Determination of the virus diversity associated with Grapevine leafroll disease

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Declaration

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Abstract

Vitis vinifera is the woody crop most susceptible to intracellular pathogens. Currently 70 pathogens infect grapevine, of which 63 are of viral origin. *Grapevine leafroll-associated virus* 3 (GLRaV-3) is the type species of the genus *Ampelovirus,* family *Closteroviridae.* It is considered to be the primary causative agent of Grapevine leafroll disease (GLD) globally; however, the etiology of GLD is not completely understood. Here we report on the viral populations present in GLD symptomatic grapevines across the Western Cape province, South Africa. A widespread survey was performed to screen 315 grapevines for 11 grapevine-infecting viruses using RT-PCR. Additionally, GLRaV-3 variant groups were distinguished with high-resolution melt (HRM) curve analysis used in conjunction with real-time RT-PCR. Members of the family *Closteroviridae* were detected with the highest frequency, particularly GLRaV-3 that was detected in 87% of tested plants. Next-generation sequencing (NGS) is capable of detecting known and novel viruses without prior knowledge of viral sequences and when used in a metagenomic approach is able to detected viral populations within diseased vines. A total of 17 grapevine samples were subjected to NGS using either an Illumina MiSeq or HiSeq 2500 instrument to determine the virome within GLD vines. Collectively, more than 190 million reads were generated through NGS. Read datasets were trimmed and filtered for quality and subjected to both read-mapping and *de novo* assembly. Contigs assembled *de novo* were analyzed with BLAST (Basic Local Alignment Search Tool) against the NCBI (National Centre for Biotechnology Information) database and it was determined that GLRaV-3 was the best-represented virus, comprising 97.5% of the assembled contigs. *Grapevine virus F* (GVF) was detected for the first time in South African vineyards through *de novo* assemblies and the complete genome sequence validated through direct Sanger sequencing. The complete genome of GVF isolate V5 spans 7 539 nucleotides and shares 89.11% nucleotide identity to existing GVF genomes. The data generated through this study will assist in further understanding the etiology of GLD, support the current hypothesis of GLRaV-3 as the primary contributor to GLD, aid in understanding virus associations in diseased vines and potentially develop systems in which to control disease spread and symptom severity.

Opsomming

Vitis vinifera is die houtagtige oes wat die mees vatbaarste is vir intrasellulêre patogene. Tans word wingerde deur 70 patogene geïnfekteer, waarvan 63 van virale oorsprong is. *Grapevine leafroll-associated virus* 3 (GLRaV-3) is die tipe spesie van die genus *Ampelovirus,* familie *Closteroviridae.* Dit word globaal beskou as die primêre oorsaak van Wingerd krulblaar-siekte (GLD), alhoewel die etiologie van GLD nie heeltemal begryp word nie. In hierdie verslag word die virale populasies teenwoordig in GLD simptomatiese wingerde oor die Wes-Kaap provinsie in Suid-Afrika gerapporteer. ‘n Wydverspreide opname was uitgevoer om 315 wingerde met 11 wingerd-infekterende virusse te ondersoek, deur gebruik te maak van tru-transkripsie polimerase ketting reaksie (PKR). Verder is variantgroepe van GLRaV-3 onderskei met hoë-resolusie smeltingskurwe-analise, tesame met die gebruik van in-tyd tru-transkripsie PKR. Die hoogste frekwensie was van die lede van die familie *Closteroviridae,* veral GLRaV-3 wat in 87% van die ondersoekte plante gevind is. Nuwe-generasie volgorderbepaling (NGS) beskik oor die vermoë om bekende en nuwe virusse te herken in virale populasies in geaffekteerde wingerde sonder vorige kennis van virale volgorderbepalings en wanneer dit in ‘n metagenomiese benadering gebruik word kan die virale bevolkings binne siek wingerde ontdek. ‘n Totaal van 17 wingerd-steekproewe was blootgestel aan NGS deur die gebruik van of ‘n Illumina MiSeq of ‘n HiSeq 2500 instrument om die virome te bepaal van GLD wingerde. In totaal is meer 190 miljoen lesings gegenereer deur NGS. Hierdie data lesings was verwerk en gefilter vir kwaliteit om onderwerp te word vir beide kartering en *de novo* samestellings. Contigs verkry deur *de novo* samestellings was geanaliseer met BLAST (Basic Local Alignment Search Tool) teenoor die NCBI (National Centre for Biotechnology Information) databasis en dit was vasgestel dat GLRaV-3 was die mees-verteenwoordigende virus, bestaande uit 97.5% van die saamgestelde contigs. *Grapevine virus F* (GVF) was vir die eerste keer in Suid-Afrikaanse wingerde waargeneem deur *de novo* samestellings en die volledige genoom volgordger is geverifieer deur middel van direkte Sanger volgorderbepaling. Die volledige genoom van GVF isoleer V5 spanwydte van 7539 nukleotiedes en deel 89.11% nukleotied identiteite van bestaande GVF genome. Die gegenereerde data van hierdie studie sal bykomende begrip van die etiologie van GLD bystaan, die huidige hipotese van GLRaV-3 as die primêre bydraer tot GLD ondersteun, verhoogde begrip van virus-assosiasies in wingerdsiektes verseker en potensiële sisteme ontwikkel om siektes en simptome te beheer.

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List of abbreviations

aa	Amino acid
ABI	Applied Biosystems
ABIC	Agricultural Bioscience International Conference
AGVd	*Australian grapevine viroid*
AlkB	2-oxoglutarate-Fe2 oxygenase
AMV	*Avian myeloblastosis virus*
ARC-BP	Agricultural Research Council Biotechnology Platform
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BWT	Burrows-Wheeler Transform
BYV	*Beet yellows virus*
CAF	Central Analytical Facilities
CCD	Charge-couple device
cDNA	Complementary DNA
CP	Coat protein
CPm	Minor capsid protein
CTAB	Cetyltrimethylammonium bromide
CTV	*Citrus tristeza virus*
cv	Cultivar
DAC-ELISA	Direct antigen-coating ELISA
DAS-ELISA	Double antibody sandwich ELISA
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DOI	Digital object identifier
dsRNA	Double-stranded RNA
EDTA	Ethylenediamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
EtBr	Ethidium Bromide
EtOH	Ethanol

FSM	First strand master mix
GB	Gigabase
GEEV	*Grapevine endophyte endornavirus*
GLD	Grapevine leafroll disease
GLRaV	Grapevine leafroll-associated viruses
GLRaV-1	*Grapevine leafroll-associated virus* 1
GLRaV-2	*Grapevine leafroll-associated virus* 2
GLRaV-3	*Grapevine leafroll-associated virus* 3
GLRaV-4	*Grapevine leafroll-associated virus* 4
GLRaV-5	*Grapevine leafroll-associated virus* 5
GLRaV-6	*Grapevine leafroll-associated virus* 6
GLRaV-7	*Grapevine leafroll-associated virus* 7
GLRaV-8	*Grapevine leafroll-associated virus* 8
GLRaV-9	*Grapevine leafroll-associated virus* 9
GLRaV-Car	*Grapevine leafroll-associated virus* Car
GLRaV-De	*Grapevine leafroll-associated virus* De
GLRaV-Pr	*Grapevine leafroll-associated virus* Pr
GNV	*Grapevine-associated narnavirus*
GRSPaV	*Grapevine rupestris stem pitting-associated virus*
GRVFV	*Grapevine rupestris vein-feathering virus*
GSyV-1	*Grapevine syrah virus* 1
GVA	*Grapevine virus A*
GVB	*Grapevine virus B*
GVD	*Grapevine virus D*
GVE	*Grapevine virus E*
GVF	*Grapevine virus F*
GYSVd	*Grapevine yellow speckled viroid*
HLV	*Heracleum latent virus*
HRM	High-resolution melt
HSP70h	Heat shock protein 70 homologue
HSVd	*Hop stunt viroid*

IC-PCR	Immunocapture PCR
ICTV	International Committee on Taxonomy of Viruses
ICVG	International Council for the Study of Virus and Virus-like Diseases of grapevine
IF	Immunofluorescence
kb	Kilobase
LAMP	Loop-mediated amplification of nucleic acids
LChV	*Little cherry virus*
LIYV	*Lettuce infectious yellow virus*
MAQ	Mapping and Assembly Quality
Mha	Million hectares
Mhl	Million hectoliters
mRNA	Messenger RNA
MV2	*Mint virus 2*
NCBI	National Centre for Biotechnology Information
NGS	Next-generation sequencing
NRF	National Research Foundation
nt	Nucleotide
nts	Nucleotides
OD	Optical density
ORF	Open reading frame
OS	Operating system
PCR	Polymerase Chain Reaction
PGM	Personal genome machine
PVP	Polyvinylpyrrolidone
qPCR	Quantitative PCR
RAM	Random access memory
RdRp	RNA-dependent RNA polymerase
RNA	Ribonucleic acid
RO-H_2O	Reverse Osmosis water
rpm	Revolutions per minute

RSA	Republic of South Africa
RT	Room temperature
RT-PCR	Reverse-transcription Polymerase Chain Reaction
SASEV	South African Society for Enology and Viticulture
SAWIS	South African Wine Industry Information and Systems
SBS	Sequencing by synthesis
SDS	Sodium dodecyl sulfate
sgRNA	Sub-genomic RNA
SMRT	Single-molecule real-time
SOLiD	Sequencing by Oligonucleotide Ligation and Detection
SRT	Superscript II reverse transcriptase
SSCP	Single-stranded conformation polymorphism
ssRNA	Single-stranded RNA
SSM	Second strand master mix
STE	Sodium/ Tris/ EDTA
TAE	Tris-acetic acid/ EDTA
Taq	*Thermus aquaticus* DNA polymerase
TAS-ELISA	Triple antibody sandwich ELISA
Tris-HCL	2-amino-2-(hydroxymethyl)-1,3-propanediol-hydrochloric acid
U	Unit
UTR	Untranslated region

