A Diverse Virome of Leafroll-Infected Grapevine Unveil by dsRNA Sequencing

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Abstract: Quebec is the third-largest wine grape producing province   in Canada, and the industry is constantly expanding. Traditionally, 90% of the grapevine cultivars grown in Quebec were rustic or semi-rustic and largely dominated by winter hardy interspecific hybrid *Vitis* sp. cultivars. Over the years, the winter protection techniques adopted by growers and climate changes have offered an opportunity to establish *V. vinifera L.* cultivars (e.g. Pinot Noir). We characterized the virome of leafroll-infected interspecific hybrid cultivar and compared it to the virome of *V. vinifera* cultivar to support and facilitate the transition of the industry. A dsRNA sequencing method was used to sequence symptomatic and asymptomatic grapevine leaves of different cultivars. The results suggested a complex virome in terms of composition, abundance, richness, and phylogenetic diversity. Three viruses, grapevine Rupestris stem pitting-associated virus, grapevine leafroll-associated virus (GLR) 3 and 2 and hop stunt viroid (HSVd) largely dominated the virome. However, their presence and abundance varied among grapevine cultivars. The symptomless grapevine cultivar Vidal was frequently infected by multiple virus and viroid species and different strains of the same virus, including GLR virus 3 and 2. Our data shows that viruses and viroids associated with the highest number of grapevines expressing symptoms included HSVd, GLR3 and GLR2, in gradient order. However, co-occurrence analysis revealed that the presence of GLR species was randomly associated with the development of virus-like symptoms. These findings and their implication for grapevine leafroll disease management were discussed.

Keywords: Grapevine leafroll disease; viromics; dsRNA extraction; virus epidemiology; virus co-occurrence; *Vitis vinifera;* interspecific hybrid

1. Introduction

The Canadian grape industry is constantly expanding, with a production area of more than 12,000 ha in 2019 for a total revenue of CAD$ 9 billion dollars[1]. Quebec is the third-largest wine grape producer in Canada in terms of acreage, tonnage and wine grape sales. Quebec's industry is also increasing the area dedicated to grapevine production. Traditionally 90% of the grapevine cultivars cultivated in Quebec were rustic (winter hardy), with nearly 50% of the grapevine production based on an interspecific hybrid *Vitis sp.* cultivar named Vidal, which was developed in the 1930s, because these hybrid cultivars were able to survive harsh winter conditions and produced mature berries in the short and warm growing season. Over the years, knowledge about the most promising cultivars and constant improvement of techniques favouring the survival of the plants led to profound change of the industry. Furthermore, growers' experience and climate changes offered an opportunity to establish *V. vinifera L.* cultivars, expanding the diversity of wine offered as much

46 for taste and flavour as for the joy of the consumers. However, this increased assortment of
47 grapevines implied various new challenges, including different susceptibility to known diseases and
48 potentially new ones, particularly viruses.

49   More than 80 viruses and viroids were discovered over the years to affect grapevine, either
50 singly or as mixed infections [2, 3]. Among perennial crops, worldwide, grapevine is considered to
51 host the highest number of viruses [3]. In north-eastern North America, tomato ring spot virus
52 (ToRSV), arabis mosaic virus (ArMV), grapevine leafroll-associated virus (GLR), the rugose wood-
53 associated viruses, comprising grapevine Rupestris stem pitting-associated virus (GRSP), grapevine
54 virus A (GVA), grapevine virus B (GVB) and grapevine virus *D* (GVD), grapevine fleck virus (GFV),
55 grapevine Red Globe virus (GRG), grapevine fanleaf virus (GFL) and grapevine red blotch-associated
56 virus (GRB) are the prominent viruses scrutinized [2]. Among these viruses, grapevine leafroll
57 disease complex (GLD), which is associated with six known viruses, is one of the most prevalent and
58 draws particular attention because of its adverse economic impacts on the grapevine yield [4-7].
59 It is known that the symptoms of GLD vary with seasons, cultivars and weather conditions [8, 9].
60 Some infected cultivars can present strong, little or no symptoms, which are well-described in *Vitis*
61 *vinifera* cultivars and include interveinal reddening, backward rolling of leaf margins and mild
62 chlorosis [8, 10, 11]. Indeed, some *Vitis vinifera* cultivars, such as Pinot Noir, are known to display
63 strong GLD symptoms, whereas the information on symptom expression on hybrid cultivars such as
64 Vidal is scarce and few studies had focused on hybrid cultivars of grapevine [12, 13]. This situation
65 is probably due to the fact that GLRs are latent in hybrid grapevines despite the significant impact
66 on fruit production [14].

67   In Canada, there are few published studies addressing the presence and incidence of grapevine
68 viruses. In 1996, MacKenzie et al. [15] surveyed grapevine viruses in four regions (British Columbia
69 [BC], Ontario [ON], Québec [QC] and Nova Scotia [NS]) across Canada and found ArMV, GFL, GLR-
70 1 and GLR-3 in BC and ON vineyards, whereas only ArMV and GLR-3 were found in QC and GLR-
71 1 and GLR-3 in NS. Incidences of infestation range from 0.06% to 12.2%. Recently, Poojari et al.
72 conducted studies on the incidence of GLR and GRBV in BC [16, 17] and surveyed for GLR, GFL,
73 GRB, *Grapevine pinot gris virus* (GPG) in NS vineyards in *V. vinifera* and *Vitis* interspecific hybrids [13].
74 Also, GLR-3 and GRB were observed in ON vineyards by Xiao et al. [18] in a study aiming at
75 improving methods to detect viruses for use in diagnostic. However, such studies have not been done
76 in QC since 1996 [15], despite that concerns were raised by viticulturists as the industry expanded by
77 more than 300% from 2003 to 2018, i.e. from 225 ha to 718 ha [19, 20]. In fact, suspicious plants
78 showing characteristic symptoms of virus infection have been seen regularly lately, and diagnostic
79 tests revealed the presence of GLR-3, GRB, GFV and ToRSV (Fall, unpublished.).

80   Throughout the scientific literature, there is limited study on the diversity of the virome of
81 grapevine interspecific hybrid cultivars in comparison with the virome of *Vitis vinifera* cultivars [9,
82 13, 21]. It is known that some *Vitis vinifera* cultivars, such as Pinot Noir, infected with grapevine
83 leafroll viruses had a complex virome and strongly expressed symptoms of GLD [9]. Quebec grape
84 production initiated a transition from the historically-dominant grapevine interspecific hybrid
85 cultivars (e.g. Vidal) to more *Vitis vinifera* cultivars such as Pinot Noir. In fact, this cultivar (Pinot Noir)
86 saw an 153.2% increase in terms of total hectares cultivated between 2012 and 2017 (Conseil des vins
87 du Québec). This situation raises concerns and research questions. For example, how will the leafroll-
88 infected asymptomatic grapevine hybrid cultivars (e.g. Vidal) that dominated QC grapevine
89 vineyards act as a reservoir and impact the diversity of the virome of the *Vitis vinifera* grapevine
90 cultivars (e.g. Pinot Noir)? As *Vitis vinifera* cultivars are more prone to express symptoms of GLD ,
91 the QC vineyard transition from hybrid cultivars to *V. vinifera* cultivars should be done with caution.
92 There are no methods to efficiently control viruses in vineyards. Chemical control against vectors is
93 costly and might not adequately regulate these populations as well as raising environmental concerns
94 about their usage. Most of our actions in agriculture to cure diseases are in fact done in a reactive
95 fashion, i.e. actions are taken to control the disease once it is established on the crop. In the case of
96 the relationship between grapevines and viruses, it is essential to switch from pathosystems to a more
97 holistic view of the system where grapevine and its virome constitute a micro-ecosystem. From this

98   standpoint, we would benefit from increasing our knowledge on virome epidemiology, anticipate
99   potential threats and design knowledge-based mitigation strategies before virus epidemics become
100   endemic and difficult to manage [22, 23]. Therefore, the objectives of the present work were to
101   characterize the virome of the interspecific hybrid cultivar and compare it to the virome of *V. vinifera*
102   cultivar. Specifically, the aims were to (i) characterize the diversity of the virome of leafroll-infected
103   leaves of different grapevine cultivars under north-eastern weather conditions, (ii) investigate the
104   association between viruses and symptom development and (iii) determine the genetic diversity of
105   viruses and viroids that were detected for the first time in QC.

