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Complete sequence and genetic characterization of *Raspberry latent virus*, a novel member of the family *Reoviridae* ☆,☆☆

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ABSTRACT

A new virus isolated from red raspberry plants and detected in the main production areas in northern Washington State, USA and British Columbia, Canada was fully sequenced and found to be a novel member of the family *Reoviridae*. The virus was designated as *Raspberry latent virus* (RpLV) based on the fact that it is symptomless when present in single infections in several *Rubus* virus indicators and commercial raspberry cultivars. RpLV genome is 26,128 nucleotides (nt) divided into 10 dsRNA segments. The length of the genomic segments (S) was similar to those of other reoviruses ranging from 3948 nt (S1) to 1141 nt (S10). All of the segments, except S8, have the conserved terminal sequences 5'-AGUU--GAUUAC-3'. A point mutation at each terminus of S8 resulted in the sequences 5'-AGUA--GAUUAC-3'. Inverted repeats adjacent to each conserved terminus as well as stem loops and extended pan handles were identified by analyses of secondary structures of the non-coding sequences. All segments, except S3 and S10, contained a single open reading frame (ORF) on the positive sense RNAs. Two out-of-frame overlapping ORFs were identified in segments S3 (ORF S3a and S3b) and S10 (ORF S10a and S10b). Amino acid (aa) alignments of the putative proteins encoded by the main ORF in each segment revealed a high identity to several proteins encoded by reoviruses from different genera including *Oryzavirus*, *Cypovirus*, and *Dinovernavirus*. Alignments of the polymerase, the most conserved protein among reoviruses, revealed a 36% aa identity between RpLV and *Rice ragged stunt virus* (RRSV), the type member of the genus *Oryzavirus*, indicating that these two viruses are closely related. Phylogenetic analyses showed that RpLV clusters with members of the genera *Oryzavirus*, *Cypovirus*, *Dinovernavirus* and *Fijivirus*. These genera belong to the subfamily *Spinareovirinae* which includes reoviruses with spiked core particles ('turreted' reoviruses). In addition, two nucleotide binding motifs, regarded as 'signature' sequences among turreted reoviruses, were also found in RpLV P8, suggesting that RpLV is a novel dicot-infecting reovirus in the subfamily *Spinareovirinae*.

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1. Introduction

The family *Reoviridae* contains viruses with genomes composed of multiple (9–12) segments of linear double-stranded RNA (dsRNA). This family is the most diverse of the dsRNA virus families, as it includes species isolated from a wide range of hosts including mammals, birds, fish, insects, arachnids, marine protists,

crustaceans, fungi and plants (Brussaard et al., 2004; Hillman et al., 2004; Mertens et al., 2005).

More than 75 virus species have been classified as members of 12 well-established genera: *Orbivirus*, *Orthoreovirus*, *Rotavirus*, *Coltivirus*, *Aquareovirus*, *Cypovirus*, *Fijivirus*, *Phytoreovirus*, *Oryzavirus*, *Seadornavirus*, *Mycoreovirus* and *Idnoreovirus* (Mertens et al., 2005). The genera *Cardoreovirus*, *Dinovernavirus*, and *Mimoreovirus*, have been established recently and include viruses isolated from crabs, mosquitoes and marine protists, respectively (Attoui et al., 2005, 2006; Zhang et al., 2004).

Currently, there are three genera of plant reoviruses; *Fijivirus*, *Oryzavirus* and *Phytoreovirus*. The genus *Fijivirus* includes members with 10 segments and is divided into 5 groups based on host, vectors and serological properties (Isogai et al., 1998a; Mertens et al., 2000). Members include viruses that infect monocots and insects (Hunter et al., 2009; Nakashima et al., 1996; Noda and Nakashima,

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1995). The genus *Oryzavirus* includes two members; *Rice ragged stunt virus* (RRSV) and *Echinochloa ragged stunt virus* (ERSV), also comprised of 10 segments (Chen et al., 1989; Holmes et al., 1995; Shikata et al., 1979; Uyeda and Milne, 1995; Yan et al., 1992). *Phytoreovirus* includes viruses with 12 segments that infect both monocots and dicots with *Wound tumor virus* (WTV), *Rice dwarf virus* (RDV) and *Rice gall dwarf virus* (RGDV) being the recognized members of the genus (Black, 1945; Mertens et al., 2000; Nuss and Dall, 1990). The recent characterization of *Tobacco leaf enation virus* (TLEV) (Picton et al., 2007; Rey et al., 1999), and *Homalodisca vitripennis reovirus* (HoVRV), isolated from the hemipteran *Homalodisca vitripennis* (Stenger et al., 2009), have expanded this genus now to include insect viruses.

In 1988, a novel reovirus was identified in red raspberry in Washington State, USA. Partial sequence was obtained and used to design detection primers (Jelkmann and Martin, 1989). The virus has been detected commonly in the main raspberry production areas in the Pacific Northwest (PNW), especially in areas where raspberry crumbly fruit disease is prominent (Murant et al., 1974; Martin, 1999).

The new reovirus does not cause symptoms when grafted onto raspberry indicator plants and thus, the name *Raspberry latent virus* (RpLV) is proposed. The characterization of RpLV has been necessary in order to elucidate possible interactions with other viruses and its implication in crumbly fruit and other raspberry diseases.