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Chapter 1

Introduction

1.1. General introduction

Vitis vinifera, commonly known as grapevine, is of the genus *Vitis*, family *Vitaceae*. Grapevine can be cultivated from tropical to temperate climatic regions in six continents of the world (Burger *et al.,* 2009). It consists of more than 5 000 varieties; however, only a fraction of these have economic value for the production of wine and table grapes (Wine & Spirits Education Trust, 2012). Grapevine fruit (grape) has been used in the production of wine since the Neolithic era, 7 000 years ago (Berkowitz, 1996) and has been used for medicinal purposes. The phytochemicals present in grapes, such as quercetin, polyphenols, proanthocyanidins, resveratrol, ellagic acid, flavonols and phenolic acids have cardio-protective properties that reduce mortality by coronary heart disease (Frankel *et al.,* 1993); furthermore they display anti-cancer properties (Jang *et al.,* 1997), antioxidant, antibacterial and antiviral activity (Pisha and Pezzuto, 1994). A study on wound healing by Nayak *et al.* in 2011, examined the medicinal qualities that grapes exhibit. The wounds of rats treated with grape-oil showed a 10% increase in the reduction of the wound area when compared to the controls (Nayak *et al.,* 2011), further signifying the medicinal properties of grapes.

In 2012, 7.5 million hectares (Mha) of the world's surface area was covered by vineyards, translating to 0.05% of the world's terrestrial capacity; with global wine production at 252 million hectolitres (Mhl), a 10% decrease in the wine production from 2000 (www.oiv.int). The global economy has placed significant focus on the viticulture industry, where the 11 major wine exporting countries (including South Africa) generated approximately R 3.3 trillion in 2012; South Africa produced 10 037 Mhl of wine which amounted to a sum of R 82.8 billion (www.oiv.int). Additional to revenue, the wine industry also generates over 275 000 jobs in South Africa (http://www.sawis.co.za/info/statistics.php).

The majority of wine produced in South Africa is from the Cape winelands; mostly from the Breedekloof region that supplies 17.7% of the wine produced. Wine is, however, produced geographically across South Africa including the provinces: KwaZulu-Natal, the Northern Cape, the Eastern Cape and Limpopo (Krige *et al.,* 2012). Currently, Stellenbosch and surrounding areas occupy the most widespread grapevine plantings, contributing 16.5% of the total hectares of vineyard in South Africa (Krige *et al.,* 2012).

South African wine is diverse, evident in the multitude of varieties available, and grown throughout the country. South Africa produces 21 red wine varieties that make up 45% of the total wine grapes; Cabernet is the most planted red grape variety occupying 12% of vineyardsl; followed by Shiraz, Pinotage and Merlot. White wine grapes comprise of 20 varieties, occupying 55% of South Africa's vineyards. Chenin blanc occupies 18% of vineyards followed by Sauvignon blanc and Chardonnay (Krige *et al.,* 2012). From 2008, an average of 1 000 hectares of wine vineyards have been uprooted opposed to planted every year, which has contributed to a general decline in the land occupied by vineyards in South Africa. Furthermore, 58.2% of total vines are over the age of 10 years while 8.2% of vines are under the age of 4 years (Krige *et al.,* 2012).

To date, more than 70 infectious agents have been identified that infect grapevine (Martelli, 2014), making it the most infected woody crop host known (Martelli, 2012). Although pesticides can control most pathogens and pests, there is little that can be done to eliminate the viruses, viroids and phytoplasmas that infect grapevine. Controlling virus spread mostly depends on management strategies. In the case of Grapevine leafroll disease (GLD) these strategies include the planting of virus-free material, effective control of the insect vectors and the rogueing of infected vines. While some grapevine infecting viruses show seasonal symptoms, others display little to no symptomology (Martelli and Boudon-Padieu*,* 2006) that may differ depending on the extent of the virus diversity in an infected host (Komar et al., 2007) or the strain of virus infecting the host (Monette and James, 1990). Methods such as enzyme-linked immunosorbent assays (ELISA) and reverse-transcription polymerase chain reaction (RT-PCR) allow for the detection of known viruses. However more recent techniques such as metagenomics, when coupled with next generation sequencing (NGS), allow for the identification of novel viruses as well as viruses present at low titre, with higher sensitivity. The recent techniques provide far greater insight into the etiology of grapevine diseases.

Grapevine leafroll disease is one of the most economically destructive viral diseases affecting grapevine production globally, and has major implications in the overall harvest and quality of both premium wine and table grapes (Wine & Spirits Education Trust, 2012). A number of grapevine leafroll-associated viruses (GLRaVs) have been associated with GLD; however *Grapevine leafroll-associated virus* 3 (GLRaV-3) remains the primary contributing agent to GLD. Disease and pests in vineyards may have a significant negative impact on the longevity of grapevine, which can cause substantial damage in the wine industry. Research regarding treatment and management of vineyards is of imperative value to the wine economy in South Africa and other wine producing countries.

1.2. Aims and objectives

The aim of this study was to identify the virus diversity associated with GLD using NGS technology as well as to determine the full complement of viruses present in grapevines that display GLD symptoms, across the Western Cape province. The following objectives were set in order to achieve these aims:

- To survey the Western Cape province to identify grapevines displaying typical GLD symptoms.
- To extract high quality double-stranded RNA (dsRNA) from a subset of vines to be used in metagenomic NGS to determine the virome of these grapevines.
- To perform bioinformatics analysis, including read mapping and *de novo* assemblies, of NGS data received.
- To determine the viral population present in grapevines collected in the survey using RT-PCR.
- To determine the complete genome of a *Grapevine virus F* isolate through direct Sanger sequencing.
- To further describe GLD and the virus diversity involved with this disease.

1.3. Chapter layout

This thesis is comprised of 6 chapters: a general introduction, a literature review, followed by three research chapters and a general conclusion. All chapters are introduced and referenced independently.

Chapter 1: Introduction

An introduction to the research and its relevance, the aims and objectives of the research study, an outline of the chapter layout as well as the research outputs accomplished throughout this study.

Chapter 2: Literature review

Information pertaining to agents infecting grapevine, GLD and virus detection methods.

Chapter 3: Identification and distribution of multiple virus infections in Grapevine Leafroll diseased vineyards.

In this chapter, a widespread survey of grapevines displaying GLD symptoms in Western Cape vineyards is described together with the virus populations identified in diseased vineyards.

Chapter 4: Determination of the virus diversity associated with Grapevine leafroll disease using next-generation sequencing.

In this chapter, the use of NGS in identifying the virus populations in 17 GLD symptomatic grapevines is described. The bioinformatics analysis used for quality control of NGS data and in determining the populations of viruses is also reported in this chapter.

Chapter 5: Detection of Grapevine virus F in South African vineyards through de novo assemblies.

In this chapter, the discovery of GVF isolate V5 is reported. The use of NGS in detecting the isolate and direct Sanger sequencing in validating the genome are described together with the genetic variation shared with existing GVF isolates.

Chapter 6: Conclusion

General concluding statements and future prospects of this study.

1.4. Research outputs

The subsequent publications, conference proceedings, international presentation as well as poster were based on results of the research study.

1.4.1. Publications

- Jooste, A.E.C., **Molenaar, N**., Maree, H.J., Bester, R., Morey, L., de Koker, W.C., Burger, J.T. (2014). Identification and Distribution of Multiple Virus Infections in Grapevine Leafroll Diseased Vineyards. *European Journal of Plant Pathology. Accepted*
- **Molenaar, N**., Burger, J.T., Maree, H.J. (2014). Detection of *Grapevine virus F* in South African vineyards through *de novo* assemblies. *Archives of Virology. Submitted*

1.4.2. Conference proceeding

- **Molenaar, N**., Maree, H.J., Burger, J.T. Determination of the virus diversity associated with Grapevine leafroll disease using next-generation sequencing. Research produced was based on work performed in Chapter 4 and was presented by N. Molenaar at the 35th South African Society for Enology and Viticulture (SASEV) congress, Somerset West, Cape Town, South Africa, 13 – 15 November 2013.

- Jooste, A.E.C., **Molenaar, N**., Maree, H.J., Bester, R., de Koker, C., Burger, J.T. Distribution of GLRaV-3 variants and other grapevine viruses in the Western Cape vineyards. Research produced was based on work performed in Chapter 3 and was presented by Dr. A.E.C. Jooste at the 35th South African Society for Enology and Viticulture (SASEV) congress, Somerset West, Cape Town, South Africa, 13 – 15 November 2013.

1.4.3. International presentation

- **Molenaar, N**. Global food security and attempts to relieve world hunger. Presented by N. Molenaar, as bursary and grant holder, at the Tomorrows Leaders Forum, Agricultural Bioscience International Conference (ABIC), Saskatoon, Saskatchewan, Canada, 5 – 8 October 2014.

1.4.4. Poster:

- **Molenaar, N**., Maree, H.J., Burger, J.T. Determination of the efficiency of next-generation sequencing as a diagnostic tool for plant viral diseases. Poster summarizing research performed in Chapter 4 was presented at the Agricultural Bioscience International Conference (ABIC), Saskatoon, Saskatchewan, Canada, 5 – 8 October 2014, by N. Molenaar. Poster won first prize in the category of emerging technologies.

