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Blueberry latent virus: An amalgam of the *Partitiviridae* and *Totiviridae*

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ABSTRACT

A new, symptomless virus was identified in blueberry. The dsRNA genome of the virus, provisionally named Blueberry latent virus (BBLV), codes for two putative proteins, one without any similarities to virus proteins and an RNA-dependent RNA polymerase. More than 35 isolates of the virus from different cultivars and geographic regions were partially or completely sequenced. BBLV, found in more than 50% of the material tested, has high degree of homogeneity as isolates show more than 99% nucleotide identity between them. Phylogenetic analysis clearly shows a close relationship between BBLV and members of the *Partitiviridae*, although its genome organization is related more closely to members of the *Totiviridae*. Transmission studies from three separate crosses showed that the virus is transmitted very efficiently by seed. These properties suggest that BBLV belongs to a new family of plant viruses with unique genome organization for a plant virus but signature properties of cryptic viruses including symptomless infection and very efficient vertical transmission.

1. Introduction

Blueberry (*Vaccinium corymbosum*) production is expanding into areas and environments where it is not native. This expansion has exposed the crop to new pathogens and/or altered blueberry habitat to make it more susceptible to known pathogens leading to the emergence and reemergence of several important diseases including mummy berry (*Monilinia vaccinii-corymbosi*) (Tarnowski et al., 2008), leaf scorch (*Xylella fastidiosa*) (Chang et al., 2009), shock (*Blueberry shock virus*) (Martin et al., 2006a,b), necrotic ring blotch (Blueberry necrotic ring blotch virus) (Brannen, 2008; Martin, personal observation), scorch (*Blueberry scorch virus*) (Wegener et al., 2006) as well as several yet uncharacterized diseases. The study presented in this communication was initiated because of a new disease that has emerged in the Pacific Northwest (Oregon, Washington and British Columbia) of North America. The new disorder, described as blueberry fruit drop, was first observed in British Columbia, Canada in 2000 and in Oregon, United States in 2003. Yield loss approaches 100% in affected bushes, as fruit is aborted when it reaches about 5 mm in diameter, however, the bushes appear to be more vigorous, without obvious foliar symptoms. Symptoms have been observed over several years and the incidence increases within fields providing evidence that a biotic agent causes the disorder. Initial work on the disorder failed to identify any fungi or bacteria in symptomatic bushes (Martin et al., 2006a,b) and therefore the possibility of virus infection was investigated. Immunological tests failed to detect any of the viruses known to infect blueberry in North America and double-stranded RNA (dsRNA) purifications were employed to investigate whether an unknown virus caused the fruit abortion symptoms. More than 10 symptomatic plants were assayed and all contained a ~3.5 kbp dsRNA band. After shotgun cloning and sequencing we determined that the dsRNA molecule belonged to a new virus with genome organization and phylogeny that bridged two major dsRNA virus families. Detection tests were developed and employed to determine whether this virus was associated with the fruit drop disease. The virus was found widespread in symptomatic and asymptomatic plants from Arkansas, Michigan, New Jersey and the Pacific Northwest. In addition to the wide geographic distribution, the virus was found in material belonging to several different cultivars and breeding accessions. Several BBLV-infected, asymptomatic plants were evaluated for a period of three years for fruit drop symptoms and none developed the disorder. In the absence of association between symptoms and virus, the agent was provisionally named Blueberry latent virus (BBLV). The unique genome organization and the number of plants carrying the virus led to a comprehensive characterization of BBLV including the complete genomic sequence of several isolates from Arkansas, Michigan and Oregon. An RT-PCR

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detection protocol was employed to study BBLV distribution in major blueberry production areas in North America, and the partial sequence of over 30 isolates was determined to evaluate virus diversity across blueberry genotypes and geographic regions.

