

Identification and molecular characterization of a marafivirus in *Rubus* spp.

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Abstract An undescribed virus with isometric particles and a diameter of *ca.* 30 nm was identified in diseased samples of wild and cultivated *Rubus* species and molecularly characterized. Its genome was 6,463 nt, excluding the 3'-terminal poly(A) tail, and contained a single open reading frame coding for a 2,035-amino-acid-long precursor polypeptide (p223). The amino terminal portion of p223, identified as a replication-associated polyprotein, contained conserved motifs of methyltransferase, endopeptidase/protease, helicase and RNA-dependent RNA polymerase. The carboxy terminus of the large polypeptide is involved in the formation of two viral coat protein subunits with deduced molecular masses of 23 and 21 kDa. Pairwise comparisons and phylogenetic analyses showed closest relationships of this virus with oat blue dwarf virus and citrus sudden death-associated virus, sharing levels of genome sequence conservation far below the species demarcation level established for tymovirids. Our data indicate that this virus, for which the name blackberry virus S (BIVS) is proposed, is a hitherto undescribed species of the genus *Marafivirus*, family *Tymoviridae*. A survey conducted in Mississippi, USA, has shown that BIVS is also present in cultivated *Rubus* germplasm. This work represents the first report of a marafivirus infecting small fruits.

Introduction

In recent years, there has been major progress in research on viruses infecting *Rubus* spp., resulting in the identification and characterization of several new viral species, as well as in first reports of infections by some known viruses [8, 10, 16, 23, 26–28]. All these viruses were isolated from symptomatic plants, indicating their possible involvement in the etiology of the respective diseases. In particular, some of the recently described viruses are components of the complex etiology of the blackberry yellow vein-disease (BYVD), an emerging problem in the southeastern United States [23, 24, 27].

This work focused on the identification and characterization of viruses/diseases affecting native *Rubus* germplasm. The research targeted blackberry germplasm present in the Great Smoky Mountains National Park (GSMNP) and represented a part of a larger study on plant viruses in ecosystems with minimal impact from human activities. Several symptomatic samples of native *Rubus* spp. were collected and analyzed for the presence of virus. Symptoms observed ranged from general chlorosis to those described for BYVD, including yellowing/mottling along the main veins (Fig. 1a).

The research on native *Rubus* germplasm in GSMNP resulted in the detection and identification of several viruses. In addition to a strain of black raspberry necrosis virus [20], tested samples of native blackberries contained at least three other viruses. While the characterization of other viruses has recently been accomplished [22], or is still ongoing (rubus virus R; Sabanadzovic et al., unpublished data), here, we report the identification and detection of a new isometric virus resembling members of the family *Tymoviridae*, for which the name blackberry virus S (BIVS) is proposed.

The nucleotide sequence data reported in this paper has been deposited in GenBank as Accession No. FJ915122.

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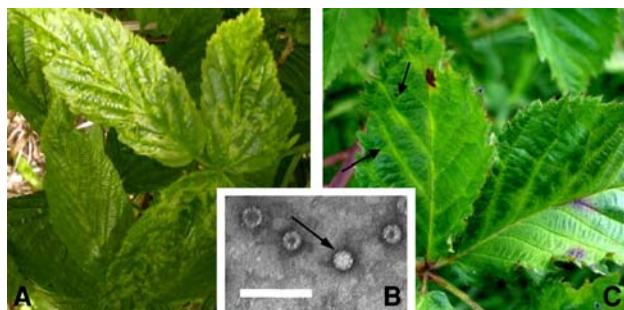


Fig. 1 **a** Original smooth blackberry sample infected by blackberry virus S (BIVS) displaying yellow vein mosaic/mottling symptoms. **b** Electron micrograph of a partially purified virion preparation from specimen GSM-8 containing scattered isometric particles. Arrow indicates putative intact virion among a few putative empty (top) particles. Bar indicates 100 nm. **c** Specimen of cultivated blackberry infected by BIVS showing symptoms resembling blackberry yellow vein disease

