Molecular Characterization of Novel *Cucurbit Aphid Borne Yellows Virus* Strains Infecting Squash and Watermelon in India

Nagendran Krishnan (krishnagendra@gmail.com)
IIVR: Indian Institute of Vegetable Research https://orcid.org/0000-0001-7473-0100
Shweta Kumari
IIVR: Indian Institute of Vegetable Research
Koshlendra Kumar Pandey
IIVR: Indian Institute of Vegetable Research
Sudhakar Pandey
IIVR: Indian Institute of Vegetable Research
Tusar Kanti Behera
IIVR: Indian Institute of Vegetable Research
Karthikeyan Gandhi
Tamil Nadu Agricultural University
Lawrence Kenyon
AVRDC World Vegetable Center

Research Article

Keywords: Cucurbit aphid-borne yellows virus (CABYV), Molecular Characterization, squash and watermelon, India

Posted Date: January 11th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1202229/v1

Abstract

The pathogen responsible for yellowing and downward rolling of leaves of squash and watermelon plants from Uttar Pradesh state, India, was identified as probably strains of *Cucurbit aphid-borne yellows virus* (CABYV) through RT-PCR using universal Polerovirus primers followed by sequencing. The full-length genome sequences of an isolate from squash (POL-SQ - 5650 nt) and one from watermelon (POL-WM - 5647nt) were determined by sequencing the products from RT-PCR with six sets of primers with overlapping products. Sequence comparison and phylogenetic analysis showed that these isolates had closest identity with a recombinant strain obtained between CABYV and *Melon aphid-borne yellows virus* (MABYV) reported from Taiwan infecting *Luffa aegyptiaca* (CABYV-R-TW82) rather than other Asian, American, or European isolates. The deduced amino acid sequences of the P0, P1 and P1-P2 proteins showed >10% variation, whereas the P3, P4 and P3-P5 proteins showed <10% variation when compared to the corresponding proteins of other strains of CABYV worldwide. Thus, according to the *Polerovirus* species demarcation threshold, these new sequences should be regarded as representing strains of a novel previously undescribed *Polerovirus* species. However, based on their sequence similarity and phylogenetic grouping with the recombinant strain from Taiwan we suggest these sequences represent recombinant strains of CABYV. These are the first full-length genome sequences for CABYV strains from India and this study adds watermelon as host for CABYV in India.

Main Text

The genus *Polerovirus* has recently been moved from the Family *Luteoviridae* to the Family *Solemoviridae* [1] and currently comprises 26 virus species formally ratified by the International Committee on Taxonomy of Viruses [2]. Poleroviruses are transmitted by specific aphid species in a circulative and non-propagative manner and are not mechanically transmitted through sap [3]. The cultivation of cucurbitaceous vegetable crops is greatly challenged by several *Polerovirus* species. In South and Southeast Asia, the main viruses are *Cucurbit aphid-borne yellows virus* (CABYV), *Melon aphid-borne yellows virus* (MABYV), and *Suakwa aphid-borne yellows virus* (SABYV) which confer severe loss in both quantity and quality [4-5]. Lecoq et al., [6] reported CABYV causing yield loss of 40-50% on melon and cucumber in France. *Pepo aphid-borne yellows virus* (PABYV) and *Luffa aphid-borne yellows virus* (LABYV), from Mali and Thailand respectively, are other poleroviruses found infecting cucurbit species [7].

Poleroviruses are positive sense single stranded RNA viruses with a genome size of about 5.7kb and comprising 6 open reading frames (ORFs). The three ORFs of the 5’ proximal end are separated by an intergenic region of about 200 nucleotides from the three ORFs in the 3’ proximal end. The proteins coded by the ORFs are a suppression of post transcriptional gene silencing (P0), a protease and genome linked protein (P1), an RNA- dependent RNA polymerase (P1-P2 fusion through ribosomal frameshift), a coat protein (P3), a movement protein (P4) and a possible aphid transmission factor (P3-P5 fusion protein as read-through translation from subgenomic mRNA). The genome of poleroviruses differs from that of enamoviruses (another genera of the Family *Solemoviridae*) by the presence of ORF4 within ORF3, and

the size of ORF5 is greater than 1200 nt [8-9]. The species demarcation threshold for poleroviruses is < 90 % identity at amino acid level of any protein [8]. Infections of poleroviruses are emerging as threats causing yellowing disease to cucurbit cultivation in the Indian subcontinent. This study presents the first full-length genome sequence of CABYV strains infecting squash and watermelon in India through reverse-transcription polymerase chain reaction (RT-PCR) and sequencing.

