



VITICULTURE ORIGINAL RESEARCH ARTICLES

Grapevine red blotch virus (GRBV) in a historical germplasm collection in south-eastern Australia

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ABSTRACT

The first detection of grapevine red blotch virus (syn. GRBV; species *Grablovirus vitis*, genus *Grablovirus*, family Geminiviridae) in Australia was initially reported in several grapevine varieties in Western Australia in 2022, but its impact and spread in the country is still currently unknown. In this study, GRBV was detected in 14 locally selected and imported wine, table and dried grapes varieties from a historical germplasm collection in Victoria ($n = 12$) and in germplasms in South Australia ($n = 2$), using a combination of a nested endpoint polymerase chain reaction (PCR) assay and tiled amplicon genome sequencing. Phylogenetic and median-joining network analyses indicated that there may have been at least three separate introductions of GRBV into the historical germplasm collection. The phylogenetic, sequence identity, median-joining network, and single nucleotide polymorphism analyses indicate a close relationship between the South Australian, Western Australian and most Victorian GRBV isolates, which appear to have emerged from an introduction of infected *Vitis vinifera* cv. Perle de Csaba in the 1960s. Spatial analyses and the known history of the 14 infected Victorian and South Australian varieties, also provide evidence of GRBV spread *via* vegetative propagation of Perle de Csaba from the historical collection to South Australia and Western Australia. The analyses also indicated that a very slow and inefficient spread of GRBV between vines in the same vineyard has also possibly occurred *via* an unknown vector. Seasonal testing of selected varieties from the Victorian collection suggests winter as the optimal time for GRBV testing under environmental conditions in north-west Victoria.

KEYWORDS: GRBV, tiled amplicon HTS, phylogenetic analysis, median-joining variant network, SNP analysis, spatial analysis

DATA SUMMARY Seven supplementary tables and two supplementary figures are available in the online version of this article. Table S1. “The GenBank accession numbers for 143 grapevine red blotch virus (GRBV; *Grablovirus vitis*) genome sequences available on NCBI and their respective country of origin”, Table S2. “PCR and tiled amplicon sequencing results of 37 cultivars or clones (total = 111 grapevines) collected from the CSIRO germplasm collection in Irymple and tested seasonally in November 2022 (spring), and February (summer), May (autumn) and July (winter) in 2023, for grapevine red blotch virus (GRBV; *Grablovirus vitis*)”, Table S3. “The number of reads generated from tiled amplicon sequencing, number of mapped reads and depth calculated using Geneious, assembled contig size of grapevine red blotch virus (GRBV; *Grablovirus vitis*), its percentage similarity to reference genome NC_022002 and GenBank accession number of each GRBV isolate for grapevine cultivars or clones that were tested seasonally and the final GRBV status based on the results of PCR and Sanger sequencing and tiled amplicon sequencing”, Table S4. “PCR, Sanger sequencing and tiled amplicon sequencing results including with mapped reads, depth, size of assembly and percentage identity to the grapevine red blotch virus (GRBV; *Grablovirus vitis*) reference genome (GenBank accession NC_022002) of the GRBV strain detected in pooled cultivars collected from the CSIRO germplasm collection and tested in May 2023 and July 2023 for GRBV”, Table S5. “The grapevine red blotch virus (GRBV; *Grablovirus vitis*) status cultivars tested a part of South Australia survey, which were known to be linked directly or indirectly or had potential linkages to cultivars or clones that were also tested in Victoria and Western Australia”, Table S6. “The grapevine red blotch virus (GRBV; *Grablovirus vitis*) status cultivars that were present in the CSIRO germplasm collection and which were known to be linked directly or indirectly or had potential linkages to cultivars or clones that were also tested in South Australia”, Table S7. “Single Nucleotide Polymorphisms (SNPs) detected across different positions in full genomes of grapevine red blotch virus (GRBV; *Grablovirus vitis*) detected from various grapevine cultivars collected from South Australia (SA), Victoria (Vic) and Western Australia (WA) in comparison to GRBV reference genome NC_022002”, Figure S1. “Seasonal testing for 111 grapevines collected from the CSIRO germplasm collection (Irymple, Victoria), representing 37 cultivars or clones across six blocks (2a, 3a, 4a, 5b, and 6a)”, Figure S2. Percentage identity for 27 full genome sequences of grapevine red blotch virus (GRBV; *Grablovirus vitis*) including 12 isolates from Victoria, 2 from South Australia and 13 from Western Australia which were assembled from tiled amplicon sequencing”, Figure S3. “Spatial analysis maps generated by join-count statistics for grapevine cultivars within the CSIRO germplasm collection that were positive and indeterminate for grapevine red blotch virus (GRBV; *Grablovirus vitis*). Analyses were done for (a) three blocks (C block 2-27, C block 55-83, and H block) of the original collection at Merbein, Victoria, Australia, and at (b) five blocks (2a, 3a, 4a, 5b, and 6a) of the current collection in Irymple, Victoria, Australia. Cultivars were analysed both with and without indeterminate GRBV cultivars. Each grapevine cultivar (consisting of 1-3 grapevines) was considered as a sampling unit and precisely mapped based on assigned coordinates (x: row number, y: grapevine cultivar)”.

INTRODUCTION

Grapevine red blotch disease (GRBD) has been observed in vineyards of North America since 2008 (Calvi, 2011). The disease is now known to be associated with infection by grapevine red blotch virus (GRBV; species *Grablovirus vitis*, genus *Grablovirus*, family Geminiviridae), a DNA virus with a circular, single-stranded, monopartite genome of approximately 3208 nucleotides (Al Rwahnih *et al.*, 2012; Krenz *et al.*, 2012; Poojari *et al.*, 2013; Varsani *et al.*, 2017; Yepes *et al.*, 2018). Detection of GRBV in an archival *Vitis* herbarium specimen collected in 1940 in California demonstrates its long-term existence in North America (Al Rwahnih *et al.*, 2015). Symptoms are characterised by red patches on the leaf blades, veins and petioles in sensitive red grape varieties and appear as irregular chlorotic regions on the leaf blades in sensitive white grape varieties (Cieniewicz *et al.*, 2017; Meng *et al.*, 2017; Rumbaugh *et al.*, 2021). GRBD infection affects fruit quality in sensitive varieties, ultimately also impacting wine quality (Rumbaugh *et al.*, 2023). GRBV infection does not usually cause vine mortality, however, it reduces the lifespan of vineyards by delaying fruit ripening and decreasing yield (Ricketts *et al.*, 2017). As GRBD symptoms are similar to those associated with grapevine leafroll disease there may be difficulties in distinguishing between these diseases when based on visual symptoms alone (Sudarshana *et al.*, 2015).