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1.6. Interent Sources

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Chapter 2

Literature review

2.1. Introduction

Grapevine is a deciduous woody species that can be cultivated in temperate regions across the globe. It is an economically important crop as its fruit has been used comprehensively for thousands of years; from eating fresh to producing jam, juice, vinegar, jelly and, most importantly, wine. Grapevine is, however, the most susceptible crop plant to intracellular pathogens, among which viroids, viruses and xylem- or phloem-limited prokaryotes remain the most significant grapevine-infecting agents. In 1964 the International Council for the Study of Virus and Virus-like Diseases of Grapevine (ICVG) was established to provide a database of methodologies and research pertaining to the viruses that affect grapevine. The ICVG has identified 70 infectious agents of grapevine, 63 of which are viruses belonging to eight separate families (Martelli, 2014). These viruses are distributed across five major diseases affecting grapevine, namely: Infectious degeneration and decline, Grapevine leafroll disease (GLD), Graft incompatibility, Rugose wood complex and Fleck complex (Martelli and Boudon-Padieu, 2006; Martelli and Digiaro, 1999). Grapevine leafroll disease, caused by viruses from the family *Closteroviridae*, remains the most extensive and economically destructive grapevine viral disease (Martelli and Boudon-Padieu, 2006; Pietersen, 2004). These diseases negatively affect grapevine in terms of yield, quality and longevity of the crop. The advances in technology have allowed research of grapevine and the associated disease etiology to be studied at a far greater depth (Martelli and Boudon-Padieu, 2006).

The development of NGS has permitted an unprecedented approach to detect viruses that are present at a low titre, new variants of viruses as well as novel viruses on a metagenomic scale. NGS offers a technology capable of producing large amounts of data at greater cost-effectiveness when compared to conventional detection techniques. Furthermore, the time taken to produce this data is significantly reduced, making the efficiency of NGS unparalleled. (Beerenwinkel and Zagordi, 2011). The use of NGS allows for the identification of the complete genetic material in an environmental sample and has been used in the determination of the virome of combined grapevine samples (Al Rwahnih *et al.,* 2011; Coetzee *et al.,* 2010).

2.2. Grapevine leafroll disease

2.2.1. Distribution and etiology

Grapevine leafroll disease was identified more than a century ago; however, nutrient deficiency was

thought to be the initial cause of the symptoms. Through the grafting of plant material displaying typical GLD symptoms onto healthy stock, the transmission of the symptoms was a clear indication of a pathogen-derived disease (Charles *et al.,* 2006a; Over de Linden and Chamberlain, 1970). It was not until 1989, after the observation of GLD spread in California, that scientists suspected mealybug as a potential vector for the transmission of the disease (Habili *et al.,* 1995; Jordan *et al.,* 1993; Engelbrecht and Kasdorf, 1990; Tanne *et al.,* 1989; Teliz *et al.,* 1989). The etiology of GLD is not well understood, however viruses from the family *Closteroviridae* are known to cause the disease. The first evidence of this occurred with the identification of closterovirus-like particles within infected plant phloem (Namba *et al.,* 1979) and with ultrastructure research done on the leaf tissue of GLD symptomatic vines (Castellano *et al.,* 1983; Faoro *et al.,* 1981). Subsequent studies revealed nine serologically unrelated clostero-like viruses that are connected with GLD (Hu *et al.,* 1990; Zimmermann *et al.,* 1990; Rosciglione and Gugerli, 1987; Rosciglione and Gugerli, 1986; Gugerli *et al.,* 1984); however this number was recently reduced to five (Martelli *et al.,* 2012). The first confirmation of GLD occurred through ELISA in 1992 at UC Davis in the Foundation vineyard (Rowhani and Golino, 1995) where-by samples displaying optical density (OD) readings of three times greater than the control were considered to be infected (Rowhani, 1992). GLD is detected in all grape-growing regions of the world, which includes Africa, Europe, the Middle East, New Zealand and North and South America (Sharma et al., 2011; Charles et al., 2009; Fuchs et al., 2009; Maliogka et al., 2009; Fiore et al., 2008; Maree et al., 2008; Mahfoudhi et al., 2008; Akbas et al., 2007; Charles et al., 2006a; Habili et al., 1995). The international exchange and distribution of infected material is responsible for the primary spread of the disease.

2.2.2. Symptomatology

GLD is an economically important disease, responsible for up to a 60% loss in grapevines product yield (Rayapati *et al.,* 2008). Symptoms may vary considerably and are dependent on a variety of conditions, which include: regional climate, geographical distribution, grape cultivar, age of vineyard, pruning techniques, virus strain and length of infection, season as well as soil composition (Mannini *et al.,* 2012; Freeborough and Burger, 2008; Rayapati *et al.,* 2008; Cabaleiro *et al.,* 1999). Physical symptoms may not always be present as, in the case of certain white-fruited cultivars (*Vitis vinifera*) and rootstocks.

Symptoms become evident in early spring, during which the development of buds and shoots are delayed. From early to midsummer, physical symptoms on the leaves become apparent. The interveinal regions of leaves of red-fruited cultivars exhibit dark-red or purple pigmentation while primary and secondary veins remain green (Figure 2.1.B). In white-fruited cultivars the symptoms

are less evident where the interveinal areas of leaves are visible in a pale green or yellow chlorotic coloration (Figure 2.1.C). For both red- and white-fruited cultivars the downward rolling along the margins of leaves remains uniform and progresses towards the end of autumn; however, the type of cultivar determines the extent of leaf-rolling. White-fruited cultivars may either show pronounced leaf-rolling by harvest, evident in Chardonnay; or may not show any signs of leaf-rolling, as evident in Sauvignon blanc and Tompson Seedless, making the visual identification of infected vines much more challenging (Rayapati *et al.,* 2008).

Other symptoms of GLD include the degeneration of phloem cells in stems and leaves which results in reduced photosynthetic activity. The degeneration of phloem cells causes an accumulation of starch caused by hindered carbohydrate movement (Charles *et al.,* 2006a; Charles *et al.,* 2006b; Cabaleiro *et al.,* 1999; Mannini *et al.,* 1996) and results in delayed ripening of the fruit, reduced levels of soluble solids and anthocyanin pigments, and an increase in titratable acidity and grapevine susceptibility in unfavorable environmental conditions (Fuchs, 2007). The viruses associated with GLD may also trigger young vine failure as well as graft incompatibility (Golino *et al.,* 2002).

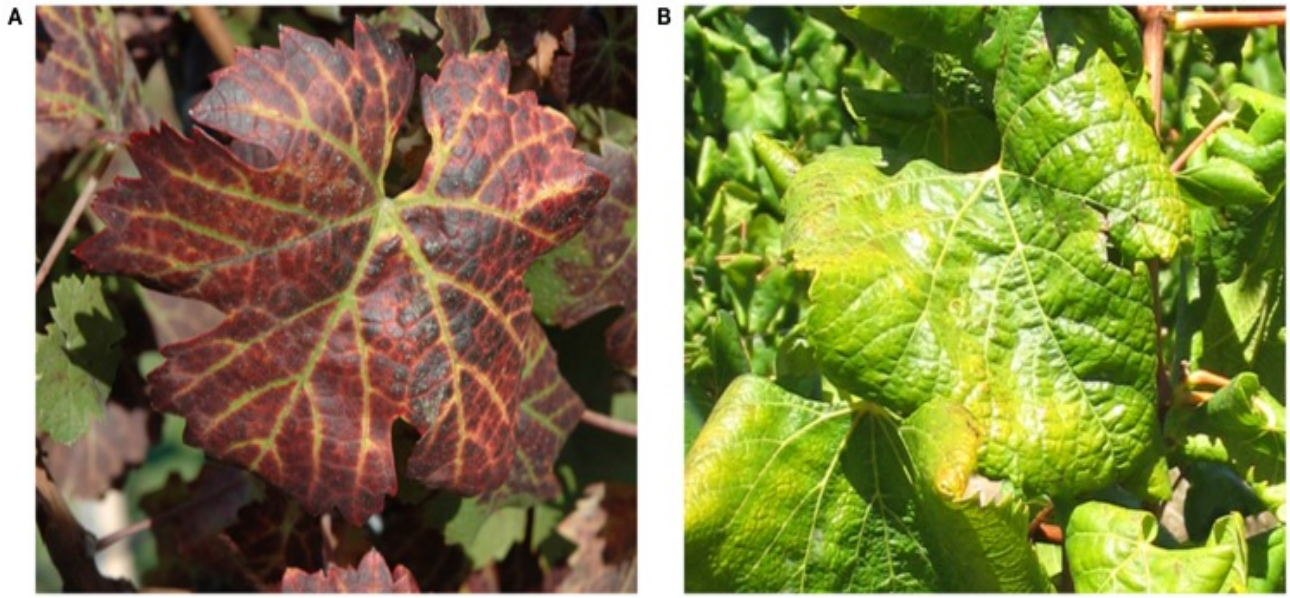

Figure 2.1: Visual symptoms of GLD. A) Infected red-fruited cultivar, *Vitis vinifera* cv Cabernet Franc. B) Infected white-fruited cultivar, *Vitis vinifera* cv Chardonnay (Maree *et al.* 2013).

