



Determination of the grapevine virome by high-throughput sequencing and grapevine viruses detection in Serra Gaúcha, Brazil

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ABSTRACT

Among grapevine (*Vitis* spp.) pathogens, viruses stand out, considering that this fruit plant is broadly susceptible to them and that they cause several diseases, reducing quality and yield of grape production. However, detecting and identifying viral infections in grapevines can be challenging. The objectives of this study were to detect grapevine viral pathogens by high-throughput sequencing (HTS) and identify grapevine viruses associated with plant propagation material from different origins. A comprehensive picture of a vine exhibiting virus-related symptoms was generated. The detected virome included four different pathogens and their variants. In addition, the incidence of seven viral pathogens was determined in samples collected in three vineyards of the main grape-growing region of Brazil. It is suggested that the observed infections could be related to the technological level of the production system adopted by these grape growers.

Keywords: RT-qPCR; HTS; NGS; *Vitis*; indexing; survey.

INTRODUCTION

The sanitary status of the grapevine (*Vitis* spp.) is determinant for growth, productivity of the grapevine and the quality of grapes. Parameters such yield and grape quality are heavily affected by viral pathogens. As a consequence, adequate diagnosis and identification of viruses is of fundamental relevance for efficient plant protection (Zherdev *et al.*, 2018).

Grapevine leafroll disease (GLRD) is widespread worldwide wherever grapes are grown and it is known as the most damaging infectious dysfunction of grapevines. The disease is often caused by a mixed infection of a complex of several leafroll-causing virus species (family *Closteroviridae*), being *Grapevine leafroll-associated virus-3* (GLRaV-3), genus *Ampelovirus*, the most prevalent and widely spread (Naidu *et al.*, 2015). Grapevine rugose wood disease (GRWD) is a complex disease that occurs worldwide which is associated with several syndromes including rupestris stem pitting; kober stem grooving; corky bark and LN33 stem grooving. Among the viral

pathogens associated with the disease, *Grapevine rupestris stem pitting-associated virus* (GRSPaV) and *Grapevine viruses A and B* (GVA and GVB), genera *Foveavirus* and *Vitivirus*, respectively, stand out as causative agents (family *Betaflexiviridae*) (Basso *et al.*, 2017). So far, *Grapevine yellow speckle viroid 1* (GYSVd-1), *Hop stunt viroid* (HSVd), and *Citrus exocortis viroid* (CEVd) have been detected in diseased grapevines in Brazil (Fajardo *et al.*, 2016; 2018). These viroids are worldwide distributed, infecting a large number of host species besides vine, except for GYSVd-1, which is restricted to grapevines. Finally, *Grapevine fleck virus* (GFkV) is a phloem-limited, positive-sense, single-stranded RNA virus belonging to the genus *Maculavirus*, family *Tymoviridae*, present worldwide and known to be associated with the grapevine fleck disease complex (Fajardo *et al.*, 2017b).

The recent improvements of nucleotide sequencing technologies, such as high-throughput sequencing (HTS), has generated great advances in prospecting for viral pathogens, including the discovery of novel viruses infecting plants. Its main advantages are sequencing

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millions of reads in a random massive parallel sequencing strategy; to allow the identification of the viruses infecting the host, including divergent viral variants, which could be more difficult to be detected using standard molecular techniques and, disease complexes; the capacity to promote detection of pathogens that are not pre-established targets of indexing and also of those pathogens whose nucleotide sequences are not available (Fajardo *et al.*, 2017b). The advent of HTS represents a great advancement for the study of grapevine viruses, given the characteristic features of these agents in terms of the difficult transmission of most of them to herbaceous plants, their usually very low titers in grapevines, and the difficulties in processing grapevine woody tissues (Saldarelli *et al.*, 2017). The implementation of HTS for diagnosis, that does not require prior information on the virus, is important for the sensitivity, comprehensiveness and reliability features attributed to the results.

