AN ABSTRACT OF THE THESIS OF

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Robert R. Martin

Raspberries are an important commodity worldwide, with the United States (U.S.) leading fourth in global production in 2017. An important factor when shipping propagated *Rubus* across the globe is the security of clean plant material verified through pathogen detection methods which is vital in preventing disease spread. A raspberry virus on the quarantine list for *Rubus* exports from the U.S. is raspberry leaf curl virus which is a causal agent of the systemic disease known as raspberry leaf curl disease (RLCD). RLCD has been reported to be caused by two separate viruses causing varying symptoms in black, purple, and red raspberry cultivars. Although the disease has been described back in 1895 and was one of the most destructive raspberry diseases throughout the U.S. and Canada, the causal agents of the disease have not been identified. The disease is known to cause severe downward curling of the leaves, noticeably stunted shoots, leaf chlorosis, and crumbly fruit; it is the only *Rubus* disease that requires bioassays for detection. The objective of this study was to identify and characterize the virus complex(es) that cause disease symptoms and develop a molecular based diagnostic tool to identify them. Five viruses, black

raspberry necrosis virus (BRNV), Rubus yellow net virus (RYNV) and three novel viruses, were identified in RLCD-like native *Rubus* and were used in single and mixed viral infections to recreate the symptoms previously described. One novel single-stranded RNA virus belonging to family *Rhabdoviridae* was taxonomically classified as the U.S. variant of raspberry vein chlorosis virus (RVCV) and named RVCV_US. The genome is 14,662 nucleotides (nt) in length and encodes for five structural proteins common to *Rhabdoviridae* and three proteins found specifically in the cytorhabdovirus genus. The genomic and phylogenetic analyses show a close evolutionary relationship of RVCV_US to Alfalfa dwarf virus (ADV) and Strawberry crinkle virus (SCV) but show a higher affinity to variants RVCV_Hutt1 and RVCV_Hutt2 isolated from the United Kingdom (U.K.). Detection primers were designed to amplify highly conserved regions of the RNA-dependent RNA polymerase (L Protein) of all three RVCV variants and were utilized in the *Rubus* survey to present information on the geographic distribution of the virus in the U.S. Single and mixed viral infections were performed using the large raspberry aphid, *Amphorophora agathonica,* and graft leaflet transmissions onto 'Columbian', 'Cumberland', and 'Cuthbert' raspberry cultivars. Varying symptoms such as leaf chlorosis, yellowing of minor veins, minor crinkling, and downward cupping of leaf tissue were observed in single and mixed infections throughout the experiment but RLCD symptoms were not replicated. The lack of classic symptoms previously reported on the same raspberry cultivars may be due to the exclusion of the novel *Luteoviridae* virus that became undetectable throughout the course of experiment despite modifying nucleic acid extraction and RT-PCR protocols.

Identification of Viruses Associated with Raspberry Leaf Curl Disease

by
Melinda Guzman Martinez

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APPROVED:

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Melinda Guzman Martinez, Author

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I've met so many special people and have made tremendous friends along the way that it'll take me at least another five pages to thank them all individually so to the entire Botany and Plant Pathology (BPP) department, thank you!!!

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CONTRIBUTION OF AUTHORS

Dr. Robert (Bob) R. Martin supervised and assisted with the overall project design, planning, and execution. He offered advice when I was stumped, suggested different routes, and helped edit my chapters. He also collected and provided the plant material utilized for Chapter 2 and Chapter 3. Patrick Di Bello assisted in HTS, the genome-assembly process, and the sequence analysis of my Chapter 2.

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DEDICATION

I dedicate all of my work to my parents, Silvia and Taurino, who without their

love, support, and constant encouragement, I wouldn't be where I am today.

Chapter 1:

Introduction and Literature Review

Melinda Guzman Martinez

An 1895 survey of raspberry cultivars grown in Minnesota fields led to the first written report of

leaf curl in red raspberry, but with few additional descriptions of the disease except noting the

devastating loss (Stewart and Eustace 1902). Growing conditions of the raspberry field were not

well documented. A few years later in 1902, a similar curling with the inclusion of yellowing

was observed and referred to as yellows disease (Stewart and Eustace 1902). The similarity of

the description of leaf curl in Minnesota, and yellows in New York and later in Connecticut

(Clinton 1904) led to conclusion that leaf curl and yellows were the same disease; this was

suggested based on experiments in red raspberry cultivars grown in Ohio (Melchers 1914), a

major raspberry producing state at the time. Additional symptoms such as a stunted appearance

of infected plants (Melchers 1914) and a reduction of yield by 20-40% with decreased fruit

quality were also observed (Bennett 1927). The disease was named raspberry leaf curl, rather

than yellows, so as to not confuse it with symptoms of mosaic disease of raspberry (Rankin and

Hockey 1922). Further characterization of raspberry leaf curl disease (RLCD) symptoms

observed in red raspberries grown in Ontario, Canada included arching and puckering between

lateral leaflet veins, with the development of curled leaves appearing 4 to 6 weeks after infection.

The symptoms were observed in mature and young leaves leading to the conclusion that leaf curl

was a systemic disease. The overall symptomology of leaf curl in raspberry was noted to be

highly similar to that potato leafroll disease (Rankin and Hockey 1922), now known to be caused

by potato leafroll virus, a virus in the family *Luteoviridae* (Rowhani and Stace-Smith 1979).

Despite being found throughout the upper Midwest and the northeastern regions of the U.S. and

southeastern Canada, little was known about the transmission of leaf curl. Through observations

made in the field, the sole possible vector regularly found in leaf curl infected fields was the

small raspberry aphid, *Aphis rubicola* Patch (Rankin and Hockey 1922). Experiments to

determine whether the leaf curl pathogen overwintered in *A. rubicola* eggs, was transmitted from adult to nymphs (viviparous reproduction), whether the pathogen could be transmitted from red to black raspberry and vice versa, and whether *A. rubicola* was the only vector of the pathogen were performed. The results of the experiments found that the virus did not overwinter in *A. rubicola* eggs nor was it transmitted in a viviparous manner from viruliferous adult aphids to their offspring. Thirdly, the virus was able to be transmitted from the black raspberry cultivar 'Hoosier' to the red raspberry 'Cuthbert', but symptoms were not observed in 'Hoosier' when transmissions were attempted from infected 'Cuthbert' plants. Lastly, of the two commonly observed aphids found in red raspberry fields, *Amphorophora rubi* Kalt. and *A. rubicola*, only *A. rubicola* aphids were determined to transmit the virus (Smith 1925), further confirming the identity of the leaf curl vector hypothesized earlier (Rankin and Hockey 1922). After the identity of the leaf curl vector was established, experiments determining type of virus transmission found that *A. rubicola* transmitted the virus in a persistent manner after feeding on an infected plant for two hours, continuing to transmit the virus (viruliferous) for up to several days (Bennett 1927). In observational field studies in parts of Michigan where an outbreak of leaf curl appeared, it was noticed that symptoms were not only observed in red raspberry cultivars but also more noticeably on black raspberry cultivars, which were previously thought to be resistant (Bennett 1930). Because the observations seen were contradictory to earlier field observations, it was hypothesized and later established that the disease known as leaf curl was caused by two separate viruses. The virus affecting red and black raspberry cultivars was designated as the beta strain, while alpha strain was given to the virus infecting only red raspberry cultivars (Bennett 1930). Experiments performed to test the susceptibility and possible resistance of different raspberry cultivars to the alpha and beta strains of leaf curl used purple raspberry 'Columbian', the black

raspberry 'Cumberland', and the red raspberry 'Cuthbert' for inoculations. The alpha strain caused severe symptoms on 'Cuthbert', mild symptoms in 'Columbian', but had no effect on the black raspberry 'Cumberland'. However, the beta strain produced severe symptoms on all red, black, and purple raspberry cultivars (Bennett 1930). Following aphid, *Aphis idaei*, and graft transmissions, the occurrence of leaf curl symptoms observed in raspberry fields in British Columbia and were identified to be caused by the alpha strain of leaf curl (Stace-Smith 1962). No clear patterns have been observed in regard to the spread of leaf curl (Bolton 1970) but the control measures of planting healthy stock, roguing infected fields, and removal of infected plants have been suggested (Rankin and Hockey 1922). Although the disease has not been reported or observed in commercial *Rubus* since 1977 (Caron et al. 1977) and not surveyed for in the native *Rubus* since then, it still poses a threat to contamination of the *Rubus* industry. RLCV is on quarantine lists for most countries for *Rubus* plants exported from the U.S. and the only disease of *Rubus* that requires bioassays for detection. The necessity to do bioassays for this virus(es) greatly limits the movement of *Rubus* germplasm from the U.S. to other parts of the world. Discovering the virus or viruses that cause RLCD and development of molecular based diagnostic tools to identify them will lead to improved certification programs and facilitate plant exports to growers and industry partners (Gergerich et al. 2015).

The objectives of this project were to: 1. Identify viruses in native *Rubus* samples that exhibited RLCD-like symptoms collected in areas where RLCD has been reported previously; 2. Characterize a novel virus detected in RLCD symptomatic plants; and 3. Determine the etiology of RLCD using single and mixed infections of the virus complex(es) identified in these symptomatic plants, by means of aphid and graft transmissions using 'Columbian', 'Cumberland', and 'Cuthbert' raspberry cultivars.

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Chapter 2:

Characterization of North American Variant of Raspberry Vein Chlorosis Virus

Melinda Guzman Martinez, Patrick L. Di Bello, and Robert R. Martin

Abstract

Raspberry vein chlorosis virus (RVCV) is a member of the *Rhabdoviridae* family, which infects *Rubus* spp. causing symptoms such as yellowing of minor leaf veins and leaf distortion. The virus has been reported to be widespread in continental Europe and the United Kingdom (U.K.). In recent surveys of wild and commercial *Rubus* in northeastern and western U.S. and southeastern Canada, a variant of RVCV was discovered in red raspberry. The complete sequence of the U.S. variant of RVCV genome is 14,662 nucleotides (nt) in length encoding five structural proteins typical of *Rhabdoviridae* viruses, and three proteins found in the cytorhabdovirus genus. Genomic organization of the novel RVCV variant is most similar to that of RVCV Hutt1 and Hutt2, both recently characterized in Scotland, and the cytorhabdoviruses Alfalfa dwarf virus (ADV), and Strawberry crinkle virus (SCV).

Introduction

Raspberries are a specialty crop of economic importance throughout the United States (U.S.), Canada, and much of northern Europe (Demchak et al. 2017). More than 30 viruses have been reported from *Rubus* spp., and in most cases virus diseases in these crops are caused by virus complexes (Martin et al. 2013, 2017). Raspberry leaf curl disease (RLCD), initially reported in 1895 (Stewart and Eustace 1902) from Minnesota, has been reported from eastern Canada and the northeastern U.S. and as far west as the Rocky Mountains by 1930. Symptoms of infection include small crumbly fruit, stunted shoots, leaf chlorosis, and downward curling of the leaves (Bolton 1970). This disease has been reported to reduce yield by 20-40% in addition to reduced fruit quality (Bennett 1927). The causal agent(s) transmitted by the small raspberry aphid, *Aphis rubicola*, transmits the disease in a persistent manner (Converse 1987). The causal agents of

RLCD have not been identified but previous studies based on the transmission properties of the viruses (Bennett 1927) indicate it may be caused by at least two viruses (Bennett 1930), varying in symptomology in red, black, and purple raspberry (Bennett 1930).

To assess the prominent viruses present in raspberry and blackberry production regions in the U.S. and Canada, a three-year *Rubus* virus survey of native habitats, nurseries, and commercial fields located throughout the northeastern and western U.S. and southeastern Canada was conducted. Native red and black raspberry plants exhibiting RLCD-like symptoms (Fig. 2.1) were collected from Maine, Pennsylvania, Wisconsin, Ontario, and Quebec and were analyzed using high-throughput sequencing (HTS). Five viruses were detected by HTS including: Black raspberry necrosis virus (BRNV), Rubus yellow net virus (RYNV), and three novel viruses, two belonging to the *Rhabdoviridae* family and the other a member of the E*namovirus* genus in the family *Luteoviridae*. One of the rhabdoviruses was further characterized and included in the raspberry virus survey.

Materials and Methods

Plant Material. Native red raspberry plants showing RLCD-like symptoms collected from Maine, Pennsylvania, Wisconsin, Ontario, and Quebec were selected for analysis by HTS. Plant material shipped (under APHIS permit P526P-16-04242) to the USDA-ARS Horticultural Crops Research Unit (HCRU) in Corvallis, Ore. and grown in a greenhouse with 16 hours of daylight at 25°C. For the survey, plant material was collected by cooperators and shipped (under permit P526P-16-04241) to the same laboratory.

High-throughput sequencing (HTS). Total nucleic acids were extracted from symptomatic

plants, digested with DNase, and polyribosomal RNA removed as previously described (Di Bello

et al. 2015). Samples were sequenced using 150bp single end kits on a HiSeq Illumina 3000

platform at the Center for Genome Research and Biocomputing at Oregon State University.

Sequences were analyzed with virus_pipe (Di Bello 2016) which automates; adapter removal and

quality trimming with Trimmomatic 36 (Bolger et al. 2014), assembly with Trinity v2.6.6

(Grabherr et al. 2011), mapping to the black raspberry genome with bwa mem (Li and Durbin

2009), unmapped sequences were searched with BLASTn with an E-value of 1 to the nt

database, sequences without a hit were then searched with BLASTx with an E-value of 10 to the

nr database (Bolger et al. 2014; Brown et al. 2012; Haas et al. 2013; Li 2013; VanBuren et al.

2016). Any virus or viroid matches were then overlaid with virus taxonomic data downloaded

from ICTV and NCBI Taxonomy database to allow the sorting of similar taxon hits (ICTV

Master Species List, 2015; Sayers et al. 2009; Benson et al. 2009). Several large contigs, largest

being 13 kb, with similarity to cytorhabdoviruses were identified. Primers were designed to fill

in the gaps in the sequence and to obtain the 5' and 3' ends. The HTS data was confirmed by

Sanger sequencing of RT-PCR products of overlapping fragments for at least a 10X coverage,

which were generated using specific primers based on the HTS assembled sequence (Di Bello et

al. 2017).

Nucleic acid extraction, reverse-transcription polymerase chain reaction (RT-PCR), 5'/3'

Rapid Amplification of cDNA Ends (RACE), and cloning. Total RNA was extracted from 100

mg leaf tissue by grinding with a 1 mL mixture of RNA extraction buffer (24.2 g Tris Base,

12.66 g Lithium Chloride, 15g Lithium dodecyl sulfate, 2.92 g EDTA, 9.42 g Deoxycholic acid,

14.3 mL 70% Tergitol-NP40, 20 g PVP, 800 mL of DI water, adjusted to pH to 8.5, and brought

to a final volume of 1 L) (Hughes and Galau 1988; Spiegel and Martin 1993) containing 20 μL

1M tris(2-carboxyethyl)phosphine (TCEP) (30 mL of sterile DI water, 11.466 g TCEP, brought

to final volume of 40 mL). The sap was collected in a 1.5 mL Eppendorf tube (Eppendorf AG,

Hamburg, Germany) and centrifuged in a Heraeus Pico 21 Microcentrifuge (Thermo Fisher

Scientific Inc., Waltham, MA, USA) at 13,000 rpm for three minutes. 500 μL of the supernatant

and 600 μL of 6 M KoAc [294.4 g potassium acetate (KoAc), 500 mL DI water, 182 mL Glacial

acetic acid, 90g Potassium hydroxide (KOH), adjust pH to 6.5 using 10M KOH, and bring to

volume of 1 L] (Sambrook and Russell 2001) were added to a new 1.5 mL Eppendorf tubes. This

mixture was then inverted 10 times, then centrifuged at 13,000 rpm for 10 minutes. Seven

hundred μL of the supernatant and 750 μL of 100% isopropyl alcohol were added into a new

Eppendorf tube, inverted 20 times to mix, and centrifuged at 13,000 rpm for 30 minutes. The

supernatant was then poured off and the liquid in the tube carefully blotted on paper towel so as

to not lose the RNA pellet. Five hundred μL of buffer containing equal amounts of wash buffer

(10 mL of 1 M Tris-HCl pH 7.5, 1 mL 0.5 M EDTA, 10 mL 5 M NaCl, and 479 mL DEPC

treated sterile H_2O) (Rott and Jelkmann 2001) and 100% ethanol were added to the Eppendorf

tube containing the pellet, followed by the addition of 10 μL of silica glass milk [60 g silica

particles (Sigma S5631), 500 mL sterile DI H_2O, adjusted to pH 2 using 1 M HCl] (Rott and

Jelkmann 2001). The sample was then vortexed to resuspend the pellet, followed by two 8-

second pulse centrifugations at 5,000 rpm. The supernatant was poured off and carefully blotted

on a new paper towel and another 500 μL of 50/50 mixture of wash buffer and 100% ethanol was

added to the pellet. The sample was again vortexed and pulse centrifuged at 5,000 rpm for two 8-

second intervals. The supernatant was then poured off and carefully blotted on a new paper

towel, with the pellet still in the Eppendorf tube. The pellet was dried in a SpeedVac vacuum concentrator (Thermo Fisher Scientific Inc.) for five minutes to dry. The dried pellet was then resuspended in 55 µL of RNA elution buffer [1 mL 10 mM of Tris-HCL pH 8.5 and 0.1 mM EDTA pH 8.0 mixture, and 49 mL molecular biology grade (MBG) water] by vortexing, then incubated at 70°C for four minutes. The heated mixture was then centrifuged for 10 minutes to pellet the glass milk, and 50 µL of the supernatant was collected and stored at -20°C.