2.2.3. Associated viruses

Viruses of the family *Closteroviridae* are associated with the onset of GLD symptoms, in particular *Grapevine leafroll-associated virus* 3 (GLRaV-3), which is believed to be the primary causative agent of the disease. Members of the family *Closteroviridae* include *Grapevine leafroll-associated viruses* 1 to 9 (GLRaV-1, -2, -3, -4, -5, -6, -9), numbered in the order that they were discovered, as well as GLRaV-Pr, GLRaV-De and GLRaV-Car. More recently it has been proposed that GLRaV-4, -5, -6, -9, -Pr, -De and -Car be grouped together (Martelli *et al.,* 2012). All members, with the exception of GLRaV-2 and GLRaV-7 that belong to the genera *Closterovirus and Velarivirus* respectively, are of the genus *Ampelovirus* (Martelli *et al.,* 2012; Al Rwahnih *et al.,* 2011; Maliogka *et al.,* 2009; Martelli and Boudon-Padieu, 2006).

2.2.4. Transmission

The primary means for transmission of GLD is through vegetative propagation; however, GLD can also be spread to adjacent vines and vineyards by mealybugs (Martelli and Boudon-Padieu, 2006; Charles *et al.,* 2006b). Soft scale insects (*Coccidae*) and certain mealybug (*Pseudococcidae*) species, particularly the vine mealybug (*Planococcus ficus*) (Figure 2.2.A), have the capacity to vector GLRaV-1, -3, -4, -5 and -9, all members of the genus *Ampelovirus* (Martelli *et al.,* 2002).

Planococcus ficus (Signoret), a small phloem-feeding, hemi-metabolous insect was first identified in the Western Cape province by Joubert in 1943 and has remained a pest in viticultural practices (Charles *et al.,* 2006a; Walton and Pringle, 2004). The average *Planococcus ficus* female will lay approximatelly 362 eggs that will overwinter as eggs or as young instar nymphs, residing on the roots and beneath the bark of vines. As the seasons progress and an optimum growth temperature between 23^{o}C and 27^{o}C is reached, the development of the mealybugs progresses through the second and third nymphal instar stages. After the third nymphal instar stage it is possible to distinguish between mealybug sexes. The males develop long filamentous anal setae and wings but lack mouthparts for transmitting GLRaVs; the females are wingless, exhibiting limited mobility (Fuchs, 2007). Upon sexual maturity, the female mealybugs release pheromones, promoting copulation through which the cycle of reproduction continues (Walton and Pringle, 2004). There are two monitoring systems used to determine the population density of mealybug species within infected vineyards; these include taking physical samples of *P. ficus* infected vines, a labour-intensive system, and pheromone-based monitoring (Walton and Pringle, 2004).

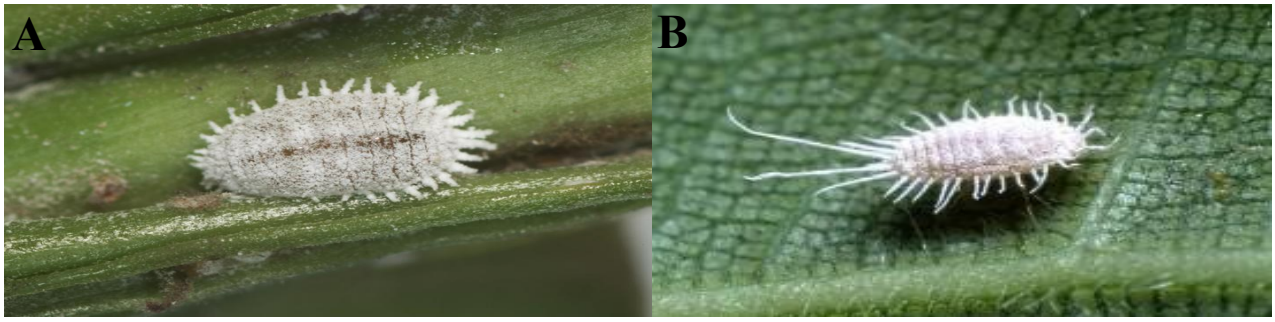

Figure 2.2: Photographs of *Vitis vinifera* mealybugs. A) *Planococcus ficus* (www.udec.ru/vrediteli/chervec.php) B) *Pseudococcus longispinus* (http://www.bcpcertis.com/Certis.bcp/English/Home/Our+Solutions/Biological+Controls/Pests+Find+out+more/Mealybug+find+out+more/page.aspx/1091).

Research by Tsai *et al.* in 2008, studied the transmission parameters involving *Planococcus ficus* and the transfer of GLRaVs to grapevine. The study determined that the first instars had greater vector efficiency when compared to mature mealybugs. The acquisition of virus transmission occurred within one hour of mealybug exposure and peaked at twenty-four hours where it remained constant for four days until individuals lost the ability to transmit GLRaV-3 (Tsai *et al.,* 2008). Tsai *et al.* noted that GLRaV-3 was not transmitted from female to progeny and that virus transmission occured in a semi persistent fashion.

The spread of GLD is dependent on the type of cultivar infected, as indicated by a study performed in 2009. GLD spread at a higher rate in young Chardonnay vines compared to young Merlot vines through the mealybug vector *Pseudococcus longispinus* (Figure 2.2.B); suggesting that Chardonnay is more susceptible to the spread of GLD through *Pseudococcus longispinus* (Charles *et al.,* 2009).

2.2.5. Disease management

No curative measures successful in eliminating GLRaV in an infected vine has been documented. The prevention of infection and of further spread of the disease is the most efficient manner in which to manage GLRaV infection (Almeida *et* al., 2013; Rayapati *et al.,* 2008). Removing and re-planting entire vineyards that contain infected vines is the most effective strategy by which to remove virus infection; however, this has associated economic implications. Selective rogueing of vines adjacent to infected individuals may act as a sensible method, but may overlook infected vines that display no visual signs of infection (Almeida *et* al., 2013; Rayapati *et al.,* 2008). Furthermore, root remnants are capable of housing the virus for up to one year, allowing newly planted material to become infected through another mealybug vector transmission (Bell *et al.,* 2009). To ensure that infected young grapevines are not planted, there are a number of methods available to eliminate virus infection. These methods include: heat therapy, somatic embryogenesis

and meristem tip cultures. Other methods applicable in the reduction of virus spread and insect control include the use of chemicals such as chlorpyrifos, imidacloprid and/ or prothiophos, in the dormant period of mealybug development, as well as implementing natural enemies, such as parasitoids (*Coccidoxenoides perminutus* and *Leptomastix dactylopii*) and predatory beetles (*Nephus angustus, N. bineavatus* and *N. quadrivittatus*) which aid in the control of mealybug circulation. The negative effects of mealybug can be further reduced through the cultural control of surrounding vegetation (Walton *et al*., 2004). Certification programs for virus-free plant material have been introduced in many countries to reduce the spread of viruses across countries and within local vineyards.

2.3. *Grapevine leafroll-associated virus* 3

2.3.1. Taxonomy

GLRaV-3 is the type species of the genus *Ampelovirus* and a member of the family *Closteroviridae* (Martelli *et al.,* 2012; Cabaleiro *et al.,* 1999). The establishment of the family *Closteroviridae*, in 1998, determined that the members of this family be distinguished based on whether the viruses involved comprised of genomes that were either monopartite or bipartite (Martelli *et al.,* 2011). In 2002, the International Committee on Taxonomy of Viruses (ICTV) reviewed the classification of viruses to distinguish between individuals based on biological and molecular properties (Martelli *et al.,* 2002). They determined that the genus *Ampelovirus* was associated with the family *Closteroviridae.* Virus members of the genus *Ampelovirus* are divided into an additional two subgroups based on genome structure and size (Table 2.1); subgroup I viruses form a coherent phylogenetic cluster and are considered divergent variants of GLRaV-4 (Martelli *et al.,* 2012; Abou Ghanem-Sabanadzovic *et al.,* 2010).

Table 2.1: Members of the genus *Ampelovirus* with corresponding subgroup number. (Abou Ghanem-Sabanadzovic *et al.*, 2010; Maliogka *et al.*, 2009; Maliogka *et al.*, 2008; Martelli *et al.*, 2002).

Genus *Ampelovirus*		
Virus Name:	**Abbreviation:**	**Subgroup:**
Grapevine leafroll-associated virus 4	GLRaV-4	I
Grapevine leafroll-associated virus 5	GLRaV-5	
Grapevine leafroll-associated virus 6	GLRaV-6	
Grapevine leafroll-associated virus 9	GLRaV-9	
Grapevine leafroll-associated virus Pr	GLRaV-Pr	
Grapevine leafroll-associated virus De	GLRaV-De	
Grapevine leafroll-associated Camelian virus	GLRaV-Car	
Pineapple mealybug wilt-associated virus 1	PMWaV-1	
Pineapple mealybug wilt-associated virus 3	PMWaV-3	
Plum bark necrosis stem-pitting-associated virus	PBNSPaV	
Grapevine leafroll-associated virus 1	GLRaV-1	II
Grapevine leafroll-associated virus 3	GLRaV-3	
Pineapple mealybug wilt-associated virus 2	PMWaV-2	
Little cherry virus 2	LChV-2	

2.3.2. Morphology and genome organization

Members of the genus *Ampelovirus* are comprised of a linear, positive sense single-stranded RNA (ssRNA), monopartite genome that ranges between 13 kb and 18.6 kb (Martelli *et al.,* 2002). The virion particles are flexible, filamentous and remarkably long (1 400 to 2 200 nm) in length with a diameter of between 10 nm and 13 nm. The body of the virion is assembled through a 34 kDa major capsid protein (CP) while the minor capsid protein (CPm) is a main component of the virion head, responsible for cell-to-cell movement (Martelli *et al.*, 2002; Ling *et al.,* 1997; Zee *et al.,* 1987).

