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BRIEF REPORT

Identification and characterization of a new potyvirus infecting cucurbits

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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 (ClYVV), 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.

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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, NIa-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 GA_6 at position nt 2967-3147 in Seq3.1and 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 $FRNKX_{12}CDN$, 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].

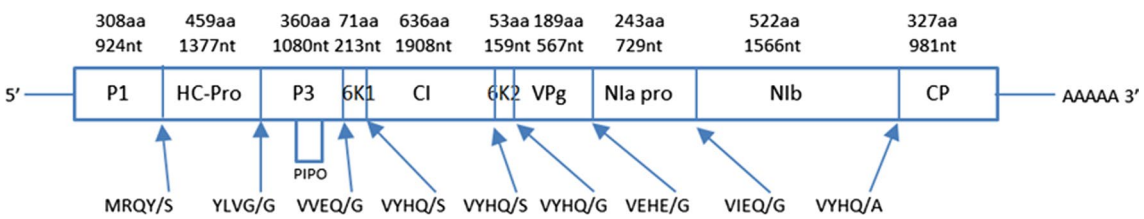

Fig. 1 Representation of the CVBV genome organization, showing the predicted cleavage site positions, based on the amino acid sequence of the Seq3.1 isolate (KY657266). Regions analyzed are: P1, HC-Pro, P3, 6K1, CI, 6K2, VPg, NIa-Pro, Nib and CP

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 MVWCIENGTSP, 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, KITC, 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).