The family *Tymoviridae* currently contains three genera (*Tymovirus*, *Marafivirus* and *Maculavirus*) of alpha-like phytoviruses and an entomovirus, *Bombyx mori maculavirus* (BmMLV) [11]. These viruses share similar virion morphology, sedimentation properties, ultrastructural effects in host cells, and a monopartite, cysteine-rich single-stranded positive-sense RNA genome [5, 15]. Unlike tymoviruses and maculaviruses reported from dicotyledonous plants, marafiviruses were associated with monocotyledonous hosts, with the exception of oat blue dwarf virus (OBDV), which has been reported from mono- and dicots [3]. Recent identification of two tentative species of this taxon from grapevines (grapevine asteroid mosaic-associated virus [GAMaV] and grapevine rupestris vein feathering virus [GRVFV]) [1, 18] and a marafivirus associated with a severe disease of citrus (citrus sudden death-associated virus [CSDaV]) [14] demonstrates that these viruses are capable of infecting the two main groups of flowering plants. However, no marafiviruses or other members of the family *Tymoviridae* have been reported from blackberry, or from any other small fruit species. In this paper, we report the characterization of an undescribed marafivirus originally identified in native *Rubus* germplasm of the GSMNP in North Carolina and Tennessee, USA.

Materials and methods

Virus sources

The original virus source used in this study was a smooth blackberry (*Rubus canadensis* L.) accession, referred to as GSM-8, collected from GSMNP, USA. This source was used for molecular characterization, purification trials and

mechanical inoculations. Additional native blackberries (11 samples) from the GSMNP and cultivated blackberries from Mississippi (25 plants) were used in some experiments (i.e., dsRNA, RT-PCR, partial genome sequencing, etc.).

Virus purification

Attempts to observe virions were essentially conducted by applying a protocol described by Lesemann et al. [13] for purification and visualization of poinsettia mosaic virus (PnMV) virions. The protocol involved clarification with *n*-butanol, precipitation with 8% polyethylene glycol (PEG) and 1% sodium chloride (NaCl) followed by further concentration by a cycle of low- and high-speed centrifugations. Partially purified preparations were negatively stained with 2% uranyl acetate and observed with a JEOL JEM 100CXII electron microscope.

Mechanical transmission

Young leaves of the accession GSM-8 were crushed in 10 volumes of 0.1 M phosphate buffer, pH 7.2, containing sodium sulphite and rubbed onto cellite-dusted basal or cotyledonal leaves of the following herbaceous seedlings: *Chenopodium amaranticolor*, *Ch. quinoa*, *Nicotiana tabacum* cv. Turkish, *N. benthamiana*, *N. rustica*, *N. glutinosa*, *Phaseolus vulgaris* cv. Bountiful, *Ph. vulgaris* cv. Cherokee Wax, *Glycine max* and *Ocimum basilicum*. The same range of test plants was challenged with a partially purified BIVS preparation. Plants were kept under greenhouse conditions at a constant temperature of 22°C and were monitored for symptom appearance daily for 45 days.

dsRNA extraction, cloning and sequencing

Collected blackberry samples were tested for the presence of viral dsRNAs by selective chromatography through CF-11 columns in the presence of STE buffer (10× STE: 1 M sodium chloride, 0.5 M Tris, 0.01 M EDTA; pH 7.5) containing 16–17% ethyl alcohol as described [29]. Extracted nucleic acids were digested with RQ DNase and RNase A for further clean-up prior to their use as templates for random-primer-generated cDNAs and cloning into pGEM-T Easy Vector (Promega, USA) as described [21]. The 3' end of the virus was amplified and cloned using the primer pair oligo(dT) and RD1 under conditions described for grapevine marafiviruses [1]. The nucleotide sequences of the viral 5' end of the BIVS genome were obtained using a 5'/3'RACE kit, essentially following manufacturer's instructions (Roche Applied Science, USA). Each nucleotide of the genome was sequenced from at least 4 independent reactions (4× coverage), and

sequencing was performed by the custom sequencing service at MWG Biotech (Huntsville, AL, USA).

Sequence data were assembled and analyzed by LaserGene software (DNAStar Inc, Madison, WI). Their comparison with sequences available in NCBI GenBank was performed using web-based BLAST resources [2]. Pairwise comparisons were performed with ClustalW2 on-line resource [12]. Phylogenetic analysis using the neighbor-joining algorithm including bootstrapping was conducted with ClustalW software [25], and trees were visualized with the TreeView program [17].

Northern blot

In order to verify the number and size of virus-associated genomic and subgenomic RNAs in infected blackberries, dsRNA extracts of two BIVS isolates were electrophoresed, denatured and neutralized prior to overnight transfer to a Hybond N⁺ nylon membrane as described [19]. Membranes were then hybridized with a digoxigenin-labeled BIVS-specific probe complementary to the central portion of the coat protein gene following the procedure outlined by the manufacturer (Roche Diagnostics, USA). The presence of hybridization signals corresponding to nucleic acid molecules homologous to the applied probes was captured on X-ray film. Relative sizes of these were estimated by comparison with references (1 kb Plus DNA Ladder; Invitrogen, USA).