2. Materials and methods

2.1. Type isolate source

Root cuttings from 'Meeker' raspberry were obtained from production fields in northern Washington State, USA. The cuttings were originated from 3- and 4-year-old plants, some of which had shown crumbly fruit symptoms in previous seasons. A total of 90 root cuttings were planted, grown in 1 gal pots and maintained in a greenhouse under standard conditions (12 h day light at 25 °C). It is important to point out that we did not know the status of each cutting at the time of planting, i.e. whether each cutting came from a symptomatic or asymptomatic plant.

Plants were tested for all reported *Rubus* viruses either by PCR or ELISA. Most plants were found to have mixed infections containing at least two of the following viruses: *Raspberry bushy dwarf virus* (RBDV), *Raspberry leaf mottle virus* (RLMV) and RpLV. A few plants were identified that contained single infections with RBDV, RLMV or RpLV. One of the plants infected only with RpLV was selected as our 'type isolate' and used for subsequent cloning and sequencing.

Our 'type isolate' plant was also used to leaf-graft inoculate the virus onto different virus-free raspberry cultivars (i.e. 'Malling Landmark', 'Norfolk Giant' and 'Munger') which are commonly used as standard *Rubus* virus indicators.

2.2. Detection

RpLV was detected by RT-PCR using primers designed from genomic segments S1, S3 and S8. Primer set S1 (F: 5'-CCACGCCCAACCTCCAAATAA-3'; R: 5'-ACCCCGTCTCCCATCTGC-3') amplifies a 465 bp fragment of the putative B spike protein gene located on the core capsid. Primer set S3 (F: 5'-GGCTGGGTACTGATCTTGG-3'; R: 5'-GCTAATCCCCGCCATC-3') amplifies a 268 bp portion of the viral RdRp gene, and primer set S8 (F: 5'-CACCGCCCAACCACAAC TCT-3'; R: 5'-CCTCGTGCTCGCTCTCCTCATA-3') amplifies a 547 bp fragment of the putative non-structural protein P8 gene.

Total RNA or viral dsRNA was heat denatured at 95 °C for 5 min and reverse transcribed by Superscript Reverse Transcrip-

tase (Invitrogen Corporation, Carlsbad, CA, USA) in a total volume of 25 µl. After denaturing the transcriptase, 2.5 µl of cDNA was immersed in a total of 25 µl of PCR mix. The PCR reactions were performed as follows: one cycle of initial denaturation at 94 °C for 4 min, 40 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 55 °C, extension for 45 s at 72 °C and a final extension for 10 min at 72 °C. PCR products were analyzed by electrophoresis through ethidium bromide-stained 2% agarose gels in 1× Tris-Phosphate-EDTA (TPE) buffer.

All primer sets were equally effective for detection of RpLV from total RNA and viral dsRNA isolated from leaf tissue (data not shown).

2.3. Double-stranded RNA extraction

Twenty grams of fresh leaf tissue was powdered in liquid nitrogen and dsRNA was extracted using phenol/STE buffer, and recovered by cellulose CF-11 chromatography, as previously described (Morris and Dodds, 1979). The dsRNA was treated with ribonuclease T1 from *Aspergillus oryzae* (Sigma-Aldrich Corp. USA) and DNase 1 from bovine pancreas (Sigma-Aldrich Corp.) under high salt conditions to remove single-stranded RNA and dsDNA, respectively. After the digestions, the dsRNA was once again purified on CF-11 cellulose columns and precipitated with EtOH.

2.4. Electrophoretic analyses

RpLV dsRNA was pelleted, dried and analyzed at room temperature by electrophoretic separation on a 1.5% agarose gel (AGE), stained with ethidium bromide and visualized with a UV light. In order to separate RpLV segments S1–S3, which co-migrated as a single band in agarose gels, dsRNA was separated on a 10% polyacrylamide gel in Tris-glycine running buffer at pH 8.3 for 8 h at 4 °C.

2.5. cDNA synthesis, cloning and sequencing

Total and gel-extracted dsRNA (the latter was done several times to obtain clones from S6) were used as template for the reverse transcription (RT) reactions. The universal random primer 5'-GCCGGAGCTCTGCAGAATTCNNNNNN-3' (Froussard, 1992) was used to generate cDNA following the methodology described by Tzanetakis et al. (2005). Briefly, a mixture containing the purified dsRNA and primers was denatured with CH₃HgOH at room temperature for 30 min. Then, a second mix, containing reverse transcription buffer, DTT, dNTPs, and Superscript III RT (Invitrogen Corp.) was added to the denaturant mix and incubated for 50 min at 50 °C. The reaction was terminated by heating at 75 °C for 10 min. The RNA template was digested with RNase H (Invitrogen Corp.), and then a PCR reaction containing the anchor primer 5'-GCCGGAGCTCTGCAGAATTC-3' was performed to amplify the cDNA products. The PCR products were cloned into the pCR4-TOPO TA vector (Invitrogen Corp.) and sequenced on an ABI 3730XL DNA analyzer by Macrogen (Seoul, Korea).