106   Here, we report the complexity and the diversity of the virome of grapevine leafroll-infected
107   cold-hardy vines cultivars (e.g. interspecific hybrids Vidal) in comparison with the cold- and virus-
108   sensitive cultivar *Vitis vinifera* (Pinot Noir) [4, 24, 25]. In the hybrid cultivar Vidal, GRSP dominated
109   the virome with 61% of viral population, whereas GLR3 and GLR2 represented 13% and 18% of the
110   viral population, respectively. In the cultivar Pinot Noir, GLR2 dominated the virome with 43% of
111   the viral population, whereas GRSP, GLR3 and HSVd represented 7%, 38% and 8% of the viral
112   population, respectively.

2. Materials and Methods

114   All statistical tests and graphics were performed in R software, version 3.6.3. The following
115   packages were used: Precrec, ggplot2, eesim, picante, ape, vegan, ade4, permute, lattice, nlme,
116   Complexheatmap (Bionconductor), Cluster, Heatplus, gplots, RcolorBrewer, circlize, colorspace,
117   Getoplong, dendextend (https://cran.r-project.org/).

2.1. Plant Material

119   Leaf samples of asymptomatic and symptomatic leafroll-infected grapevine were collected from
120   three different vineyards in Quebec, Canada (Fig. 1). The presence of GLR was confirmed by RT-PCR
121   using primers described by Xiao et al. [26] and Poojari et al. [27]. The first vineyard was at the
122   Agriculture and Agri-Food Canada's experimental farm in Frelighsburg (Fr, latitude 45° 03′ 12″ N;
123   longitude 72° 51′ 42″ W), the second at a commercial vineyard in Hemmingford (Hem, latitude 45°
124   02′ 50.99″ N; longitude 73° 35′ 4.04″ W), and the third at a commercial vineyard in Saint-Jacques-le-
125   Mineur (SJM, latitude 45° 16′ 39″ N; longitude 73° 25′ 4.82″ W). The frequencies of sampled grapevine
126   cultivars were 50%, 36%, 7%, 4% and 3% of Vidal (hybrid), Pinot Noir (*Vitis vinifera*), Marechal Foch
127   (hybrid), DM85 (hybrid), Seyval blanc (hybrid), respectively. A total of 140 composite samples from
128   28 grapevine plants (five entire leaves with petiole per plant) were collected per plant between July
129   and September 2018 and 2019 and placed in a plastic bag or a sterile 50-mL centrifuge tube and
130   brought back to the laboratory for cold storage at -20 C. Leaves were washed with distilled water and
131   roughly crushed before being homogenized in a liquid-nitrogen-cooled 50-mL stainless-steel (SS)
132   grinding jar with one 20–25-mm SS ball with a Retsch tissue lyser MM 400 (Retsch, Haan, Germany).
133   The powdered leaves (2.5–3 g), were then transferred in sterile 50-mL centrifuge tubes and kept at -
134   80°C until proceeding with double-stranded RNA (dsRNA) extraction.

2.2. dsRNA extraction, libraries construction and sequencing

136   Extraction of dsRNA from grapevine leaves was performed based on a modified version of the
137   technique from Kesanakurti et al. [28]. Differences brought to the protocol were: black bean (*Phaseolus
138   vulgaris*, Black Turtle Baking Beans (Vesey, York, PEI, Canada) was added as positive control to the
139   homogenized sample-extraction buffer mix at a final concentration of 1% (W/W), and DNase I and
140   RNase T1 digestions were made after the final dsRNA elution. The cultivar of *P. vulgaris* contains
141   distinct endornavirus species, including *Phaseolus vulgaris endorvirus1* (PvEV1), which was used to
142   assess the efficiency of dsRNA extraction and for monitoring of correctness of high throughput
143   sequencing results. Then digestion was stopped with EDTA 50 mM for 10 min at 65 °C. The dsRNA
144   was denatured at 99 °C for 5 min in the presence of 1 μL of 60 μM random primers and 1 μL of 10
145   mM dNTP and 4 μL First Strand buffer, and then reverse transcribed with 400 U of Superscript III

146   (Invitrogen) in a final volume 20 μL for 50 min at 65 °C. The second-strand DNA was synthetized
147   using Klenow polymerase DNA (Promega) treated with RNase H to get rid of any remaining RNA
148   hybrids, and then cleaned using Agencourt AMPure XP magnetic beads (Beckman-Coulter).
149   Libraries (28, 300bp mean insert size) were constructed using the Nextera XT DNA library prep kit
150   (Illumina) with 1 ng of input double-stranded cDNA. MiSeq 2 × 250 cycle paired-end sequencing was
151   conducted using MiSeq Reagent Nano Kits v2 in the Illumina Miseq sequencer. The black bean added
152   to all grapevine samples was sequenced alone to differentiate its virome from the grapevine virome
153   associated with each sample.

2.3. Bioinformatic and statistical analysis

2.3.1 Raw data treatement

156   The raw data fastq files were demultiplexed and checked for sequencing quality using fastQc
157   (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Removal of adapter sequencing and
158   reads quality trimming (minimum quality score of 20) were performed using Trimmomatic V.0.32
159   [29]. Paired fastq files were imported in two different pipelines developed to detect and discover
160   viruses. The first pipeline used was Virtool, an in-house implemented pipeline developed by Rott et
161   al. [30], and the second pipeline was VirFind, an online tool developed by Ho and Tzanetakis [31].
162   From Virtool, the depth, coverage and weight were deducted. The depth represents the number of
163   times a genome was covered by mapped reads, the coverage measures percentage of the mapped
164   reads that cover the viral genome, and the weight represents the proportion of reads mapping to a
165   virus that is proportional to the titer. Consensus virus detected by both pipelines was considered as
166   a positive detection. In general, a coverage greater than 0.5 and a weight greater than 0.001 were
167   considered as positive detection of a given virus. In addition, only samples with positive detection of
168   the positive control virus (PvEV1) were conserved for further analysis. The mean proportion of viral
169   read that mapped for a given virus (MPVR), total number of symptomatic leaves (TNSL) associated
170   with a given virus, mean depth (MD), mean depth relative to the depth of the positive control virus
171   (MDRC), mean relative abundance (MRA) and mean weight (MW) were calculated for each virus.
172   The depth was used as a measure of absolute abundance and, since many multivariate methods are
173   sensitive to the total abundance [32], the relative abundance of each detected virus in each sample
174   was calculated using a function of the vegan package. Vegan functions (specpool, estimateR) were
175   used to estimate virus species richness based on incidence in sample plants. The richness Z score and
176   the richness of each detected virus relative to the richness of the positive control virus (PvEV1) were
177   calculated.

2.3.2 Analysis of the diversity of the virome and association between viruses and symptom development

179   Because grapevine is subject to infection by more than 70 different viruses and viroids, having
180   the ideal number of samples to capture the diversity is challenging. Therefore, the virus species
181   accumulation and abundance as function of virus species rank curves were made using the vegan
182   and ade4 packages to assess the robustness of our sampling effort.
183   After standardization of the data using the generic function "scale" (R built-in command) to
184   make variables comparable, the distance matrix was calculated, and functions of the
185   ComplexHeatmap package with clustering were used to visualize the heatmap made from Pearson
186   correlation analysis showing the association between variables (MPVR, MW, TNSL, MDRC, MD,
187   MRA and genome size [GS]) and virus species. The pam function of the cluster package was used for
188   partitioning of the data into k clusters around Medoids (k-Medoids, [k = 3]) because it is less sensitive
189   to outliers compared to k-means [33]. In addition, the matrix of data displaying the normalized
190   relative abundance with virus species in columns and the grapevine samples in rows was used to
191   generate a heatmap and a hierarchical clustering to classify virus species and grapevine samples into
192   different groups. The Bray-Curtis dissimilarity matrix and average linkage distance were generated
193   using the vegan package.

