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Detection and molecular characterization of *Grapevine yellow speckle viroid 1* isolates infecting grapevines in Brazil

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Abstract Presently, *Hop stunt viroid* (HSVd) and *Citrus exocortis viroid* (CEVd) are the only viroids reported to infect grapevines (*Vitis* spp.) in Brazil, among the seven viroid species already reported infecting this host in other countries. All grapevine viroid diseases are graft-transmissible and can induce losses especially when associated with viruses. The aim of this work was to confirm infection by *Grapevine yellow speckle viroid 1* (GYSVd-1) in grapevine samples exhibiting yellow speckle symptoms in the leaves and in asymptomatic samples sequenced by next generation sequencing (NGS). The occurrence of this viroid in Brazil was further investigated in a second study. Total RNAs and dsRNAs were extracted from five symptomatic plants and 16 asymptomatic samples, respectively. Specific primers were used for RT-PCR and amplified DNA fragments were cloned and sequenced by the Sanger method. Eleven complete nucleotide sequences of GYSVd-1 isolates (366–367 nt) were obtained from NGS and from RT-PCR amplicons. Comparisons showed high identities (95.9–100 %) among ten isolates and an identity of 87.2–90.4 % with a divergent isolate (RM-BR). Phylogenetic analyses placed GYSVd-1 isolates in four clusters (types 1, 2, 3 and 4). All GYSVd-1 infections were confirmed by conventional RT-PCR and RT-qPCR using specific oligonucleotides and a labeled probe. This is the first report and molecular characterization of GYSVd-1 infecting grapevines in Brazil, and our survey indicates that this viroid could be widespread in the major grape producing regions of Brazil.

Introduction

The grapevine (*Vitis* spp.) is one of the main fruit crops grown in the world, considering its socioeconomic importance and cultivated area. It is susceptible to several graft-transmitted agents that cause relevant diseases (Martelli 2014). Its vegetative propagation has contributed to worldwide spread of these pathogens, and its perennial life cycle accelerated the mixing and introduction of several pathogens into a single vine. The major vegetatively transmissible diseases cause crop losses, reduced plant vigor, reduced longevity of grapevines, delayed ripening and reduced quality of berries, with a negative impact on sugar content, pigments and acidity of wines. Currently, approximately 65 viruses and several viroids belonging to different families have been reported infecting grapevines (Maliogka et al. 2015).

Viroids are non-encapsidated, non-protein-coding, small (246–401 nucleotides) covalently closed, circular single-stranded RNAs, adopting a compact folding as a result of their high self-complementarity. They replicate autonomously and depend on the interaction with host factors to complete their infectious cycle (Flores et al. 2015). Viroids are currently classified according to their molecular and biological properties into the families *Pospiviroidae* and *Avsunviroidae* (Di Serio et al. 2014). Seven viroid species have been reported from grapevine. *Grapevine yellow speckle viroid 1* (GYSVd-1, genus *Apscaviroid*) and *Hop stunt viroid* (HSVd, genus *Hostuviroid*) are distributed worldwide, whereas *Grapevine yellow speckle viroid 2* (GYSVd-2),

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Australian grapevine viroid (AGVd) (both in genus *Apscaviroid*) and *Citrus exocortis viroid* (CEVd, genus *Pospiviroid*) are found sporadically. Recently, *Grapevine latent viroid* (GLVd) genus *Apscaviroid* and Grapevine hammerhead viroid-like RNA (GHVd) (unclassified) were also detected infecting grapevines (Gambino et al. 2014; Flores et al. 2015). So far, HSVd and CEVd are the only viroids that have been detected in diseased grapevines in Brazil (Eiras et al. 2006b). These two viroids are worldwide distributed and infect a large number of hosts besides grapevine, while the other three members of the genus *Apscaviroid* (AGVd, GYSVd-1 and GYSVd-2) occur exclusively in grapevine (Little and Rezaian 2003). Usually, grapevine-infecting viroids do not induce symptoms in this host, with the exception of the synergism reported between GYSVd-1 or GYSVd-2 and *Grapevine fanleaf virus* (GFLV, a nematode-transmitted virus), which results in severe vein banding (Szychowski *et al*. 1995).

Yellow speckle (YS) is a disease distributed widely in grapevine growing regions worldwide. The observed symptoms are small chlorotic spots on the entire surface of the leaves which are most abundant in infected plants during days with high temperatures (Rezaian 1992; Sano et al. 2000; Elleuch et al. 2002). Expression of YS is ephemeral and mostly evident at the end of the summer, indicating that symptoms are strongly influenced by climatic conditions (Szychowski et al. 1998; Salman et al. 2014).