2.6. Deep sequencing

Illumina sequencing technology was also implemented to advance the sequencing of RpLV genome. cDNA was first generated using the primer 5'-TTATCATCTGAGTGAGGTACCNNNNNN-3' from dsRNA as described above (bolded bases represent KpnI restriction site). Random fragments were PCR-amplified using the anchor primer 5'-TTATCATCTGAGTGAGGTACC-3' and subsequently digested by KpnI at 37 °C for 24 h. From this point, sample preparation was performed as recommended by Illumina. Briefly, amplified DNA was randomly fragmented to less than 800 bp by

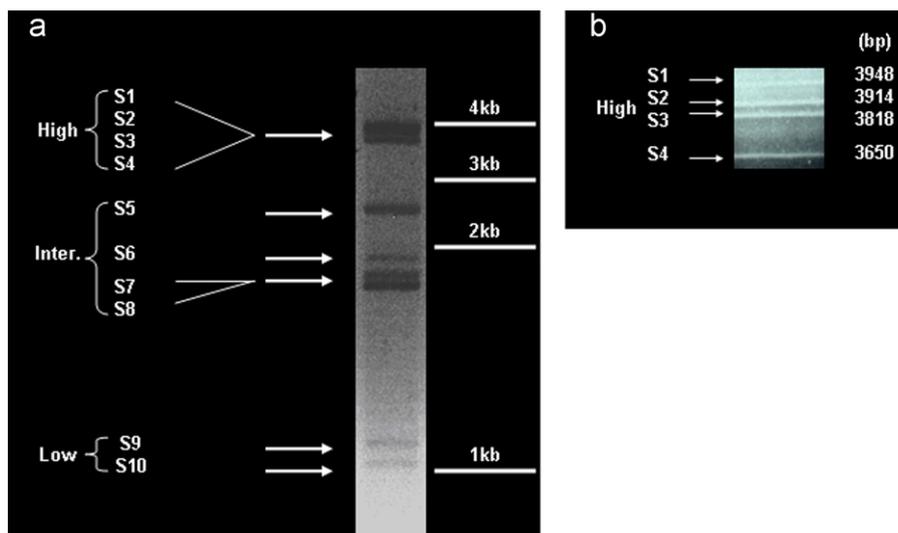


Fig. 1. Electrophoretic profile of dsRNA genomic segments of *Raspberry latent virus*. Arrows indicate the location of each dsRNA band. (a) Complete genome separated in 1.5% agarose gel. High, Inter., and Low refer to segments in groups of high, intermediate and low molecular mass, respectively. Note that band S6 is much less intense than S5, S7 and S8. (b) Segments S1–S4 separated in 10% PAGE.

a nebulization technique which generates dsDNA pieces that are blunt-ended or have 5'/3' overhangs. An end repair reaction was then carried out that removed the 3' overhangs and filled in the 5' overhangs, followed by the addition of adenosine to the 3' ends.

Adapters were then ligated to the ends of the DNA fragments and PCR-amplified using primers complementary to the adapters. Lastly, the PCR products were hybridized to the flow cell of the Illumina platform and sequenced.

2.7. Genome assembly

Sequences obtained from regular cloning were assembled into contigs using the assembly program CAP3 (Huang and Madan, 1999). The contigs were compared to the database at the National Center for Biotechnology Information (NCBI) GenBank using BLASTx and aligned with the nearest relative in order to design primers to fill the gaps. After getting the ends of each segment (see below) open reading frames (ORFs) were identified by the ORF finder software at the NCBI website (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>).

The Illumina sequencing yielded about 3 million paired-end reads of 36 bases each which were processed and assembled into contigs using Velvet (Zerbino and Birney, 2008) and CodonCode Aligner (CodonCode Corp., Dedham, MA, USA). Contigs obtained from these programs were aligned to those obtained from conventional cloning by CAP3 and ClustalW (Thompson et al., 1994).

2.8. Terminal regions

The 5' and 3' termini were obtained by poly(A) tailing of the 3' ends of dsRNAs, as described (Isogai et al., 1998b), followed by RT-PCR using primers developed to known sequences near the ends and oligo dT. To reconfirm the first (5'-end) and last (3'-end) bases of each segment, a 3' blocked DNA oligonucleotide was ligated to both 3'-ends of the dsRNAs followed by RT-PCR using its complementary primer and specific primers for each end as described (Attoui et al., 2000).

2.9. Sequence comparison and phylogeny

ClustalW was used for alignment of amino acid sequences corresponding to the polymerase of representative members of each

genus in the *Reoviridae* (the genus *Idnoreovirus* was not included in the analysis because the RdRp sequence has not been determined to date). Alignment performed by ClustalW was also used for phylogeny analyses. The evolutionary distances were computed by MEGA software version 4.1 (Tamura et al., 2007) using Maximum parsimony and Neighbor-Joining methods with the bootstrap test (500 replicates) (Felsenstein, 1985; Saitou and Nei, 1987). GenBank accession numbers of the amino acid sequences used in this analysis are provided in Fig. 3.

3. Results

3.1. Electrophoretic analysis of double-stranded RNAs

Double-stranded RNA from RplV singly-infected plants was separated by 1.5% agarose gel electrophoresis (AGE) and 10% PAGE. The AGE profile was similar to those of other reoviruses and revealed the presence of multiple segments arranged in three groups, based on their molecular masses (high, intermediate and low) (Fig. 1). The PAGE profile allowed for the separation of three distinct dsRNA segments that co-migrated as a single band in AGE. S1–S4 form the high molecular mass group, the intermediate class contains segments S5–S8 and the low molecular mass group includes segments S9 and S10 (Fig. 1). S6 was found to be less intense compared to the rest of the intermediate and high bands. However, semi-quantitative RT-PCR using primers developed to this segment were as effective as detection primers from other genomic segments when total RNA was used as template (data not shown).

The electrophoretic pattern was observed consistently in dsRNA preparations from different raspberry cultivars that had been leaf-graft-inoculated with RplV (i.e. 'Malling Landmark', 'Norfolk Giant' and 'Munger'), ruling out the presence of additional dsRNAs associated with RplV or belonging to other viruses.

