Evidence of sympatric speciation of elderberry carlaviruses

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Abstract

Five new carlaviruses infecting elderberry were characterized and tentatively named as elderberry virus A-E (EIVA-EIVE). Their genome organization is similar to that of other carlaviruses with size ranging from 8540 to 8628 nucleotides, excluding the polyadenylated tails. EIVA, EIVB and EIVD share a common ancestor as do EIVC and EIVE, indicating that speciation may be sympatric with all viruses having emerged in elderberry. Analyses of the carlavirus conserved domains indicate that the 2-oxoglutarate and the Fe(II)-dependent oxygenase motifs are reliable indicators of virus phylogenetic classification with recombination playing a significant role in the evolution of the genus. A universal RT-PCR assay that detects all the elderberry carlaviruses and potentially other members of the genus has been developed. This tool can be used for research and regulatory purposes as elderberry cultivation is rapidly expanding to new areas where the viruses may be absent.

Keywords: discovery, evolution, universal carlavirus detection

Elderberry (genus *Sambucus*, family Adoxaceae) is gaining popularity as fresh market fruit but also in the culinary industry and medicine (Grbic et al., 2011; Samuels et al., 2012). Several viruses are known to cause detrimental symptoms in both American and European elderberry (Way, 1981) including members of the family *Bromoviridae* [cucumber mosaic virus (Poláka et al., 1990)], *Secoviridae* [arabis mosaic virus, cherry leaf roll virus, strawberry latent ringspot virus, tobacco ringspot virus, tomato black ring virus, tomato ringspot virus (Jones, 1972; Jones and Murrant, 1971; Lister, 1964; Poláka et al., 1990; Uyemoto, 1970; Way, 1981)], and *Tombusviridae* [elderberry latent virus, tobacco necrosis virus, tomato bushy stunt virus (Jones, 1972; Poláka et al., 1990; Uyemoto and Gilmer, 1971)].

Blueberry scorch virus (BIScV), elderberry symptomless virus (EIBSV) and several other putative members of the genus *Carlavirus* (family *Betaflexiviridae*) have also been reported in elderberry (Cooper, 1993; Ellis et al., 1992; Van Lent et al., 1980). BIScV has been characterized at the molecular level (Cavileer et al., 1994), but little is known about other elderberry carlaviruses. This fact makes discrimination between other elderberry carlaviruses a challenging undertaking. In the past, the elderberry carlaviruses were grouped as either of North American or European type; still those 'types' could be isolates or strains of the same species (Ellis et al., 1992).

Preliminary data established the presence of several carlaviruses in elderberry in Missouri (Keller et al., 2015) and Oregon (Ho and Tzanetakis, 2014). In this study the genomes of five elderberry carlaviruses, tentatively named as Elderberry virus A, B, C, D and E (EIVA-EIVE, respectively), are analyzed in regards to their phylogenetic placement and evolution within the genus.

As elderberry is clonally propagated in commercial settings, clean, virus-tested germplasm is of great value for the expanding industry in North America

(Keller et al., 2015). For this reason a detection test for all elderberry carlaviruses and potentially all members of the genus was developed. This tool could efficiently be used for identification of the viruses in a lab or nursery setting, allowing for the elimination of the infected material from the propagation pipeline.

Virus discovery was described in Ho and Tzanetakis (2014) as a proof-of-concept as the complete genomes of the viruses were not presented nor analysis of the genomes was provided. Briefly, five elderberry genotypes showing virus-like symptoms including mosaic, leaf yellowing and malformation and plant dwarf were collected in 2011 (Table 1) from the elderberry germplasm collection of the National Clonal Germplasm Repository in Corvallis, Oregon. The genomic sequences were obtained using dsRNA as template. Leaf tissue (20g) were processed as described (Tzanetakis and Martin, 2008) to enrich in viral nucleic acids. DsRNA was denatured and subject to random reverse transcription and PCR amplification before high throughput sequencing (Ho et al., 2015). Virus contigs were assembled from large scale sequence data from 'Golden', 'Hwy O' and 'G-17321' samples using VirFind (version 1.1) with updated GenBank nucleotide and amino acid databases (downloaded September 03, 2015). Sequence gaps were obtained using virus-specific PCR reactions (Supplementary Table 1 Supplementary Table) with amplicons cloned and sequenced (at least three independent clones/site) as described earlier (Thekke-Veetil et al., 2013). The virus termini were obtained using 5' and 3' Rapid Amplification of cDNA Ends according to manufacturer's instructions (Thermo Fisher Scientific). ORFs identified by NCBI ORF Finder and Blastp (Altschul et al., 1997) were used for identification of conserved protein domains (Marchler-Bauer et al., 2015).