194   To investigate the degree to which viruses species, leafroll symptoms and grapevine cultivars
195 are negatively or positively associated with each other, a recently-published co-occurrence model
196 that is metric-free, distribution-free and randomization-free was used [34, 35]. The model determined
197 the probability that observed frequency of co-occurrence between two events (e.g. virus or symptoms
198 presence in a given sample) is significantly greater (large, Pgt) than expected (meaning a positive
199 association), significantly less (small, Plt) than expected (meaning negative association), or not
200 significantly different and approximately equal to expectation (meaning random association) [34, 35].
201 Therefore, Pgt < 0.05 is considered to highlight a positive co-occurrence between the two events, and
202 Plt < 0.05 is interpreted as a negative co-occurrence between the two events. The R package co-occur
203 was used to determine the co-occurrence, and the probability (P) that two species (viruses or
204 grapevine species) co-occur at j number of sampling units (a grapevine plant) is calculated as follow:
205

206
$$P_j = \frac{\binom{N_1}{j} X \binom{N - N_1}{N_2 - j}}{\binom{N}{N_2}}$$

207   where N_1 is the number of sampling units where the first event occurs, N_2 is number of sampling
208 units where the second event occurs, and N is the total number of sampling units. To gain more
209 robustness, additional data were gathered from five published manuscripts using the same NGS
210 methodologies to characterize the virome of grapevine plants [9, 36-39]. In summary, a total of
211 66 sampling units and 1452 points of comparison were obtained and used in this analysis. Also, any
212 event pairs that were expected to share less than one sampling unit were removed from the analysis.
213   To determine the genetic diversity of important viruses and viroids that were detected for the
214 first time in QC, phylogenetic trees were generated from nucleotide alignments of partial
215 (concatenated, details in Tables S2 and S3 respectively) or complete sequences of the genome from
216 samples that were positive for a given virus. Maximum likelihood algorithms were carried out using
217 bootstrap (1000 pseudo-random iterations) to assess the confidence of the branching pattern, and the
218 phylogram package in R was used. Full genomes from different clades and geographical regions were
219 downloaded from GenBank and added to tree. Selection of right reference genomes was done by
220 running a BLAST in GenBank using an isolate as query and from the result page, the genome with
221 the highest alignment scores was chosen. One genome of the same cluster with selected isolate and
222 at least two other genomes from different clusters were selected. The Newick format file was exported
223 to the interactive tree of life platform for better visualization and creation of high-quality figures [40].
224 To display an overview of the diversity among the sequences, pairwise nucleotides comparison
225 analysis was used to generate percentage of identity and distance for each pair of sequences using
226 CLC Main Workbench software (V 20.0.3). The percentage of identity was calculated by dividing the
227 number of identical residues in the alignment positions with the total number of overlapping
228 alignment positions between the two sequences. The distance was measured using the Jukes-Cantor
229 distance between the two sequences and is the proportion between identical and overlapping
230 alignment positions between two sequences.

231 **3. Results**

232 *3.1. Diversity of virome in leafroll-infected leaves of different grapevine cultivars*

233   The grapevine plants infected with leafroll expressed different intensity of symptoms. The
234 cultivar Vidal, an interspecific hybrid, expressed little to no visible leafroll symptoms in comparison
235 with other cultivars of *Vitis vinifera* (e.g. Pinot gris) (Fig. 1).

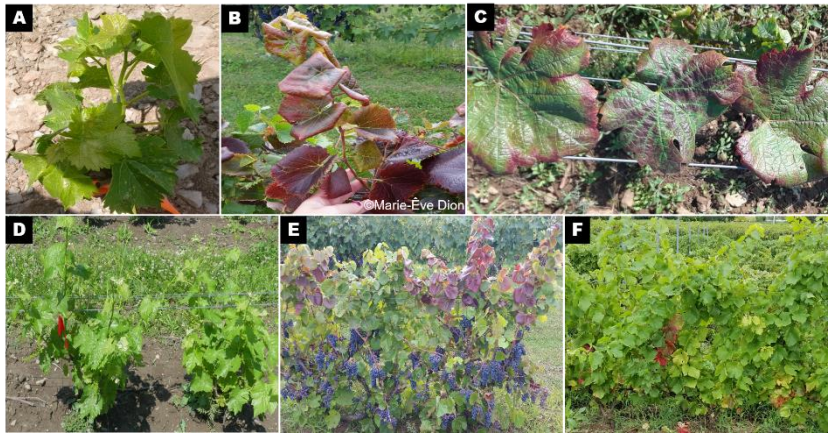
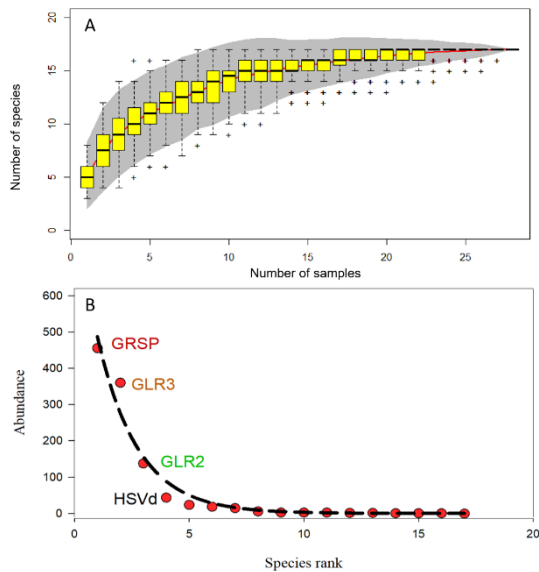
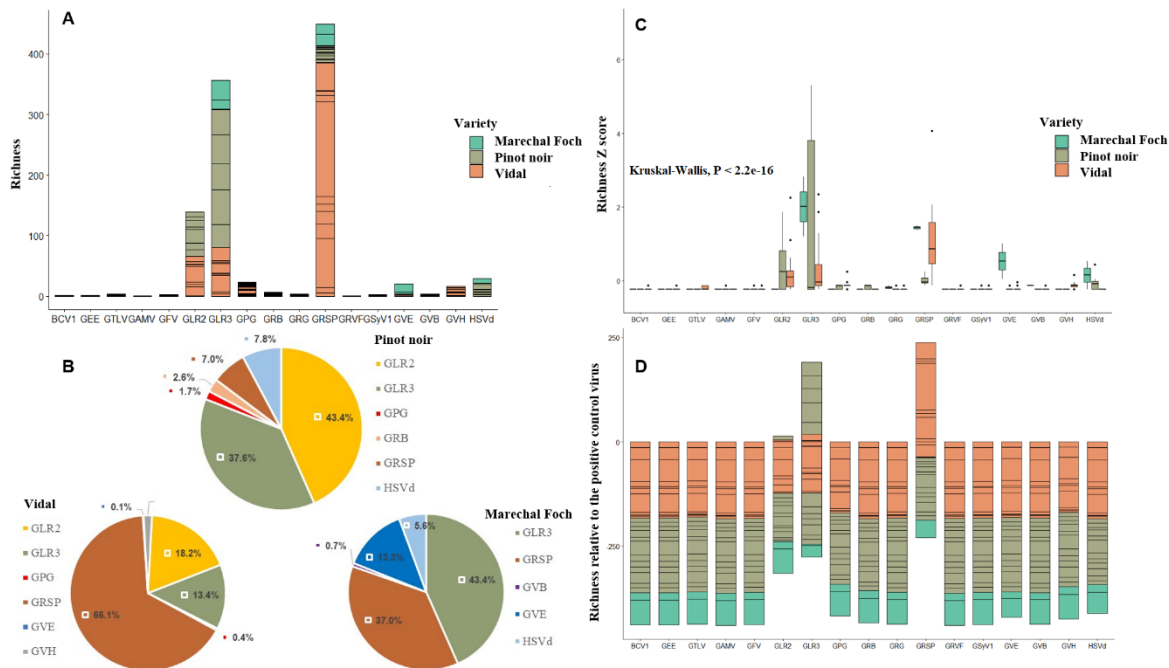



Figure 1. Asymptomatic and symptomatic leaves observed in different cultivars that were positive for grapevine leafroll-associated virus (GLR 2 or 3 or both) in Quebec, Canada. Cultivar Vidal positive to GLR without symptom expression (A and D), cultivar DM85 positive to GLR with leafroll symptom expression (B and E) and cultivar Pinot Noir positive to GLR with leafroll symptom expression (C and F).

