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First report of *Hop stunt viroid* infecting *Vitis gigas*, *V. flexuosa* and *Ampelopsis heterophylla*

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Abstract

Hop stunt viroid (HSVd) is one of the most common viroids that infect grapevine (*Vitis* spp.) worldwide. Sixteen sequences of the HSVd genome were obtained from infected grapevines in Brazil by next generation sequencing (NGS). Multiple alignments of the sequences showed nucleotide identities ranging from 94.6% to 100%. This is the first report of HSVd infecting two wild grape species and *Ampelopsis heterophylla*. These HSVd isolates along with others from *V. vinifera* and *V. labrusca* were phylogenetically analyzed.

Keywords Next generation sequencing · HSVd · Incidence · Genetic variability · *Vitis*

Grapevine (*Vitis* spp.) is a globally important fruit crop considering its socioeconomic importance and cultivated area. Among graft-transmissible grapevine pathogens, viruses and viroids can reduce plant vigor, yield, productivity and fruit quality. Losses are especially significant in mixed infections (Basso et al. 2017). Viroids are naked, non-protein-coding, small (246–401 nt) covalently closed, circular single-stranded RNAs that adopt a compact folding due to their high self-complementarity. Despite being self-replicating plant pathogens, viroids depend on the interaction with host factors to complete their infection cycle (Flores et al. 2016; Steger and Perreault 2016). Distinguishing features such as secondary structure, replication site, rolling circle mechanism, ribozyme activity, and host range, help determine viroid classification and taxonomy (Flores et al. 2015; Lee et al. 2015). *Hop stunt viroid* (HSVd), one of the most common viroids worldwide, is the type species of the genus *Hostuviroid*, family *Pospiviroidae* (Steger and Perreault 2016). Its complete genome, consisting of 297 nucleotides (nt), was first sequenced by Ohno et al. (1983). HSVd has a wide range of hosts including trees, shrubs and herbaceous plants, with the majority of isolates to date identified from citrus species, followed by grapevine and stone fruits (*Prunus* spp.). HSVd causes disease symptoms, such as hop stunt, dappled fruits in plum and peach trees, and citrus cachexia (Jo et al. 2017). The viroid can be transmitted vegetatively, mechanically, or via grape seeds (Wan Chow Wah and Symons 1999).

HSVd, *Citrus exocortis viroid* (CEVd), and *Grapevine yellow speckle viroid 1* (GYSVd-1) have been detected in diseased grapevines in Brazil (Eiras et al. 2006; Fajardo et al. 2016). Information about variability of HSVd isolates from Brazilian grapevines is limited (Eiras et al. 2006). Therefore, the objective of the current research was to analyze the transcriptome data generated from commercial and wild grapevine genotypes using next generation sequencing (NGS), and subsequently characterize the HSVd genomes.

Seventeen genotypes were sampled (one sample/genotype) from three grapevine collections, maintained by Brazilian research institutions in the States of Rio Grande do Sul, São Paulo, and Pernambuco, Brazil. The evaluated *V. vinifera* plants were symptomatic, exhibiting general virus-like leaf symptoms; other genotypes were asymptomatic (Table 1). HSVd isolates were characterized in enriched dsRNAs extracted from 30 g of bark scrapings per sample using CF11 cellulose. Sequencing data was generated from a complementary DNA library, constructed by Macrogen (Korea) or Eurofins (USA). The Illumina HiSeq2000 platform was used to generate the paired-end reads. CLC Genomics Workbench software was used for quality trimming, and de novo contig

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Table 1 Sequences of HSVd obtained by NGS in the current study