RT-PCR

A set of primers, designed to amplify a 434-nt portion of the viral coat protein gene, was used to investigate the presence of this virus in cultivated blackberries. Before their application in the general survey, the validity of the primer set BIVS-CP was preliminarily tested on several BIVS-infected specimens of wild blackberries from GSMNP and on cDNAs of several tymovirids available at the time in the MSU Plant Virology Lab (i.e., GFkV, GRVfV, PnMV, physalis mosaic virus).

For that purpose, total nucleic acids were extracted using a protocol combining the Qiagen RNeasy protocol with the procedure described by Foissac et al. [7]. Briefly, 0.15 g of foliar tissue was ground in a plastic extraction bag with mesh (Bioreba AG, Switzerland) in the presence of 10–12 volumes of extraction buffer (4 M guanidine thiocyanate; 0.2 M sodium acetate, pH 5.0, 25 mM EDTA, 2.5% PVP-40 and 1% β -mercaptoethanol). After thorough grinding, 600 μ l of the homogenate was mixed with 60 μ l of sodium lauryl sarkosine (20%) and incubated at 65–70°C for 10 min with intermittent shaking. Samples were then mixed with an equal volume of chloroform, vortexed and centrifuged before transferring the supernatant to the

QIAshredder spin columns and proceeding as per manufacturer's instruction (Qiagen Inc., USA). Total RNAs were eluted with 75 μ l RNase and DNase-free water. Extracted total RNAs were reverse transcribed with random primers and M-MLV reverse transcriptase as described. Five microliters of RT reaction were used as a template in PCR. Amplification of the target genome portion was performed with primers BIVS-CPF (5'AATGTCACCTCCCAGGTCCG3') and BIVS-CPR (5'ATGCGGCTCACGTC AAGAGG3') for 40 cycles at an annealing temperature of 52°C. Generated PCR products were analyzed by electrophoresis in a 1.5% agarose gel, stained with ethidium bromide and visualized under a source of ultraviolet light.

Etiological investigations

In order to investigate the possible association of BIVS with observed symptoms, all plants resulting positive for BIVS infections were tested for infections by other viruses. For this purpose, they were tested in ELISA using antibodies specific for the following viruses: raspberry bushy dwarf virus (RBDV), arabis mosaic virus (ArMV), tobacco ringspot virus (TRSV), tomato ringspot virus (ToRSV), cucumber mosaic virus (CMV), tomato spotted wilt virus (TSWV), impatiens necrotic spot virus (INSV), peanut stunt virus (PSV), tobacco mosaic virus (TMV) and raspberry ringspot virus (RpRSV), as well as with the broad-spectrum monoclonal antibody to potyviruses (all Agdia Inc., USA). In addition, the same samples were further tested via RT-PCR for possible infections with blackberry virus Y (BVY) [23], blackberry yellow vein-associated virus (BYVaV) [27], beet pseudo yellows virus (BPYV) [26], grapevine virus Q [22] and few viruses still under characterization: blackberry virus X (Tzanetakakis and Martin, unpublished data), blackberry virus E and rubus virus R (Sabanadzovic et al., unpublished data).

Results and discussion

Using the degenerate primer set RD [18], designed to detect a portion of the viral RNA-dependent RNA polymerase of viruses belonging to the family *Tymoviridae*, amplicons of the expected size were generated from 4 out of 12 tested specimens of native blackberries (not shown). Nucleotide sequence identities among clones from these four samples ranged from 96 to 99%, indicating they were the same virus. Thus, the specimen GSM-8 was used for further genome sequencing and for attempts to purify and visualize virus particles. The purification protocol adopted [13] allowed the fortuitous observation of scattered putative isometric virions (Fig. 1b) of *ca* 30 nm in

diameter in a background of impurities of plant cell origin (not shown). The quantity of purified virus was limited and did not form detectable bands in sucrose gradients. However, it allowed visualization of two types of putative virions, resembling top and bottom fractions (Fig. 1b). Very few apparently intact particles exhibited the prominent surface structures characteristic for the members of the family *Tymoviridae* (Fig. 1b, arrow). Attempts to transmit the virus on a range of herbaceous test plants failed, regardless of the inocula used (plant tissue or partially purified virion preparations).

