Comparison of the Coat Protein Gene of Raspberry Bushy Dwarf Virus from Raspberry and Blackberry

by

Crystle J. Chamberlain

A Thesis Submitted to Oregon State University Bioresource Research Program

In partial fulfillment of requirements for the Bachelors of Science in Bioresource Research

Presented May 31, 2002
Commencement June 2002
Raspberry bushy dwarf virus (RBDV) infects red raspberry and black raspberry worldwide. Infection causes reduced vigor and drupelet abortion leading to crumbly fruit and reduced yields. While this crumbly fruit can still be used in juice, jam, and preserves, it cannot be used for the more valuable fresh and individually quick frozen (IQF) markets. In 1997, RBDV was first detected in blackberry plants in Aurora, Oregon. When grafting, infected blackberry scions were able to transmit RBDV to healthy raspberries, and infected raspberry scions were able to transmit the virus to healthy blackberries. Sequencing of cloned coat protein genes of the NWREC RBDV isolates revealed little to no difference between the RBDV isolates from raspberry and blackberry. The consensus coat protein gene of the NWREC RBDV is different from that of RBDV isolates from France, Canada, Russia and Scotland. The lack of variability among coat protein genes of RBDV from raspberry and blackberry suggests that mutation in the coat protein did not cause the recent appearance in blackberry.
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Approved:

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6-6-02

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6/6/02

Bioresource Research Director: Dr. Kate Field

6-7-02

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.

Crystle J. Chamberlain

6-11-02

Date
Acknowledgments

I thank Dr. Robert Martin for supervision and support throughout this entire project. I thank Dr. Jennifer Kraus for guidance in lab work, companionship, and daily patience. I also thank everyone in the Martin lab for their time, energy and support.
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Introduction

The Pacific Northwest is the largest producer of blackberries and raspberries in the world. In 2000, 36,070 million pounds of blackberries, primarily ‘Marion’, on 6,140 acres were produced in Oregon (Oregon Agricultural Statistics Service). The goal of the growers is to produce a high quality berry that is sold in the individually quick frozen (IQF) berry market and as purée. IQF berries are sold for a higher price than berries sold to make juices and jams; thus, a higher yield of “perfect” berries is extremely important.

Raspberry bushy dwarf virus (RBDV) infects both *Rubus idaeus* L. (red raspberry) and *R. occidentalis* L. (black raspberry). RBDV infection leads to reduced yields as well as drupelet abortion leading to crumbly fruit in red raspberry (Martin, 1998). While this crumbly fruit can still be used in juice, jam and preserves, it cannot be used for the valuable IQF market. Loss of yield and IQF fruit due to RBDV infection can cost growers $1,000-$3,000 per acre due to reductions in fruit quality and size. In addition, replanting a field of raspberries can cost $3,500 per acre and fields are out of production 1.5 years each time they are replanted (Martin, personal communication).

Historically, RBDV has not been observed in blackberries (*Rubus L.*) (Murant, 1987). In 1997, however, ‘Marion’ and a number of other trailing blackberry cultivars at the North Willamette Research and Extension Center (NWREC) in Aurora, Oregon were found to be infected with RBDV. Martin and Strik (2002, in press) found that RBDV also reduces yields, fruit size and drupelet number in blackberry.
RBDV is classified in the genus *Idaeovirus* (Jones *et al.*, 1996). RBDV can be transmitted horizontally (to plants in the same generation or throughout a field) via pollen and vertically by seed to next generation. It can also be transmitted mechanically to herbaceous host. The virions are semi-spherical, and not enveloped (Jones *et al.*, 1996). RBDV contains a bi-partite single-stranded RNA genome. The RNA-1 of this genome contains one open reading frame (ORF) that encodes a polymerase and a second overlapping but out-of-frame ORF encoding the 2b protein (Wood *et al.*, 2001). The RNA-2 has two ORFs encoding the movement protein at the 5' end and the coat protein at the 3' end. The coat protein is expressed from a sub-genomic RNA, RNA-3 (Natsuaki *et al.* 1991)(Fig. 1a).