The association of a viroid with this disease was first observed by Koltunow and Rezaian (1988). Heterogeneity in natural GYSVd-1 populations has been demonstrated (Polivka et al. 1996). Based on sequence variations and possible symptom-inducing abilities, GYSVd-1 populations are classified as types 1, 2, 3 and 4 (Szychowski et al. 1998; Hajizadeh et al. 2012). YS isolates have been identified and characterized in different grapevine-growing areas. For example, types 1, 2 and 3 are present in Australia, but only types 1 and 3 are symptomatic (Szychowski et al. 1998; Little and Rezaian 2003; Salman et al. 2014), while type 4 was discovered in Iran and has both symptomatic and asymptomatic variants (Hajizadeh et al. 2012). GYSVd-1 possesses a genome of 366–368 nucleotides (nt). Unlike other known viroids infecting grapevines, usually asymptomatic, it is directly associated with symptoms and consequent damage to the crop (Szychowski et al. 1998).

The aims of this work were to confirm infection by GYSVd-1 in five grapevine sources exhibiting yellow speckle symptoms and in asymptomatic samples found positive for GYSVd-1 by NGS, and to perform the molecular characterization of these isolates. Also, the occurrence of this viroid was investigated in a survey with samples collected in grapevine collections in Brazil.

Material and methods

Symptomatic leaves of five grapevine sources, *V. labrusca* cv. Niagara Rosada (two sources), *V. vinifera* cv. Semillon and cv. Moscato Bailey and a hybrid ('BRS Rúbea' x 'IAC 1398-1') cv. Violeta, displaying conspicuous yellow speckles, were collected from the *Vitis* collections at Embrapa Uva e Vinho (Bento Gonçalves, Rio Grande do Sul state) and Embrapa Agropecuária Oeste (Dourados, Mato Grosso do Sul state) in Brazil (Fig. 1, Table 1). Total RNA extractions were performed using the RNeasy Plant Mini kit (Qiagen) from 100 mg of petioles or veins of symptomatic leaves or adsorption of nucleic acids on silica particles from 1 g of grapevine tissues (Rott and Jelkmann 2001), grinding plant tissue in liquid nitrogen, and following the mentioned protocols. The primer pairs used to amplify GYSVd-1 for one-step RT-PCR were GYSVd-1-AS (5′-GCG GGG GTT CCG GGG ATT GC-3′) and GYSVd-1-S (5′-TAA GAG GTC TCC GGA TCT TCT TGC-3′), complementary and viral, respectively (Elleuch et al. 2013). RT-PCR in a single step was carried out using the One Step RT-PCR kit (Qiagen) according to the manufacturer's instructions with 4 μL of total RNA. The amplification cycle was 50 °C for 30 min, 95 °C for 15 min, followed by 35 cycles of 94 °C for 50 s, 50 °C for 50 s, 72 °C for 1 min, and a final extension of 72 °C for 10 min. RT-PCR products were analyzed in 1.5 % agarose gels prepared in TBE pH 8.0 in the presence of ethidium bromide, visualized under UV light. The expected DNA bands were cut from the gels, eluted using the Wizard SV Gel and PCR Clean-Up System kit (Promega) (Sambrook and Russell 2001) and cloned into the pGEM-T Easy vector (Promega). Automatic nucleotide sequencing (Sanger method) was performed with two or three clones per isolate. Multiple sequence alignments of nucleotides (nt) and the matrix of nucleotide identities were performed using Clustal X 1.8 (Thompson et al. 1997) and BioEdit 7.2.5 (Hall 1999) softwares, respectively. The sequences obtained in this work were aligned with those of the representative four types of GYSVd-1 (type 1, GenBank access number AF462159; type 2, Z17225; type 3, NC_001920; type 4, JN008866 and JQ686713), as well as other isolates, using MEGA 6.0 (Tamura et al. 2013). GenBank access numbers of the nucleotide sequences of the full-length viroids used for phylogenetic analysis are listed in Table 1. Phylogenetic relationships were determined from the aligned sequences using the Neighbor-Joining (NJ) method (1,500 bootstrap replications) implemented in MEGA 6.0.

