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BRIEF REPORT

Molecular characterization and detection of a novel vitivirus infecting blackberry

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Abstract

Blackberries exhibiting yellow vein disease symptoms were found to be infected by a new virus, a putative member of the genus *Vitivirus*. Recombination assessment of several vitiviruses revealed multiple events involving the newly identified virus isolate. Occurrence in areas of high disease pressure was investigated and the population structure was studied using the *movement* and *coat protein* genes; both under purifying selection. This information was exploited in the development of a detection protocol for routine screening and *Rubus* certification programs around the globe.

Blackberry (*Rubus* spp) has become a major fruit crop in the United States [1, 2]. Blackberry yellow vein (BYV), a disease caused by coinfection with two or more viruses, affects cultivated and wild blackberry alike [3] and is a major constraint to production in the Southeastern United States. There are several reports on the characterization, population structure and epidemiology of viruses associated with the disease [3–11]. Yet, there are still cases where none or only one of those viruses are present in BYV-affected plants [12, Tzanetakis et al. unpublished], indicating that additional viruses may be involved in symptom development. Such was the case of 'Osage' blackberries that inspired the study presented here (Supplemental Fig. 1).

Double-stranded RNA-enriched material was extracted from symptomatic leaves using the protocol described by Tzanetakis and Martin [13] and used as a template for high throughput sequencing as described previously [14]. The sequences were analyzed using VirFind [14], with several of the assembled reads showed homology to vitiviruses. The virus genome reconstructed from several overlapping RT-PCR products was sequenced again using the Sanger method. The 5' and 3' termini were obtained using FirstChoice RLM-Race Kit (ThermoFisher, Scientific, USA) and poly A-tailing, essentially as described previously [7]. All contigs were assembled using the VirFind and BioEdit bioinformatics tools [14, 15] and the 7,285 nucleotide (nt) long genome of the virus, sequenced to at least 3x coverage by Sanger sequencing, was deposited in GenBank under accession no MG254193.

Open reading frame (ORF) identification was done on ORFfinder [16] whereas phylogenetic analyses were conducted on matrices of aligned sequences using neighbor-joining phylogenetic inference with 1000 bootstrap pseudo-replicates [17]. Potential recombination events were identified using RDP4 v.4.95, implementing seven algorithms; RDP, GENECONV, BOOTSCAN, MAXIMUM CHI SQUARE, CHIMAERA, SISTERSCAN, and 3SEQ [18]. Analysis was performed on default settings with a p-value cut-off of 0.05. Recombination events were considered significant if identified by at least four algorithms. Selection pressure on the movement and coat genes was calculated using the Synonymous/ Non-synonymous analysis program (SNAP) [19].

The population structure of the virus was studied targeting a 1667 nt genome fragment flanking the MP and CP using primers BVA5348F (5'-AGGTTTGAGCCTAGC AGGGTTGTAC-3')/BVA7015R (5'-TTTAGCAGCACT

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CTTAGACTCCCC-3'). Three hundred and nine (309) samples, exhibiting BYVD-like symptoms, collected from Arkansas (190), Florida (20), Georgia (41), Mississippi (5), North Carolina (37) and South Carolina (16) were assessed. Amplicons were cloned and sequenced, with at least 3x coverage, and sequences were edited and assembled using BioEdit [15]. Sequences from 12 isolates were submitted to GenBank under accession numbers MG893877-88.

The area targeted for the population structure study was selected so it would represent highly expressed genes; representing a better target for a PCR-based detection protocol. Primers were selected based on the following criteria: that they had a) the primer target sequence was 100% identical between isolates; b) they could be multiplexed with internal control primers primers NADHbF/NADHbR [13]; and c) that they shared no nt sequence identity with other plant viruses or the blackberry genome. Based on those criteria, several primers sets were designed (data not shown); BVA6435F (5'-ACGGTACTAGTTCTAAAGTGAGT-3')/ BVA6978R (5'-TTAGCAGCACTCTTAGACTCCCC-3'), which amplify a 543 nucleotide fragment of the virus genome, gave consistent, reliable results so were chosen for virus-specific RT-PCR detection (Supplemental Fig. 2). The PCR amplification conditions included an initial denaturation for 3 min at 94 °C; followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 55 °C for 25 sec, and extension at 72°C for 45 sec followed by a final extension at 72 °C for 10 min. The detection assay was used to rescreen the 309 samples from the population structure study.

