A new, widespread emaravirus discovered in blackberry

Mohamed Hassan^{a,b*}, Patrick L. Di Bello^{a*}, Karen E. Keller^c, Robert R. Martin^c, Sead Sabanadzovic^d and Ioannis E. Tzanetakis.^{a*}

^a Department of Plant Pathology, Division of Agriculture, University of Arkansas. Fayetteville, AR 72701.

^b Agricultural Botany Department, Faculty of Agriculture, Fayoum University, Egypt

^c USDA-ARS Horticultural Crops Research Laboratory, Corvallis, OR 97330.

^d Department of Biochemistry, Molecular Biology, Entomology and Plant Pathology, Mississippi State University, Mississippi State, MS, 39762.

* These authors contributed equally to this work

** Corresponding author: itzaneta@uark.edu

A new virus was identified in blackberry plants exhibiting leaf mottling, chlorotic ringspots and curved midribs, symptoms associated with blackberry yellow vein disease (BYVD). The genome of the new virus, provisionally named blackberry leaf mottle-associated virus (BLMaV), was characterized and phylogenetic analysis revealed its close relationship to recognized members of the genus *Emaravirus*. BLMaV was transmitted by a yet to be described eriophyid mite species, further reinforcing its placement in the genus. Detection protocols were developed and used to determine the presence of the virus in plants collected from several areas in the U.S.A. The incidence of BLMaV was greater than 40% in BYVD-affected material.

Keywords: *Rubus*, emaravirus, detection, transmission, yellow vein disease

1. Introduction

Over 40 viruses infect *Rubus* spp. (L., blackberry, raspberry and their hybrids; Khadgi, 2015; Martin et al., 2013; Shahid et al., 2017; Thekke-Veetil et al., 2013). More than ten are associated with blackberry yellow vein disease (BYVD), the most economically important virus disease in the southeastern United States. Symptoms observed on BYVD-affected plants include yellowing of the main veins, mottling, oak leaf patterns and chlorotic ringspots. The disease affects fruit quality, yield and causes plant decline (Martin et al., 2013). BYVD-free plants can be productive for more than 20 years, whereas affected blackberries become unproductive and uneconomical to the producer, leading to replanting 5-7 years after the onset of the disease (Martin et al., 2013).

In an effort to better understand the etiology of BYVD, affected plants were tested for viruses commonly associated with the disease (Khadgi, 2015). When tested negative, material was further evaluated for the presence of possible new agents using different large sequencing approaches and a virus, tentative member of the genus *Emaravirus*, reported in this paper, was identified.

Emaraviruses are negative strand RNA viruses with a segmented genome, related to members of the family *Bunyaviridae* and transmitted by eriophyid mites (Mielke-Ehret and Mühlbach, 2012). Six viruses are currently recognized as members of the genus: European mountain ash ringspot-associated virus, fig mosaic virus (FMV), wheat mosaic virus (WMoV), pigeon pea sterility mosaic virus (PPSMV), raspberry leaf blotch virus (RLBV) and rose rosette virus (RRV) (ICTV Master Species List, 2015 v1). Three recently characterized viruses; actinidia chlorotic ringspot-associated virus (AcCRaV), pigeon pea sterility mosaic virus 2 (PPSMV-2) and redbud yellow ringspot-associated virus (RYRSaV) are *bona fide* members of

the genus and await official recognition (Di Bello et al., 2016; Elbeaino et al., 2015; Zheng et al., 201x).

The virus, provisionally named blackberry leaf mottle-associated virus (BLMaV), is widespread and found in a significant number of BYVD-affected samples tested in this study. The objectives of this work included molecular characterization of the virus, development of a reliable detection protocol, evaluation of incidence of the virus in BYDV-affected plants in the southeastern U.S. and eriophyid mite transmissibility.