A possible cause for the appearance of RBDV in blackberry is an increased overlap in bloom periods of raspberries and blackberries due to new cultivars and cultural practices that extend the fruiting seasons of these crops. Pollen carried by bees can transmit RBDV (Murant 1987). This simple overlap could be what has allowed RBDV to make the jump from raspberries to blackberries. Alternatively, RBDV may have infected blackberries years ago and simply spread so slowly that it was not detected until now. RBDV appears to spread more slowly through blackberry fields than through red raspberry fields (Martin & Strik, 2000). Another possibility is that the RBDV in blackberries represents a new strain of the virus. Mutations in the movement protein, coat protein, as well as other viral genes that resulted in altered host range have been identified (Yasushi Okinaka *et al.*, 2001). To address the possibility that the RBDV of blackberry is a variant strain, we tested for graft-transmissibility of RBDV from blackberry and raspberry to healthy raspberries and blackberries. We also
cloned, sequenced and compared the coat protein genes from RBDV infected blackberry plants of various cultivars and RBDV infected raspberry plants.
Materials and Methods

Sample Collection

Samples from eight different *Rubus* hosts were collected from the Oregon State University North Willamette Research and Extension Center (NWREC) in Aurora, Oregon in spring, summer and autumn of 2001 (Table 1). Samples of an RBDV infected ‘Favorite’ raspberry from France and an RBDV infected blackberry from an Oregon grower were included in this study. These plants were maintained in a greenhouse. Approximately two grams of rapidly growing tissue was collected from each plant, stored at 4°C and processed within 5 days. Samples were tested by a Triple Antibody Sandwich – Enzyme Linked ImmunoSorbent Assay (TAS-ELISA) for the presence of RBDV (Martin 1998). All reagents were used at 100ul per well in Nunc flat-bottom microtiter plates, except for the blocking step, which was 200ul per well. Coating antibody, purified polyclonal IgG, was diluted in coating buffer (carbonate pH 9.6). Plates were coated for 2 to 6 hours at room temperature. Plates were then blocked with phosphate-buffered saline (PBS) containing 0.05% Tween-20 and 0.1% nonfat dried milk powder (blocking buffer) for 1 hour at room temperature. Leaves were homogenized (approximately 1:40, wt/vol) in blocking buffer containing 2% polyvinylpyrrolidone (mol wt 44,000) and 100ul samples loaded onto the duplicated plates and incubated overnight at 4°C. After washing 3 times with half strength PBS, monoclonal antibodies in the form of culture fluid diluted 1:200 in blocking buffer was combined with goat anti-mouse (IgG + IgM, Sigma Chemical Co., St Louis, MO) alkaline phosphatase conjugate at a dilution of 1:5000 and added to the plates and incubated at 26°C for 2 to 3 hours. Substrate (p-nitrophenyl phosphate, Sigma
Chemical Co., St. Louis, MO) at 0.5mg/ml in 10% diethanolamine, pH 9.8, was added and the plates incubated at room temperature. Absorbance of each well at 405nm was read in an ELISA plate reader (Molecular Devices, Sunnyvale, CA) after 1-4 hours and again after overnight incubation at room temperature. Reactions were considered positive if the A405 values were greater than 5 times the values obtained for healthy controls and greater then 0.1 (Martin, 1998).

**Transmission to *Chenopodium quinoa* Willd.**

RBDV infected tissue was homogenized with a mortar and pestle in a 0.05M phosphate buffer, pH 7, containing 2% PVP. Four-week-old *C. quinoa* plants were dusted with 600 mesh carborundum and each leaf was rubbed with the homogenate. Plants were rinsed with water 30 minutes later and maintained in a greenhouse with 14 hours of daylight.

**RNA Extraction**

RNA was extracted from samples of raspberry, blackberry and *C. quinoa* that tested positive for RBDV, using the Qiagen RNeasy® Plant Mini Kit (Chatsworth, CA) according to the manufacturer’s protocol.

An aliquot of the purified RNAs were used immediately in a reverse transcription reaction. The remainder of the samples was snap frozen in liquid nitrogen and stored at -70°C.

**Reverse Transcription and Polymerase Chain Reaction**

The reverse transcription (RT) reaction contained 10 ul of 5X buffer, 50 U RNaseOUT, 5 ul of 0.1 M DTT, 2.5 ul of 10 mM dNTP’s, 21 ul water, and 1 ul Superscript II (Invitrogen, Carlsbad, CA) in a 50 ul reaction. Five ul of RNA was used
as template and the RT reaction was primed with 10 pmoles of the oligonucleotide RC2 (CACAATTTGTGAGATAAACGGC) located 3′ of the coat protein gene (Fig. 1a). The entire reaction mix was placed in the thermocycler at 42°C for 1 hour.