3.2. Sequence analysis

The 10 RplV segments were sequenced completely and deposited in the GenBank under accession numbers (HQ012653–HQ012662). The complete genome consisted of 26,128 nucleotides with segments ranging from 3948 bp (S1) to 1141 bp (S10) (Table 1). Reovirus segments normally encode a

Table 1
Genome organization of *Raspberry latent virus* ('X' denotes the G + C content average).

RNA segment	Segment size (nt)	G + C (%)	ORF position	Protein size (aa)	Protein molecular weight (kDa)	Accession numbers
S1	3948	42.6	30–3860	1276	143.1	HQ012653
S2	3914	43.9	65–3820	1251	140.6	HQ012654
S3	3818	42.6	a. 45–3761 b. 506–1468	1238 320	140.6 35.3	HQ012655
S4	3650	41.6	22–3585	1187	134.9	HQ012656
S5	2563	41.3	109–2265	718	80.4	HQ012657
S6	1996	41.5	21–1928	635	72.8	HQ012658
S7	1957	43.7	34–1890	618	68.4	HQ012659
S8	1936	39.8	53–1858	601	67.9	HQ012660
S9	1205	42.9	121–1125	334	36.8	HQ012661
S10	1141	46.7	a. 12–1040 b. 269–871	342 200	38.8 22.2	HQ012662
	X=	42.7				

single open reading frame (ORF) which spans almost the entire length of the segment. However, there have been several cases where reovirus segments encode more than one ORF (Bodelon et al., 2001; Mattion et al., 1991; Soo et al., 1998; Suzuki et al., 1996; Upadhyaya et al., 1998).

For RplV, all segments except S3 and S10 contain a single ORF. Non-coding regions (NCR) range from 11 to 120 bp for the 5' ends and 57 to 298 bp for the 3' ends (Table 1). Additional out-of-frame overlapping ORFs were identified in segments S3 (ORF S3b) and S10 (ORF S10b). The ORF locations and predicted size for the putative proteins are shown in Table 1. Examination of the context of the initiator codons in RplV ORFs S3b and S10b indicated that ORF S3b has a weak context (AGCAUGC) compared to the one in S10b (ACGAUGC). However, studies have demonstrated that initiator codons flanked by an adenine in position –3 regardless of the nucleotide at +4, can be considered strong initiators (Kozak, 1984, 1991). Leaky scanning mechanisms have been shown to operate for initiation at multiple AUG initiator sites in polycistronic segments in reoviruses (Suzuki et al., 1996; Bodelon et al., 2001).

Using BLASTx default parameters, several RRSV proteins showed the highest amino acid identity to the putative RplV orthologs including ORFs S1, S2, S3a, S4, S5, S8, S9 and S10a. Therefore, the putative protein functions of the respective ORFs are proposed based on the homology to the RRSV counterparts (Table 2).

S6 and S7 ORFs did not show significant amino acid sequence identity to RRSV orthologs. However, when the search was limited to dsRNA viruses only, the mammalian orthoreovirus-2 (mORV2) core protein, namely μ 2, returned as the closest hit (33% aa identity; e-value: 0.13) for the region between nt 301 and 474 of RplV S7.

Likewise, the region between nt 60 and 305 of RplV S6 showed 28% identity to the putative P8 structural protein of RRSV when the search was limited to dsRNA viruses. This was further analyzed by using PSIPRED software (McGuffin et al., 2000) to predict secondary structures of RplV ORF S6 and RRSV P8. The analysis revealed sim-

ilar folding patterns between the two proteins (Supplemental Fig. S1), suggesting that these proteins may have diverged at a faster rate compared to other proteins. Similar folding patterns were not observed between RplV ORF S7 and mORV2 μ 2 when the same analysis was performed (data not shown).

BLASTp (PSI- and PHI-BLAST) analyses also failed to identify orthologs for RplV ORFs S3b and S10b. It is noteworthy that RRSV segment 4 (orthologous to RplV S3) also contains an internal ORF (RRSV ORF 4b) located at positions similar to that of RplV S3b (Upadhyaya et al., 1998). However, comparison of folding patterns between the two ORFs did not reveal significant similarities (data not shown).

Two subfamilies: *Spinareovirinae* and *Sedoreovirinae* have been created for reoviruses based on their core structure. The former includes the 'turreted' viruses, which have protrusions (spikes) located atop the core particle, and the latter includes the 'non-turreted' viruses, without spikes resulting in particles with a smooth surface appearance (Miyazaki et al., 2008; Reinisch et al., 2000). Recent observations have shown that viruses in the turreted group contain two nucleoside triphosphate (NTP) binding motifs (Nibert and Kim, 2004). These motifs were also identified in the S8 ORF of RplV (Supplemental Table S2). Studies conducted on mORV2 μ 2 protein (which also contains these two NTP binding motifs) have suggested that reovirus proteins containing these two motifs should be minor components of the inner core capsid and possibly involved in the viral transcription complex (Nibert and Kim, 2004).

3.3. Analysis and comparison of terminal sequences

Two characteristic features located in the terminal regions have been observed in reoviruses; genus-specific conserved terminal sequences and segment-specific inverted repeats. Both features are believed to have evolved as recognition signals for replication and packaging of reoviruses

Table 2
Putative functions of *Raspberry latent virus* (RplV) proteins based on amino acid homology to *Rice ragged stunt virus* (RRSV). N.A. indicates no orthologous protein identified.