2. Material and methods

2.1 Genome assembly

The project spanned five years and different approaches were applied to determine the genomic sequences based on the available technology at the time. The initial approach was similar to that of Laney et al. (2011) using Illumina large scale sequencing, which yielded small genome fragments. In order to obtain additional sequences, an adenosine-tail was added to dsRNA, extracted as described in Tzanetakis and Martin (2008), using the Poly-A Tailing Kit [Ambion]. After ethanol precipitation, the tailed dsRNA was reverse transcribed and subjected to PCR with primers comprised of the 13 conserved bases complementary to conserved terminal sequences in emaravirus genomic RNAs and a dT₁₈ track (Laney et al., 2011). The resulting amplicons corresponding to RNAs 2-4 were cloned into the pCRTM2.1-TOPO[®] TA vector, as described by the manufacturer (Invitrogen) and sequenced. Due to the size of RNA1, PCR amplification did not yield a product. For this reason, a primer walking approach using degenerate primers was employed (Supplemental Table 1). Virus specific primers were designed to fill sequence gaps within the RNA (Supplemental Table 1). Amplicons of expected sizes were

cloned and sequenced. Three clones of each PCR product were sequenced to obtain a 3x genome coverage. RNA 5 was obtained using the 454-Junior platform as previously described (Di Bello et al., 2015). The VirFind pipeline was used for identification and *de novo* assembly of the original Illumina and filtered 454-Junior reads into larger contigs as previously described (Ho and Tzanetakis, 2014).

The 5' and 3' ends of RNA3 were verified by RACE as described for RRV (Laney et al., 2011). The nucleotide sequence of the BLMaV genome is deposited in GenBank under accession numbers KY056657- KY056661.

To determine whether the five segments belong to the same virus, RT-PCR was implemented using primers specific to each segment (Supplemental Table 1). Thirty two (32) BLMaV-infected blackberries, collected from different US states and BLMaV cleft-grafted black raspberries (*R. occidentalis*, L.) were tested. Representative amplicons were sequenced to confirm their identity.

Phylogenetic analysis conducted on aligned amino acid sequence, using the Neighbor-Joining (NJ) and Maximum Likelihood (ML) methods of phylogenetic inference with 1000 bootstrap replicates options as implemented in MEGA 6.0 (Tamura et al., 2013).

2.2 Detection and geographical distribution

Total nucleic acids were extracted from 524 samples collected from commercial and wild blackberries from Arkansas, Florida, Georgia, North Carolina, Maryland, Mississippi, Oklahoma, Oregon, and South Carolina (Table 1). Nucleic acid extractions and reverse

transcription (RT) were conducted according to Poudel et al. (2013). The integrity of the RNA and absence of PCR inhibitors were evaluated using an internal control as described by Thekke-
Veetil et al. (2016).

In order to develop a robust detection system, the nucleoprotein gene of 32 representative BLMaV variants were sequenced and aligned using CLUSTALW embedded in BioEdit (Hall, 1999). Different primers were designed based on consensus sequence of BLMaV-P3 having 100% nucleotide identity to all variants. Different primer combinations were tested under several PCR conditions (data not shown). Primer set P3-For358 (5'-TACAGTTGCATCTTACC TTTCG-3') and P3-Rev706 (5'- GAATTCATACCCAGGAACAATC-3') that amplify a 349 nt long fragment of RNA 3 provided higher sensitivity and specificity when compared to other primers and thus used for routine detection. The PCR program was initiated with 94°C for 2 min followed by 40 cycles of 94°C for 30 sec, annealing at 58°C for 15 sec, and extension at 72°C for 30 sec; followed by final extension of 10 min at 72°C.

