The mountain papaya: is a possible reservoir of the Kashmir bee virus?

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Background: Kashmir bee virus (KBV) is an RNA virus that affects hymenopteran species such as bees, wasps, and other pollinators, potentially contributing to population declines. KBV can cause death of bees from larvae to adult stage. KBV can propagate by vertical transmission, for example from queen to larvae, but also horizontally by food contamination. Pollinated plants constitute a source of KBV intra- and inter-species horizontal transmission, particularly by the contamination of pollen and flowers by feces of KBV-infected pollinators. **Result:** This study investigates the presence of KBV sequences in the transcriptomes of *Vasconcellea pubescens*, a commercially valuable plant species known as mountain papaya. We mapped transcriptome reads originated from fruit, leaves, and root tissues to the KBV reference genome obtaining 91% coverage, from which we produced a consensus sequence denominated Kashmir bee virus Ch. Phylogenetic analysis revealed that KBV Ch shares 97% nucleotide identity with the reference genome, and groups with other KBV strains isolated from Spain, Chile and New Zealand. Additionally, by using RT-PCR we detected KBV in *in vitro* cultivated plants, suggesting that *V. pubescens* may serve as a reservoir for KBV. **Conclusion:** This study marks the first detection of KBV outside of floral tissues in plants. The presence of KBV in mountain papaya highlights implications for ensuring virus-free propagation materials through in vitro cultivation practices .

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Abstract

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Conclusion: This study marks the first detection of KBV outside of floral tissues in plants. The presence of KBV in mountain papaya highlights implications for ensuring virus-free propagation materials through in vitro cultivation practices

Introduction

Kashmir bee virus (KBV) is a positive-sense, single-stranded RNA virus classified in the *Dicistroviridae* family within the *Cripavirus* genus (Mazzei et al., 2019). KBV primarily infects bee species such as *Apis cerana*, *Apis mellifera*, and bumblebees (*Bombus spp*) within the *Apidae* family. It has also been identified in wasps (*Vespula germanica*) from the *Vespidae* family (de Miranda et al., 2004). Notably, KBV can co-infect with closely related viruses, such as acute bee paralysis virus (ABPV) and Israel acute paralysis virus (IAPV), forming the AKI viral complex (de Miranda et al., 2004; Evans & Schwarz, 2011). KBV isolates have been reported globally, including Spain, New Zealand, South Korea, North America, Australia, and Chile (Berényi et al., 2006; Nanetti et al., 2021; Riveros et al., 2018; Tentcheva et al., 2004). The KBV reference genome spans 9,524 base pairs and contains two open reading frames (ORFs) that encode nonstructural and structural proteins (de Miranda et al., 2004). The 5' ORF encodes the non-structural polyprotein, comprising three domains corresponding to a helicase, a 3C protease domain, and 8 RNA polymerase domains that includes an RNA-dependent RNA polymerase. The 3' ORF encodes a structural polyprotein composed of two capsid protein domains belonging to the VP4 superfamily (de Miranda et al., 2004)

In honeybees, KBV primarily infects worker bees, but can spread throughout the colony under stressful conditions. Transmission occurs vertically from queen to offspring, and horizontally among bees through contaminated food, affecting individuals from the larval stage to adulthood (Meeus et al., 2014). Additionally, *Varroa destructor*, a common mite pest of bees, serves as a vector and activator of KBV and other viruses (Brødsgaard et al., 2000). Specifically, KBV has been identified in *V. destructor* (Shen et al., 2005). Survival of pathogenic KBV particles in mites can explain why different mite species may serve as potential routes of KBV infection and/or transmission (Carreck et al., 2010; Tixier, 2018). Moreover, increased stress levels in mite-infected hives further enhance KBV transmission dynamics (de Miranda et al., 2010).

Plants serve as potential sources of entomopathogenic viruses that affect pollinators through contamination of pollen and flowers. Recently the pollen virome has been explored (Fetters et al., 2022), revealing a wide diversity of plant and insect pathogenic viruses. The shared use of flowers by different pollinator species can facilitate horizontal transmission routes (Durrer & Schmid-Hempel, 1994), often through feces deposition (Figueroa et al., 2019). Recent research has emphasized the role of flowers (Alger et al., 2019), nectar, and pollen in facilitating inter-species viral transmission among pollinators, suggesting that plants may act as reservoirs for entomopathogenic viruses (Mcart et al., 2014). KBV has been detected in honeybee feces (Hung, 2000), parasitic mites (Shen et al., 2005), and pollen (Singh et al., 2010). Similarly, the closely related ABPV and IAPV have been found in flowers.