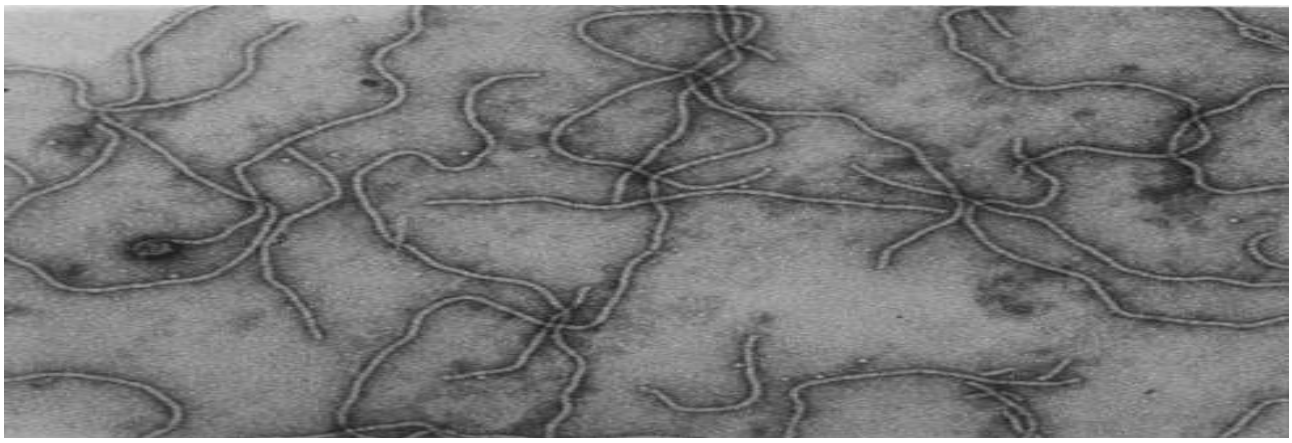

Figure 2.3: Purified GLRaV-3 virion particles, isolated from a GLD vine, visualized with a transmission electron microscope and stained with 1% (w/v) aqueous uranyl acetate. (Picture by G.G.F. Kasdorf).

GLRaV-3 isolate GH11 has the second largest genome, of 18 671 nt in size (NCBI Accession: JQ655295.1), of all plant infecting viruses, second to *Citrus tristeza virus* (19 296 nt) (NCBI Accession: NC_001661.1). The first reporting of a GLRaV-3 genome (isolate NY-1) occurred in 2004; NY-1 possessed 13 open reading frames (ORFs) and included 5' and 3' untranslated regions (UTRs) of 158 and 277 nts, respectively (Ling *et al.,* 2004). A study performed in 2008 disproved isolate NY-1 as being a complete genome due to a shortened 5' UTR (Maree *et al*., 2008); the evidence based on the findings that GLRaV-3 isolates GP18 (NCBI Accession: EU259806.1), 621 (NCBI Accession: GQ352631.1) and 623 (NCBI Accession: GQ352632.1) have 737 nucleotide 5' UTRs (Jooste *et al.,* 2010; Maree *et al.,* 2008). Subsequent findings revealed that GLRaV-1 has the longest 5' UTR (780 nt) of any representative of the family *Closteroviridae*. Other *Closteroviridae* members and corresponding 5' UTR lengths include: *Little cherry virus* (LChV-2; 539 nt) (NCBI Accession: AF531505); *Citrus tristeza virus* (CTV; 107 nt) (NCBI Accession: DQ272579); *Grapevine leafroll-associated virus* 2 (GLRaV-2; 105 nt) (NCBI Accession: AY881628) and *Lettuce infectious yellow virus* (LIYV; 97 nt) (NCBI Accession: NC_003617) (Martelli *et al.,* 2002). The 5' UTR of the members of the family *Closteroviridae* has little known function, however, remains highly variable between variants of GLRaV-3, a valuable characteristic for the studies of GLRaV-3 variation.

GLRaV-3 has a genome organization consistent with that of the conventional closterovirus described by Agranovsky *et al.* (1994). It comprises of 13 ORFs labeled 1a, 1b and 2 to 12 (Figure 2.4) as well as a lengthy GC-rich intergenic region situated between ORFs 2 and 3, which is uncharacteristic of members of the family *Closteroviridae*. ORFs 1a and 1b house the replication genes that encode the replication-associated proteins. More specifically, ORF 1a contains four conserved domains: a papain-like protease, a viral methyltransferase, AlkB and a Viral RNA helicase protein. These encode a large polypeptide, while ORF 1b encodes the RNA-dependent RNA-polymerase (RdRp) expressed through a +1 translational frameshift due to an overlapping nucleotide with ORF 1a (Maree *et al.,* 2008; Ling *et al.,* 2004; Ling *et al.,* 1998). ORF 2 is believed to encode a small peptide (p6), however, it is absent in GLRaV-3 group VI isolates. Homologs have been detected within the closteroviruses, however the functionality of this protein is unknown (Bester *et al.,* 2012; Peremyslov *et al.,* 2004a). ORF 3 encodes a small hydrophobic transmembrane protein (p5) whose equivalent in *Beet yellows virus* (BYV) is responsible for cell-to-cell movement and is contained within the endoplasmic reticulum (Peremyslov *et al.,* 2004a). The ORF 4-encoded homolog of cellular HSP70 molecular chaperones (HSP70h) is responsible for cell-to-cell movement (Peremyslov *et al.,* 1999) and the assembly of small virion heads (Peremyslov *et al.,* 2004b; Alzhanova *et al.,* 2001). The 60 kDa protein (p55) encoded by ORF 5 shares little sequence similarity when compared to other members of the family *Closteroviridae*; however, it shares a similar function to HSP70h and is believed to assist in cell-to-cell movement as well as in the assembly of virion heads (Alzhanova *et al.,* 2007). Open reading frame 6 encodes a coat protein (CP) that aids in cell-to-cell movement (Alzhanova *et al.,* 2000), while ORF 7 encodes a minor capsid protein (CPm; p53), a major component of the virion head (Agranovsky *et al.,* 1995). Open reading frames 3 through to 7 encode five genes conserved throughout the family *Closteroviridae* and are collectively implicated in virus cell-to-cell movement (Dolja *et al.,* 2006). Open reading frames 8 (p21), 9 (p19.6), 10 (19.7), 11 (p4) and 12 (p7) do not have determined function due to their conservation exclusively within the genus *Ampelovirus*. However, ORFs 8 to 10 are believed to play a role in viral transport as well as RNA silencing suppression (Dolja *et al.,* 2006). The p19.7 protein encoded by ORF 10 exhibits varying suppressor activity, as a result of amino acid substitutions, across GLRaV-3 variants and is strongly believed to have an association with RNA silencing (Gouveia and Nolasco, 2012). ORFs 11 and 12 remain unique to GLRaV-3 (Ling *et al.,* 1998).

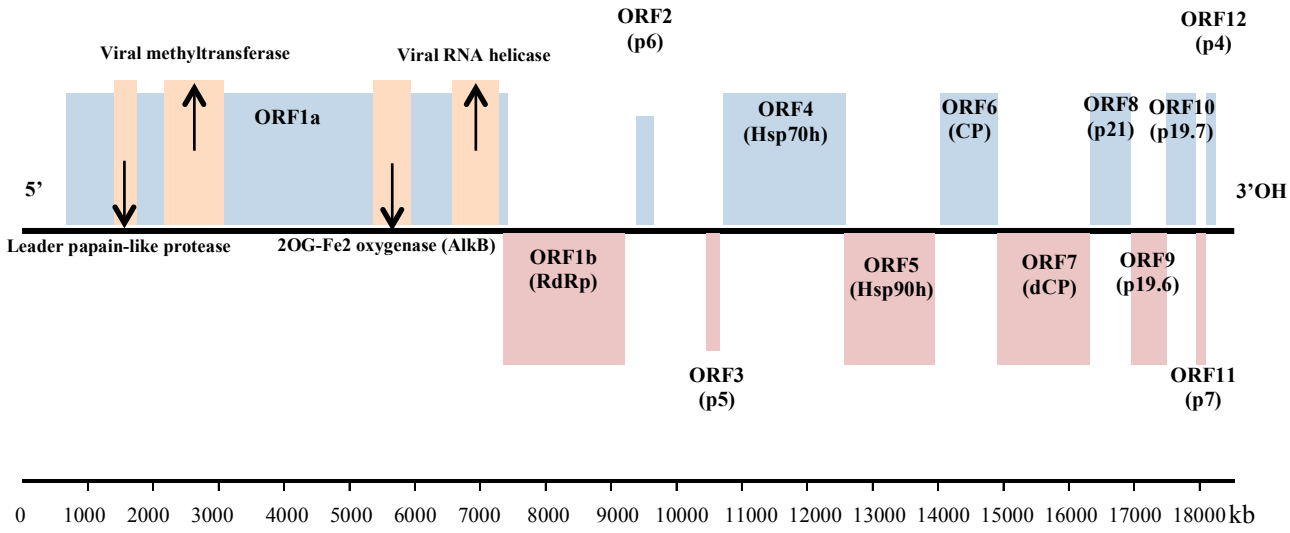

Figure 2.4: Schematic representation, drawn to scale, of the GLRaV-3 isolate GP18 genome. Outlined are the ORF sizes and positions (nt) and corresponding encoded proteins as well as the untranslated regions (UTRs) adapted from Maree *et al.,* 2008.