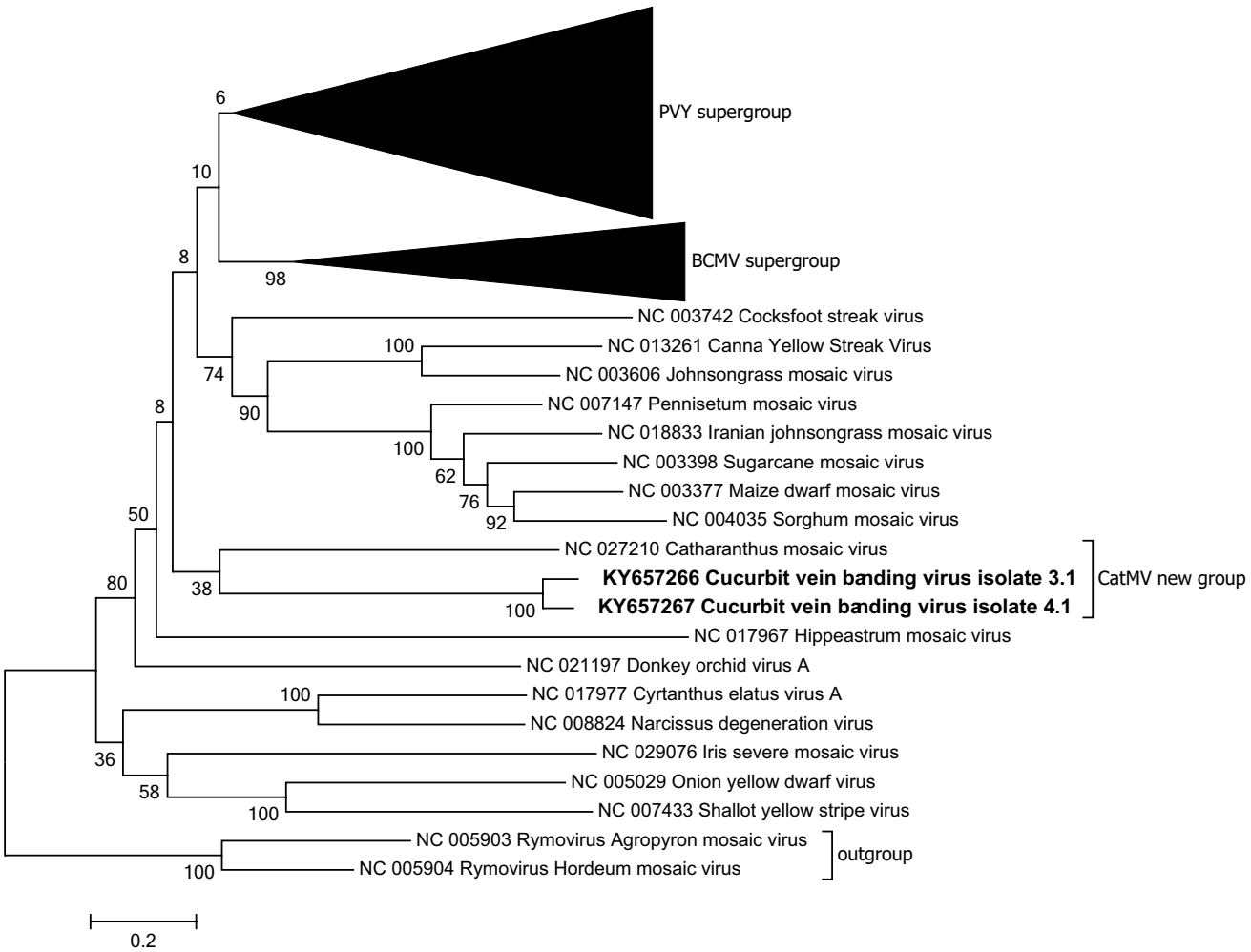

Fig. 2 Condensed maximum likelihood tree inferred from full potyvirus amino acid sequences. A Maximum Likelihood tree was then constructed using the JTT model (+G+I). The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with 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

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′-GCAAGGTCGGTTTGGCATTC-3′) for amplification of a 357pb fragment of the CP region (conserved). P1F (5′-CAAACATTCAGCGCAAACT-3′) and PIR (5′-CCATTTTTGTCACGCTGGTT-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 (CVYV).

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.

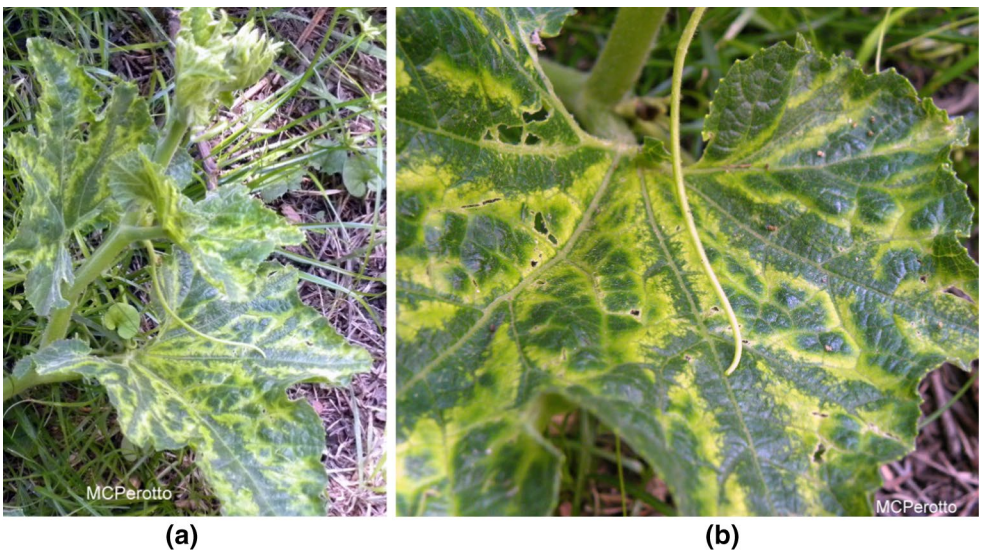

Fig. 3 (a) Squash plant showing virus disease symptoms, (b) strong vein banding observed in a leaf infected with CVBV

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.

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Compliance with ethical standards

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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.

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