The predicted secondary structures of minimal free energy of the Brazilian GYSVd-1 isolates reported here were obtained with the Mfold program for circular molecules (Zuker 1989) and visualized with the RnaViz program (De Rijk and De Wachter 1997). The identified mutations between the eleven Brazilian GYSVd-1 isolates and the reference isolates X06904 (Koltunow and Rezaian 1988) and NC_001920

Fig. 1 Leaves of grapevines infected by GYSVd-1 showing yellow speckle symptoms. (**a**), Healthy and (**b**), Infected *Vitis vinifera* cv. Semillon; (**c**), *V. labrusca* cv. Niagara Rosada (source: Rio Grande do Sul state); (**d**), *V. labrusca* cv. Niagara Rosada (source: Mato Grosso do Sul state); (**e**), *V. vinifera* cv. Moscato Bailey; (**f**), Hybrid grapevine cv. Violeta
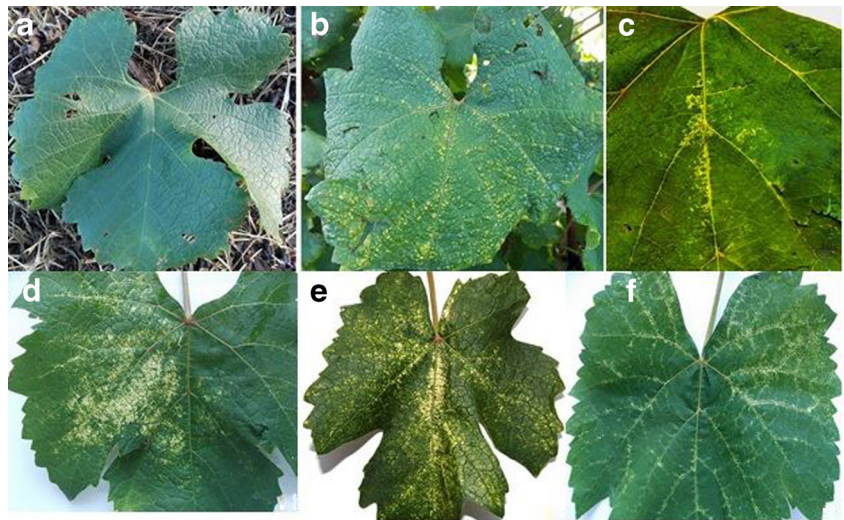
(Szychowski et al. 1998) were mapped using the same programs.

Sixteen grapevines were sampled in three grapevine collections in Rio Grande do Sul, São Paulo and Pernambuco states. The evaluated plants, with unknown viroid infection status, exhibited symptoms related to virus diseases (leafroll, red, yellow or coriaceous leaves, stem pitting) or were asymptomatic (Table 1, sample numbers 6–21). Enriched double-stranded RNAs were extracted from 30 g of bark scrapings per sample using CF11 cellulose (Valverde et al. 1990). Sequencing data was generated from a complementary DNA library that was constructed by Macrogen Inc. (Seoul, South Korea) or Eurofins Genomics Company (Huntsville, USA) from those extractions. The Illumina HiSeq2000 platform was used to generate the paired-end reads. CLC Genomics Workbench software v.6.0.3 (CLC Bio, Qiagen) was used for quality trimming and *de novo* contig assembly from the reads. All contigs were analyzed using NCBI's Blast algorithm (http://www.ncbi.nlm.nih.gov/blast) against the viral and viroid RefSeq databases. To further confirm the results, all contigs identified as GYSVd-1 were individually analysed using Blastn against the GenBank database.

For the survey, total RNAs extracted from 98 grapevine samples by the silica method were indexed to GYSVd-1 by real-time quantitative RT-PCR (RT-qPCR). These samples were collected from the *Vitis* collections maintained by six Brazilian research institutions in six states (Rio Grande do Sul, Santa Catarina, Paraná, São Paulo, Minas Gerais and Pernambuco) in Brazil. In all performed analyses, RNAse-free water, healthy grapevines and positive controls were included. RT-qPCR reactions (One Step RT-PCR) were carried out in 96-well plates using the TaqMan Fast Virus 1-Step Master Mix kit (Life Technologies): 3 µL of the 4X TaqMan Fast Virus 1-Step Master Mix, 0.6 µL of the mixture of primers and probe (415 nM primer and 85 nM probe), 3 µL of total RNA (ca. 300 ng) to a final volume of 12 µL.