2.3.3. Virus replication and genome expression

GLRaV-3 replication, unlike other members of the family *Closteroviridae* (CTV, BYV), has not been studied comprehensively and is assumed to follow similar modes of replication to these closteroviruses (Maree *et al.*, 2013; Dolja *et al.,* 2006). The majority of closteroviruses are transmitted through insect vectors to the host plant whereby positive stranded RNA viruses will follow a four-stage replication cycle. Replication commences with virus uncoating, which is followed by primary and secondary translation of the exposed viral nucleic acids to construct both structural as well as non-structural proteins. Genomic replication then occurs and finally the formation of capsids around genomic RNA progenies, which allows for the spread of virions through the plant (Dolja *et al.,* 2006). Viral replication is mediated through the RdRp, which initially synthesizes a complementary negative RNA strand serving as the template for subsequent synthesis of subgenomic RNAs (sgRNAs) and genomic RNA progenies. The number of sgRNAs synthesized by a virion is equivalent to the number of ORFs enclosed within that virion, allowing for a monocistronic translation of the ORFs beginning with the 5'-proximal ORF (Dolja *et al.,* 2006). The synthesis of sgRNAs occurs in a timely fashion to optimize virion competence. In order to achieve greater efficiency the host defense systems need to be disrupted, therefore sgRNAs responsible for the translation of RNA silencing suppressors are effectively formed in the initial phases of infection (Dolja *et al.,* 2006). Once the translation of the genome has commenced, a number of replication complexes will form, giving rise to multitudes of structural proteins aiding in the assembly of the virion (Dolja *et al.,* 2006). Studies performed, in 2010, by Maree and Jarugula

et al. indicated that sgRNAs can be used to translate additional ORFs and that ORFs 6, 8, 9 and 10 occurred in greater quantities during infection. Their results demonstrate the differences in the levels of expression of sgRNAs during various stages of replication in grapevine.

2.3.4. Distribution and genetic variants

Geographically, GLD is distributed across all regions of the world and as the primary infectious agent contributing towards GLD, GLRaV-3 is also assumed to be present in the listed regions (Sharma *et al.,* 2011; Charles *et al.,* 2006a). The distribution of GLRaV-3 variants within vineyards varies as a range of factors determines the level of distribution. These include: viticulture practices, wind direction, introduced infected material, vector transmission of the virus as well as the range of GLRaV-3 variants already present in the vineyard. GLRaV-3 variant group II and VI have been shown to be most prevalent in South African vineyards (Bester *et al.,* 2012; Jooste *et al.,* 2011). In China group I variants were found most common (Farooq *et al.,* 2012) and GLRaV-3 variant group II and I most frequent in Portugal (Gouveia *et al.,* 2009). The occurrence of single and mixed variant infection is, likewise, dependent on a multitude of factors. A study performed in the vineyards of Napa Valley in 2011 found that 21% of the diseased vines had multiple infections (Sharma *et al.,* 2011).

Currently, 11 distinct isolates of GLRaV-3 have been fully sequenced (Table 2.2). Previous research, regarding the genetic variability of GLRaV-3, made use of single-stranded conformation polymorphisms (SSCP) along with sequence comparisons of the RdRp, CP and HSP70h genes (Jooste and Goszczynski, 2005; Turturo *et al.,* 2005). The results revealed that the above-mentioned regions shared greater than 90% nucleotide identity when compared and that GLRaV-3 isolates 623 and 621 fall within the divergent variant group II and I, respectively (Jooste and Goszczynski, 2005). Further research performed in 2009 revealed nucleotide differences in the range of 74.1% to 100% and amino acid identity differences in the range of 85.9% to 100% when comparing the HSP70h gene of various GLRaV-3 isolates, confirming a potential five variant groups numbered I to V (Fuchs *et al.,* 2009). Successive research, making use of phylogenetic analysis, revealed an additional two variant groups (VI and VII^{P}) (Sharma *et al.,* 2011; Bester *et al.,* 2012a; Chooi *et al.,* 2009; Seah *et al.,* 2012). However divergent, the greatest variation among isolates of GLRaV-3 remains in the 5' UTR regions of the genomes, whereby isolates 621, 623 and PL-20, representatives from variant group I, II and III, respectively, show between 22% and 33% variation (Jooste *et al.,* 2010).

Table 2.2: Fully sequenced GLRaV-3 isolates with corresponding genome size, variant group and GenBank accession numbers.

Virus Name:	Isolate:	Genome Size (nt):	Variant Group:	Accession Number:
Grapevine leafroll-associated virus 3	LN	18 563	III	JQ423939.1
Grapevine leafroll-associated virus 3	GH30	18 576	VI	JQ655296.1
Grapevine leafroll-associated virus 3	GH11	18 671	VI	JQ655295.1
Grapevine leafroll-associated virus 3	GP18	18 498	II	EU259805.1
Grapevine leafroll-associated virus 3	CI-766	17 919	I	EU344893.1
Grapevine leafroll-associated virus 3	PL-20	18 433	III	GQ352633.1
Grapevine leafroll-associated virus 3	623	18 498	II	GQ352632.1
Grapevine leafroll-associated virus 3	621	18 498	I	GQ352631.1
Grapevine leafroll-associated virus 3	WA-MR	18 498	I	GU983863.1
Grapevine leafroll-associated virus 3	3138-07	18 498	I	JX559645.1
Grapevine leafroll-associated virus 3	GH24	18 493	-	KM058745

2.4. A means to viral diagnostics

Virus detection in diseased plants is complicated by various factors, including: mixed viral infection, low virus titre in early infections, comparable disease symptoms from unrelated viruses, uneven viral spread in infected vines as well as less visible symptoms in certain cultivars and rootstock plants. Viruses are detectable in most regions of a plant, including the veins of leaves, roots, trunk and canes; phloem remains the most useful tissue in which to sample GLRaV-3 (Ling *et al.,* 2001; Teliz, 1987). Currently, there are a number of techniques available for the detection of viruses in plant material, these include: biological indexing, serology, electron microscopy, nucleic-based techniques as well as next-generation sequencing.

2.4.1. Biological indexing

Biological indexing was one of the initial techniques developed in order to detect grapevine virus diseases (Nicholas, 2006; Weber *et al.,* 2002). Before molecular detection techniques became available in the late 1980s, biological indexing was used to identify a plant's response to pathogen-derived diseases through the inoculation of a test vine with a control vine, either through grafting or rub-inoculation (Constable *et al.,* 2010). There are three primary types of biological indexing for grapevine viruses, herbaceous indexing, green grafting and hardwood indexing. Sensitive indicator plants are used in each technique that includes the *Vitis vinifera* cultivars Cabernet Franc, Cabernet

Sauvignon, Barbera or Pinot Noir. Herbaceous indexing makes use of rub-inoculation practices whereby extracts from a test vine are rubbed onto the leaves of an indicator plant, inside a greenhouse. Within several weeks, diagnostic symptoms in the test plant will develop upon virus infection (Weber *et al.,* 2002). Green-grafting makes use of undeveloped scions grafted onto young test shoots. The advantages of this technique are evident in the graft success rate as well as in the ability of the graft to overcome incompatibility with distant *V. vinifera* species (Walker and Golino, 1999). The most labor-intensive and time-consuming means to index is through hardwood or woody indexing; the practice required to determine the diseased state of plants infected with GLRaV-3. Woody indexing involves the grafting of test plants onto indicator plants in a field whereby the symptoms of the disease are observed over two consecutive growing seasons (Weber *et al.,* 2002). In order for the results obtained through woody indexing to have significance, test fields should be geographically isolated as to minimize interference with external pathogens and test plants should be healthy (Constable *et al.,* 2010). Results obtained through indexing may vary depending on unpredictable criteria, which may include: strain of virus, virus distribution and titre as well as minimal symptom expression (Constable *et al.,* 2010; Rowhani *et al.,* 1997). Other complications remain in the ambiguous symptom expression of certain viruses in indicator plants as well as non-specific symptoms that may be evident due to environmental stress or viruses that display similar visual symptoms (Monis and Bestwick, 1996). However successful biological indexing may be as a viral disease-associated detection technique, the practice of disease confirmation requires skilled virologists with reliable observations.

2.4.2. Serological assays

Various immunodiagnostic methodology designs exist whose primary approach for viral detection is through antigen-antibody reactions, a few of these techniques involve: Enzyme-linked ImmunoSorbent assays (ELISA), Immuno-strip tests and immunofluorescence (IF) (Schaad *et al.,* 2003). ELISA offers a robust, simple, cheap and high throughput approach in the detection of viruses and remains the most frequently used immunodiagnostic method since the 1970s (Ward *et al.,* 2004; Clark and Adams, 1977; Engvall and Perlmann, 1971). There are four forms of ELISA used under different conditions, namely: direct antigen-coating (DAC) ELISA, indirect ELISA, triple antibody sandwich (TAS) ELISA and double antibody sandwich (DAS) ELISA. The latter is one of the most common forms of the technique used in the detection of viruses. The mechanisms involved in each technique remain similar while the detection of the antigen-antibody complex varies (Koenig and Paul, 1982). DAS-ELISA functions through the attachment of a virus antigen to a particular antibody attached to a microtitre plate, after which the plate is washed leaving behind the desired antigen-antibody complex. A color change occurs with the addition of a second enzyme-

labeled antibody that binds to the attached antigen, signifying the presence of a certain virus. Color intensity, upon viral occurrence, permits virus detection as well as virus titre quantification (Ward *et al.,* 2004; Clark and Adams, 1977).