2.3 Mite transmission and identification

'Natchez' blackberry plants, tested positive for BLMaV and naturally infested with eriophyid mites were used as the virus source in transmission experiments. In two independent experiments, adult mites were collected under a dissecting microscope with a human eyelash attached to a dissecting needle (De Lillo et al., 2010) as routinely done in our laboratory and transferred onto leaves of two groups of five BLMaV-free 'Natchez' blackberries, 50 mites per plant. Blackberries were inspected for symptoms and assessed for BLMaV infection by RT-PCR three months of post infestation. For mite identification, the ITS region was amplified from about 20 mites collected from BLMaV-infected blackberries as described previously (Lava Kumar et al., 2001). Briefly, mites were boiled in 20 µl H₂O for one minute before fast chilling

in liquid nitrogen. One µl of crude extract was used for rDNA amplification using primers MITS1 (5'-CCGTAGGTGAAC CTGCGGAAGG-3') and MITS4 (5'-CCACCGTTAATTG TGATTTATTTTGTC-3') (Fenton et al., 1997; Lava Kumar et al., 2001) and amplicons were directly sequenced. Mites belonging to *Phyllocoptes fructiphilus* (Keifer) were used as a control in the experiments. The nucleotide sequence of ITS1 region of eriophyid mites used in BLMaV transmission has been deposited in GenBank under accession number KY095116.

2.4 Experimental Host range

BLMaV-infected leaf tissue homogenized in 0.05 M phosphate or 0.05 M phosphate containing 2% nicotine (pH 7.2) was used to mechanically inoculate carborundum-dusted leaves of plants belonging to twelve species: *Cucurbita pepo*, *Glycine max* cv. Lee, *Pisum sativum* cv. Wando, *Nicotiana occidentalis*, *Nicotiana benthamiana*, *Cucumis sativus* cv. National Pickling, *Phaseolus vulgaris* var. Black Valentine, *Gomphrena globosa*, *Spinacia oleracea* cv. Bloomsdale, *Chenopodium amaranticolor*, *Chenopodium quinoa*, *Beta vulgaris* cv. Ruby Queen. Two different inoculation experiments for a total of 12 plants per species were performed and plants were tested for the presence of BLMaV by RT-PCR one month post-inoculation.

Material from a RT-PCR positive 'Natchez' blackberry were cleft-grafted onto twenty *Rosa multiflora* seedlings and 10 virus-tested black raspberry 'Munger' plants. Material was kept in a moist chamber for two weeks and then grown in a greenhouse and tested monthly for six months for the presence of the virus by RT-PCR.

3. Results

3.1. Genome organization and phylogenetic analysis

Five negative-sense RNAs with homology to other emaraviruses were discovered to constitute the BLMaV genome. Each RNA encodes a single ORF located on the complementary strand of the genomic RNA. RNA 1 is 7050 nt long (Fig. 1) and encodes the ORF between CAU₇₀₀₀₋₇₀₀₂ and UUA₉₂₋₉₄. The ORF encodes a putative 2302 amino acid (aa) protein (p1) with a predicted molecular mass of 268 kDa. P1 is orthologous to the conserved Emaravirus/*Bunyaviridae* RNA-dependent RNA polymerase (RdRp) (Bunya_RdRp superfamily; pfam04196, E-value: 4.48e-45) with the A-E enzymatic motifs mapped between aa₁₁₃₂₋₁₃₃₂. Motifs A (DASKWSARD₁₁₃₂₋₁₁₄₀) and C (SDDSTYDF₁₂₅₈₋₁₂₆₅) are 100% conserved across all emaraviruses sequenced to date (Di Bello et al., 2016; Zheng et al., 2016). Motif D of all emaraviruses include two Lys₁₃₁₀₋₁₃₁₁ residues with the exception of AcCRaV whereby its motif D sequence is NK. BLMaV Motif B shares the same sequence (QGNLNMISS₁₂₁₇₋₁₂₂₅) as PPSMV-2, FMV, and RRV whereas motif E₁₃₂₀₋₁₃₃₂ is only divergent in RLBV where the Thr is substituted to a Ser (Supplemental Fig. 1).