<i>Rice ragged stunt virus</i> (RRSV) genome organization		<i>Raspberry latent virus</i> (RplV) homology to RRSV cognate proteins		
Segment	Protein function	Segment	Query coverage (%)	Amino acid identity (%)
S1	P1: spike protein B	S1	98	36
S2	P2: inner core capsid	S4	93	26
S3	P3: core capsid	S2	81	27
S4	P4A: polymerase P4B: unknown	S3a N.A.	94 N.A.	36 N.A.
S5	P5: capping enzyme	S5	39	27
S6	Pns6: movement/NTP-bind	N.A.	N.A.	N.A.
S7	Pns7: non-structural	S8	89	24
S8	P8: outer capsid	S6	12	28
S9	P9: spike protein	S10a	51	24
S10	Pns10: non-structural	S9	68	23

Table 3

Conserved terminal sequences and inverted repeats (shaded areas) located at both termini of *Raspberry latent virus* (RpLV). Note the point mutations at S8 termini (bolded nucleotides).

RpLV terminal sequences			
RNA segment	5' end		3' end
	Inverted repeats		
S1	AGUUUUUUCUC		GAGAAUAGCGAAUAC
S2	AGUUUUUACG		CGUAAAAGCGAAUAC
S3	AGUUUUAAUCC		GGAUUAGCGAAUAC
S4	AGUUUAUUCUUU		AAAGAAUAGCGAAUAC
S5	AGUUUUUCCACG		CGUGGGAAAAAGUGAAUAC
S6	AGUUUUAAUCCUA		UAGGAUUAAGCGAAUAC
S7	AGUUUAAUCC		GGAAUUAGCGAAUAC
S8	AGU AAAA UCUCUA		UAGAGGUUUGCGAUUAC
S9	AGUUAAAACC		GGUUUUGUGAAUAC
S10	AGUUAAAUCCA		UGGAAUUUGCGAAUAC
Consensus	AGU ^{u/a}		G ^{g/w} GA ^{a/b} UAC

and possibly other segmented RNA viruses (Anzola et al., 1987; Gaillard et al., 1982; Kudo et al., 1991; Ni et al., 1996).

Analysis of RpLV terminal sequences revealed the conserved tetranucleotide AGUU at the 5' termini and hexanucleotide GAAUAC at the 3' termini for all segments except S8, where a point mutation at both ends (5'-AGUA--GAUUAC-3') was observed (Table 3). Comparison of the 5' termini revealed conservation of the first three nucleotides (AGU) between members of the genera *Cypovirus*, *Fijivirus* and *Dinovernavirus*. The triplet starting at the second base of RpLV (GUU, except for S8) resembles those of the genera *Orbivirus*, *Seadornavirus*, and *Aquareovirus*. The last three bases at the 3' termini (UAC) were identical to those of some species in the genus *Orbivirus*, a more distantly related group (Supplemental Table S3).

RpLV terminal sequences also contain 6–11 nt-long inverted repeats adjacent to the conserved termini of each segment (Fig. 2 and Table 3). The analysis of the secondary structure of non-coding sequences revealed the presence of stem loops and extended pan handles that start at the complementary sequences near the ends of each segment (Supplemental Fig. S4). These features were shown

to be involved in segment packaging and template recognition for initiation of minus-strand RNA synthesis inside the core particle of rotaviruses and are hypothesized to function in a similar fashion for other reoviruses (Guglielmi et al., 2010).

The inverted repeats of RpLV contain a high A + U content (above 70%) (Table 3). Based on the suggested functions for these inverted motifs (Anzola et al., 1987; Guglielmi et al., 2010; Patton and Spencer, 2000), this high A+U content may indicate the need for a weak and transient interaction between the complementary sequences during segment encapsidation and RNA synthesis.

3.4. Phylogenetic analysis

Maximum parsimony (MP) and neighbor-joining (NJ) methods were used to construct phylogenetic trees using the complete amino acid sequences of the RdRp from representative species of each recognized genus in the *Reoviridae*. Both methods yielded identical trees. Fig. 3 shows a radial MP-tree where RpLV clustered with members of the group of the turreted reoviruses, particularly with those belonging to the genera *Oryzavirus*, *Cypoviruses* and *Dinovernavirus*.

Further analysis of the full-length amino acid sequence of the RdRp revealed a 36% identity between RpLV and RRSV. The most conserved region was located between aa 420 and 796 of RpLV (438–808 for RRSV) (Supplemental Fig. S5a). Several other reoviruses belonging to the genera *Cypovirus*, *Dinovernavirus* and *Fijivirus* also showed aa identities ranging from ~20% to 28% (data not shown). Multiple sequence alignment of the RdRp aa sequences from members of the genera closest to RpLV revealed highly conserved regions distributed along the entire sequence, suggesting a common origin for these viruses (Supplemental Fig. S5b).

Analysis of the inner core capsid protein, also conserved in reoviruses (Attoui et al., 2002), supported the close relatedness between RpLV and members of the genera *Oryzavirus* and *Cypovirus*, as the closest relatives, and *Dinovernavirus* and *Fijivirus* as more distant viruses (data not shown). Accordingly, the G + C content of RpLV was found to be 42.7% (Table 1) similar to those of cypoviruses (43%) and the oryzavirus RRSV (44.7%). Whereas members of the *Dinovernavirus* and *Fijivirus* genera have G + C contents significantly lower, i.e. 34.4% for *Aedes pseudoscutellaris dinovernavirus* and 34.8% for *Nilaparvata lugens fijivirus*.