2.4.3. Nucleic-acid based techniques

Ever since the invention of the Polymerase Chain Reaction (PCR) in 1985 (Mullis *et al.,* 1986), genetic studies have increasingly advanced. Currently more than 25 variations in the rudimentary PCR technique exist, a few of which include: immunocapture PCR (IC-PCR), Spot-PCR, Nested PCR, Nanoparticle-Assisted PCR (nanoPCR) (Cenchao, 2013; Cenchao *et al.,* 2009), Miniprimer PCR (Isenbarger *et al.,* 2008), Simple Sequence Repeat (SSR)-anchored PCR (Zietkiewicz *et al.,* 1994), Inverse PCR (Ochman *et al.,* 1988), Ligation-mediated PCR (Mueller and Wold, 1988), Methylation-specific PCR (Herman *et al.,* 1996), Touchdown PCR (Don *et al.,* 1991), Quantitative PCR (qPCR), Multiplex-PCR and, most relevant to this study, reverse-transcription PCR (RT-PCR). A majority of the viruses infecting grapevine possess RNA genomes and GLRaV-3 is no exception (Schaad and Frederick, 2002). The use of RT-PCR in the diagnostic detection of viruses associated with grapevine is an effective strategy. Initially, in the application of RT-PCR, primers specific to genomic regions of interest, derived from viral RNA, need to be designed and when used amplify complimentary DNA (cDNA) exponentially. The product of this amplification is then visualized on a stained gel after electrophoresis; however the results are not definite as the molecular weight of the product alone is not conclusive as to the products identity (Schaad and Frederick, 2002). Sequencing needs to be applied to validate the amplified sequence. PCR-based techniques have the advantages of being highly sensitive, specific to a region of interest and have the ability to detect several pathogens at a time (Ward *et al.,* 2004). Disadvantages of PCR-based techniques include the inability to calculate virus titre as well as the challenge of designing specific primers aimed at variants with high mutation rates. To overcome the inability of calculating virus titre and post-reaction analysis of PCR products, quantitative RT-PCR (RT-qPCR) was developed. RT-qPCR releases fluorescence with each consecutive PCR cycle making it possible to detect and quantify a virus-containing sample in real time (Ward *et al.,* 2004).

PCR-based techniques can be time consuming when the detection of multiple viruses is required, as in the case of numerous grapevine infections. To overcome this difficulty, oligonucleotide microarray analysis was developed to identify numerous genes or viruses simultaneously. In 2010, a study identified ten grapevine viruses when using a 570-probe microarray. This was designed to detect the conserved species-specific regions of 44 virus genomes affecting plants (Engel *et al.,* 2010). A study by Thompson *et al.,* in 2012, developed a macroarray platform for grapevine viruses

that successfully detected multiple viruses in a single run. Microarray analyses offer a relatively simple and robust method for the detection of multiple viruses at a time. However, this technology requires a great deal of post-operation analysis, is limited by available sequence data, is relatively expensive and remains less sensitive than techniques such as qPCR.

2.5. Metagenomics and next-generation sequencing

2.5.1. Introduction

In the past, techniques such as ELISA and nucleic acid-based methods have been the most suitable in identifying viruses associated with plant diseases (Adams *et al.,* 2009). Despite how specific and consistent these techniques may be, they do not reflect the entire complement of viruses that may be involved in certain disease etiology and may even neglect the presence of dissimilar virus variants. Metagenomic sequencing has overcome these restrictions through the identification of the complete compliment of viruses present in any given sample (Coetzee *et al.,* 2010; Adams *et al.,* 2009; Al Rwahnih *et al.,* 2009).

Metagenomics or environmental genomics refers to the analysis of genetic material sampled directly from the environment and takes into account the complete composition of all organisms inhabiting the shared environment sampled (Hugenholtz and Tyson, 2008). Multiple metagenomic studies have been performed to investigate the population of organisms in a number of environments. These environments include salt water (Williamson *et al.,* 2008), terra firma (Kim *et al.,* 2008; Fierer *et al.,* 2007), plant matter (Coetzee *et al.,* 2010; Adams *et al.,* 2009; Al Rwahnih *et al.,* 2009) and the excrement of *Homo sapiens* (Zhang *et al.,* 2005). The process through which metagenomic studies function involves the collection of nucleic acids from a designated area, after which fragmentation of the genetic material occurs. The smaller fragments are then cloned into vectors, producing a library of clones, and sequenced to determine the total organismal composition (Hugenholtz and Tyson, 2008). Initially metagenomic studies made use of Sanger sequencing, a capillary-based method. However, limitations regarding the requirements of known sequence information as well as the construction of cloned fragments makes this technique cost and time-inefficient (Adams *et al.,* 2009). In 2005 the introduction of NGS, also known as second generation sequencing, massively parallel or deep sequencing, delivered an unparalleled approach in identifying the genetic components of numerous organisms at a time; providing faster and more sensitive data than before (Adams *et al.,* 2009; Margulies *et al.,* 2005).

NGS occurs with the random fragmentation of nucleic material collected from a desired sample. Fragments are then attached to universal adaptors immobilized on specific sequencing beads or a

flow cell, depending on the NGS platform selected. The fragments, along with universal adaptors, are clonally amplified to produce clusters of identical sequences that show no bias towards a particular organism, essential in the discovery of novel viruses. NGS avoids chain termination chemistry and electrophoresis, as used in Sanger sequencing. This is carried out by employing chemistries such as sequencing-by-ligation, sequencing-by-synthesis and pyrosequencing (Ware *et al.,* 2012) to sequence the fragments. The use of NGS results in the detection of novel virus genomes before identification and characterization of the virus is initiated, as established in previous NGS studies (Koonin and Dolja, 2012).

Previous metagenomic studies have been unsuccessful in determining the complete pool of viruses present in a specified sample. Metagenomics, when combined with NGS, can successfully identify the complete set of viruses given in any environmental sample whether the viruses are known or novel (Mokili *et al.,* 2012). Coupling metagenomic and NGS techniques provides a sensitive manner in which to detect low titre viruses and establish genetic variation within strains of virus variants (Al Rwahnih *et al.,* 2009). Furthermore, specific viruses within the community can be quantified according to the frequency at which the reads related to that virus occur (Mardis, 2008b).

NGS comprises of three primary elements, which include the preparation of the sample, high-throughput sequencing and the bioinformatics analysis of the data generated (Mokili *et al.,* 2012). NGS technology does not require a large amount of initial material, however it does require high quality material in order to construct suitable libraries through RT-PCR (Yuzuki, 2012). RNA viruses, viroids, ribosomal RNA (rRNA), messenger RNA (mRNA) and replicating DNA viruses all mark RNA as the preferred form of starting material. In addition to this, the use of RNA eliminates host genomic DNA that may be present in the sample (Adams *et al.,* 2009). Double-strand RNA (dsRNA) is an intermediate produced from complementary RNA during the replication of either a positive or negative ssRNA viral genome (Martelli *et al.,* 2012). dsRNA, when used as the starting material for NGS applied to grapevine, selects the detection of sequences derived from viruses.

2.5.2. Next-generation sequencing platforms

Various next-generation sequencing technologies are commercially available from a number of companies; however, Illumina Genome Analyzer, ABI SOLiD and Roche 454 FLX are the three most extensively employed (Liu *et al.,* 2012; Zhang *et al.,* 2011; Mardis, 2008a). These platforms provide larger output data in a high-throughput manner compared to traditional sequencing techniques. The Illumina MiSeq, Life Technologies Ion Torrent PGM and Roche 454 GS Junior are examples of personal genome machines (PGM) that generate less output data at a faster rate

(Loman *et al.,* 2012). The above-mentioned platforms require PCR amplification, which complicates the process of genotyping by incorporating sequencing errors as well as producing duplicate template molecules (Kozarewa *et al.,* 2009). The development of third-generation sequencers, such as Pacific Biosciences PacBio RS, allows researchers to overcome these problems by eliminating the PCR amplification process; instead a single DNA molecule is used whereby emitted signals during sequencing are observed in real time (Liu *et al.,* 2012).

Illumina offers innovative technologies that allow the analysis of genetic variation and function, making studies like this possible across the globe. Illumina provides the largest output data per run with high accuracy across short reads and was selected as the instrument of choice based on these facts. The Illumina Genome Analyzer / HiSeq System makes use of sequencing by synthesis (SBS) chemistry. The sequencing process begins with fragmented DNA of less than 800 bps, to which universal adaptors are ligated and single-stranded DNA molecules generated through denaturation (Zhou *et al.,* 2010). The DNA templates are then distributed on a flow cell and immobilized on a glass slide, where clonal DNA fragments are generated through bridge amplification (Mercier *et al.,* 2003). A reaction mixture containing four fluorescently labelled reversible terminator nucleotides (2',3'-dideoxynucleotide triphosphates), is then added to the glass slide during sequencing. The 2',3'-dideoxynucleotide triphosphates contain a 3'-block that, when incorporated, terminates the sequencing cycle; a charge-couple device (CCD) sensor detects the position of the incorporated nucleotide through the uniquely labelled fluorescent dye (Zhou *et al.,* 2010). Once the cycle is complete, the fluorescent dye and terminators are chemically removed to prepare complementary strands for the following sequencing cycle. The number of cycles is determined based on the focus of the sequencing reaction. Once the sequencing has completed, reads that show poor quality are removed and a base-calling algorithm is used to determine the cluster sequences (Liu *et al.,* 2012; Mokili *et al.,* 2012; Zhang *et al.,* 2011; Mardis, 2008a; Mardis, 2008b). Commercially available Illumina sequencers include the MiSeqDx, NextSeq 500, HiSeq 2500, HiSeq X Ten and the HiScan (Table 2.3).