The identified virus, tentatively named as blackberry virus A (BVA), codes for five ORFs similar to other vitiviruses (Fig. 1) [20]. The identities and similarities between the BVA proteins and their vitivirus orthologs are presented in Table 1. ORF1 codes for the viral replicase with four conserved domains: a methyltransferase at its N-terminus (aa_{45-292}), a helicase ($aa_{743-893}$), a DNA alkylation damage repair protein (AlkB; $aa_{938-1023}$), and an RNA-dependent RNA polymerase (RdRp) located at the C-terminus ($aa_{1360-1596}$) [21, 22]. ORF2 encodes a 17 kDa putative protein of unknown function, orthologs of which have been hypothesized to play a role in transmission in grapevine-infecting vitiviruses [23]. The 272 aa, 31KDa product of ORF3 shares significant homology with vitivirus movement proteins (MP) [24]. The BVA MP contains a conserved motif of the 30K MP superfamily (aa_{59-186}; pfam01107). ORF4 encodes the putative coat protein (CP) of 199 aa (22kDa). A more detailed analysis of this ORF identified two in-frame start codons similar to other vitivirus CPs [25–27]. Similar to its orthologs, the protein contains the highly conserved $RQ_{108-109}$/$FDF_{150-152}$, speculated to form a salt-bridge and stabilize the virions of filamentous viruses [28] . ORF5 encodes a putative 109 aa protein of 12.5 kDa. This highly polar protein shares significant similarity with the nucleic acid binding protein (NABP) found in other vitiviruses (Table 1). Recent studies have shown that the vitivirus ORF5-encoded protein is a RNA silencing suppressor [29, 30], and the arginine-rich motif (ARM) affects pathogenesis by binding nucleic acids in a non-specific manner [23]. BVA has an ARM at the amino terminus of its ORF5, though there is some sequence variation when compared to other vitiviruses: $KRRRARR_{15-21}$ in GVA, $AKRRAKR_{13-19}$ in GVE and $AKRRARR_{13-19}$ in BVA.

Phylogenetic analysis, based on the complete sequences of selected vitiviruses and members of other *Betaflexiviridae* taxa (*Trichovirus*, *Foveavirus*, *Carlavirus, Citrivirus, Capillovirus* and unassigned species classified within the family) is presented in Fig. 2. BVA groups with vitiviruses, and clusters with agave tequilana leaf virus (AgTLV) in one clade. The same was observed when using CP sequences (Fig. 2). Based on data presented here and the fact that the virus has <60% nt and <70% aa identity to other vitiviruses in any of the encoded proteins (Table 1), identities much below the species demarcation criteria for

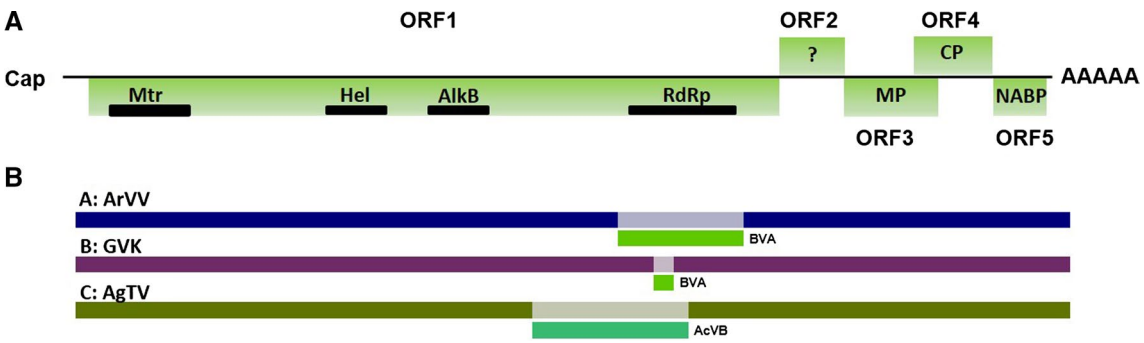

Fig. 1 Genome organization of blackberry virus A. Abbreviations: Mtr: methyltransferase; Hel: helicase; AlkB: 2OG-Fe(II) oxygenase domain; RdRp: RNA dependent RNA polymerase; MP: movement protein; CP: coat protein: NABP nucleic-acid-binding protein. ORF2 encodes a hypothetical protein of unknown function. (B) Scheme of the recombinant genomes from RDP analysis of three interspecies recombination events (A, B and C)