For the reverse-transcriptions, 5 µL of the RNA was transferred into a single well in a non-skirted PCR plate (Thermo Fisher Scientific Inc.). In a 1.5 µL Eppendorf tube, 29.2 µL of H_2O, 10 µL 5X First-Strand Buffer (Invitrogen Corp., Carlsbad, CA, USA), 2 µL 10mM dNTPs (GenScript Biotech Corp., Piscataway, NJ, USA), 2 µL 2% PVP [2 g polyvinylpyrrolidone(PVP) MW 40,000 added to 100 mL MBG H_2O], 1 µL 0.3 µg/µL random primers (100 µL of 3 µg/µL Invitrogen random primers, diluted with 900 µL MBG H_2O), 0.5 µL of 40 U/µL Ribolock RNase Inhibitor (Thermo Fisher Scientific Inc.) and 0.3 µL 200 U/µL Maxima Reverse Transcriptase (Thermo Fisher Scientific Inc.) were mixed; this 45 µL RT mixture was then transferred into the same PCR plate well containing the 5 µL RNA. After the Veriti 96-well thermal cycler (Thermo Fisher Scientific Inc.) heated to 50°C, the RT reaction mixture was inserted and incubated at 50°C for 60 minutes followed by denaturation of the enzyme by heating at 85°C for 5 minutes. The generated cDNA was stored in a -20°C for later use in PCR.

For the PCR reaction, 2 µL of the cDNA from the RT reaction was added to a 18 µL PCR mixture containing 15.08 µL H_2O, 2 µL 10X Taq buffer (GenScript Biotech Corp.), 0.4 µL 10mM dNTPs (GenScript Biotech Corp.), 0.4 µL 50 µM NAD5 primer mixture, and 0.12 µL 5 U/µL Green *Taq* DNA polymerase (GenScript Biotech Corp.). For the PCR reaction there was an initial denaturation at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 30 s,

annealing at 55°C for 45 s, and extending at 72°C for 30 s, with final 10 min extension at 72°C

before being cooled down to 15°C. To check the quality of the extraction, NAD5 primers were

used to amplify the ND2 subunit of NADH(B) dehydrogenase (Mannerlöf and Tenning 1997).

Gel electrophoresis was performed in an electrophoresis chamber (Bio-Rad Laboratories Inc.,

Berkeley, CA, USA) through a 2% agarose gel stained with 4 µL of ethidium bromide (10

mg/mL in H$_2$O) run at 90 V for 30 min. Nucleic acid bands were then visualized by exposure to

UV light on the AlphaImager Gel Imaging System (ProteinSimple, San Jose, CA, USA).

To obtain the ends of the viral genome, the SMARTer 5'-and-3'-rapid amplification of cDNA

ends (RACE) Kit (Takara Bio Inc., Mountain View, CA, USA) was utilized. To obtain the 5'

end, cDNA was produced using a virus specific primer (5'-GTG CTT GAA GGT CGA CTA

GTC ACT GTG G-3') located 70 bases from the end. PCR amplification of the 5' end was

performed according to the manufacturer's instructions of 5'-RACE cDNA amplification with

random primers using the SMARTer II A Oligonucleotide (Takara Bio. Inc.). For obtaining the

3' end, cDNA was synthesized using a primer (5'-ACC TAA ACC TGA GCG CTA TCT CAT

GGG-3') located 177 bases from the end of the RNA. The 3' end was amplified by PCR using a

modified oligo (dT) primer. The resulting cDNA products of the 5' and 3' ends were purified

using the Zymoclean Gel DNA Recovery Kit (Zymo Research Corp., Irvine, CA, USA) and

cloned using the TOPO TA Cloning Kit (Thermo Fisher Scientific Inc). Cloning was carried out

according to the manufacturer's instructions (Thermo Fisher Scientific Inc).

Plasmid purifications were carried out using the Zyppy Plasmid Miniprep Kit according to

manufacturer's instructions (Zymo Research Corp.). Direct sequencing of inserts from 10

colonies for each cloning reaction was done in the 5' and 3' directions using universal primers

M13F (5'-TGT AAA ACG ACG GCC AGT-3') and M13R (5'-CAG GAA ACA GCT ATG

AC-3'). Sequencing was carried out at Eton Bioscience (Eton Bioscience Inc., San Diego, CA, USA).

Resequencing. To validate the sequence and correct any residual errors of the novel PA *Cytorhabdovirus*, the virus genome was fully sequenced 10 times using 22 overlapping primer sets amplifying the entire genome, except for the 5' and 3' ends, in 700 bp fragments with a 50°C annealing temperature. The PCR products were then purified using the DNA Clean and Concentrator-5 Kit (Zymo Research Corp.), cloned into the pCR4 TOPO vectors with the TOPO TA Cloning Kit (Thermo Fisher Scientific Inc.) and transformed into competent *E. coli* cells according to the manufacturer's instructions (Thermo Fisher Scientific Inc.). Plasmids were sequenced as described above.

Detection. Three primer pairs were designed for PCR detection by aligning the sequences of the available raspberry vein chlorosis virus isolates: RVCV_Hutt1; MK240091; RVCV_Hutt2; MK257717; and raspberry vein chlorosis virus US variant (RVCV_US) using the ClustalW (M. A. Larkin et al. 2007) with a gap open cost of 10 and a gap extend cost of 5 alignment in Geneious v7.1 (Table 2.1). Tissue samples of the two UK isolates of RVCV were imported under APHIS permit. The PCR, using the RVCV-specific primers, was done by adding 2 μL of positive control RVCV cDNA to a PCR mixture containing 15.1 μL H_2O, 2 μL 10X Taq buffer (GenScript Biotech Corp.), 0.4 μL 10mM dNTPs (GenScript Biotech Corp.), 0.4 μL 50 μM NAD5 primer mixture, and 0.1 μL 5 U/μL Green *Taq* DNA polymerase (GenScript Biotech Corp.) in a final volume of 20 μL. The PCR temperature conditions were optimized on a Veriti 96-well thermal cycler fitted with a temperature block (Thermo Fisher Scientific Inc.). The PCR

conditions for the L1 primers were: one cycle at 95°C for 5 min, then 40 cycles at 95°C for 30 s,
50°C for 45 s, and 72°C for 30 s, with final 10 min extension at 72°C before being cooled down
to 15°C. The PCR cycles for the L2 and L3 primers were identical to that of the L1 primers
expect that the annealing temperatures were 50°C and 56°C respectively (Table 2.1). The PCR
products were analyzed by electrophoresis in a 2% agarose gel stained with ethidium bromide.

Genomic analysis of novel cytorhabdovirus. The nucleotide and translated amino acid sequence
of the novel Cytorhabdovirus were compared with other sequences in GenBank using BLASTn,
BLASTx, and later BLASTp (Madden 2013). Open Reading Frames (ORFs) were identified
using the ORF Finder tool (Rombel et al. 2002). The molecular mass was calculated using the
Protein Molecular Weight calculator (https://www.bioinformatics.org/sms/prot_mw.html) and
conserved domains were identified using the CD-Search tool (Marchler-Bauer and Bryant 2004).
All sequence alignments were done with ClustalW (Larkin et al. 2007) with a gap open cost of
10 and a gap extended cost of 5; the alignment was performed in Geneious v7.1.

Phylogenetic analysis. Phylogeny trees for each of the ORFs were created using (Neighbor-
Joining algorithm, 1,000 bootstrap replications) comparing the three RVCV isolates and strains
of cytorhabdoviruses and selected nucleorhabdoviruses. Five canonical conserved regions of the
Rhabdoviridae family, and three additional proteins unique to the cytorhabdoviruses (Walker et
al. 2018) were estimated using the Blosum62 cost matrix with the Jukes-cantor amino acid
model of substitution, with no outgroup, using global alignment; phylograms were visualized on
Geneious v7.1.

Results

Virus characterization. The viral genome of the RVCV_US cytorhabdovirus consists of 14,662 nucleotides (nt) revealing a genome organization of 3'-N-P'-P-P3-M-G-P6-L-5' (Fig. 2.2). This U.S. variant of RVCV shares 82% and 73% percent identity to RVCV_Hutt2 and RVCV_Hutt1, respectively. Its query coverage is 90% and 82% to the Hutt2 and Hutt1 isolates. Using BLASTn, two additional viruses were closely related to the U.S. variant: Alfalfa dwarf virus (ADV) (NC_028237) with 68% identity (32% coverage and 0.0 E-value) and Strawberry crinkle virus (SCV) (MH129616) with 69% identity (25% coverage and 0.0 E-value). Where viruses belonging to the *Rhabdoviridae* family encode anywhere from six to nine proteins, this variant like the RVCV isolates characterized in Scotland, encodes for eight.

ORF 1 encodes for the nucleoprotein (N) with 485 amino acids (aa), has a molecular mass of 54.2 kDa, and shares 86% aa sequence identity to ORF 1 of RVCV_Hutt2. ORF 2 overlaps with ORF 3 and encodes a short polypeptide containing 80 aa, with a molecular mass of 9.58 kDa, sharing 80% identity to Hutt2. ORF 3 encodes for 340 aa phosphoprotein (P) and shares 85% sequence identity to Hutt2, with a molecular mass of 38.9 kDa. ORF 4 encodes for a putative movement protein (P3) with 263 aa, sharing 77% sequence identity to Hutt2 and has a molecular mass of 29.5 kDa. ORF 5 shares 88% homology of the matrix protein (M) to that of the RVCV_Hutt2 isolate and has a molecular mass of 21.3 kDa and is 197 aa in length. ORF 6 encodes for the glycoprotein (G) sharing 84% to the G-protein of the Hutt2 isolate, at a length of 567 aa and a molecular mass of 62.4 kDa. ORF 7 encodes for the P6 gene of unknown function at 64 aa in length, a molecular mass of 7.27 kDa, and 94% similarity to Hutt2 P6 protein. The final ORF encodes for the largest protein, the polymerase (L), with a length of 2088 aa and a molecular mass of 239 kDa, sharing 93% aa sequence identity to ORF 8 or RVCV_Hutt2 (Table

2.2). This eighth ORF contains three main conserved domains of the super family

Mononegavirales and one G-7-MTase superfamily domain. The first Mononegavirales conserved

domain is the RNA-dependent RNA polymerase which is made of two subunits, a large protein

(L) and a phosphoprotein (P) where the P protein functions as a transcription factor and the L

protein provides the RNA polymerase activity on the complex (De et al. 1997). The second

Mononegavirales domain is the mRNA-capping region V. The V domain of the L protein

synthesizes the mRNA cap (Li et al. 2008). The final conserved domain is the mRNA (guanine-

7-)methyltransferase (G-7-MTase) which is a domain that aides in the formation of viral mRNAs

with methylated cap structure (Gopinath and Shaila 2009).

Taxonomic placement. Phylogenetic analyses using the five ORFs present in all *Rhabdoviridae*

viruses and three ORFs present in cytorhabdoviruses depicted RVCV_US consistently being

most closely related to RVCV_Hutt1 and RVCV_Hutt2, sharing the most recent common

ancestor (Fig. 2.3-2.10). The sister taxons of the RVCV clade throughout all the phylogenetic

trees were other cytorhabdoviruses, Alfalfa dwarf virus (ADV) and Strawberry crinkle virus

(SCV), as previously reported (Jones et al. 2019). The proteins used for these analyses from the

3' end were: the nucleocapsid (N), a 5'-end protein found overlapping part of the P protein ORF

(P'), phosphoprotein (P), putative movement protein (P3), matrix protein (M), glycoprotein (G),

unknown P6 protein, and the RNA-dependent RNA polymerase (L) protein. The phylogram

results not only confirm the relationship between the novel cytorhabdovirus found in the U.S.

and RVCV variants from Scotland, but also the existence of divergent variants of RVCV in the

genus *Cytorhabdovirus*.

Virus detection. Leaf tissue samples containing RVCV_Hutt1 and RVCV_Hutt2 were sent to the

USDA-ARS HCRU from Dr. Stuart MacFarlane of the James Hutton Institute (Dundee,

Scotland, UK) to evaluate the primers developed to amplify the RNA-dependent RNA

polymerase (L) protein after the ClustalW alignment of all three RVCV variants. The third leaf

tissue sample used to evaluate the primers was that of the Pennsylvania plant, the same plant

where the U.S. variant of RVCV was isolated from, kept and maintained in the greenhouse at the

USDA-ARS HCRU in Corvallis, Ore. Nucleic acid extractions, as previously described, were

performed on all three leaf tissue samples and RT-PCRs were later performed using the L1, L2

and L3 primer pairs (Fig. 2.11).

Discussion

Viruses belonging to the *Rhabdoviridae* family are enveloped bacilliform-shaped negative-sense

single-stranded RNA viruses with 20 recognized genera, four that infect plant hosts (Walker et

al. 2018). The novel *Rhabdoviridae* virus found in Pennsylvania was determined to be a strain of

raspberry vein chlorosis virus (RVCV) with a genome of 14,662 nt and is designated as

RVCV_US. RVCV is known to affect wild and cultivated red raspberries with symptoms of

vein-clearing and leaf distortion (Jones et al. 1977).

The taxonomic classification of the U.S. variant of RVCV as a cytorhabdovirus was supported

by genomic and phylogenetic analysis of the amino acid (aa) sequences of all five *Rhabdoviridae*

proteins, and the three cytorhabdovirus-specific proteins (Table 2.2). These analyses show a

close evolutionary relationship of RVCV_US to Alfalfa dwarf virus (ADV) and Strawberry

crinkle virus (SCV), but also shows RVCV_US having a higher affinity to both variants

RVCV_Hutt1 and Hutt2. From a previous report, RVCV_Hutt1 and Hutt2 were determined to be

most like ADV and SCV, both of which belong to the *Cytorhabdovirus* genus (Jones et al. 2019).

The relationship between these cytorhabdoviruses at the aa level is shown for each putative

protein in terms of % amino acid sequence identity in Table 2.3.

All three of the RVCV variants encoded for the P' overlapping protein, which was also found to

be encoded by two isolates of SCV (Fig. 2.4). This gene is found to be encoded between the P

and N genes, starting at location 1998 nt and overlapping with the P protein through 2240 nt

(Fig. 2.2). The RVCV_US and RVCV_Hutt2 share an 80% aa similarity within the P' protein

(Identities: $64/80 = 80\%$, Gaps: $0/80 = 0\%$) (Fig. 2.12). The conserved *Rhabdoviridae* matrix

protein (M) consisted of the widest percentage of aa identity, ranging from 17.5% to that of

Persimmon virus A (PVA) (Table 2.3) to 88.3% of RVCV_Hutt2 (Identities: $173/196 = 88\%$,

Gaps: $0/196 = 0\%$) (Fig. 2.12).