2. Materials and methods

2.1. Plant material

Plant material was collected from several commercial farms in Arkansas, British Columbia, Florida, Georgia, Michigan, New Jersey, Oregon and Washington, and breeding accession lines from Arkansas and Oregon (Suppl. Table 1). To avoid uneven distribution in plants as observed with other viruses in blueberry (MacDonald et al., 1991), four leaves from single bushes were collected in a X pattern and combined before tested by reverse transcription polymerase chain reaction (RT-PCR). For seed transmission studies, 17 seedlings representing three crosses of BBLV-positive parents were tested for the presence of the virus. The possibility that BBLV is a fungal virus was investigated as infected material was surface sterilized, placed in tissue culture and grown on Anderson's woody media (Anderson, 1980). Plants in tissue culture, free of visual fungal or bacterial contamination, were used for virus and endophytic fungi detection with RT-PCR and PCR, respectively.

2.2. Virus purification

Virus purifications were performed as described for the ilarvirus *Blueberry shock virus* (BlShV) (MacDonald et al., 1991) and the carlavirus *Blueberry scorch virus* (BlScV) (Martin and Bristow, 1988). Briefly, for the BlShV protocol, tissue was homogenized in 0.03 M phosphate buffer, pH 8.0, expressed through cheesecloth and centrifuged at 16,000 × *g* for 20 min (low speed centrifugation). The supernatant was adjusted to pH 5.0 using 6 M HCl, then centrifuged as above, the supernatant was collected and polyethylene glycol and NaCl added to 8% and 1% (w/v), respectively. Then after 1 h at 4 °C, the sample was centrifuged again as above, and the pellet resuspended in 1/10 the original volume and stirred overnight at 4 °C. After another low speed centrifugation, the supernatant was subjected to a high speed centrifugation at 200,000 × *g* for 2 h. The pellet was resuspended overnight at 4 °C and after another low speed centrifugation, the supernatant was examined by electron microscopy. For the BlScV protocol, the tissue was homogenized in a borate buffer, pH 8.2, containing 2% (w/v) polyvinyl pyrrolidone-44, 0.1 M EDTA, 0.5% nicotine alkaloid and 0.1% 2-mercaptoethanol. After a low speed centrifugation, the supernatant was centrifuged at 200,000 × *g* for 90 min and the pellets resuspended in 0.01 M phosphate buffer, pH 7.5, subjected to another cycle of low speed and ultra centrifugation, the pellets were then suspended in phosphate buffer and examined by electron microscopy.

2.3. Microscopy

Light microscopy was employed to examine for the presence of endophytic fungi in virus-infected leaf material after clearing and staining of the tissue. Leaves were cleared using a method that has been reported for azaleas, which belong to the same family as blueberries (Linderman, 1973). The tissue was cleared in 5% NaOH for one week, with a change of solution each day. Once cleared the leaves were rinsed with water, transferred to 100% methanol overnight, then stained with 0.01% trypan blue. Leaf pieces were then observed under a stereo microscope for blue staining of fungal cell walls. Thin sections of infected and BBLV-free plant tissue, including pollen, were observed under a Philips CM-12 scanning transmission electron microscope for evidence of cytopathology and/or virus particles.