A universal test able to detect all known elderberry carlaviruses and potentially **other** member of the genus was developed based on sequence data available in Genbank as of July 2015. Thirty seven (37) carlavirus genomes were used to design oligonucleotide primers for the assay (Supplementary Table 2). Sequence alignments were generated using ClustalW (Thompson et al., 1994) through BioEdit software version 7.2.5 (Hall, 1999) with default parameters. Two conserved regions at the amino acid level were back translated using EMBOSS 6.3.1:backtranambig (Rice et al., 2000; Supplementary Table 3) for primer design. Reverse transcription was done using an oligo dT primer and PCR reactions included a 2 min denaturation at 94 C followed by 40 cycles of 15 sec at 94C, 15 sec at 53C and 30 sec at 72C. The reaction terminated with 4 min incubation at 72C. Amplicons were separated on a 1.5% agarose gel, stained in GelRed solution (Biotium) and visualized in a UV transilluminator. Virus specific primers were also used to confirm individual infections (Supplementary Table 1).

Phylogenetic analyses of **the replicase** (REP) and **coat protein** (CP) amino acid sequences were performed on EIVA-EIVE and all orthologs of the ICTV-recognized carlaviruses (Supplementary Table 2) using the Neighbor-Joining method (Saitou and Nei, 1987) with 1000 bootstrap replicates (Felsenstein, 1985) on MEGA (version 6.06; Tamura et al., 2013). The evolutionary distances were computed using the JTT matrix-based method (Jones et al., 1992). Analysis of recombination patterns was performed using RDP4 with seven methods based on different assumptions (Posada, 2002): RDP, GENECONV, BootScan, MaxChi, Chimaera, SiScan and 3Seq with default parameters (Martin et al., 2015). Statistical significance was obtained using a Bonferroni-corrected cut-off of $P \leq 0.05$. Only recombination signals detected by five

or more methods ($P < 0.05$) were considered as true events to minimize false positives.

The reference genomes of EIVA, EIVB and EIVC were validated from 'Golden', EIVD from 'Hwy O' and EIVE from 'G-17321'. It is noteworthy to indicate that EIVA-EIVD were present in multiple samples (Table 1). The genome lengths ranged between 8540-8628 nt excluding the polyadenylated tails (GenBank accessions KJ572560-4 for EIVA-EIVE, respectively; [Supplemental Table 4](#)). The 5' and 3' untranslated regions are 60-70 and 42-87nt long respectively. All carlaviruses, including those described herein, have six open reading frames ([ORF; Supplemental Table 4](#)). ORF 1 codes for the viral replication-associated protein (REP) which has conserved domains for a methyltransferase, 2-oxoglutarate and Fe(II)-dependent oxygenase (2OG-FeII_Oxy, which includes AlkB - Alkylated DNA repair protein), carlavirus endopeptidase, ATP binding cassette transporter nucleotide-binding, superfamily 1 viral RNA helicase, and RNA dependent RNA polymerase ([Figure 1; Supplemental Table 5](#)). ORFs 2, 3, and 4 encode the triple gene block (TGB), proteins involved in virus movement. TGB p1 includes a superfamily 1 viral RNA helicase. TGB p2 includes a movement protein domain, whereas TGB p3 has a 7kD coat protein superfamily domain ([Supplemental Table 5](#)). The coat protein (CP) is encoded by ORF5 [and](#) has two consecutive carlavirus coat protein domains namely Flexi_CP_N superfamily and Flexi_CP. ORF 6 encodes a nucleic acid binding protein (NAB) involved in gene silencing and includes a carlavirus homonymous domain (Zhou et al., 2006). A major difference between these viruses is the presence of an OTU-like cysteine protease domain (Makarova et al., 2000), mapped only in the EIVC and EIVE REP orthologs (Gly₉₄₅-Phe₁₀₄₀ and Gly₉₀₃-Phe₉₉₈, respectively, [Figure 1; Supplemental Table 6](#)).