Reactions were performed in a StepOnePlus real-time PCR System (Applied Biosystems) as follows: 45 °C for 35 min (for reverse transcription), 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The reaction data were analyzed as presence/absence assays and graphically, using the StepOne Software v.2.3 (Applied Biosystems), by determining the Cq (quantification cycle). The primers and probe used for GYSVd-1 detection by real-time RT-PCR were named GYSVd-1 PozFa (5′-GTG GTT CCT GTG GTT TCA CC-3′), GYSVd-1 PozFb (5′-GTG GTT CCT GTG GTT ACA CC-3′), GYSVd-1 PozR (5′-GAC GTC GAC CAG CTC AGG-3′) and GYSVd-1P probe (5′-AGA AGA AGA TAG GGG CAG AGG GG-3′, labeled with 6-FAM and TAMRA at the 5′ and 3′ ends, respectively), based on the GYSVd-1 GenBank accession X87917 and designed by Bianchi et al. (2015). The primer pairs and probe used to index GFLV by RT-qPCR in 21 grapevine samples were GFLV 769 F / GFLV 868R and GFLV 799P (probe) (Osman and Rowhani 2006).

Results and discussion

GYSVd-1 was detected in all plants exhibiting yellow speckles, thus the symptoms observed in the infected plants can be attributed to viroid infection. Apparently, GYSVd-1-infected plants did not exhibit any other kind of symptoms (such as decreased vegetative vigor) besides those that were observed in the leaves. It is difficult to perform visual diagnosis in grapevine infected by viruses and/or viroids because frequently mixed pathogen infections are present in vines, and specific symptoms may not be exhibited. Thus, reliable GYSVd-1 detection is of fundamental importance. Besides, determining the identity of viroid species that infect grapevines is important for taxonomic purposes and constitutes an

Table 1 Evaluation of the presence of GYSVd-1 in different Brazilian grapevine species and cultivars by symptom observation, RT-PCR, RT-qPCR and sequencing

Sample number	State of collection[a]	Grapevine species and cultivar	Yellow speckle presence	GYSVd-1 infected samples (RT-PCR and RT-qPCR)	Genbank access number (isolate name, genome length)	Type
1	RS	*Vitis labrusca* cv. Niagara Rosada	yes	+	KU668661 (NR-RS-BR, 367 nt)	4
2	MS	*Vitis vinifera* cv. Moscato Bailey	yes	+	KU668662 (MB-BR, 367 nt)	4
3	MS	*Vitis labrusca* cv. Niagara Rosada	yes	+	KU668663 (NR-MS-BR, 367 nt)	4
4	MS	Hybrid grapevine cv. BRS Violeta	yes	+	KU668664 (VI-BR, 366 nt)	1
5	RS	*Vitis vinifera* cv. Semillon	yes	+	KU668665 (SE-BR, 367 nt)	1
6	RS	*Vitis vinifera* cv. Cabernet Sauvignon	no	-		
7	RS	*Vitis flexuosa* (wild grapevine)	no	+	KU880712 (VF-BR, 367 nt)	1
8	RS	*Vitis labrusca* cv. Isabel (source 1)	no	-		
9	RS	*Ampelopsis heterophylla* (ancestral grapevine)	no	-		
10	SP	*Vitis labrusca* cv. Isabel (source 2)	no	+ (GFLV+)	not deposited at GenBank (237 nt, incomplete genome)	
11	SP	*Vitis gigas* (wild grapevine)	no	+	not deposited at GenBank (344 nt, incomplete genome)	
12	SP	*Vitis vinifera* cv. RedMeire	no	+ (GFLV+)	KU880713 (RM-BR, 366 nt)	3
13	SP	*Vitis vinifera* cv. Moscato de Hamburgo	no	+ (GFLV+)	KU880714 (MH-BR, 366 nt)	1
14	PE	*Vitis vinifera* cv. Syrah	no	+	KU880715 (SY-BR, 367 nt)	2
15	PE	*Vitis vinifera* cv. Tempranillo	no	+	not deposited at GenBank (344 nt, incomplete genome)	
16	RS	*Vitis tillifolia* (wild grapevine)	no	-		
17	RS	*Vitis vinifera* cv. Italia (Pirovano 65)	no	-		
18	RS	*Vitis vinifera* cv. CG 90450	no	-		
19	RS	*Vitis labrusca* cv. Tardia de Caxias	no	+	not deposited at GenBank (345 nt, incomplete genome)	
20	RS	*Vitis vinifera* cv. Trajadura	no	+	KU880716 (TR-BR, 366 nt)	1
21	RS	*Vitis vinifera* cv. Cabernet Franc	no	+	KU880717 (CF-BR, 367 nt)	2

[a] RS, Rio Grande do Sul; MS, Mato Grosso do Sul; SP, São Paulo; PE, Pernambuco

essential requirement for the development of more precise and sensitive detection methods.