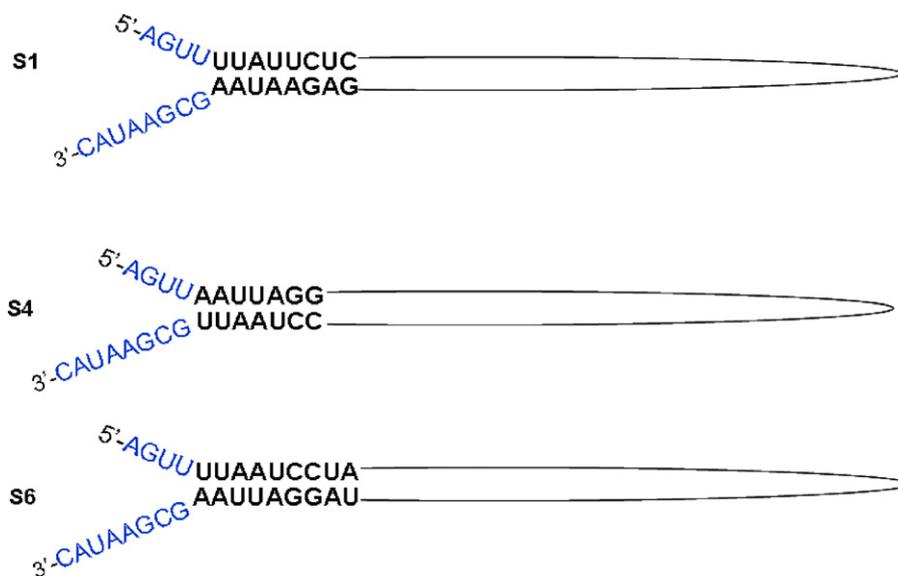


Fig. 2. Inverted repeats of three of the 10 segments of *Raspberry latent virus*. Bolded letters in black represent perfect complements located adjacent to the conserved terminals in blue. (For interpretation of the references to color in the figure caption, the reader is referred to the web version of the article.)