Primers were synthesized by Invitrogen or the Center for Gene Research and Biotechnology (CGRB) at Oregon State University. Briefly, 4 ul of the RT reaction product was placed in a 40 ul PCR reaction containing 4 ul of 10X buffer, 2.4 ul of 1.5 mM MgCl₂, 0.8 ul of 10 mM dNTP’s, 2 U of Taq (Gibco BRL), 21 ul water, 8 pmoles primer FC1 (CTTAGTTGTTAATGCATTGAATGC), and 8 pmoles primer RC2. This reaction was placed in the thermocycler (RoboCycler® Grad ient 96 Temperature Cycler, Stratagene, La Jolla, CA) at 94°C for 4 min followed by 30 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 2 min, then ending at 72°C for 4 min. A 10 ul sub-sample from each PCR reaction was electrophoresed through a 2% agarose gel in TPE buffer (containing 100 ng/ml of ethidium bromide), at 65V for 1 hour, viewed under UV light, and photographed. Molecular weight standards BST E I cut λ DNA (Invitrogen) were used as markers. If no product was visible, 1ul of the first PCR reaction was used as a template with the nested primers FC2 (GAATGCATATGACACATAG) and RC1 (GTGGGCATATCTCACAATTTG) in a second PCR reaction. These primers are located just inside primers FC1 and RC2 but still outside the coding region of the coat protein gene (Fig. 1b).

Cloning

PCR products were cloned into the plasmid vector pCR 2.1 using a TOPO TA Cloning® kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol.
After isolation the plasmids were used to transform DH5α cells and plated on LB Agar containing 75 mg/ml Kanamycin and 40 mg/ml X-Gal. Plasmids were purified using the eppendorf Perfectprep® Plasmid Mini preps (Hanover Park, IL) according to the manufacturer’s recommendations. The plasmids were digested with Eco RI and double digested with Eco RI, Sal I to identify inserts that contained the Sal I site in the coat protein gene (Fig. 2a)

Sequencing

Plasmids were sequenced using Applied Biosystems capillary 3100 Genetic Analyzer, at the CGRB. Forward and reverse M13 primers and primers CAACGAGATGAAGGAGAGGT and GCTGTGAGGTCATTGAAGGT internal to the coat protein gene were used for sequencing (Fig. 3). Sequences were assembled using Staden software (Staden, Beal & Bonfield, 1998). A consensus sequence was determined for each isolate and sequences were aligned using PILEUP (Genetics Computer Group, Wisconsin Package version 10.3.). Phylogenetic analysis was performed with Vector NTI (Vector NTI Suite 7.0 including Xpression NTI) software, using the neighbor-joining method (Saitou & Nei, 1987).

Grafting

To test for graft-transmissibility of RBDV, canes of infected plants were collected from NWREC and placed in water to force budding. These canes were used as donors along with the RBDV infected blackberry and ‘Favorite’ raspberry maintained in a greenhouse. Healthy blackberries and raspberries were the recipients in an approach graft.
Four to six weeks after grafting, leaf samples were tested by ELISA to determine if RBDV was transmitted from infected blackberry and raspberry to healthy raspberries and blackberries respectively.
Results

Most of the coat protein sequences collected from NWREC (blackberry and raspberry), were identical and formed the consensus sequence (Fig. 4). Isolate Q, from a 'Jerrell' blackberry showed one nucleotide difference from the consensus at position 412. This is a base change of an adenine to a guanine resulted in the change of a lysine to glutamic acid in the amino acid sequence (Fig. 5). Isolate U, from an 'Adrienne' blackberry showed one nucleotide difference located at position 312. This is a base change of a thymine to a cytosine that had no effect on the amino acid sequence.

