Identification and characterization of a new potyvirus infecting cucurbits

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Received: 16 August 2017 / Accepted: 20 October 2017
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Abstract
A new potyvirus, tentatively named cucurbit vein banding virus (CVBV), was identified in crops of cucurbits in San Pedro (Buenos Aires, Argentina). The complete genome sequences of two isolates of CVBV were obtained by next-generation sequencing (Illumina). The genomic RNA consisted of 9968 and 9813 nucleotides, respectively, and displayed typical potyvirus organization. The percentage identity for these two genome sequences, using BLASTn, was 77% to sweet potato virus c and 73% to tomato necrotic stunt virus. BLASTx analysis of the complete polyprotein showed that the most closely related virus is plum pox virus, with 48% amino acid sequence identity for both isolates. Sequence comparisons and phylogenetic analyses indicate that CVBV belongs to a previously undescribed species in genus Potyvirus.

Keywords Potyvirus · Squash · Viruses · Illumina sequencing

Diseases caused by viruses represent a constant threat to the production of cucurbits in all cultivated areas worldwide. In recent years, the number of identified virus species that infect cucurbits has been steadily increasing. This is probably due to improved methods of identification of plant viruses and an increased exchange of plant materials between globalized economies [18–20, 28, 34]. Some viruses induce significant damage to crops, affecting both the yield and quality of the fruit. In Argentina, viral diseases, especially those spread by aphids, are considered the main limiting factors in terms of yield and quality of fruit for some crops [27].

In Argentina, three of the four most common cucurbit-infecting potyviruses occur: watermelon mosaic virus (WMV), papaya ringspot virus (PRSV) and zucchini yellow mosaic virus (ZYMV) [6, 10, 11, 26].

Among viruses that infect cucurbits, potyviruses are some of the most important because of the damage they cause and the number of species that have been reported, which is constantly increasing. Twelve potyviruses have been reported to naturally infect cucurbits: Algerian watermelon mosaic virus (AWMV), clover yellow vein virus (CIYVV), melon vein-banding mosaic virus (MVBMV), Moroccan watermelon mosaic virus (MWMV), papaya ringspot virus (PRSV), turnip mosaic virus (TuMV), watermelon leaf mottle virus (WLMV), watermelon mosaic virus (WMV), zucchini yellow fleck virus (ZYFV), zucchini yellow mosaic virus (ZYMV) and more recently zucchini tigré mosaic virus (ZTMV) and zucchini shoestring virus (ZSTV) [12, 18, 29].

Plant tissue was collected from squash (Cucurbita maxima) samples displaying severe symptoms, collected during 2012 surveys done in the San Pedro, Buenos Aires province of Argentina.

A RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) was used, following the manufacturer’s instruction, to extract total RNA from 100 mg of tissue. This was submitted to INDEAR (Genomics and Bioinformatics Platform, INDEAR Inc., Rosario, Argentina) for synthesis of cDNA from polyadenylated RNA followed by deep sequencing using an Illumina HiSeq 1500 with a read length average of 100 bp.
Contigs were de novo assembled as part of the INDEAR bioinformatics services [24]. Virus consensus sequences were constructed using contigs from Mira 4. In addition, manually curated assemblies were made using Geneious 9.1 (Biomatters) before proceeding with the annotation [14]. Reads for the full-length genome of two isolates were assembled with coverage of 8823 and 2430 reads/nt. Two large viral contigs of 9968 and 9813 nt were thereby generated, Seq3.1 and Seq4.1 and the sequences were deposited in GenBank under accession numbers KY657266 and KY657267.

The complete sequences of 103 potyviruses, obtained from the NCBI Viral database, were used for comparison against the two novel CVBV isolates using BLAST. The percentage identity obtained for these two contigs, using BLASTn and the GenBank data, was 77% with a 523-nt segment of sweet potato virus c isolate SPVC-Arg (KF386015.1) (5% coverage) and 73% nt sequence with an 884-nt segment of tomato necrotic stunt virus (JX846918.1) (9% coverage). On the other hand, BLASTx analysis of the complete encoded polyprotein showed that the most closely related virus is plum pox virus, with 48% amino acid sequence identity for both isolates.