The putative P6 protein, encoded from 7645 nt to 7839 nt between the G and L proteins, which

has no known function (Figure 2.2), but is found in five other cytorhabdoviruses, varying in aa

percent identity of 37.5% to 93.8% (Table 2.3).

Notably, the RVCV_Hutt2 ORFs using BLASTp (Madden 2013) consistently scored the lowest

e-value for proteins encoded by RVCV_US (Table 2.2), with the highest aa identity being in the

highly conserved L gene at 93.3% (Madden 2013) (Identities: $1947/2088 = 93\%$, Gaps: $0/2088 =$

0%). In contrast, the partial RNA-dependent RNA polymerase (L gene) sequence of RVCV

(FN812699) previously obtained from RNA extract of *Aphis idaei* (McGavin et al. 2011),

showed to have a 90.4% aa identity to that of RVCV_US (Identities: $969/1010 = 90\%$, Gaps:

$0/1010 = 0\%$) (Table 2.3).

Due to the high percent similarity of the L gene among all RVCV variants, the three detection

primers were designed to target this specific region of the genome. The primer pairs L1, L2, and

L3 (Table 2.1) were utilized in the *Rubus* survey and so far only two samples so far have tested positive. Further testing using these RVCV primers for virus detection in *Rubus* from native habitats, nurseries, and commercial fields should present information on the diversity of RVCV and may show that these three strains are the result of geographic isolation between the U.S. and U.K. In conclusion, this research provided the complete genome sequence of the U.S. variant of RVCV from *Rubus* showing RLCD-like symptoms. In the HTS analysis of approximately 25 different *Rubus* samples exhibiting RLCD-like symptoms this virus has been detected only in Pennsylvania.

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Figure 2.1 RLCD-like symptoms on native raspberry collected from Pennsylvania during *Rubus* virus survey.

Table 2.1 PCR primers designed to amplify segments of conserved regions of the RNA-dependent RNA polymerase (L protein) of all three variants of raspberry vein chlorosis virus (RVCV). Their sequences, predicted amplicon size, and annealing temperatures. L3 primers are degenerate primers.

Primer name	Primer sequence	Amplicon size (bp)	Tm (°C)
L1: Forward	5' ACA TTG TTT ACA GTG TGT GC 3'	703	50
Reverse	5' CAG GAA ATC CTC TCA TCA TCA 3'		
L2: Forward	5' ATG GGA ATG GGG GAT AAT CA 3'	640	50
Reverse	5' CAG GAA ATC CTC TCA TCA TCA 3'		
L3: Forward	5' TGG TTT GTY CCR GAR GAT TC 3'	764	56
Reverse	5' AAC CAR CTC CAC TCW GCA CA 3'		
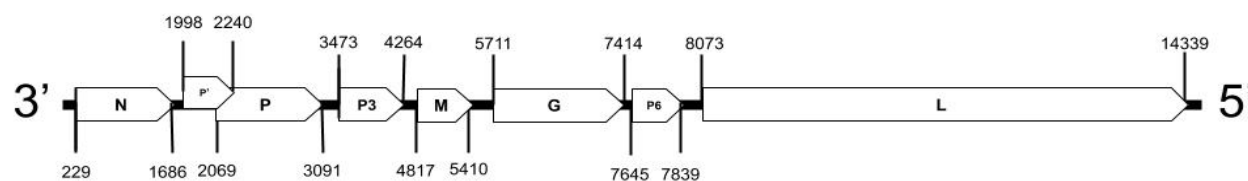
Figure 2.2 Schematic diagram of RVCV_US genome organization. Open reading frames (ORFs) are shown as pentagons where the nucleocapsid (N), P' overlapping protein (P'),phosphoprotein (P), movement protein (P3), matrix protein (M), glycoprotein (G), unknown function protein (P6), and RNA-dependent RNA polymerase protein (L) genes are identified. Numbers above or below each ORF are first and last nucleotides indicating their location on the genome.

Table 2.2. Characteristics of proteins encoded by US variant of the RVCV genome determined by predictive algorithms.

ORF No.	Gene name	Calculated kDa	Amino acid (aa) length	Putative function	Highest scoring virus protein/E-value (BLASTx)
1 (+)	N	54.2	485	Nucleocapsid	RVCV_Hutt2 N/0.0
2 (+)	P'	9.58	80	P'-overlapping protein	RVCV_Hutt2 P'/3e-37
3 (+)	P	38.9	340	Phosphoprotein	RVCV_Hutt2 P/0.0
4 (+)	P3	29.5	263	Movement protein	RVCV_Hutt2 P3/1e-135
5 (+)	M	21.3	197	Matrix protein	RVCV_Hutt2 M/7e-111
6 (+)	G	62.4	5657	Glycoprotein	RVCV_Hutt2 G/0.0
7 (+)	P6	7.27	64	Unknown	RVCV_Hutt2 P6/1e-34
8 (+)	L	239	2088	Polymerase	RVCV_Hutt2 L/0.0
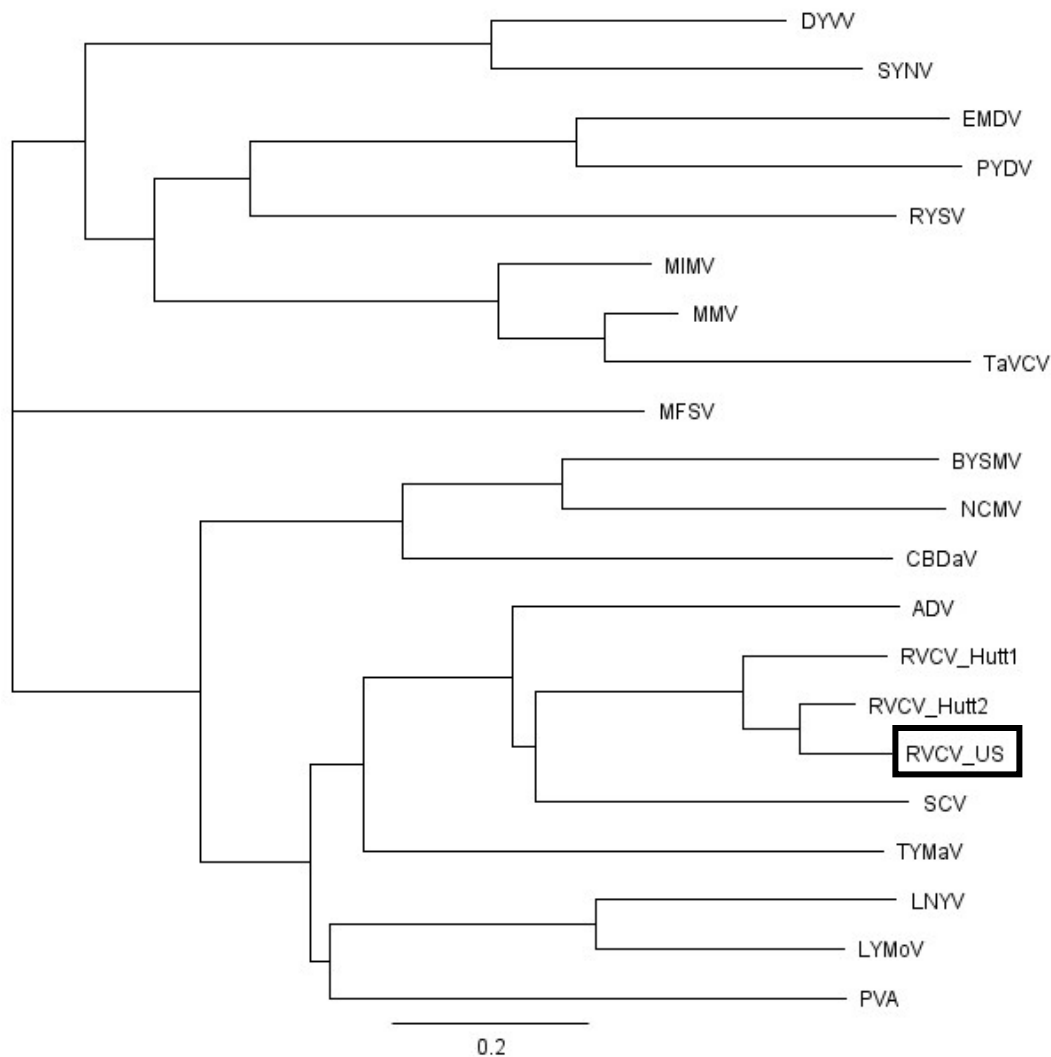
Figure 2.3. Neighbor-joining phylogenetic tree showing relationship of the amino acid sequences of the nucleocapsid (N) protein of RVCV_US and corresponding sequences of other plant cytorhabdoviruses and selected nucleorhabdoviruses. The viruses and their accession numbers are: alfalfa dwarf virus (ADV; YP_009177015), barley yellow striate mosaic cytorhabdovirus (BYSMV; YP_009177222), colocasia bobone disease-associated virus (CBDaV; YP_009362275), datura yellow vein nucleorhabdovirus (DYVV; YP_009176972), eggplant mottled dwarf nucleorhabdovirus (EMDV; YP_009094352), lettuce necrotic yellows virus (LNYV; Q86134), lettuce yellow mottle virus (LYMoV; YP_002308371), maize fine streak nucleorhabdovirus (MFSV; YP_052843), maize Iranian mosaic nucleorhabdovirus (MIMV; AYN79151), maize mosaic virus maize (MMV; Q6E0X1), northern cereal mosaic cytorhabdovirus (NCMV; Q9JGU1), persimmon virus A (PVA; YP_006576501), potato yellow dwarf nucleorhabdovirus (PYDV; YP_004927965), raspberry vein chlorosis virus hutt1 (RVCV_Hutt1; QBS46629), raspberry vein chlorosis virus hutt2 (RVCV_Hutt2; QBS46637), rice yellow stunt nucleorhabdovirus (RYSV; Q86523), strawberry crinkle cytorhabdovirus (SCV; AWK49426), sonchus yellow net nucleorhabdovirus (SYNV; P10550), taro vein chlorosis nucleorhabdovirus (TaVCV; Q5GA90), and tomato yellow mottle-associated virus (TYMaV; YP_009352242). Bootstrap values of 1000 replicates are indicated at the branch points.
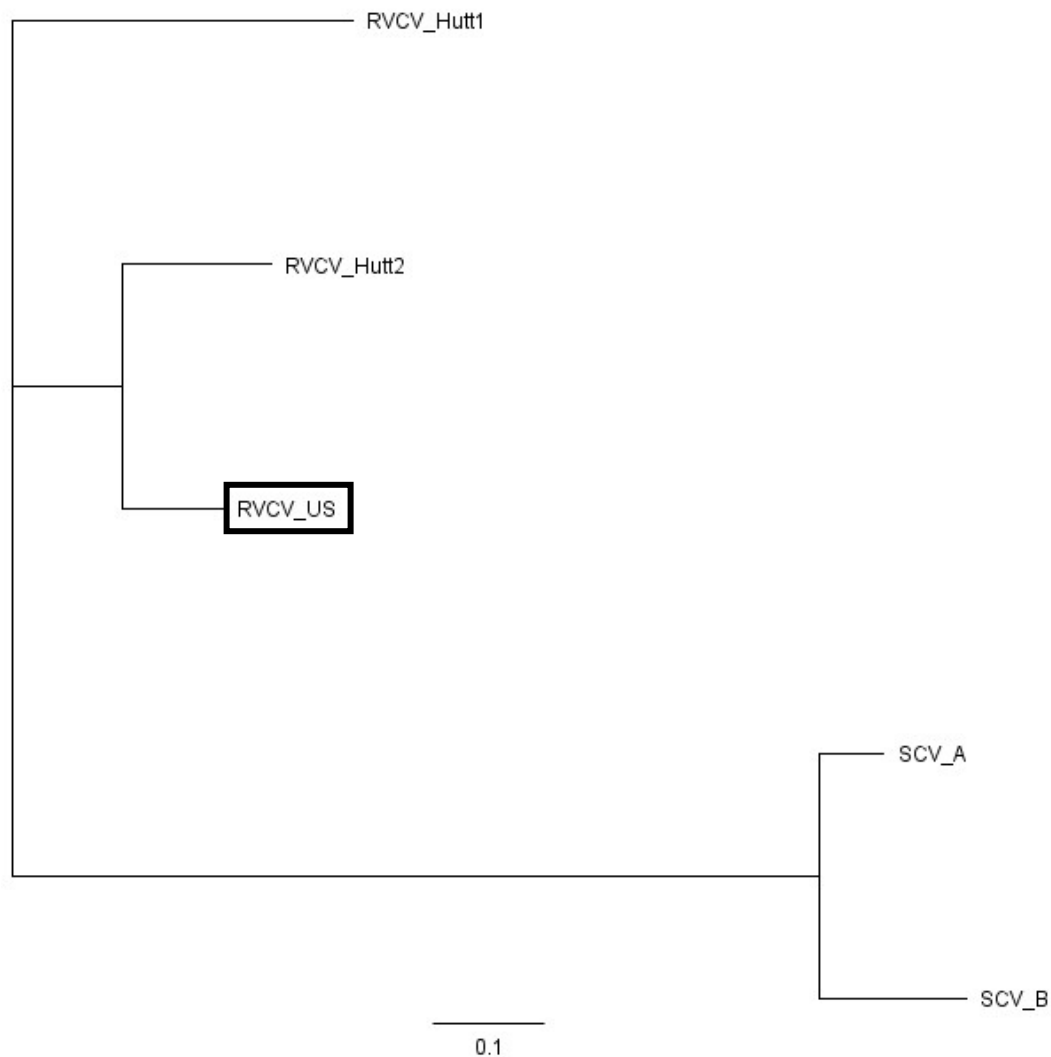
Figure 2.4. Neighbor-joining phylogenetic tree showing relationship of the amino acid sequences of the P' overlapping protein of RVCV_US and corresponding sequences of other plant cytorhabdoviruses. The viruses and their accession numbers are: raspberry vein chlorosis virus hutt1 (RVCV_Hutt1; QBS46630), raspberry vein chlorosis virus hutt2 (RVCV_Hutt2; QBS46638), strawberry crinkle virus isolate A (SCV_A; AWK49427), and strawberry crinkle virus isolate B (SCV_B; AWK49434). Bootstrap values of 1000 replicates are indicated at the branch points.

Figure 2.5. Neighbor-joining phylogenetic tree showing relationship of the amino acid sequences of the phosphoprotein (P) of RVCV_US and corresponding sequences of other plant cytorhabdoviruses and selected nucleorhabdoviruses. The viruses and their accession numbers are: alfalfa dwarf virus (ADV; YP_009177016), barley yellow striate mosaic cytorhabdovirus (BYSMV; YP_009177223), colocasia bobone disease-associated virus (CBDaV; YP_009362276), citrus necrotic spot virus (CNSV; AGQ21977), datura yellow vein nucleorhabdovirus (DYVV; YP_009176973), eggplant mottled dwarf nucleorhabdovirus (EMDV; YP_009094354), lettuce necrotic yellows virus (LNYV; YP_425088), lettuce yellow mottle virus (LYMoV; YP_002308372), maize fine streak nucleorhabdovirus (MFSV; YP_052844), maize Iranian mosaic nucleorhabdovirus (MIMV; AJW77720), maize mosaic virus maize (MMV; Q6E0X0), northern cereal mosaic cytorhabdovirus (NCMV; Q9JGU0), persimmon virus A (PVA; YP_006576502), potato yellow dwarf nucleorhabdovirus (PYDV; YP_004927967), raspberry vein chlorosis virus hutt1 (RVCV_Hutt1; QBS46631), raspberry vein chlorosis virus hutt2 (RVCV_Hutt2; QBS46639), rice yellow stunt nucleorhabdovirus (RYSV; O70790), strawberry crinkle cytorhabdovirus (SCV; AWK49428), sonchus yellow net nucleorhabdovirus (SYNV; P21299), taro vein chlorosis nucleorhabdovirus (TaVCV; Q5GA89), and tomato yellow mottle-associated virus (TYMaV; YP_009352241). Bootstrap values of 1000 replicates are indicated at the branch points.

