Epidemiology and Genetic Diversity of Grapevine Leafroll-Associated Viruses in British Columbia

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Abstract

Grapevine leafroll disease (GLD) is a complex associated with one or more virus species belonging to the family *Closteroviridae*. The majority of viruses in this complex are vectored by one or more species of mealybugs (*Pseudococcidae*) and/or scale insects (*Coccidae*). Grape-growing regions of British Columbia (BC), including Okanagan, Similkameen, and Fraser valleys and Kamloops (BC central interior), Vancouver, and Gulf islands, were surveyed during the 2014 and 2015 growing seasons for the presence of four major grapevine leafroll-associated viruses, including *Grapevine leafroll-associated virus 1* (GLRaV-1), GLRaV-2, GLRaV-3, and GLRaV-4. In total, 3,056 composite five-vine samples were collected from 153 *Vitis vinifera* and three interspecific hybrid vineyard blocks. The results showed GLRaV-3 to be the most widespread, occurring in 16.7% of the composite

samples, followed by GLRaV-4 (3.9%), GLRaV-1 (3.8%), and GLRaV-2 (3.0%). Mixed infections of two or more GLRaVs were found in 4.1% of the total samples. The relative incidence of GLRaVs differed among regions and vineyard blocks of a different age. Characterization of partial *COI* region from a total of 241 insect specimens revealed the presence of *Pseudococcus maritimus*, *Parthenolecanium corni*, and other *Pulvinaria* sp. in BC vineyards. Spatial patterns of GLRaV-3 infected grapevines in three vineyard blocks from three different regions in the Okanagan Valley showed variable degrees of increase in disease spread ranging from 0 to 19.4% over three growing seasons. Regional differences in the relative incidence and spread of GLD underline the need for region-based management programs for BC vineyards.

Grapevine leafroll disease (GLD) is long known to affect grapevine (*Vitis vinifera* L.) and is still regarded as one of the most important biotic constraint to grapevine health across grape-growing regions around the world (Alabi et al. 2016; Atallah et al. 2012; Komar et al. 2010; Martelli and Boudon-Padiou 2006; Oliver and Fuchs 2011). To date, five GLD-associated virus species belonging to three genera have been characterized, namely *Grapevine leafroll-associated virus 1* (GLRaV-1), GLRaV-3, GLRaV-4 and its strains -5, -6, -9, -Car, -De, and -Pr in the genus *Ampelovirus*, GLRaV-2 in the genus *Closterovirus*, and GLRaV-7 in the genus *Velarivirus* (Al Rwahnih et al. 2012; Naidu et al. 2014). Among all GLRaVs, GLRaV-3 is known to be the most widespread worldwide (Maree et al. 2013; Martelli 2012; Naidu 2011). Characteristic symptoms of GLRaVs include interveinal reddening and, in some cases, backward rolling of leaf margins in red-fruited *V. vinifera* cultivars whereas in white-fruited *V. vinifera* cultivars the symptoms are limited to mild chlorosis and backward rolling of leaf margins. The negative effects of GLD on vine vigor and berry quality have been demonstrated in several commercial grapevine cultivars from distinct geo-climatic conditions (Basso et al. 2010; Blaisdell et al. 2016; Fuchs et al. 2009; Golino et al. 2009; Lee et al. 2009; Santini et al. 2011). In addition, region-specific crop losses due to GLD infection are estimated in the range of 10 to 40% (Atallah et al. 2012; Nimmo-Bell 2006; Ricketts et al. 2015).

Primary dissemination of GLD occurs through propagation and distribution of infected planting material. Accordingly, certification programs to produce grapevine nursery material free of leafroll-associated viruses have been established in several countries (Golino et al. 2008; Komínek and Holleínová 2003; Martin et al. 2005; Nimmo-Bell 2006). With the exception of GLRaV-2, secondary spread of GLRaVs within and between vineyards is known to be

mediated by one or more species of mealybugs (Hemiptera: *Pseudococcidae*) and/or soft scale insects (Hemiptera: *Coccidae*) in a semipersistent manner (Daane et al. 2012; Tsai et al. 2010). Mealybugs and soft scale insects are considered to be major vineyard pests and are known to be responsible for secondary damage to fruit by promoting fungal infections through secretion of honeydew (Flaherty et al. 1992). Viticulture practices that favor the movement of viruliferous insects and wind currents are thought to play a critical role in the spatial and temporal distribution of GLRaVs within and between vineyards under natural conditions (Cabaleiro et al. 2008; Daane et al. 2012; Grasswitz and James 2008; Habili and Nutter 1997).