In 2018, during a survey conducted in Varanasi district of Uttar Pradesh, virus-like symptoms of yellowing and downward rolling of leaves with stunted plant growth reminiscent of *Polerovirus* infection were observed in squash and watermelon crops (Fig 1). Eight leaf samples each of squash and watermelon were collected from different farmer's field. Total RNA was extracted from 100 mg of each fresh plant sample using the TRI reagent® (Sigma Aldrich, USA) according to the manufacturer's instructions. A two-step RT-PCR assay was performed to determine the presence of polerovirus. In the first step, cDNA was synthesized from the extracted RNAs using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Reactions were performed at 42 °C for 60 min followed by incubation at 70 °C for 5 min. For preliminary *Polerovirus* detection, PCR was performed with the cDNAs using universal *Polerovirus* primer pair (PolGenUp2/Down2) targeting part of the RdRp gene of the *Polerovirus* genome with an amplification product size of 593bp [10]. When a subset of eight samples of each crop was tested by RT-PCR, five watermelon samples and four squash samples showed positive for the presence of *Polerovirus* RNA. The symptoms in these *Polerovirus* negative samples might be attributed to other cucurbit infecting viruses such as criniviruses, begomoviruses, potyviruses etc., or abiotic stresses such as nutritional deficiency or senescence. The results were further validated by directly sequencing the amplicons (partial RdRp gene) which showed that the virus detected in the squash and watermelon samples had >92% identity by BLASTn analysis with previously reported CABYV strain sequences. The serological detection of CABYV by ELISA was not used in this study because the available CABYV antisera are reported to give unreliable results and may be virus strain and host specific [11-13]. Based on the partial sequencing, CABYV has been reported earlier in India on bitter gourd and teasel gourd [14-15]. Recently, we reported the occurrence of the three poleroviruses, CABYV from squash, MABYV from ivy gourd (*Coccinia grandis*), and LABYV from pumpkin, bitter gourd, and sponge gourd, in Uttar Pradesh state, India, based on partial nucleotide sequence of the RdRp gene [16].

To amplify the complete genome, PCR was performed with 6 sets of primer pairs designed from multiple alignments (using ClustalW) of different CABYV isolate sequences retrieved from NCBI/GenBank (Table 1) to produce six overlapping products that together would cover the whole *Polerovirus* genome. The amplified PCR products were purified using QIAquick® gel extraction kit (Qiagen, Germany) and cloned in the pTZ57r/T vector system (Thermo Scientific) and clones were sequenced in both directions by Delhi University-South Campus (New Delhi, India). Sequences were assembled using ClustalO (https://www.ebi.ac.uk/Tools/msa/clustalo/) and the ORFs were annotated using the ORF finder tool (https://www.ncbi.nlm.nih.gov/orffinder/). When the nucleotide sequences of the six overlapping RT-PCR products were assembled, the complete genome sequence of the virus from a squash sample (POL-SQ) and from a watermelon sample (POL-WM) were determined to be 5650 nt and 5647 nt, respectively (Deposited as GenBank Accession numbers MN688219 and MN688220, respectively). Both the

sequences possessed the typical genome organization of poleroviruses and presence of the ORF0 and ORF4 in both the isolates clearly distinguishes them from the species of the genus *Enamovirus*. All the predicted ORFs and the deduced protein sequences are of the expected size and possess the conserved regions typical of other *Polerovirus* sequences available in the NCBI/GenBank database.

The slippery sequences and fusion protein prediction for both the newly determined full-length sequences were performed with available diverse cucurbit infecting *Polerovirus* sequences from the NCBI/GenBank database. The slippery heptamer sequence, GGGAAAC, with the corresponding pseudo-knot structure was predicted at genome position 1476. These predicted slippery heptamer sequences allow the translation of the P1–P2 fusion proteins by shifting the P2 sequence into the correct reading frame. The read-through translation of ORF5, which follows the translation of ORF3 and results in the P3–P5 protein, was found to be in-frame for both full-length Varanasi sequences. In addition, for P0, which was identified as the most variable protein sequence of the *Polerovirus* genome (Supplementary Table 1. and 2.), the start of the F-box consensus sequence (LPxxI/ L) implicated in silencing suppressor function was also identified at nt 186–212 for both the isolates based on the previous reports.