RNA 2 is 2271 nt long and encodes a putative protein (P2) CAU₂₂₂₂₋₂₂₂₄-UUA₂₆₄₋₂₆₆ of 652 aa (75 kDa). *In silico* analysis revealed that P2 contains essential sequence elements of the glycoprotein precursor of bunyaviruses, six putative glycosylation sites and four transmembrane helices. The tetrapeptide (ADDN₁₉₆₋₁₉₉) sequence, may present the cleavage site that yields the two mature glycoproteins, Gn (23 kDa) and Gc (52 kDa). This tetrapeptide was found in other emaraviruses and is identical to that of EMARaV, FMV, and RRV (Elbeaino et al., 2009; Laney et al., 2011; Mielke and Muehlbach, 2007). Like other emaraviruses, BLMaV P2 contains a phlebovirus glycoprotein motif (GCX₂CX₂G₄₈₂₋₄₈₉).

RNA3 is 1510 nt in size and contains a single ORF (CAU₁₄₁₁₋₁₄₁₃-UUA₄₆₁₋₄₆₃) coding for the putative nucleocapsid protein (p3) of 35kDa. Two conserved amino acid blocks

(NX₂SXNX₃A₁₃₁₋₁₄₀ and NXLA₁₇₉₋₁₈₂) (Supplemental Fig. 2) were found in BLMaV p3; however, there is a substitution from Arg to Lys₁₈₀ in BLMaV p3, also present in RYRSaV, WMoV, and RLBV (NKLA₁₇₉₋₁₈₂). These regions were previously reported as possible emaravirus nucleocapsid motifs (Di Bello et al., 2016; Elbeaino et al., 2009; Laney et al., 2011).

RNA4 is 1504 nt long and contains a single ORF (CAU₁₄₂₂₋₁₄₂₄-UUA₃₃₁₋₃₃₃) of 363 codons. The putative protein with an estimated molecular mass of 41 kDa is postulated to be involved in cell-cell movement based on its homology to RRV, FMV and RLBV p4s (Ishikawa et al., 2013; Yu et al., 2013).

RNA5 is 1224 nt long and codes for a putative protein (CAU₁₁₅₄₋₁₁₅₆-UCA₄₇₄₋₄₇₆) of 226 aa and molecular mass of 26 kDa. The secondary structure of BLMaV p5 (Supplemental Fig. 3) makes it a likely ortholog of EMARaV p4, FMV p6, PPSMV2 p6, RRV p6, and RYRSaV p5. The aa identity between the BLMaV proteins and their emaravirus orthologs are presented in Table 2.

Several attempts to identify additional BLMaV genomic RNAs using multiple sequencing approaches and technologies/platforms, including Sanger, Illumina and 454, were unsuccessful. As confirmed by specific RT-PCR test, the five RNAs were present in all 32 tested BLMaV variants collected from different states in the U.S. In addition, the five RNAs were detected in wild blackberry (8 samples) and in four graft-inoculated BLMaV black raspberry. Sequencing of PCR products obtained using specific primers for all five segments showed nucleotide identity of 90 to 100% between variants (data not shown), supporting the notion that the RNAs belong to the same species.

Phylogenetic trees were constructed using the complete amino acid sequences of RdRp, glycoprotein precursor and nucleocapsid proteins and orthologous sequences of BLMaV, other emaraviruses and selected recognized members of the family *Bunyaviridae* (Fig. 2).

The virus consistently clustered within the genus *Emaravirus* with high bootstrap values. Phylogenetic trees created using Neighbor-Joining and Maximum Likelihood methods gave similar topologies, depicting the evolutionary relatedness between BLMaV and emaraviruses.

3.2 Detection and occurrence of blackberry leaf mottle-associated virus

Primers were designed based on multiple alignments of 32 p3 gene sequences. The selected primers showed 100% nt identity to all variants used (data not shown). The specificity of the assay was assessed using several BLMaV-infected or -free plants. The presence of BLMaV was investigated in 524 BYVD-affected blackberry plants collected from U.S. states using the RT-PCR protocol and was detected in 43% of the samples. In Arkansas, the state that was assayed for infection in both wild and cultivated material, the infection rate was higher in wild blackberries (59%) than in commercial fields (38%; Table 1). The highest infection rates were found in the samples collected from North Carolina (83%).