The determination of virus identity that infect grapevines is important for etiology, but also because this achievement will result in more detailed knowledge of the pathogen and therefore more precise and accurate detection methods can be developed and adopted. In this context, many grapevine viruses have been already biologically, serologically and molecularly characterized in Brazil (Basso *et al.*, 2017). Grapevine disease control is inevitably based on determining the sanitary status of the vines. Since many grapevine viruses are transmitted by several types of vectors (nematodes, mealybugs, mites, among others), the new vineyards should be established with certified plant propagation material to prevent against disease spread. Since it is impossible to restore plant health of a virus-infected plant in the field, prophylactic measures such as the use of healthy plant propagation material during planting has outstanding importance (Basso *et al.*, 2017). The objectives of this study were to detect grapevine viruses by high-throughput sequencing (HTS) and identify grapevine viruses associated with plant propagation material of different origins.

MATERIAL AND METHODS

HTS

To achieve a more comprehensive picture of the hybrid grapevine cv. BRS Nbia virome, an adult plant exhibiting severe virus-related symptoms i.e. down rolling and reddening of leaves with the green main veins and coriaceous leaves in early fall (Figure 1), maintained in the experimental field at Embrapa Grape & Wine in Bento Gonalves, RS, Brazil, was analysed by high-throughput sequencing (HTS).

Double-stranded RNAs (dsRNA) were extracted from 30 g of bark scrapings of mature grapevine canes. The

resulting supernatant (nucleic acid solution) was enriched for dsRNA using Whatman CF11 cellulose affinity purification (chromatography) (Valverde *et al.*, 1990). The complementary DNA library was constructed utilizing TruSeq Stranded mRNA kits (Illumina, USA) according to the manufacturer's instructions. After quantification and qualification during the quality control step, the sample library was sequenced on the BGISEQ-500 platform (paired-end reads). Sequencing was performed using a service from Beijing Genomics Institute (BGI, China). CLC Genomics Workbench v. 8.5.1 (CLC Bio, Qiagen, USA) was used for quality trimming and *de novo* contig assembly of the reads. All contigs were annotated by BLASTn and BLASTx (<http://www.ncbi.nlm.nih.gov/blast>) against the nr or protein GenBank databases using a cut-off e-value of 10^{-4} .

Nucleotide sequences obtained by HTS were deposited in the GenBank and aligned with the reference sequences of the viruses as well as with some of the most similar sequences among the homologous viruses available in the GenBank using the NCBI's Blastn program (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The GenBank accession codes of the nucleotide sequences of the isolates used for the homology analysis are shown in Table 1 and Figure 2. Multiple nucleotide (nt) sequence alignments were performed using ClustalX 2.1 program. Phylogenetic relationships were determined from the aligned sequences by the neighbor-joining (NJ) method using Kimura 2-parameter method with gamma distribution (G) and 1,000 bootstrap replications implemented in Molecular Evolutionary Genetics Analysis (MEGA 7.0.21) software package.

Virus survey

A total of thirty-six grapevine plants cvs. Nigara Branca, Chardonnay and BRS Carmem, exhibiting virus symptoms or symptomless (Figure 1), were analysed for seven pathogens [*Grapevine leafroll-associated virus 3* (GLRaV-3), *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), *Grapevine fleck virus* (GFkV), *Grapevine rupestris stem pitting-associated virus* (GRSPaV), *Grapevine leafroll-associated virus 4* (GLRaV-4) and *Grapevine yellow speckle viroid 1* (GYSVd-1)] by real-time RT-PCR (RT-qPCR) (Dubiel *et al.*, 2013) and additionally by conventional RT-PCR for *Hop stunt viroid* (HSVd). Twelve samples per vineyard were analysed: two asymptomatic samples, two samples with virus-like symptoms and eight randomly collected samples. The complementary information about the three sampled vineyards (V) were: **V1:** scion cultivar (cv.) Nigara Branca (17 years old), undefined rootstock; **V2:** cv. Chardonnay (one year old), rootstock cv. 1103P, and **V3:** cv. BRS Carmem (two years old), rootstock cv. 1103P,

respectively in pergola, trellis and pergola training systems. The three areas sampled in municipalities of Serra Gaúcha, Rio Grande do Sul State, Brazil, in March 2019 were: vineyard 1 - Farroupilha; vineyard 2 - Santa Tereza and vineyard 3 - Pinto Bandeira.