A full-length sequence from squash and watermelon sample were aligned with the cucurbit infecting polerovirus sequences from different parts of the World using ClustalW and their phylogenetic relationships were determined by the neighbour joining method in MEGA X with 1000 bootstrap replicates [17]. Sequence identity percentage at the nucleotide and aminoacid levels were calculated using the species demarcation tool (SDT Version 1.2) [18]. Phylogenetic analyses revealed that the two Indian cucurbit *Polerovirus* isolates were placed within a distinct clade with CABYV-R-TW82 (JQ700306) and CABYV-CZ (HQ439023) from Taiwan and China, respectively, both identified as recombinant strain between CABYV and MABYV (Fig 2.). When the molecular variability among the two Varanasi CABYV isolate sequences along with 16 CABYV, 2 MABYV, 2 PABYV, 3 LABYV and 1 SABYV isolate sequences from GenBank were compared at complete nucleotide and deduced amino acid level, variations were detected throughout the CABYV genomes. Over the entire genome sequence, both the Varanasi isolates showed the greatest nucleotide identity (>87.5%) to CABYV isolates HQ439023 and JQ700306 (Fig 3; Supplementary Table 1.). Among the different genomic regions of the CABYV isolates, high nucleotide diversity was observed over the 5’ region whereas the 3’ regions showed relatively low diversity values (Supplementary Table 1.).

The deduced amino acid sequences of the Varanasi squash and watermelon isolates had 71.0 to 71.9% identity at P0 and 82 – 83.8% identity at P1 with the isolate from Zucchini (*Cucurbita pepo*) in China (HQ439023); 88.1 - 89.9% identity at P1-P2 with the recombinant isolate from Luffa (*Luffa aegyptiaca*) in Taiwan (JQ700306); 89.6 - 92.5% identity at P3-P5 with the isolate from squash (*Cucurbita* spp.) in China (GQ221223); 96 - 97% identity at P3 with the isolate from cucumber (*Cucumis sativa*) in Papua New Guinea (MG780352) and the isolate from bitter gourd (*Momordica charantia*) in Taiwan (JQ700305); and >91% at P4 with the isolate from squash in Spain (JF939814) (Supplementary Table 2.). The genome 5’ proximal ends were highly conserved having 100% nucleotide identity between the Varanasi isolates and several other CABYV isolates, whereas more significant nucleotide sequence variations were observed in

the 3’ proximal end, with the Varanasi isolates having highest nucleotide sequence identity of 94% with the Zucchini-China isolate (HQ439023) (Supplementary Table 1.). The current species demarcation threshold for poleroviruses is that if there is >10% amino acid variation in any of the six ORFs the sequence should be considered as representing a different *Polerovirus* species [9]. Although the other properties of the Varanasi isolates such as host range, vector transmission and serological affinity were not determined in this study, the finding of 11-29% variation in the amino acid sequences of the P0, P1, P1-P2, and P3-P5 proteins observed when compared to other CABYV isolates suggests that the Varanasi isolates should be considered as representing two strains of a distinct, previously unreported, species. Since the coat protein gene (P3) deduced amino acid sequences shared >93% identity with other CABYV isolates and the Varanasi isolates were more closely related to the recombinant strain of CABYV (R-TW82) reported earlier from Taiwan [19] than other species and strains of Polerovirus. Hence the Varanasi isolates were classified as strains of CABYV as described by Knierim et al., [19].

In conclusion, the *Polerovirus* strains infecting squash and watermelon in Varanasi were identified as closely related to previously described recombinant strains of CABYV. However, more intensive field surveys, particularly in unexplored areas, coupled with genetic diversity studies, will provide a better understanding of the distribution of different species, strains and recombinants of *Polerovirus* in India, and could shed light on when the virus(es) were introduced into India and how they have evolved and recombined. This greater understanding should lead to the development of more specific diagnostic tools for cucurbit-infecting *Polerovirus* species/ strains in India and the development of effective and sustainable measures for their control. There are still many questions regarding cucurbit-infecting *Polerovirus* host range, vector transmission, and geographic distribution/prevalence, as well as symptom induction and production losses caused in India, that should be addressed, and these will be the focus of future studies.