3.3. Mite transmission and molecular identification

Four out of ten ‘Natchez’ plants infested with a homogeneous population of mites transferred from a BLMaV-infected plant were BLMaV–positive as assessed by RT-PCR with plants remaining symptomless for at least six months post infestation.

Amplicons generated by ITS1-PCR from 20 gall mite sampled from BLMaV-infected blackberries were 340 bp long whereas the control, *Phyllocoptes fructiphilus* gave amplicons of ~550 base pairs (Supplemental Fig. 4). The difference in product size is not unusual as noted in the original publication describing the primers used in the study (Fenton et al., 1997).

The mite is distinct from *Phyllocoptes fructiphilus* and *P. gracilis*, vectors of RRV and RLBV in rose and raspberry, respectively (Di Bello et al., 2015; McGavin et al., 2012). Phylogenetic analysis indicates that it is distantly related to the genus *Aceria*, representing a possible new species or even genus within the *Eriophyidae* (Supplemental Fig. 5).

3.4 Experimental Host Range

BLMaV was successfully graft-transmitted onto four black raspberry ‘Munger’ plants as confirmed by RT-PCR using the primer pair P3-For358/ P3-Rev706 and sequencing. Amplicons obtained from the four plants were 99% identical to the BLMaV RNA 3 of the type isolate. None of the grafted roses showed symptoms or tested positive for BLMaV as was the case with the twelve herbaceous species used in the host range study.

4. Discussion

BYVD is attributed to mixed infection of RNA viruses (Khadgi, 2015; Martin et al., 2013; Thekke-Veetil et al., 2013), and here we describe a new negative-strand RNA virus, a putative emaravirus, found in a significant number of diseased plants.

Confirmed or putative emaraviruses have been reported with number of genomic RNA segments ranging between four and eight (Di Bello et al., 2016, 2015, Elbeaino et al., 2015, 2014; Ishikawa et al., 2012; Lu et al., 2015; Mielke and Muehlbach, 2007; Tatineni et al., 2014; Zheng et al., 2016). Several attempts to identify additional BLMaV genomic RNAs using a similar approach applied for RRV and RYRSaV (Di Bello et al., 2015; 2016) failed to identify additional segments. RNAs 1-4 encode essential proteins, involved in virus replication, virion formation and movement. The function of the protein encoded in RNA 5 remains ambiguous, even though orthologs exist in other emaraviruses. Yet, the possibility of BLMaV having

additional segments cannot be definitely excluded, given the lack of extensive homology of RNAs 5-8 among emaraviruses proven to have more RNAs (Lu et al., 2015; Tatineni et al., 2014).

There are two emaraviruses known to infect *Rubus* spp., RLBV and BLMaV. The two viruses belong to distinct evolutionary lineages supporting the allopatric evolution of the genus (Di Bello et al., 2016). This polyphyletic nature of the genus is also evident in the case of PPSMV and PPSMV-2, both of which infect the same species, but do not share a recent common ancestor (Elbeaino et al., 2015).

Sensitive reliable detection tests are essential for virus disease management schemes. We developed a detection protocol based on data from 32 virus variants collected from several states in the United States. This protocol is based on the nucleoprotein gene which is assumed to be expressed at high levels; allowing for reliable detection of the virus. Using this test, BLMaV presence was verified in the vast majority of states surveyed. However, plants were frequently co-infected with other viruses (Hassan et al., unpublished; Khadgi, 2015) making BYVD epidemiology and management even more complex than previously thought (Martin et al., 2013). The incidence of BLMaV was 43% among all samples tested. The high incidence of the virus in wild blackberry in Arkansas (59%) indicates that they may act as reservoir for the virus, a major consideration in developing management strategies for BYVD.