Real time and conventional RT-PCR

Total RNA extractions were performed using the adsorption of nucleic acids on silica particles from 1 g of petioles or veins of leaves or scrapings of mature stems, grinding plant tissues in liquid nitrogen (Dubiel *et al.*, 2013). RT-qPCR reactions (one step RT-qPCR) were carried out in 96-well plates using the thermocycler StepOnePlus Real-Time PCR System (Applied Biosystems) and the kit TaqMan Fast Virus 1-Step Master Mix (Applied Biosystems) following the manufacturer's recommendations and as described by Fajardo *et al.* (2016). The reaction data were analysed as presence/absence assays and graphically, using the StepOne Software v2.3 (Applied Biosystems), by determining the Cq (quantification cycle). Specific primers and TaqMan TAMRA probes used for viruses detections by RT-qPCR were previously described (Osman *et al.*, 2007; Dubiel *et al.*, 2013).

The presence of all viruses/viroids identified by HTS was confirmed in the cv. BRS Núbia sample by conventional RT-PCR just as performed for HSVd detection in the field survey. The RT-PCR was carried out using OneStep RT-PCR kit (Qiagen) and reactions were performed according to the manufacturer with 4 µL (ca. 400 ng) of total RNA. Specific primers for GLRaV-3, GRSPaV, GYSVd-1 and HSVd used for pathogen detections by RT-PCR were previously described (Basso *et al.*, 2010; Fajardo *et al.*, 2016; 2018).

RESULTS AND DISCUSSION

HTS

Four pathogens [*Grapevine leafroll-associated virus 3* (GLRaV-3), *Grapevine rupestris stem pitting-associated virus* (GRSPaV), *Grapevine yellow speckle viroid 1*

(GYSVd-1) and *Hop stunt viroid* (HSVd)] were disclosed by HTS in cv. BRS Núbia exhibiting virus-like symptoms thus confirming the broad capacity of the technique in detecting pathogens from different taxa in a concurrent infection. This complex symptomatology hampered the visual diagnosis. The presence of these pathogens infecting the analysed plant material was confirmed by conventional RT-PCR. Complete or near-complete genomes corresponding to these pathogens were obtained and deposited at GenBank: GLRaV-3 complete genome, GLRaV-3.NUB-BR isolate, 18498 nucleotides (nt), MK804765; GRSPaV, three distinct isolates corresponding to nearly complete genomes, lacking only 30-36 nucleotides in the translatable portion of the genome (5' ORF1 - coding for replicase): NUB1-BR, 8627 nt, MK804766; NUB2-BR, 8633 nt, MK804767 and NUB3-BR, 8665 nt, MK804768; GYSVd-1 complete genome, GYSV1.NUB-BR isolate, 366 nt, MK804769 and HSVd complete genome, HSV.NUB-BR isolate, 298 nt, MK804770. Referring to the virus populations in grapevines, Saldarelli *et al.* (2017) considered the "concept of a virome" more appropriate to characterize the presence of multiple infections as a result of viticultural procedures that lead to accumulation of virus and viroid pathogens in connection with the long-term exposure of the grapevine to these pathogens.

According to Saldarelli *et al.* (2017), grapevine plants harbor a core assembly of viruses and viroids which they call the "background" virome, consisting of GRSPaV, HSVd, GYSVd-1 as well as some viral species belonging to the genera *Maculavirus* and *Marafivirus*. As a result, these authors consider a "virus-free" grapevine as an inappropriate naming. In this perspective, the presence of this core group of pathogens, although probably involved as co-determinants in a synergistic system, is irrelevant and not determinant of disease etiology. Disease itself is triggered by the really damaging viral species that disrupt the balance of the "natural" virome present in the plant. In fact, previous surveys with samples of grapevines collected in Brazil detected high incidences of these "background" viruses: up to 80% GRSPaV (Catarino *et*



Figure 1: Grapevine samples collected and evaluated: (a) hybrid grapevine cv. BRS Núbia exhibiting virus-related symptoms; symptomatic vines (b) cv. Niágara Branca and (c) cv. Chardonnay and, (d) asymptomatic cv. BRS Carmem.