2.4. Virus characterization

DsRNA was extracted by either the Yoshikawa and Converse (1990) or Tzanetakis and Martin (2008) methods, both of which include RNAse and DNAse digestions steps and subjected to shotgun cloning as previously described (Tzanetakis et al., 2005a). BLASTX and BLASTP searches on the shotgun cloned fragments revealed the presence of several virus-like sequences that were assembled to contigs using CAP3 (Huang and Madan, 1999). Sequence gaps were filled in after development of specific primers in the known sequence and PCR amplification and cloning of the fragments. Genome ends determined using the methodology described in Tzanetakis and Martin (2004). Three additional isolates of the virus were sequenced after RT-PCR amplification and cloning. Virus dsRNA was denatured for 10 min in the presence of 20 mM methyl mercury hydroxide and 2 μM primers BBLV5′RT (5′-ATATATGTATTTTTATTT TCGGACACCGAGGTTC-3′) and BBLV3′RT (5′-ATATATGGATAGTGACACACGTACCGG TGAC-3′). Genomes were amplified as previously described (Tzanetakis et al., 2005b) in two overlapping regions using primers BBLV5′ (5′-GTATTTTTATTTTCGGACACCGAGGTTC-3′) and BBLVmidR (5′-CCGTCTGTATGCTCCTAACAA-3′) and BBLVmidF (5′-CCTTCTTCATT GATGTAATTCTT-3′) and BBLV3′ (5′-GGATAGTGACACACGTACCGGTGAC-3′) that amplify 2106 and 1405 bp fragments of the 5′ and 3′ ends, respectively. The PCR reactions were performed with LA Taq (Takara) according to manufacturer's recommendations and the program consisted of 2 min denaturation at 94 °C, followed by 40 cycles of 30 s denaturation at 94 °C, 15 s annealing at 55 °C and 2 min extension at 72 °C. The fragments were cloned onto pCRII or pCR2.1 vector (Invitrogen) and sequenced at either at the University of Arkansas DNA Resource Center or Macrogen Inc., Seoul, S. Korea. The sequences of four BBLV genomes were deposited in Genbank under accession numbers EF442779 (Oregon) and HM029246–HM029248 (for the Arkansas and the two Michigan isolates, respectively) and represent an at least 3× coverage of the genome. In addition, a region of 1052 bases spanning the overlapping region of the two open reading frames (ORF) of the virus were directly sequenced from PCR products of 33 isolates from Arkansas, British Columbia, New Jersey and Oregon to further investigate the diversity of the virus between different geographic regions and cultivars (Suppl. Table 1). The accession numbers for these isolates are HM756695–HM756727. Protein structural analysis was performed using PSIPRED (Jones, 1999) whereas phylogenetic analysis on the polymerase conserved motifs identified by Marchler-Bauer et al. (2007) was performed using CLUSTALW, using the neighbor-joining algorithm, Kimura's correction and bootstrap consisting of 1000 pseudoreplicates (Thompson et al., 1994).

RNAse protection assays were performed to investigate genome composition (ss or dsRNA), the possible function of the ORF1 product and the presence of an A-tail at the 3′ terminus of the virus. The assays involved grinding of BBLV infected tissue (0.15 g) in 1 ml phosphate buffered saline (PBS) or 0.4 M NaCl containing 1% mercaptoethanol. Genome composition was determined after centrifugation of the extracts for 2 min at 5000 × *g*, and phenol/chloroform clarification of the supernatant. The water soluble fraction was then treated with RNAseA to a final concentration of 0.5 μg/ml for 1 h at 37 °C. The extract was clarified twice with phenol/chloroform and ethanol precipitated. The pellet underwent further purification to eliminate enzymatic inhibitors (Tzanetakis et al., 2007a). Genome protection was investigated as described above except for the initial phenol/chloroform clarification. Material was tested for BBLV and internal control primers for the NADH

dehydrogenase ND-2 subunit (Tzanetakis et al., 2007b) were used in the reactions to confirm RNAse activity. The presence of an A-tail was evaluated by performing reverse transcription on RNAseA-treated BBLV dsRNA (in 0.4 M NaCl) using an oligo dT primer or random nucleotide hexamers.

2.5. Detection

Total RNA was extracted as described previously (Tzanetakis et al., 2007a) from 194 blueberry samples. Reverse transcription was performed using Reverseaid reverse transcriptase (Fermentas) according to the manufacturer's instructions and PCR using Taq polymerase (Genscript). Amplification was performed using primers BBLVdetF (5′-CTGAGGCGGTGAA GCATTATAG-3′) and BBLVmidR (5′-CCGTCTGTATGCTCCTAACAA-3′) and the PCR program that consisted of 2 min denaturation at 94 °C, followed by 40 cycles of 30 s denaturation at 94 °C, 15 s annealing at 55 °C and 1 min extension at 72 °C. The program terminated with 10 min incubation at 72 °C. The presence of fungi, possible carriers of the virus, was determined after DNA extraction using DNAeasy Kit (Qiagen) and primers ITS primers ITS 1/4 (White et al., 1990). *Botrytis cinerea* DNA, extracted as above, was mixed (1:10) with DNA extracted from BBLV-infected blueberry and used as a positive control. For Northern blot analysis, the 257 terminal nt of the virus were amplified using BBLVendF (5′-GATCAGAGGAGGCCAGT ACG-3′) and BBLV3′. The amplicon was labeled using 100 ng of purified PCR template and DIG-High Prime (Roche) according to manufacturer's instructions. Blots were performed using dsRNA, extracted from a British Columbia and an Oregon isolate, essentially as described in Tzanetakis et al. (2009). Briefly, dsRNA was run in a 0.8% agarose gel at 60 V for 3 h, incubated for 30 min in 0.2N NaOH and transferred by capillary action using 0.2N NaOH to Amersham Hybond™-N+ (GE Healthcare) nylon membrane. Washes and incubation with the probe was performed as recommended by the manufacturer (Roche). Blots where incubated in X-ray film (Kodak) and developed and fixed as described in Sambrook (2001).