The mountain papaya (*Vasconcellea pubescens*), also known as Chamburo, Chilean papaya, or wild papaya (Salvatierra-González & Jana-Ayala, 2016), belongs to the *Caricaceae* family, which includes six genera and comprises a total of 21 species. *V. pubescens* is a perennial, herbaceous, and trioecious species capable of cross-pollination between male and female plants, occasionally exhibiting hermaphroditism (Chong-Pérez et al., 2018). The fruit is smaller in comparison to other papaya species, typically displaying a spherical to cylindrical shape with a green-yellow hue (Briones-Labarca et al., 2015). Additionally, the primary products derived from papaya include candies, preserves, juice, syrup, jams, and beers (Dotto & Abihudi, 2021). Moreover, it finds industrial applications in health, cosmetics, and pharmaceuticals (Gaete-Eastman et al., 2009).

Well-described phytopathogenic viruses infecting *Carica papaya* include papaya ringspot virus, papaya leaf distortion mosaic virus, papaya lethal yellowing virus, and papaya mosaic virus, among others (Abreu et al., 2015; Adams et al., 2005; Chávez-Calvillo et al., 2016; Razean Haireen & Drew, 2014; Yang et al., 2012). However, the virome of *V. pubescens* remains largely unexplored. Documenting viruses affecting this species is crucial due to their potential impact. Additionally, although not directly affecting papaya, the presence of entomopathogenic viruses could impact pollinating insects, such as bees, ~~which~~ interact with papayas (Badillo-Montaño et al., 2019). Therefore, establishing a viral library of mountain papaya could benefit both the agroindustrial and apiculture sectors. In this study, we conducted transcriptome analyses of leaf, root, and fruit tissues to establish a virome library of the mountain papaya collected from fields in Chile central region. Our findings reveal, for the first time, the presence of Kashmir bee virus (KBV) in mountain papaya. The detection of KBV viral sequences in roots, leaves, and *in vitro* cultivated plants suggests that papaya may act as a reservoir or vector for KBV infection.

Materials & Methods

Material Collection

Plant samples were collected from *V. pubescens* orchards in Lipimávida, Licantén, Vichuquén, Chanco, Pelluhue, Putú, Iloca, Duao, Constitución, and Curanipe, located in the coastal areas of the Maule region, Chile (Figure 1). Plant material was randomly selected using a zigzag pattern, covering the entire area of the orchard. Each tissue sample, including leaves, roots, and fruits, was collected in triplicate. This work was conducted as part of the project "Innovation Fund for Regional Competitiveness (FIC)", titled “Fortalecimiento de la Competitividad en la Industria Papayera de la Región del Maule Mediante el Desarrollo de Herramientas Biotecnológicas”, BIP: 40.001.007-0.

RNA Extraction and Sequencing

RNA extraction from papaya fruit, leaves, and roots was performed using the Spectrum™ Plant Total RNA Kit (SIGMA). Quantification of concentration and purity was carried out using the NanoDrop 2000 equipment (Thermo Scientific). Nine transcriptomes from leaves, roots, and fruits were sequenced. These transcriptomes were processed using the TruSeq Stranded mRNA

LT Kit (Illumina) at Genoma Mayor (Chile) and sequenced with Illumina technology. Sequencing data has been deposited in NCBI SRA under the PRJNA1142012 accession number.

Preliminary Treatment of Transcriptomes

FastQC was employed to assess the quality of raw sequences obtained with Illumina (v0.12.0) (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Trimmomatic was utilized to remove adapter sequences and low-quality reads (reads with ambiguous bases 'N' and reads with more than Q<20 bases) to obtain high-quality reads (v0.33) (Bolger et al., 2014; González et al., 2020). The Trinity software was used for *de novo* assembly from the concatenated high-quality reads of these libraries (v2014-04- 13) (Grabherr et al., 2011), utilizing default parameters.