Another essential component of disease management is vector identification, also part of this communication. Using ITS sequencing, methodology that is used to distinguish between eriophyid mites species and genera (Fenton et al., 1997; Hillis and Dixon, 1991; Navajas et al., 2001), we determined that the BLMaV vector possibly represents a new species or even genus within the *Eriophyidae*. It remains to be investigated whether the mite can transmit BLMaV to

other *Rubus* species. This is of particular importance for raspberries, as they are susceptible to the virus, as demonstrated in the cleft-grafting experiments.

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References

- De Lillo, E., Craemer, C., Amrine Jr, J., Nuzzaci, G., 2010. Recommended procedures and techniques for morphological studies of Eriophyoidea (Acari: Prostigmata), in: Eriophyoid Mites: Progress and Prognoses. Springer, pp. 283–307.
- Di Bello, P.L., Ho, T., Tzanetakis, I.E., 2015. The evolution of emaraviruses is becoming more complex: seven segments identified in the causal agent of Rose rosette disease. *Virus Res.* 210, 241–244.
- Di Bello, P.L., Laney, A.G., Druciarek, T., Ho, T., Gergerich, R.C., Keller, K.E., Martin, R.R., Tzanetakis, I.E., 2016. A novel emaravirus is associated with redbud yellow ringspot disease. *Virus Res.* 222, 41–47.
- Elbeaino, T., Digiario, M., Martelli, G.P., 2009. Complete nucleotide sequence of four RNA segments of fig mosaic virus. *Arch. Virol.* 154, 1719–1727.
- Elbeaino, T., Digiario, M., Uppala, M., Sudini, H., 2015. Deep sequencing of dsRNAs recovered from mosaic-diseased pigeonpea reveals the presence of a novel emaravirus: pigeonpea sterility mosaic virus 2. *Arch. Virol.* 160, 2019–2029.
- Elbeaino, T., Digiario, M., Uppala, M., Sudini, H., 2014. Deep sequencing of pigeonpea sterility mosaic virus discloses five RNA segments related to emaraviruses. *Virus Res.* 188, 27–31.
- Fenton, B., Malloch, G., Moxey, E., 1997. Analysis of eriophyid mite rDNA internal transcribed spacer sequences reveals variable simple sequence repeats. *Insect Mol. Biol.* 6, 23–32.
- Hall, T., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* 41, 95–98.
- Hillis, D.M., Dixon, M.T., 1991. Ribosomal DNA: molecular evolution and phylogenetic inference. *Q. Rev. Biol.* 411–453.
- Ho, T., Tzanetakis, I.E., 2014. Development of a virus detection and discovery pipeline using next generation sequencing. *Virology* 471-3, 54–60.

ICTV Master Species List, 2015.

Ishikawa, K., Maejima, K., Komatsu, K., Kitazawa, Y., Hashimoto, M., Takata, D., Yamaji, Y., Namba, S., 2012. Identification and characterization of two novel genomic RNA segments of fig mosaic virus, RNA5 and RNA6. *J. Gen. Virol.* 93, 1612–1619.

Ishikawa, K., Maejima, K., Komatsu, K., Netsu, O., Keima, T., Shiraishi, T., Okano, Y., Hashimoto, M., Yamaji, Y., Namba, S., 2013. Fig mosaic emaravirus p4 protein is involved in cell-to-cell movement. *J. Gen. Virol.* 94, 682–686.

Khadgi, A., 2015. Blackberry Virosome: A Micro and Macro Approach. University of Arkansas.

Kumar, P.L., Fenton, B., Duncan, G., Jones, A., Sreenivasulu, P., Reddy, D., 2001. Assessment of variation in *Aceria cajani* using analysis of rDNA ITS regions and scanning electron microscopy: implications for the variability observed in host plant resistance to pigeonpea sterility mosaic disease. *Ann. Appl. Biol.* 139, 61–73.