3. Results and discussion

The viral etiology of blueberry fruit drop, an emerging disorder in the Western United States was investigated and a new virus was detected in a significant number of samples from diseased and symptomless bushes, but not from all samples. Although BBLV was not associated with blueberry fruit drop, its widespread distribution in the primary survey, led to the further characterization of the agent and evaluation of its distribution in blueberry production areas in North America.

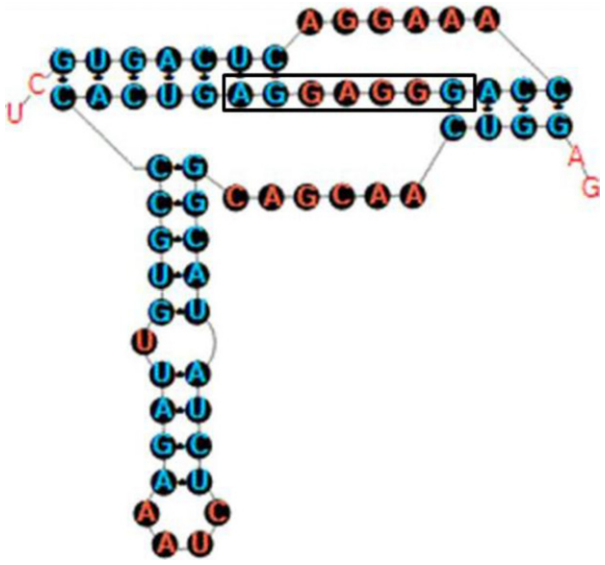

Fig. 2. RNA structural analysis of the area in proximity to the ribosomal frameshift. The boxed area indicates the putative sequence where the ribosomal frameshift takes place.

BBLV genome consists of 3431 nt (A+T: 53%) and encodes two partially overlapping ORFs, similar to the genome organization of Southern tomato virus (STV) (Fig. 1; Sabanadzovic et al., 2009). Untranslated regions are the first 166 and the last 98 bases of the 5′ and 3′ termini, respectively. The first ORF extends to nucleotide 1294, encoding a putative protein of 375 aa with predicted molecular mass of 41 kDa (p41). The protein is most similar to STV p42, Vicia cryptic virus-M p43 (Liu and Chen, 2009) and a putative protein found in *Populus trichocarpa* that probably belongs to a similar virus infecting this host. In all cases, BBLV p41 shares less than 20% identical residues with its three counterparts. Whereas there is no significant primary sequence similarity between the four proteins, structural analysis indicates that they have very similar secondary

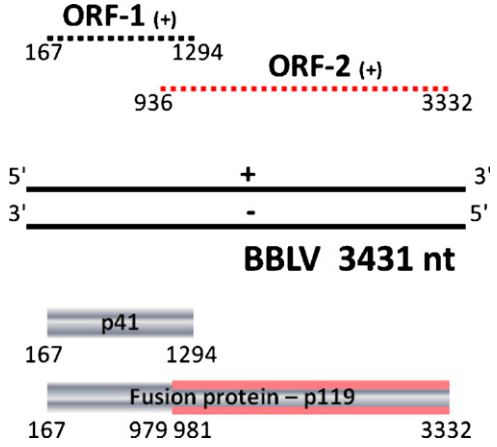

Fig. 1. Genomic representation and putative protein products of Blueberry latent virus (BBLV). Numbers represent the coordinates of the start and stop codons of open reading frames (ORF) and the putative frameshift site that produces for the fusion protein.

