Diversity of viroids infecting grapevines in the South African *Vitis* germplasm collection

Seamus W. Morgan[1], David A. Read[2,3], Johan T. Burger[1], Gerhard Pietersen[1]

[1] Department of Genetics, Stellenbosch University, Stellenbosch, 7600, South Africa

[2] Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0002, South Africa

[3] Agricultural Research Council - Biotechnology Platform, Onderstepoort, Pretoria, 0110, South Africa

Abstract

Seven viroid species and one putative viroid species have been reported to infect grapevine namely, hop stunt viroid (HSVd), grapevine yellow speckle viroid 1 (GYSVd-1), grapevine yellow speckle viroid 2 (GYSVd-2), Australian grapevine viroid (AGVd), Japanese grapevine viroid (JGVd), grapevine latent viroid (GLVd), and citrus exocortis viroid (CEVd), as well as a grapevine hammerhead viroid-like RNA (GHVd), so far. In this study, RNA sequence (RNA-Seq) data, from 229 *Vitis* accessions from the field-maintained vineyard of the South African *Vitis* germplasm collection, were analysed to determine the diversity of the viroids present. Five of the seven known grapevine infecting viroids and one putative grapevine-infecting viroid species were very commonly found, with 214 of the 229 samples containing at least one viroid species. HSVd, GYSVd-1, GYSVd-2, AGVd, and JGVd, as well as GHVd, were identified in the RNA-Seq data of the samples and confirmed using RT-PCR and Sanger sequencing. The HSVd sequences indicated the presence of two variants, with one showing multiple nucleotide insertions. AGVd and GYSVd-2 did not display significant sequence diversity, confirming past international studies. GYSVd-1 occurs as four major variants worldwide and representatives of all four variants were identified in this vineyard. This is first report on the diversity of viroids infecting grapevine in South Africa and the first report of JGVd outside of Japan and GHVd in South Africa. Further studies are needed to fully assess the population and to identify potentially new viroid species.

Keywords Viroids · diversity · RT-PCR · Next Generation Sequencing · Grapevine

Introduction

Viroids are RNA molecules which are small (245-400 nucleotides), single stranded, circular, non-encapsidated and non-protein-coding. They possess high levels of self-complimentary sequences which result in the formation of secondary structures. Viroids are classified into two families based on structural features and functionality: the *Pospiviroidae* and the *Avsunviroidae* (1–4). Viroids of the *Pospiviroidae* family contain a central conserved region (CCR) within a rod-like secondary structure (2,5) and replicate in the infected host cell's nucleus (6). In contrast, viroids of the *Avsunviroidae* family do not possess a CCR, adopt a secondary branch-like structure, contain hammerhead ribozymes, and replicate in the host cells plastids (2,7–9).

Seven species of viroids are known to infect grapevines, namely hop stunt viroid (HSVd) (10), citrus exocortis viroid (CEVd) (11), Australian grapevine viroid (AGVd) (12), grapevine yellow speckle viroid 1 (GYSVd-1) (13), grapevine yellow speckle viroid 2 (GYSVd-2) (14), grapevine latent viroid (GLVd) (15), and Japanese grapevine viroid (JGVd) (16). A hammerhead viroid-like RNA (GHVd) was recently detected in grapevines in California, Italy, France and Greece, however its true viroid nature has yet to be determined, and is regarded as a putative viroid species in this study (17–20). GYSVd-1 and GYSVd-2 are the causal agents of yellow speckle disease which is characterised by yellow spots on the grapevine leaves (14). These two viroids also exhibit a synergism with grapevine fanleaf virus which results in severe vein banding in grapevine (21). The remaining viroid species do not result in disease symptoms in grapevines (22), though CEVd and HSVd do cause disease symptoms in other host plants. It is thought that these latently infected plants may be inoculum sources for other susceptible hosts, such as HSVd moving into hops from HSVd infected grapevines, resulting in outbreaks of hop stunt disease in Japan (23).

Both GYSVd-1 and GYSVd-2 have a global distribution and are found in nearly all grape growing regions (22) though GYSVd-1 has a greater level of diversity when compared to GYSVd-2 (24,25). GYSVd-1 possesses a heterogenous population which is divided into four types based on variation in nucleic acid sequences and symptom-inducing ability (26). The variation in symptom inducing ability may be due to the sequence variation in the pathogenicity domain of the viroid genome (26,27).