Large scale surveys to understand the incidence of GLRaVs and species of insect vectors are the first step for the development of region-based GLD management practices as recently shown in several grapevine-growing regions (Fuchs et al. 2009; Jones et al. 2015; Jooste et al. 2015; Liu et al. 2013; Milkus and Goodman 1999; Sharma et al. 2011, 2015). Although grapes have long been cultivated in British Columbia (BC), it was not until the implementation of the North American Free Trade Agreement in 1994 that significant changes in grapevine production were adopted. One of the most relevant changes was the substitution in the late 1980s of approximately 3,000 acres of hybrid varieties with *V. vinifera* cultivars under the “pull-out” program supported by the Canadian government. Accordingly, the BC wine-grape industry has since witnessed a steady increase in *V. vinifera* acreage, with major plantings occurring between the late 1990s and mid-2000s. At present, over 11,000 acres of grapevines are cultivated in BC, of which 99.9% are *V. vinifera* according to the British Columbia Wine Institute (2015). This substantial shift has resulted in the importation of a large amount of grapevine propagation material from Canadian Food Inspection Agency (CFIA)-approved international sources in France, Germany, and the U.S.A. GLRaVs are no longer regulated viruses in Canada since the mid-1990s, and thus, BC has been exposed to a high risk of primary spread through the various sources of grapevine nursery material.

A survey conducted in all major grape-growing regions of Canada during the 1994 and 1995 growing seasons recorded the occurrence of *Arabis mosaic virus* (ArMV), *Grapevine fanleaf virus* (GFLV),

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GLRaV-1, and GLRaV-3 in BC in a limited number of vineyards, but no information was provided about potential insect vectors and temporal spread of detected viruses (MacKenzie et al. 1996). Moreover, recent studies revealed the low incidence of *Grapevine red blotch virus* in BC vineyards (Poojari et al. 2017). Besides, there is a need to re-evaluate the status of GLRaVs in Canada given the inflow of planting materials since the previous survey and to determine their genetic diversities. Therefore, the objectives of this study were to i) conduct large scale field surveys to determine the incidence of the major GLRaVs (GLRaV-1, -2, -3, and -4) in BC vineyards; ii) understand their genetic diversity; iii) identify and characterize potential mealybug and soft scale insect vector species in commercial vineyards; and iv) determine the temporal spread of GLRaV-3 in commercial vineyard blocks in the Okanagan Valley, the main production area of BC.

Materials and Methods

Field survey and sample collection. To assess GLD incidence and document potential high disease pressure regions for GLRaVs, commercial vineyards (established between 1990 and 2015) were randomly selected from all five major grape growing regions in BC, including the Okanagan, Similkameen, and Fraser valleys, Vancouver Island, and the Gulf Islands (Fig. 1). A total of 153 *V. vinifera* vineyard blocks and three interspecific hybrid blocks were surveyed from August to late October of 2014 and 2015 corresponding to the post-*véraison* phase of berry development and maturity (Table 1). The majority of the vineyards surveyed were located in the Okanagan Valley, where nearly 82% of the total vineyard acreage is located. Border rows of vineyard blocks adjacent to other blocks were preferred to determine possible edge effect of virus movement. A 4 × 5 quadrat sampling strategy with a stratified regular quadrant distribution was adopted (Fuchs et al. 2009) with the exception that four composite samples were collected from the border rows. Each composite sample consisted of four leaf samples per vine and five vines per sample (total 20 leaves per sample). The sampling pattern was adjusted to fit the sampling size in case of small vineyard blocks. Thus, 10 to 20 composite samples were collected per block, representing 50 to 100 vines. To minimize error in detection due to uneven distribution of virus within the vines, the four leaf tissue samples from each vine were collected from all four cardinal directions. Samples were collected with no attempt to prioritize characteristic leafroll symptoms (Fig. 2A to F). All composite samples were transported to the laboratory on ice packs for immediate processing or storage at -20°C until further use.

Detection of GLRaVs. Each composite sample was ground in extraction buffer using a semiautomated HOMEX 6 homogenizer and tested in duplicate wells by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) using antibodies specific to GLRaV-1, -2, -3, and -4 (BIOREBA, Reinach, Switzerland) following the manufacturer's protocol. Optical density (OD) was measured using a BioTek ELx808 microplate reader (BioTek, Winooski, VT) at the excitation wavelength of 405 nm 1 h after addition of substrate (*p*-nitrophenylphosphate). Positive and negative controls for respective GLRaV supplied with the DAS-ELISA kits were included in each ELISA plate. Mean OD ratios were calculated for each plate from the average negative control OD values, plus two standard deviations from the mean. Positives were scored based on a threshold of $\geq 2 \times$ mean absorbance of the negative control for the corresponding GLRaV. Samples with reactions that varied considerably between replicates or reactions close to the negative-positive threshold were retested by respective GLRaV-specific DAS-ELISA or by reverse transcription-polymerase chain reaction (RT-PCR) using virus-specific primers as described below.