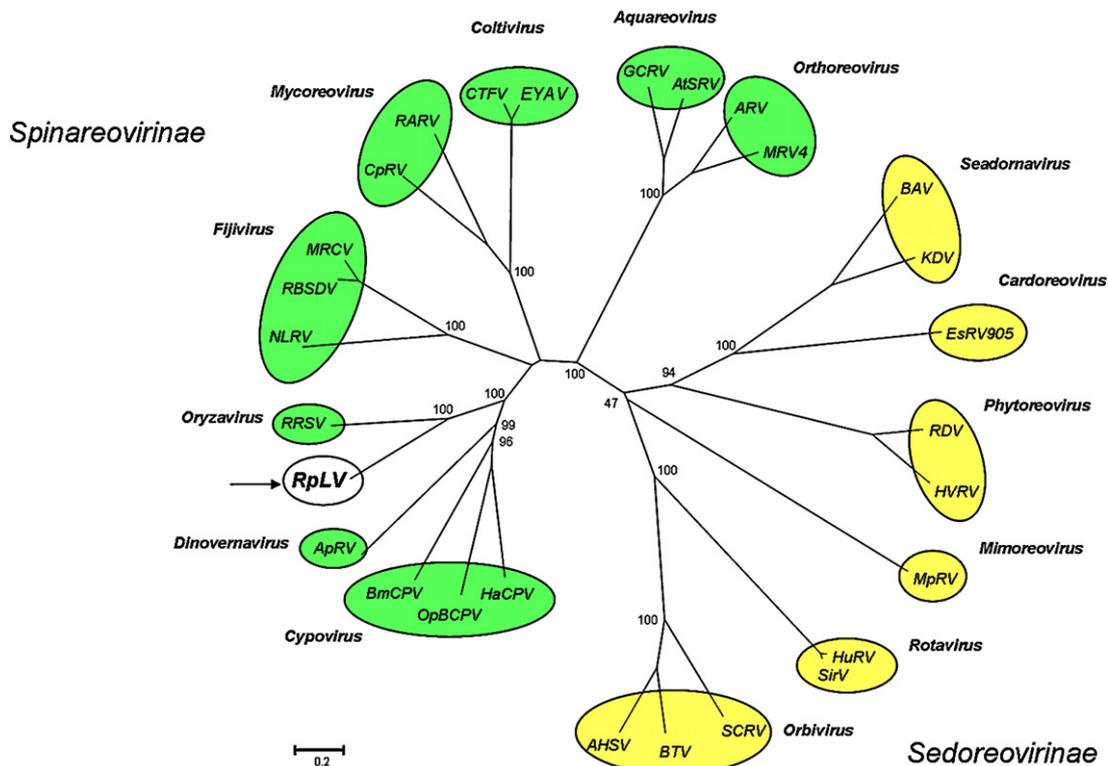


Fig. 3. Neighbor-joining tree of the RdRp amino acid sequences from representative members of the *Reoviridae*. Bootstrap percentage values are indicated on each branching site. The arrow indicates the location of *Raspberry latent virus* (RpLV) in the tree. Abbreviations and Genbank accession numbers: RpLV: HQ012655; RRSV: *Rice ragged stunt virus*, NP.620541; HaCPV-5: *Heliothis armigera cypovirus* 5, YP.001883321; BmCPV1: *Bombyx mori cypovirus* 1, AAR88092; ObRV: *Operophtera brumata cypovirus*, ABB17221; ApRV: *Aedes pseudoscutellaris reovirus*, YP.443936; RBSDV: *Rice black streaked dwarf virus*, NP.620452; MRCV: *Mal del rio cuarto virus*, AAO73182; NLRV: *Nilaparvata lugens reovirus*, BAA08542; CpRV: *Cryphonectria parasitica mycoreovirus-1*, AAP45577; RARV: *Rosellinia anti-rot virus*, BAC98431; CTFV: *Colorado tick fever virus*, NP.690891; EYAV: *Eyach virus*, AAM18342; GCRV: *Grass carp reovirus*, AAG10436; AtSRV-TS, *Atlantic salmon reovirus*, ABO32573; ARV: *Avian orthoreovirus*, ABF82230; MRV-4: *Mammalian orthoreovirus 4*; RDV: *Rice dwarf virus*, NP.620544; HVRV: *Homalodisca vitripennis reovirus*, YP.002790884; BTV: *Blue tongue virus*, ADD62087; AHSV: *African horse sickness virus*, AAC40586; SCRv: *St Croix river virus*, AAG34363; HuRV: *Human rotavirus*, ACV73799; SiRV: *Simian rotavirus*, P22678; MprV: *Micromonas pusilla reovirus*, YP.654545; BAV: *Banna virus* NP.694469; KDV: *Kadipiro virus*, NP.694468; EsRV905: *Eriocheir sinensis reovirus*, AAT11887.

4. Discussion

RpLV was first isolated from raspberry in 1988 and has been found at high incidence in the main production areas in the northern Washington State, USA and British Columbia, Canada. The suggested name derives from the fact that RpLV is symptomless in single infections in standard *Rubus* virus indicators or in the red raspberry cultivars tested. However, RpLV is implicated in a new virus complex responsible for causing severe crumbly fruit in red raspberries (Quito-Avila et al., 2009). The high incidence of mixed virus infections in red raspberry plants containing RpLV, *Raspberry bushy dwarf virus* (RBDV) (Daubeny et al., 1982) and *Raspberry leaf mottle virus* (RLMV) (Jones, 1982; McGavin and MacFarlane, 2010; Tzanetakis et al., 2007) in production areas where raspberry crumbly fruit is most severe has led to the speculation that RpLV plays a role in this disease. In order to elucidate possible interactions between RpLV and other raspberry viruses, the complete characterization of RpLV was necessary.

Partial sequence showed that RpLV was closely related to *Rice ragged stunt virus* (RRSV), the type member of the genus *Oryzavirus* (Holmes et al., 1995; Shikata et al., 1979; Yan et al., 1992). Here we report the complete nucleotide sequence of RpLV and highlight important genetic features showing that RpLV is indeed a new member of the family *Reoviridae* and constitutes the first reovirus reported to infect *Rubus* and one of only a few that are known to infect dicots.

Reoviruses have been classified based on morphological and genetic features. Two important criteria considered for classification purposes to the genus level are the amino acid identity above

30% in the polymerase, and the presence of conserved terminal nucleotide motifs (Anzola et al., 1987; Mertens et al., 2000, 2005).

The aa alignment of the polymerase between RpLV and RRSV showed an identity of 36%. This places the viruses in the same genus. However, the analysis of the conserved terminal motifs of RpLV revealed a complete lack of conservation with members of the *Oryzavirus* (RRSV and ERSV), which share the terminal oligonucleotide sequences 5'-GAUAAA and GUGC-3' (Yan et al., 1992, 1994). The first three nucleotides at the 5'-end of RpLV (AGU) are shared among members of the genera *Cypovirus*, *Fijivirus* and *Dinovernavirus*, which are also phylogenetically related to RpLV. The three terminal nucleotides (UAC) show conservation with their counterparts of members of the genus *Orbivirus*, a well-characterized non-turreted reovirus genus that includes species transmitted by different insects (Mertens et al., 2005). Another feature is the conservation of the first and last nucleotides in some genera. RpLV, along with *Dinoverna*-, *Fiji*-, and *Cypovirus* have an adenosine as the first nucleotide whereas members of the *Orbi*-, *Seadorna*-, *Aqua*-, *Ortho*-, *Oryza*-, *Mimo*-, and *Phytoreovirus* have a guanosine. The last cytosine appears to be conserved across most reovirus species, manifesting the evolutionary linkage between species in this family and suggesting a possible monophyletic origin (Supplemental Table S3).

Despite the high aa identity in the RdRp between RpLV and RRSV, the complete lack of conserved terminal sequences between these two viruses, and other features discussed below, may indicate that RpLV represents a new genus of plant reoviruses that infects dicots. To our knowledge, aquareoviruses and orthoreoviruses, having an aa identity of 42% in the RdRp, have constituted the only case

where reoviruses with such a high aa identity (above 30%) in the RdRp are placed in two separate genera (Attoui et al., 2002). The conserved terminal motifs may also differ between members of the same genus as is the case with mycoreoviruses (Suzuki et al., 2004) and cytopoviruses (Rao et al., 2003). The inverse, species with the same terminal motifs that belong to different genera, has also been observed, i.e. *Reptilian orthoreovirus* (RRV) and *Aquareovirus C* (AQR-C) (Mohd Jaafar et al., 2008).

In addition, analyses of the non-coding regions of RplV revealed a pattern that is consistent for each segment. This includes uniformity of the conserved termini, inverted repeats adjacent to each conserved terminus and the predicted secondary structures formed by non-coding regions (Fig. 2; Supplemental Fig. S4).

As in the case of aquareoviruses and orthoreoviruses (42% aa identity in the RdRp), other parameters must be considered rather than basing virus classification solely on the amino acid identity of the polymerase (Attoui et al., 2002). One of these parameters is the host range of the virus. *Oryzaviruses* have only been reported to infect monocots whereas RplV has been isolated from raspberry, a dicot.