Types 1, 2 and 3 have been reported from Australia, with only type 1 and 3 inducing disease, while type 4 has been reported from Iran with both symptomatic and asymptomatic variants occurring in the population (22,27,28). AGVd is one of the least studied of the grapevine viroids, however population studies suggests that it occurs as a series of variants clustering into three possible groups based on geographical origin and introduction route (29–32). CEVd occurs as a range of variants falling in two groups with no association to host or geographical origin (33). Population studies on HSVd have demonstrated a series of variants which often cluster together based on host origin with grapevine isolates clustering together (23,33). Phylogenetic analyses have suggested that HSVd orginated in grapevine and 'jumped' into hops resulting in hop stunt disease (23). Sano et al. (23) has also suggested that HSVd in hops is in the process of becoming adapated to this new host and is therefore virulent in hops. GLVd was first reported from symptomless grapevines in China (15) and Italy (34). JGVd was first reported from symptomless grapevines in Japan (16).

No vectors are known for the grapevine viroids, and dissemination occurs through mechanical inoculation and distribution of infected propagation material (35). The first report of grapevine-infecting viroids in South Africa was in 2017 in the Western Cape grape growing region (36,37). The South African grapevine industry is largely based on planting material derived from nuclear vines which have been subjected to heat therapy and meristem tip culture. This method of virus elimination has been shown to be ineffective at removing viroids (38). As no testing is done for viroids in the South African Certification Scheme of grapevines, it is not known which viroid species are present locally, and the genetic diversity of these in South African vineyards. This study has provided some insight into the presence and diversity of the grapevine infecting viroids in the South African *Vitis* germplasm collection, Agricultural Research Council (ARC), Stellenbosch, Western Cape.

Materials and Methods

During December 2019, petioles of all replicates of the 229 *Vitis* species, cultivars, and interspecific crosses, were collected from the *Vitis* Germplasm collection Block D2 at the ARC, Stellenbosch. Each cultivar accession is made up ranging from one to five replicate vines, which were sampled and pooled as a single sample. Total RNA

was isolated from these pooled samples using the method of White et al. (39). RNA quality control and quantification was performed using an Implen N60 spectrophotometer (Implen, Munich, Germany). A total of 300ng from each sample was used to prepare RNAtag-seq libraries (40), with 32 samples per library and subjected to Illumina RiboZero (plant leaf) ribo-depletion (Illumina, San Diego, CA, USA). The resulting libraries were sequenced using an Illumina HiSeq 2500 (Illumina, San Diego, CA, USA) at the Agricultural Research Council, Biotechnology Platform, Pretoria, South Africa. Sequencing data was demultiplexed using the Je suite (41). Sequence data was trimmed, filtered, and assembled using CLC Genomics Workbench (CLC) (Qiagen Bioinformatics, Aarhus Denmark). Assembled contigs of between 200 and 400 bp in size were extracted and subjected to BLAST analysis (E-value cut off: $1e^{-5}$; query coverage: ≥ 80%) against the NCBI GenBank RefSeq of viruses and viroids using the CLC BLASTn function. Contigs were then extracted subjected to BLASTn analysis at NCBI Genbank to confirm identity.

In order to capture viroid diversity potentially overlooked by the analysis of 200-400bp contigs, reads were first mapped to the *Vitis vinifera* genome (GCF_000003745.3) using CLC Workbench mapping tool (Qiagen Bioinformatics, Aarhus Denmark) with default parameters (match score-1, mismatch cost-2, length fraction-0.5, similarity fraction-0.8) (42). Unmapped reads were then mapped to reference sequences of all known viroid (www.ncbi.nlm.nih.gov/genome/viruses) species using the CLC Workbench reference mapping tool (Qiagen Bioinformatics, Aarhus Denmark) using the following parameters, match score-1, mismatch cost-2, length fraction-0.9, similarity fraction-0.9. Consensus sequences were extracted from CLC and aligned with the reference sequences, using CLUSTALW 1.6 in MEGA7.0 (43).

In cases where only a partial viroid genome sequence was obtained, these isolates were analysed again using reference mapping using representative datasets of each viroid species. These representative datasets were constructed as follows; all existing full-length genome sequences of the five viroids infecting grapevine were downloaded from NCBI Genbank and aligned using CLUSTALW 1.6 in MEGA7.0 (43). Only a single representative was selected amongst sources with near identical nucleotides (98-99% identity) to serve as reference sequences in further analysis.

Phylogenetic relationships were determined from the aligned sequences using the Maximum Likelihood method (1000 bootstrap replications, General Time Reversible

model) implemented in MEGA 7.0. Nucleotide identity scores were determined using the Sequence Demarcation Tool (SDTv1.2) (44). Representative samples were selected based on sequence alignments and phylogenetic analysis, along with the type sequences of each viroid species, and analysed to determine the predicted secondary structures of minimal free energy were determined using the web-based RNA secondary structure prediction tool RNAstructure (29,44).