The molecular taxonomic position of CVBV was estimated through Pairwise Sequence Comparison (PASC) of the two CVBV isolates with 1273 non-redundant sequences representing the family Potyviridae, using BLAST-based alignments [4]. The complete CVBV genome had 50.85% and 50.32% pairwise identity, for isolate Seq3.1 and Seq4.1 respectively, with other members classifiable within the genus Potyvirus (http://www.ncbi.nlm.nih.gov/sutils/pasc/).

When aligned with each other, the two sequences shared 87.82% nt identity and 93.07% aa identity. In addition, Seq3.1 was the closest genome to Seq4.1 and showed 86.23% identity using the BLAST-based alignment method performed by PASC. The species demarcation criteria for potyviruses suggested by ICTV is <76% nucleotide identity and <82% amino acid identity [2]. Taking into account our analyses suggests that Seq3.1 and Seq4.1 belong to the same species [4] which are members of a distinct and novel species in the genus Potyvirus.

Possible recombination events within the genome sequence of CVBV were tested using complete genomes of 26 potyviruses and the RDP4 package. Seven programs included in the package were used with default parameters and a Bonferroni corrected P-value cut-off of 0.01; specifically, RDP, GENECONV, MaxChi, Chimaera, 3Seq, BootScan and SiScan [22]. A recombination pattern is considered to be a positive event if it is detected by four or more of these programs with high probability [15, 31]. No evidence of recombination was discovered within the genome sequence of CVBV.

Open reading frames (ORFs) within the CVBV genome were predicted using ORF Finder (http://www.ncbi.nlm.nih.gov/). The data provided by Adams et al. [1] was used for the identification of the cleavage sites and information from Chung et al. [5] was useful in identifying the PIPO sequence.

A single open reading frame was found in the nucleotide sequences of both isolates, confirming a typical potyvirus genomic organization. This large ORF (9507nt) encodes a polyprotein of 3168 aa with an estimated MW of 359.5 kDa (http://web.expase.org/compute_pi/) (169-9675 Seq4.1 and 164-9670 Seq3.1). This large polyprotein is likely cleaved into a set of ten functional proteins: P1, HC-Pro, P3, 6K1, CI, 6K2, VPg, Nla-Pro, Nib and CP, by three virus-encoded proteases with a gene order that is conserved throughout the family. Nine putative cleavage sites in CVBV were identified through reference to a study of protease cleavage sites by Adams et al. [1] (Fig 1). Also, the PIPO ORF (60aa) was identified downstream of the highly conserved motif GA6 at position nt 2967-3147 in Seq3.1 and nt in 2963-3143 Seq4.1.

Conserved amino acid motifs were identified, including Hx8Dx32SG21RG. Downstream FVLRG in Seq3.1 and FILRG in Seq 4.1 were present in P1, instead of the more common FIVRG, which has proteinase activity and structural features. The conserved motif FRNKX12CDN, which is involved in symptom developments [8, 30] and KITC, which is involved in aphid transmission, were present at the N terminal regions of the HC-Pro protein and were found in both isolates. Moreover, as observed in other potyviruses the highly conserved PTK domain present in the HC-Pro region, was changed to PTR. The PTR motif is involved in transmission by aphids and its presence has been observed in MWMV and AWMV, two potyviruses that infect cucurbit crops [32, 33].

Fig. 1 Representation of the CVBV genome organization, showing the predicted cleavage site positions, based on the amino acid sequence of the Seq3.1 isolate (KY657266). Regions analyzed are: P1, HC-Pro, P3, 6K1, CI, 6K2, VPg, Nla-Pro, Nib and CP

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The motifs GAVGSGKST and EPTRPL located in the CI protein region, are involved in NTP binding and helicase activity, respectively. The conserved motifs QPSTVVDN and GDD were observed in the nuclear inclusion b (NIb) protein in both CVBV isolates, a motif essential for RNA polymerase activity. Also, the DAG motif, involved in aphid transmission, is present at the N-terminus of the CP [3]. Finally, other consensus motifs found in the CP were MVW-CIENGTP, AFDF, and QMKAAAL [21, 23].