Over 2 228 600 reads were obtained with a percentage of mapped viral reads ranging between 3.12 and 9.50 % of the total number of reads The viral communities were relatively well-sampled across the three sampling sites. The plateau in terms of number of virus species was detected after a cumulus of 17 samples, and 60% of the viral species were detected after a cumulus of five samples, suggesting that many of the viral species were present in the majority of sampled plants (Fig. 2A). The three most abundant virus species in the leafroll-infected samples were grapevine ruspestris stem pitting virus (GRSP), grapevine leafroll-associated virus 3 (GLR3) and grapevine leafroll-associated virus 2 (GLR2). Hop stunt viroid (HSVd) was the fourth most abundant species across the grapevine samples (Fig. 2B). Using the dsRNA sequencing method, it was possible to detect RNA and DNA viruses and viroids. Except for GLR3, all the viruses and viroids reported in Fig. 2C were detected for the first time in Quebec (Table 2). Even though three viruses (GRSP, GLR and GLR2) and one viroid (HSVd) largely dominated the virome, their presence and abundance can be sorted by grapevine cultivars (Fig. 3). In the hybrid cultivar Vidal, GRSP dominated the virome with 61% of the viral population, whereas GLR3 and GLR2 represented 13% and 18% of the viral population, respectively, and the other viruses occupied between 0.001% and 0.1% of the virome. Grapevine virus H was only present in the hybrid cultivar Vidal. In the cultivar Pinot Noir (*Vitis vinifera*), GLR2 dominated the virome with 43% of the viral population, whereas GRSP, GLR3 and HSVd represented 7%, 38% and 8% of the viral population, respectively, and the other viruses occupied between 0.01% and 2.7% of the virome. In the cultivar Marechal Foch (hybrid cultivar), GLR3 dominated the virome with 43% of the viral population, whereas GRSP, GVE and HSVd represented 37%, 13% and 5.6% of the viral population, respectively, and the other viruses occupied between 0.01% and 0.7% of the virome. The HSVd was only detected in the Pinot Noir and Marechal Foch cultivars, whereas grapevine red blotch-associated virus (GRB) was only detected in the Pinot Noir cultivar (Fig. 3 A, B, C and D). When considering the species abundance relative to the abundance of the positive control virus (PvEV1) that was added to the samples, only GLR2, GLR3 and GRSP were more abundant than PvEV1. In Marechal Foch grapevine cultivar, none of the detected viruses seem to be more abundant than PvEV1 (Fig. 3 D).

269

270   **Figure 2.** An accumulation curve of the viral population in grapevine plant samples (A). The red line
271   represents the number of virus species as a function of number of samples collected. The gray area
272   displays the standard deviation, and the yellow box plots show the species richness based on linear
273   interpolation of random permutation. The virus rank abundance curve (B) displays the virus
274   abundance as a function of virus species rank. The four virus and viroid species (GRSP GLR3, GLR2,
275   HSVd) in terms of abundance are shown. R packages vegan, gridExtra, ggplot2 and ade4 were used
276   (see materials and methods section for more details).

277

278   **Figure 3.** Virus richness (A), virus relative abundance (B), virus richness Z score (C) and virus richness
279   relative to the richness of the positive control (PvEV1) (D), sorted by the three major grapevine
280   cultivars that constituted 93% of the total plants sampled (Marechal Foch, Pinot Noir and Vidal). R
281   package ggplot2 was used (see materials and methods section for details on how richness and richness
282   Z score was calculated)

283   The optimal number of clusters based on independent variables (MPVR, MW, TNSL, MDRC,
284   MD, MRA and GS) used as inputs was 2.One cluster was composed by GLR3, GLR2 and GRSP, and

285   the other consisted of the remaining viruses. However, the latter cluster can be divided into two
286   subclusters, one subcluster composed of grapevine asteroid mosaic virus (GAMV), grapevine Fleck
287   virus (GFV), HSVd and grapevine pinot gris virus (GPV) and a second composed of the rest of the
288   viruses (Fig. 4). When we collapsed the variables to have two dimensions using discriminant
289   principal component analysis, the viral population was divided into four groups (GRSP, GLR3, GLR2
290   and rest of the viruses) (Fig. 1S). Viruses and viroids that were associated with the highest number of
291   plants expressing symptoms (variable TNSL) were GRSP, HSVd, GLR3, GPG, GRB and GLR2 in
292   gradient order (Fig. 4). However, this association may be random and not directly linked to the
293   symptoms because many NGS studies described GRSP as one of the viruses of the background
294   virome. Nevertheless, the analysis of the association between virus and leafroll symptom
295   development will bring more insights.

296

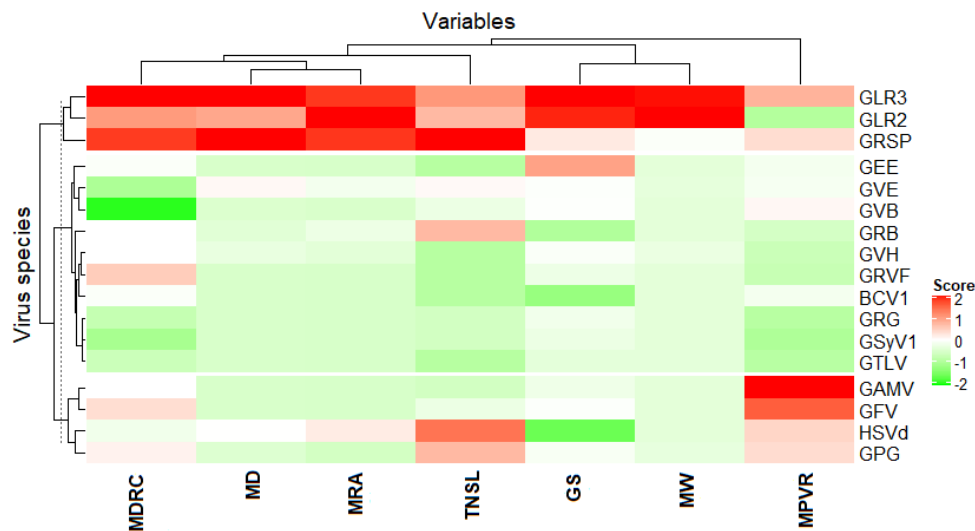



Figure 4. Heatmap displaying the characteristics and association between detected viruses and the
mean proportion of viral reads that mapped for a given virus (MPVR), total number of symptomatic
leaves (TNSL) associated with a given virus, mean depth (MD), mean depth relative to the depth of
the positive control virus (MDRC), mean relative abundance (MRA), mean weight and genome size
(GS). The pam function of the cluster package was used for partitioning the data into k cluster around
Medoids (k-Medoids, [k = 3]) because it is less sensitive to outliers compared to k-means [33]. R
packages ComplexHeatmap, circlize, colorspace, dendextend, cluster and GetoptLong were used (see
materials and methods section for more details). For virus names and abbreviations please see table
1.