Table 1 Comparisons of the predicted amino acid identity and similarity (in parentheses) of five genes encoded by blackberry vitivirus A and related vitiviruses

Vitivirus	GenBank Acc	Amino acid identity (similarity)					Size (aa)/molecular weight (kDa)				
		ORF1	ORF2	ORF3	ORF4	ORF5	ORF1	ORF2	ORF3	ORF4	ORF5
Blackberry virus A	MG254193	ID	ID	ID	ID	ID	1688/192	148/17	272/31	199/22	109/13
Actinidia virus A	JN427014	28 (53)	6 (33)	25 (52)	38 (56)	10 (38)	1710/195	219/25	297/33	198/21	105/12
Actinidia virus B	JN427015	28 (54)	6 (29)	22 (51)	36 (56)	10 (42)	1707/195	231/27	290/32	198/21	105/12
Agave tequilana leaf virus	KY190215	**46 (63)**[b]	**44 (68)**	**64 (54)**	**70 (87)**	**57 (68)**	1539/176	151/17	267/30	199/22	126/12
Arracacha virus V	KY392781	27 (52)	NA[c]	27 (80)	38 (60)	28 (52)	1705/194	NA[c]	293/33	192/21	110/13
Grapevine virus A	DQ855083	27 (53)	7 (39)	27 (57)	45 (62)	13 (39)	1707/195	176/19	278/31	198/22	90/11
Grapevine virus B	EF583906	29 (53)	7 (33)	20 (47)	40 (58)	41 (54)	1708/195	179/20	321/37	197/22	124/14
Grapevine virus E	KF588015	43 (64)	17 (33)	40 (63)	61 (79)	42 (63)	1698/192	191/21	265/29	199/22	108/13
Grapevine virus F	JX105428	28 (52)	11 (42)	27 (58)	39 (59)	9 (42)	1726/197	159/18	271/30	198/22	109/12
Grapevine virus G	MF405923	43(64)	16 (45)	41 (65)	63 (83)	35 (52)	1703/193	154/17	286/32	201/22	117/14
Grapevine virus H	MF521889	28 (54)	5 (41)	30 (60)	40 (57)	23 (48)	1717/195	162/18	265/29	197/22	106/12
Grapevine virus K	NC_035202	28 (54)	16 (39)	29 (56)	45 (62)	12 (37)	1700/195	161/18	273/30	197/22	91/10
Grapevine virus J	NA[d]	28 (54)	9 (38)	28 (54)	44 (65)	11 (42)	1702/195	164/18	273/31	197/22	103/12

[a]Number of predicated amino acids/predicted molecular mass (kDa)

[b]Amino acid sequence identity and, similarity in parenthesis; the highest sequence identities and similarities are shown in bold

[c]Not applicable, as ORF2 was not predicated at Arracacha virus V (ArVV)