Although the viral vector has not yet been identified, the presence of the above-mentioned highly conserved aphid transmission motifs, KIIC, PTR (HC-Pro) and DAG (CP), suggest that CVBV is most likely transmitted by aphids.

Phylogenetic analyses were conducted using MEGA 7 [17]. Complete sequences of the two CVBV isolates and 103 complete potyvirus genome sequences from GenBank were used to construct a phylogenetic tree. Agropyron mosaic virus and Hordeum mosaic virus were used as outgroups since they are classified in the Rymovirus genus, considered the closest relative of potyviruses [7]. A Maximum Likelihood tree was then constructed based on the JTT matrix-based (+G+I) model and 500 bootstrap replicates [13]. The JTT +G+I model was selected using the Akaike information criterion (AIC), GTR +G+I was also selected for nucleotide data (not shown) and both substitution models were inferred with MEGA.

When analyzed phylogenetically, CVBV does not fit into an existing group but is most closely related to Catharanthus mosaic virus (CatMV) which until now was a stand-alone (singleton), mentioned by Koh [16]. Now, these viruses form a new cluster within the genus; a cluster which has high bootstrap support (100) (Fig. 2).

![Fig. 2 Condensed maximum likelihood tree inferred from full potyvirus amino acid sequences. A Maximum Likelihood tree was then constructed using the JTT model (+G+I). The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 103 potyviruses amino acid sequences. Sequences of Agropyron mosaic virus and Hordeum mosaic virus (genus Rymovirus, family Potyviridae) were used as outgroups. Supergroups PVY and BCMV are indicated as triangles.](image-url)

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It has been suggested that there was an initial radiation of potyviruses 7000 years ago as a result of adaptation to agriculture. Subsequently, all potyviruses appear to evolve at the same rate, showing the same distance from this point of initial radiation [9]. Taking into account this new cluster and analyzing all complete potyvirus sequences available in GenBank (103), the lineage succession proposed by Gibbs [9] can now be improved. The initial divergence gave rise to the onion yellow dwarf virus (OYDV) group, followed by donkey orchid virus A, Hippeastrum mosaic virus and the new CatMV group, followed by the sugarcane mosaic virus group, and the two super groups, PVY and BCMV (Fig 2).

Of note, CVBV does not belong to either of the two most common groups of cucurbit potyviruses; the PRSV or BCMV groups.

To verify the sequence and confirm the presence of CVBV in field samples, RT-PCR was performed using two pairs of CVBV specific primers. The primers were designed based on the sequences of the two isolates, CPF (5′-CAACAGGCACATTCTCGCTC-3′) and CPR (5′-GCAAGGTCCGTTTGGCATT-3′) for amplification of a 357pb fragment of the CP region (conserved). PIF (5′-CAACATTACGCGAAA-3′) and PIR (5′-CCATTGTTTGCAGGCT-3′) were designed for amplification of a 400pb fragment of the P1 region (variable). Total RNA was extracted from symptomatic leaves with CTAB methods. A one-step RT-PCR protocol was performed in a total volume of 25 µl as described in Mohammed [25]. The resulting RT-PCR products were purified (Genomic DNA Clean & Concentrator Zymo) and sequenced via conventional Sanger dideoxy sequencing at the Genomics Unit of the Biotechnology Institute-INTA (Argentina).

In addition, a DAS-ELISA test was performed for the samples collected, using antisera against ZYMV, PRSV, WMV, squash mosaic virus (SqMV) and cucurbit yellow stunting disorder virus (CYSDV). The serological testing kits were all specific commercial antisera from BiorebaAG (Switzerland). In order to test the putative presence of other viruses, PCR was performed with universal begomovirus primers and specific primers for cucurbit aphid-borne yellows virus (CABYV) and cucumber vein yellowing virus (CVYY).

Samples were chosen from different regions of Argentina and different Cucurbitaceae species, all of them showing virus-like symptoms. Bands of the expected size were observed by RT-PCR with specific CVBV primers. The sequences obtained from RT-PCR were identical to that gained from the contigs, confirming the presence of CVBV in the nine samples from San Pedro, Buenos Aires, collected in 2012. All these samples were also co-infected with the other potyviruses tested. CVBV was also detected in two samples from Córdoba province, one in San José de la Dormida and another in the peri-urban area of Córdoba. These results confirm the presence of the CVBV in the Buenos Aires and Córdoba province, Argentina. A vein banding symptom was observed in the sample from the peri-urban area. Surprisingly, this sample result was negative for all other viruses tested. As such we proposed cucurbit vein banding virus as the name for this new virus (Fig 3).