Table 1: Information about sequences of GLRaV-3, GRSPaV, GYSVd-1 and HSVd included in the study and available in GenBank and nucleotide identities among them

Virus species and Brazilian isolates	Isolate	Country	Genome length (nucleotide)	GenBank accessions (genome)	Genome nucleotide identity between isolates	
GLRaV-3 (GLRaV-3.NUB-BR)	Kat255	USA	18498	MH521108	99.5%	
	WA-MR	USA	18498	GU983863	99.4%	
	LN3204	USA	18498	MH521112	99.4%	
	GLRaV3-ID46	USA	18498	MH796135	99.4%	
	3138-07	Canada	18498	JX559645	99.4%	
	Bla223	USA	18498	MH521090	99.4%	
	Cha106	USA	18497	MH521093	99.3%	
	reference sequence	USA	17919	NC_004667	97.8%	
GRSPaV (NUB1-BR)	35 clone 4	France	8728	MG938308	98.4%	
	1050-02	Canada	8716	JX513892	98.4%	
	AMCF clone 2	France	8735	MG938310	98.3%	
	P70	France	8726	MG925334	98.2%	
	34 clone 2	France	8691	MG938304	98.2%	
	-	USA	8725	AF026278	98.1%	
	-	USA	8744	AF057136	98.1%	
		reference sequence	USA	8744	NC_001948	98.1%
		NUB2-BR	Brazil	8633	MK804767	79.7%
	NUB3-BR	Brazil	8665	MK804768	79.5%	
GRSPaV (NUB2-BR)	Clone 2	France	8725	MG925335	92.3%	
	LSL	China	8754	KR054735	92.3%	
	35 clone 2	France	8676	MG938306	92.0%	
	EVC42	France	8716	MG938332	80.1%	
	EVB47	France	8726	MG938328	80.1%	
	EVC60	France	8713	MG938340	80.1%	
	EVC53	France	8716	MG938335	80.1%	
		reference sequence	USA	8744	NC_001948	79.7%
		NUB3-BR	Brazil	8665	MK804768	79.0%
GRSPaV (NUB3-BR)	VD-102	Croatia	8706	MF979534	97.7%	
	34 clone 1	France	8706	MG938303	93.7%	
	strain Syrah	USA	8742	AY368590	93.3%	
	SWTi clone 2	France	8723	KX035000	92.5%	
	SWT6 clone 1	France	8741	KX034997	92.5%	
	MBE10 clone 2	France	8737	MG938343	92.5%	
	SWT2 clone 2	France	8741	KX034991	92.4%	
		reference sequence	USA	8744	NC_001948	79.7%
GYSVd-1(GYSV1.NUB-BR)	Cari 2-4	Tunisia	366	AF462162	92.8%	
	UDSV1-4	Nigeria	366	MF576400	92.6%	
	VI-BR	Brazil	366	KU668664	92.5%	
	DgSV1-8	Nigeria	366	MF576407	92.3%	
	Cari 1-1	Tunisia	366	AF462167	92.3%	
	variant VIII	-	366	X87922	92.3%	
	Cari 2-5	Tunisia	367	AF462161	92.3%	
		reference sequence	-	366	NC_001920	83.0%
HSVd (HSV.NUB-BR)	VL-TC	Brazil	298	MG431974	99.3%	
	VV-CF	Brazil	298	MF774875	99.3%	
	VV-CG	Brazil	298	MF774872	99.3%	
	VV-IT	Brazil	298	MF774871	99.3%	
	VV-CS	Brazil	298	MF774862	99.3%	
	cultivar Riesling	-	298	X06873	99.3%	
	HSVd.PN.12	Hungary	298	GQ995466	99.0%	
		reference sequence	-	302	NC_001351	94.4%

al., 2015), 71.4% GYSVd-1 (Fajardo *et al.*, 2016) and, 94% HSVd (Fajardo *et al.*, 2018). In the present study, the “background” virome (GRSPaV, HSVd and GYSVd-1) and a damaging virus (GLRaV-3) were detected, of which the latter could be the causal agent of the observed leafroll symptoms in the sampled vine (Figure 1). Thus, we conclude, our results are in agreement with the above information.