306   The viral signature (virome composition) was clustered by sampling site (Fig. 5). Sample names
307   starting with "Bac" were from the first commercial vineyard (Hem), those started with "DSJ" were
308   from the second commercial vineyard (SJM), and the rest of the samples were from the experimental
309   farm (Fr, see Plant Material section). Samples from the same site were in the same cluster, except for
310   three samples from Hem vineyard, and the grapevine cultivar type seems to have no impact on it
311   (Fig. 5). Virome in the Hem and SJM commercial vineyards were dominated by GLR3 and GLR2,
312   respectively, whereas it was dominated by GRSP at the experimental vineyard, except for four
313   samples (Fig. 5). The GRB was only detected in the SJM commercial vineyard, and the new grapevine-
314   associated tymo-like virus (GTLV) was only detected in the Fr vineyard, whereas the grapevine
315   asteroid mosaic-associated virus (GAMV) was only detected in the Hem commercial vineyard (Fig.
316   5, Table 1).

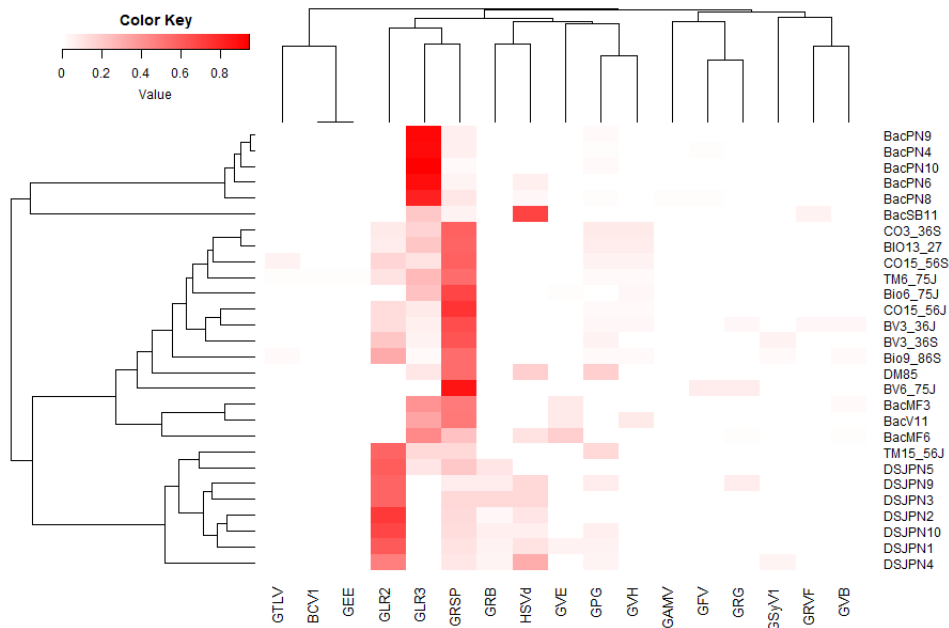



Figure 5. Heatmap of hierarchical clustering of grapevine virome composition profiles represented by the normalized relative abundance per grapevine sample. Heatmap colour (white to dark red) displays the row-scaled relative abundance of each virus across all samples. The Bray-Curtis dissimilarity matrix and average linkage distance were used. R packages Vegan, Heatplus, gplots, RcolorBrewer and readxl were used (see materials and methods section for more details).

Table 1. Detection and description of viruses detected by dsRNA sequencing and confirmed by PCR reaction from composite samples of grapevine leaves collected at the two commercial vineyards (Hem and SJM) and the experimental vineyard (Fr).

Virus name	Species (ICTV)[a]	Abbreviation	NGS[b]	PCR[c]	Sampling sites		
					Fre	Hem	SJM
Beet cryptic virus 1	Beet cryptic virus 1	BCV1	+	NT	+	-	-
Grapevine endophyte endornavirus	Grapevine endophyte alphaendornavirus	GEE	+	NT	+	-	-
Grapevine associated tymo like virus	Not been approved yet	GTLV	+	NT	+	-	-
Grapevine asteroid mosaic associated virus	Not been approved yet	GAMV	+	NT	-	+	-
Grapevine fleck virus	Grapevine fleck virus	GFV	+	+	+	+	-
Grapevine leafroll associated virus 2	Grapevine leafroll-associated virus 2	GLR2	+	+	+	+	+
Grapevine leafroll associated virus 3	Grapevine leafroll-associated virus 3	GLR3	+	+	+	+	+
Grapevine Pinot gris virus	Grapevine Pinot gris virus	GPG	+	+	+	+	+
Grapevine red blotch associated virus	Grapevine red blotch virus	GRB	+	+	-	-	+
Grapevine Red Globe Virus	Grapevine red globe virus	GRG	+	NT	+	-	+
Grapevine rupestris stem pitting associated virus	Grapevine rupestris stem pitting-associated virus	GRSP	+	NT	+	+	+
Grapevine rupestris vein feathering virus	Not been approved yet	GRVF	+	NT	+	+	-
Grapevine syrah virus 1	Not been approved yet	GSyV1	+	+	+	-	+
Grapevine virus E	Grapevine virus E	GVE	+	NT	-	+	+
Grapevine virus B	Grapevine virus B	GVB	+	NT	+	+	-
Grapevine virus H	Not been approved yet	GVH	+	NT	+	+	-
Hop stunt viroid	Hop stunt viroid	HSVd	+	+	-	+	+

[a]ICTV virus species names following guideline of the international committee on taxonomy of viruses (ICTV), [b]next generation sequencing technology using dsRNA extraction method, [c]polymerase chain reaction; NT for not tested.

3.2. Association between viruses and virus-like symptom development

The co-occurrence analysis removed 75 pairs (32.7%) that had an expected co-occurrence < 1, and 156 pairs of combination were analyzed. Sixteen positive associations, 17 negative associations and 123 random associations were found (Table 2). Only those significant of the most important associations were displayed, and the events that did not have sufficient occurrence data were removed (Fig. 6). Grapevine leafroll symptom expression had a significant positive association with the cultivar Vitis vinifera (Pgt < 0.00000) and a significant negative association with the cultivar Vidal

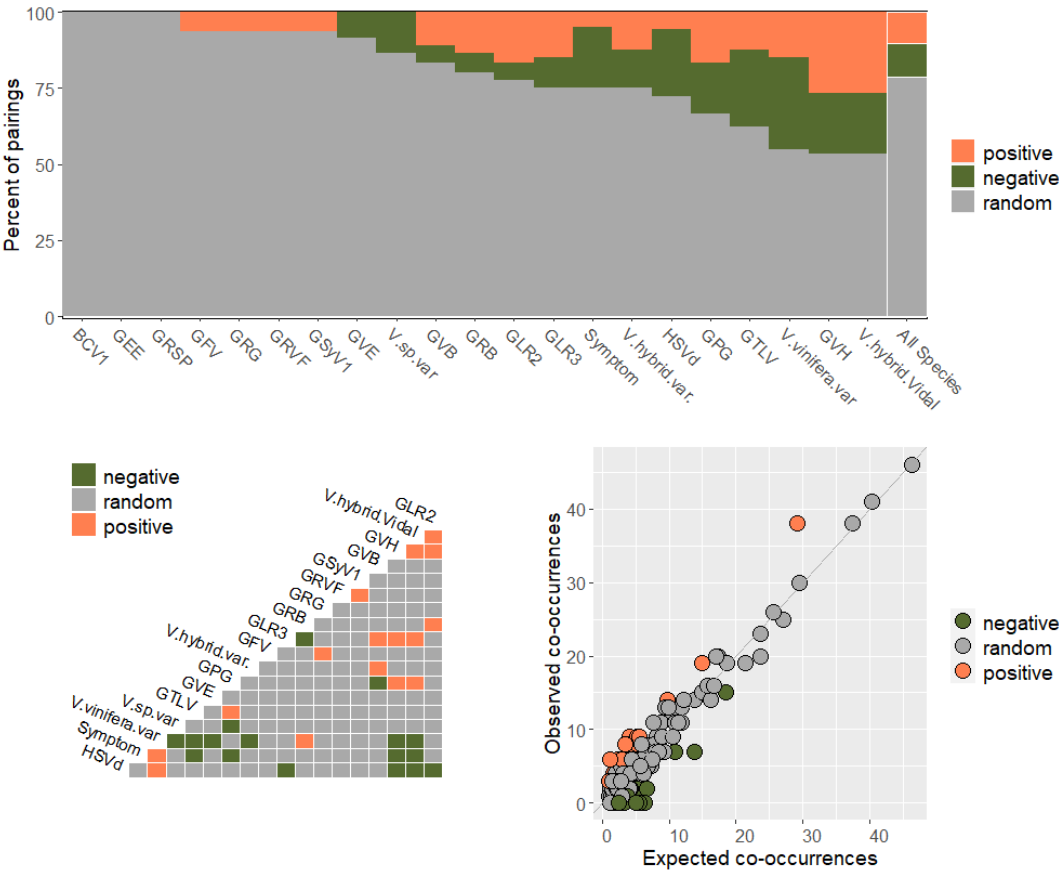
334 (Vitis sp. Hybrid) (Plt < 0.00000) (Table 2, Fig. 6, Fig. 2S, Table S1). Presence of GLR species (GLR2
335 and GLR3) was randomly associated with symptom development. When the analyses of co-
336 occurrence were done without the cultivar Vidal, the association between GLD species and symptom
337 expression was still random, and Grapevine leafroll-associated virus 3 (GLR3) was significantly and
338 negatively associated with Hop stunt viroid (Pgt < 0.00050) and Grapevine red blotch-associated virus
339 (GRB). However, the number of plants that were infected with GRB was low (9), and therefore the
340 negative association between GRB and GLR3 was considered as weak (Table 2, Fig. 6).