Figure 2.6. Neighbor-joining phylogenetic trees showing relationship of the amino acid sequences of the putative movement (P3) protein of RVCV_US and corresponding sequences of other plant cytorhabdoviruses and selected nucleorhabdoviruses. The viruses and their accession numbers are: alfalfa dwarf virus (ADV; AKD44213), datura yellow vein nucleorhabdovirus (DYVV; AKH61403), eggplant mottled dwarf nucleorhabdovirus (EMDV; AHL89004), lettuce necrotic yellows virus (LNYV; Q9E7N8), maize Iranian mosaic nucleorhabdovirus (MIMV; ATY38956), maize mosaic virus maize (MMV; Q6E0W9), potato yellow dwarf nucleorhabdovirus (PYDV; ADE45271), raspberry vein chlorosis virus hutt1 (RVCV_Hutt1; QBS46632), raspberry vein chlorosis virus hutt2 (RVCV_Hutt2; QBS46640), rice yellow stunt nucleorhabdovirus (RYSV; Q98663), strawberry crinkle cytorhabdovirus (SCV; AWK49429), sonchus yellow net nucleorhabdovirus (SYNV; P31332), taro vein chlorosis nucleorhabdovirus (TaVCV; Q89914), and tomato yellow mottle-associated virus (TYMaV; Q5GA88). Bootstrap values of 1000 replicates are indicated at the branch points.
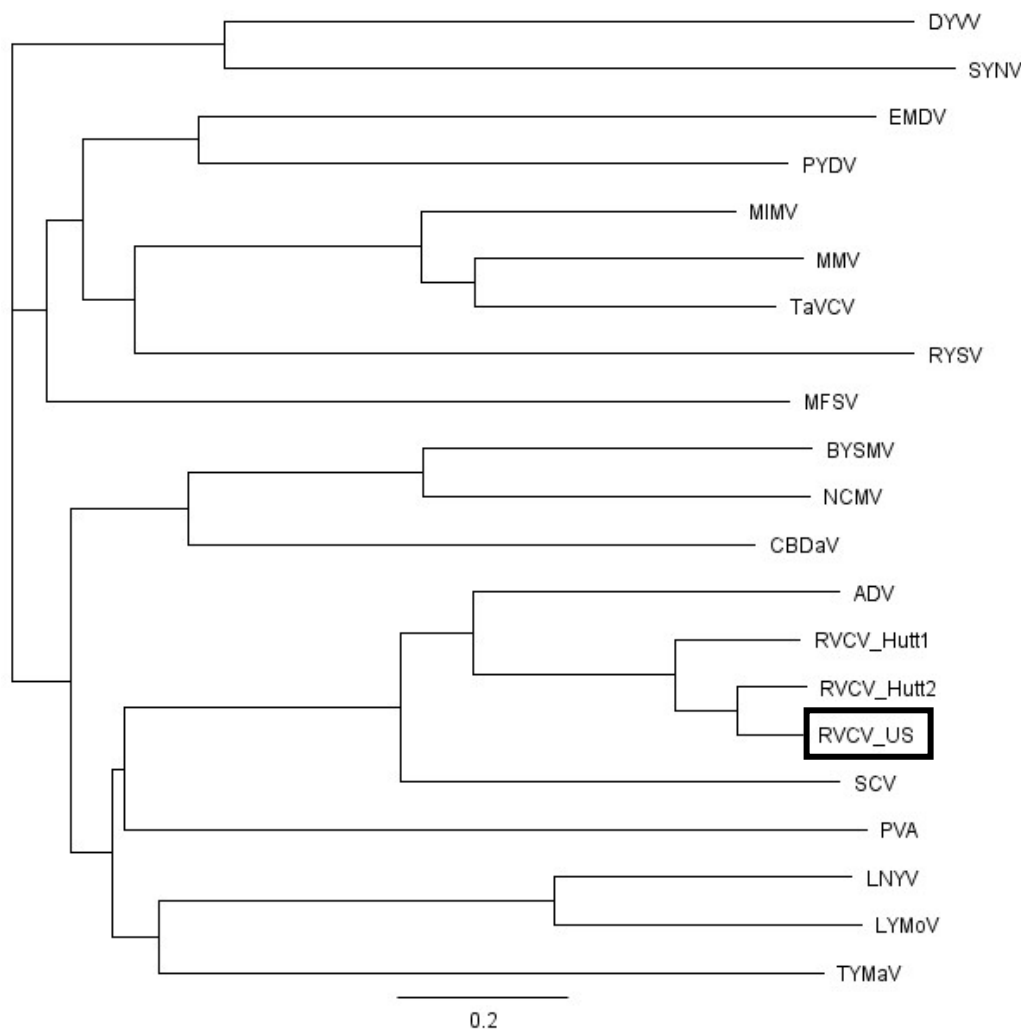
Figure 2.7. Neighbor-joining phylogenetic trees showing relationship of the amino acid sequences of the matrix protein (M) of RVCV_US and corresponding sequences of other plant cytorhabdoviruses and selected nucleorhabdoviruses. The viruses and their accession numbers are: alfalfa dwarf virus (ADV; YP_009177018), barley yellow striate mosaic cytorhabdovirus (BYSMV; YP_009177228), colocasia bobone disease-associated virus (CBDaV; YP_009362278), citrus necrotic spot virus (CNSV; AGQ21977), datura yellow vein nucleorhabdovirus (DYVV; YP_009176975), eggplant mottled dwarf nucleorhabdovirus (EMDV; YP_009094356), lettuce necrotic yellows virus (LNYV; YP_425090), lettuce yellow mottle virus (LYMoV; YP_002308374), maize fine streak nucleorhabdovirus (MFSV; YP_052847), maize Iranian mosaic nucleorhabdovirus (MIMV; YP_009444711), maize mosaic virus maize (MMV; Q6E0W8), northern cereal mosaic cytorhabdovirus (NCMV; Q9JGT5), persimmon virus A (PVA; YP_006576504), potato yellow dwarf nucleorhabdovirus (PYDV; YP_004927969), raspberry vein chlorosis virus hutt1 (RVCV_Hutt1; QBS46633), raspberry vein chlorosis virus hutt2 (RVCV_Hutt2; QBS46641), rice yellow stunt nucleorhabdovirus (RYSV; NP_620499), strawberry crinkle cytorhabdovirus (SCV; AWK49437), sonchus yellow net nucleorhabdovirus (SYNV; P19692), taro vein chlorosis nucleorhabdovirus (TaVCV; Q5GA87), and tomato yellow mottle-associated virus (TYMaV; YP_009352239). Bootstrap values of 1000 replicates are indicated at the branch points.
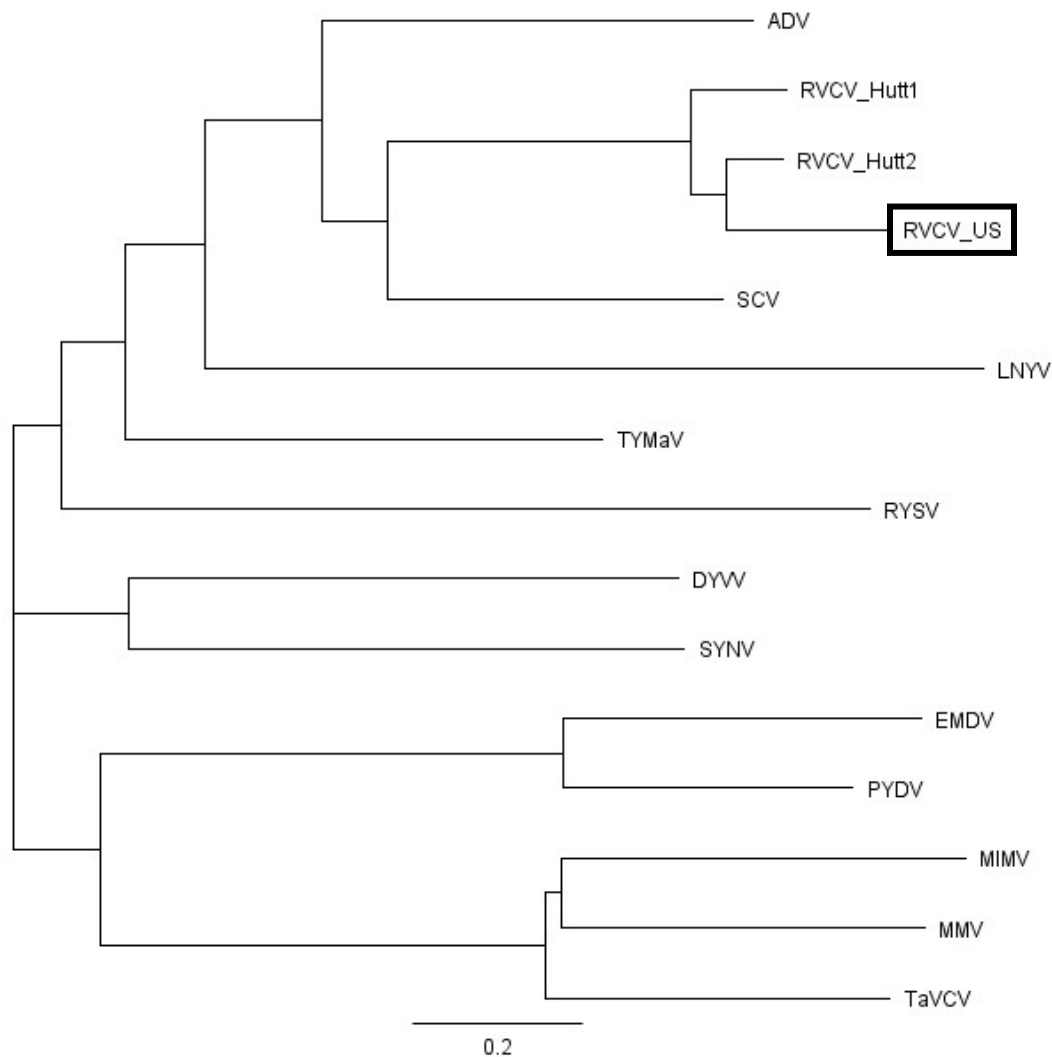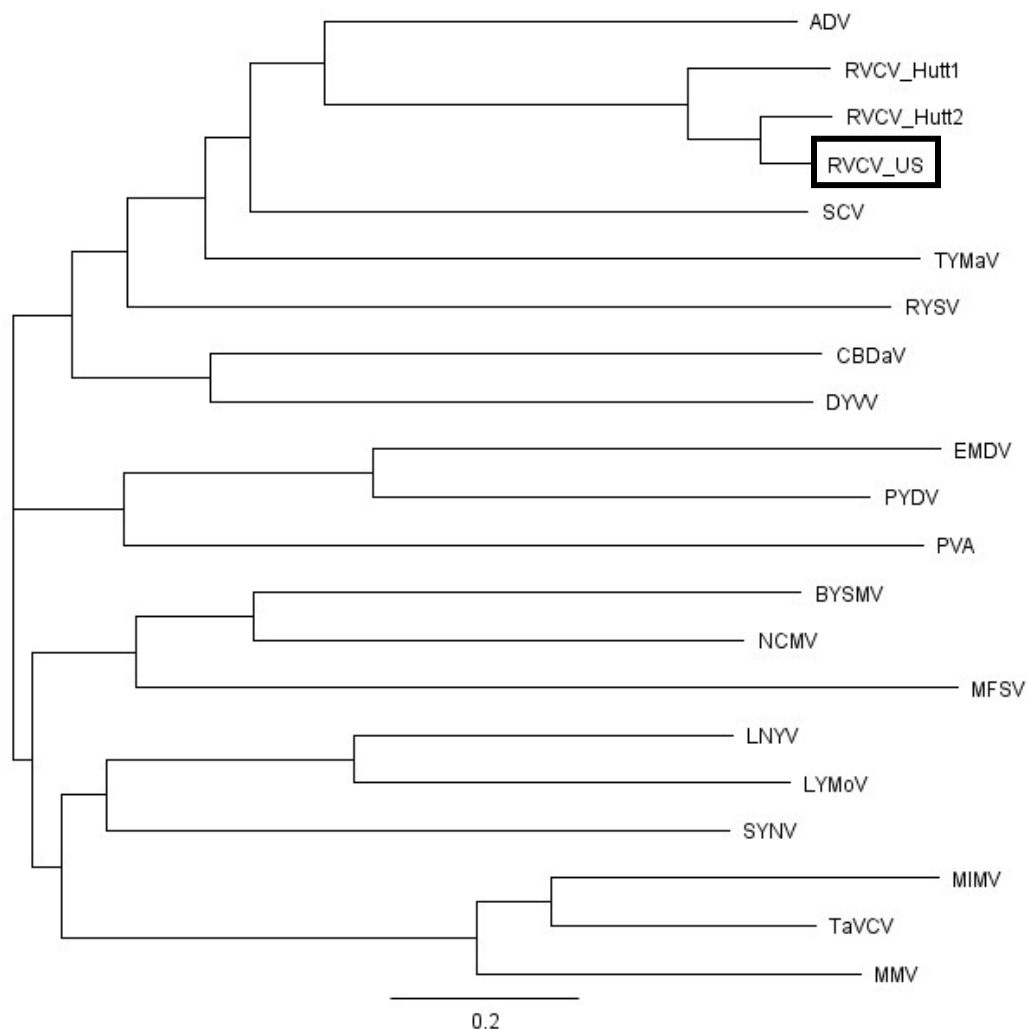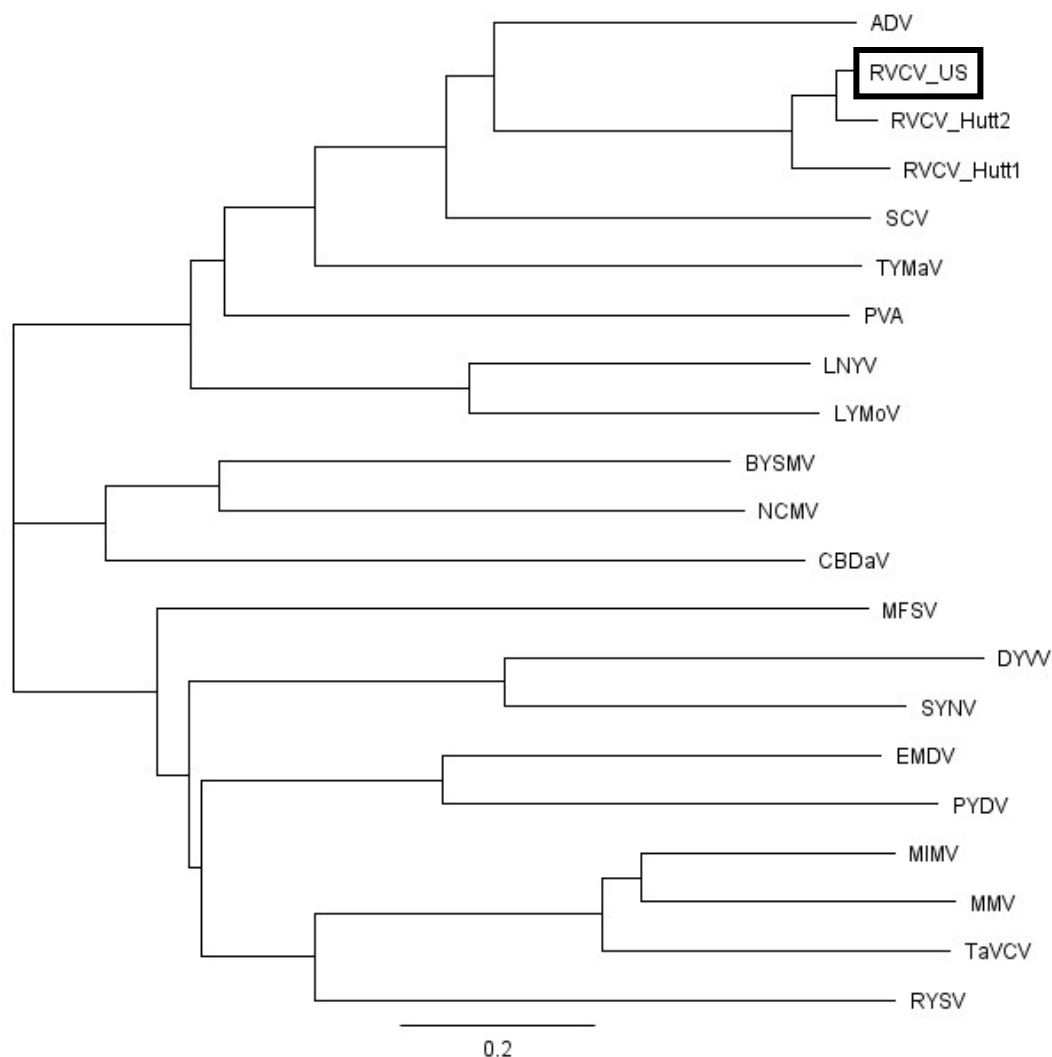
Figure 2.8. Neighbor-joining phylogenetic trees showing relationship of the amino acid sequences of the glycoprotein (G) of RVCV_US and corresponding sequences of other plant cytorhabdoviruses and selected nucleorhabdoviruses. The viruses and their accession numbers are: alfalfa dwarf virus (ADV; YP_009177019), barley yellow striate mosaic cytorhabdovirus (BYSMV; YP_009177229), colocasia bobone disease-associated virus (CBDaV; YP_009362279), datura yellow vein nucleorhabdovirus (DYVV; YP_009176976), eggplant mottled dwarf nucleorhabdovirus (EMDV; YP_009094357), lettuce necrotic yellows virus (LNYV; Q9DIC6), lettuce yellow mottle virus (LYMoV; YP_002308375), maize fine streak nucleorhabdovirus (MFSV; YP_052848), maize Iranian mosaic nucleorhabdovirus (MIMV; AYN79074), maize mosaic virus maize (MMV; Q6E0W7), northern cereal mosaic cytorhabdovirus (NCMV; Q9JGT4), persimmon virus A (PVA; YP_006576505), potato yellow dwarf nucleorhabdovirus (PYDV; YP_004927970), raspberry vein chlorosis virus hutt1 (RVCV_Hutt1; QBS46634), raspberry vein chlorosis virus hutt2 (RVCV_Hutt2; QBS46642), rice yellow stunt nucleorhabdovirus (RYSV; O10236), strawberry crinkle cytorhabdovirus (SCV; AWK49438), sonchus yellow net nucleorhabdovirus (SYNV; NP_042285), taro vein chlorosis nucleorhabdovirus (TaVCV; Q5GA86), and tomato yellow mottle-associated virus (TYMaV; AQY17507). Bootstrap values of 1000 replicates are indicated at the branch points.