A comparison of the NWREC consensus and database sequences of RBDV coat protein genes showed that the NWREC coat protein sequence was nearly identical to that of the M strain, originally isolated from black raspberry (Table 2, Table 3). RBDV isolates from Scotland (D1, D200) Canada (Can, Can-S) and Russia (RB) showed between 1 and 28 nucleotide differences from the NWREC consensus resulting in differences among the amino acid sequence of the coat proteins of between one and five residues (Table 2, Table 3). Isolate F1, from France, was found to be very similar to the D1 isolate from Scotland. Phylogenetic trees also show a relationship between isolates D1 and F1 at both the nucleotide and amino acid level (Fig. 6, Fig. 7). There also is a close relationship between the NWREC consensus, the Q and U isolates, and the M strain.

In the graft-transmission study, six out of twenty-one combinations of grafts tested were positive for RBDV. Positive grafts from blackberry donors onto raspberry recipients include, ORUS 1122-1, 'Olallie', and the Suavie Island blackberry all grafted
onto ‘Meeker’ red raspberry, and ‘Silvan’ grafted onto ‘Amity’ red raspberry. They also include a ‘Kotata’ blackberry grafted onto a ‘Favorite’ raspberry and a ‘Meeker’ raspberry grafted onto a ‘Favorite’ raspberry used as a control.

The cultivar ‘Willamette’ contains the Bu genes that confer natural resistance to RBDV. Only the RB strain of RBDV has been shown to infect ‘Willamette’. In this study, none of the ‘Willamette’ graft recipients tested positive for the virus, thus indicating that the strain of RBDV present in blackberry in Western Oregon is not the RB strain found in Europe.
Discussion

The coat protein genes of all NWREC samples were nearly identical to each other regardless of whether they were from raspberry or blackberry. Therefore, the recent appearance of RBDV in blackberries was not caused by mutation in the coat protein gene.

Isolates of RBDV have been assigned to three categories based on *Rubus* host range and serology (Jones *et al.*, 2001). The three categories are: 1) the resistance breaking strain (RB), a natural variant of RBDV that is able to overcome resistance conferred by the *Bu* gene present in some red raspberry cultivars; 2) group B, best characterized by isolate M, a serological variant from black raspberry; and 3) group S, including the Scottish type isolate D200, the type culture described by Barnett and Murant (1970). The consensus NWREC coat protein gene sequence was nearly identical to that of strain M, which places the NWREC isolates in RBDV group B.

The differences found among the isolates are best illustrated in the analysis of the phylogenetic trees. At the nucleotide and amino acid level, there is a relationship between isolates D1 and F1. Also, at the nucleotide level there is a relationship between the consensus and isolates Q and U.

RBDV was graft transmissible from infected blackberries to healthy raspberries, and from infected raspberries to healthy blackberries. This suggests that the recent appearance of RBDV in blackberries is not due to the appearance of a variant of RBDV with an altered host range. We propose that the recent appearance of RBDV in
blackberries may have been caused by pollinator mediated pollen transfer from raspberry to blackberry.

RBDV has been observed in many regions of the world. Phylogenetic analysis of coat protein genes may be useful in determining the source of RBDV present in South America, Australia, China or other parts of the world, as well as aid in our understanding of evolution and the spread of plant viruses.
TABLE 1. RBDV isolates used in this study.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Sourcea</th>
<th>Originb</th>
<th>PCR/Nestedd</th>
<th>C. quinoae</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>Raspberry 'Meeker'</td>
<td>NWREC</td>
<td>PCR</td>
<td>Yes</td>
</tr>
<tr>
<td>D</td>
<td>Raspberry 'Meeker'</td>
<td>NWREC</td>
<td>Nested</td>
<td>No</td>
</tr>
<tr>
<td>F1</td>
<td>Raspberry 'Favorite'</td>
<td>France</td>
<td>PCR</td>
<td>Yes</td>
</tr>
<tr>
<td>A</td>
<td>Blackberry 'Marion'</td>
<td>NWREC</td>
<td>PCR</td>
<td>Yes</td>
</tr>
<tr>
<td>B</td>
<td>Blackberry 'Marion'</td>
<td>NWREC</td>
<td>PCR</td>
<td>Yes</td>
</tr>
<tr>
<td>X</td>
<td>Blackberry 'Marion'</td>
<td>NWREC</td>
<td>PCR</td>
<td>No</td>
</tr>
<tr>
<td>I</td>
<td>Blackberry</td>
<td>Sauvie Island</td>
<td>PCR</td>
<td>No</td>
</tr>
<tr>
<td>N</td>
<td>Blackberry 'Olallie'</td>
<td>NWREC</td>
<td>Nested</td>
<td>No</td>
</tr>
<tr>
<td>Q</td>
<td>Blackberry 'Jerrell'</td>
<td>NWREC</td>
<td>PCR</td>
<td>No</td>
</tr>
<tr>
<td>S</td>
<td>Blackberry 'Silvan'</td>
<td>NWREC</td>
<td>PCR</td>
<td>No</td>
</tr>
<tr>
<td>U</td>
<td>Blackberry 'Adrienne'</td>
<td>NWREC</td>
<td>PCR</td>
<td>No</td>
</tr>
</tbody>
</table>