Declarations

Acknowledgement

Authors are thankful to the ICAR-IIVR, Varanasi for providing facilities in conducting the research activity. Resources of chemicals and sequencing services has been utilized from the budget of the project ECR/2016/000182 (Early Carrier Research Award) funded by Science and Engineering Research Board (Government of India) awarded to Nagendran Krishnan.

Funding

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Conflict of interest

The authors declare that they have no conflict of interest.

Author's contribution

Conceptualization: NK, KG, KKP

Methodology and investigation: NK, SK

Writing - Original draft preparation: NK, SK

Writing – Review and editing- LK, KG, TKB, SP

All authors have read and agreed to publish the manuscript.

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Table

Table 1. Details of newly designed overlapping primers to amplify full-length genome of CABYV

Primer ID	Sequence (5’-3’)	Location*	Annealing Temp. (°C)	Size (bp)
NS POL F1	ACAAAAGATACGAGCGGG	1-18	47	1126
NS POL R1	CATTTGATGACTCCCAATC	1108-1126		
NS POL F2	GCTAAAATAGTCTCCACTCGG	907-927	49	1061
NS POL R2’	GTGTTGYCGGCATATCTTG	1949-1967		
NS POL F3	CCTGGATTCAAACAGGTC	1875-1892	48	1190
NS POL R3	GAAACCTCGACTTTRAAACC	3045-3064		
NS POL F4	GATTCGAGTGATGGCTG	2939-2955	49	1151
NS POL R4	GACCTGGCACTTGATGG	4073-4089		
NS POL F5	CCATCTCTTATGAGTTGGACC	3869-3889	50	983
NS POL R5	CATTRCCCCCTTACGTG	4835-4851		
NS POL F6	GACCTTTGTRGCDGGAC	4566-4582	48	1054
NS POL R6	CGTTGCCTATCCTTTCG	5603-5619		

*Based on nucleotide sequence of CABYV isolate (JQ700305)

Figures

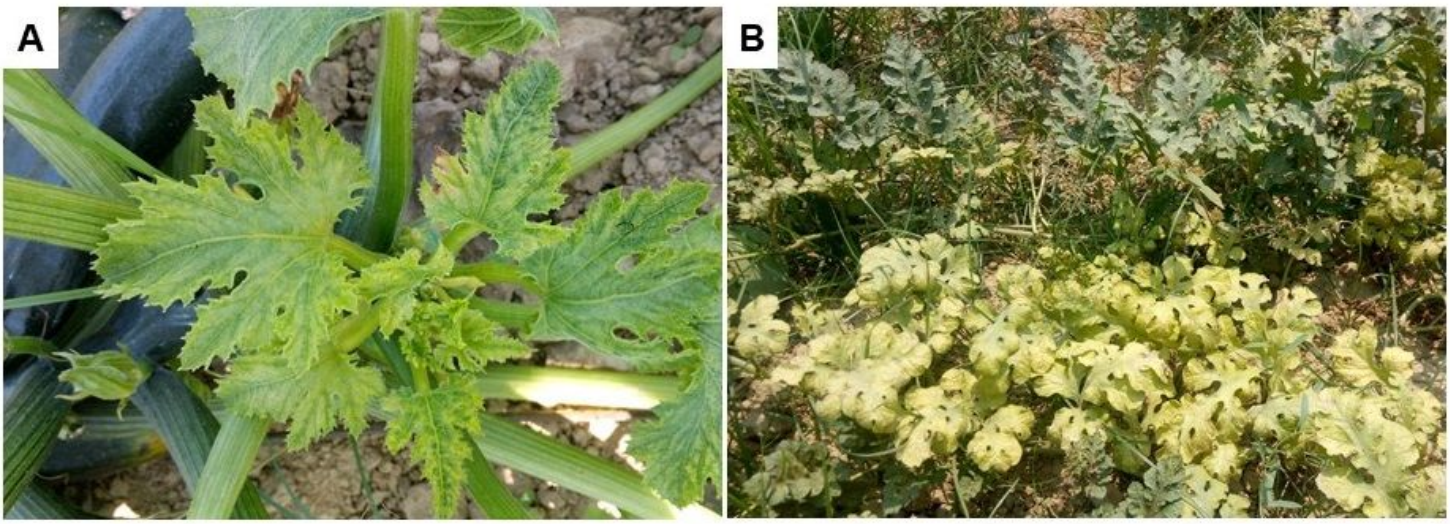

Figure 1

Yellowing and leaf rolling on Squash (A) and Watermelon (B) by infection of CABYV