Figure 2.9. Neighbor-joining phylogenetic trees showing relationship of the amino acid sequences of the unknown function of the P6 protein of RVCV_US and corresponding sequences of other plant cytorhabdoviruses and selected nucleorhabdoviruses. The viruses and their accession numbers are: alfalfa dwarf virus (ADV; YP_009177020), northern cereal mosaic cytorhabdovirus (NCMV; Q9JGT6), raspberry vein chlorosis virus hutt1 (RVCV_Hutt1; QBS46635), raspberry vein chlorosis virus hutt2 (RVCV_Hutt2; QBS46643), rice yellow stunt nucleorhabdovirus (RYSV; O70791), strawberry crinkle cytorhabdovirus (SCV; AWK49432), and tomato yellow mottle-associated virus (TYMaV; YP_009352237). Bootstrap values of 1000 replicates are indicated at the branch points.
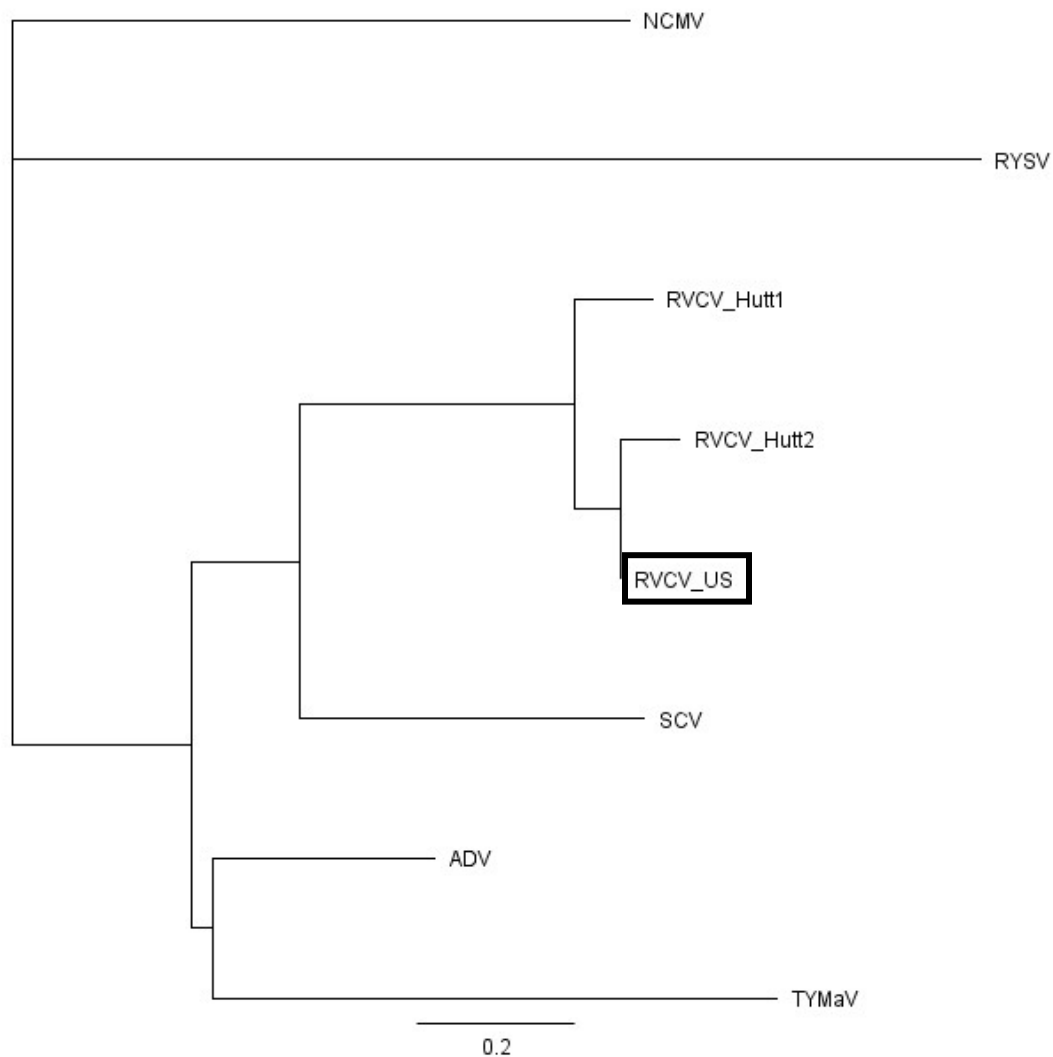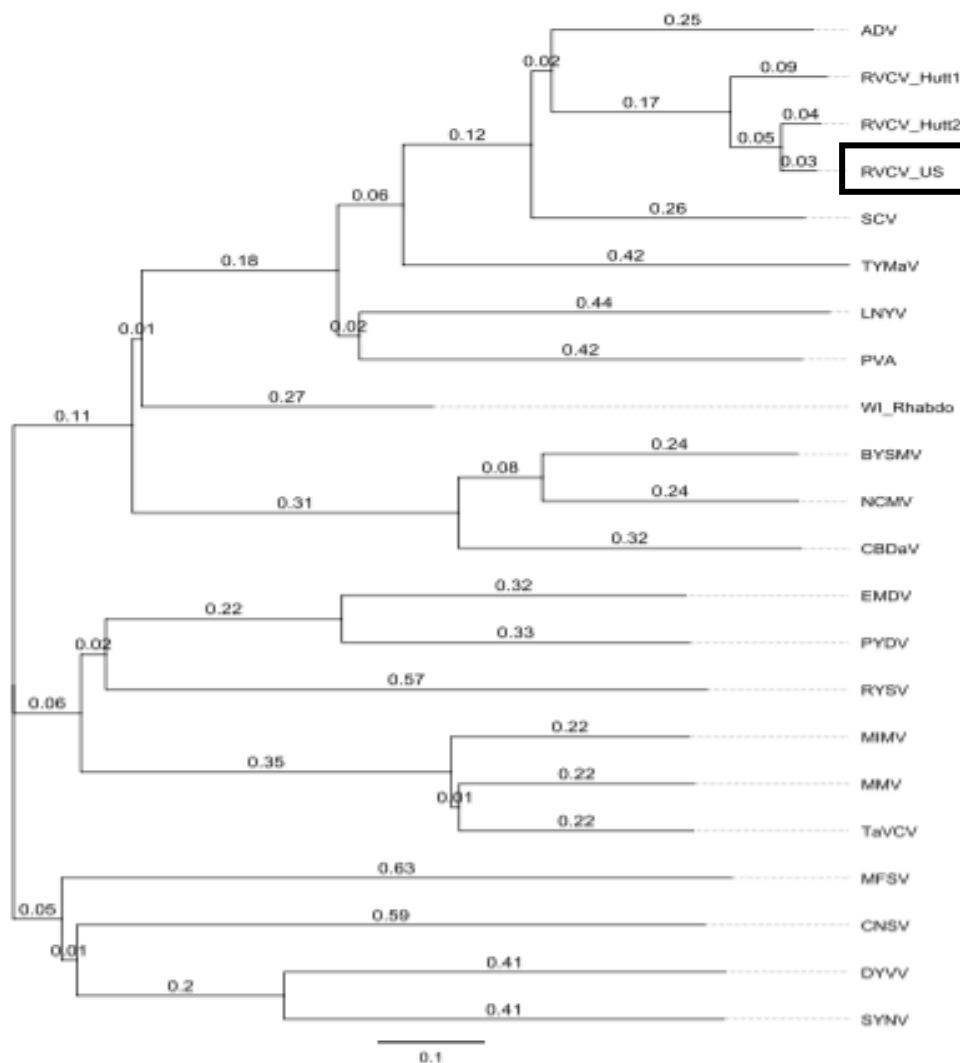
Figure 2.10. Neighbor-joining phylogenetic trees showing relationship of the amino acid sequences of the RNA-dependent RNA polymerase (L) protein of RVCV_US and corresponding sequences of other plant cytorhabdoviruses and selected nucleorhabdoviruses. The viruses and their accession numbers are: alfalfa dwarf virus (ADV; YP_009177021), barley yellow striate mosaic cytorhabdovirus (BYSMV; YP_009177231), colocasia bobone disease-associated virus (CBDaV; YP_009362280), citrus necrotic spot virus (CNSV; AGQ21977), datura yellow vein nucleorhabdovirus (DYVV; YP_009176977), eggplant mottled dwarf nucleorhabdovirus (EMDV; YP_009094358), lettuce necrotic yellows virus (LNYV; Q4W382), maize fine streak nucleorhabdovirus (MFSV; YP_052849), maize Iranian mosaic nucleorhabdovirus (MIMV; YP_009444713), maize mosaic virus maize (MMV; Q6E0W6), northern cereal mosaic cytorhabdovirus (NCMV; NP_597914), persimmon virus A (PVA; YP_006576506), potato yellow dwarf nucleorhabdovirus (PYDV; YP_004927971), raspberry vein chlorosis virus hutt1 (RVCV_Hutt1; QBS46636), raspberry vein chlorosis virus hutt2 (RVCV_Hutt2; QBS46644), rice yellow stunt nucleorhabdovirus (RYSV; O10378), strawberry crinkle cytorhabdovirus (SCV; AWK49433), sonchus yellow net nucleorhabdovirus (SYNV; P31332), taro vein chlorosis nucleorhabdovirus (TaVCV; Q5GA85), tomato yellow mottle-associated virus (TYMaV; YP_009352236), and uncharacterized *Rhabdoviridae*-like virus from Wisconsin (WI_Rhabdo). Bootstrap values of 1000 replicates are indicated at the branch points.
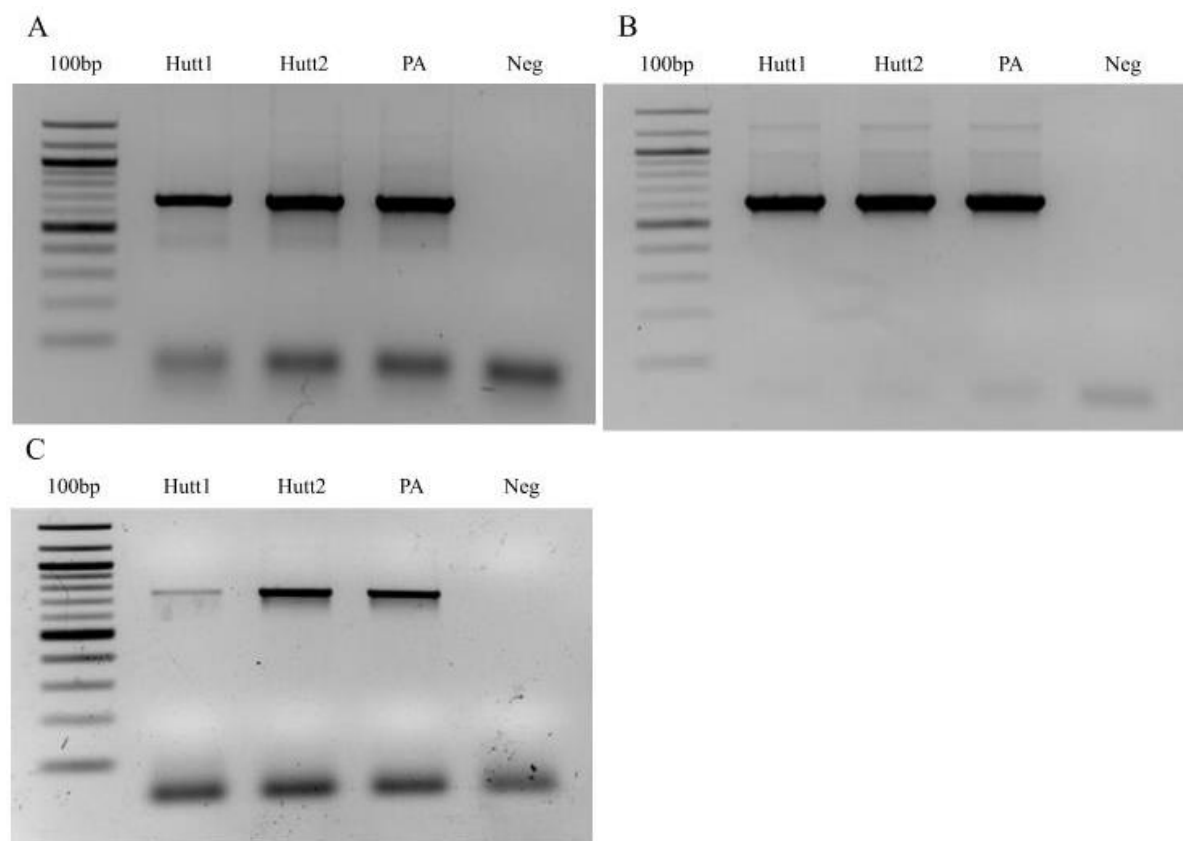
Figure 2.11. RT-PCR amplification of the RNA-dependent RNA polymerase (L protein) using leaf samples containing isolates of RVCV_Hutt1, RVCV_Hutt2, and RVCV_US on 2% agarose gel. (A) L1 primers amplify 703bp of the L protein, (B) L2 primers amplify 640bp, and (C) L3 primers amplify 764bp. First lanes represent 100-bp ladder (NEB) and the fifth lanes represent the negative MBG water control.