Samples containing divergent viroid strains as determined by referencing mapping, de novo assembly, sequence alignments, and phylogenetic analyses were sampled again. Leaf and petiole tissue was sampled from up to 5 vines per cultivar, total RNA was extracted using a modified CTAB extraction method and pooled (39). One step RT-PCR (45) was performed using the primers listed in Table 1. A one-step RT-PCR reaction was conducted using 1 µl total RNA in a mixture containing 10 µM of each respective forward and reverse primer (Table 1), 5 X GoTaq® buffer, 100 mM molecular grade DDT, 25 mM $MgCl_2$, 10 mM dNTP mix, 200 U/µl Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT), 40 U/µl Recombinant RNasin® ribonuclease inhibitor and 5 U/µl GoTaq® DNA polymerase (all reagents were sourced from New England BioLabs, Massachusetts, USA). The remainder of the 25 µl total volume was made up of nuclease-free water. Amplification was done using the following program: one cycle of reverse transcription for 45 min at 37 ˚C, 2 min at 50 ˚C, 5 min at 94 ˚C, and 35 cycles of 30s at 94˚C, 30s at each primer annealing temperature, 30 s at 72 ˚C, followed by a terminal extension of 5 min at 72 ˚C. The final elongation step was conducted for 5 min at 72 °C. PCR products were analysed by electrophoresis on a 3% agarose gel. PCR products were submitted for bidirectional Sanger sequencing at the Central Analytical Facility at Stellenbosch University.

Table 1: Primers used for RT-PCR of five viroid species.

Species	Primer Sequence	Band size	Annealing Temp	Reference
HSVd	HSVdI-5'-GCGTCTCATCGGAAGAGCC-3'	296	56˚C	(46)
	HSVdII-5'-GACCGGTGGCATCACCTCT-3'			
GYSVd-1	AS-5'-GCGGGGGTTCCGGGGATTGC-3.	367	55˚C	(26)
	S-5'-TAAGAGGTCTCCGGATCTTCTTGC-3'			
GYSVd-2	P1-5'-ACTAGTACTTTCTTCTATCTCCCGAAGC-3'	370	60˚C	(47)
	P2-5'-ACTAGTCCGAGGACCTTTTCTAGCGCTC-3			
AGVd	AS-5'-GTCGACGACGAGTCGCCAGGTGAG-3'	370	64˚C	(48)
	S-5'-GTCGACGAAGGGTCCTCAGCAGAG-3'			
JGVd	JG-R2 - 5'-AGCCTCTCTCTGTCCATCGG-3'	290	55˚C	(16)
	JG-F2 – 5'-GAGGCGCTTTTTCTTTTCTCCTA-3'			
GHVd	Vir-F2-5'-GTGTGGTGCTCCTGACGAGTCCA-3'	320	60˚C	(17)
	Vir-R2-5'-CTAGCCTAGGAGCGAATCTGCCA-3'			

Results

Using both reference mapping and BLASTn analysis, full viroid sequences were identified in 214 of the 229 (93.4%) accessions analysed. HSVd was the most abundant viroid, present in 170 samples (74.2%), followed by GYSVd-1, present in 106 samples (46.3%), then GYSVd-2, present in 74 samples (32.3%), AGVd, present in 19 samples (8.3%), and JGVd, in one sample (0.3%). Viroid sources were named after their location in the germplasm collection and the host cultivar. Samples 06-04 Bourboulenc, 05-02 Azal Branco, 11-02 Dauphine, and 14-07 Gobernador Benegas were infected with the four main viroid species. Full sequences of grapevine hammerhead viroid-like RNA was identified in 33 of the 229 cultivars (14.4%) (Supplementary Data table 1). Figure 1 shows the number of vines with various combinations of mixed infections of GYSVD-1, GYSVd-2, HSVd, and AGVd.