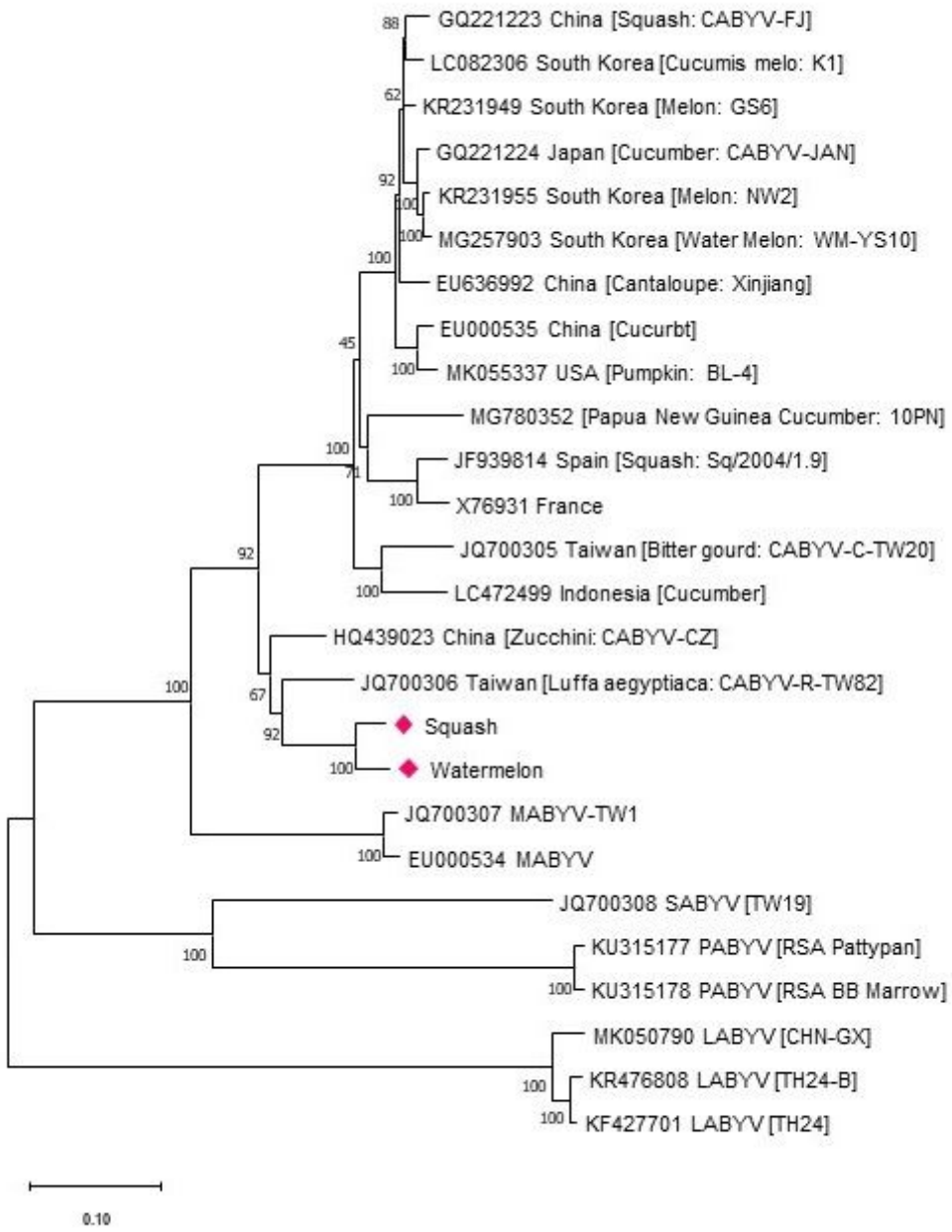

Figure 2

Phylogenetic analysis of *Polerovirus* species infecting cucurbits world-wide along with CABYV isolates of India infecting squash and watermelon based on full-length nucleotide sequences. This tree was generated using Neighbour-joining method in MEGA X. The bootstrap consensus tree values from 1000 replicates are given at the branch nodes.