GRBV has been detected in Argentina (Luna *et al.*, 2019), Canada (Xiao *et al.*, 2015), France (Reynard *et al.*, 2022),

Italy (Bertazzon *et al.*, 2021), India (Marwal *et al.*, 2019), Mexico (Gasperin-Bulbarela *et al.*, 2019), South Korea (Lim *et al.*, 2016) and Switzerland (Reynard *et al.*, 2018). It is thought that the global dissemination of this virus is primarily due to the movement of infected plant material and grafting (Al Rwahnih *et al.*, 2012; Krenz *et al.*, 2012; Poojari *et al.*, 2013). In North America transmission by *Spissistilus festinus*, commonly known as the three-cornered alfalfa treehopper, has been demonstrated to result in the spread of the virus within and between vineyards (Poojari *et al.*, 2013; Bahder *et al.*, 2016; Kahl *et al.*, 2021; LaFond *et al.*, 2022). Other possible insect vectors have also been suggested in North America but are yet to be proven (Wilson *et al.*, 2022). Vector-borne transmission of GRBV has not yet been demonstrated in countries outside of North America. *S. festinus* is currently not known to occur in Australia.

In 2022, GRBV was detected in several grapevine varieties in Western Australia during routine testing of a germplasm collection (Kinene *et al.*, 2025; Eppo, 2023). Tracing linked one infected variety, Perle de Csaba, to have originated from a germplasm collection located in Victoria in the south-east of Australia. The Victorian germplasm collection was established in the 1960s and the variety Perle de Csaba was introduced to Australia from the USA in 1969 (Dry *et al.*, 2022). The aim of this study was to undertake surveillance of the Victorian germplasm collection to determine the prevalence of GRBV and to determine if the local spread of the virus by an insect vector had occurred during the last six to seven decades.

Tracing of infected varieties was also done to estimate the risk of spread of GRBV into South Australia, which has the largest vineyard area and wine production in Australia (Wine Australia, 2023).

MATERIALS AND METHODS

1. Study site

The Commonwealth Scientific and Industrial Research Organisation (CSIRO) germplasm collection based at Irymple in the Sunraysia horticultural district (Victoria, Australia), and referred to as the “CSIRO germplasm collection” was used for this study. The CSIRO germplasm collection contains table, wine and dried fruit grapevine varieties, cultivars, clones, wild *Vitis* species and rootstocks that were imported into Australia from Europe and the USA since the 1800s. The collection also includes locally bred varieties and rootstocks. The district is warm and semiarid, with a long hot growing season and a cool winter. The average annual mean maximum and minimum temperatures are 24 °C and 10.4 °C, respectively. The highest and lowest mean maximum daytime temperatures are 32.5 °C in January (summer) and 15.5 °C in July (winter), respectively; the highest and lowest mean minimum night-time temperatures are 16.9 °C in January (summer) and 4.4 °C in July (winter), respectively (Bureau of Meteorology Australian Government, 2022). Grapevines are grown on their own roots on a single-wire vertical trellis. Row and grapevine spacings are 3 m and 2 m, respectively and the grapevines in the Victorian germplasm collection are regularly watered through a systematic irrigation system.

The CSIRO germplasm collection at Irymple was established in 2011 using cuttings from the original collection that was previously established during the 1960s at Merbein, also located in the Sunraysia horticultural district (Dry *et al.*, 2022). The two sites are approximately 11 kilometres apart. The current collection at Irymple does not hold all the varieties, cultivars and clones, wild *Vitis* species and rootstocks that were held at Merbein.

2. Sample collection

2.1. Seasonal testing – CSIRO germplasm collection

To determine the most reliable season to test for GRBV, samples from 111 grapevines from the CSIRO germplasm collection, representing 37 varieties/clones (3 grapevines each), were collected in November 2022 (spring), and February (summer), May (autumn) and July (winter) in 2023. The 111 grapevines included 60 grapevines (20 varieties/clones) that were located together in a group across three adjacent rows in the block (Figure S1). Two clones of the variety Perle de Csaba that were known to be infected with GRBV were located in the middle row of this group. The remaining 51 grapevines (17 varieties/clones, 3 grapevines each) that were tested were dispersed across the collection and were located approximately 20-250 meters from the group of 60 grapevines (Figure S1). These grapevines

included the variety Kandahar (3 grapevines each) that had tested positive for GRBV in the French national repository at Vassal, France (Reynard *et al.*, 2022); and six varieties (3 grapevines each) imported from UCD between 1964 to 1971, that were reported to be infected with GRBV in the UCD collection (Al Rwahnih *et al.*, 2015). The remaining grapevines included ten varieties (30 grapevines) that were previously grown adjacent to Perle de Csaba in the original Merbein collection but were planted next to Perle de Csaba in the Irymple collection. The three grapevines of the same clone or variety were pooled for testing.

2.2. CSIRO germplasm collection survey

In May 2023 (autumn), samples were collected from 2,823 individual grapevines from the CSIRO germplasm collection, including the 111 seasonally tested grapevines, to determine the incidence and distribution of GRBV in the CSIRO germplasm collection. These grapevines comprised of 944 wine, table or dried grapes varieties, clones, and rootstocks. The grapevines were tested as composite samples of up to nine grapevines (up to 3 varieties/clones) per composite and a total of 312 composite samples were prepared. If a composite sample tested positive by PCR and Sanger sequencing the individual grapevine variety, clone or rootstock was re-tested using the same collected samples. If a negative pool contained a known GRBV-positive variety or had a potential or known link to a GRBV-positive or GRBV-indeterminate variety located in South Australia, those individual varieties were also retested. Grapevines were visually assessed for GRBD symptoms. Re-sampling and confirmatory testing were also done in July 2023 for some varieties that tested negative from a positive pool.

2.3. South Australian survey

In May 2023, 526 grapevines were sampled from germplasms located in different regions of South Australia (representing 58 varieties/clones) and the grapevines were either tested individually or as composite samples of up to five grapevines per composite. There were 22 varieties/clones (Opuzensia Rana; Parsley leafed Chasselas; 4 clones of Muscat Gordo Blanco; 3 clones of Dolcetto; 12 clones of Semillon; Muscat Cannon Hall) that were known or suspected to occur in the CSIRO germplasm collection. In addition, four varieties (Brachetto, Harslevelu, Kadarka and two clones of Perle de Csaba) that were known to be positive for GRBV in Western Australia (Kinene *et al.*, 2025) and also known to occur in the CSIRO germplasm collection (Tables S5 and S6) were also sampled.

2.4. Sampling

The basal (mature) 20-30 cm portion of three to four shoots or canes (with leaves attached, if present), nearest to the trunk, were collected from each grapevine. Shoots with leaves were collected during November 2022 (spring) and February 2023 (summer). Canes were collected during May (autumn) and July (winter) in 2023. Shoots or canes of individual grapevines were placed into a single ziplock bag and stored at 4 °C until processing.

3. DNA extraction and polymerase chain reaction (PCR) conditions

Each shoot of an individual grapevine was sampled for DNA extraction and, wherever possible, at least one petiole from each shoot along with the vascular tissues of shoots were also sampled. Between each individual, or pooled sample, benches were decontaminated, and gloves and scalpel blades were disposed, to prevent contamination. The shoot tissues, 0.3 g (fresh weight) for individual grapevines and up to 2.7 g for composite samples (= number of grapevines × 0.3 g fresh weight) were placed into separate disposable extraction bags (Bioreba) homogenised in a modified lysis buffer at a ratio of 1:10 *w/v* (Green *et al.*, 1999). The DNeasy® Plant Mini Kit (Qiagen) was used for DNA extraction and DNA was eluted in 200 µl Buffer AE. Each extract was stored at –20 °C until further use.