Laney, A.G., Keller, K.E., Martin, R.R., Tzanetakis, I.E., 2011. A discovery 70 years in the making: characterization of the Rose rosette virus. *J. Gen. Virol.* 92, 1727–1732.

Lu, Y., McGavin, W., Cock, P.J., Schnettler, E., Yan, F., Chen, J., MacFarlane, S., 2015. Newly identified RNAs of Raspberry leaf blotch virus encoding a related group of proteins. *J. Gen. Virol.* 96, 3432–3439.

Martin, R.R., MacFarlane, S., Sabanadzovic, S., Quito, D., Poudel, B., Tzanetakis, I.E., 2013. Viruses and Virus Diseases of Rubus. *Plant Dis.* 97, 168–182.

McGavin, W.J., Mitchell, C., Cock, P.J.A., Wright, K.M., MacFarlane, S.A., 2012. Raspberry leaf blotch virus, a putative new member of the genus Emaravirus, encodes a novel genomic RNA. *J. Gen. Virol.* 93, 430–437.

Mielke, N., Muehlbach, H.-P., 2007. A novel, multipartite, negative-strand RNA virus is associated with the ringspot disease of European mountain ash (*Sorbus aucuparia* L.). *J. Gen. Virol.* 88, 1337–1346.

Mielke-Ehret, N., Muehlbach, H.-P., 2012. Emaravirus: a novel genus of multipartite, negative strand RNA plant viruses. *Viruses* 4, 1515–1536.

Navajas, M., Gutierrez, J., Williams, M., Gotoh, T., 2001. Synonymy between two spider mite species, *Tetranychus kanzawai* and *T. hydrangeae* (Acari: Tetranychidae), shown by ribosomal ITS2 sequences and cross-breeding experiments. *Bull. Entomol. Res.* 91, 117–123.

Poudel, B., Wintermantel, W.M., Cortez, A.A., Ho, T., Khadgi, A., Tzanetakis, I.E., 2013. Epidemiology of Blackberry yellow vein associated virus. *Plant Dis.* 97, 1352–1357.

Shahid, M.S., Aboughanem-Sabanadzovic, N., Sabanadzovic, S., Tzanetakis, I.E., 2016. Genomic characterization and population structure of a badnavirus infecting blackberry. *Plant Dis.* 101, 110–115.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* mst197.

Tatineni, S., McMechan, A.J., Wosula, E.N., Wegulo, S.N., Graybosch, R.A., French, R., Hein, G.L., 2014. An eriophyid mite-transmitted plant virus contains eight genomic RNA segments with unusual heterogeneity in the nucleocapsid protein. *J. Virol.* 88, 11834–11845.

Thekke-Veetil, T., Ho, T., Moyer, C., Whitaker, V.M., Tzanetakis, I.E., 2016. Detection of Strawberry necrotic shock virus using conventional and TaqMan® quantitative RT-PCR. *J. Virol. Meth.* 235, 176–181.

Thekke-Veetil, T., Sabanadzovic, S., Keller, K.E., Martin, R.R., Tzanetakis, I.E., 2013.
Molecular characterization and population structure of Blackberry vein banding associated
virus, new ampelovirus associated with yellow vein disease. *Virus Res.* 178, 234–40.
Yu, C., Karlin, D.G., Lu, Y., Wright, K., Chen, J., MacFarlane, S., 2013. Experimental and
bioinformatic evidence that raspberry leaf blotch emaravirus P4 is a movement protein of
the 30K superfamily. *J. Gen. Virol.* 94, 2117–2128.
Zheng, Y., Navarro, B., Wang, G., Wang, Y., Yang, Z., Xu, W., Zhu, C., Wang, L., Di Serio, F.,
Hong, N., 201x. Actinidia chlorotic ringspot-associated virus: A novel emaravirus
infecting kiwifruit plants. *Mol. Plant Pathol.*, DOI: 10.1111/mpp.12421