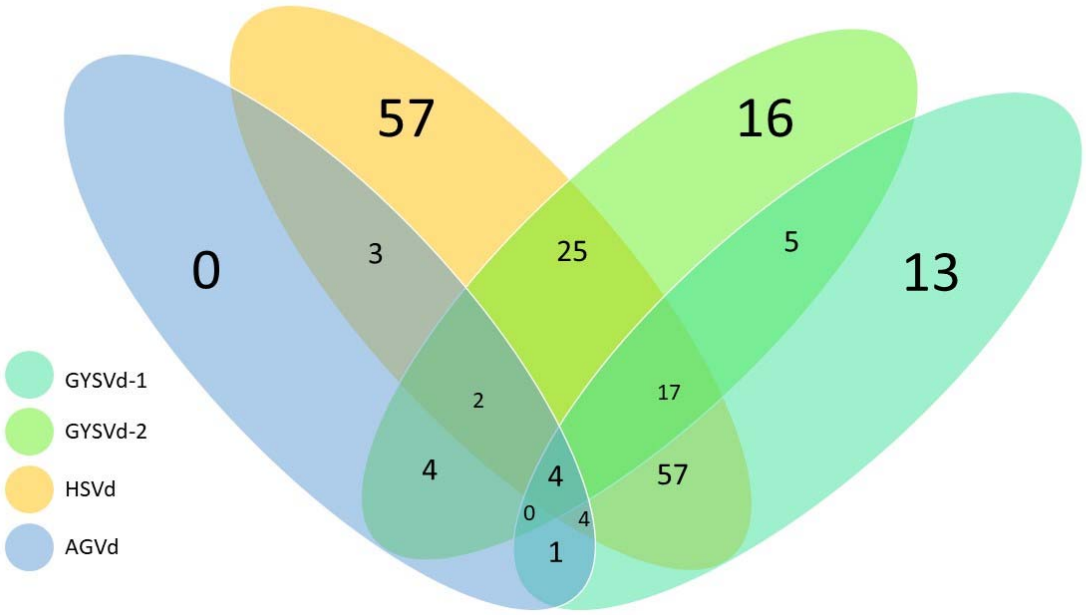

Figure 1: Venn diagram representing the number of samples positive for GYSVd-1, GYSVd-2, HSVd, and AGVd, and displaying the occurrence of mixed infections of the four viroid species.

Sequence analysis and comparisons revealed that the HSVd isolates were divided into two groups, with the majority of HSVd isolates obtained in this study being most similar to HSVd isolate PGH-2 (GenBank accession KR909028) with high levels of nucleotide identity (97.7-100%) (Supplementary data table 2). However, 34 samples proved to be the exception with these isolates sharing 98.3-100% nucleotide identity to HSVd isolate SDLY-23 (GenBank accession KY270463). Sample 11-15 Donzillinho do Galego was most similar to sequence HSVd VL-IS2 (GenBank accession MF774866) with 94.6% nucleotide identity. The presence of both sequence variants was confirmed using RT-PCR. Modifications in the CCR, P, and V domains have resulted from a number of nucleotide changes with a minor difference in the modifications seen in the CCR between the two variants.