127 Virus specific primers are traditionally designed for detection of newly
128 identified viruses. In the case of the elderberry carlaviruses, there is no knowledge
129 on the population structure of the newly identified viruses, and thus a virus-specific
130 detection test may only identify a subset of the isolates that circulate in the wild. For
131 this reason, a RT-PCR protocol able to detect all carlaviruses infecting elderberry
132 and potentially other carlaviruses was developed and successfully tested against
133 EIVA-EIVE and BIScV (Figure 2).

Phylogenetic analyses based on REP and CP amino acid sequences indicated that viruses originated from two distinct ancestors with EIVA, EIVB and EIVD evolving from one and the remaining two **viruses** from another (**Error! Reference source not found.3**).

The genomes share 42-66% nucleotide (nt) identity between EIVA-E, and 40-58% to reference genomes of other carlavirus species (Supplementary Table 7). REP proteins shared the highest aa identities (36-58%) to members of the genus *Carlavirus*, and 40-71% among themselves (Supplementary Table 4). According to the 9th Report of International Committee on Taxonomy of Viruses (ICTV), species demarcation is at the 80% amino acid (aa) identity level between the respective REP/CP genes (King et al., 2012). REP pairwise comparisons with all carlavirus orthologs were lower than the ICTV threshold for species demarcation, confirming that all five viruses are tentative new members of the genus, and are distinct species from each other. However, the CPs of EIVA and EIVB share 89% aa identities, opening potential future discussions about the species demarcation in the genus (Supplementary Table 7).

As mixed infections occurred in most elderberry samples (Keller et al., 2015), recombination was evaluated using the full length genomes of EIVA-EIVE and 32 ICTV-recognized carlaviruses. EIVA was found to be a recombinant of garlic common latent virus-like and sweet potato chlorotic fleck virus-like viruses as major and minor parents, respectively, whereas EIVE or an EIVE-like virus was predicted to be one of the parent viruses for cowpea mild mottle virus. Interspecies recombination was also detected for daphne virus S, lily symptomless virus, nerine latent virus,

phlox virus B, potato latent virus and red clover vein mosaic virus (Supplemental Figure 1).

Elderberry has a complex virome. For example, 'Maxima' was infected with six viruses; EIVA-EIVD, CLRV and EILV (Ho and Tzanetakis, 2014). Elderberry carlavirus group 1 (EIVA, EIVB, EIVD) and group 2 (EIVC and EIVE) appear to have emerged from two distinct lineages, containing closely related viruses that infect the same host, indicative of sympatric speciation (Figure 3). This, in addition to the recombination analysis, imply that elderberry, along with hop, phlox and potato (respectively infected by hop latent virus and hop mosaic virus; phlox virus B and phlox virus S; potato virus P and potato virus S), are major contributors of the carlavirus evolution (Supplemental Error! Reference source not found.1).

AlkB, a member of the 2OG-Fell_Oxy superfamily, has often been used in conserved domain analysis of viruses in the family *Betaflexiviridae* (Martelli et al., 2007). There are several viruses in the *Betaflexiviridae* that contain the 2OG-Fell_Oxy domain (Supplemental Table 6) and we propose that 2OG-Fell_Oxy should be used for phylogenetic placement as it appears to incorporate more diversity and provide better phylogenetic resolution compared to AlkB. Additionally, the OTU domains of chrysanthemum virus B, garlic common latent virus and lily symptomless virus were discovered with expected values of ≥ 0.01 in conserved domain search, indicating that evolutionarily, these viruses might be on course to lose this domain due to genetic drift caused by absent or weak selective forces.