Full-length amplicons of GYSVd-1, with 366–367 nucleotides (nt), were successfully amplified by RT-PCR from five symptomatic samples with speckle symptoms (data not shown). Sequence comparisons revealed low genetic variability (97.2–100 % nt identities) among these Brazilian isolates, and also significant variability (89.1–91 %) with the reference variant 3 of GYSVd-1 from grapevine deposited in GenBank (NC_001920). Besides these five Brazilian isolates (named NR-RS-BR, MB-BR, NR-MS-BR, VI-BR and SE-BR) which were sequenced by the Sanger method, the complete sequences of six additional isolates (VF-BR, RM-BR, MH-

BR, SY-BR, TR-BR and CF-BR) and incomplete sequences (237–345 nt) of four isolates were obtained by NGS, totalizing fifteen GYSVd-1 sequenced isolates (Table 1). All these 15 samples were also confirmed positive for GYSVd-1 in indexing by conventional RT-PCR and RT-qPCR (data not shown).

There is no genetic resistance against viruses and viroids that infect grapevine, but tolerance is verified in some genotypes. In these cases, the infected vine shows restricted symptoms or even is asymptomatic and damage is reduced (Laimer et al. 2009). In this work, it was shown by sequencing that GYSVd-1 was able to infect a range of 15 different grapevine genotypes out of 21 analysed ones, including cultivars of

V. labrusca, *V. vinifera*, a hybrid grapevine and wild grapevines (*V. flexuosa* and *V. gigas*) (Table 1).

Even from asymptomatic vines, it was possible to obtain specific sequences of 10 isolates of GYSVd-1 using NGS (Table 1). Several new viral species or strains of known viruses, mycoviruses, viroids and phytoplasmas have been identified using NGS of RNA, siRNA or dsRNA samples isolated from specific tissues of numerous grapevine cultivars worldwide. NGS is a powerful technology that allows detection and discovery of viruses and viroids without any prior knowledge of their genome and other properties of the pathogens (Navarro et al. 2009; La Notte et al. 2012). Thus, besides the Sanger sequencing of RT-PCR-amplified and cloned GYSVd-1 isolates, NGS was used in this work to take advantage of its aforementioned characteristics.

Comparative analysis of the sequences of ten Brazilian GYSVd-1 isolates (KU668661-5, KU880712 and KU880714-7) and the reference type 1 isolate (X06904) showed high identities (95.9–100 %) among them. Moreover, an identity of 99.4 % was found between the RM-BR isolate (KU880713) and the reference type 3 isolate (NC_001920). However, the identity between these two groups was relatively low and ranged from 87.2 to 91 %. Collectively, the ten above mentioned isolates possess a relatively broad host range, including different wine and table grape species and cultivars. Nevertheless, it seems that these isolates are not under environmental pressure to suffer significant diversification. On the other hand, the isolate RM-BR could be under some kind of environmental pressure that induced the meaningful variability observed. Szychowski et al. (1998) mentioned that a higher sequence identity exists between the nonsymptom- and symptom-inducing GYSVd-1 variants (86–98 %) than between GYSVd-1 symptomatic variants and GYSVd-2 (75–78 %). Therefore, the broad diversity observed in the symptom-inducing GYSVd-1 variants may contribute to the variability and ephemeral expression of the yellow speckle-vein banding symptoms.

The predicted secondary structure of minimum free energy of the Brazilian GYSVd-1 isolates was obtained (Fig. 2). We observed that residue changes were distributed through all domains, but more concentrated in the region corresponding to the Variable (V) domain, either at the upper or at the lower part of the molecule (Fig. 2). Most observed mutations occurred in loops not involved in base-pairing, and most of them had their complementarity restored, thus not affecting the secondary structure of the RNA. Compared to the other Brazilian GYSVd-1 variants sequenced here, two main divergent regions were observed in the isolate RM-BR and also in the reference type 3 isolate (NC_001920): GUGC→AGCU at positions 125–128 and GCCACC→CUGCUG at positions 245–250 (Fig. 2). These changes were observed only in the RM-BR isolate, and apparently they are specific motifs of GYSVd-1 type 3.