The similarity between RRSV ORF S8 and RplV ORF S6 was lower compared to those observed for RplV ORFs S1, S2, S3a, S4, S5, S8, S9 and S10a (Table 2). RRSV P8 has been suggested to be a component of the outer capsid. As such, it may be involved in vector transmission along with other capsid proteins, i.e. P1 and P9. The analysis of secondary structures revealed similar folding patterns for RRSV ORF S8 and RplV ORF S6 (Supplemental Fig. S1), indicating that the two proteins may have evolved from a common ancestor and such a low similarity between them may be vector-related. RRSV is persistently transmitted by the brown planthopper *Nilaparvata lugens* and other *Nilaparvata* spp. (Hibino, 1996); whereas preliminary transmission studies failed to transmit RplV using several hopper species commonly found in *Rubus*. However, we cannot rule out this possibility until RplV vector is identified.

RRSV orthologs were not identified corresponding to RplV ORF S7. Instead, a short alignment region (33% aa identity; e-value: 0.13) was found between this sequence and mORV2 μ 2 protein when BLASTx searches were limited to dsRNA viruses only. The comparison of the secondary structures between these two proteins failed to identify similar folding patterns, minimizing the possibility of function similarities between them. Interestingly, members of the genera *Aquareovirus* (i.e. *Grass carp reovirus*) and *Cypovirus* (i.e. *Dendrolimus punctatus cypovirus*) also show a very short region with low similarity to mORV2 μ 2 protein (Quito-Avila, personal observation). However, the alignments for each of these viruses are located at different regions of the μ 2 protein, and belong to proteins with different functions. In addition, RplV P8 but not P7 shares the two 'signature' motifs also found in mORV2 μ 2 (Supplemental Table S2). These findings obscure the possibility of an evolutionary linkage of the mammalian orthoreovirus μ 2 protein and other reovirus proteins.

The two 'signature' motifs have been found in all reoviruses that belong to the subfamily *Spinareoviridae* ('turreted' reoviruses). Proteins containing such motifs are speculated to have NTP-binding functions and be involved in the viral replication complex as minor components of the inner core capsid (Nibert and Kim, 2004). These 'signature' motifs were identified in RplV P8 and its ortholog RRSV P7 (Supplemental Table S2). Interestingly, recent studies demonstrated that RRSV P6 but not P7 was involved in NTP binding (Shao et al., 2004) and also in virus cell-to-cell movement (Wu et al., 2010). Furthermore, RRSV P7 was reported to be a non-structural protein (Pns7) with no NTP-binding activity (Upadhyaya et al., 1997).

We were unable to identify a RRSV P6 orthologous protein in RplV. We hypothesize that RplV S7 may encode a protein with viral movement functions similar to that of RRSV P6, and the lack

of homology between the two proteins might be related to the different host systems in which each virus operates, i.e. monocots for RRSV and dicots for RplV. It has been reported that movement proteins (MPs) play a significant role in host specificity in some closely related viruses. Mise et al. (1993) demonstrated that the monocot-adapted *Brome mosaic virus* and the dicot-adapted *Cowpea chlorotic mottle virus* were not able to systemically infect their hosts when the MPs were exchanged between the two bromoviruses. Furthermore, RplV ORF S7 has a high serine (12%) and threonine (8%) content. Proteins with these characteristics are usually O-glycosylated and involved in interactions with cell walls (adhesins) (Zhou and Wu, 2009). At this point, however, we can only speculate about the putative functions of RplV P7 and its evolutionary divergence from RRSV P6 until experimental data is obtained.

RplV ORF S3b and ORF S10b did not show homology to any of the RRSV ORFs. In addition, the predicted secondary structures for these two proteins did not reveal similar patterns to any of the proteins reported in data bases (data not shown), making difficult to hypothesize about their possible functions. Despite the similarity in positions and size of the predicted proteins encoded by RplV ORF S3b and RRSV ORF S4b, the functions for these two proteins seem to be different as evidenced by the lack of homology and secondary structure similarities.

Two methods have been described by Uyeda et al. (1998) for isolation of three important reoviruses: RDV, RBSDV and RRSV. Several trials using both methods failed to yield RplV particles from infected raspberry plants, hampering the analysis of the virion morphology. However, the presence of conserved NTP-binding domains and the phylogenetic clustering of RplV with turreted viruses suggest that RplV belongs to the subfamily of *Spinareovirinae* (Appendix A Supplemental Table S2; Fig. 3).

Modifications to the described methods, as well as cytology studies by electron microscopy are needed to elucidate the virion structure and any cytoplasmic inclusions associated with RplV.

In summary, comparisons between RplV and RRSV suggest that the two viruses have different genetic features i.e. terminal sequences, inverted repeats and the absence of RRSV homologs for RplV segments S3b, S7 and S10b, which may have emerged as responses to the type of host and transmission mechanisms. The 36% aa identity in the polymerase between the two viruses may justify that RplV be placed in the genus *Oryzavirus*. However, at the time this paper was being prepared, another reovirus with identical terminal sequences to RplV was isolated from a different dicotyledonous host in Southeastern United States and is now being characterized by Dr. Sead Sabanadzovic at Mississippi State University (Sabanadzovic, personal communication). Comparison of the RdRp sequences indicated that the two viruses are very closely related but yet distinct species. This finding adds more evidence for a possible new genus for the classification of reoviruses isolated from dicotyledonous hosts that contain the conserved terminal sequences 5'-AGUU and GAAUAC-3'.

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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.2010.11.008.

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