 | 58.5 |
| 13 Z29370_1_Turnip_vein-clearing_virus                | 45.2        | 43.7 | 44.9 | 47.1 | 45.1 | 44.6 | 45.6 | 45.2 | 46.6 | 82.2 | 81.7 | 86.9 |      | 58.9 | 58.5 | 57   | 56.5 | 57.3 | 56.6 | 56.4 | 56   | 56   | 58.5 |
| 14 KX434725_1_Hoya_chlorotic_spot_virus               | 45.9        | 44.6 | 44.6 | 46.6 | 44.5 | 44.2 | 45.1 | 45   | 47.2 | 58.8 | 58.9 | 59.2 | 58.9 |      | 56.8 | 56.1 | 55.5 | 55.7 | 55.1 | 55.4 | 54.1 | 55.6 | 56.3 |
| 15 AB078435_1_Tobacco_mild_green_mosaic_virus         | 45.9        | 44.8 | 45   | 47.4 | 45   | 44   | 45.8 | 47.2 | 47.3 | 57.8 | 57.6 | 57.8 | 58.5 | 56.8 |      | 64.1 | 63.1 | 63.4 | 63.8 | 63.4 | 60.8 | 63   | 60   |
| 16 AB083196_1_Tomato_mosaic_virus                     | 45.3        | 44.4 | 44   | 47.2 | 44.8 | 43.9 | 45.6 | 45.8 | 47.2 | 56.7 | 56.7 | 57.2 | 57   | 56.1 | 64.1 |      | 79.1 | 76.6 | 68.8 | 68.7 | 62.1 | 63.3 | 60   |
| 17 FR878069_1_Tobacco_mosaic_virus                    | 45.3        | 44.1 | 44.5 | 46.8 | 44.2 | 43.7 | 46.2 | 45.7 | 47.9 | 56.4 | 56.4 | 56.6 | 56.5 | 55.5 | 63.1 | 79.1 |      | 74.3 | 68   | 68.2 | 62.1 | 62.2 | 59   |
| 18 DQ355023_1_Bell_pepper_mottle_virus                | 44.7        | 43.5 | 43.9 | 46.9 | 44.1 | 43.4 | 45.9 | 45.5 | 46.6 | 56.3 | 56.6 | 57.2 | 57.3 | 55.7 | 63.4 | 76.6 | 74.3 |      | 68.2 | 67.8 | 61.5 | 62.2 | 60.3 |
| 19 KU659022_1_Tropical_soda_apple_mosaic_virus        | 45.2        | 44.9 | 43.8 | 47.2 | 44.9 | 43.5 | 46   | 46   | 47.1 | 56.5 | 56.3 | 56.3 | 56.6 | 55.1 | 63.8 | 68.8 | 68   | 68.2 |      | 79.4 | 61.6 | 63.1 | 60.4 |
| 20 KX063611_1_Pepper_mild_mottle_virus                | 45.6        | 44.2 | 43.5 | 47.1 | 44.9 | 43.8 | 46.5 | 46.3 | 47.6 | 56.5 | 57   | 56.6 | 56.4 | 55.4 | 63.4 | 68.7 | 68.2 | 67.8 | 79.4 |      | 61.5 | 62.5 | 60.2 |
| 21 NC_003852_1_Obuda_pepper_virus                     | 45.1        | 44.1 | 43.7 | 46.8 | 45   | 43.5 | 45.5 | 45.8 | 46.1 | 55.5 | 55.5 | 55.7 | 56   | 54.1 | 60.8 | 62.1 | 62.1 | 61.5 | 61.6 | 61.5 |      | 67.2 | 57.2 |
| 22 KF495564_1_Yellow_taiflower_mild_mottle_virus      | 45.4        | 44.9 | 44.8 | 46.9 | 44.6 | 43.4 | 46.2 | 46.6 | 46.9 | 56   | 56.5 | 56.3 | 56   | 55.6 | 63   | 63.3 | 62.2 | 62.2 | 63.1 | 62.5 | 67.2 |      | 58.6 |
| 23 X82130_1_Odontoglossum_ringspot_virus              | 44.6        | 43.7 | 43.6 | 46.1 | 44.1 | 42.8 | 45.4 | 45   | 46.4 | 57.9 | 58.5 | 58.5 | 58.5 | 56.3 | 60   | 60   | 59   | 60.3 | 60.4 | 60.2 | 57.2 | 58.6 |      |

\* In gray, all identity percentages of opuntia virus 2 where the two highest values, corresponding to tobamoviruses identified in cacti, are in bold.



Supp. Table 2. Primers used for RACE, RT-PCR, sequencing and full genome amplification of opuntia virus 2.

| Primer name              | Forward/<br>Reverse | Prime sequence 5'-3'            | Location (accession<br>MF434821) |
|--------------------------|---------------------|---------------------------------|----------------------------------|
| VP3374AsTobamo_5'end     | Reverse             | ACTTAGGACGGCGGTCTTGA            | 180-199                          |
| VP3373sTobamo_3'end      | Forward             | AGTTTCCTTATAACTGCACG            | 6280-6299                        |
| Tobamo_2nopF             | Forward             | CATTCCAAAGGCTGCCAACC            | 5488-5507                        |
| Tobamo_2nopR             | Reverse             | ACAGAGCGTAAACCAGACGG            | 6383-6364                        |
| VP3379sTobamo_genome     | Forward             | GTTTTTTATTACAAGAACAAGCACATTTTAC | 1-31                             |
| VP3380AsTobamo_genome    | Reverse             | TGGGCCCTACCCGGGGTAA             | 6453-6424                        |
| VP33401sTobamo_sequence  | Forward             | TCACAGCCTTTATGACATC             | 648-666                          |
| VP33402sTobamo_sequence  | Forward             | CAGCTTACCTATGAGAGTG             | 1288-1306                        |
| VP33403sTobamo_sequence  | Forward             | GCCTTCTGCTTCAACTCCTA            | 1929-1948                        |
| VP33404sTobamo_sequence  | Forward             | AAGCTGTGAGTATGCTGCG             | 2591-2609                        |
| VP33406AsTobamo_sequence | Reverse             | CCTAAGACGCAACGACCCTA            | 3807-3788                        |
| VP33407AsTobamo_sequence | Reverse             | CCTGACCTGAACATCGTCA             | 4503-4485                        |
| VP3390AsTobamo_sequence  | Reverse             | GGCGAACAGTCTTGACACAG            | 5033-5013                        |
| VP3353AsTobamo_sequence  | Reverse             | GCAAGCTGGGATCGCAACT             | 5779-5761                        |