Transcriptome Annotation

ORFinder (https://www.ncbi.nlm.nih.gov/orffinder/), blastn, and blastp (https://blast.ncbi.nlm.nih.gov/Blast.cgi) against nt/nr from NCBI were utilized to predict open reading frames (ORFs) from the assembled sequences (https://www.ncbi.nlm.nih.gov/orffinder/). ORFs were compared with reference genomes to identify the start (methionine) and stop codon. The following tools were employed for functional annotation: CDD (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi/) (Marchler-Bauer et al., 2015), PFAM (https://pfam.xfam.org/) (El-Gebali et al., 2019), SMART (http://smart.embl-heidelberg.de/) (Letunic & Bork, 2018), and PROSITE (https://prosite.expasy.org/) (Sigrist et al., 2013) for predicting conserved protein domains; PLACE (https://www.dna.affrc.go.jp/) (Higo et al., 1999) and Neural Network Promoter Prediction (https://www.fruitfly.org/seq_tools/promoter.html) (Reese, 2001) for predicting promoters and regulatory regions; and ELM (http://elm.eu.org/) (Dinkel et al., 2012; Elkhaligy et al., 2021) for profile identification.

Viral Identification

Viruses were first identified in the transcriptome by exploring the taxonomy of the annotated sequences. Four putative viral contigs were compared with sequences from the NCBI database, which showed a match with Kashmir bee virus (AY275710.1).

Reference Mapping

Mapping to the KBV reference genome (AY275710.1) was conducted for the nine transcriptome samples using BWA with default parameters. Subsequently, a consensus sequence for the putative KBV was obtained from the bam alignment of the fruit replicate 3 sample using IVAR consensus tool (-n N -m 1 -t 0.5). The consensus sequence was named “Kashmir bee virus Chile” or “Kashmir bee virus Ch”, and it was submitted to NCBI with the accession PP103295.

Phylogenetic analysis

A phylogenetic analysis was conducted to compare KBV Ch sequence to other KBV Chilean isolates using a 320 bp region that comprises the 3’ end of the intergenic region and the 5’ end of

the structural polyprotein. In addition, a BLASTN of this 320 bp region to the nt database was performed to identify other KBV sequences to include in the phylogenic analysis. A total of 30 sequences were aligned using Muscle (default parameters), after which a phylogenetic tree was constructed using the Neighbor-Joining method in MEGA (Kimura 2 parameter method, 1000 bootstraps). The Israel Acute Paralysis Virus was included as an outgroup. The sequences used were EU122368.1 (USA), EU122369.1 (USA), EU122370.1 (USA), EU122371.1 (USA), EU122372.1 (USA), EU122373.1 (USA), NC_004807.1 (USA), MT096516.1 (Spain), KC833152.1 (Chile), KC833158.1 (Chile), KC833142.1 (Chile), KC833149.1 (Chile), AY641447.2 (New Zealand), MW314660.1 (Spain), PP103295 (Chile), MN226368.1 (Nigeria), EU122374.1 (USA), MN226367.1 (USA), HM067845.1 (USA), KP965377.1 (South Korea), KP965379.1 (South Korea), KP965382.1 (South Korea), KP965383.1 (South Korea), KP965378.1 (South Korea), KP965376.1 (South Korea), KP965381.1 (South Korea), KP965380.1 (South Korea), KP965375.1 (South Korea), KP965374.1 (South Korea), KP965373.1 (South Korea), KF219804.1 (Israel).