Sample number	State (Brazil)	Host species	Cultivar	Length (nt)	GenBank accession	Isolate name
1 M	RS	*Vitis vinifera*	Cabernet Sauvignon	298	MF774862	VV-CS
2 M	RS	*V. flexuosa*	wild grapevine	298	MF774863	VF
1	RS	*V. labrusca*	Isabel	298	MF774864	VL-IS1
2	RS	*Ampelopsis heterophylla*	synonym *Parthenocissus heterophylla*	298	MF774865	AH
3	SP	*V. labrusca*	Isabel	301	MF774866	VL-IS2
4	SP	*V. gigas*	wild grapevine	301	MG431973	VG
5	SP	*V. vinifera*	Red Meire	298	MF774867	VV-RM
6	SP	*V. vinifera*	Muscat of Hamburg	299	MF774868	VV-MH
7	PE	*V. vinifera*	Syrah	297	MF774869	VV-SY
8	PE	*V. vinifera*	Tempranillo	297	MF774870	VV-TE
9	RS	*V. tillifolia*	wild grapevine		–	not detected
10	RS	*V. vinifera*	Italia	298	MF774871	VV-IT
12	RS	*V. vinifera*	CG90450	298	MF774872	VV-CG
16	RS	*V. vinifera*	Semillon	297	MF774873	VV-SEM
17	RS	*V. labrusca*	Tardia de Caxias	298	MG431974	VL-TC
18	RS	*V. vinifera*	Trajadura	297	MF774874	VV-TRAJ
19	RS	*V. vinifera*	Cabernet Franc	298	MF774875	VV-CF

Legend: Brazilian states are designated as RS (Rio Grande do Sul), SP (São Paulo), and PE (Pernambuco)

assembly of the reads. All contigs were analyzed using NCBI Blast algorithm (http://www.ncbi.nlm.nih.gov/blast) against the viral and viroidal RefSeq databases. Subsequently, all contigs identified as HSVd were individually analyzed using Blastn against the GenBank database (Fajardo et al. 2017).

Multiple sequence alignments of nucleotides (nt) were performed using ClustalX 2.1 and a pairwise nucleotide sequence identity matrix was generated using Sequence Demarcation Tool version 1.2 (SDT v1.2). The matrix generation of nt identities was performed using BioEdit v.7.2.5 software. The 16 sequences obtained were aligned among themselves, and with three previously characterized Brazilian HSVd isolates (Eiras et al. 2006) and two reference grapevine HSVd isolates, retrieved from GenBank (X06873 and X87927). The accession numbers of the nt sequences used for phylogenetic analysis are presented in Table 1 and Fig. 1a, b. Phylogenetic relationships were determined from the aligned sequences by using neighbor-joining (NJ) method, using Kimura 2-parameter with gamma distribution (G) and 5000 bootstrap replications implemented in Molecular Evolutionary Genetics Analysis (MEGA 7.0.21) software package.

To confirm the results obtained by NGS, the 17 samples (grapevines and *Ampelopsis*) (Table 1) were resampled for total RNA extractions, using the adsorption of nucleic acids on silica particles from 1 g of tissue that had been ground in liquid nitrogen. The primer pairs used to amplify HSVd by one-step RT-PCR were HSVd (+) and HSVd (−) (Eiras et al. 2006) or VP1181 (5'AATTCTCGAGTTGCCGCAACA3') and VP1182 (5'CACAGTCGACAGGGGCTCAA GAGAGGATC3'), viral and complementary, respectively (personal communication by J.A. Sánchez-Navarro). The RT-PCR was performed using the One Step RT-PCR kit (Qiagen), according to the manufacturer instructions, with 4 μL of total RNA (ca. 400 ng). RT-PCR reactions and amplification cycling, amplified DNA analysis, elution of DNA fragments, cloning, purification of recombinant plasmids from *Escherichia coli* and nucleotide comparisons were performed as described by Fajardo et al. (2016). The Sanger nucleotide sequencing was performed using two clones per isolate.

The viral and viroidal communities in the samples included, at least, 16 other pathogens: Grapevine enamovirus-1, Grapevine Cabernet Sauvignon reovirus, *Grapevine vein clearing virus*, *Grapevine Red Globe virus*, *Grapevine Syrah virus 1*, *Grapevine leafroll-associated virus 2, 3, 4* and *4* strain *5*, *Grapevine rupestris stem pitting-associated virus*, *Grapevine virus A, B* and *D*, *Grapevine fleck virus*, *Grapevine rupestris vein feathering virus*, and GYSVd-1 (Fajardo et al. 2016, 2017). It was not possible to associate specific symptoms or disorders with HSVd infections because all positive viroid samples also included various combinations of the above mentioned viruses. Although symptoms are not typically observed in grapevines infected by HSVd (Eiras et al. 2006), this viroid might contribute to disease and losses resulting from synergistic effects associated with mixed infections. Kappagantu et al. (2017) reported that HSVd alters host metabolism, physiology, and plant defense responses.