Before GRBV testing, the presence and quality of extracted DNA were assessed using a quantitative PCR assay targeting a conserved region of the plant *cytochrome oxidase (cox)* gene (Weller *et al.*, 2000). The GoTaq® Probe qPCR kit (Promega) was used as per the manufacturer's instructions, with the addition of 2 µl template DNA to each reaction and a 20 µl total reaction volume.

A *Taq* DNA Polymerase kit (Invitrogen) was used for all PCR assays and the total reaction volume of 25 µl contained 2.5 µl of 10X PCR Buffer, 0.75 µl of MgCl₂ (50 mM), 1 µl of dNTPs (10 mM), 1 µl of each primer (10 µM), 0.1 µl of *Taq* polymerase (1 U/µl) and 3 µl of DNA template. Each sample was tested for GRBV with a nested endpoint PCR assay. The first PCR used was an in-house designed pair of primers, GRBV-992-F1 (5'-TTGCATGATATTTATTTTGGGA-3') and GRBV-1562-R1 (5'-CTCCGCGCTCAGATC-3') (this study), which generated a 570 bp amplicon. The first round PCR product was used as the template for nested PCR. The nested PCR used primers C_{pf}/C_{pr} (Krenz *et al.*, 2014) and 3 µl of PCR product from the first PCR as a template, generating a 231 bp amplicon. The amplicons were analysed by gel electrophoresis as described previously (Kaur *et al.*, 2023) and were Sanger sequenced bi-directionally to confirm GRBV detection.

4. Genome sequencing of grapevine red blotch virus isolates

Tiled amplicon sequencing was used to generate genome sequences of GRBV from PCR-positive samples (Kinene *et al.*, 2025). Two multiplex PCR reactions were prepared for each sample, using two 10 µM primer pools of forward and reverse primers for alternate regions of the GRBV genome. The MyFi™ DNA Polymerase kit (Meridian Bioscience) was used following the manufacturer's instructions and 5 µl of template DNA was added to each reaction. The PCR cycling conditions were 30 s at 98 °C for initial denaturation followed by 30 cycles of 15 s at 98 °C and 5 min at 65 °C for annealing and extension. For each sample, the PCR products from both multiplex assays were combined and purified using AMPure XP beads (Beckman Coulter). The concentration of the purified amplicons was quantified

using a spectrophotometer (Nanodrop, Thermo Fisher Scientific) and Qubit™1X dsDNA HS Assay Kit (Thermo Fisher Scientific). The resulting amplicons were normalised ~100 fM per flow cell and 50 ng nucleic acid was used for library preparation with rapid barcoding sequencing protocol using the SQK-RBK004 kit (Oxford Nanopore Technologies), according to the manufacturer's instructions. The libraries were sequenced on the MinION (Oxford Nanopore Technologies) using an R.9.4.1 flowcell and MinKNOW software v.4.3.25 (Oxford Nanopore Technologies).

All the reads from the raw data of each sample were base-called with high accuracy and demultiplexed using Guppy (version 6.4.6), resulting in FASTQ files used for downstream analysis. The Minimap2 (version 2.28) algorithm was used for reference mapping, utilizing the data against the GRBV reference genome available on GenBank (NC_022002). The resulting reads were then processed through sorting, alignment, and polishing stages using default settings with SAMtools (version 1.19.2) and Racon (version 1.4.3). Subsequently, a consensus sequence was generated through Medaka consensus (version 1.11.3) which was viewed in Geneious (version 11.0) to assess coverage, mapped reads and average depth for generated genomes of each sample. An arbitrary threshold for confirming a positive GRBV result by tiled amplicon sequencing was established, requiring more than 60 % coverage of the genome, at least 15-fold depth and a minimum number of 250 mapped sequence reads by the assembled GRBV genome. A total of 13 GRBV sequences, 11 GRBV isolates from Victoria and 2 isolates from South Australia, were deposited into GenBank (Table S1).

5. Results interpretation

A grapevine variety was designated as positive for GRBV if both PCR/Sanger sequencing and tiled amplicon sequencing (in accordance with the above threshold) generated a GRBV sequence. A variety was considered indeterminate (*i.e.*, neither positive nor negative) if the GRBV sequence was detected by PCR/Sanger sequencing but the tiled amplicon sequencing did not generate a sufficient sequence, above the threshold. A variety was considered negative if neither assay generated a GRBV sequence.

6. Sequence analysis

Phylogenetic analysis using full genome sequences of 12 GRBV isolates from Victoria and 2 isolates from South Australia generated in this study and 13 isolates from Western Australia (Kinene *et al.*, 2025) were aligned with 143 GRBV genome sequences available in GenBank (Table S1) using MUSCLE alignment software (Kumar *et al.*, 2018). The genetic distances within the isolate groups were calculated as previously described (Kaur *et al.*, 2023).

7. Single nucleotide polymorphisms (SNPs) analysis

Sequence analysis of the 27 full genomes of the Australian GRBV isolates to identify single nucleotide polymorphisms (SNPs) was performed with the iVar program

(version 1.9.5) (Grubaugh *et al.*, 2019) using the output of SAMtools mpileup command (version 1.15-GCC-11.2.0) (Danecek *et al.*, 2021). The remaining sequences were compiled into a “.csv” file for manual comparison across different nucleotide positions and identification of SNPs. These SNPs identified in genomes originating from Victoria, South Australia, and Western Australia were compared to evaluate similarities and potential relationships.

8. Median-joining (MJ) variant network analysis

Variant networks were created of the 27 full genomes of the Australian GRBV isolates using the Median-joining (MJ) algorithm and visualized using the PopART software (<http://popart.otago.ac.nz>), with default settings, as described previously (Kaur *et al.*, 2023) to evaluate potential relationships amongst the Australian GRBV isolates.

9. Spatial analyses

For the original Merbein collection, the virus status of each variety was based on the known positive or indeterminate status of the same variety in the Irymple collection. Therefore, each individual grapevine was considered as a sampling unit and precisely mapped based on assigned coordinates (x: row number, y: grapevine). For Irymple, each grapevine cultivar (consisting of 1-3 grapevines) was considered as a sampling unit and precisely mapped based on assigned coordinates (x: row number, y: grapevine cultivar).

Spatial analyses were performed using GRBV-positive grapevine varieties only and were repeated using both GRBV-positive and indeterminate grapevine varieties. Their occurrence was analysed using their original location in the CSIRO germplasm collection at Merbein, across three separate blocks (C block 2-27, C block 55-83, and H block; Figure S3a) and in their current location at Irymple across six blocks (2a, 3a, 4a, 5b, and 6a; Figure S3b). The spatial analyses utilized data indicating the presence (1) or absence (0) of GRBV in a variety.

Moran’s index (Moran’s I) (Moran, 1950) was employed in R studio (version 2023.12.0) to assess aggregation and spatial dependence of GRBV-positive and indeterminate grapevines in each block. Moran’s I measures the strength and significance of the spatial correlation, particularly

suitable for nominal variables such as healthy (0) and diseased (1). Moran’s I value ranges from -1 (indicating perfect dispersion) to +1 (indicating perfect correlation; that is, strong clustering).