Except for GYSVd-1 and GYSVd-2, grapevine-infecting viroids do not induce symptoms. The association of these viroids with GFLV is thought to elicit vein banding, a syndrome characterized by chrome-yellow flecks localized along the main veins and progressing into the interveinal areas of affected vines (Hajizadeh et al. 2015). The samples of *V. labrusca* cv. Isabel (source 2), *V. vinifera* cv. RedMeire and *V. vinifera* cv. Moscato de Hamburgo were infected with GFLV according to indexing by RT-qPCR. However, co-infection by GYSVd-1 did not induce visible symptoms of vein banding or yellow speckles (Table 1). The correlation between increased viroid titer and symptom expression might be related to any number of stress factors, such as high temperatures, day length or virus infection. Whether this phenomenon is a cause or effect of symptom expression has not been defined (Szychowski et al. 1998). Therefore, it is not possible to diagnose GYSVd-1 infections based exclusively on symptomatology.
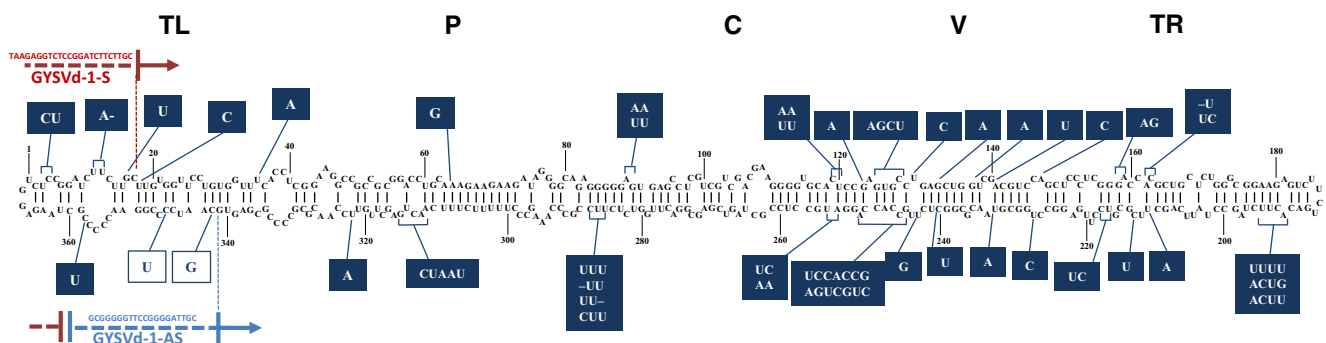

Fig. 2 Predicted secondary structure of the GYSVd-1 variant SE-BR, sequenced in this work from *V. vinifera* cv. Semillon, with 367 nucleotides (GenBank access number KU668665). Changes in the residues among Brazilian GYSVd-1 isolates sequenced in this work (KU668661-KU668664 and KU880712-KU880717) and the reference type 1 GYSVd-1 isolate (X06904) are indicated in the blue boxes. Changes in the residues between the Brazilian GYSVd-1 isolate RM-BR (KU880713) and the reference type 3 GYSVd-1 isolate (NC_001920) are indicated in the white boxes. The primers used for RT-PCR are indicated in red (GYSVd-1-S) and blue (GYSVd-1-AS) letters, respectively. The five domains (TL, P, C, V and TR) are also indicated

The status of GYSVd-1 infection of grapevines in Brazil was unknown. As this viroid is present worldwide in grapevine, it should be presumably also widespread in Brazilian vineyards. Accordingly, 98 grapevine accessions assayed by RT-qPCR revealed a high level of GYSVd-1 infection (71.4 %). These results were corroborated by visualizing 126 bp amplicons in agarose gels (data not shown). These infected samples could be infected by multiple types of GYSVd-1 (1 through 4), as specifically demonstrated for the 11 isolates characterized in this work (Fig. 3). This is supported by the fact that the sampled grapevines exhibited low vegetative vigor and general symptoms of virus infections as a consequence of long-term maintenance in collections under field conditions. Based on these findings, there is a need of further evaluation of commercial vineyards to precisely determine the geographical distribution of this pathogen in Brazil.