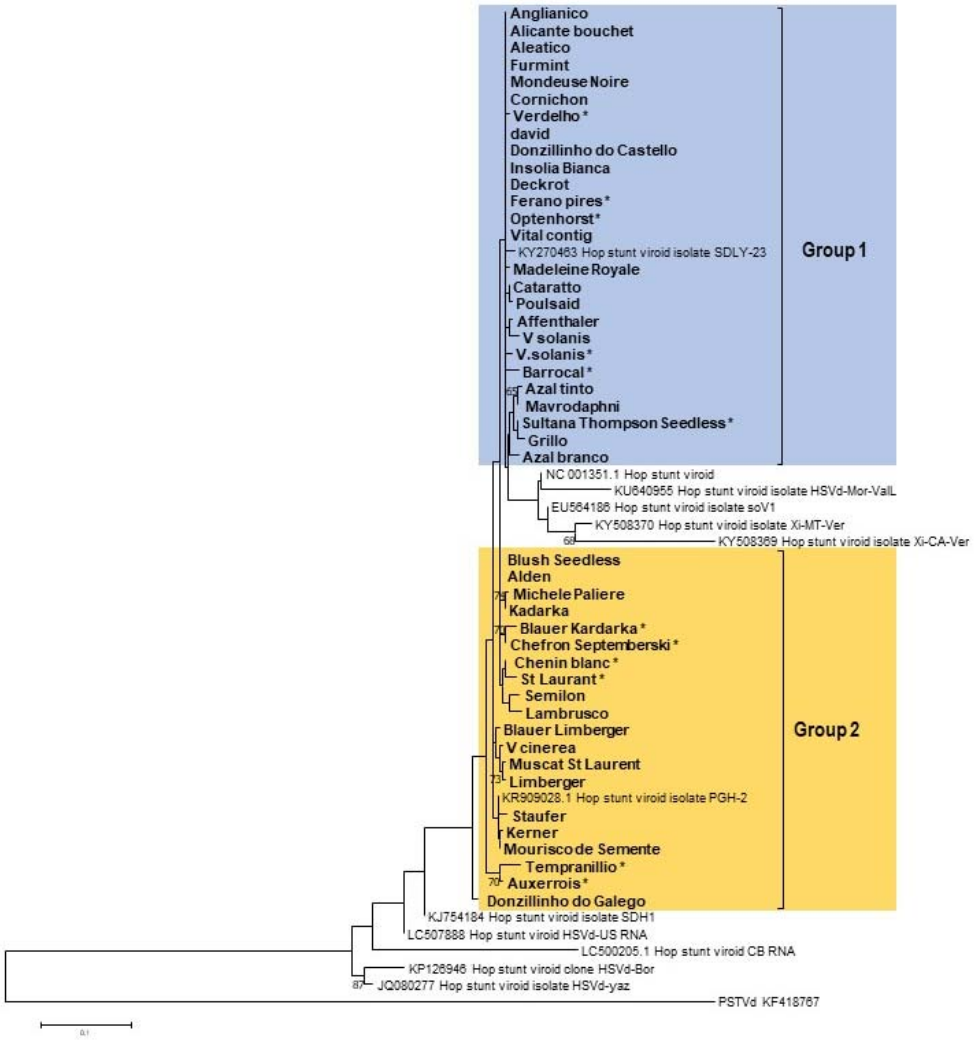

Figure 2. Phylogenetic tree based on the alignment of nucleotide sequences of HSVd isolates obtained in this study (Bold,*RT-PCR derived sequences) and from NCBI Genbank. Each group is indicated to the side. Sequences that were between 98-100% in similarity were removed and one representative was selected. Tree was constructed in MEGA 7.0 using Maximum Likelihood analysis at 1000 bootstraps. Only bootstraps above 60 are shown. Potato Tuber Spindle Viroid was used as an outgroup.

Sequence analysis revealed that each of the four types of GYSVd-1 is present in the D2 vineyard. This can be observed in the phylogenetic analyses (Figure 3), as well as sequence comparisons between the four types of GYSVd-1 with isolates sharing identities ranging between 81.6-99.9% (Supplementary data table 3). The presence of GYSVd-1 was confirmed using RT-PCR. Mixed infections of the four GYSVd-1 types were confirmed through reference mapping and BLASTn analysis using reference sequences for the four types of GYSVd-1. Three isolates of GYSVd-1 type

3, and two isolates of GYSVd-1 type 1 were analysed using RNAstructure. No changes in nucleotide bases were observed in CCR and limited variation was observed in the remaining domains. Sample 47-01 Deckrot possesses a number of nucleotide changes throughout the genome which have resulted in minor structural changes. Samples 01-03 V. candicans and 12-02 Fernao Pires, which represent GYSVd-1 type 1 differ significantly from 05-02 Azal Branco, however, both samples possess similar secondary structures.

Figure 3. Phylogenetic tree based on the alignment of nucleotide sequences of GYSVd-1 isolates obtained in this study (Bold*RT-PCR derived sequences) and from NCBI Genbank. Each type is

indicated to the side. Sequences that were between 98-100% in similarity were removed and one representative was selected. Tree was constructed in MEGA 7.0 using Maximum Likelihood analysis at 1000 bootstraps. Only bootstraps above 60 are shown. Pepper Chat Fruit Viroid was used as an outgroup.

Sequence comparisons reveal that the isolates of GYSVd-2 are most similar to four references sequences, GYSVd-2 isolate 1-5 (GenBank accession DQ377126), GYSVd-2 isolate Maragheh (GenBank accession JN008867), GYSVd-2 isolate clone 1 (GenBank accession MG780425) and GYSVd-2 isolate Sup4 (GenBank accession LR735996), ranging from 98.1-100% in nucleotide identity. The overall identity at nucleotide level between the isolates obtained in this study range from 95.1-100% (Supplementary data table 4). Phylogenetic analyses shows that there are low levels of diversity amongst the GYSVd-2 isolates (figure 4). Infection of GYSVd-2 was confirmed using RT-PCR. Sequence comparisons revealed that the GYSVd-2 strains amplified were most like reference isolate GYSVd-2 clone 1 (MG780425). Sample 08-01 Chenin Blanc has shown to have at least two variants of GYSVd-2 based on sequence comparisons between the sequences obtained from the NGS data and the sequences obtained from RT-PCR. Five isolates were analysed using RNAstructure and minor changes in secondary structure were observed in the TL and P domain of two of the selected samples, 12-02 Fernao pires and 19-09 Madeleine Royale.