Partial sequencing of HSP70h gene and phylogenetic analysis. Representative individual vines from ELISA-positive panel samples were further confirmed by single tube RT-PCR. Total nucleic acids (TNAs) were extracted from petioles as described in Rowhani et al. (2000). TNAs isolated from petioles of a GLRaV-infected grapevine maintained at the Summerland Research and Development Center (SuRDC) greenhouse was used as a positive control. Virus-specific primers for the detection of heat shock protein 70 homolog (HSP70h)

gene for GLRaV-2 (Bertazzon and Angelini 2004), GLRaV-3 (Osman and Rowhani 2006), GLRaV-4 (Osman et al. 2007; Poojari et al. 2016), and coat protein duplicate 2 (CPd2) genes of GLRaV-1 (Alabi et al. 2011) were used in a single-tube RT-PCR (Fig. 3) as described by Rowhani et al. (2000). RT-PCR amplicons were purified using QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and were cloned into the pCR2.1 vector using a TOPO TA cloning kit (Life Technologies, Grand Island, NY) following manufacturer's instructions. Plasmids were isolated from two independent recombinant clones and sequenced in both directions. Nucleotide sequences were generated using the ABI PRISM BigDye Terminator Cycle Sequencing Kit v.3.1 (Applied Biosystems, Waltham, MA) and run on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Overlapping nucleotide sequences of respective clones were assembled using Vector NTI software (Invitrogen, Grand Island, NY) and deposited into GenBank (Table 2). Phylogenetic trees were built using the maximum-likelihood method in MEGA7 software (Tamura et al. 2013) with 1,000 bootstrap replicates. Corresponding sequences of GLRaV isolates from different geographical locations were retrieved from GenBank and included in the analyses.

Field survey and DNA barcoding of mealybug and scale insect species. DNA barcoding based on partial mitochondrial *COI* regions was performed at the Barcode of Life Data Systems (BOLD) (Ratnasingham and Hebert 2007) for a total of 225 specimens of mealybugs and soft scale insects collected from 71 vineyard blocks located in the Okanagan and Similkameen valleys during the growing seasons of 2013 to 2016. Nucleotide sequences, collection dates, specimen photographs, trace files, and corresponding GenBank accession numbers are available in the project files entitled "PAFRC" and "SCMB" in BOLDsystems (www.boldsystems.org). These sequences were further compared with corresponding sequences of mealybug and scale insect species available in BOLD and GenBank to determine the extent of homology.

Assessment of GLRaV-3 temporal spread. One red-fruited vineyard block from each of the north (NO), central (CO), and south (SO) Okanagan regions were mapped for the presence of GLD symptoms (Fig. 2A to C). Single-visit ground surveys were carried out post-*véraison* and before harvest; the incidence of GLD based on visual symptoms was recorded on a vine by vine basis during three consecutive years (2014 to 2016). In all three vineyard blocks, petiole samples from a minimum of 60 vines (20 symptomatic and 40 asymptomatic) were collected and tested for the presence of GLRaV-1, -2, -3, and -4 by one-step RT-PCR.

Statistical analyses. The incidence of GLRaVs was compared and contrasted among the four BC regions. Percent positives for each composite sample with respect to each GLRaV were analyzed using PROC GENMOD (SAS version 8.1, SAS Institute, Cary, NC). Data were fit to a general lineal model with binomial distribution (Sharma et al. 2011, 2015). A vineyard block was considered as a random variable and region as a fixed factor. If the prevalence was significant among the regions, a single step posthoc test was used to contrast the differences. PROC MIXED (SAS version 8.1) was used to determine the effect of region and age of the vineyard with regard to the prevalence of the viruses. Region and age were fixed factors with cultivar and block being the random variables. Statistically significant interactions ($P < 0.05$) of fixed factors with prevalence were later subjected to Tukey-Kramer mean separation test. Relative percent incidence of a viral strain with respect to region and age is reported.

Results

Incidence of GLRaVs in BC. Of the total 3,056 field-collected composite samples, ELISA results showed that 3.8% were positive for GLRaV-1, 3.0% for GLRaV-2, 16.7% for GLRaV-3, and 3.8% for GLRaV-4. Thus, GLRaV-3 was the most widespread virus, followed by GLRaV-4, GLRaV-1, and GLRaV-2. Low incidence (4.1%) of mixed infections of two or more GLRaVs were also detected, including GLRaV-3 + GLRaV-1 (2.3%), GLRaV-3 + GLRaV-2 (0.9%), GLRaV-3 + GLRaV-4 (0.6%), and GLRaV-1 + GLRaV-4 (0.07%) (Table 1). None of the samples tested positive for three or more GLRaVs. Analysis

Fig. 1. Areas surveyed in the major vine growing regions of British Columbia. Four main sampling zones shown in dotted line drawings in black were depicted according to their growing degree days and agro-ecological conditions. The red-dotted areas represent geolocated vineyards.