Join-count statistics (Cliff & Ord, 1971) were applied in R studio (version 2023.12.0) to the grapevine varieties, offering insights into spatial autocorrelation of binary data and the occurrence of clustering. This analysis classifies adjacent units (Diseased: D or Healthy: H) by the type of join linking them (D-D, H-H, or H-D), enabling assessment of whether neighbouring units (along rows, across rows, or diagonally) are more likely to share the same or opposite disease status compared to random positioning in the vineyard. For each block in the CSIRO germplasm collection, the observed and expected number of join-counts, along with the standard deviation of the expected value, were determined. The standard normal statistic (Z) was then calculated, with a high positive Z value indicating the clustering of infected plants.

RESULTS

1. Seasonal testing for the presence of grapevine red blotch virus

A summary of the results of seasonal testing of the 111 grapevines from the CSIRO Irymple germplasm collection is presented in Table 1. Over the four seasons, a total of 21 out of 37 varieties tested positive by PCR for GRBV and detection was confirmed in the 21 varieties by Sanger sequencing of the PCR product during at least one of the seasons (Table S2). Based on PCR results only, the least number of positive results were obtained in summer (3/37) and the majority of GRBV-positive detections were obtained in winter (19/37).

However, when based on the definition of a GRBV-positive result as described above, which requires sufficient sequence generated by PCR/Sanger sequencing and tiled amplicon sequencing, only 8/21 varieties/clones were considered true GRBV-positives including Chasselas Parsley IC8424, Chasselas Parsley IC8423, Kandahar, Opuzensia Rana, Peloursin, Perle de Csaba FSAC, Perle de Csaba L6V13 and Primera (Table S3). The total reads generated from genome

TABLE 1. The number of grapevine varieties or clones collected from the CSIRO Irymple germplasm collection that were tested by PCR and tiled amplicon sequencing and found to be positive for grapevine red blotch virus (GRBV; *Glabrovirus vitis*) in November 2022 (spring), and February (summer), May (autumn) and July (winter) in 2023.

Seasonal testing		Spring November 2022	Summer February 2023	Autumn May 2023	Winter July 2023
Total positive varieties	GRBV-indeterminate (PCR and Sanger sequencing only)	0/37*	0/37	2/37	13/37
	GRBV-positive (PCR and Sanger sequencing and tiled amplicon sequencing)	4/37	3/37	4/37	6/37

* Number positive/37 varieties (total = 111 grapevines) that were tested.

sequencing for the eight samples ranged from 255-60,434 and the genome size ranged from 2,577 to 3,212 nucleotides (Table S3). The remaining 13 varieties were considered indeterminate because, although they were positive by PCR/Sanger sequencing, insufficient sequence was generated by tiled amplicon sequencing. Only 3/8 GRBV-positive varieties (two clones of Perle de Csaba, Kandahar) tested positive for GRBV in each season. One variety (Opuzensia Rana) was positive for GRBV in spring and autumn, and four varieties (Peloursin, two clones of Chasselas Parsley, Primera) were positive in winter.

2. CSIRO germplasm collection survey

Most of the sampled grapevines in the CSIRO germplasm collection were asymptomatic across all seasons, although some displayed symptoms such as reddening or yellowing of leaves, often associated with leafroll-like symptoms and possibly with senescence or abiotic stress.

Nineteen of the 312 pooled grapevine samples tested in autumn (May 2023), representing 64 of 944 grapevine varieties, were positive by PCR and Sanger sequencing and tiled amplicon sequencing (Table S4). The total reads generated from the genome sequencing of the 19 GRBV-positive pools ranged from 1,156-109,393 and the genome size from the consensus sequence ranged from 3,206 to 3,210 nucleotides (Table S4). When the 64 grapevine sample pools were processed and tested individually in autumn (May) or winter (July) 2023, only four varieties tested positive by PCR/Sanger sequencing and tiled amplicon sequencing (Couderc Noir; Dolcetto; Kandahar; Semillon). The Kandahar clone detected in this survey is the same clone that tested positive for GRBV during seasonal testing.

Seven pools of grapevine samples collected in May (autumn) initially tested negative for GRBV by PCR. However, when the individual varieties and clones within these pools were tested in both May and July, ten varieties were found to be GRBV-positive. These included Perle de Csaba FSAC, Perle de Csaba L6V13, Opuzensia Rana, five clones of Muscat Gordo Blanco, Muscat Ottonel, and Muscat Cannon Hall. The varietal clones of Opuzensia Rana, Perle de Csaba, Muscat Ottonel and Muscat Cannon Hall are the same as those that tested positive for GRBV during seasonal testing. GRBV was not detected in pools containing the three varieties Brachetto, Harslevelu or Kadarka, nor in each of the varieties when they were tested individually, which were known to be GRBV-positive in the Western Australian germplasm collection.

Combining the seasonal testing and survey results, there were 12 varieties/clones from the CSIRO germplasm collection in which GRBV was confidently detected by PCR/Sanger sequencing and tiled amplicon sequencing. The total reads generated from the 12 varieties/clones ranged from 255-60,434 and the genome size ranged from 2,577 to 3,210 nucleotides (Table S3). There were 19 varieties/clones in which GRBV results were considered indeterminate (Table S3).

3. South Australian survey

Five of the eight South Australian varieties/clones that were tested were positive for GRBV by PCR/Sanger sequencing and tiled amplicon sequencing (Muscat Cannon Hall; Parellada; Muscat Gordo Blanco AS.70.2266; Opuzensia Rana and Perle de Csaba L6V13; Table S3). The Opuzensia Rana and Perle de Csaba clone L6V13 in which the presence of GRBV was detected by PCR/Sanger sequencing was not put through tiled amplicon sequencing because it was assumed to be positive based on the Victoria and Western Australia positive results. The total reads generated from the remaining three GRBV-positive samples ranged from 500-11,370 and the genome size ranged from 2,058 to 3,209 nucleotides (Table S3). Indeterminate results were obtained for a further three (Kishmishi; Montepulciano; White Vernaccio) of the 22 varieties/clones tested in this survey.

4. Linkages between GRBV-positive varieties or clones in South Australia and the CSIRO germplasm collection in Victoria

Three varieties sampled in South Australia *i.e.* Muscat Cannon Hall, Opuzensia Rana and Perle de Csaba, in which GRBV was detected or returned an indeterminate outcome have known or potential links to varieties or clones that were positive or indeterminate in the CSIRO germplasm collection (Table S6). There were also five varieties from the CSIRO germplasm collection that were designated as GRBV-positive or indeterminate in that did not test positive in varieties collected in South Australia, even though they are known or likely to have similar origins (Table S6). Similarly, there were five varieties from South Australia that were GRBV-positive or indeterminate that did not test positive in the CSIRO germplasm collection, even though they are known or likely to have similar origins (Table S6). GRBV was not detected in the three varieties Brachetto, Harslevelu or Kadarka from the CSIRO germplasm collection which are known to be positive in Western Australia (Kinene *et al.*, 2025).