a Identifies if isolate came from raspberry or blackberry and cultivars.
b Location where isolate originated
c Whether amplification was by PCR or Nested PCR.
d RNA was isolated from C. quinoa
TABLE 2. Percent identity at the nucleotide level between coat protein genes of various isolates

<table>
<thead>
<tr>
<th></th>
<th>Nucleotide Similarity (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>China</td>
</tr>
<tr>
<td>RB</td>
<td>87.6</td>
</tr>
<tr>
<td>M</td>
<td>88.3</td>
</tr>
<tr>
<td>Can-S</td>
<td>87.2</td>
</tr>
<tr>
<td>Can</td>
<td>87.8</td>
</tr>
<tr>
<td>D200</td>
<td>87.3</td>
</tr>
<tr>
<td>D1</td>
<td>86.9</td>
</tr>
<tr>
<td>F1</td>
<td>87.6</td>
</tr>
<tr>
<td>U</td>
<td>88.4</td>
</tr>
<tr>
<td>Q</td>
<td>88.6</td>
</tr>
<tr>
<td>Consensus</td>
<td>88.6</td>
</tr>
</tbody>
</table>
TABLE 3. Percent identity at the amino acid level between coat protein genes of RBDV

<table>
<thead>
<tr>
<th></th>
<th>Amino Acid Similarity (%)</th>
<th></th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>China</td>
<td>RB</td>
<td>M</td>
<td>Can-S</td>
<td>Can</td>
</tr>
<tr>
<td>RB</td>
<td>92.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>94.1</td>
<td>98.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Can-S</td>
<td>92.4</td>
<td>97.5</td>
<td>98.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Can</td>
<td>93.7</td>
<td>99.2</td>
<td>99.2</td>
<td>98.4</td>
<td></td>
</tr>
<tr>
<td>D200</td>
<td>92.7</td>
<td>98.2</td>
<td>98.1</td>
<td>97.1</td>
<td>98.8</td>
</tr>
<tr>
<td>D1</td>
<td>93.5</td>
<td>98.2</td>
<td>98.5</td>
<td>97.1</td>
<td>99.6</td>
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<td>98.2</td>
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<tr>
<td>Q</td>
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<td>98.2</td>
<td>99.3</td>
<td>97.8</td>
<td>99.2</td>
</tr>
<tr>
<td>Consensus</td>
<td>93.4</td>
<td>98.5</td>
<td>99.6</td>
<td>98.2</td>
<td>99.6</td>
</tr>
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Table 4. Combinations of graft donors and recipients.

<table>
<thead>
<tr>
<th>Raspberry Donors</th>
<th>Blackberry Recipient</th>
<th>RBDV Transfer</th>
</tr>
</thead>
<tbody>
<tr>
<td>'Meeker'</td>
<td>'Kotata'</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>'Silvan'</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>'Black Butte'</td>
<td>-</td>
</tr>
<tr>
<td>'Favorite'</td>
<td>'Kotata'</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>'Waldo'</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>'Arapaho'</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>'Silvan'</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>'Black Butte'</td>
<td>-</td>
</tr>
<tr>
<td>Blackberry Donors</td>
<td>Raspberry Recipient</td>
<td></td>
</tr>
<tr>
<td>'Marion'</td>
<td>'Willamette'</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>'Qualicum'</td>
<td>-</td>
</tr>
<tr>
<td>'Arapaho'</td>
<td>'Willamette'</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>'Meeker'</td>
<td>-</td>
</tr>
<tr>
<td>ORUS 1122-1</td>
<td>'Willamette'</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>'Meeker'</td>
<td>+</td>
</tr>
<tr>
<td>'Olallie'</td>
<td>'Meeker'</td>
<td>+</td>
</tr>
<tr>
<td>'Jerrell'</td>
<td>'Amity'</td>
<td>-</td>
</tr>
<tr>
<td>'Ardenberry'</td>
<td>'Willamette'</td>
<td>-</td>
</tr>
<tr>
<td>'Silvan'</td>
<td>'Amity'</td>
<td>+</td>
</tr>
<tr>
<td>'Kotata'</td>
<td>'Qualicum'</td>
<td>-</td>
</tr>
<tr>
<td>GH Blackberry</td>
<td>'Willamette'</td>
<td>-</td>
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<tr>
<td></td>
<td>'Meeker'</td>
<td>+</td>
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<tr>
<td>Raspberry Donors</td>
<td>Raspberry Recipient</td>
<td></td>
</tr>
<tr>
<td>'Favorite'</td>
<td>'Meeker'</td>
<td>+</td>
</tr>
</tbody>
</table>