Table 1. Detection of grapevine leafroll associated viruses (GLRaV-1, GLRaV-2, GLRaV-3, and GLRaV-4) from composite (five vines; 20 leaves) grapevine leaf samples collected from vineyard blocks in British Columbia during 2014 and 2015 growing seasons

Cultivar	# of vineyard blocks	# of composite samples					# of multiple virus infections	% positive for at least one virus
		Collected	GLRaV-1	GLRaV-2	GLRaV-3	GLRaV-4		
White-fruited								
Bacchus	1	20	0	0	0	0	0	0.0
Chardonnay	21	420	86	3	130	3	77	34.5
Gewurztraminer	3	65	0	0	5	0	0	7.7
Kerner	1	20	0	0	0	0	0	0.0
Moscato Giallo	1	20	0	0	0	0	0	0.0
Muscat Ottonel	3	60	0	0	2	0	0	3.3
Pinot blanc	4	80	3	0	0	0	0	7.5
Pinot gris	13	262	10	0	20	22	4	18.3
Pinot Meunier	1	20	0	0	0	0	0	0.0
Riesling	12	245	3	3	39	12	1	22.9
Sauvignon blanc	5	100	3	3	20	35	15	46.0
Semillon	1	20	0	0	20	0	0	100.0
Viognier	2	40	1	2	1	0	0	10
Red-fruited								
Cabernet franc	11	220	0	14	64	0	3	34.5
Cabernet Sauvignon	11	220	0	61	42	0	3	46.4
Gamay noir	2	40	5	0	0	13	2	40.0
Merlot	30	512	4	7	149	13	6	32.6
Pinot noir	22	446	0	0	11	20	15	3.6
Syrah	5	110	2	0	0	0	0	21.8
Tempranillo	1	20	0	0	5	0	0	25.0
Zinfandel	3	60	0	0	0	0	0	31.7
Interspecific hybrids								
Marquette	1	20	0	0	0	0	0	0
La Crescent	1	16	0	0	1	0	0	6.3
Frontenac noir	1	20	0	0	0	0	0	0

of the results by cultivar type indicated that of 1,372 composite samples collected from 13 different white-fruited *V. vinifera* cultivars, GLRaV-3 infections were the most prevalent (17.3%), followed by GLRaV-1 (7.7%), GLRaV-4 (5.2%), and GLRaV-2 (0.8%). Similarly, out of 1,628 composite samples collected from eight different red-fruited *V. vinifera* cultivars, GLRaV-3 was the most prevalent (16.6%), followed by GLRaV-2 (5.0%), GLRaV-4 (2.8%), and GLRaV-1 (0.7%). In the case of interspecific hybrids, only one of 56 composite

samples (1.8%) tested positive for GLRaV-3 (Table 1). None of the interspecific hybrids composite samples tested positive for GLRaV-1, -2, or -4.

Relative incidence of GLRaVs differ by geographical regions and age of the vineyards. The relative incidence of the four grapevine leafroll-associated viruses (GLRaV-1, -2, -3, and -4) differed among the regions ($z = 39.25$, $P < 0.0001$, $df = 604$). Although there were no differences in relative incidence of GLRaVs when

Fig. 2. Characteristic symptoms associated with *Grapevine leafroll associated virus-3* observed during harvest time in British Columbia. Symptoms on red fruited cultivar Merlot include interveinal reddening and backward rolling of leaf margins (**A**, **B**) leading to the entire canopy (**C**), and on white fruited cultivar Chardonnay, backward rolling of leaf margins (**D**, **E**) and mild yellowing of the bottom portion of the canopy (**F**). Adult female colonies of *Pseudococcus maritimus* (**G**) and *Parthenolecanium corni* (**H**), the commonly found insect species in British Columbia vineyards.

comparisons were made between North Okanagan, central Okanagan, and Similkameen (Fig. 1), each region was statistically different from South Okanagan (Table 3). The descending order of percent infection rate for GLRaVs among all regions ($F_{3, 12E3} = 110.93, P < 0.0001$) was South Okanagan > Similkameen = Central Okanagan > North Okanagan. Among GLRaVs ($F_{3, 12E3} = 156.99, P < 0.0001$), the descending order of incidence noted was GLRaV 3 > GLRaV 2 > GLRaV 1 = GLRaV 4 in all four regions. Among all the regions, South Okanagan recorded the highest incidence of GLRaV-3 (Table 3).

In an age-based analysis, there were significant differences ($F_{2, 12E3} = 22.91, P < 0.0001$) in GLRaV infection levels for younger vines (vineyard blocks planted between 2011 and 2015) in comparison with older vines (vineyard blocks planted between 1990 to 2000 and 2001 to 2010; Table 4). The low levels of GLRaV-1 and GLRaV-4 across the regions and ages of vines is noteworthy. Also, it is relevant to note the absence of GLRaV-1 in the Similkameen region (Table 3) and in the oldest vineyard blocks surveyed (1990 to 2001; Table 4). Overall, the interactions of region ($F_{9, 12E3} = 38.43, P < 0.0001$) and age of the vines ($F_{6, 12E3} = 22.91, P < 0.0001$) on the occurrence of GLRaV were statistically different. All possible combinations of mean GLRaV incidences with respect to regions and age are reported in Tables 3 and 4.