Altogether, our results lead us to conclude that CVBV is a member of a distinct Potyvirus species present in squash in the Cordoba and Buenos Aires provinces of Argentina. CVBV appears to be often found in mixed infections with other potyviruses, such as WMV, ZYMV and PRSV.

Fig. 3 (a) Squash plant showing virus disease symptoms, (b) strong vein banding observed in a leaf infected with CVBV
Further studies are needed in order to develop a complete biological characterization of CVBV. Issues such as host range, vector transmission, epidemiology and other properties are relevant for the proper management of viral disease in cucurbits in order to reduce damage.

Acknowledgements This study was carried out at IPAVE-CLAP-INTA and was partially supported by INTA and CONICET. Dr. Luis Conci kindly provided positive samples.

Compliance with ethical standards

Funding This study was funded by CONICET (PIP no. 112-20110111016) and INTA (PNHFA 1106072, PNPV 1135024).

Conflict of interest All 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.

References

1. Adams MJ, Antoniw JF, Beaudoin F (2005) Overview and analysis of the polyprotein cleavage sites in the family Potyviridae. Mol Plant Pathol 6:471–487
2. Adams MJ, Antoniw JF, Fauquet CM (2005) Molecular criteria for genus and species discrimination within the family Potyviridae. Arch Virol 150:459–479
3. Atreya PL, Atreya CD, Pirone TP (1991) Amino acid substitutions in the coat protein result in loss of insect transmissibility of a plant virus. Proc Natl Acad Sci USA 88:7887–7891
4. Bao Y, Chetvernin V, Tatusova T (2014) Improvements to pairwise sequence comparison (PASC): a genome-based web tool for virus classification. Arch Virol 159:3293–3304. https://doi.org/10.1007/s00705-014-2197-x
5. Chung BY, Miller WA, Atkins JF, Firth AE (2008) An overlapping essential gene in the Potyviridae. Proc Natl Acad Sci USA 105:5897–5902
6. Feldman JM, Gracia O (1992) Un nuevo virus de cucurbitáceas en Argentina: el virus de la mancha anillada de la papaya. Revista de Investigaciones Agropecuarias 23:63–67
7. French R, Stenger D (2005) Genome sequences of Agropyron mosaic virus and Hordeum mosaic virus support the reciprocal monophyly of the genera Potyvirus and Rymovirus in the family Potyviridae. Arch Virol 150:299–312
8. Gal-On A (2000) A point mutation in the FRNK motif of the potyvirus helper component-protease gene alters symptom expression in cucurbits and elicits protection against the severe homologous phytopathology 90:467–473
9. Gibbs AJ, Ohshima K (2010) Potyviruses and the digital revolution. Annu Rev Phytopathol 48:205–223
10. Gracia O (2000) First Report of Zucchini yellow mosaic virus in Argentina. Plant Dis 84:371
11. Gracia O, Feldman JM (1986) Virus identifieds in cultivos of cucurbitáceas. IDIA 445–448:1–6
12. Ibaba JD, Laing MD, Gubba A (2016) Zucchini shoestring virus: a distinct potyvirus in the papaya ringspot virus cluster. Arch Virol 161:2321–2323
13. Jones DT, Taylor WR, Thornton JM (1992) The rapid generation of mutation data matrices from protein sequences. Comput Appl Biosci 8:275–282
14. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Mientjes P, Drummond A (2012) Geneious basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28:1647–1649
15. Keohé MA, Coutts BA, Buirchell BJ, Jones RAC (2014) Split personality of a potyvirus: to specialize or not to specialize? PloS One 9(8):e105770