341 **Table 2.** Co-occurrence table displaying the events that are significantly associated with leafroll-like
342 symptom development and presence of *Grapevine leafroll-associated virus 3* (GLR3).

	Event[a]	Ev_inc[b]	obs_cooccur[c]	prob_cooccur[d]	exp_cooccur[e]	p_lt[f]	p_gt[g]
Symptom expression	Hybrid Vidal	9	0	0.097	6.4	0.00000	1.00000
	V.vinifera cvs	41	38	0.442	29.2	1.00000	0.00000
	GTLV	4	0	0.043	2.8	0.00538	1.00000
	GPG	26	15	0.281	18.5	0.04747	0.98688
	GVH	9	0	0.097	6.4	0.00000	1.00000
GLR3 presence	GRB	9	2	0.079	5.2	0.02587	0.99662
	GVB	17	14	0.148	9.8	0.99742	0.01517
	GVH	9	9	0.079	5.2	1.00000	0.00440
	HSVd	24	7	0.209	13.8	0.00050	0.99994
	Hybrid Vidal	9	8	0.079	5.2	0.99560	0.04140

[a]represent virus presence, symptom presence in a given sample or a given variety.
[b]represent number of sampling sites where a given event occurs
[c]represent observed number of sampling sites having two given events
[d]represent probability that the two given events occur at a sampling site
[e]represent expected number of sampling sites having the two given events
[f]represent probability that the two events would co-occur at a frequency less than the observed number of co-occurrence sampling sites if the two events were distributed randomly
[g]represent probability of co-occurrence at a frequency greater than the observed frequency

343

344

345 **Figure 6.** The percent of total pairings for virus species, leafroll-like symptoms and grapevine
346 cultivars that are positive, negative and random (boxplot top graph). The bar outline in white shows
347 the assemblage-wide percentages. A heatmap of the positive, negative and random associations

348 (bottom-left graph) displays the probabilistic co-occurrence that observed frequency of co-occurrence
349 between two events (e.g. virus, viroids or symptoms presence in a given sample) is significantly
350 greater (large) than expected (meaning a positive association), significantly less (small) than expected
351 (meaning negative association), or not significantly different and approximately equal to expectation
352 (meaning random association)). Events are positioned to indicate the columns and the rows that
353 represent their pairwise relationships. Graphic displaying the scatter plot of observed versus expected
354 co-occurrence (bottom-right graph). Each event pair in the analysis is represented by a circle coloured
355 based on whether it was classified as positive (orange), negative (dark green) or random (gray). Any
356 event pairs that were expected to share less than one sampling unit were removed from the analysis.
357 R package co-occur was used (see materials and methods section for more details).

358 *3.3. The genetic diversity of viruses and viroids detected for the first time in Quebec*

359 Among all viruses that were detected, it was possible to recover consensus sequences for GLR2,
360 HSVd and GRSP phylogenetic analyses, and these sequences were deposited in GenBank (MT899925-
361 MT899930, MT769768-MT769774, MT832848-MT832891 and MT855968-MT855978). For GLR2, partial
362 sequences (5788 bp) from six samples, all from the cultivar Pinot Noir, were recovered and used for
363 the phylogenetic analysis. Four corresponding sequences from full genomes of GLR2 from China,
364 Brazil and Canada (BC) available in GenBank were aligned and used to construct a phylogenetic tree.
365 All our isolates clustered in the same group with isolates originating from China and BC (Canada).
366 Within this cluster, two subgroups were supported by a significant bootstrap value >70%.
367 Comparative analysis revealed a nucleotide (NT) identities in the range of 98.78% to 99.98% between
368 our isolates and those from BC (Canada, MH814500) and China (KU508672). Our isolates were
369 dissimilar to the isolates from Brazil (KX774192) and BC (Canada, MH814498) in terms of NT identity
370 and Jukes-Cantor distance (70.78% to 78.30% NT identity, Fig. 7A and B). For HSVd, seven whole
371 genomes (297–299 bp) from five samples were used for the phylogenetic analysis. Three full genomes
372 of HSVd from USA, China and India retrieved from GenBank were added for the alignment and the
373 construction of the phylogenetic tree (Fig. 7C). The phylogenetic relationship within HSVd isolates
374 could not be determined due to the low bootstrap support for the clustering (Fig. 7C). Comparative
375 analysis revealed that HSVd sequences were highly similar to the isolate from China (HM357802) in
376 terms of NT identity and Jukes-Cantor distance (97.66% to 100% NT identity, Fig. 7D) and slightly
377 dissimilar to HSVd isolate from citrus plant in USA in 1988 (NC001351). Overall, our HSVd full
378 genomes were highly similar to isolates from China, India and USA (92.01% to 100% NT identity)
379 (Fig. 7 C and D). For GRSP, a total of 14 sequences, 11 from our isolates (5244 bp) and three
380 corresponding sequences from GRSP full genome (one from USA and two from France) obtained
381 from GenBank, were aligned and used to construct a phylogenetic tree (Fig. 8). Two main well-
382 supported GRSP phylogenetic groups were detected in the analysis of our isolates. Most of our
383 isolates grouped into distant large subclusters. The first cluster was closely related (94.96% to 96.13%
384 NT identity) to the GenBank sequences of GRSP from France (MG938295) and USA (AY368590) (Fig.
385 8). The second cluster was phylogenetically diverse (79.85% to 92.95% NT identity) with some isolates
386 (BacPN4, DM85, BacMF6 and Co15_56J) closely related (89.84% to 92.29% NT identity) to the
387 GenBank sequence of GRSP isolate from France (MG938303). Presence of any GRSP phylo-groups
388 was not associated with grapevine cultivars (Fig. 8). Overall, all isolates of GRSP were in the same
389 Clade 1 with recombinant genomes of isolates from France (MG938295 and MG938303) and USA
390 (AY368590).