Table 2.3. Amino acid sequence identities (%) of RVCV_U.S. proteins compared with those of other cytorhabdoviruses using BLASTp. Dashes indicate absence of an encoded viral protein.

	N	P'	P	P3	M	G	P6	L
ADV	47.2	-	45.4	45.3	44.4	48.7	41.3	61.3
BYSMV	24.2	-	80.0	-	23.4	83.3	-	30.4
LNYV	31.1	-	19.6	-	36.6	31.6	-	40.6
NCMV	23.0	-	25.6	-	23.0	24.5	37.5	30.7
PVA	34.1	-	23.5	-	17.5	50.0	-	42.9
RVCV_Hutt1	75.0	66.2	75.3	75.5	79.1	84.5	81.3	83.9
RVCV_Hutt2	86.0	80.0	85.3	77.3	88.3	94.2	93.8	93.3
RVCV_partial	-	-	-	-	-	-	-	90.4
SCV	48.5	44.9	37.2	53.1	35.3	39.1	39.3	58.7
TYMaV	22.6	-	19.6	30.5	26.8	58.3	66.7	46.4

A

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RVCV_US     MIFDIDPELLFKLLSRFISPTSLKWTILILTVYQTLSLMWRCLKLIRSVILTTLWLIKVG     60
RVCV_Hutt2  MLSDIDPELLFKLFARFISPTSLKWIILILTAYQTLSLTWRCLKLIRSVILITLWLIRIG     60
               **          **          *     *        *             *      **
RVCV_US     RRIWTWATSINLRMGARKKR      80
RVCV_Hutt2  ARMWTWARTLNLRMGARKRR      80
            *  *     ***             *
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B

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RVCV_US     MSHTDEAGSSGKQGGVMAWRGISILYKSASLDFKKGFGPIKLTHNGEISSAIGTLLSEAG     60
RVCV_Hutt2  MAQIEDNGASGSQRGIMAWRGISIIYKSASLDFKKGYGPIKLTHNGEISSAIGALLSEAG     60
            *  **  *       *          *              *

RVCV_US     GTKPVVTILRSMIDHRHARNFVDHYTSPLLGPKTQRLNFVFPKFVVVPFPANIPCVHEKI     120
RVCV_Hutt2  GTKTVVTILRSMIDHKHARNFVDHYTSPLLGPKTQRLNFVFPKFVVVPFPANIPCVHEKI     120

RVCV_US     TAIGKRGKIGGRDVVSAFDIDVVITEIDPGKIKKLLSETPEWFIGELELPYGPPIPDLSS     180
RVCV_Hutt2  TAIGKRGKIGGRDVVSAFDIDVVITEIDPGKVKKLLSETPEWFIGELELPYGPPTIDPST     180
                                           *                    **  *  *

RVCV_US     VGQSLVQTMVDGVKKL     196
RVCV_Hutt2  SGPSLVQTVVDGVKKL     196
            * *     *
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Figure 2.12. Alignments of two ORFs present in RVCV_US and RVCV_Hutt2 genomes using the ClustalW alignment tool. (A) 80% aa identity found in ORF 2, P' overlapping protein, between RVCV_US and Hutt2 and (B) 88.3% aa identity of the matrix proteins. Asterisks denote different amino acids.

Chapter 3:

Single and Mixed Viral Infections to Recreate Raspberry Leaf Curl Disease

Melinda Guzman Martinez and Robert R. Martin

Abstract

To determine the virus complex(es) that cause raspberry leaf curl disease (RLCD), five viruses

identified by high-throughput sequencing (HTS) on native *Rubus* exhibiting RLCD-like

symptoms were utilized in single and mixed viral infections in black, purple, and red raspberry

through aphid and graft transmissions. The five viruses identified were black raspberry necrosis

virus (BRNV), rubus yellow net virus (RYNV), a novel enamovirus, and two novel viruses

belonging to family *Rhabdoviridae*. The viruses used in the experiments were BRNV, RYNV,

raspberry leaf mottle virus (RLMV), the novel enamovirus, and one of the novel rhabdoviruses.

The addition of RLMV into the virus complex mixture was because it was vectored by the same

aphid, *Amophorophora agathonica,* as BRNV, RYNV, and the novel enamovirus. After single

and mixed infections of the viruses were created, none replicated the symptoms previously

described. This may be because throughout the course of the experiment, the enamovirus was no

longer detectable using RT-PCR.

Introduction

Although current major United States (U.S.) raspberry production occurs in California, Oregon,

and Washington (NASS 2017), some of the first established raspberry sites were found to be in

the regions of Michigan, New York, Ohio, Pennsylvania, and in the British Columbia and

Ontario regions of Canada (Demchak et al. 2017). More than 30 viruses have been reported from

Rubus spp., and in most cases virus diseases in these crops are caused by virus complexes

(Martin et al. 2013, 2017). Raspberry leaf curl disease (RLCD), initially reported in 1895

(Stewart and Eustace 1902) in Minnesota, has been reported from eastern Canada and the

northeastern U.S. and as far west as the Rocky Mountains by 1930. Symptoms of infection

include small crumbly fruit, stunted shoots, leaf chlorosis, and downward curling of the leaves

(Bolton 1970). This disease has previously been reported to reduce yield by 20-40% in addition

to reduced fruit quality, with the infected plant dying after only a few seasons (Converse 1987).

The symptomology parallels between RLCD on *Rubus* to symptoms of potato leaf roll disease

(Rankin and Hockey 1922), now known to be caused by a virus in the family *Luteoviridae*, has

led to the speculation that one of the viruses involved in RLCD may also be caused by a virus in

the same family (Matthews 1982).

The small raspberry aphid, *Aphis rubicola,* was regularly found within infected areas of leaf curl

(Rankin and Hockey 1922), and later used for transmission studies leading to the determination

that *A. rubicola* transmitted the disease in a persistent manner (Converse 1987).

The spread of RLCD was determined to move slowly (Rankin and Hockey 1922) with no clear

pattern, despite the increased population of *A. rubicola* (Bolton 1970). The causal agent(s) of

RLCD have not been identified but previous studies based on the transmission properties of the

viruses indicate it may be caused by at least two viruses, varying in symptomology in red

'Cuthbert', black 'Cumberland', and purple 'Columbian' raspberry cultivars (Bennett 1930).

Throughout the course of a three year *Rubus* virus survey to assess prominent viruses of native

habitats, nurseries, and commercial fields in prominent raspberry and blackberry production

regions in the U.S. and Canada (Martin 2019), several native red raspberry plants exhibiting

virus-like symptoms were collected in Arkansas, California, Delaware, Idaho, Maine, Maryland,

Massachusetts, Michigan, Minnesota, Montana, New York, North Carolina, Ohio, Oregon,

Pennsylvania, South Carolina, Virginia, Washington, West Virginia, Wisconsin, and Wyoming

as well as in British Columbia, Ontario and Quebec Canada. Additional plants from the USDA-

ARS National Clonal Germplasm Repository in Corvallis, Ore. collected from several of the

previously listed states but with the inclusion of New Jersey, were also included in the *Rubus*

survey. The plants that exhibited RLCD-like symptoms (Maine, Pennsylvania, Wisconsin,

Ontario, and Quebec) were subsequently analyzed using high-throughput sequencing (HTS) (Di

Bello et al. 2017). After HTS analysis, five viruses were detected including: Black raspberry

necrosis virus (BRNV), Rubus yellow net virus (RYNV), and three novel viruses, two belonging

to the *Rhabdoviridae* family and the other a member of the E*namovirus* genus in the family

Luteoviridae.

Aphids belong to order *Homoptera* and are known to be one of three classes of Insecta that

transmit plant viruses. Viruses can be transmitted by vectors in three manners: non-persistently

where aphids only need to feed on an infected plant for a few seconds before being able transmit

the acquired virus; semi-persistently where the aphid requires longer periods, up to hours, of

feeding to transmit a virus successfully; and persistently where aphids need anywhere from a few

hours or days to acquire and virus, but will not be able to immediately transmit the virus as the

virus will need to circulate within the aphid vector before it is excreted through the salivary

system and persistent propagative where the virus infects the aphid and replication in the aphid is

required before the virus can be transmitted. The last method listed often has a latent period of

days to weeks between acquisition and ability to transmit the virus. The feeding time that an

aphid requires to uptake a plant virus from an infected plant is known as the acquisition access

period (AAP), while the period of time given for the vector to transmit the virus is known as the

inoculation access period (IAP) (Dijkstra and de Jager 2012).

Both BRNV and RYNV are known to be transmitted in a semi-persistent manner by the large

raspberry aphid, *Amphorophora agathonica,* and are known to be part of a separate virus

complex that cause Raspberry mosaic disease (RMD) (Stace-Smith and Jones 1987; Halgren et

al. 2007). The novel Luteoviridae-like virus, hereafter referred to as 'enamovirus', was found to be graft-transmissible to raspberries and blackberries and determined to be vectored by *A. agathonica* in a persistent manner through a previous study (Di Bello et al. 2017). The transmission properties of the novel Rhabdoviridae-like viruses, one found in Wisconsin and the other in Pennsylvania, were not yet determined however viruses belonging to the family *Rhabdoviridae* are known to be persistent-propagatively transmitted (Jackson et al. 2005). The novel Rhabdoviridae-like virus isolated from Pennsylvania is hereafter referred to as 'cytorhabdovirus'.

Noting the previous studies suggesting that RLCD is caused by an unknown virus complex, the viruses identified through HTS (BRNV, RYNV, enamovirus, and cytorhabdovirus), with the inclusion of the *A. agathonica* semi-persistently transmitted raspberry leaf mottle virus (RLMV) (Quito-Avila and Martin 2012), were used in single and mixed infections through aphid and leaflet graft transmissions to recreate RLCD symptoms and identify the viruses involved in the complex.

Materials and Methods

Plant Material and virus isolates. dsRNA-free purple raspberry cultivar 'Columbian' (PI number 553328), black raspberry cultivar 'Cumberland' (PI number 553739), and red raspberry cultivar 'Cuthbert' (PI number 553363) were obtained from the USDA-ARS National Clonal Germplasm Repository (NCGR) in Corvallis, Ore. The 'Columbian', 'Cumberland', and 'Cuthbert' were propagated by cuttings and planted in 4" x 4" pots for use in aphid and graft transmissions studies. Isolates of black raspberry necrosis virus (BRNV), raspberry leaf mottle virus (RLMV), Rubus yellow net virus (RYNV), the enamovirus, and the cytorhabdovirus were

obtained from *Rubus* collection maintained at the USDA-ARS Horticultural Crops Research Unit (HCRU) in Corvallis, Ore. and from plant material shipped (under APHIS permit P526P-16-04242) to the same laboratory.

The healthy and infected plants were kept and grown in separate greenhouses with 16 hours of daylight at 25°C.

Nucleic acid extraction and reverse-transcription polymerase chain reaction (RT-PCR).

Nucleic acid extractions and reverse-transcription polymerase chain reaction (RT-PCR) testing were performed to identify the plants in the infected greenhouse that contained BRNV, RLMV, RYNV, enamovirus, and the cytorhabdovirus; same protocol was also used for confirmation of aphid and graft transmission studies. Total RNA was extracted from 100 mg leaf tissue by grinding with a 1 mL mixture of RNA extraction buffer (24.2 g Tris Base, 12.66 g Lithium Chloride, 15g Lithium dodecyl sulfate, 2.92 g EDTA, 9.42 g Deoxycholic acid, 14.3 mL 70% Tergitol-NP40, 20 g PVP, 800 mL of DI water, adjusted to pH to 8.5, and brought to a final volume of 1 L) (Hughes and Galau 1988; Spiegel and Martin 1993) containing 20 µL 1M tris(2-carboxyethyl)phosphine (TCEP) (30 mL of sterile DI water, 11.466 g TCEP, brought to final volume of 40 mL). The sap was collected in a 1.5 mL Eppendorf tube (Eppendorf AG, Hamburg, Germany) and centrifuged in a Heraeus Pico 21 Microcentrifuge (Thermo Fisher Scientific Inc., Waltham, MA, USA) at 13,000 rpm for three minutes. 500 µL of the supernatant and 600 µL of 6 M KoAc [294.4 g potassium acetate (KoAc), 500 mL DI water, 182 mL Glacial acetic acid, 90g Potassium hydroxide (KOH), adjust pH to 6.5 using 10M KOH, and bring to volume of 1 L] (Sambrook and Russell 2001) were added to a new 1.5 mL Eppendorf tubes. This mixture was then inverted 10 times, then centrifuged at 13,000 rpm for 10 minutes. 700 µL of the supernatant

and 750 µL of 100% isopropyl alcohol were added into a new Eppendorf tube, inverted 20 times

to mix, and centrifuged at 13,000 rpm for 30 minutes. The supernatant was then poured off and

carefully blotted on paper towel so as to not lose the RNA pellet. A 500 µL mixture containing

equal amounts of wash buffer (10 mL of 1 M Tris-HCl pH 7.5, 1 mL 0.5 M EDTA, 10 mL 5 M

NaCl, and 479 mL DEPC treated sterile H_2O) (Rott and Jelkmann 2001) and 100% ethanol were

added to the Eppendorf tube containing the pellet, followed by the addition of 10 µL of silica

glass milk [60 g silica particles (Sigma S5631), 500 mL sterile DI H_2O, adjusted to pH 2 using 1

M HCl] (Rott and Jelkmann 2001). The sample was then vortexed to resuspend the pellet,

followed by two 8-second pulse centrifugations at 5,000 rpm. The supernatant was poured off

and carefully blotted on a new paper towel and another 500 µL of 50/50 mixture of wash buffer

and 100% ethanol was added to the pellet. The sample was again vortexed, and pulse centrifuged

at 5,000 rpm for two 8-second intervals. The supernatant was then poured off and carefully

blotted on a new paper towel, with the pellet still in the Eppendorf tube. The pellet still inside the

Eppendorf tube was placed in the SpeedVac vacuum concentrator (Thermo Fisher Scientific

Inc.) for five minutes to dry. The dried pellet was then resuspended in 55 µL of RNA elution

buffer [1 mL 10 mM of Tris-HCL pH 8.5 and 1 mM EDTA pH 8.0 mixture, and 49 mL

molecular biology grade (MBG) water] by vortexing, then incubated at 70°C for four minutes.

The heated mixture was then centrifuged for 10 minutes to pellet the glass milk, then 50 µL of

the supernatant was collected and used as template for reverse-transcription reactions; stored at -

20°C in a VWR Standard Series Auto Defrost Laboratory Freezer (VWR International LLC.,

Radnor, PA, USA).

For the reverse-transcriptions, 5 µL of the RNA was transferred into a single well in a non-

skirted PCR plate (Thermo Fisher Scientific Inc.). In a 1.5 µL Eppendorf tube, 29.2 µL of H_2O,

10 µL 5X First-Strand Buffer (Invitrogen Corp., Carlsbad, CA, USA), 2 µL 10mM dNTPs (GenScript Biotech Corp., Piscataway, NJ, USA), 2 µL 2% PVP [2 g polyvinylpyrrolidone(PVP) MW 40,000 added to 100 mL MBG H_2O], 1 µL 0.3 µg/µL random primers (100 µL of 3 µg/µL Invitrogen Random Primers, diluted with 900 µL MBG H_2O), 0.5 µL of 40 U/µL Ribolock RNase Inhibitor (Thermo Fisher Scientific Inc.) and 0.3 µL 200 U/µL Maxima Reverse Transcriptase (Thermo Fisher Scientific Inc.) were mixed; this 45 µL RT mixture was then transferred into the same PCR plate well containing the 5 µL RNA. After the Veriti 96-well thermal cycler (Thermo Fisher Scientific Inc.) heated to 50℃, the RT reaction mixture was inserted and ran at 50℃ for 60 minutes followed by denaturation of the enzyme by heating at 85℃ for 5 minutes. The generated 50 µL cDNA was stored in a -20℃ auto defrost freezer (VWR International LLC.) for later use in PCR.