Table 1. Incidence of blackberry leaf mottle-associated virus in cultivated and wild blackberries collected from USA states.

		infected/ tested
	State	
	Arkansas	25/94 (38%)
	Arkansas-W ¹	73/123 (59%)
	Florida	2/32 (6%)
	Georgia	12/58 (21%)
	North Carolina	52/63 (83%)
	Maryland	0/20 (0%)
	Mississippi	9/42 (21%)
	Oklahoma	5/10 (50%)
	Oregon	1/9 (11%)
	South Carolina	47/73 (64%)
	Total	226/524 (43%)

W¹ : Wild blackberry

Table 2 Percent amino acid identities between blackberry leaf mottle-associated virus (BLMaV) putative proteins and emaraviruses orthologs. Highest scores are indicated in bold.

Emaraviruses	BLMaV proteins				
	P1	P2	P3	P4	P5*
FMV	67	54	61	55	26
RRV	67	51	58	52	29
PPSMV-2	66	54	62	54	27
PPSMV	53	41	41	39	-
EMARaV	48	33	31	-	10
RYRSaV	46	36	38	32	14
AcCRaV	46	35	35	31	-
RLBV	32	21	19	17	-
WMoV	30	20	15	16	-

Abbreviations: fig mosaic virus (FMV), rose rosette virus (RRV), pigeon pea sterility mosaic virus-2 (PPSMV-2), pigeon pea sterility mosaic virus (PPSMV), European mountain ash ringspot-associated virus (EMARaV), redbud yellow ringspot-associated virus (RYRSaV), Actinidia chlorotic ringspot-associated virus (AcCRaV), raspberry leaf blotch virus (RLBV), wheat mosaic virus (WMoV).

P5* - BLMaV p5 was compared to its orthologs; EMARaV p4, FMV p6, RRV P6, and RYRSaV p5.

Figure 1

Fig. 1. Schematic representation of the blackberry leaf mottle associated virus genome. Expression product of each RNA is presented by a gray box; their aa length, estimated molecular mass (kDa), and function of the putative protein are indicated. Figure not drawn to scale.

Figure 2

P1

P2

P3

Fig 2. Neighbor joining phylogenetic trees generated on the deduced amino acid sequences of the RNA-dependent RNA polymerase (p1), glycoprotein precursor (p2) and nucleoprotein (p3) of blackberry leaf mottle associated virus (BLMaV), recognized and putative members of the genus *Emaravirus* and orthologs of representative members in the family *Bunyaviridae* and genus *Tenuivirus*. The bar represents the number of amino acid replacements per site. The shaded-boxed region highlights the genus *Emaravirus* clade. Position of BLMaV within this clade is indicated by a black dot (●). Abbreviations: Iris yellow spot virus (IYSV, genus *Tospovirus*, family *Bunyaviridae*); Dugbe virus (DUGV, genus *Nairovirus*, family *Bunyaviridae*); Puumala virus (PUUV, genus *Hantavirus*, family *Bunyaviridae*); Rift valley fever virus (RVFV) and Aguacate virus (AGUV) both genus *Phlebovirus*, family *Bunyaviridae*; bunyamwera virus (BUNV) and Akabane virus (AKAV) both genus *Orthobunyavirus*, family *Bunyaviridae*; rice grassy stunt virus (RGSV) is unassigned (genus *Tenuivirus*). GenBank accession numbers are indicated. Members of the genus *Emaravirus* include actinidia chlorotic ringspot-associated virus (AcCRaV); European mountain ash ringspot-associated virus (EMARaV); fig mosaic virus (FMV); wheat mosaic virus (WMoV); pigeon pea sterility mosaic virus (PPSMV); pigeon pea sterility mosaic virus 2 (PPSMV-2); raspberry leaf blotch virus (RLBV); redbud yellow ringspot-associated virus (RYRSaV); rose rosette virus (RRV).