RNA extraction from *in vitro* plants and RT-PCR analysis

Four *in vitro* cultured explants of *V. pubescens* were used for RT-PCR identification of KBV Ch. 100 mg of each sample was processed with liquid nitrogen to extract RNA using the Spectrum TM Plant Total RNA Kit (Sigma-Aldrich, USA) according to the manufacturer's instructions, then cDNA was synthesized using the Thermo Scientific TM Revert Aid First Stand cDNA Synthesis Kit (Sigma-Aldrich, USA). Subsequently, a solution of 12 µL of 1X PCR Buffer, 1.5 mM PCR Buffer (Winkler), 2.5mM MgCl2, 0.2 mM dNTPs, along with 300pM of each primer and 1.5u Taq DNA polymerase (Agilent) was prepared. The MultiGene™ OptiMax thermocycler (Labnet™) was used and an initial denaturation program of 5 minutes at 95°C, followed by 35 cycles of denaturation for 30 seconds at 95°C, alignment for 30 seconds at 58°C and a final extension of 30 seconds at 72°C, remaining at 4°C until verification by 2.3% m/v agarose gel electrophoresis in 1X TAE buffer (Winkler). GeneRuler 50 bp DNA Ladder molecular weight marker (Thermo Fisher TM). The primers for amplify the KBV sequence were forward 5'-ATGATTGGGGGGCGGTGTAATA-3' and reverse 5'- TGCCTGTGTGAAAAGCTGTC-3' to obtain a 200bp amplicon. In addition, primers for *V. pubescens* ribosomal 18s RNAr were used as positive control (Gambino & Gribaudo, 2007).

Results

Detection of Kashmir bee virus sequences in *V. pubescens* transcriptomes

To explore the virome of the mountain papaya *V. pubescens*, *de novo* assemblies were conducted on root, leaf, and fruit transcriptomes from samples collected from multiple orchards in the Maule region (Chile). To find assembled viral sequences, a BLAST annotation of the transcriptome contigs was performed, after which we identified contigs with match to Kashmir Bee Virus (KBV). Subsequently, we assessed the viral load across different tissues by mapping transcriptome reads to the KBV reference genome (Table 1). Notably, one replicate of fruit

samples showed 820 mapped reads, covering 92% of the reference sequence with an average depth of 12X. In addition, root and leaf samples ~~also exhibited low-level detection~~, ranging from 2 to 6 reads per sample (Table 1), suggesting the presence of KBV in plant tissues typically less frequented by pollinators. From the reads aligned to the KBV reference genome, we constructed a consensus sequence named "KBV Ch (Chile)", ~~which revealed a total of~~ 199 single nucleotide polymorphisms (SNPs).

Phylogenetic analysis

KBV has been previously reported in honeybee samples in Chile (Riveros et al., 2018). To determine the genetic relationship of KBV Ch with these previously detected Chilean isolates and others, we conducted a phylogenetic analysis using a 320 bp region spanning the intergenic region and the 5' start of the polyprotein region. ~~This analysis revealed that KBV Ch is closely related to a KBV isolate from Spain (Figure 2), sharing a clade with isolates from Spain, New Zealand, and additional Chilean samples.~~

Detection of KBV Ch in Laboratory and Field Plants

Given the detection of KBV sequences in root and leaf tissues, suggesting potential vascular tissue incorporation of the virus, we investigated the presence of KBV in *in vitro* cultivated plants derived from *V. pubescens* explants collected from the field. We performed RT-PCR using primers specific for KBV Ch on RNA extracted from these *in vitro* tissues. These plant tissues were exclusively produced in laboratory conditions without exposure to field-derived materials. ~~The results revealed the presence of a 200 bp amplicon in one sample of an *in vitro* grown plant (Figure 3).~~

Discussion

Through transcriptome analysis of leaf, root, and fruit tissues of the mountain papaya, *V. pubescens*, we successfully identified a near-complete genome sequence of the Kashmir bee virus (KBV), and detected KBV sequences in *in vitro* grown plants. The presence of KBV was notably higher in one replicate of fruit transcriptome, suggesting an occasional ocurrence of the virus in *V. pubescens* orchards. As a trioecious species capable of cross-pollination, *V. pubescens* produces male, female, and hermaphrodite plants (Carrasco et al., 2009). Pollination can occur via wind or insects, with self-pollination observed in commercial varieties of *Carica papaya* (Carrasco et al., 2022). Infected pollen presents a potential source of KBV particles in fruits. There are viruses capable of infecting pollen, which can pass to the plant ovule and produce virus-infected fruit. Pollen can be infected by different vectors, such as abiotic factors (like wind) or biotic factors (like insects) (Bhat & Rao, 2020). In addition, Figueroa et al. (2019) studied the mechanisms involved in pathogen deposition, persistence and acquisition in flowers. Bees, known to spend extended periods feeding on flowers, deposit and acquire feces from other pollinators, promoting transmission rates.