(+ ) Indicates a positive ELISA test
(- ) Indicates a negative ELISA test
Fig. 1a Schematic diagram of genomic organization of RBDV

- RNA 1
  5' [Polymerase] 3'

- RNA 2
  5' [Movement protein] 3'

- RNA 3
  5' [Coat protein] 3'

Fig. 1b Schematic diagram of RBDV RNA-3 showing the location of primers used for RT-PCR and Nested PCR.
Fig. 2a  Schematic representation of plasmid and coat protein gene restriction sites used for the double digest reaction.

![Diagram of plasmid and coat protein gene restriction sites](image)

Fig. 2b  Agarose gel viewed under UV light of a double digest with Eco RI and Sal I. Molecular weight standards BST E I cut λ DNA and 100bp DNA ladder were used as markers.

![Agarose gel](image)
Fig. 3 Schematic representation showing the location of the primers used for sequencing.

M13 F  |  Coat protein  |  M13 R
5'  Plasmid  |  Coat protein  |  Plasmid  3'
Fig. 4 Nucleotide sequence alignments of the coat protein genes.*

<table>
<thead>
<tr>
<th>NWREC Consensus</th>
<th>1</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q</td>
<td>CTTAGTTGT</td>
<td>AATGCATTGA</td>
</tr>
<tr>
<td>U</td>
<td></td>
<td></td>
</tr>
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**Fig. 5** Multiple sequence alignment of coat protein sequence of the RBDV isolates used in this study.

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Fig. 6  Phylogenetic tree analysis of 20 coat protein gene sequence using the neighbor-joining method.
Fig. 7  Phylogenetic tree of 20 RBDV coat proteins (translated from gene sequence) using the neighbor-joining method.


Oregon Agricultural Statistics Service (2001), 2000 Berry production up 3 percent http:www.oda.state.or.us/oass.html


Vector NTI Suite 7.0 including Xpression NTI, © 2001, InforMax, INC. 7600 Wisconsin Avenue, Suite #1100 Bethesda, MD 20814
Appendix

Recipes followed:

**Reverse Transcription**
10 ul 5X buffer  
50 U RNaseOUT  
5 ul 3’ primer (RC2 2µm stock)  
5 ul of 0.1 mM DTT  
2.5 ul of 10 mM dNTP  
21 ul Water  
1 ul Superscript II  
5 ul Template  
50 ul total volume

**Polymerase Chain Reaction (PCR)**
4 ul Template  
4 ul Primer (FC1 2µm stock)  
4 ul Primer (RC2 2µm stock)  
4 ul 10X buffer  
2.4 ul of 25 mM MgCl₂  
0.8 ul of 10 mM dNTP  
0.4 ul Taq (2 U)  
21 ul Water  
40 ul total volume

**Nested PCR**
4 ul Template  
4 ul Primer (FC2 2µm stock)  
4 ul Primer (RC1 2µm stock)  
4 ul 10X buffer  
2.4 ul of 25 mM MgCl₂  
0.8 ul of 10 mM dNTP  
0.4 ul Taq (2 U)  
21 ul Water  
40 ul total volume

**Double Digest**
3 ul plasmid  
1.5 ul 10X buffer (NEB buffer #3)  
0.5 ul Eco RI (5 U)  
0.5 ul Sal I (5 U)  
9.5 ul Water  
15 ul total volume