Genetic diversity of GLRaVs. Multiple nucleotide sequence alignments of the partial HSP70h gene of 11 GLRaV-2 isolates showed a high level of identity (98.7 to 100%) among BC isolates. Pair-wise comparison of BC isolates with global variants of GLRaV-2 resulted in 69.5 to 86.9% identities with H4, 93/95, PV20, BD, and RG variants. Phylogenetic analysis of GLRaV-2 isolates with global GLRaV-2 variants belonging to six (H4, 93/955, PN, PV20, BD, and RG) previously described variant groups (Jarugula et al. 2010) showed that GLRaV-2 BC isolates grouped with PN lineage, indicating the presence of

a single variant of GLRaV-2 in BC vineyards (Fig. 4B). In the case of GLRaV-3, multiple nucleotide sequence alignments of the partial HSP70h gene of 15 isolates showed that BC isolates are highly identical, sharing 95.7 to 100% identity among themselves. Comparative analysis with GLRaV-3 global isolates revealed 88.7 to 99.8% nucleotide identity level. In phylogenetic analyses, GLRaV-3 isolates from BC grouped into two distinct groups (group I and II; Fig. 4C). Similarly, two GLRaV-4 isolates shared 98.4 to 100% and GLRaV-4 strain 5 isolates shared 99.4 to 100% identities among them and grouped with respective global isolates (Fig. 4D). On the other hand, partial Cpd2 gene four GLRaV-1 isolates shared 95.9 to 97.8% identity among them and 82.5 to 97.7% identity with nine global isolates belonging to two groups (Fig. 4A).

Identification of vineyard mealybug and scale insects. Mealybug and soft scale insects were present at low population levels in most of the surveyed blocks. Of the 241 specimens DNA bar-coded for partial mitochondrial *COI* region, 38 were identified as *Pseudococcus maritimus* (Ehrhorn) (Hemiptera: Pseudococcidae), all of which were collected from vineyard blocks in the Central ($n = 3$) and South ($n = 35$) Okanagan. No other mealybug species were found in the collected specimens in this study. Among the soft scale species, *Parthenolecanium corni* (Bouché) was found in all four regions viz., North ($n = 7$), Central ($n = 21$), and South ($n = 57$) Okanagan and Similkameen ($n = 19$). Both *Ps. maritimus* and *Pa. corni* showed nucleotide percent identities in the range of 99.2 to 99.5 with reference sequences in both

Table 2. Details of isolates of grapevine leafroll-associated viruses (GLRaV-1, -2, -3, and -4) characterized in this study

Species	Isolate	Cultivar	Location	GenBank acc. no.
GLRaV-1	LR1BC1	Pinot gris	North Okanagan	KY381128
	LR1BC2	Pinot blanc	Central Okanagan	KY381129
	LR1BC3	Cabernet franc	South Okanagan	KY381130
	LR1BC4	Chardonnay	South Okanagan	KY381131
GLRaV-2	LR2BC1	Gamay noir	North Okanagan	KY381132
	LR2BC2	Chardonnay	North Okanagan	KY381133
	LR2BC3	Sauvignon blanc	North Okanagan	KY381134
	LR2BC4	Merlot	Central Okanagan	KY381135
	LR2BC5	Riesling	North Okanagan	KY381136
	LR2BC6	Riesling	North Okanagan	KY381137
	LR2BC7	Cabernet franc	South Okanagan	KY381138
	LR2BC8	Merlot	North Okanagan	KY381139
	LR2BC9	Chardonnay	South Okanagan	KY381140
	LR2BC10	Chardonnay	South Okanagan	KY381141
	LR2BC11	Cabernet Sauvignon	South Okanagan	KY381142
GLRaV-3	LR3BC1	Gamay noir	North Okanagan	KY381143
	LR3BC2	Cabernet franc	North Okanagan	KY381144
	LR3BC3	Chardonnay	North Okanagan	KY381145
	LR3BC4	Chardonnay	Central Okanagan	KY381146
	LR3BC5	Merlot	Central Okanagan	KY381147
	LR3BC6	Merlot	South Okanagan	KY381148
	LR3BC7	Merlot	South Okanagan	KY381149
	LR3BC8	Riesling	South Okanagan	KY381150
	LR3BC9	Chardonnay	South Okanagan	KY381151
	LR3BC10	Tempranillo	South Okanagan	KY381152
	LR3BC11	Riesling	South Okanagan	KY381153
	LR3BC12	Gewurztraminer	South Okanagan	KY381154
	LR3BC13	Zinfandel	Similkameen Valley	KY381155
	LR3BC14	Merlot	Victoria Islands	KY381156
GLRaV-4	LR4BC1	Gamay noir	Lower Mainland	KY381157
	LR4BC2	Merlot	North Okanagan	KY381158
-strain-5	LR5BC1	Merlot	Central Okanagan	KY381159
	LR5BC2	Chardonnay	South Okanagan	KY381160
	LR5BC2	Cabernet franc	South Okanagan	KY381161

Fig. 3. High resolution capillary gel electrophoresis of RT-PCR amplicons specific to Grapevine leafroll-associated virus (GLRaV)-1, -2, -3, and -4. Highlighted in green are 3,000 bp (top) and 15 bp (bottom) QX Alignment Markers (Qiagen). Lane A1: QX DNA Size Marker 50 bp to 1.5 kb (Qiagen) was used to compare the DNA size amplicons. Lane A2-A5: GLRaV. Lane A6: Negative control.