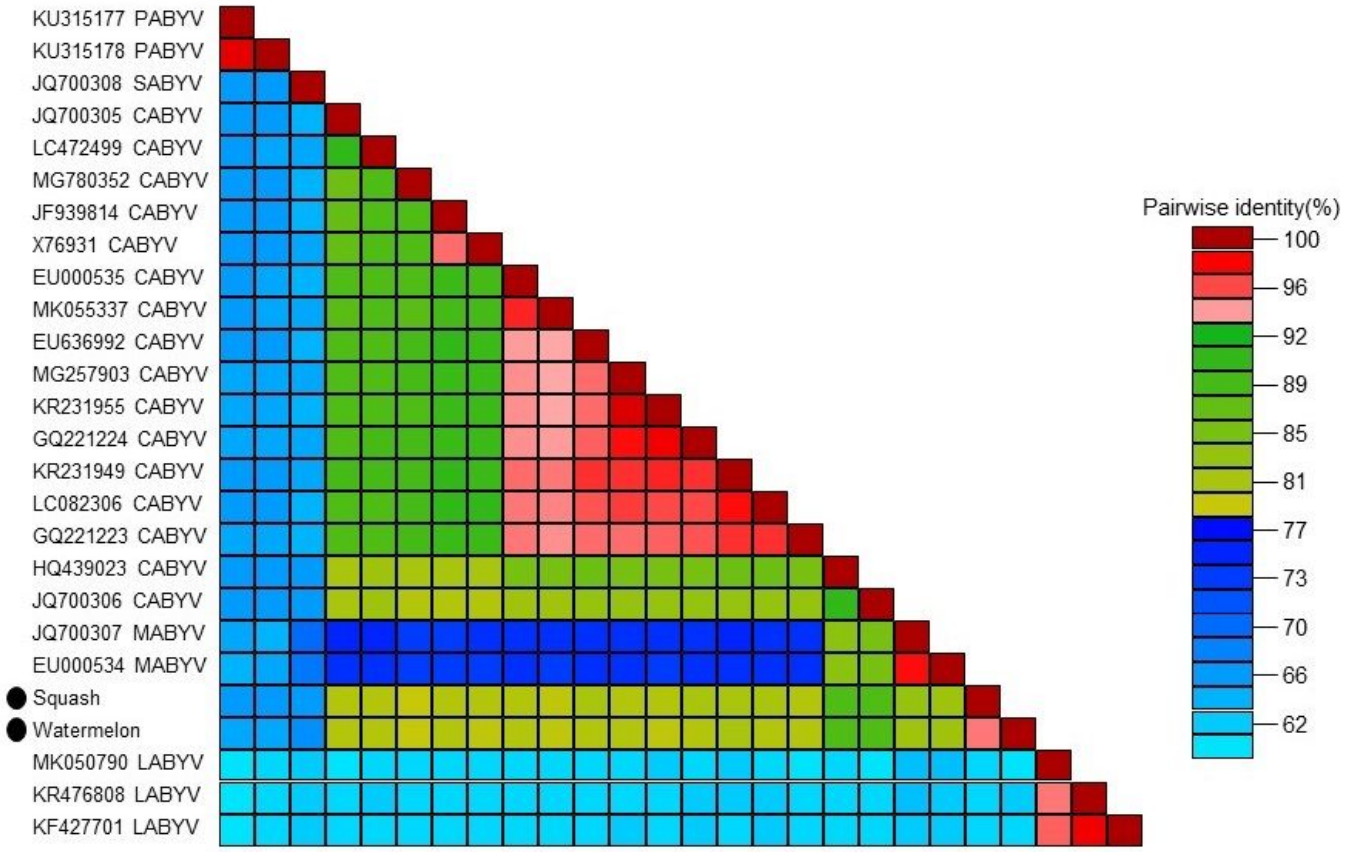

Figure 3

Pairwise nucleotide identity matrices of polerovirus (CABYV) infecting squash and watermelon in India along with other cucurbit infecting polerovirus reported previously worldwide based complete genome

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- ESMTable1.xlsx
- ESMTable2.xlsx