The presence of GRBV was indeterminate in one of four clones of Muscat Gordo Blanco collected in South Australia. GRBV was also detected in two of eight clones in the CSIRO germplasm collection. Three of the eight CSIRO Muscat Gordo Blanco clones were indeterminate for GRBV. There are no known linkages between the South Australian and CSIRO Muscat Gordo Blanco clones.

5. Phylogenetic and sequence identity analysis

Phylogenetic analysis including full genome sequences of 12 GRBV isolates from Victoria, 2 from South Australia, 13 from Western Australia and 143 isolates from overseas (Cieniewicz *et al.*, 2020), demonstrated that all Australian isolates fall into Clade II. The Australian isolates share 99-100% nucleotide identity with each other, although isolates from Victoria, South Australia and Western Australia each cluster in separate groups (Figure 1). Victorian isolates from the varieties Kandahar, Perle de Csaba FSAC and Opuzensia Rana, are less closely related to other Australian isolates

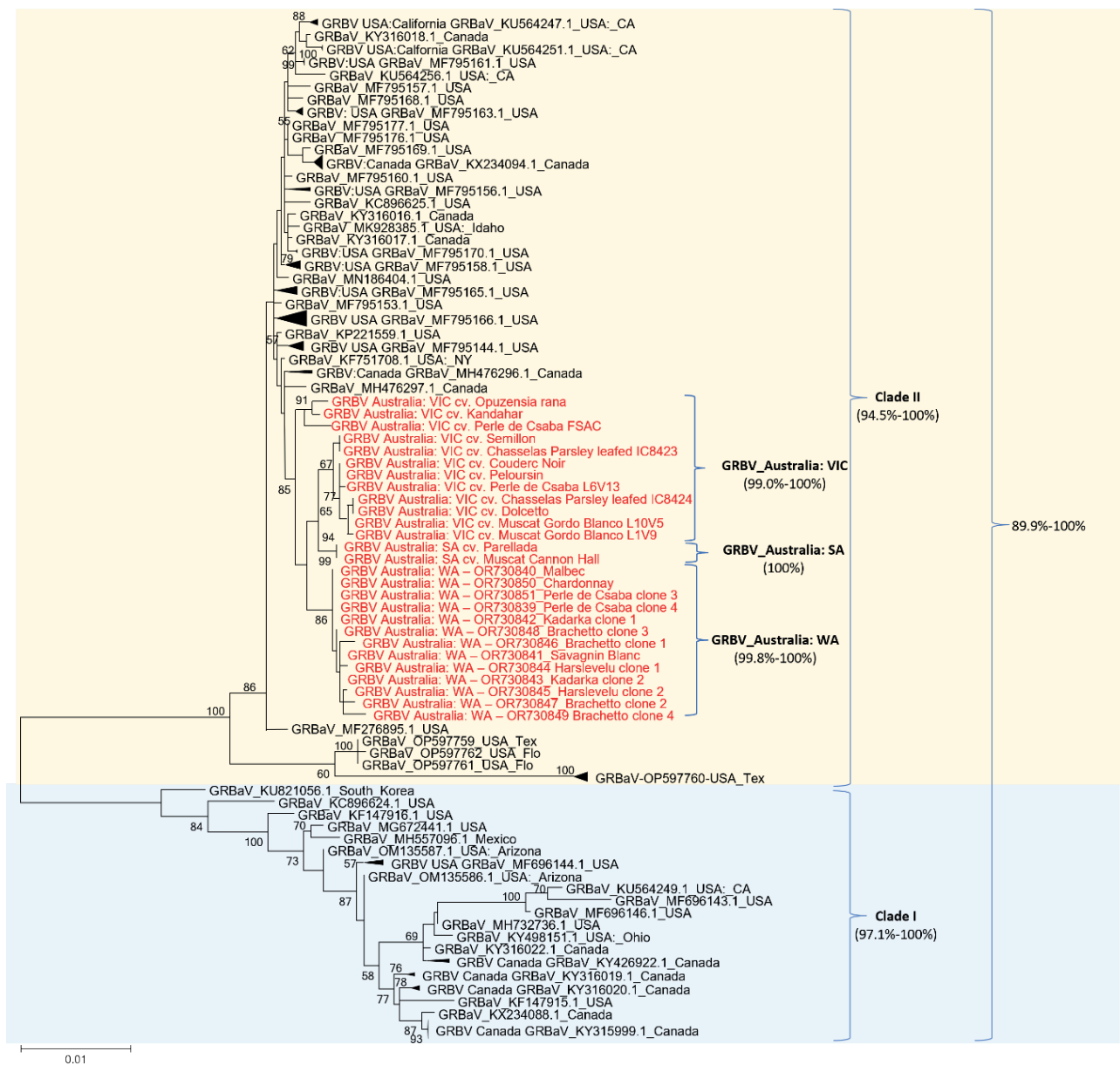


FIGURE 1. Maximum likelihood tree inferred from 27 Australian grapevine red blotch virus (GRBV; *Grabovirus vitis*) full genome sequences, which were assembled from tiled amplicon sequencing, and whole genome sequences of 143 overseas GRBV isolates published on GenBank. The number at each node indicates bootstrap percentages based on 1,000 replicates. The scale bar corresponds to the number of substitutions per site. The percentage (%) identity to each other for each clade is on the right. Australian isolates are shown in red font. SA = South Australia, WA = Western Australia and Vic = Victoria.

and share 99.2-99.4 % nucleotide identity with each other. The two clones of Perle de Csaba from Western Australia shared 99.3 % nucleotide identity with Perle de Csaba FSAC and 99.6 % nucleotide identity with Perle de Csaba L6V13 from Victoria (Figure S2).

6. Median-joining (MJ) variant network analysis

The MJ network constructed from full genome sequences of 12 Victorian GRBV isolates, 2 South Australian isolates and 13 isolates from Western Australia (total = 27 isolates) showed two distinct variant clusters, one containing isolates from the CSIRO germplasm collection and South Australia,

the other containing isolates from Western Australia (Figure 2). The largest cluster of Western Australian isolates are linked to isolates from CSIRO and South Australia by missing intermediate variants (unlabelled black dots).