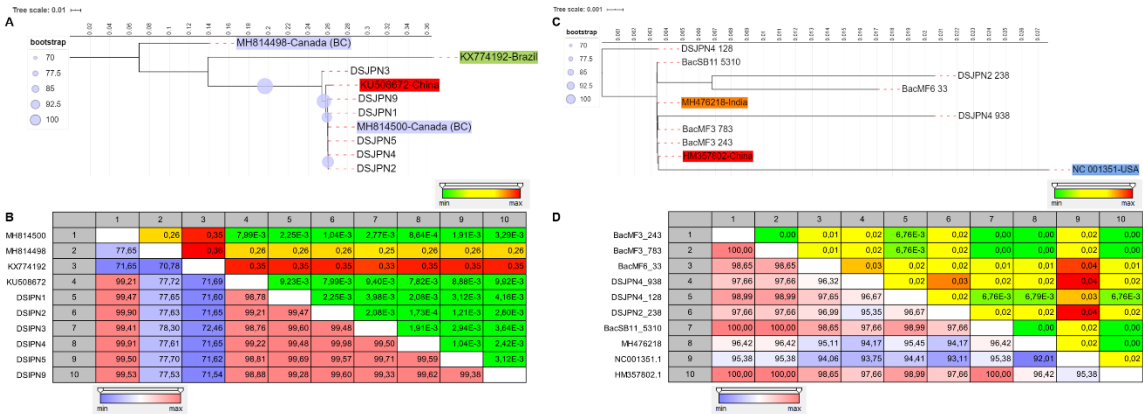



Figure 1. Maximum likelihood phylogenetic tree constructed from recovered consensus partial sequences of (**A**) grapevine leafroll-associated virus 2 (GLR2) and full genome of (**C**) hop stunt viroid (HSVd). Neighbor-joining construction method with 1000 bootstrap replicates was used. Branch length represents phylogenetic distances determined with distance matrices of nucleotide sequences. Purple dots above critical branches are significant bootstrap values (>70%). GenBank accession number of fully-sequenced genomes from different countries are highlighted for reference. Isolates from this study are represented and are not highlighted. The percent of identity (purple-red scale) and *Jukes-Cantor* distance (yellow-red scale) are presented for each pair of sequences of (**B**) GLR2 and (**D**) HSVd. The percent of identity is the proportion of identical residues in the alignment positions to overlapping alignment positions between the two sequences. The distance was calculated using the *Jukes-Cantor* distance between the two sequences and is the proportion between identical and overlapping alignment position between two sequences. R package phylogram and the interactive tree of life platform was used to generate the tree (see the materials and methods section for more details).

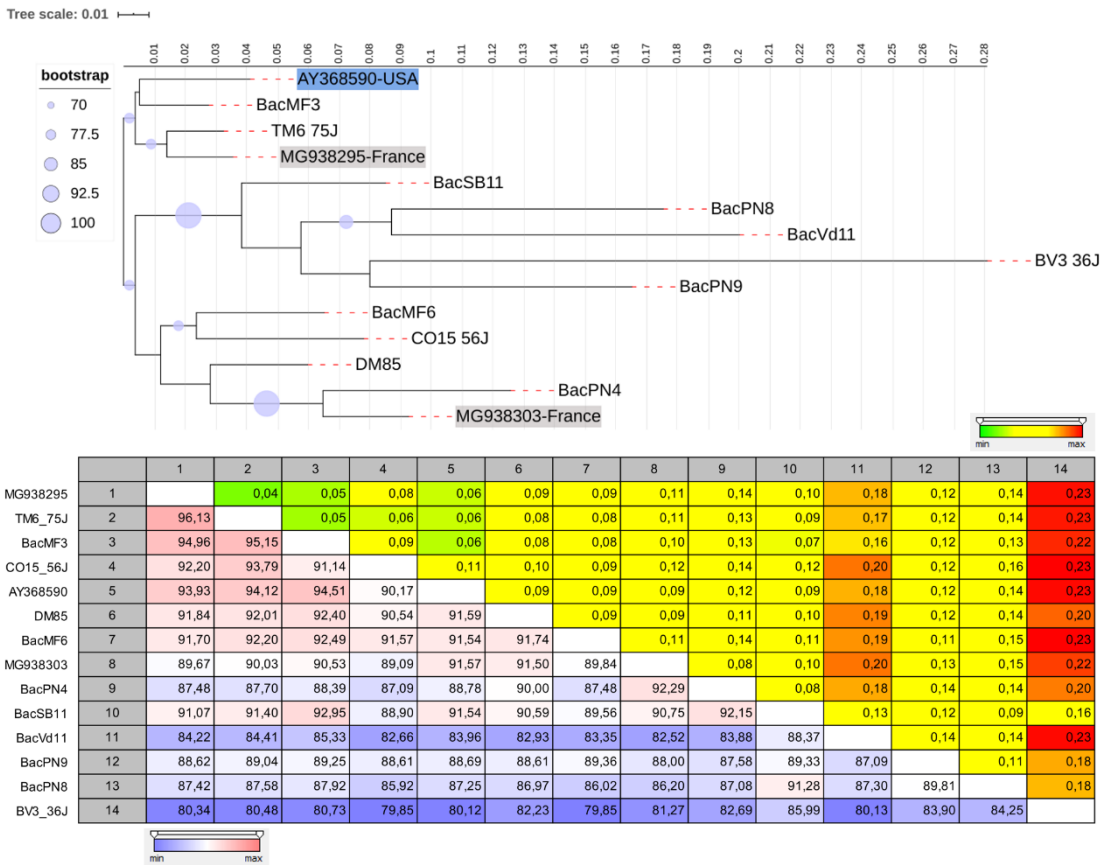



Figure 8. Maximum likelihood phylogenetic tree constructed from recovered consensus partial sequences of grapevine Ruspestris stem pitting-associated virus *(*GRSP*)*. Neighbor-joining construction method with 1000 bootstrap replicates was used. Branch length represents phylogenetic distances determined with distances matrices of nucleotide sequences. Purple dots above critical branches are significant bootstrap values (>70%). GenBank accession numbers of fully-sequenced genomes from different countries are highlighted for reference. Isolates from this study are represented and are not highlighted. The percent of identity (purple-red scale) and *Jukes-Cantor* distance (yellow-red scale) are represented for each pair of sequences of GRSP. The percent of identity is the proportion of identical residues in the alignment positions to overlapping alignment positions between the two sequences. The distance was calculated using the *Jukes-Cantor* distance between the two sequences and is the proportion between identical and overlapping alignment position between two sequences. . R package phylogram and the interactive tree of life platform was used to generate the tree (see the materials and methods section for more details)

4. Discussion

Growers' increasing experience and expertise in Northern viticulture and climate change represent an opportunity for major change in Quebec's grapevine industry by allowing a shift from traditionally-cultivated hybrid cultivars (e.g. interspecific hybrids Vidal) into cultivars with lower cold hardiness and disease tolerance, such as *Vitis vinifera* (Pinot Noir). However, this situation comes with potential risk including grapevine leafroll disease (GLD), one of the most economically-damaging grapevine diseases. Considering that the disease is latent in some grapevine hybrid cultivars (e.g. Vidal) and that these cultivars will continue to be grown, they can serve as a virus reservoir [14]. In this study, we characterized the virome of the interspecific hybrid cultivars and compared it to the virome of a *V. vinifera* cultivar to guide grapevine producers during the transition.