GenBank and BOLD database. Sequences specific to the genus *Pulvinaria* but not robust enough to define species (nts percent identities in the range of 82.0 to 95.0 with available NCBI GenBank/BOLD database) were detected in all four regions viz., North ($n = 3$), Central ($n = 21$), and South ($n = 30$) Okanagan and Similkameen ($n = 45$).

GLRaV-3 spread in the Okanagan Valley. Spatial patterns of leafroll-associated virus infections in three red-fruited *V. vinifera* vineyard blocks (South, Central, and North Okanagan; Fig. 5A to C) showed variable degrees of disease spread over the span of three growing seasons (2014 to 2016). In the case of the Central Okanagan vineyard block ($n = 480$; Fig. 5B), an increase of 19.4% was observed in vines expressing symptoms (Fig. 2A to C) of GLRaV-3 infection over three years. Similarly, in the South Okanagan vineyard block ($n = 500$; Fig. 5A), an increase of 8.6% was observed in GLRaV-3 symptom expression over the same time period indicating secondary spread of GLRaV-3 in both Central and South Okanagan blocks. In contrast, no disease progression was recorded in the North Okanagan vineyard block ($n = 344$; Fig. 5C) and infection remained constant at 5.8% over the three years. RT-PCR and PCR testing of 60 representative vines (20 symptomatic and 40 asymptomatic) in each block revealed the presence of GLRaV-3 in symptomatic vines in both Central and South Okanagan blocks, whereas for the North block, 15 out of 20 symptomatic vines were positive for GLRaV-3 and the remaining five symptomatic vines were positive for GLRaV-2. All asymptomatic vines in the three vineyard blocks tested negative for the presence of GLRaV-1, -2, -3, and -4, indicating the accuracy of the visual survey in these blocks. Comparative sequence analysis of RT-PCR amplicons specific to partial HSP70h gene of GLRaV-3 from individual adult female *Ps. maritimus* populations (Fig. 2G) sourced from vines showing GLD symptoms as well as from source vines from both Central and South Okanagan vineyard blocks showed 100% identity at nucleotide level, indicating the likely insect vector-mediated movement of GLRaV-3 under natural conditions.

Discussion

This study represents a comprehensive assessment of the incidence and distribution of four GLRaVs (GLRaV-1, -2, -3, and -4) in grape-growing regions of BC and is the first of its kind in Canada. Based on laboratory virus test, it is evident that GLRaV-3 is the predominant species in BC vineyards irrespective of cultivar, region, and age of the vines. Similar trends of GLRaV-3 prevalence were observed in previous studies in several wine grape-growing regions (Fuchs et al. 2009; Jones et al. 2015; Jooste et al. 2015;

Liu et al. 2013; Sharma et al. 2011, 2015). In comparison with the other wine grape-growing regions, GLRaV-3 occurs at a significantly higher level in parts of the South Okanagan where the highest vineyard acreage concentration in BC is located. Our results also indicated limited presence of GLRaV-3 in targeted samples collected from Fraser Valley, Kamloops, and Vancouver and Gulf islands (data not shown). The majority of the *V. vinifera* cultivars grown in BC are grafted onto rootstocks, which might play an initial role in the primary distribution of GLRaVs that occur via infected rootstock or scion combinations. Analysis of partial HSP70h gene sequences from representative GLRaV isolates showed close relationships with GLRaV isolates reported from other geographical origins indicating that the predominant mode of entry of these viruses into the BC vineyards likely occurred via planting materials from outside sources. Although Canada imports grapevine planting material from certified sources in France, Germany, and the U.S.A., it is very probable that infected material might be introduced in undetectable levels using standard assays and moreover, GLRaVs are not listed as quarantinable viruses in Canada. The presence of GLRaVs in certain vineyards with absence of mealybug and scale insect incidence stresses the need and importance to commence a domestic clean plant program for Canada to improve the sanitary status of grapevines.

The acreage of wine grapes in BC has increased from 4,184 acres in 1999 to 9,066 acres (53.8%) in 2008, with a further increase to 10,260 acres in 2014 (11.6% increase) (British Columbia Wine Institute 2015). Among all grape-growing regions of BC, the South Okanagan represents 56% of the total acreage followed by Central (13.8%) and North Okanagan (12.6%). The Similkameen Valley represents 6.4% of the total BC acreage. The Okanagan and Similkameen valleys in BC (Fig. 1) are considered relatively young and expanding grape-growing regions with significant differences in varietal performance, viticultural practices, and climatic conditions (Bowen et al. 2005). These factors might play a crucial role in pest and disease pressure. The negative effects of GLRaVs depend on virus, insect-vector pressure, and viticultural practices, which would vary from region to region and vineyard to vineyard. Several studies have reported the adverse effects of GLRaVs on fruit color and quality (Cabaleiro et al. 1999; Credi and Babini 1997; Over de Linden and Chamberlain 1970). However, the cool-climate conditions of BC put it at great risk of higher negative impacts of GLD as reported by Alabi et al. (2016). Limited studies in cool climatic conditions have focused on the significant reduction in grower's income levels from GLD infections (Atallah et al. 2012; Cabaleiro et al. 1999).