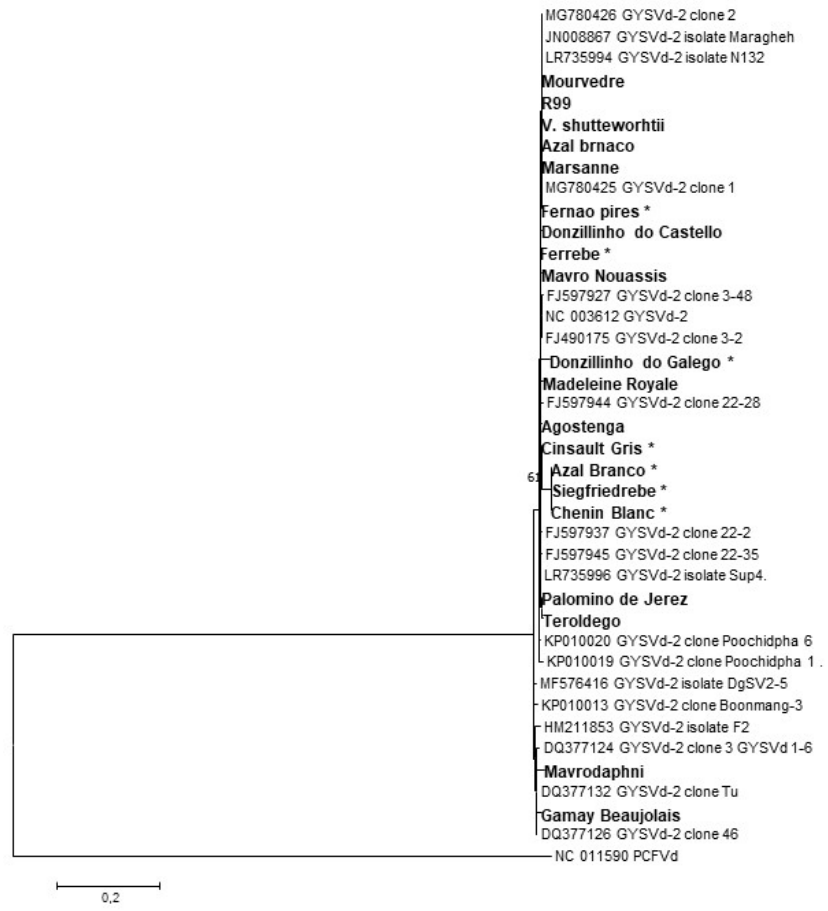

Figure 4. Phylogenetic tree based on the alignment of nucleotide sequences of GYSVd-2 isolates obtained in this study (Bold,*RT-PCR derived sequences) and from NCBI Genbank. Sequences that were between 98-100% in similarity were removed and one representative was selected. Tree was constructed in MEGA 7.0 using Maximum Likelihood analysis at 1000 bootstraps. Only bootstraps above 60 are shown. Pepper Chat Fruit Viroid was used as an outgroup.

Sequence comparisons reveal that the majority of the AGVd isolates are most like references sequence AGVd isolate WM35-VII (GenBank accession MH521286) ranging from 98.4-100% in nucleotide identity (Supplementary data table 5), while two isolates are most similar to AGVd isolate 6061 (GenBank accession KF007271) (99.9%) and AGVd isolate Xatam (KF876037) (99.9%). The nucleotide identity between these isolates and the AGVd reference genome (GenBank accession NC003553) range from 96.1-99.5%. The overall identity at nucleotide level between the isolates obtained in this study range from 95.1-99.5%. All AGVd isolates obtained in this study cluster together apart from 09-03 Cinsault Gris (Figure 5). RT-PCR confirmation confirmed the presence of AGVd in samples 05-02 Azal Branco

and 09-03 Cinsault Gris and comparison of these sequences to the sequences obtained from the NGS data reveal no significant differences between the sequences. Three isolates were analysed using RNAstructure. No structural changes were observed, apart from the TL domain of sample 08-08 Chasselas Blanc, were minor changes occurred due to a number of nucleotide changes.