Grapevine leafroll-infected plants had a diverse virome in terms of composition, abundance and richness. Three viruses (GRSP, GLR3 and GLR2) and one viroid (HSVd) largely dominated the overall virome. However, their presence and abundance can be sorted by grapevine cultivars. Indeed, in the hybrid cultivar Vidal, GRSP dominated the virome with 61% of the viral population, and GLR3 and GLR2 represented 13% and 18% of the viral population, respectively, whereas in the cultivar Pinot Noir (*Vitis vinifera*), GLR2 dominated the virome with 43% of the viral population, and GRSP, GLR3 and HSVd represented 7%, 38% and 8% of the viral population, respectively. The virome composition was clustered by sampling site and not by grapevine cultivars. These results suggested that GLR2 and GLR3 were widely distributed in Quebec and in the cultivar Vidal even though the relative abundance of these viruses was lower in Vidal than in Pinot Noir. Therefore, Vidal cultivar could serve as a virus reservoir and play a key epidemiological role during the transition. In addition, Vidal cultivar did not express any notable visual symptoms despite the presence of both GLR2 and GLR3. Indeed, leafroll symptom expression had a significant positive association with the cultivar *Vitis vinifera* and a significant negative association with cultivar Vidal (*Vitis sp*. Hybrid). These results are supported by Beuve et al. [9] and Kovacs et al. [14], who noticed strong symptom expression associated with grapevine cultivar Pinot Noir and that the grapevine leafroll viruses are latent in Vidal cultivar. Therefore, from a strictly GLD disease management decision-making standpoint, Vidal should be classified with cultivars such as Thompson Seedless and Sauvignon Blanc, in which GLD is nearly impossible to detect visually [41]. When establishing new cultivars (e.g. *Vitis vinifera*), growers cannot rely only on clean planting materials   derived from virus-tested stocks. They should also test their vineyards for at least the presence of GLR (2 and 3), and the testing should particularly include the symptomless Vidal cultivar. In vineyards where it is not possible to randomly test enough Vidal plants, managers should consider maximizing the distance between new plantings and the existing grapevine plants to reduce potential GLD spread.

Most of the virus and viroid species detected, including GRSP, HSVd, grapevine syrah virus 1 (GSyV1) grapevine fleck virus and grapevine Red Globe, can be considered as part of the "background" virome. These background viruses and viroids are not primarily critical in the expression of virus-like symptoms [42]. Indeed, the symptom expressions are believed to be associated with additional infection of rarely-damaging viruses (GLR3, GLR2, grapevine pinot gris virus, grapevine red blotch, etc.) that disrupt the existing virome, leading to the expression of symptoms [42]. Our data show that viruses and viroids associated with the highest number of plants expressing leafroll symptoms (variables TNSL) were GRSP, HSVd, GLR3, GPG, GRB and GLR2, in gradient order. However, co-occurrence analysis revealed that the presence of GLR species (GLR2 and GLR3) was randomly associated with symptom development. This conclusion derived from analysis of 1452 points of comparison resulting from compilation of our data and data from five other published studies. In white grapevine cultivars such as Vidal, the symptoms are often subtle and difficult to diagnose, which can induce a bias in the co-occurrence analysis. However, even when the co-occurrence analyses were done without Vidal cultivar, the association between GLD virus species and symptom expression was still random. A possible explanation of this unexpected result may be that the link between GLR viruses (2 and 3) and the symptoms that have been demonstrated in many published studies is more likely related to the virus titer in a given plant than its presence or absence. Indeed co-occurrence analyses are based on presence and absence and do not consider the virus titer.

473 Hence, this is not an evidence of quantitative interaction between GLR viruses and symptom
474 expression [43]. Monis and Bestwick demonstrated that symptomatic leafroll-infected leaves of
475 grapevine had high titers of the virus and while undetectable virus titers was obtained from no
476 leafroll symptoms samples [44].

477 Because symptoms result from complex abiotic and biotic interactions [8], the role of abiotic
478 factors (e.g. weather) and the different genetic variants of GLR and other viruses and viroids that
479 were detected may also explain this unexpected result. In fact, association between GLR2 and
480 symptoms were linked with the phylogenetic clustering of the genetic variants [45], and Thompson
481 et al. [46] discovered a novel genetic variant of GLR3 that induces no foliar symptoms. However, we
482 did not have enough consensus sequences of GLR3 to study the phylogenetic diversity of GLR3
483 strains associated with our samples. The phylogenetic analysis of GLR2 strains associated with our
484 samples revealed a single monophyletic group that is highly similar to some isolates from BC and
485 also significantly dissimilar to others isolates from BC. As in BC, this monophyletic group was
486 associated with the cultivar Pinot Noir, which indicated possible infection via planting materials [16].
487 However, as highlighted by Poojari et al. [16], there is a need to understand the impact of GLR2
488 genetic variants in Canadian vineyards. In summary, more research is needed to shed light on GLD
489 symptom expression resulting from the interaction of different genetic variants of GLR (2 and 3) and
490 grapevine cultivars. Moreover, the role of genetic variability among strains of the most abundant
491 viruses/viroids of the background virome, such as GRSP and HSVd, should be studied. Indeed, our
492 isolates of GRSP were grouped into distant large clusters and were all classified in the same clade
493 (clade 1) with other recombinant genomes from France [47]. Until today, no published studies have
494 shown a relationship between strains from the four main clades of GRSP and symptom development
495 even though it is known that some strains of GRSP can cause symptoms on certain grapevine cultivar
496 (*Vitis Rupestris* cv. St. George) [48]. Implication of HSVd in symptom development, which is not yet
497 proven, cannot be related to the genetic variability among our HSVd strains because our HSVd
498 sequences were highly similar and no significant dissimilarities of our HSVd full genomes were
499 observed when compared to known grapevine HSVd. Nevertheless, as these two members of the
500 background virome are frequently observed in grapevine infected plants [9, 42, 49], more studies are
501 needed to understand the corpus of these viruses and their potential role in symptom expression.

5. Conclusions

503 This study highlights the complexity of the grapevine virome associated with leafroll-infected
504 plants of different cultivars. Independently of the grapevine cultivar, the plants were infected by
505 multiple viral and viroid species and different variants of the same virus. These results are supported
506 by Beuve et al.. However, these authors described this complex virome only on the cultivar Pinot
507 Noir [9]. The symptomless hybrid cultivar Vidal presents a complex virome which, in contrast, was
508 dominated by grapevine Rupestris stem pitting-associated virus despite the presence of grapevine
509 leafroll disease (GLD) virus species. Therefore, removing and destroying of symptomatic grapevines
510 and possibly vines immediately adjacent to those symptomatic grapevines, which is proven to be
511 efficient to control GLD spread [8, 50, 51], will not be effective in vineyards where Vidal grapevines
512 are grown because this latter will hamper the management efforts. During the transition and
513 establishment of new less winter hardy cultivars such as *Vitis vinifera* (e.g. Pinot Noir) in Quebec,
514 growers cannot rely only on certified material derived from virus-tested stocks, they also need to
515 periodically test their vineyards for at least the presence of GLR viruses (2 and 3), and the testing
516 should particularly include the symptomless Vidal cultivar.

517 **Supplementary Materials:** The following are available online at www.mdpi.com/xxx/s1, **Figure S1**:
518 Discriminant principal component analysis of the virome and association between detected viruses and the
519 mean proportion of viral reads that mapped for a given virus (MPVR), total number of symptomatic leaves
520 (TNSL) associated with a given virus, mean depth (MD), mean depth relative to the depth of the positive control
521 virus (MDRC), mean relative abundance (MRA), mean weight, and genome size (GS). Showing groups of species
522 that induce similar response. **Table S1**: Co-occurrence table displaying association between all events. **Table S2:**
523 GenBank accession numbers of *Grapevine leafroll-associated virus* 2 and their nucleotide positions of sequences

524 used for generation of concatenated sequences in this study. **Table S3**: GenBank accession numbers of *Grapevine*
525 *rupestris stem pitting-associated virus* and their nucleotide positions of sequences used for generation of
526 concatenated sequences in this study.

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528 formal analysis, M.L.F., D.X. and P.L.; investigation M.L.F., D.X. and P.L.; resources, D.X., P.L. and M.L.F.; data
529 curation, M.L.F. and D.X.; writing—original draft preparation, M.L.F.; writing—review and editing, M.L.F., P.L.,
530 D.X., I.E.B.M, O.C. and C.B.; visualization M.L.F. and D.X.; project administration, M.L.F; funding acquisition,
531 M.L.F., O.C. and B.C. All authors have read and agreed to the published version of the manuscript.

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542 relationships that could have appeared to influence the work reported in this paper.

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