[d]Virus sequence not yet available in GenBank

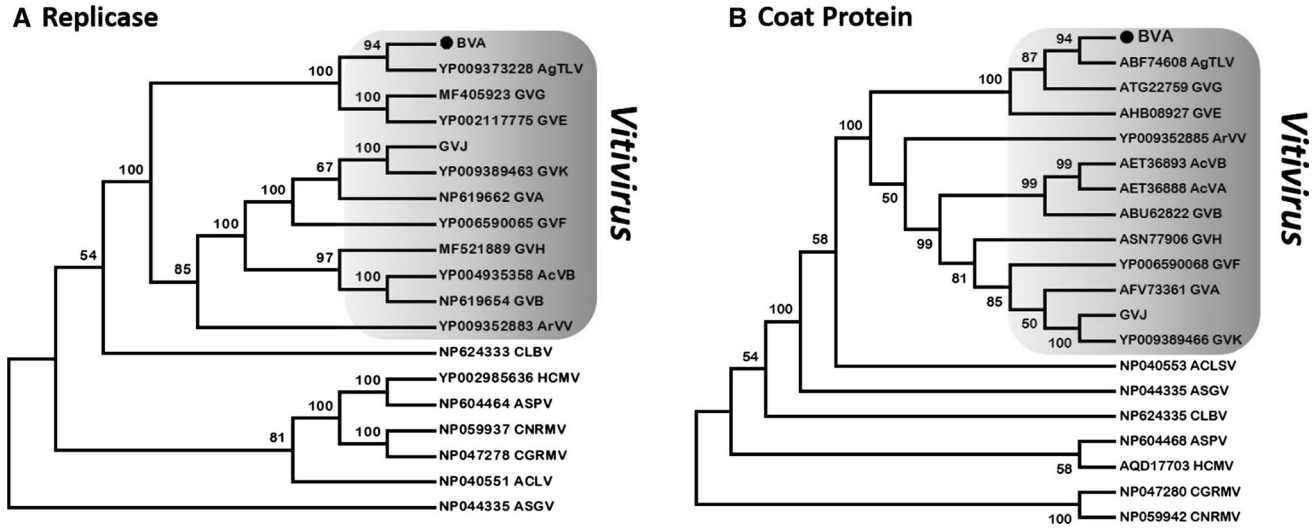

Fig. 2 Phylogenetic analyses using the replicase (A) and coat protein (B) of selected members of each genus in the family *Betaflexiviridae* and vitiviruses. Multiple sequence alignments were generated by Clustal W, and the unrooted cladograms were constructed using neighbor joining. The bootstrap consensus trees were inferred from 1,000 bootstrap pseudoreplicates and presented as percentage values. Virus acronyms are as follows: grapevine virus A (GVA; *Vitivirus*); grapevine virus B (GVB; *Vitivirus*); grapevine virus E (GVE; *Vitivirus*); grapevine virus F (GVF; *Vitivirus*); grapevine virus G (GVG; *Vitivirus*); grapevine virus H (GVH; *Vitivirus*); grapevine virus K (GVK; *Vitivirus*); grapevine virus J (GVJ; *Vitivirus*); actinidia virus A (AcV A; *Vitivirus*); actinidia virus B (AcVB; *Vitivirus*); agave tequilana leaf virus (AgTLV; *Vitivirus*); arracacha virus V (ArVV; *Vitivirus*); apple chlorotic leaf spot virus (ACLSV; *Trichovirus*); apple stem pitting virus (ASPV; *Foveavirus*); hydrangea chlorotic mottle virus (HCMV; *Carlavirus*); citrus leaf blotch virus (CLBV; *Citrivirus*); cherry necrotic rusty mottle virus (CNRMV) and cherry green ring mottle virus (CGRMV) are unnassigned species in family *Betaflexiviridae)*

the genus [20], we conclude that BVA represents an isolate of a new species in that taxon.

The genome of BVA and 12 other recognized vitiviruses were scanned for putative recombination events. RDP4 analysis suggested several putative recombination events (Supplemental Table 2). One of the putative recombination events suggests that AgTLV is a product of BVA or a BVA-like virus acting as the major parent. Moreover, the recombination events of arracacha virus V and grapevine virus K (an isolate of grapevine virus D) include BVA or a BVA-like virus acting as a minor parent (Supplemental Table 1). Interspecies recombination is documented for many plant RNA viruses such as vitiviruses, nepoviruses and prunevirus [31–33]. Interestingly, the putative recombination events (A, B and C) were detected in and around the RdRp domain, similar to other vitiviruses (Fig 1) [31, 34].

To gain insight into the population structure of BVA, the sequence of the *MP* and the *CP* genes was determined for 12 isolates. The identity among the MP sequences of BVA isolates ranged between 98%-100% (nt) and 99%-100% (aa) whereas for the CP it was 98-100% (nt) and 97-100% (aa) (Supplemental Tables 2 and 3). No evidence of recombination events between the isolates was identified using RDP4 (data not shown). The selection pressure was estimated using the mean diversity and substitution ratio; both genes were <1, suggesting overall purifying selection, in agreement to previous reports for other vitiviruses [20].

Out of the 309 plants tested BVA was detected in thirteen plants of different genotypes from Arkansas, North Carolina and South Carolina. The role of BVA in BYV disease is not clear as all samples tested were symptomatic and co-infected with at least BLMaV [3, 6]. Yet this study provides important information for the development of a diagnostic assay that can be used for field screening and certification purposes. The robustness and reliability of an assay are important factors for any diagnostic tool and the one presented here possesses both as it was developed based on the population structure of the virus in the southern United States, the only area in the world that BYV disease is known to impact the crop.

There is limited data available on vitiviruses, although a few have been studied in more depth [35–37]. This is the first vitivirus found in blackberry. The importance of BVA to the industry is unclear but the multiplicity of different viruses in blackberry is of concern due to the possibility of more virulent virus combinations emerging, thus decreasing production and potentially affecting other crops.

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Compliance with ethical standards

Conflicts of interest The authors declare no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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