Many viruses and viroids have been identified using NGS (Brass et al. 2017; Pecman et al. 2017), including several

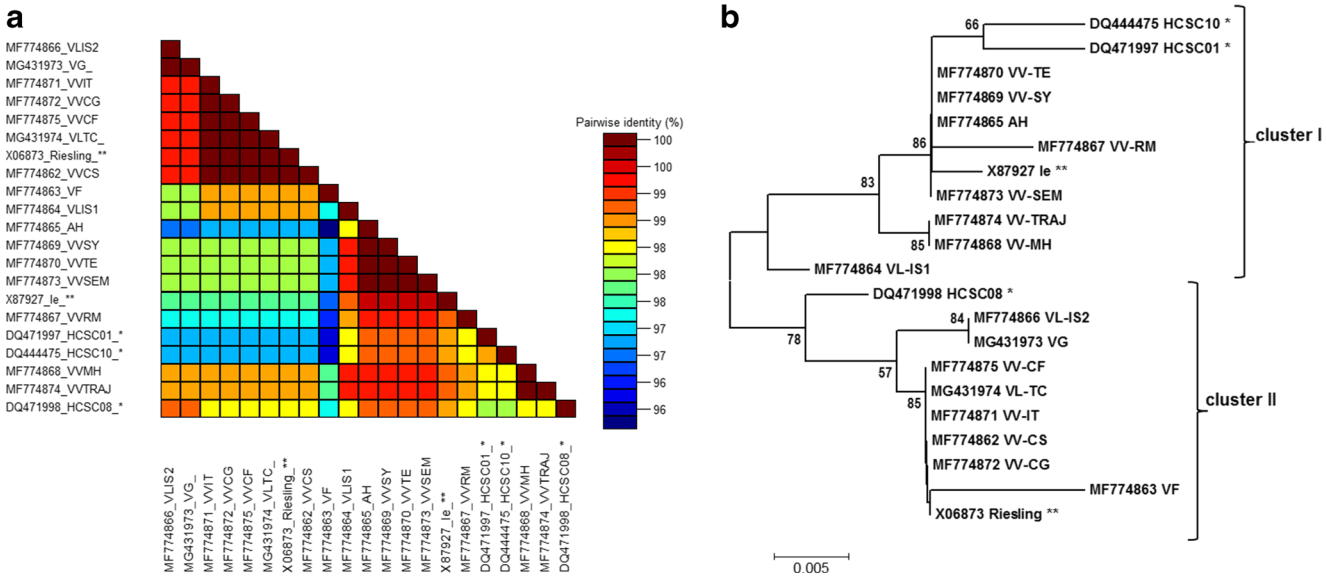

Fig. 1 **a** Pairwise nucleotide identity matrix of 21 HSVd isolates generated using SDT software: 16 Brazilian isolates characterized in this work, three previously characterized by Eiras et al. (2006), assigned with *, and as reference two grapevine isolates retrieved from GenBank, assigned with **. **b** Phylogenetic relationship of the 21 HSVd isolates based on the multiple alignment of their complete genome sequences. Clustering results are indicated. Tree was constructed by the neighbor-joining method using Kimura 2-parameter with gamma distribution (G) and validated by 5000 bootstrap replicates, implemented in the MEGA 7 software package. Bootstrap values (>50) are reported at the nodes. GenBank accession codes and isolate names are shown as GenBank accession information. Bar: number of nucleotide substitutions per site

replicating circular RNAs in grapevine, apple and other hosts by small RNA sequencing (Jo et al. 2017). The technique is especially amenable to detecting RNA viruses and viroids. Because dsRNA is synthesized by RNA viruses and viroids, as replicative intermediates, and plants do not normally produce dsRNA, sequencing of total dsRNA dramatically increases the proportion of reads specific for these pathogens (Wu et al. 2015).