As there is no molecular data or known isolate of EIBSV, a recognized member of the genus *Carlavirus*, and given that anti-EIBSV antibodies or any other elderberry virus for this matter could always cross-react to other viruses (ICTVdB Management,

2006), it is impossible to determine whether any of the new elderberry viruses could be an EIBSV isolate. Considering that these viruses are routinely found in mixed infections, there is also a possibility that the virus was indeed a mixture of different viruses, and viruses previously identified as EIBSV (using polyclonal antiserum), in fact, were mixed carlavirus infections. Antibodies developed against the elderberry carlavirus-North America isolate could not detect either EIVC or EIVD (Keller et al., 2015) which belong to two different phylogenetic clades, indicating distant relationship of the new viruses and the one characterized by Ellis et al. (1992). We suggest against using the 'strains' classification of elderberry carlaviruses and propose that their identity be based only on molecular data.

Mixed infections did not allow for the assessment of the role of individual viruses in pathogenicity or economic impact on elderberry. Other carlaviruses such as BIScV can cause severe disease in several hosts (Martin et al., 2012) but is symptomless in elderberry (Kalinowska et al., 2013). Given that the effect of the new viruses in single or mixed infections is unknown and while the biological characterization is on-going (Postman, Martin and Tzanetakis, unpublished), efforts should focus on eliminating those viruses from plants used in nursery propagation. Given the diversity of elderberry viruses and the lack of information on their vectors, it is imperative to use virus-tested, clean stock for field establishment rather than attempting to control all potential vectors (Charlebois et al., 2010). In other berry crops such as strawberry and *Rubus* spp., it is well documented that symptomless viruses can act synergistically to cause severe diseases when they occur in mixed infections (Martin et al., 2013; Martin and Tzanetakis, 2013). The universal detection protocol developed will be of great assistance for the nursery system when elderberry production expands to new areas as it provides reliable, unbiased, detection which is

not affected by the population structure of individual viruses having been developed from the consensus of 37 carlavirus genomes.

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Tables

Table 1. List of samples and viruses in this study. Abbreviations: elderberry virus A-E (EIVA-EIVE); elderberry latent virus (EILV); cherry leaf roll virus (CLRV)

No.	Plant species	Genotype	Origin	Virus	Additional viruses
1	<i>S. nigra</i> subsp. <i>canadensis</i>	Golden	America	EIVA, EIVB, EIVC	EILV
2	<i>S. nigra</i> subsp. <i>canadensis</i>	Hwy O	America	EIVD	CLRV
3	<i>S. racemosa</i>	G-17321	Japan	EIVC, EIVD, EIVE	CLRV
4	<i>S. nigra</i> subsp. <i>canadensis</i>	Maxima	America	EIVA, EIVB, EIVC, EIVD	CLRV, EILV
5	<i>S. racemosa</i> subsp. <i>sibirica</i>	Tashkent #2149	Uzbekistan	EIVC, EIVD	None

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Figures

Figure 1. Graphic representation of the genome organization of elderberry virus A (EIVA) and elderberry virus C (EIVC), representatives of the two elderberry carlavirus groups. The putative enzymatic motifs of the virus replicase (REP) are shaded. Abbreviations: MT, Viral methyltransferase; 2OG-FeII_Oxy, 2-oxoglutarate and Fe(II)-dependent oxygenase; OTU, ovarian tumour-like cysteine protease; EP, Carlavirus endopeptidase; Hel, Superfamily 1 viral RNA helicase; Pol, RNA dependent RNA polymerase; TGB, triple gene block; CP, coat protein; NABP, nucleic acid binding protein. Coordinates of the open reading frames and enzymatic motifs can be found in supplemental tables 4 and 5.

Figure 2. Agarose gel electrophoresis of elderberry carlavirus detection using universal primers. Lanes 1-5: elderberries 'Golden', 'Hwy O', 'G-17321', 'Maxima' and 'Tashkent #2149'; 6: blueberry infected with blueberry scorch virus; 7: uninfected elderberry; 8: reverse transcription control (RT without template); 9: PCR control (water); M: molecular weight marker HyperLadder IV (Bioline, MA).

Figure 3. Phylogenetic relationship between elderberry virus A-E and other members of the *Carlavirus*. The relationship is based on the amino acid sequences of (A) replication protein, and (B) coat protein. Phylogenetic trees were constructed using Mega software's Neighbor-joining method with 1000 bootstrap replicates. Bootstrap values lower than 70% are eliminated as they are considered untrustworthy. Virus abbreviations and Genbank accessions can be found in supplemental table 2.