The highest nucleotide identities among the studied Brazilian virus and viroid isolates and some homologous isolates retrieved from GenBank ranged from 92.0% to 99.5% allowing unambiguous identification of the pathogens that were infecting the grapevine cv. BRS Nbia (Table 1). In addition, the phylogenetic analysis of nearly complete

genomes (Figure 2) highlighted the identification of three widely variable isolates of GRSPaV showing 79.0% to 79.7% nucleotide identities among them (Table 1). Considering that the capsid protein gene is usually conserved because it encodes a structural protein, genetic variability studies based only on this gene can not allow to fully explore the existing variability between isolates (Moura *et al.*, 2018). Fajardo *et al.* (2017a) reported the occurrence of three molecular variants of GRSPaV infecting different grapevine genotypes in Brazil based on the analysis of the viral capsid protein nucleotide sequences. On the other hand the present study demonstrates, based on partial genome variabilities, the occurrence of three different GRSPaV isolates in one single plant whereas Fajardo *et al.* (2017a)

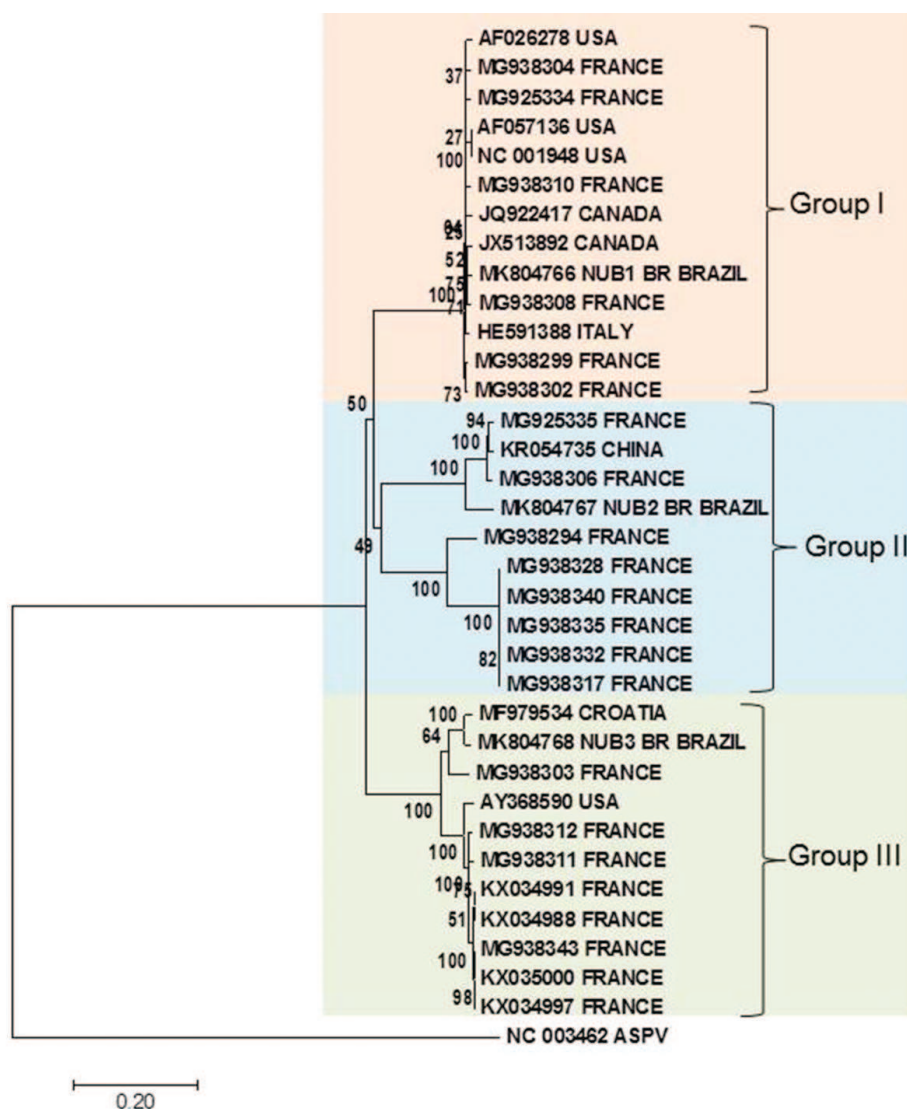


Figure 2: Phylogenetic relationship among GRSPaV isolates based on the multiple alignment of the complete genome or near-complete genome nucleotide sequences of three isolates from Brazil and other isolates retrieved from the GenBank. The tree was constructed by the neighbor-joining method using Kimura 2-parameter with gamma distribution (G) and 1,000 bootstrap replications implemented using MEGA 7.0.21 software package. Accessions of GRSPaV and origins were included according to the GenBank. Phylogenetic clusters (groups) are indicated. Apple stem pitting virus (ASPV) was used as outgroup. Bar: number of nucleotide substitutions per site.

demonstrated the presence of up to two different isolates (variants) in the same plant (genotype). The occurrence of different isolates, variant sequences, strains or groups of GLRaV-3, GYSVd-1 and HSVd infecting grapevines was previously reported (Fajardo *et al.*, 2016, 2017b, 2018, Moura *et al.*, 2018). This study may contribute to expand the understanding of the variability and diversity of these viral/viroidal species in Brazil, although this was not specifically the focus of this study, and to elucidate the meaning of symptom expression considering the grapevine genotype and the pathogens/strains present in the pathosystem. Another contribution of this study was that by determining the nucleotide sequences of local viral isolates, diagnostic tools can be customized to detect these pathogens, *e.g.* RT-PCR primers or probes could be designed specifically for the detection of these isolates, thus increasing the probability of reliable diagnosis.