Fig. 3 Phylogenetic tree based on the alignment of nucleotide sequences of 11 GYSVd-1 isolates from Brazil (red asterisks) and other countries. The tree was constructed by the Neighbor-Joining method using MEGA6.0, and bootstraped with 1,500 replications. Names of GYSVd-1 variants and origins were included according to GenBank, and specific clusters are indicated. *Grapevine yellow speckle viroid 2* (GYSVd-2) was used as outgroup. Bar = number of substitutions per site
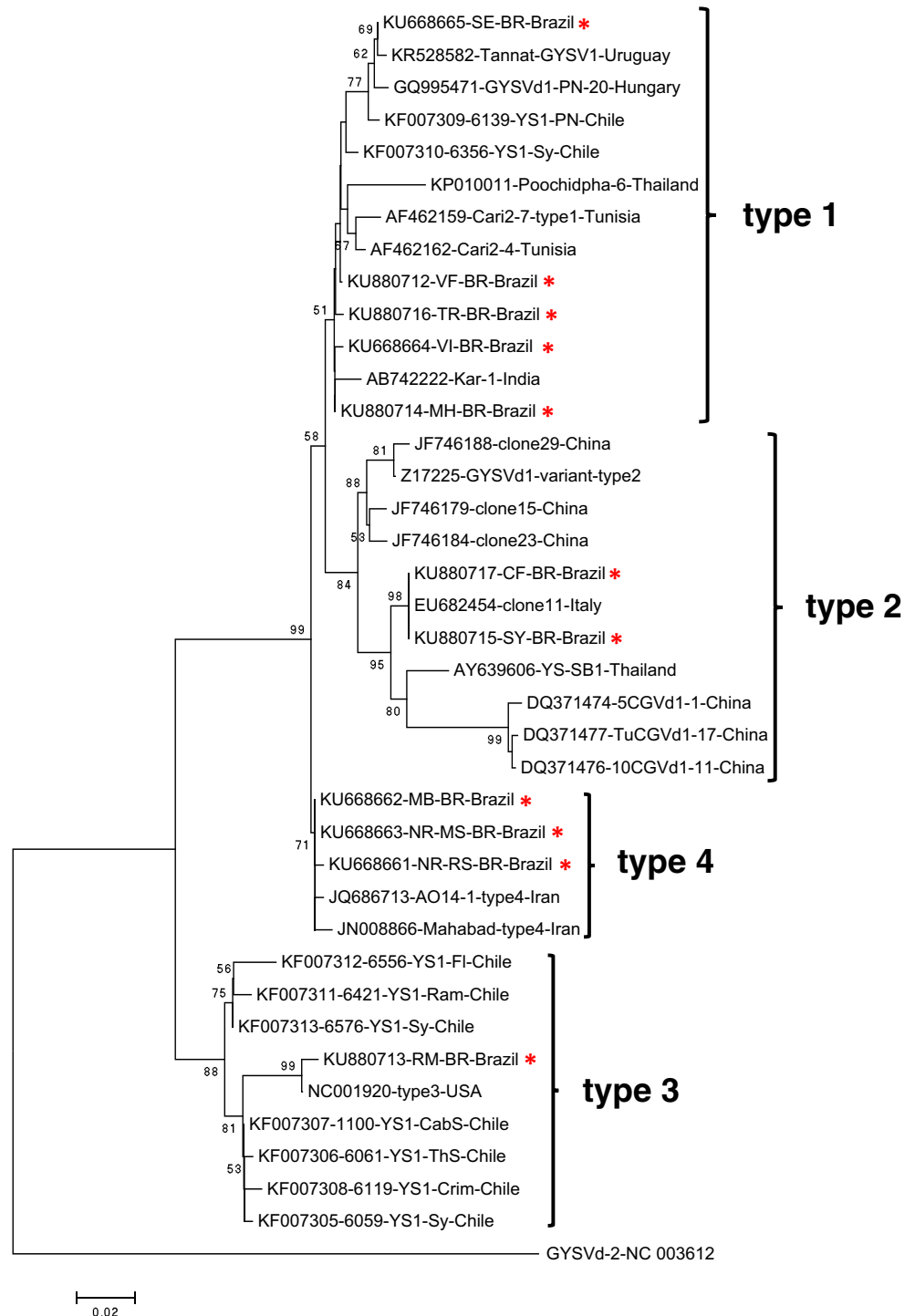
Besides conventional RT-PCR, RT-qPCR was used in this work to evaluate its capacity to detect a range of GYSVd-1 isolates regardless of their variability. This variability was demonstrated by identifying all four types of GYSVd-1 among the Brazilian isolates (Fig. 3). Moreover, this result demonstrates that the primers/probe defined by Bianchi et al. (2015) were suitable to diagnose GYSVd-1 and its sequence variability in the 98 assayed samples.