7. Single Nucleotide Polymorphism (SNP) analysis.

A total of 63 SNPs were detected across the full genomes of 10 GRBV isolates from Victoria and 4 isolates from South Australia identified in this study along with 13 isolates from Western Australia (total = 27 isolates) (Table S7). At positions 108 and 164, the SNPs are shared by all isolates

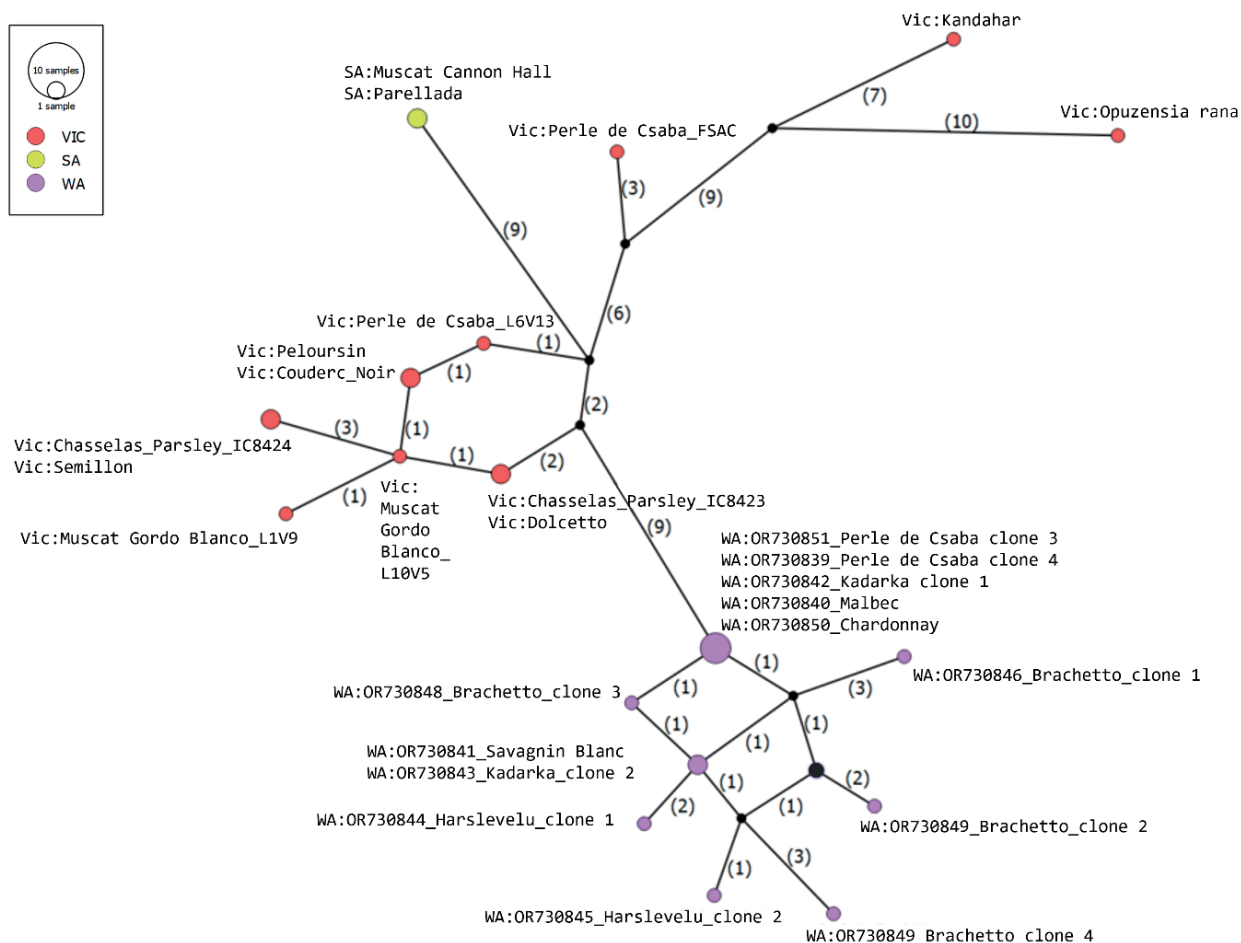


FIGURE 2. Median-joining network constructed from 27 Australian grapevine red blotch virus (GRBV; *Grablovirus vitis*) full genome sequences showing GRBV variants dividing the isolates into two distinct clusters including 27 GRBV isolates from Australia. The sizes of the circles are proportional to the number of times each variant was observed. Unlabelled black dots (median vectors) are missing intermediate variants connecting the variant groups. Numbers in brackets infer the number of mutations separating the variants. Geographical distribution is represented by different colours: SA = South Australia, yellow; WA = Western Australia, purple; and Vic = Victoria, orange.

from Western Australia and South Australia along with five isolates from Victoria including the two clones of Perle de Csaba. At position 241, the SNP is found in isolates obtained from Kandahar, Perle de Csaba FSAC from Victoria and all isolates from Western Australia. Another SNP at position 962 is shared by 19/27 Australian isolates. At position 1237, the SNP is shared by all isolates from Western Australia and South Australia along with seven isolates from Victoria including the two clones of Perle de Csaba. An SNP at position 1611 was present solely in isolates from Victoria and South Australia was detected at position 1611, except for the GRBV isolate from the variety Kandahar. Additionally, a SNP at position 2519 was exclusively present only in Western Australia isolates of GRBV.

8. Estimating spread in the germplasm collection with spatial analysis

The CSIRO germplasm collection at Merbein across C block 2-27, C block 55-83 and H block exhibit positive Moran’s I values (0.089-0.186), which indicate significant

clustering of GRBV affected varieties when the indeterminate and true GRBV-positive results were combined and when only true GRBV-positive varieties were analysed. These results were supported by high positive Z-scores (10.39-24.68), obtained from join-count statistics (Table 2, Figure S3).

The CSIRO germplasm collection at Irymple across blocks 2a, 3a, 5b, and 6a show positive Moran’s I values (0.004-0.194), indicating clustering of GRBV affected varieties when the indeterminate and true GRBV-positive results were combined and when only true GRBV-positive varieties were analysed. These findings were reinforced by positive Z-scores (4.38-12.06), signifying significant clustering of infected plants within these blocks. Conversely, block 4a exhibited a Moran’s I value (−0.021) and a Z-score (1.55) close to 0, suggesting a random distribution (Table 2, Figure S3). In block 2a with indeterminates as GRBV positive, and C block 55-83 and block 5b with GRBV positive only, although clustering is indicated by the analysis it cannot be inferred as in each block there was only one GRBV positive and/or indeterminate unit.

TABLE 2. Spatial analysis of grapevine red blotch virus (GRBV; *Grablovirus vitis*) prevalence and incidence among grapevine varieties initially distributed across three blocks (C block 2-27, C block 55-83, and H block) of the CSIRO germplasm collection in Merbein (Victoria, Australia), followed by relocation to five blocks (2a, 3a, 4a, 5b, and 6a) in Irymple (Victoria, Australia). Analysis was conducted using Moran's Index (Moran's I), *p*-values, Z-scores derived from join-count statistics and resulting patterns of the infected grapevines.

Spatial analysis		With indeterminates as positive					Without indeterminates as positive				
Location	Blocks	Number of positive and indeterminate units*	Moran's I	<i>p</i> -value	Z-score	Pattern	Number of positive units	Moran's I	<i>p</i> -value	Z-score	Pattern
CSIRO germplasm collection at Merbein	C block 2-27	18	0.154	< 2.2e-16	18.98	Clustered	8	0.165	< 2.2e-16	20.4	Clustered
	C block 55-83	13	0.186	< 2.2e-16	23.2	Clustered	1	0.158	< 2.2e-16	24.68	Clustered
	H block	10	0.089	< 3.1e-5	10.39	Clustered	3	0.133	< 2.5e-10	11.03	Clustered
CSIRO germplasm collection at Irymple	2a	1	0.154	0.5	5.52	Clustered	No confirmed positive in block 2a				
	3a	11	0.091	0.004	3.65	Clustered	4	-0.022	0.695	0.87	Random
	4a	2	-0.021	0.605	1.55	Random	No confirmed positive in block 4a				
	5b	2	0.004	0.5625	4.38	Clustered	1	0.194	0.955	8.97	Clustered
	6a	16/375	0.141	2.968e-8	12.06	Clustered	7/375	0.091	0.001	6.22	Clustered

* For Merbein, a unit is a variety and comprises of 1-4 grapevines. For Irymple, a unit is a variety and comprises of 1-3 grapevines.