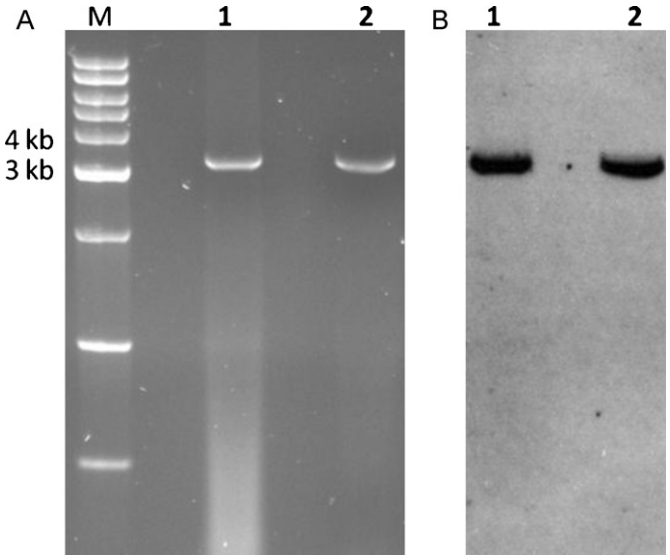

Fig. 3. (A) Agarose gel electrophoresis of double-stranded RNA extracted from two blueberry plants from British Columbia (1) and Oregon (2). M: One-kilobase ladder (Bioline). (B) Northern blot analysis of the gel electrophoresis presented in (A) using a probe against the last 257 bases of Blueberry latent virus.