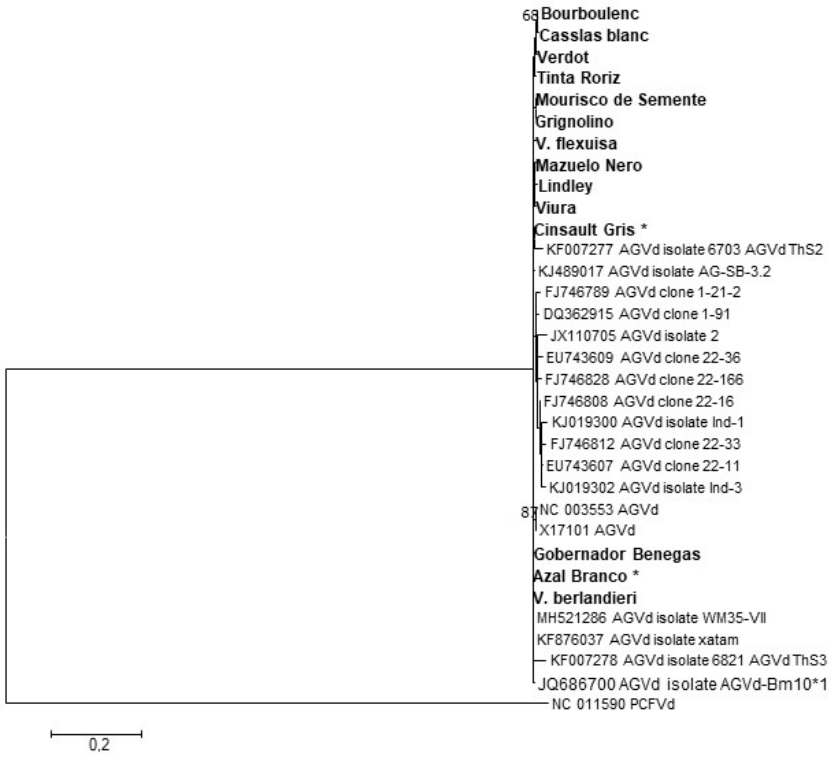

Figure 5. Phylogenetic tree based on the alignment of nucleotide sequences of AGVd isolates obtained in this study (Bold, *RT-PCR derived sequences) and from NCBI Genbank. Sequences that were between 98-100% in similarity were removed and one representative was selected. Tree was constructed in MEGA 7.0 using Maximum Likelihood analysis at 1000 bootstraps. Only bootstraps above 60 are shown. Pepper Chat Fruit Viroid was used as an outgroup.

Discussion

From the 229 accessions in the South African *Vitis* germplasm collection, 370 full viroid sequences of five of the seven known grapevine infecting viroid species, as well as 33 full sequences of GHVd were obtained. This study is the first in-depth study on the diversity of grapevine infecting viroids in South Africa, and the first report of GHVd and JGVd in South Africa.

Differences between nucleotide sequences of isolates obtained for HSVd suggest that it follows a quasispecies model (33,49). These unique HSVd isolates can be divided into two groups, one similar to HSVd isolate SDLY-23, which originates from strawberry, and the other sharing nucleotide identity to HSVd isolate PGH-2, which originates from grapevine. Several *Vitis* accessions may contain a mixed infection of different strains of HSVd and the observed difference may be a result of recombination between strains (2,28). The HSVd sequences have several SNPs distributed across the genomes, which have led to changes in the secondary structure of the molecule. Changes in secondary structure may reflect in other properties such as host interaction and disease expression (26,27,50).

GYSVd-1 occurs in a naturally heterogenous population and can be separated into four types (22,28,50). Results of this study indicate that GYSVd-1 type 1, GYSVd-1 type 2, GYSVd-1 type 3, and GYSVd-1 type 4 are present in the South African *Vitis* germplasm collection, with type three being the more abundant of the two. Results also indicate that some vines may contain mixed infections, such as sample 05-02 Azal Branco containing type 1 and type 3. Types 1, 3, and 4 have been implicated in diseases (26, 28, 45, 46), however the nucleotide identity difference between these three is relatively low, ranging from 89.8% to 91.8%. These differences may contribute to the variability and transient expression of the yellow speckle-vein banding symptoms (26,51). The occurrence of mixed infections is important for diagnostic testing as disease symptoms are type-specific. Therefore, cultivars which have tested positive for GYSVd-1 may need to be tested using type specific RT-PCR. Yellow speckle disease is more prominent during the height of the summer temperatures, and therefore surveys for disease expression and sampling for leaf and petiole tissue should occur during this time. Differences between nucleotide sequences of the isolates of the four GYSVd-1 types obtained in this study as well as phylogenetic analyses support the suggestion that GYSVd-1 follows a quasispecies model (28). The GYSVd-1 sequences identified here have SNPs distributed throughout the genomes. This has resulted in modifications to the secondary structure of the viroids, and this may influence disease expression (8). Changes in secondary structure may reflect in other properties such as host interaction (51). Collectively, these GSYVd-1 isolates were obtained from a variety of *Vitis* hosts

including different table and wine grape species. The lack of significant variation between isolates suggests that they are not under diversifying selection (25).