DISCUSSION

This study provides recommendations for optimal sampling times to maximise GRBV detection, an evaluation of PCR protocols for the detection of GRBV, updated information on the prevalence of GRBV in south-eastern Australia and a phylogenetic analysis of GRBV isolates detected in Australia. Both seasonal testing and surveillance studies highlight the complexity of GRBV ecology and diagnosis under some Australian grape-growing conditions. Seasonal testing across spring, summer, autumn and winter suggested winter was a more suitable time for GRBV testing in north-west Victoria due to the higher number of true positive results as compared to other seasons.

A preliminary evaluation and application of several published GRBV PCR assays (Al Rwahnih *et al.*, 2013; Krenz *et al.*, 2014; Poojari *et al.*, 2016) by several Australian diagnostics laboratories demonstrated that they generated off-target amplification or had poor sensitivity when testing grapevines grown in different Australian regions (data not shown). Consequently, an in-house nested PCR, which uses first round primers GRBV-992-F1/GRBV-1562-R1 designed in this study, coupled with the CPfor/CPprev primer pair (Krenz *et al.*, 2014) in the nested step improved test sensitivity and specificity. Nevertheless, to have complete confidence in the survey results for this study, a grapevine was only deemed GRBV-positive if Sanger sequencing of the nested PCR amplicon and sufficient tiled amplicon sequence (≥ 60 % coverage of the genome, ≥ 5 -fold depth, ≥ 250 mapped sequence reads) was generated. If an insufficient GRBV genome sequence was generated by the tiled amplicon

sequence method, the result was deemed indeterminate. Using these criteria, a total of 24 individual grapevine varieties, 21 from the CSIRO germplasm collection and three from South Australia, were considered indeterminate and likely associated with low virus titre in the grapevine sample tested.

Variability in the virus status of cultivars or grapevines tested as individuals was observed between sampling time points. Seasonal effects on GRBV detection and the effect of uneven distribution, and possibly uneven titre, within infected vines has been previously reported (Krenz *et al.*, 2014; Setiono *et al.*, 2018) and could explain these results. Environmental factors, particularly temperature, have been known to significantly influence virus-host interactions and therefore detection of GRBV in the USA and Canada (Rumbaugh *et al.*, 2022; Kahl *et al.*, 2022). These effects could compromise the results of the large composite samples used in this study, leading to false negative results and an underestimation of the number of positive varieties occurring in the germplasms that were surveyed, even though prior studies have shown that GRBV can be detected in pools of up to ten vines using sensitive assays (Reynard *et al.*, 2018). Further research is underway to improve reliability of GRBV testing in Australia and will further evaluate grapevine sampling, pooling and diagnostics assays and seasonal testing over several years and to improve diagnostics outcomes for Australian viticulture.

From the survey of the CSIRO germplasm collection in autumn there were 19 pooled samples, each containing at least three varieties or clones and representing 64 varieties

or clones in total, that were true GRBV-positives. However, the presence of GRBV was not indicated by PCR in any of the varieties or clones from 14/19 of the positive pools when they were retested individually (Table S4). Decontamination was done between samples to prevent contamination, and controls were included for PCR and sequencing to ensure there were no false positive results. The reason for the negative results is uncertain but may have been due to low virus titre or virus degradation during the approximate two-month gap between testing of the pools and the stored samples of individual varieties and clones. Similarly, observations of negative GRBV results after long-term storage of previously positive samples have been made in two other Australian diagnostics laboratories (personal comm. Affinity labs, South Australia and Department of Primary Industries and Regional Development, Western Australia) and these further highlights the complexity of testing for GRBV. GRBV was detected in 12 wine, table or dried fruit varieties in the CSIRO germplasm collection and in two varieties from South Australia vineyards, and there were indeterminate results for 24 other varieties. Further work is required to confirm the detection of GRBV in these varieties and clones. There is also a need to further trace the distribution and GRBV status of any distributed materials, especially for varieties originating from the CSIRO germplasm collection, to better understand the incidence and risk to the industry. However, GRBV was not detected in high throughput sequencing (HTS) based grapevine virome studies of grapevines from commercial vineyards Victoria ($n = 75$) and South Australia ($n = 52$) (Habibi *et al.*, 2023; Kaur *et al.*, 2023; Wu *et al.*, 2023; Wycliff Kinoti, personal communication), although none of the varieties or clones were the same as those which were positive or indeterminate for GRBV in this study, and the sensitivity of the HTS methodology may not have detected the low titre infections of this virus. Nevertheless, the combined results from Victoria and South Australia surveys support the hypothesis that the virus is not widespread in either region.

Phylogenetic and MJ variant network analyses of these GRBV isolates indicate the possibility of three separate introductions of GRBV into the CSIRO germplasm collection, in Kandahar, Opuzensia Rana and Perle de Csaba, even though they share > 99 % nucleotide identity. Based on CSIRO records, we know that the key GRBV-infected variety Perle de Csaba was brought into Australia from the National Clonal Germplasm Repository in California UC Davis, USA in the 1960s, at the same time as Kandahar, while Opuzensia Rana was imported in the 1980's. The high degree of similarity between the Western Australia Perle de Csaba isolate and an isolate from the same variety in the USA (Kinene *et al.*, 2025) supports the hypothesis that the introduction of GRBV-infected Perle de Csaba into Australia. Planting material of Perle de Csaba was distributed from the CSIRO collection in Victoria to other regions including Western Australia and South Australia. The high similarity and clustering between Perle de Csaba isolates in Victoria, South Australia and Western Australia, along with the known history of its importation and subsequent distribution, provides

evidence that spread through infected planting material of this variety has occurred from the CSIRO germplasm collection to the other two states. Interestingly Kishmishi, which was also imported from the UC Davis collection in the 1960s and in which GRBV was detected in the UC Davis repository (Al Rwahnih *et al.*, 2015), was negative in the CSIRO germplasm collection and indeterminate in South Australia (Tables S5 and S6). Kishmishi was likely to have been distributed to South Australia from the CSIRO germplasm collection. This could suggest that this variety is infected at both Australian sites but the ecology of the virus in these regions leads to potentially false negative results.