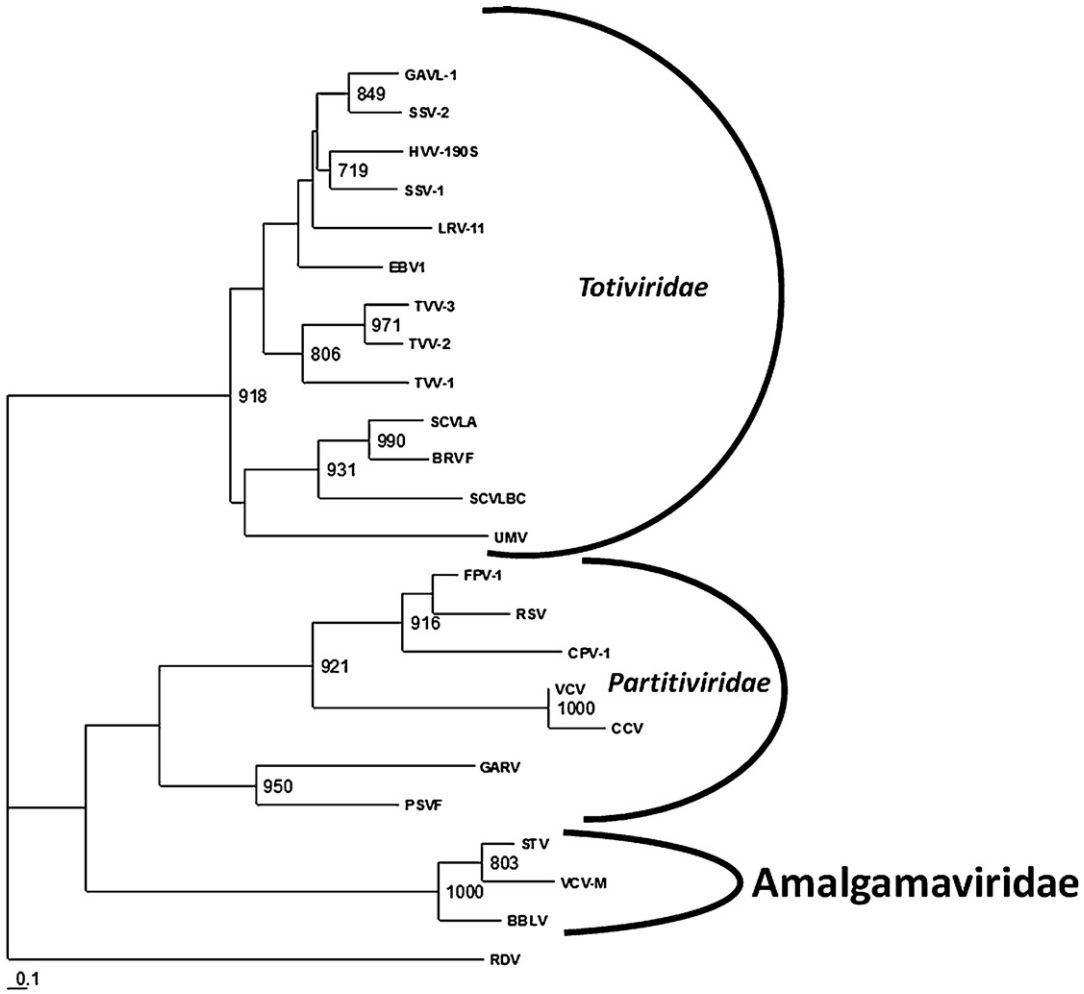

Fig. 4. Phylogenetic analysis of the conserved polymerase motifs (Marchler-Bauer et al., 2007) using Blueberry latent and related viruses. Abbreviations and Genbank accession numbers: Black raspberry virus F (BRVF; NC009890); Blueberry latent virus (BBLV; EF442779); *Carrot cryptic virus* (CCV; FJ550604); Ceratocystis polonica partitivirus (CPV; NC_010705); Eimeria brunetti RNA virus-1 (EBV-1; AF356189); *Fusarium poae virus-1* (FPV-1; NC003883); Gremmeniella abietina RNA virus MS1 (GARV; NC_004018); *Helminthosporium victoriae virus 190S* (HVV-190S; U41345); *Leishmania RNA virus-1* (LRV-1; NC002063); P*enicillium stoloniferum virus F* (PSVF; NC007221); *Rhizoctonia solani virus* (RSV; NC003801); *Rice dwarf virus* (RDV; NC003773); *Saccharomyces cerevisiae virus L-A* (SCVLA; NC003745); *Saccharomyces cerevisiae virus L-BC* (SCVBC; NC001641); Southern tomato virus (STV; NC011591); *Sphaeropsis sapinea RNA virus-1* (SSV-1; NC001963); *Sphaeropsis sapinea RNA virus-2* (SSV-2; NC001964) *Trichomonas vaginalis virus-1* (TVV-1; NC003824); *Trichomonas vaginalis virus-2* (TVV-2; NC003873); *Trichomonas vaginalis virus-3* (TVV-3; NC004034); *Ustilago maydis virus H1* (UMV; NC003823); *Vicia cryptic virus* (VCV; NC007241); Vicia cryptic virus-M (VCV-M; EU371896). The numerical values of the nodes with bootstrap values of less than 70% are not shown, as they are not considered significant. RDV is used as an outgroup. The bars represent 0.1 amino acid changes per site.

structure comprising primarily of α-helices with a noticeable lack of extended β-sheet structures (Suppl. Fig. 1). The overlapping area of the two ORFs extends for 363 nucleotides. In STV, the heptapeptide $GGGAAGA_{984–990}$, was identified as a potential ribosomal slippage site of the fusion protein. In BBLV, a similar heptapeptide ($GGGAGGA_{979–985}$) found in all isolates sequenced, was identified, and, as in the case of STV, a pseudoknot was predicted in the proximity of the site (Fig. 2; Sperschneider and Datta, 2008). The possibility of an alternative expression strategy of the polymerase was investigated by Northern blot analysis. A probe at the 3′ terminus of the virus was used and a single signal corresponding to the genomic RNA was obtained indicating that the virus does not use subgenomic RNA as an expression strategy for ORF2 (Fig. 3). The putative fusion protein is 1054 aa long with a predicted molecular mass of 119 kDa and contains signature polymerase motifs (active, metal binding and RNA binding sites) between residues 586 and 782 (Marchler-Bauer et al., 2007). This area has the highest similarities to other proteins in Genbank exceeding 58% and 52% for the orthologous regions of STV and VCV-M, respectively.