HTS allowed obtaining of complete or near-complete genomes of four viral and viroidal pathogens, including different isolates in a single sequencing step. The application of HTS, for this aim, overcomes the eventual difficulties of conventional RT-PCR amplifications of several DNA fragments, which require extensive cloning and sequencing of recombinant clones to obtain complete viral genomes (Souza *et al.*, 2017). In this study, the complete translatable nucleotide sequences of viruses such as GLRaV-3, one of the largest genomes among plant-infecting viruses, have been obtained. The high contents of closteroviruses usually found in metadata produced from dsRNA could be determined by the fact that many RNA viruses, as closteroviruses, show a higher tendency to accumulate double-stranded replicative intermediate forms of RNA (Saldarelli *et al.*, 2017) as compared to other viruses.

The restriction on the use of a particular molecular diagnostic test lies on the fact that it is only directed to the detection of pathogens with prior known nucleotide sequences and to specific pathogens that would be predefined reaction targets. This makes it impossible to detect pathogens with unknown nucleotide sequences or genomes, pathogens with sequences that vary from the standard isolate or that are not predefined detection targets. These three limitations are overcome by HTS as an investigation tool (Saldarelli *et al.*, 2017) as also demonstrated in this study. As the grapevine can be simultaneously infected by several viral pathogens, the expression of symptoms would not be elucidative, and thus, the selection of probable pathogens that would need to be tested as etiological agents of the disease would become an impaired task (Basso *et al.*, 2017). Therefore, HTS is an extremely relevant and interesting option to apply since it allows to determine the full range of pathogens present in a diseased plant (Villamor *et al.*, 2019).

Additionally, the information generated with this technique can be used to define more efficient strategies for virus control and management.