The complete genome of the virus consisted of 6,463 nt, excluding the 3' poly(A) tail, and was characterized by a high overall cytosine content (38.14%), similar to genomes of other tymovirids. Computer analysis of the nucleotide sequences revealed the presence of a single, long open reading frame (ORF), with a genome organization suggestive of a marafivirus (Fig. 2a). This ORF started with an AUG codon at position 195 and extended for 6105 nucleotides. The putative product of this ORF had an estimated molecular mass of 222.5 kDa (p223) and contained conserved domains of the tyoviral methyltransferase superfamily (pfam 10641), tymovirus endopeptidase (peptidase_C21; pfam 05831), viral helicase superfamily 1 (pfam01443), RNA-dependent RNA polymerase superfamily 2 (RDRP_2; pfam00978) and tymovirus coat protein (pfam00983).

The replication-associated polyprotein shared 60% amino acid (aa) sequence identity with CSDaV, 59% with OBDV [6] and 56% with maize rayado fino virus (MRFV) [9]. The same polyprotein shared 43% overall identity with grapevine fleck virus replicase [19] and similar levels of conservation (43–45%) with various tymoviruses used in comparisons. When examined separately, individual domains showed different levels of conservation.

Methyltransferase and helicase domains shared the highest levels of identity (in both cases 68%) with OBDV and CSDaV. RNA-dependent RNA polymerase was the most conserved part of the BIVS genome and shared 85% identity with OBDV, 82% with CSDaV, 80% with GAMaV and a few percentages less with MRFV and GRVfV. Generally speaking, maculaviruses and tymoviruses shared ca 47–55% identical amino acids with BIVS (depending on the virus and genome portion used in comparisons), except in the case of RdRp, when identities rose to 63–68%.

The two carboxy co-terminal viral coat proteins, with deduced molecular masses of 23 and 21 kDa, appeared to be the less conserved parts of the genome and shared identities with marafiviruses ranging from 44–62% (Table 1), 28–29% with maculaviruses and 21–24% with tymoviruses. However, BIVS shared extensive CP amino acid sequence conservation with OBDV, CSDaV and GAMaV, particularly in a 45-aa-long portion located in the central part of these proteins (Fig. 2b). Identity levels ranged from 78% with GAMaV to 84% with CSDaV.

The BIVS genome also contained a highly conserved string of 16 nucleotides preceding putative CP sequences, a tymo/marafibox, involved in synthesis of subgenomic RNA [4] (Fig. 2a). These sequences in the BIVS genome were identical (100%) to the corresponding region in GAMaV, OBDV and BELV (not shown).

Phylogenetic trees constructed on viral polymerases or coat proteins (Figs. 3a, b) showed a clear grouping of BIVS with other members of the genus *Marafivirus* in a separate clade within the family. In both scenarios, BIVS appeared closer to OBDV, CSDaV (and with GAMaV in available genomic portions) than with monocot-infecting marafiviruses (MRFV and BELV).

Northern blot hybridization performed on virus-associated dsRNAs revealed the presence of two distinct molecules

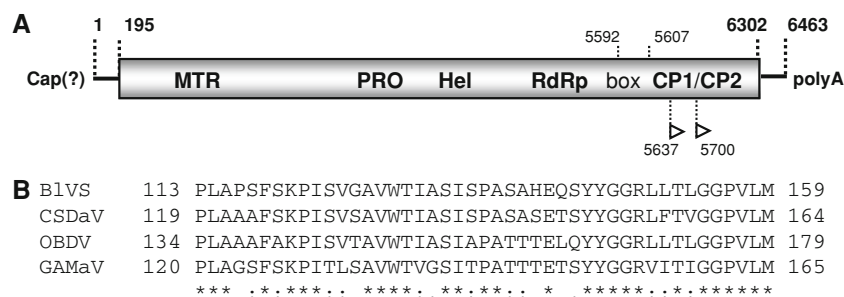


Fig. 2 a Diagrammatic representation of the BIVS genome with key nucleotide coordinates. *Box* depicts large ORF and corresponding putative polyprotein, *lines* represent untranslated genomic regions at the genome extremes. *MTR* Methyltransferase, *PRO* endopeptidase/protease, *Hel* helicase, *RdRp* RNA-dependent RNA polymerase, *CP* coat protein, *box* marafibox. **b** Highly conserved 45-amino-acid-long central portions of viral coat proteins of blackberry virus S (BIVS), oat blue dwarf virus (OBDV), citrus sudden death-associated virus