While reports on bee viruses in pollinated plants typically focus on flowers and pollen, other viruses from the *Dicistroviridae* family have been detected circulating in other plant tissues, among which most examples are from aphid viruses (Jones, 2018). For instance, the *Rhopalosiphum padi virus* (RhPV) spreads systemically in barley plants including roots, potentially infecting healthy individuals that consume RhPV-carrying plants (Ban et al., 2007). Similarly, Aphid lethal paralysis virus has been found in cucumber leaf transcriptomes, suggesting a comparable transmission route (Maina et al., 2017). In our study, we detected KBV RNA in roots and leaves, albeit in lower quantities compared to fruits, suggesting than KBV could circulate from contaminated sexual organs to other plant tissues. Despite rigorous surface cleaning of plant material before RNA extraction, residual KBV RNA could stem from superficial contamination with feces, corpses, or other insect sources carrying KBV particles. Importanly, KBV RNA was also detected in *in vitro* grown plants, which underwent stringent disinfection procedures, and only novel tissue was used for RNA extraction. Virus in *in vitro* plant cultures can originate from the source vegetative plant (Li et al., 2013). Such is the case of babaco plants (*Vasconcellea* x *heilbornii*), which are often propagated from cuttings without virus elimination protocols typically resulting in *in vitro* plants infected with viruses (Muñoz et al., 2023). Therefore, it is possible that KBV could systemically circulate through the plant as it was shown for other members of *Dicistroviridae*, utilizing pollinated plants as reservoirs and passive vectors. The phenomenon of horizontal transmission of entomopathogenic viruses by host plants as passive vectors remains understudied yet holds significant implications for pest control and conservation of wild pollinators (Jones, 2018).

While the exact mechanism of KBV introduction into *V. pubescens* populations in Chile remains uncertain, previous studies conducted by Chilean researchers have identified partial KBV sequences *Apis mellifera* populations across several regions in Chile (Riveros et al., 2018). Phylogenetic analysis of the KBV sequence obtained in our study reveals close genetic proximity to these previously reported Chilean isolates, as well as isolates from Spain and New Zealand, suggesting reintroduction of genetically different KBV isolates across continents. Furthermore, the interaction between bees and papaya trees is noteworthy (Badillo-Montaño et al., 2019). This interaction implies two key aspects: firstly, bees in the studied locations may act as vectors for KBV transmission, potentially disseminating the virus during pollination of *V. pubescens*; and secondly, there is a critical need to monitor bee populations in the coastal areas of the Maule Region for KBV presence. Such surveillance could benefit beekeepers and lay the groundwork for forthcoming investigations.

Conclusions

This study represents a significant advancement by documenting, for the first time, the detection of “Kashmir bee virus Chile” within *Vasconcellea pubescens* transcriptomes, marking a milestone in the agro-industrial and apiculture sectors of Chile’s Maule region. Transcriptome analysis uncovered widespread presence of KBV across various *V. pubescens* tissues, notably abundant in fruit tissues, suggesting a potential role as a reservoir or vector of infection. Further

investigations are warranted to elucidate transmission mechanisms and assess the impact of this virus on agriculture and beekeeping. Such efforts are crucial for developing strategies to manage viral diseases affecting both crops and pollinators.

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Table 1(on next page)

RNA-seq of mountain papaya reveals presence of KBV RNA

Table 1: RNA-seq of mountain papaya reveals presence of KBV RNA

	Number of reads mapped to KBV genome	*Total reads*	*% of total reads*
Root R1	6	46.122.074	0,000
Root R2	2	47.440.504	0,000
Root R3	2	39.754.722	0,000
Leaf R1	1	55.401.252	0,000
Leaf R2	0	42.124.523	0,000
Leaf R3	0	72.734.557	0,000
Fruit R1	0	51.194.927	0,000
Fruit R2	0	45.857.234	0,000
Fruit R3	820	43.542.557	0,002

Figure 1

Map of the Maule Region with *Vasconcellea pubescens* cultivars depicted.

Red stars from top to bottom represent the cities of Lipimávida, Duao, Iloca, Putú, Constitución, Chanco, Pelluhue, and Curanipe. Green stars from top to bottom represent the cities of Vichuquen and Licanten. The red circle indicates the samples obtained from each locality, corresponding to roots, leaves, and fruits. The map of the Maule Region was obtained from the National Curriculum, Ministry of Education, Government of Chile.