Virus survey

The TaqMan RT-qPCR and conventional RT-PCR were sensitive, specific and reliable tests to evaluate the presence of the six viruses and two viroids in a limited survey of field-sampled vines. The average rates of viral and viroidal infections considering 12 samples/vineyard were 61.4% (V1), 37.4% (V2) and 53.1% (V3). The observed incidences (%) of each pathogen in each vineyard (V) were as follows: **V1:** 75% (GLRaV-3), 66.6% (GVA), 33.3% (GVB), 91.6% (GFkV), 66.6% (GRSPaV), 91.6% (GYSVd-1), 66.6% (HSVd); **V2:** 25% (GLRaV-3), 16.6% (GVA), 0% (GVB), 16.6% (GFkV), 75% (GRSPaV), 91.6% (GYSVd-1), 75% (HSVd) and; **V3:** 75% (GLRaV-3), 33.3% (GVA), 50% (GVB), 100% (GFkV), 0% (GRSPaV), 100% (GYSVd-1), 66.6% (HSVd). None of the evaluated samples were infected with GLRaV-4. These results confirm other results reporting high incidence of these pathogens in vineyards evaluated in other grape-growing regions of Brazil (Catarino *et al.*, 2015; Moura *et al.*, 2018). Probably, the infection levels found are determining detrimental effects in the yield potential and product quality (reductions in total soluble solid content and berry color intensity) of the three evaluated cultivars and vineyards as reported when healthy vines were compared to those with viral infections (Mannini & Digiario, 2017), although this issue was not evaluated in this study.

Multiple viral and viroidal infections were frequent. Infection rates by pathogens were increasing considering the origin of the grafted-vine cuttings (V2: imported grafted-vine cuttings with official certification; V3: grafted-vine cuttings purchased in Brazilian commercial nursery that adopts certain practices of phytosanitary improvement, and V1: grafted-vine cuttings produced by the grape grower with his own plant propagation material, without any certification or without adoption of cultural practices that could significantly contribute to the improvement of the vineyard phytosanitary condition) suggesting that the origins of the grafted-vine cuttings may have influenced the determined virus incidences. In other words, the incidence of viruses would have varied according to the level of technology adoption by the grape growers, specifically regarding the phytosanitary quality of the grafted-vine cuttings used for the establishment of vineyards. This is an inference because the evaluated plants had been in the field for different times after planting and in this condition could have suffered viral reinfection to a greater or lesser extent. Also, the viral incidence increased with the age of vineyards suggesting transmission of some evaluated pathogens

by vectors, for *e.g.* mealybugs and scale insects that can transmit GLRaV-3, GVA and GVB; mechanical transmission occurrence (in the case of viroids) or dissemination through vegetative propagation using infected material (in the case of viruses that do not have vectors: GFkV and GRSPaV) (Catarino *et al.*, 2015; Fajardo *et al.*, 2016). These possibilities could be acting in the evaluated vineyards over time. Asymptomatic samples [in particular American grapevine (*V. labrusca*) cv. Niágara Branca or hybrid cv. BRS Carmem] were also infected similarly to symptomatic vinifera vines. Thus, there was no evident difference in viral infection incidences considering asymptomatic and symptomatic samples (data not shown). This is relevant because asymptomatic plants can function as indistinguishable viral inoculum source and even they may have their quality and yield of production negatively affected by viral infection, but usually at lower levels than those verified in symptomatic plants (Basso *et al.*, 2010; Mannini & Digiario, 2017). Taken together, the information generated on viral incidence, even though limited, could be useful for the management of viruses in the evaluated regions, avoiding further dissemination and dispersion.

The HTS is the most sensitive and comprehensive diagnostic tool for the study of undiagnosed viral diseases presently available; it is very cost effective compared to the traditional detection methods and allows to investigate viral genome diversity, virus-host interaction and virus epidemiology (Saldarelli *et al.*, 2017; Villamor *et al.*, 2019). Also, the determination of the pathogen incidence in certain regions represents relevant information that enables the proposition of more efficient management and control techniques, including the determination of the actual phytosanitary status of grafted-vine cuttings used by growers for the establishment of new vineyards.

CONCLUSIONS

The use of high-throughput sequencing to determine the total viral content of a sample resulted in the identification of a complex disease, including viruses, viroids and their strains in the analysed grapevine. Thus, HTS was a very sensitive, comprehensive and accurate diagnostic tool, allowing to investigate thoroughly grapevine viral genome diversity and constituted a reliable tool to assess the phytosanitary status, overcoming some limitations encountered in grapevine virus diagnosis.

The phytosanitary status determination which consisted of identification of viral pathogens associated with grapevines in vineyards contributed to understand some aspects related to the disease (*e.g.* virus transmission through vegetative propagation of infected grapevines, virus epidemiology). In this perspective it is an appropriate approach to target virus control and management.

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