(CSDaV) and grapevine asteroid mosaic-associated virus (GAMaV). Accession numbers of sequences used for comparisons are the same as in Fig. 3 (*asterisk* denotes identical residues in all compared sequences; *colon-* indicates conserved substitutions; *dot* indicates semi-conserved substitutions). *Numbers* denote aa positions of the highly conserved portions counting from the putative start of the larger CP subunits in each of the viruses used for comparison

Table 1 Amino acid sequence identity levels (%) between BIVS and some members of the family *Tymoviridae*

| | Identity (%) | | | |
|-------------|-----------------|-----------|-----------|-----------|
| | MTR | HEL | RdRp | CP |
| OBDV | 68 | 66 | 85 | 62 |
| MRFV | 61 | 62 | 77 | 51 |
| CSDaV | 65 | 68 | 82 | 62 |
| GAMaV | 67 ^a | NA | 80 | 57 |
| GRVFV | 55 | 64 | 74 | 44 |
| GFkV | 47 | 55 | 68 | 28 |
| Tymoviruses | 51–55 | 49–53 | 63–68 | 21–27 |

The highest values for each genomic segment are in bold

^a Only partial sequences available for GAMaV

in extracts from BIVS-infected sources, but not in the controls (Fig. 4a). Their sizes were estimated at 6.5 and 0.8 kbp, which correspond to replicative forms of the full-genomic size molecule and subgenomic RNA, the template for 21 K CP translation, as in other marafiviruses [5].

The primer set, designed on the viral coat protein gene, proved to be BIVS-specific, as it detected homologous virus in all tested BIVS sources from GSMNP and did not generate any visible amplicon with cDNAs synthesized from genomes of related tymovirids under tested conditions (not shown). Thus, the primer set BIVS-CP allowed detection of BIVS in two symptomatic samples of cultivated blackberries collected from backyards and/or from commercial orchards in Mississippi (Fig. 4b). Symptoms observed on BIVS-positive plants included vein clearing (Fig. 1c), ring spots and vein yellowing. Unfortunately, none of the BIVS sources, either from GSMNP or Mississippi, were infected only by this virus, thus making difficult to understand its real etiological role.

Data generated in this work clearly demonstrate that BIVS represents a new species in the genus *Marafivirus* (family *Tymoviridae*). Although it was originally found in samples of smooth blackberry growing spontaneously in the GSMNP, further studies involving virus-specific detection tools proved that this virus also infects commercial *Rubus* germplasm. Despite being identified in