16. Koh SH, Li H, Admiraal R, Jones MGK, Wylie SJ (2015) Catharanthus mosaic virus: a potyvirus from a gymnosperm, Welwitschia mirabilis. Virus Res 203:41–46. https://doi.org/10.1016/j.virusres.2015.03.007
17. Kumar S, Stecher G, Tamura K (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for larger datasets. Mol Biol Evol 33:1870–1874
18. Lecoq H, Desbiez C (2012) Viruses of cucurbit crops in the Mediterranean region: an ever-changing picture. Adv Virus Res 84:67–126
19. Lecoq H, Katis N (2014) Cucurbit virus control. In: Loebenstein G, Katis N (eds) Advances in virus research control of plant virus diseases: seed-propagated crops. Academic Press is an imprint of Elsevier, San Diego, London, pp 255–296
20. Lecoq H, Wisler GC, Pitrat M (1998) Cucurbit viruses: the classics and emerging. In: McCreight JD (ed) Cucurbitaceae ‘98, evaluation and enhancement of cucurbit germplasm. ASHS, Alexandria, pp 126–142
21. Maciel SC, Silva RF, Da Reis MS, Jâdão AS, Rosa DD, Giampan JS, Kitajima EW, Rezende JAM, Camargo LE (2011) Characterization of a new potyvirus causing mosaic and flower variation in Catharanthus roseus in Brazil. Sci Agric 68:687–690
22. Martin DP, Murrell B, Golden M, Khoosal A, Muhire B (2015) RDP4: detection and analysis of recombination patterns in virus genomes. Viruses 1:1–5
23. Miglino R, Druffel KL, Pappu HR (2010) Identification and molecular characterization of a new potyvirus infecting Triteleia species. Arch Virol 155:441–443
24. Minoche AE, Dohm JC, Himmelbauer H (2011) Evaluation of genomic high-throughput sequencing data generated on Illumina HiSeq and Genome Analyzer systems. Genome Biol 12:R112. https://doi.org/10.1186/gb-2011-12-11-r112
25. Mohammed HS, Zicca S, Mangili A, Mohamed ME, El Siddig MA, Tomassoli L, El Hussein AA (2014) Identification and phylogenetic analysis of common pumpkin viruses in Sudan. J Plant Pathol 96:77–84
26. Nome SF, March GJ, Giorda LM (1974) Disminución de la productividad de plantas de zapallito de tronco (Cucurbita maxima Duch var. zapallito (Carr. Millán) infectadas con el virus del mosaico de la sandía, raza 2 (Watermelon Mosaic Virus-2). IDIA, pp 321–324
27. Perotto MC, Celli MG, Pozzi EA, Luciani CE, Conci VC (2016) Occurrence and characterization of a severe isolate of Watermelon mosaic virus from Argentina. Eur J Plant Pathol 146:213–218
28. Provvidenti R (1996) Diseases caused by viruses. In: Zitter TA, Hopkins DL, Thomas CE (eds) Compendium of cucurbit diseases. American Phytopathological Society, St. Paul, pp 37–45
29. Romay G, Lecoq H, Desbiez C (2014) Zucchini tigre mosaic virus is a distinct potyvirus in the papaya ringspot virus cluster: molecular and biological insights. Arch Virol 159:277–289
30. Shibolet Y, Haronsky E, Leibman D, Arazi T, Wassengger M, Whitham SA, Gaba V, Gal-On A (2007) The conserved FRNK box in HC-Pro, a plant viral suppressor of gene silencing, is required for small RNA binding and mediates symptom development. J Virol 81:13135–13148
31. Wylie SJ, Jones RAC (2009) Role of recombination in the evolution of host specialization within *Bean yellow mosaic virus*. Phytopathology 99(5):512–518

32. Yakoubi S, Desbiez C, Fakhfakh H, Wipf-Scheibel C, Marrakchi M, Lecoq H (2008) Biological characterization and complete nucleotide sequence of a Tunisian isolate of *Moroccan watermelon mosaic virus*. Arch Virol 153:117–125

33. Yakoubi S, Lecoq H, Desbiez C (2008) *Algerian watermelon mosaic virus* (AWMV): a new potyvirus species in the PRSV cluster. Virus Genes 37:103–109

34. Zitter TA, Hopkins DL, Thomas CE (1996) Compendium of cucurbit diseases. APS Press, St. Paul