GYSVd-2 has been shown to be less genetically diverse than GYSVd-1 (Jiang et al., 2009b) but also forms a quasispecies (8,28,52). GYSVd-2 isolates obtained in this study showed limited genetic variation with sequence identities between isolates ranging from 97.5-100%. Several samples contain SNPs however there was a single dominant sequence present throughout most of the samples. Those isolates containing nucleotide changes may experience a change in secondary structure. The rod-shaped secondary structure of the viroid has been shown to serve as functional motifs which interact with cellular factors of the host to accomplish various aspects of replication and disease induction (51). These GSYVd-2 viroids were obtained from a variety of hosts including different table and wine grape species and cultivars, and lack of variation between isolates suggests that they are not under diversifying selection (47). Only GYSVd-1 and -2 result in disease in grapevine (35), symptoms were not surveyed during this study due to the presence of grapevine leaf roll-associated viruses and vitiviruses in the majority of the 229 accessions.

AGVd variants can be distinguished from one another by geographic origin (8,28,52). Several isolates obtained in this study possess a number of SNPs. These isolates containing nucleotide changes experience a change in secondary structure when compared to the reference NC_003553. These changes in secondary structure may reflect in other properties such as host interaction (18,30). Sample 09-03 Cinsault Gris is distinguished from the other isolates, this may be because the sequence is shorter than the others. A similar effect can be seen with KY114494, which is shorter in length and groups outside of the AGVd sequences (29). Phylogenetic analyses and nucleotide sequence identity show that AGVd does not possess a high degree of genetic variability (30), though wider testing may reveal more diversity within the South African population especially if AGVd was introduced through multiple sources (31,32). These AGVd isolates were obtained from a variety of hosts including different table and wine grape cultivars and lack of variation between isolates suggests that they are not under diversifying selection (35,50).

JGVd is a tentative new viroid species of 367bp detected in symptomless grapevines in Japan (16) and was identified in sample 44-02 Katta Khurgan and confirmed using

RT-PCR. This isolate of JGVd has a 96% sequence similarity with the available genome of JGVd on Genbank (LC500206). Katta Khurgan is an Asian grapevine variety, therefore JGVd is potentially more widespread in Asia and has simply not been reported. It was only reported from one grapevine in Japan (16) and not reported during a recent NGS study of grapevine in Russia (53). JGVd is thought to have originated from sequence recombination between viroid species of the apscaviroid group, similar to how AGVd is thought to have originated through sequence recombination of viroid species of both the apscaviroid and pospiviroid groups (16,54).

GHVd is a poorly characterized circular, single-stranded RNA molecule discovered through the bioinformatic analysis of small interfering RNAs of grapevine in Italy (19). It is 375 nucleotides in length and shares many structural characteristics of viroids or of some small circular satellite RNA molecules. GHVd has been detected in Italy, France (17), California (19), and Greece (20), and potentially more widespread than previously thought, however as it is not tested for this is unknown. GHVd was detected and confirmed using RT-PCR for the first time in South Africa during this study but further studies are needed to understand the exact nature of this molecule.

Two grapevine viroids were not identified in this study, CEVd and GLVd. GLVd has only been identified in two countries so far, China (15) and Italy (34), thus this viroid does not appear to have a widespread presence in the grapevine industry though further testing may shed some light on this. CEVd is found in almost all citrus growing regions of the world but it has only been identified in a handful of grape growing regions and therefore it is not unexpected to for CEVd to not be present in this study (35). Testing vineyards planted near citrus orchards would be a potential avenue to determine whether CEVd is in South Africa.

This study has provided the first insight into the diversity of grapevine infecting viroids in South Africa. Given the high level of stringency applied during this study, further studies are needed to determine if any new viroid species are potentially present in South African vineyards. With the increasing temperatures across the winelands, especially over the summer period, more expression of yellow speckle disease may be observed (35,50). It may therefore to be prudent to consider adding

GYSVd-1 and GYSVd-2 to the list of viral agents targeted for elimination in the industry.

Author contributions SWM, and GP contributed to the study design. DR and GP contributed towards sample collection and processing. SWM, DR, JTB, and GP, wrote the manuscript.

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Supplementary data table 1: Results of NGS analysis, using both Reference mapping and nBLAST, of block D2 indicating positive viroid infection.
Supplementary data table 2: percentage identity scores between HSVd isolates determined by SDTV1.2
Supplementary data table 3: percentage identity scores between GYSVd-1 isolates determined by SDTV1.2
Supplementary data table 4: percentage identity scores between GYSVd-2 isolates determined by SDTV1.2
Supplementary data table 5: percentage identity scores between AGVd isolates determined by SDTV1.2
Supplementary data table 6: Genbank Accessions of viroid sequences obtained in this study

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards The manuscripts constitutes original research and has not been previously published. All authors are in agreement for manuscript submission, which complies with the ethical standards of the journal.

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