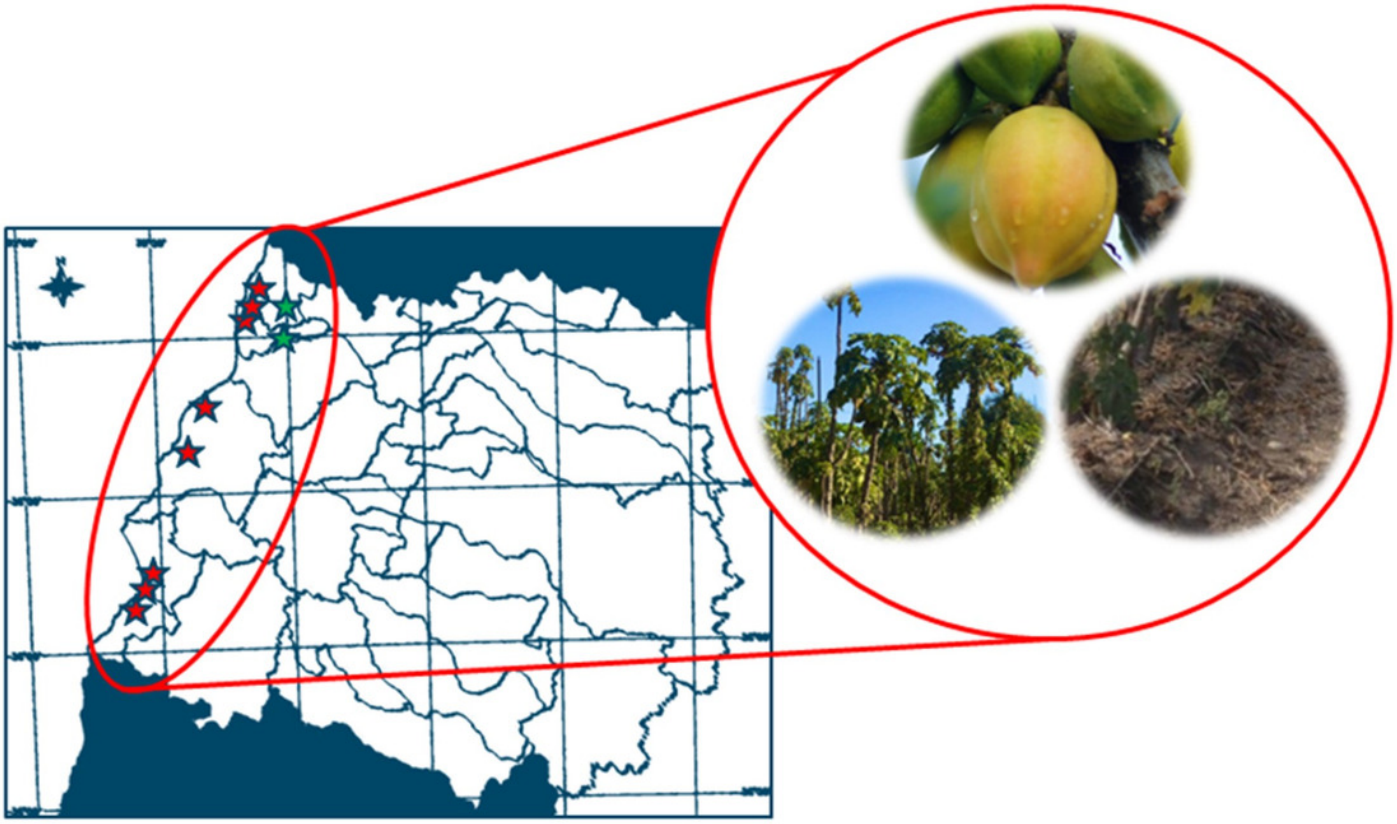

Figure 2

Phylogenetic analysis groups KBV Ch with other isolates from Chile, New Zealand, and Spain.