For the PCR reaction, 2 µL of the cDNA from the RT reaction was added to a 20 µL PCR mixture containing 15.08 µL H_2O, 2 µL 10X Taq buffer (GenScript Biotech Corp.), 0.4 µL 10mM dNTPs (GenScript Biotech Corp.), 0.4 µL 50 µM NAD5 primer mixture, and 0.12 µL 5 U/µL Green *Taq* DNA polymerase (GenScript Biotech Corp.); the 20 µL PCR mixture was loaded into a single well of a non-skirted PCR plate (Thermo Fisher Scientific Inc.). The PCR plate was inserted into the thermal cycler (Thermo Fisher Scientific Inc.) after it heated to 95℃. For the PCR reaction there was an initial denaturation at 95℃ for 5 min followed by 40 cycles of denaturation at 95℃ for 30 s, annealing at 55℃ for 45 s, and extending at 72℃ for 30 s, with final 10 min extension at 72℃ before being cooled down to 15℃. To check the quality of the extraction, NAD5 primers were used to amplify the ND2 subunit of NADH(B) dehydrogenase (Mannerlöf and Tenning 1997). Gel electrophoresis was performed in an electrophoresis chamber (Bio-Rad Laboratories Inc., Berkeley, CA, USA) through a 2% agarose gel stained with

4 µL of ethidium bromide (10 mg/mL in H_2O) run at 90 V for 30 min. Nucleic acid bands were then visualized by exposure to UV light on the AlphaImager Gel Imaging System (ProteinSimple, San Jose, CA, USA).

Oligonucleotide forward and reverse (F and R) primers (Table 3.1) were used for RT-PCR detection of BRNV, RLMV, RYNV, enamovirus, and cytorhabdovirus in the virus infected plants. Each virus primer mixtures contained equal amounts of F and R primers with a final concentration of 50 µM for each primer. The BRNV primers used amplified a 415 bp fragment the RNA-dependent RNA polymerase of BRNV RNA1 viral genome (DQ344639); RLMV primers amplified a 452 bp fragment of the coat protein homolog (ABC8779); RYNV primers amplified 463 bp fragment of the polyprotein (AF468454); enamovirus primers amplified 209 bp fragment RNA-dependent RNA polymerase (unpublished primers); cytorhabdovirus primers amplified a 700 bp fragment of RNA-dependent RNA polymerase protein (unpublished primers); and the internal control NAD5 amplified an 812 bp fragment of the gene encoding NADH dehydrogenase (Mannerlöf and Tenning 1997) (Table 3.1).

Aphid colonies. Aphids, *Amphorophora agathonica*, were obtained from northern Washington in May 2018. After collection, the aphids were placed on moist filter paper overnight and a newly born aphid was collected to start a virus-free colony from a single female mother. *A. agathonica* were reared in an incubation chamber (Percival Scientific Inc. Perry, IA, USA) at 26°C with 16 hours of light, feeding on dsRNA-free healthy black raspberry cv. 'Munger' obtained from tissue culture at the USDA-ARS HCRU. Once the colony grew, adult wingless (apterous) *A. agathonica* aphids were used in experiments to transmit BRNV from a mixed virus infected plant into single infections and were also used to determine the transmission properties of the

cytorhabdovirus. Throughout the course of aphid rearing, the colony collapsed several times due to the entrance of an aphid parasitoid into the growth chamber. Fortunately, other colonies formed from the same single female aphid mother were kept in two separate growth chambers; aphids from these colonies were then later used.

Aphid transmission experiments. Before beginning RLCD graft transmissions, all viruses were required to be in single virus infected *Rubus* plants. After RT-PCR testing was performed, BRNV was found to be in the virus mixed infection plant, AHB14, also containing blackberry chlorotic ringspot virus (BCRV) and strawberry necrotic shock virus (SNSV). The other viruses used to create mixed infections were present in plants with single infections; red raspberry plant infected singly with RLMV, referred to as RLMV 3/16 ADL; a red raspberry plant containing cytorhabdovirus referred to as 09 Penndot; and a red raspberry plant of selection WSU 991 containing RYNV and did not require aphid transmissions to isolate the viruses into single infections. The isolation and detection of the enamovirus will be discussed later.

As previously mentioned, BRNV was found in the mixed virus infected AHB14 plant and therefore needed to be separated into single infections. BRNV was the only virus in the AHB14 source plant that was semi-persistently transmissible by the large raspberry aphid, *A. agathonica* (Halgren et al. 2007). Viruses that are semi-persistently transmitted only require an aphid to feed on an infected plant anywhere from a few minutes to hours for virus uptake (Halgren et al. 2007) and to separate BRNV from AHB14, a one hour AAP and one hour IAP intervals were utilized (Fig. 3.1 A). Aphids were starved for at least 30 minutes prior to feeding to ensure uptake of the virus (Gray 2008). Five groups, consisting of 10 aphids each, were placed in a clip cage and placed on the underside of the leaf along the midribs of different leaves throughout the BRNV

PCR-positive AHB14 plant. This was performed to restrict the movement of the aphids (Fig. 3.1

B). The clip cages were each composed of a metal double prong hair clip (50 mm long), one

piece of plastic tube (10 mm high), and a rubber backed washer. Aphids were moved using a

soft-bristle paint brush. A one-hour feeding period was utilized for BRNV acquisition (Halgren

et al. 2007). Each aphid group were then moved using the soft-bristle paint brush and placed onto

five separate healthy 'Columbian' plants, meaning the 50 aphids that fed on the AHB14 plant

were kept in 10-aphid groups and moved onto five different 'Columbian' plants. An inoculation

access period of one hour was used to allow the aphids to transfer virus to healthy test plants

(Halgren et al. 2007). The aphids were then removed and the plants treated with insecticides. The

BRNV aphid transmissions were repeated twice using the same BRNV AHB14 source plant, but

with two new batches of 50 virus-free *A. agathonica* aphid groups to transfer BRNV into five

'Cumberland' and five 'Cuthbert' plants. After all the BRNV aphid transfers, five replicates of

'Columbian', 'Cumberland', and 'Cuthbert' contained only a single virus, BRNV. All aphid

transmission studies occurred within the clip cages inside a Bugdorm insect rearing tent

(MegaView Science Co., Taiwan) in the infected greenhouse with 16 hours of daylight at 25°C.

Detection of novel enamovirus. The *Rubus* plants previously used for successful enamovirus

aphid and graft transmissions (Di Bello et al. 2017) no longer tested PCR-positive despite

utilizing the same nucleic acid extraction and RT-PCR protocols. The final PCR-positive

detection of the virus occurred in the spring following the initial transmissions.

Luteoviridae viruses phloem associated (Brault et al. 2001) and it was possible that seasonal

fluctuations of virus titer, which has been reported for other plant viruses such as Grapevine leaf

roll associated viruses (Narayanasamy 2005), led to the undetectability of the enamovirus during

the late summer when RT-PCR testing occurred. To initiate false spring conditions during the fall months, all the plants initially testing PCR-positive for the enamovirus were cut down to short leaf-less stems and subjected to a 40-day cold treatment at 4°C (de Capite 1955). After the cold treatment limit was reached, the plants were moved back into the greenhouse conditions of 16 hours of daylight at 25°C to regrow. Mature plant leaves were subjected to the same nucleic acid extraction and RT-PCR protocols as before, yet all remained enamovirus PCR-negative. The next phase for finding luteovirus containing plants led to the USDA-ARS NCGR, where some accessions of native *Rubus* plants had also previously tested PCR-positive for the luteovirus (Di Bello et al. 2017). Plant tissue was collected from four separate *Rubus* plants and nucleic acid extraction and RT-PCR was performed, again resulting in no luteovirus detection. The lack of luteovirus detection led to PCR modifications of forward and reverse (F+R) primer pair concentrations (μM), amounts of cDNA template used (μL), and PCR reaction volumes (μL) while maintaining the same annealing temperature of 54°C. The different luteovirus primer pair concentrations used were 20 μM F+R and 50 μM F+R concentrations. It is common to use 1/10th of cDNA template of the PCR reaction volume for efficient amplification (Manual) but to remove possible inhibitors affecting the PCR (Bessetti 2007), the volumes of cDNA template were manipulated. The cDNA template volume was either diluted to half the typical amount of 1/10th of PCR reaction volume (½ X), concentrated to double the amount (2X), or kept at the typical volume of cDNA template (X). The third and final modification was employing reaction volumes of 10 μL, 20 μL, and 25 μL for PCR reactions. After testing the 18 different combinations of primer pair concentrations, cDNA template amounts, and PCR reaction volumes, all samples from the USDA-ARS HCRU and NCGR still tested negative for the luteovirus. Therefore, the luteovirus was not used in the RLCD graft transmissions.

Graft transmissions for single and mixed viral infections. After single infections of BRNV

were established through aphid transmissions, RLMV, RYNV and the cytorhabdovirus were still

in their source plants and not yet in single infections on dsRNA-free 'Columbian',

'Cumberland', and 'Cuthbert' plants. The leaflet grafting method, using donor leaves of virus

infected plants and grafting them onto virus-free plants (Converse 1987), was utilized for graft

transmissions onto healthy plants. Young and vigorous 'Columbian' leaves were removed except

for three sets of compound leaves that would accept donor terminal leaflet grafts from the 09

Penndot plant, which contains a single virus infection of the cytorhabdovirus. A mature and

vigorous donor terminal leaflet was removed from the 09 Penndot plant. Holding the donor

leaflet from the terminal petiolule, the leaflet was cut, using a single edge razor, to the size of a

quarter about 24.3 mm in diameter ensuring the midrib of the leaflet was still attached to the

petiolule. Next, the petiolule was held on its 'side' on a water-soaked paper towel where it was

sharpened to a long, thin point exposing the xylem; this sharpening was repeated on the other

side of the petiolule. Focusing on one of the three compound leaves of the 'Columbian' recipient,

the petiolule and terminal leaflet were removed in order to accept the donor leaflet from the 09

Penndot plant. Using the single edge razor, the petiole was cut directly through the xylem

between the two other leaflets of the test 'Columbian' plant. The donor quarter-sized sharpened

petiolule leaflet was then inserted, following the natural pattern of the recipient petiole, into the

split recipient petiole and was tightly wrapped with a Sealtex Bit Wrap Latex Bandage (Sealtex

Company, Boyds, MD, USA) (Fig. 3.2) This was repeated using the same 09 Penndot plant to

donate two more leaflets to the other two compound leaves of the same 'Columbian' plant. In

total, three leaflet grafts were performed on a single recipient plant. To keep the moisture level

high for the graft, a 2-liter soda bottle, with the removal of the label and bottom cut off, was

placed over the 4"x4" pot for two weeks then removed. The leaflet graft process was repeated to have five of each 'Columbian', 'Cumberland', and 'Cuthbert' plants grafted with leaflets of the same 09 Penndot plant. Overall, this process was repeated to obtain single infections of RLMV, using RLMV 3/16 ADL, and RYNV, using the WSU 991 plant, and grafting the donor leaflets into 'Columbian', 'Cumberland', and 'Cuthbert' plants. To ensure that aphid and graft transmissions were successful, nucleic acid extractions and RT-PCR, as previously described, were utilized first using NAD5 primers to determine the success of the nucleic acid extraction (Fig. 3.3). The plants that did not test positive for their singly infected virus (Figure 3.4 A-C) were again used for another round of the same grating method after additional compound leaves had formed and matured. After five of each 'Columbian', 'Cumberland', and 'Cuthbert' cultivars tested RT-PCR positive for BRNV, RLMV, RYNV (Fig. 3.5), and the cytorhabdovirus, the same plants were then used in mixed infections as double, triple, and quadruple infections in the three *Rubus* cultivars (Table 3.2). All grafted plants were kept in the infected greenhouse with 16 hours of daylight at 25°C where observations were made to track the development of symptoms.

Results

Symptoms of single and mixed viral infections. Despite obtaining single and mixed infections of the BRNV, cytorhabdovirus, RLMV, and RYNV, not all the plants used for transmissions survived long enough after being infected with different viruses to perform nucleic acid extractions. This doesn't necessarily mean that the virus mixture within them were the sole cause of their death. Symptoms of the cytorhabdovirus grafted onto 'Cuthbert' resulted in leaf chlorosis (Fig. 3.6 A) and displayed some minor leaf crinkling in 'Columbian' (Fig. 3.6 B). Single infections of RYNV showed yellowing between minor veins in 'Columbian' and

minor yellow net-like symptoms in 'Cuthbert' and (Fig. 3.7 B). When mixed infections of

RYNV and RLMV were in the red raspberry 'Cuthbert', an overall downward cupping of leaf

tissue was observed (Fig. 3.8). In a triple virus infection containing RLMV, RYNV, and

cytorhabdovirus in red raspberry 'Cuthbert' showing symptoms of cupping of leaves, chlorotic

spots, and yellowing of veins. Lastly, in the virus complex containing viruses BRNV, RLMV,

RYNV, and cytorhabdovirus in red raspberry 'Cuthbert' expressed symptoms of mosaic and

crinkling of leaves.

Discussion

Although single and mixed infections of BRNV, cytorhabdovirus, RLMV, and RYNV were

transmitted successfully into black, purple, and red raspberry, the varying mixture of these

viruses did not recreate classic raspberry leaf curl disease (RLCD) symptoms previously

reported. Such symptoms include severe curling of the leaves and considerably stunted shoots

(Bennett 1927). The absence of symptoms may be due to the lack of the luteovirus used in the

aphid and graft transmissions. Through earlier studies, one viral strain of RLCD was

hypothesized to be part of the family *Luteoviridae* because of its' persistent transmissibility

through the small raspberry aphid, *A. rubicola* (Rankin and Hockey 1922; Matthews 1982).

Because viruses belonging to *Luteoviridae* are systemic (Gray 2008), it is possible that lack of

detection of the luteovirus was due to fluctuating virus titer throughout the year. Other studies

have shown that virus prevalence within the host plant can be underestimated if within-host virus

distribution is not homogenous, especially in wild-collected plants (Lacroix et al. 2016). It may

be also possible that the relationship between the aphid used, *Amphorophora agathonica,* is not

the natural vector of the luteovirus. Unfortunately, experimental evidence for the causal agent(s)

of RLCD cannot be provided because RLCD-like symptoms were not observed in the *Rubus*

cultivars.

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Table 3.1. List of PCR oligonucleotide primers used for detection of BRNV, RLMV, RYNV, luteovirus, and cytorhabdovirus.