Six genetic variants (PN, 93/955, H4, BD, RG, and PV20) of GLRaV-2 (Fig. 4B) were reported majorly based on their genetic variability and association with graft incompatibility

Table 3. Region based percent relative incidence of grapevine leafroll associated viruses (GLRaV-1, -2, -3, and -4) in British Columbia vineyards during the 2014 and 2015 growing seasons. Means sharing the same letters are not significantly different from each other ($P_c > 0.05$).

Region	Composite samples (n)	Virus	% Incidence
North Okanagan	858	GLRaV-1	1.89 ± 0.85 GHI
		GLRaV-2	1.98 ± 0.85 FGHI
		GLRaV-3	7.59 ± 0.85 CDE
		GLRaV-4	2.55 ± 0.85 FGHI
Central Okanagan	385	GLRaV-1	3.71 ± 1.37 DEFGHI
		GLRaV-2	6.94 ± 1.37 CDEFG
		GLRaV-3	12.93 ± 1.37 C
		GLRaV-4	1.08 ± 1.37 FGHI
South Okanagan	1,237	GLRaV-1	5.72 ± 0.71 DEF
		GLRaV-2	8.64 ± 0.71 CD
		GLRaV-3	33.09 ± 0.71 A
		GLRaV-4	4.87 ± 0.71 EFGH
Similkameen Valley	576	GLRaV-1	0.00 ± 1.24 I
		GLRaV-2	8.48 ± 1.24 CDE
		GLRaV-3	21.16 ± 1.24 B
		GLRaV-4	0.28 ± 1.24 HI

Table 4. Age based percent relative incidence of grapevine leafroll associated viruses (GLRaV-1, -2, -3, and -4) in British Columbia vineyards during 2014 and 2015 growing seasons. Means sharing the same letters are not significantly different from each other ($P_c > 0.05$).

Vineyard age	Composite samples (n)	Virus	% Incidence
>1990 to 2000	480	GLRaV-1	0.00 ± 1.25 DE
		GLRaV-2	15.23 ± 1.25 BC
		GLRaV-3	19.35 ± 1.25 AB
		GLRaV-4	2.88 ± 1.25 DE
2001 to 2010	806	GLRaV-1	4.55 ± 0.98 D
		GLRaV-2	4.01 ± 0.98 DE
		GLRaV-3	22.70 ± 0.98 A
		GLRaV-4	1.80 ± 0.98 DE
2011 to 2015	1,770	GLRaV-1	3.94 ± 0.60 D
		GLRaV-2	0.29 ± 0.60 E
		GLRaV-3	14.03 ± 0.60 C
		GLRaV-4	1.91 ± 0.60 DE

(Bertazzon et al. 2010; Zhu et al. 1998). The PN variant was reported to be most widespread and associated with leafroll symptoms and graft incompatibility (Bertazzon et al. 2010) on grapevines. Although more studies are required to understand

the presence of other genetic variants of GLRaV-2 in BC vineyards, the predominance of PN isolates in the current study indicates the possibility of GLRaV-2 distribution occurred though infected planting material.

Fig. 4. Phylogenetic tree depicting the evolutionary relationships among coat protein duplicate 2 (CPd2) genes of GLRaV-1 (A) isolates and partial heat-shock protein 70 homolog (HSP70h) gene sequences of GLRaV-2 (B), GLRaV-3 (C), and GLRaV-4 (D) isolates from British Columbia with representative global isolates. The evolutionary history was inferred using the maximum-likelihood (ML) method using the "Find Best DNA/Protein Models." The bootstrap consensus tree inferred from 1,000 replicates and branches with less than 70% bootstrap replicates are collapsed and the percentage of replicate trees are shown next to the branches. Evolutionary analyses were conducted in MEGA7. Isolates characterized in this study are highlighted in bold font and details of isolates are mentioned in Table 2.

The spatial distributions of vector-mediated virus diseases in crop plants are often influenced by various environmental factors as well as by the mode of insect vector dispersal under natural conditions. Wind-aided dispersal of viruliferous mealybug or scale insect crawlers

Fig. 5. Spatial distributions of GLRaV-3 in three red-fruited vineyard blocks South (**A**; cv. Cabernet franc); Central (**B**; cv. Merlot), and North (**C**; cv. Merlot) Okanagan blocks observed during the 2014 to 2016 growing seasons. Colored circles with numbers represent individual vines showing leafroll symptoms in respective rows. Percent increase in symptom expression is shown in the parenthesis for each season.