The genome organization of BBLV is similar to that of totiviruses but phylogenetic analysis clearly places BBLV along with STV and VCV-M closer to partitiviruses (Fig. 4), strengthening the notion that viruses of the group belong to a new taxon that may be the 'go between' of partiti- and totiviruses (Sabanadzovic et al., 2009).

The genome of BBLV is unusual given that almost all plant viruses encode for a polymerase, coat and movement proteins. This property leads to the obvious question whether this is a plant virus or based on phylogeny, a fungal virus. We used several approaches to test the possibility that BBLV is a fungal virus, although fungal viruses, because of mycelial incompatibility, are very host-specific even to the isolate level (Anagnostakis, 1982; Ghabrial, 1998; Chiba et al., 2009) and BBLV was detected in areas found several thousand miles apart. Notwithstanding, light and electron microscopy was used to search for endophytic fungi without success. In addition, sterilized plants were grown in tissue culture in the presence of antibiotics, but the virus was still easily detectable by RT-PCR (data not shown). In all cases, PCR using the universal ITS1/4 primers (White et al., 1990) failed to yield any amplicons, whereas a *Botrytis cinerea* isolate used as control in the experiments did yield amplicons of the expected size.

Partiti- and totiviruses have spherical virions. The genome organization and the putative fusion protein expressed via a +1

ribosomal frameshift lead to the assumption that BBLV forms particles similar in form and function to totiviruses, with the polymerase being part of the virion as a gag-pol-like fusion. Structural analysis of ORF1 (Jones, 1999) showed that the putative protein does not fold into a jelly-roll β-barrel, a common coat protein structure among isometric viruses, but it is more similar to the *Penicillium stoloniferum virus S*, a partitivirus (Ochoa et al., 2008) with small stretches of β-barrel sheets near the C-terminus of the protein (Suppl. Fig. 1). Attempts using purification protocols optimized for spherical and elongated viruses in blueberry failed to provide any particles as assessed by the absence of any bands in the ultracentrifuge gradient or under the electron microscope. In addition, no virions or abnormal structures were observed in pollen or leaf EM thin sections. Those results are similar to those obtained for STV and indicate that BBLV is probably present in low titer in plants.

RNAse protection assays were used to determine the genome properties and the possibility that the genome is protected, presumably by the product of ORF1. When there was not protein removal before RNAse treatment, virus amplicons were obtained under all conditions. When there was phenol/chloroform extraction before RNAse treatment, only the NaCl-extracted material gave an amplicon (Fig. 5). In parallel experiments, purified BBLV dsRNA could only withstand RNAseA treatment in 0.4 M NaCl but not PBS (data not shown). Those results indicate that the genome is protein protected and can only withstand the RNAse treatment under high salt concentrations but not in a weak salt buffer like PBS, indicative of the dsRNA composition of the genome.

Attempts to amplify the 3′ terminus of the virus without A-tailing using an oligo dT primer were unsuccessful. Those results were further validated after performing the reverse transcription with either a dT primer or random nucleotide hexamers and tested by PCR using the standard detection primers BBLVdetF/BBLVmidR. There were no amplicons obtained when priming the reverse transcription with an oligo dT primer, in contract to the random nucleotide hexamers (Fig. 5).