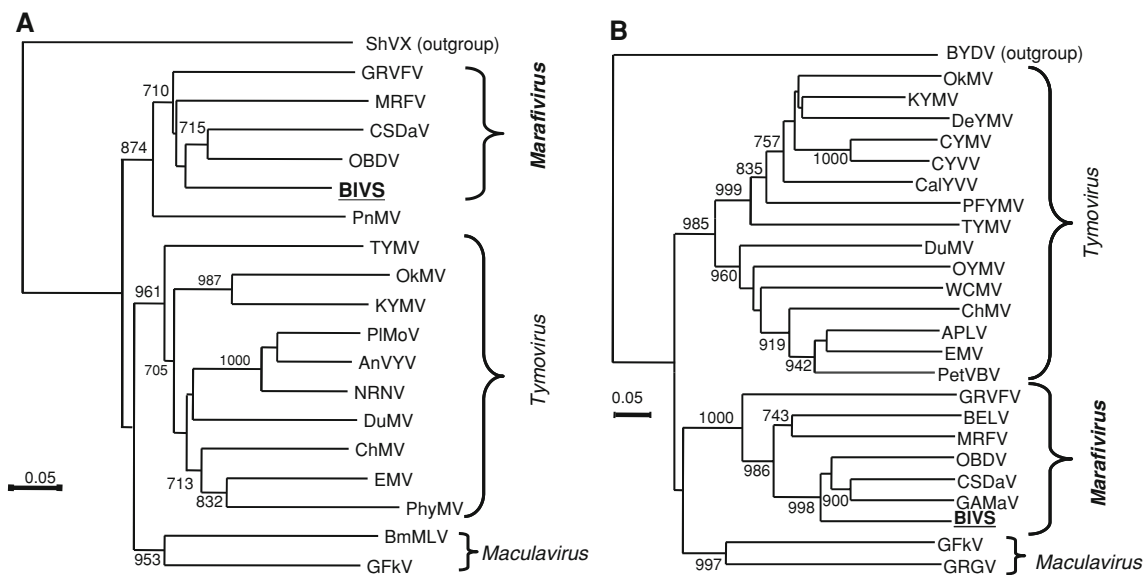


Fig. 3 Phylogenetic tree constructed with amino acid sequences of the **a** replicases and **b** coat proteins of members of the family *Tymoviridae*. Viruses used in the phylogenetic analyses and their NCBI/GenBank/EMBL accession numbers are: Anagryis vein yellowing virus (AnVYV; NC_011559), Andean potato latent virus (APLV, AF035402), Bermuda grass etched-line virus (BELV, AY040531), *Bombyx mori* macula-like latent virus (BmMLV, AB186123), cacao yellow mosaic virus (CYMV, X54354), Calopogonium yellow vein virus (CalYVV, AAC58458), chayote mosaic virus (ChMV, AF195000), citrus sudden death-associated virus (CSDaV, NC_006950), Clitoria yellow vein virus (CYVV, AF035200), dulcamara mottle virus (DuMV, NC_007609), eggplant mosaic virus (EMV, J04374), grapevine asteroid mosaic-associated virus (GAMaV, AJ249358), grapevine fleck virus (GFkV, NC_003347), grapevine red globe virus (GRGV, AJ249360),

grapevine rupestris vein feathering virus (GRVFV, AY128949), Kennedy yellow mosaic virus (KYMV, NC_001746), maize rayado fino virus (MRFV, AF265566), Nemesia ring necrosis virus (NRNV, NC_011538), oat blue dwarf virus (OBDV, U87832), okra mosaic virus (OkMV, AF035202), Ononis yellow mosaic virus (OYMV, J04375), passion fruit yellow mosaic virus (PFYMV, AF467107), petunia vein banding virus (PetVBV, AF210709), Physalis mottle virus (PhyMV, Y16104), Plantago mottle virus (P1MoV, NC_011539), poinsettia mosaic virus (PnMV, NC_002164), Scrophularia mottle virus (SrMV, NC_011537), turnip yellow mosaic virus (TYMV, NC_004063), and wild cucumber mosaic virus (WCMV, AF035633). The replicase and coat protein sequences of shallot virus X (ShVX, NC_003795) and barley yellow dwarf virus-PAV (BYDV-PAV, X07653), respectively, were used as outgroups

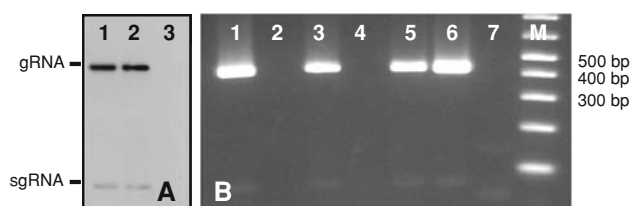


Fig. 4 **a** Northern blots. Two hybridization signals, corresponding to replicative forms of full-genomic size and subgenomic RNA molecules, obtained with a specific probe to viral coat protein gene. Signals are present in dsRNA extracts of both tested BIVS isolates (lanes 1 and 2), but not in the negative control (lane 3). **b** RT-PCR detection of BIVS in wild (lanes 2 and 3) and cultivated *Rubus* spp (lane 5) using the BIVS-CP primer set. Blackberry samples in lanes 1 and 4 apparently do not contain the target virus. Positive control GSM-8 is in lane 7. Healthy control is in lane 6. DNA ladder (1 kb Plus, Invitrogen, USA) is in lane M

symptomatic samples in both *Rubus* genotypes, its etiological role could not be clarified due to inability to isolate the virus from infected plants and on-going multiple virus infections. The virus sources identified to date, in addition to BIVS, contained at least one of the recently described viruses from *Rubus* spp. [8, 22, 23, 26] and/or viral entities still under characterization. However, its presence in symptomatic samples suggests that it may be a component of the disease.

The symptoms observed on two BIVS-infected cultivated blackberries resembled BYVD, which is triggered by co-infection and interaction between various viruses [24]. Thus, it is possible that BIVS in mixed infections with other viruses (primarily BVYav, BVY and BIVE) could be responsible for BYVD-like symptoms in cultivated blackberries, especially considering that the disease symptoms are comparable to the effects ascribed to other related viruses (i.e., GAMaV and GRVfV) in some grapevine genotypes (i.e., vein-clearing, feathering, mosaics along the veins, etc.). The results of our investigation represent the first report of a marafivirus from *Rubus* spp. and from small fruits in general.

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