The finding that all Australian GRBV isolates cluster within Clade II is consistent with a limited number of introductions into the country. It likely reflects stringent plant quarantine and biosecurity regulations in Australia, restricting new introduction based on observations of symptoms or by testing during selection of varieties at their origin or during Australian post entry quarantine. It may also reflect a lower distribution of GRBV in the USA collection at the time selections for the CSIRO collection were made between the 1960's to the 1980's, although both GRBV clades occur in the UC Davis collection (Al Rwahnih *et al.*, 2015). This hypothesis is further supported by the negative GRBV results in this study for four cultivars in the CSIRO collection (Calmeria, Early Muscat, Kishmishi, Monukka; tested individually or as part of a composite sample) that were imported from the UC Davis collection in the 1960's. It is also possible that uneven distribution of GRBV (Krenz *et al.*, 2014; Setiono *et al.*, 2018) led to provision of uninfected imported dormant canes in some instances.

It is hypothesised that GRBV was introduced into the French germplasm collection at Vassal in 1982 by propagation material of the varieties Husseine and Kandahar that was provided from the CSIRO germplasm collection (Reynard *et al.*, 2022). CSIRO records indicate that both varieties were imported into the CSIRO germplasm collection from the USA in the 1960s and were tested in this study. Kandahar was infected with GRBV in the CSIRO germplasm collection, partly supporting this hypothesis, although there is no GRBV sequence from the French collection to compare and confirm the linkage. However, GRBV was not detected nor was an indeterminate result obtained in the Husseine clone in the CSIRO germplasm collection. Therefore, the possibility exists that GRBV in Husseine in the Vassal collection became infected *via* a different pathway. It is also possible that a false negative result was obtained in the CSIRO accession.

Several lines of evidence support the likelihood that the spread of GRBV within Australia may have occurred *via* an unknown vector. The first is the clustering of Australian GRBV isolates, except Kandahar and Opuzensia Rana isolates, in the phylogenetic analysis and their high percentage sequence identity. The second piece of evidence is the occurrence of GRBV in the variety Dolcetto in the CSIRO germplasm collection which was unlikely to have been infected when introduced into Australia because of

its European origin and importation in the 19th century, possibly before American varieties were introduced to Australia (Table S6). Thirdly, the lack of detection of GRBV in Dolcetto in South Australia, which was derived from the same original importation in the 19th century (Table S6). Another line of evidence is the detection of GRBV in Semillon SA82 in the CSIRO germplasm collection, but not in grapevines of Semillon SA82 tested in South Australia, even though they were derived from the same original source in the 1980s (Table S6; Nicholas, 2006). These results support the similar observations made in other varieties from Western Australia that were unlikely to have been infected when imported into Australia (Kinene *et al.*, 2025).

Further evidence of GRBV spread within the CSIRO germplasm collection is derived from the statistically significant clustering of GRBV-confirmed infected varieties as well as both infected and indeterminate varieties at the original Merbein germplasm collection site and the current location of the collection at Irymple in the spatial distribution analyses. It is hypothesised that a transient species of Auchenorrhyncha taxa may serve as a potential vector, exhibiting rapid arrival on the grapevines, short feeding periods, quick acquisition of GRBV and subsequent movement to neighbouring grapevines or other plant species. The three-cornered alfalfa hopper (*Spissistilus festinus*) is the only confirmed vector of GRBV (Bahder *et al.*, 2016; Flasco *et al.*, 2021). However, recent research suggests that there may be other potential insect vectors capable of transmitting GRBV which include other species within the family Membracidae and closely related Auchenorrhyncha taxa such as *Colladonus reductus* (Cicadellidae), *Osbornellus borealis* (Cicadellidae), and *Melaniolarus* sp. (Cixiidae) (Cieniewicz *et al.*, 2019; Dalton *et al.*, 2020; Kahl *et al.*, 2021; LaFond *et al.*, 2022; Wilson *et al.*, 2022). While these species are not known to occur in Australia, there are many other Auchenorrhyncha species that are found in Australia which may be potential vectors (Atlas of Living Australia, 2024). Therefore, surveillance efforts are required to assess their prevalence and determine their potential as vectors.

The phylogenetic and MJ network analyses further show that most isolates from Victoria, South Australia and Western Australia are closely clustered and more distant from overseas isolates. However, Western Australia isolates cluster away from both Victoria and South Australia suggesting that after the introduction the Western Australia isolates may have diverged from the Victoria and South Australia isolates with time. This is also supported by distinct SNPs occurring in Western Australia isolates compared to many of the South Australia and Victoria isolates and vice versa (Table S7). For example, distinct SNPs occur at genome positions 1611 and 2159 in all but one South Australia/Victoria GRBV isolate and all Western Australia isolates, respectively. There are also other SNPs that occur in all Western Australia GRBV isolates but less frequently in Victoria and South Australia isolates, particularly genomes positions 108, 164 and 241. Conversely, at genome position 1507, most South Australia and Victoria isolates exhibit

SNPs less frequently compared to Western Australia isolates. The occurrence of SNPs that are common in both South Australia and Victoria but distinct from Western Australia isolates, and *vice versa*, are different from overseas strains providing further evidence of spreading events after the introduction of GRBV into each region. The remaining SNPs occurring in Australian GRBV isolates could have occurred due to the presence of quasispecies within a grapevine variety. Perle de Csaba is likely to be the primary source for the spread of GRBV in Western Australia and the CSIRO germplasm collection, given the closer relationship of isolates in other varieties to those in Perle de Csaba and the close relationship between Victoria, South Australia and Western Australia Perle de Csaba isolates, demonstrated by the MJ network analysis. There does not appear to be any evidence of the spread from GRBV in Kandahar and Opuzensia Rana. Fortunately, Perle de Csaba is not widespread nor grown commercially in Australia, thus it is unlikely to contribute to significant GRBV spreading events.

CONCLUSION

Further work is needed to understand the potential impact of GRBV in Australia. However, it seems unlikely that the virus is widespread in Australia, due to the limited distribution of most of the known and potentially infected varieties, the lack of detection in commercial vineyards in other Australian studies and the likelihood that if there is an Australian vector, it is inefficient. Therefore, based on the findings from this study and research done in Western Australia (Kinene *et al.*, 2025), it is recommended to retain GRBV as a regulated pest and continue testing for GRBV in *Vitis* species at the Australian border. GRBV testing should also be included in comprehensive virus testing for grapevines in certification programs and routine testing of grapevine production at vineyards that suspect virus-associated diseases such as leafroll, Shiraz disease and red blotch or when growers intend to top work their vineyards. The Australian viticulture industry should be encouraged not to propagate from GRBV infected grapevines, to prevent further dissemination of the virus. If there is a low prevalence of GRBV in Australia, there is an opportunity for its incidental eradication through testing and removal of infected grapevines.

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Author contributions

All authors have read and agreed to the published version of the manuscript. Conceptualization, K. P. K. and F. C.; methodology, K. P. K., D. L., T. K. and F. C.; formal analysis, K. P. K.; investigation, K. P. K.; resources, K. P. K., D. L.,

T. K., A. C., I. D., M. K. and F. C.; data curation, K. P. K., and F. C.; writing—original draft preparation, K. P. K.; writing—review and editing, K. P. K., A. R., M. K., B. R. and F. C.; visualization, K. P. K.; supervision, F. C., A. R. and B. R.; project administration, F. C.; funding acquisition, F. C. and B. R.

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Conflicts of interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of the data; in the writing of the manuscript; or in the decision to publish the results.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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