originating from neighboring leafroll infected vineyards has been suspected for GLRaVs (Barrass et al. 1994). The presence of low numbers of insect vectors can result in significant spread of leafroll viruses (Golino et al. 2008; Wallingford et al. 2015). Three years of spatial mapping of GLRaV-3 in two commercial vineyards in the Central and South Okanagan valley showed a linear increase in spread with both random and aggregated patterns. In the South Okanagan vineyard block, a distinct edge effect with an increase in disease gradient of symptomatic vines originating from an adjacent older vineyard block with severe leafroll infection was observed from the western side of the block toward the eastern side. The percent of symptomatic vines increased to 70.1% (80 to 114) in the first five rows adjacent to the older, leafroll-infected block in comparison with 50% (8 to 16) increase in the last five rows to the east of the vineyard block, suggesting rapid short distance movement of viruliferous insect vectors as demonstrated by Geiger and Daane (2001). An increase of 55.9% (118 to 211) in the number of vines infected with GLRaV-3 for the Central Okanagan vineyard block over three years suggests strong secondary infections in random and aggregated patterns. This block is surrounded by vineyard blocks with a significant incidence of leafroll infection that can act as a source of virus for secondary spread by movement of mealybugs or scale insects. Random patterns of GLRaV-3 in vineyard blocks were previously reported as a result of possible aerial dispersion of viruliferous vectors and frequent replacement of infected vines with GLRaV-3-free vines (Cabaleiro et al. 2008; Charles et al. 2009; Jones and Nita 2016). A much higher spread of GLRaV was also observed in certain South Okanagan vineyard blocks than North and Central vineyard blocks. GLRaV infection rates increased up to 10% during a single growing season. It can be speculated that high initial introduction of infected planting material along with high incidence of vector populations and vineyard management tactics might have contributed to comparative high incidence of GLD in pockets of South Okanagan region. Similar observations were made in Australia (Habibi and Nutter 1997), California (Golino et al. 2008), and New York (Wallingford et al. 2015). The high nucleotide identity of the partial HSP70h gene of GLRaV-3 in vineyard blocks and from *Ps. maritimus* isolated from South and Central Okanagan blocks and their neighboring blocks indicates the likely vector-mediated movement of GLRaV-3 under natural conditions. In contrast, no increase in the number of symptomatic vines was observed for the North Okanagan vineyard block. Although we found scale insect species in a few pockets of North Okanagan regions, there were no indications mealybug or scale populations within or in neighboring vineyard blocks during our three-year survey for this particular block.

The bar coding results revealed that *Ps. maritimus* (Ehrhorn) and *Pa. corni* (Bouche) are present in BC vineyards. Molecular characterization of partial COI regions also revealed the presence of two other *Pulvinaria* sp. present in all four regions of BC, with BLAST results for these specimens providing 83 to 95% identity with other species of scale insects belonging to *Pulvinaria* sp. Availability of sequence based information in public databases for *Pu. innumerabilis* (Rathvon) and *Pu. vitis* (L) would improve the accuracy of molecular characterization at species level. Further morphological and genomic studies are currently underway to identify these soft scale species.

Populations of *Ps. maritimus* (Fig. 2G) and *Pa. corni* (Fig. 2H) were shown to be highly variable between vineyard blocks and regions. *Ps. maritimus* is considered an economically damaging pest if present in high populations in the vineyards (Charles 1982), but its ability to transmit multiple grapevine viruses exceeds the economic damage if found in complex with other known mealybug species such as *Planococcus ficus*, *Ps. longispinus*, and *Pl. citri* (Wallingford et al. 2015). The absence of other mealybug species, including *Pl. ficus*, which is considered to be the primary vector of GLRaV in Californian vineyards, differentiates the epidemiology of GLRaVs in the cool climatic regions of BC. Our results also agree with previous observations supporting the predominance of *Ps. maritimus* in Washington and Oregon, the two main grape-growing

regions in the Pacific Northwest (Rayapati et al. 2008; Skinkis et al. 2009). Epidemiological importance and transmission efficiencies of *Ps. maritimus* for GLD have been studied (Daane et al. 2012; Golino et al. 2008; Wallingford et al. 2015). Similarly, transmission capabilities of *Pa. corni* for GLRaV-1 (Fortusini et al. 1997) and GLRaV-3 (Bahder et al. 2013) were reported indicating the epidemiological significance of these insect species for GLD.

Regional management strategies developed and implemented in recent times highlighted the importance of collective and a multidirectional approach for effective and long-term management of GLD (Almeida et al. 2013; Bell et al. 2009; Daane et al. 2008; Le Maguet et al. 2013; Naidu et al. 2009; Pietersen and Walsh 2012; Pietersen et al. 2013). Successful approaches for GLD management include, but are not limited to, educating growers and stakeholders, implementing a virus-free certification program capable of large-scale supply of clean propagative plant material, and developing regional strategies for the control of insect vector populations. The importance of region-based knowledge on the life cycle of mealybug and scale insect species and their population size plays a crucial role in developing effective management of secondary spread of GLD (Almeida et al. 2013). In this regard, studies to understand the seasonal population dynamics of mealybug and soft scale insect species in BC are underway.

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