Primer name		Primer sequence (5' - 3')	Amplicon size (bp)	Tm (°C)
BRNV:	Forward	ATG CTG AGC CAC TTG TGA	415	55
	Reverse	ATC TGG TGT GTT CCG CAT		
NAD5:	Forward	TAG CCC GAC CGT AGT GAT GTT AA	812	55
	Reverse	ATC ACC GAA CCT GCA CTC AGG AA		
RLMV:	Forward	CGA AAC TTY TAC GGG GAA C	452	58
	Reverse	CCT TTG AAY TCT TTA ACA TCG T		
RYNV:	Forward	CGT GAT AAC GGC TTG GTT TT	463	56
	Reverse	CGT AAG CGC AGA TTT CTT CC		
Luteo:	Forward	GAA ACC GCG ACT TTG ACT GTC AGG A	209	54
	Reverse	CTT ACA TCC CAG CCT GCC GAA TCT T		
Cytorhab:	Forward	TGT TCC CTG ATG ATC ACA AG	700	50
	Reverse	TTC ATC TCC TTT AAG CCC TC		
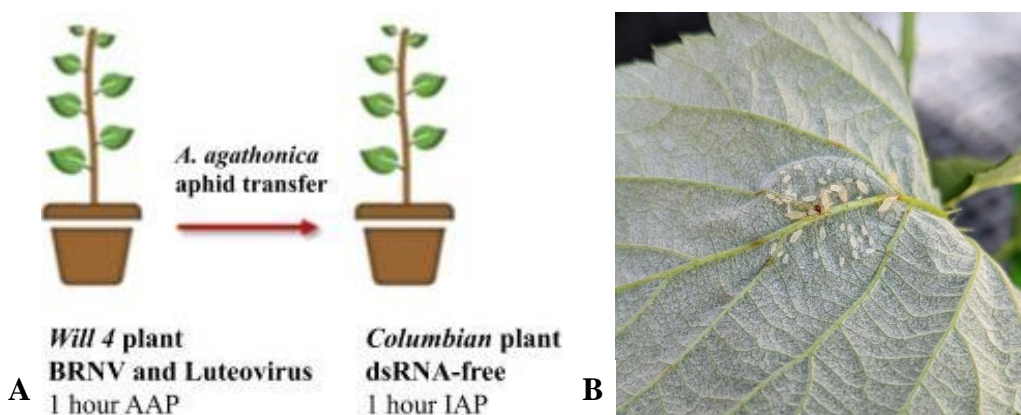
Figure 3.1. (A) Schematic used for BRNV aphid transfers to isolate BRNV into single infection from AHB14 plant that also contained blackberry chlorotic ringspot virus (BCRV) and strawberry necrotic shock virus (SNSV). (B) Aphids were kept in clip-cages to ensure feeding and limit their movement on the leaf. Adult and nymph aphids are shown.

Figure 3.2. Leaflet grafting using (A) donor leaf of 09 Penndot plant and grafting onto (B) 'Columbian' recipient with latex bandage.
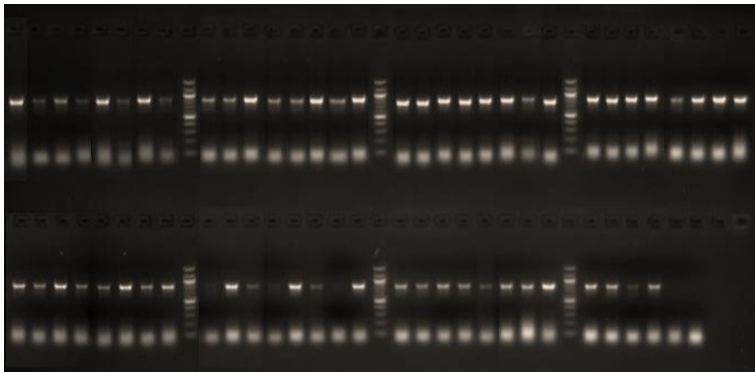
Figure 3.3: Agarose gel electrophoresis of NAD5 (812 bp) internal control to determine success of nucleic acid extraction of single infections from aphid and graft transmissions.
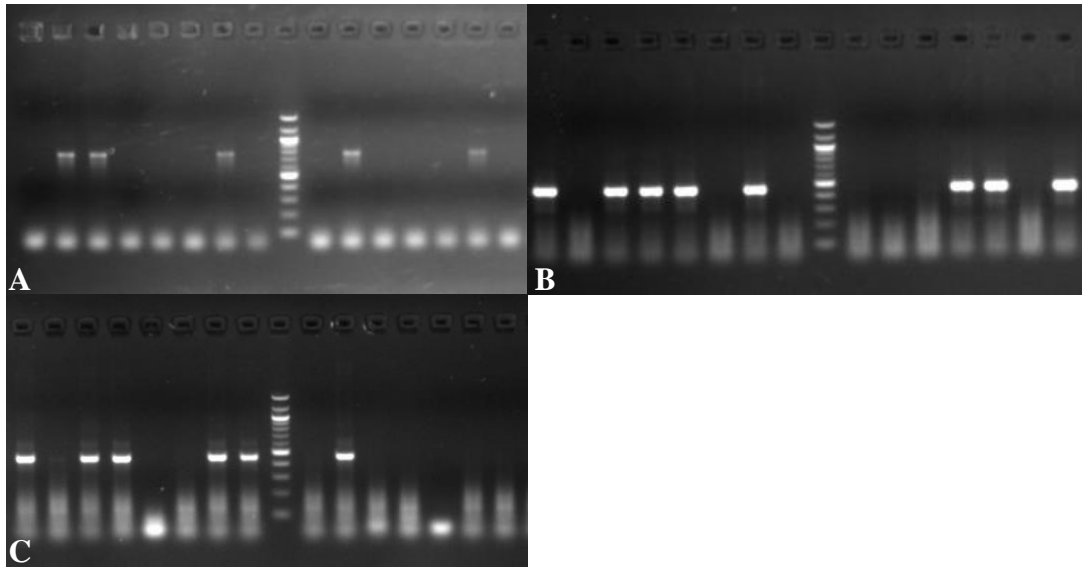
Figure 3.4. Agarose gel electrophoresis of RT-PCR showing single infections of (A) cytorhabdovirus (700 bp), (B) RLMV (452 bp), and (C) RYNV (463 bp) after they have been graft transmitted from the source plant carrying the respective virus into healthy plants. The first five lanes in gel photos are of 'Columbian'; the second five lanes are of 'Cumberland'; and the final five lanes are of 'Cuthbert'.
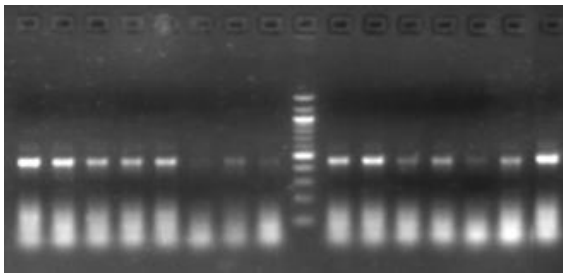
Figure 3.5. Agarose gel electrophoresis of RT-PCR products showing all 'Columbian', 'Cumberland', 'Cuthbert' plants single infected RYNV (463 bp).

Table 3.2. Single and mixed virus combinations used to recreate RLCD-like symptoms in 'Columbian', 'Cumberland', and 'Cuthbert' cultivars.

Single	Double	Triple	Quadruple
BRNV	BRNV, Cytorhab	BRNV, Cytorhab, RLMV	BRNV, Cytorhab, RLMV, RYNV
Cytorhab	BRNV, RLMV	BRNV, Cytorhab, RYNV	
RLMV	BRNV, RYNV	Cytorhab, RLMV, RYNV	
RYNV	Cytorhab, RLMV	BRNV, RLMV, RYNV	
	Cytorhab, RYNV		
	RLMV, Cytorhab		

Figure 3.6. Single infection of cytorhabdovirus in (A) 'Cuthbert' showing chlorosis of the recipient leaf and (B) minor crinkling symptoms seen in 'Cumberland'.
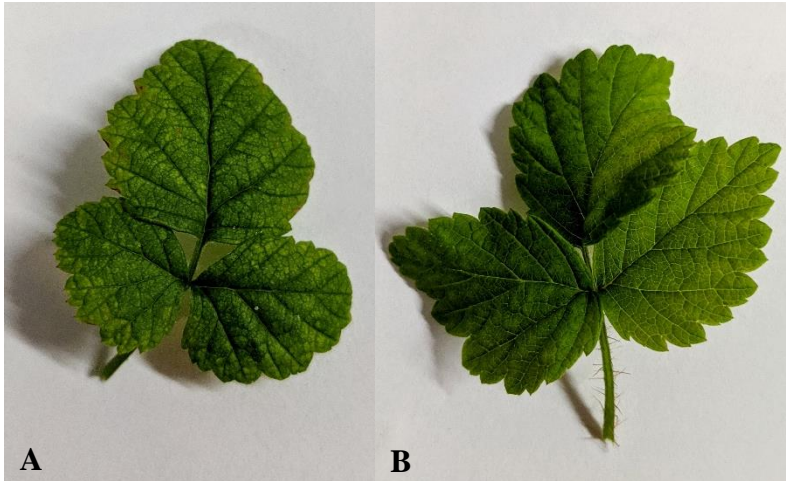
Figure 3.7 Single infection of RYNV in (A) 'Columbian' showing yellowing between minor veins and (B) minor net-like appearance showing in 'Cuthbert'.

Figure 3.8. Red raspberry 'Cuthbert' plant containing RLMV and RYNV. Downward cupping of the leaves can be seen.

Figure 3.9. Triple virus infection containing RLMV, RYNV, and cytorhabdovirus in red raspberry 'Cuthbert' showing symptoms of cupping of leaves, chlorotic spots, and yellowing of veins.

Figure 3.10. Quadruple virus infection containing BRNV, RLMV, RYNV, and cytorhabdovirus in red raspberry 'Cuthbert' showing symptoms of mosaic and crinkling of leaves.

Chapter 4:

Conclusions

Melinda Guzman Martinez

Plant viruses rely on the plant host machinery for survival; some viruses are even able to replicate both within their vector and plant host (Sanfaçon 2017). With more than 30 viruses known to cause disease in *Rubus* spp., many of them do not express viral symptoms in single infections. These viruses are often present in mixed infections in the field that lead to many of the virus diseases of *Rubus* (Martin et al. 2013, 2017). The disease known as raspberry leaf curl disease (RLCD), first reported in 1895 (Stewart and Eustace 1902), has been thought to be caused by a virus complex because of the varying severity of symptoms across different *Rubus* cultivars (Bennett 1930). Despite previous studies performed, the causal agent(s) of RLCD have not been identified (Bolton 1970) yet they still pose a threat to the *Rubus* industry and the requirement for biological indexing restricts plant exports from the U.S. (Gergerich et al. 2015). It has been suggested that a member of the *Luteoviridae* family (OEPP/EPPO 1978) is the causal agent of RLCD based on its' aphid transmission properties.

As part of a three-year effort to assess prominent viruses present in *Rubus,* high-throughput sequencing (HTS) was performed on plants exhibiting RLCD-like symptoms resulting in the detection of black raspberry necrosis virus (BRNV), Rubus yellow net virus (RYNV), two novel viruses belonging to family *Rhabdoviridae,* and a novel *Enamovirus* in the family *Luteoviridae* (Di Bello et al. 2017). These viruses served as possible candidates of the causal agent(s) of RLCD. The novel member of the family *Rhabdoviridae* from native red raspberry in Pennsylvania was selected for characterization since it was present in symptomatic plants in single infections.

The classification of the novel 14,662 bp *Rhabdoviridae* virus as a member of the *Cytorhabdovirus* genus, and a strain of raspberry vein chlorosis virus (RVCV) was supported by genomic and phylogenetic analyses of the amino acid (aa) sequences of all eight viral encoded

proteins, three of which were cytorhabdovirus-specific. Eighty percent aa similarity of the

cytorhabdovirus-specific P' overlapping protein was seen between the RVCV variant Hutton2

(RVCV_Hutt2) and the novel cytorhabdovirus (Identities: $64/80 = 80\%$, Gaps: $0/80 = 0\%$). The

sister taxons of the RVCV clade throughout all the phylogenetic trees were other

cytorhabdoviruses, Alfalfa dwarf virus (ADV) and Strawberry crinkle virus (SCV), as previously

reported (Jones et al. 2019). The RVCV variants Hutton1 (RVCV_Hutt_1) and RVCV_Hutt2

showed a close evolutionary relationship to the novel cytorhabdovirus, with RVCV_Hutt2

consistently sharing more than 77% aa identity of all eight proteins. Both RVCV_Hutt1 and

RVCV_Hutt2 have been isolated in the U.K. and have not been detected in the U.S. but with the

supported taxonomic classification of this virus and because of the high affinity to both variants

of RVCV, the novel virus was determined to be the first isolated U.S. variant of RVCV. RVCV

is known to affect wild and cultivated red raspberries with symptoms of vein-clearing and leaf

distortion (Jones et al. 1977). Detection primers for this virus were created based on the sequence

of the three strains of RVCV and used in *Rubus* survey testing to determine presence of the virus

throughout the U.S. and Canada; this virus was also later used as one of the candidate viruses for

RLCD.

The detection of BRNV, RYNV, the two novel family *Rhabdoviridae* viruses, and the family

Luteoviridae virus in RLCD-like symptomatic plants suggested these virus(es) as possible causal

agent(s) of RLCD. Obtaining single and mixed infections of these viruses in different

combinations through aphid and leaflet graft transmissions within cultivars 'Columbian',

'Cumberland', and 'Cuthbert' were to recreate the symptoms previously described in literature. It

was noted that the symptomology of RLCD was profoundly similar that of potato leafroll disease

(Rankin and Hockey 1922); a disease now known to be caused by a virus in the genus

Polerovirus within the family *Luteoviridae* (Rowhani and Stace-Smith 1979). The use of the viruses in different combinations did not result in RLCD symptoms, theorized because transmissions continued without the leading virus candidate, the enamovirus.

Unfortunately, no current experimental evidence to determine if the enamovirus was indeed a causal agent of RLCD was determined because of the undetectability of the virus throughout the course of the study, despite previously being aphid and graft transmissible (Di Bello et al. 2017). First, the virus was undetectable through RT-PCR testing occurring in late summer and was hypothesized to be in low titer throughout the phloem (Narayanasamy 2005). To institute similar conditions to the last detection of enamovirus, plants were cut down and given a 40-day cold treatment at 4°C (de Capite 1955) then moved back into greenhouse conditions of 16 hours of daylight at 25°C. The RT-PCR testing resulted in no enamovirus detection. Additional plant tissue collected from the USDA-ARS National Clonal Germplasm Repository (NCGR) that formerly tested positive for the virus (Di Bello et al. 2017) also were retested and gave negative results. The consistently negative RT-PCR testing of the enamovirus led to adjustments of the nucleic acid extraction method and RT-PCR protocol (Manual; Bessetti 2007) all of which resulting in no enamovirus detection. The manipulations made throughout the study to detect the enamovirus were unsuccessful.

At this stage, the enamovirus detected through HTS cannot be excluded as a possible causal agent of RLCD. Previous studies have shown that the systemic movements of RNA plant viruses can serve as a population bottleneck reducing the population size (Sacristán et al. 2003). It may be possible that throughout the extractions performed, the enamovirus titer was too low to detect despite seasonal testing, and modifications made to the methods of extraction and RT-PCR protocol. Electron microscopy (EM) has been used in the discovery and description of viruses

since 1939 (Goldsmith and Miller 2009) and can be used as a secondary verification tool of the virus in the plants that previously tested positive for the enamovirus. Ideally, collecting additional plant material showing RLCD-like symptoms from the same location enamovirus was first collected from and testing them with the current enamovirus primers would be a step towards confirming the presence of the virus. Continuing investigation in determining the etiology of RLCD is still required. The same HTS detected viruses still have a possibility of being involved in RLCD. Further investigations using the same cultivars 'Columbian', 'Cumberland', and 'Cuthbert' and large raspberry aphid, *Amphorophora agathonica*, should be used in further studies along with BRNV, RYNV, the novel cytorhabdovirus, and the enamovirus with the addition of the small raspberry aphid, *Aphis rubicola,* as it was used to successfully transmit the virus (Rankin and Hockey 1922).

Previous studies determined that RLCD was transmitted in a persistent manner through the small raspberry aphid, *A. rubicola*, which continued to transmit the virus for up to several days (Bennett 1927). Under field conditions, symptoms of RLCD did not always appear in the first year of infection, but by the second year characteristic and marked symptoms of curl appeared on both young and older plant tissue (Bennett 1927). It was later reported that RLCD showed different symptoms in red and black raspberry which led to the hypothesis that RLCD was caused by two separate persistently transmitted virus strains, where one strain only infected red raspberry and the other infected both red and black raspberry (Bennett 1930).

In conclusion, a novel virus belonging to family *Rhabdoviridae* was characterized as the U.S. variant of RVCV, with low disease incidence surveyed across the U.S. possibly due to changes in vector populations or resistant raspberry cultivars currently grown. The overall transmission

properties of the virus strains described to cause RLCD (Bennett 1930) match those of viruses belonging to family *Luteoviridae* and *Rhabdoviridae*.

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