Sixteen complete sequences of the HSVd genome (297 to 301 nucleotides in length) were obtained by NGS from seventeen different grapevine genotypes including wild and commercial cultivars of wine and table grapes (Table 1). Each HSVd genome sequence was derived from only one contig per sample and this contig represents a consensus of the viroidal population. HSVd genomes typically range from 294 nt to 306 nt, most frequently 297 to 299 nt (Jo et al. 2017). The majority of HSVd sequences deposited in GenBank is from citrus species (Jo et al. 2017). Among grapevine isolates, almost all were isolated from *V. vinifera* and, approximately six isolates are now from *V. labrusca*, including the three sequenced here (Table 1). To the best of our knowledge, this is the first report of HSVd infecting genotypes of wild grapevine (*V. gigas*, synonym *V. aestivalis* and *V. flexuosa*) and *Ampelopsis heterophylla* (synonym *Parthenocissus heterophylla*), species of *Vitaceae* (GRIN 2015). HSVd was detected by NGS in 94% (16 out of 17) of analyzed samples (Table 1), which is consistent with a high prevalence in grapevine reported by others (Fiore et al. 2016).

The sequences obtained for HSVd Brazilian isolates were deposited in GenBank under accession numbers MF774862 through MF774875, and MG431973-MG431974 (Table 1). Multiple nucleotide alignments among these sequences showed identities ranging from 94.6% to 100% (Fig. 1a), suggesting limited variability among the isolates. Nucleotide identities among the 16 Brazilian HSVd isolates and other Brazilian isolates from grapevine (HCSC01, HCSC08 and HCSC10), previously characterized by Eiras et al. (2006), showed similar identity levels (94.6 to 98.9%) (Fig. 1a). The phylogenetic analysis of the full-length genome data (Fig. 1b) clustered the isolates in two clades, regardless of the grapevine genotype or the geographic origin (i.e. Brazilian states) from where the HSVd isolate was obtained. As previously determined by Amari et al. (2001), some grapevine HSVd isolates are phylogeneticaly grouped with hop isolates, while others are grouped only with those from grapevines, as shown in Fig. 1b, considering clusters I and II, respectively. The hop isolates are not shown in the dendrogram, since our analysis focused on the variability of grapevine isolates.

The viroid was demonstrated to comprise a range of molecular variants (Fiore et al. 2016). A specific relationship between some of the viroidal variant groups and distinct host species appears to exist (Amari et al. 2001), although the issue was not studied in-depth in the current work. Amari et al. (2001) proposed the classification of HSVd variants based on phylogenetic analyses into five groups, including the three major groups: citrus-, hop- (including grapevine isolates) and,

plum-type, along with two groups formed as a result of recombination events between members of the plum- and the citrus-type (P-C group), or of the plum- and hop-type, or cit3 sequence variant (P-H/cit3 group). In the genome-based phylogenetic relationships of 16 Brazilian isolates of HSVd and isolates from other host species retrieved from GenBank, we verified the previously discussed clustering, with three distinct major groups: grapevine/hop, citrus/cucumber and *Prunus* isolates (data not shown).

The amplicons from the HSVd-positive cv. Cabernet Sauvignon and *V. gigas* grapevines obtained using VP1181/VP1182 primers were Sanger sequenced to confirm NGS results. Sequencing of the DNA fragments of 291–292 nt, covering the partial HSVd genome, showed 96–99% nt identities with the VV-CS (GenBank MF774862) and the VG (MG431973) isolates (Table 1).

In this work, sixteen HSVd isolates were identified by bioinformatics analysis of NGS results and the sequences were phylogenetically analyzed. The presence of HSVd in some analyzed samples was also confirmed by RT-PCR followed by Sanger sequencing. Furthermore, to our knowledge, HSVd was reported for the first time from three hosts, two wild grapevine genotypes, and one species of *Vitaceae* that does not belong to the *Vitis* genus. In addition, the molecular variability of the viroid infecting a broad range of cultivars of *Vitis* spp. was determined. This information might support improved management strategies of grapevine diseases in Brazil.

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