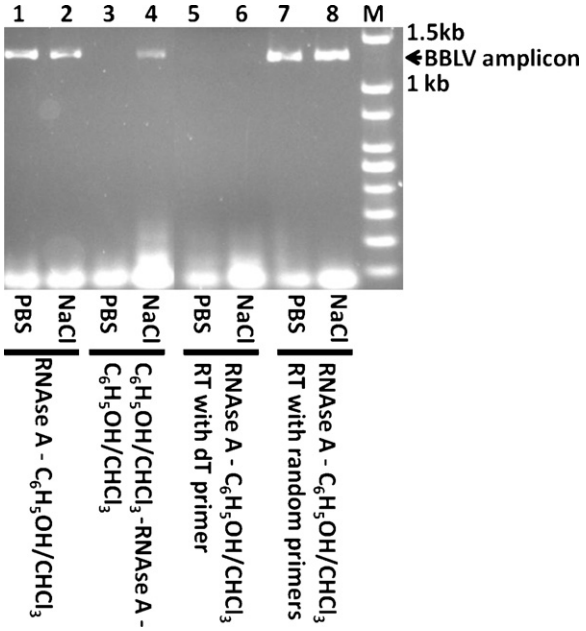

Fig. 5. Analysis of Blueberry latent virus (BBLV) genome properties. Lanes 1–4: RT-PCR amplification to evaluate whether the genome is protected. Both PBS and NaCl yielded amplicons (1.3 kb) when treated with RNAse before protein removal. Only the 0.4 M NaCl treatmentgave an amplicon when proteins were removed before RNAse treatment, an indication that the genome is protected and resistant to nuclease degradation; lanes 5–8. Presence of A-tail at the end of BBLV genome: RT-PCR products of BBLV-infected material using an oligo dT primer or random hexanucleotide primers in the reverse transcription step. Amplicons were obtained only when using random hexanucleotide primers suggestive of the absence of an A-tail at the end of the genome. M: One-kilobase ladder (Bioline).

The lack of a movement protein led to the assumption that BBLV is moving within the plant with cell division as in the case with plant cryptic viruses or the product of ORF1 has multiple functions including that of a movement protein. One of the features of cryptic viruses is the high percentage of seed transmission (Mink, 1993). We tested this with BBLV using seedlings that were obtained from three different crosses of all infected parents: (1) 'Georgiagem' × 'Aurora'; (2) 'Ozarkblue' × selection ORUS 380-2 and (3) 'Powderblue' × selection MS614. RT-PCR amplification showed that 5/5, 6/6 and 6/6 seedings, respectively were infected with BBLV. Several of the amplicons were sequenced and all were BBLV-specific. The results indicate that BBLV is transmitted with seed at a very high rate.

Cryptic viruses do not cause symptoms and are transmitted by cell division. These properties lead to the assumption that they are not under the genetic pressure to evolve and avoid host defense mechanisms. We examined the molecular evolution and ecology of the virus by testing the presence and obtaining the complete sequence of four isolate and partial sequences of 33 isolates from diverse areas, environments and blueberry accessions in North America. The four genomes were obtained for areas separated by several hundred miles and isolated from different cultivars (Duke – AR, Bluecrop – MI and OR). Comparisons between the four genomes showed minimal differences with only 5 bases differing between genomes (99.85% nt identity). In a larger scale we investigated the diversity of the virus between a large number of cultivars and breeding accessions. We targeted the junction of the two ORFs to determine the validity of the putative frameshift site and all sequenced isolates had 100% identity in the putative slippery hexanucleotide. In addition, this area does not contain conserved enzymatic motifs that may be under negative evolutionary pressure and should give a better representation of virus diversity. The sequence results indicate that the virus is very homogeneous as the identities between isolates were over 99% (Suppl. Table 2).

There are new viruses found continually. In most cases the new viruses are found in a small number of specimens and are usually associated with a disorder or disease. Our quest to understand blueberry fruit drop disease led us to the discovery of BBLV, a virus that is prevalent in all major blueberry producing areas of the United States, found in more than 50% of the samples tested (Suppl. Table 1) and is very efficiently seed transmitted. The virus does not cause any obvious symptoms in single infections but its role, if any, in mixed infections is to be determined.

BBLV genome properties place it in a new virus taxon with members recently discovered in tomato, vicia, redbud, rose and rhododendron (Tzanetakis, unpublished results; Sabanadzovic, in press). With the previous work and the work presented here it is obvious that a new virus family needs to be formed and the name Amalgamaviridae is proposed as the viruses of the group seem to be a amalgam between the *Partitiviridae* (protein phylogeny) and the *Totiviridae* (genome composition).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2010.09.020.

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