Thirty nucleotide sequences corresponding to a 320 bp region spanning the intergenic region and 5’ start of the structural protein. Isolates origins are shown: 10 from the USA, 10 from North Korea, 4 from Chile, 2 from Spain, 1 from Nigeria, and 1 from New Zealand. The outgroup was the Israel Acute Paralysis Virus. The sequence found in papaya transcriptomes, designated as KBV Ch, are highlighted in red. The dendrogram was constructed using the Neighbor-Joining method and Bootstrap method (n=1000).

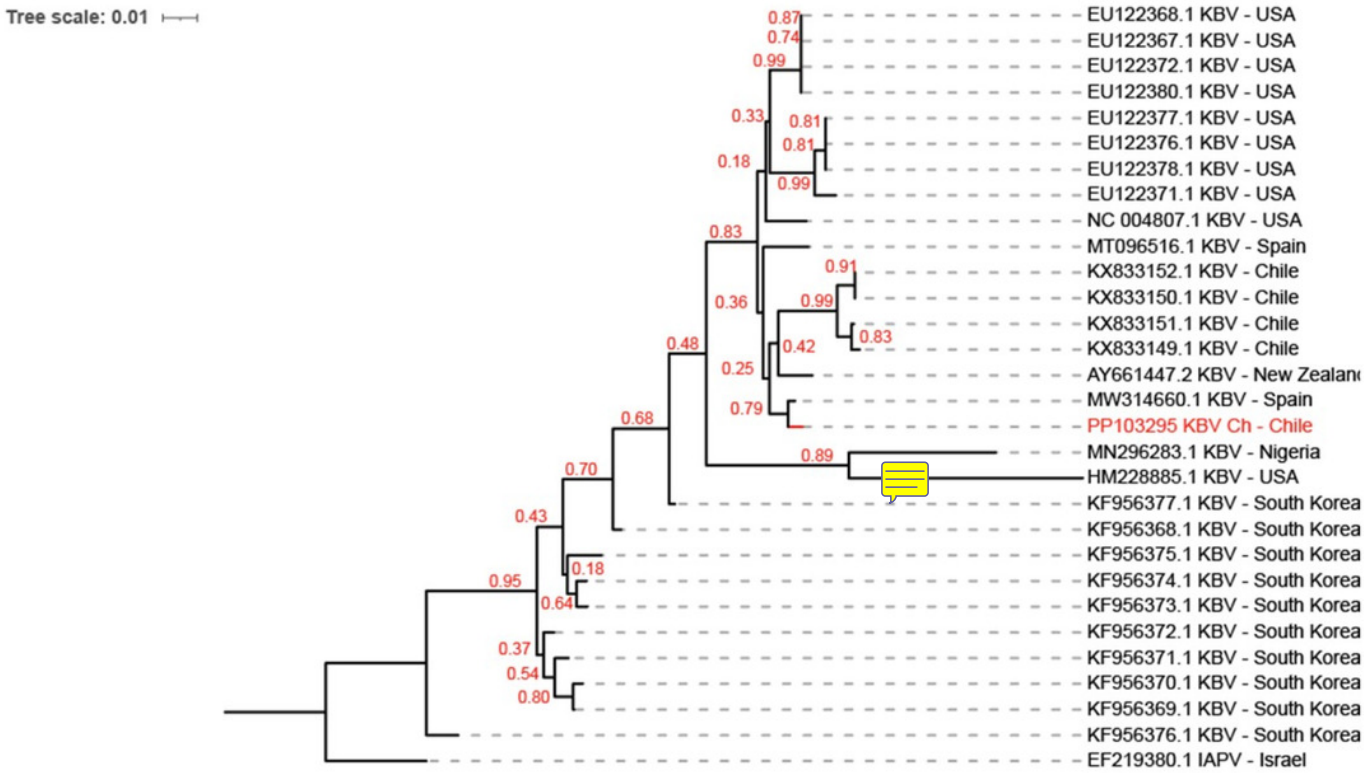

Figure 3

KBV sequences are detected in *in vitro* grown wild papaya.

(Left) Housekeeping gene 18s RNAr with amplicon sizes between 800 and 900 bp for samples 1, 2, 3, and 4. The first lane contains a 100 bp molecular marker, and the last lane contains a negative control. (Right) Amplicon for KBV of approximately 200 bp for samples 1, 2, 3, and 4. The first lane contains a 50 bp molecular marker, and the last lane contains a negative control. Images were obtained using a transilluminator.