Phylogenetic analyses of GYSVd-1 isolates showed an association of Brazilian isolates, independently of cultivar or geographic distribution, to types 1, 2, 3 and 4 (Table 1, Fig. 3). Type 3 was previously described as symptomatic, in contrast with type 1 described as asymptomatic (Szychowski et al. 1998). Interestingly, isolate RM-BR clustered with variant 3, but no YS symptoms were observed in the corresponding plant at the time of collection. Isolates of GYSVd-1 clustered in four groups (types) closely related with Chinese, Iranian, Tunisian and Chilean isolates (Fig. 3). Jiang et al. (2012) and Hajizadeh et al. (2012) identified geographic influence on the variability of GYSVd-1, although the role of sequence diversity in pathogenicity and evolution of the viroid was not determined.

The sequence variability of GYSVd-1 isolates was previously noticed for Chilean and Iranian GYSVd-1 isolates (Hajizadeh et al. 2012; Zamorano et al. 2015). The characterization of genetic structure and diversity of viroid variants is important to understand viroid evolution and to assess the real status of the population of viroid RNA molecules in the plant. As demonstrated here, GYSVd-1 seems to follows a *quasispecies* structure. In spite of this variability, the long term infection of this viroid in grapevine suggests that viroid-host interactions may play a selective pressure for viable RNA molecules, preserving the main conserved motifs. These aspects were demonstrated in the analyzed genomes of GYSVd-1, which presented regions of the molecule with a high degree of conservation, while some regions were more variable (Fig. 2). It has been confirmed that different sequence variants of GYSVd-1 can coexist in a single plant (Polivka et al. 1996), and that the majority of sequence variation occurs in the pathogenicity domain (PD) of the GYSVd-1 genome (Little and Rezaian 2003). This knowledge is important to connect the effects of nucleotide sequence variation in symptom expression, and to evaluate different molecular techniques for viroid detection that can be used for indexing programs (Eiras et al. 2006a).

The sequenced Brazilian isolates of GYSVd-1 presented several nucleotide changes dispersed in all structural domains of the molecule (Fig. 2), which could influence symptom expression (Sano et al. 1992; Salman et al. 2014) and the secondary structure of the molecule. These mutations could eventually lead to new interactions among nucleotides, redefining the secondary structure, which in turn would reflect in other properties of the viroid (Flores et al. 2005).

Information about sequence diversity among variants may help to select regions of the viroid genome targeted for specific detection. Thus, the occurrence of mutations may limit the suitability of RT-PCR for viroid detection. Intraspecies sequence diversity in GYSVd-1 makes the selection of conserved regions for primer design rather difficult, affecting detection by PCR. These results highlight the relevance of knowledge of sequence variability when designing primers for a reliable detection of all known viroid variants (Jiang et al. 2012).

The presence of GYSVd-1 infecting Brazilian grapevines can be attributed to the introduction of infected propagative material from other countries, since this viroid has no vectors associated with its transmission. The control of this pathogen in other countries is based on indexing programs using molecular methods for viroid detection and a clean stock program specifically designed to collect, maintain and distribute viroid-free propagation material for the farmers (Eiras et al. 2006a). The main sanitation techniques to eliminate grapevine viruses and viroids are thermotherapy *in vivo* or *in vitro*, chemotherapy, meristem and shoot tip culture, somatic embryogenesis, electrotherapy and cryotherapy (Gambino et al. 2011), but the efficiency of these techniques in obtaining viroid-free materials is restricted in some cases. This could be a result of the great stability of the viroid molecule conferred by its complementary structure.

A survey for GYSVd-1 allowed us to first detect and further evaluate the genetic variability and geographical distribution of this pathogen in Brazil. These results provide relevant information for the development of improved detection and control strategies in the framework of programs for production of healthy grapevine propagative materials. Our study revealed that genetic diversity of GYSVd-1 is also present in Brazilian grapevine hosts, and this information should be taken into consideration in symptomatological assessments and biological and molecular indexing.

The current knowledge about incidence of grapevine viruses and viroids indicate that some diseases such as grapevine leafroll and rugose wood complex are a real problem in Brazilian grape cultivation. Others, such as grapevine red blotch, which are not present in the country, may become serious risks if proper preventive measures are not taken. Considering the increase in cultivar introduction from diverse foreign sources, virus and viroid diseases may become more serious. Since some of these diseases are transmitted by several species of nematodes, mealybugs, scale insects or even mechanically (in the case of viroids), new vineyards must be established with pathogen-free propagative materials.

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