Table 2.3: The comparison of next-generation sequencers available on the market.

Company:	Instrument:	Sequencing Mechanism:	Read Length (bp):	Reads/ Run:	Output Data/ Run:	Time/ Run:	Advantages:	Disadvantages:
Conventional Sequencing (Sanger):								
Life Technologies	3730xl DNA Analyzer	Dideoxy Chain Termination	400 - 900	96	0.7 - 2 Mb	2 hrs	High Quality, Long Reads	High Cost, Low Output
Second-Generation Sequencers:								
Illumina	HiSeq 2500	Sequencing-by-Synthesis	2 x 100	640 M[a]	600 Gb	11 d[b]	High-Throughput	Short Read Assembly
	HiScanSQ	Sequencing-by-Synthesis	2 x 100	1.5 B[a]	150 Gb	8.5 d		
Applied Biosystems	SOLiD 5500xl	Sequencing-by-Ligation	Paired End: 75 x 35 Mate Pair: 60 x 60 Fragment: 75	2.8 B[a]	180 Gb	7 d	Accurate, Low Cost	Short Read Assembly
454 Sequencing	GS FLX Titanium XL+FLX System	Pyrosequencing	1000	1 M	700 Mb	23 hrs	Long Reads, Accurate	High Cost, Long Run Time
Third-Generation Sequencer:								
Pacific Biosciences	PacBio RS (SMRT)	Real-Time Flourescent DNA Polymerization	2200	10 K	10 Mb	2 hrs	Short Run Time, Long Reads	High Cost, Low Read/ Run
Personal Genome Machines:								
454 Sequencing	GS Junior	Pyrosequencing	400	0.1 M	35 Mb	10 hrs	Short Run Time	Low Output, Low Read/ Run
Illumina	MiSeq	Sequencing-by-Synthesis	2 x 250	34 M[a]	7.5 - 8.5 Gb	39 hrs	High Output	Long Run Time
Life Technologies	Ion Torrent 314 chip	Proton sensing by Semiconductor	200	0.1 M	20 Mb	2.4 hrs	Short Run Time	High Cost, Long Sample Preparation Time
	Ion Torrent 316 chip			1 M	200 Mb	3 hrs		
	Ion Torrent 318 chip			5 M	1 Gb	4.5 hrs		
	Ion Proton I			80 M	10 Gb	4 hrs		

[a] Number of paired-end reads.
[b] Dual flow cell time/ run.
Data was acquired from the following websites:
Applied Biosystems: http://www.appliedbiosystems.com/absite/us/en/home/applications-technologies/solid-next-generation-sequencing/next-generation-systems.html
Illumina: http://www.illumina.com/technology/sequencing_technology.ilmn
Life Technologies: http://www.lifetechnologies.com/global/en/home.html
Pacific Biosciences: http://www.pacificbiosciences.com/products/smrt-technology/
Roche: http://454.com/products/gs-flx-systems/index.asp

2.5.3. Bioinformatics associated with next-generation sequencing

The invention of NGS has enabled the scientific community to produce vast amounts of sequencing data at an increased rate. The data, however, requires extensive interpretation that requires a suitable amount of storage not only to supply the capacity of produced raw data but in analysis and comparisons of the sequences generated (Zhang *et al.,* 2011; Hugenholtz and Tyson, 2008). In the order of 100 GB storage is needed to store NGS data with a compute capacity of 2-4 multicore CPUs and roughly 500 GB of random access memory (RAM) for processing the data generated. Difficulties in bioinformatics occur with the immense memory needed for read assemblies and the independent formats of output data produced through different sequencing platforms. As a result there are no universal bioinformatics tools that can be applied for all platforms (Zhang *et al.,* 2011).

The preliminary step in analyzing NGS data is trimming the short read datasets with low quality scores to generate high quality reads. Following this is a filtering process whereby the reads that match unwanted sequences, such as host and adaptor sequences, are removed from the database. Once the reads have been trimmed and filtered the process of either read-mapping or *de novo*

assemblies can be performed (Mokili *et al.,* 2012). Generated reads can be aligned to a set of reference sequences or genomes, located in GenBank databases or in personal archives, through read-mappings or assembled through *de novo* assemblies to produce contiguous sequences (contigs) that can be analyzed using BLAST searches. Both processes functioning in identifying the origin of the NGS reads generated. The most common practice in sequence alignment is to compare the translated query sequences generated with translated sequences in a database. Through this approach a greater hit count is obtained and limitations associated with base-calling can be overcome. However, comparing sequences in this manner requires a greater computing power and additional time (Edwards and Rohwer, 2005).

The process of aligning short reads to a reference genome is termed read-mapping. Performing mapping assemblies determines the percentage of the reference genome covered, the number of reads aligning to a reference and the depth of reference coverage by aligned reads. Read-mapping can be performed using either a Burrows-Wheeler Transform (BWT) or spaced seed indexing based algorithm (Schbath *et al.,* 2012). Spaced seed indexing requires greater RAM on computers and generates alignments at a lower rate. This algorithm is employed by MAQ (Mapping and Assembly Quality), where BWT is used for Bowtie (Trapnell and Salzberg, 2009).

There are substantial numbers of metagenomic reads or contigs that are non-homologous to known sequences present in databases. To determine where these sequences originate, *de novo* assemblies can be used. Through *de novo* assemblies, raw reads are distributed into groups of fragments, termed k-mers, of a defined length (hash length). Overlapping reads are then concatenated to form longer contigs made possible with de Bruijn graphs (Scholz *et al.,* 2012; Zerbino and Birney, 2008). The above-mentioned process requires between 256 and 512 GB of RAM to construct a medium sized genome; however, the assembly time is significantly reduced when using the k-mer based assembly (Scholz *et al.,* 2012).

There are a number of open source bioinformatics tools available online (Supplementary Table S.1); however a majority make use of UNIX-like operating systems which has limitations in user graphical interface ability. CLC Genomics Workbench (CLC Bio) offers a user-friendly analysis package for visualizing, comparing and analyzing NGS data. CLC Genomics Workbench supports the major NGS platforms and is available for Mac OS X, Windows and Linux. The functions of CLC are versatile, as one can check for quality, trim reads, assemble reads either through read-mapping or *de novo* assemblies, providing users with a complete set of analyses (CLC Bio). Data can also be imported and exported in a multitude of formats, allowing versatility in subsequent analysis.

2.5.4. Application of next-generation sequencing to grapevine diseases

There are many viruses infecting grapevine, including those that cause diseases of unknown etiology and the latent viruses with unknown disease association. The association of viruses to form virus complexes causing disease is less known and may contribute to symptoms more severe than with single infections. With the advent of NGS, a more comprehensive understanding in disease etiology can be attained. There have been multiple studies on grapevine disease using NGS (Maree *et al.,* 2012; Al Rwahnih *et al.,* 2011; Coetzee *et al.,* 2010; Al Rwahnih *et al.,* 2009), each of which employs a different approach. These approaches differ in the type of starting material used, NGS technology applied, sampling methods and bioinformatics analysis. The following information links to previously performed research on grapevine diseases using NGS.

Al Rwahnih *et al.* (2009) made use of the 454 GS FLX NGS platform to sequence dsRNA extracted from the bark scrapings of two healthy and two Syrah decline symptomatic vines. Direct comparison of generated reads to GenBank databases revealed that *Grapevine leafroll-associated virus type* 9 (GLRaV-9), *Grapevine Yellow speckled viroid* (GYSVd), *Australian grapevine viroid* (AGVd) and *Hop stunt viroid* (HSVd) existed in the diseased vines; *Grapevine rupestris stem pitting-associated virus* (GRSPaV) and *Grapevine rupestris vein-feathering virus* (GRVFV) were present in all of the vines sampled. The study also detected a novel virus named *Grapevine syrah virus-1* (GSyV-1) (Al Rwahnih *et al.,* 2009).

In 2010, Coetzee *et al.* performed a survey of 44 random Merlot samples in a South African diseased vineyard. dsRNA was extracted from phloem and subsequently sequenced as a combined sample to generate paired-end reads using the Illumina Genome Analzyer II. Velvet was used to perform *de novo* assemblies, after which generated scaffolds were subjected to homology BLAST searches; MAQ was used to map reads to existing virus genomes. They identified GLRaV-3 as the most prevalent virus, representing 59% of the generated reads, followed by GRSPaV and GVA. GVE and mycoviruses were detected for the first time in South African grapevines (Coetzee *et al.,* 2010).

Maree *et al.* (2012) made use the Illumina HiScanSQ NGS platform to sequence the dsRNA extracted from five vines displaying either shiraz disease or leafroll disease (control) symptoms. Through read-mappings and homology searches using BLAST, eight viruses and virus variants were identified along with 46 mycoviruses and four viroids (GYSVd-1, GYSVd-2, HSVd and AGVd). GLRaV-3 was detected in all of the tested vines and GVA was present in 80% of the tested vines; further signifying the hypothesis that GVA group II variants are coupled with Shiraz disease (Maree *et al.,* 2012).

2.6. Conclusion

GLD has been documented to cause up to 60% loss in product yield in some vineyards; placing significant limitations on the production of premium quality wines and delaying wine sales. Extensive research has shown that the most significant of the GLRaVs in GLD infection is GLRaV-3. However, grapevine is host to 70 infectious agents and the association of these viruses to different diseases has yet to be identified. The symptom expression of GLD differs considerably between cultivars creating difficulties in the visual selection of diseased vines. As a result, virus screening of infected plants remains the basis of accurate detection for the development of disease management programs.

The genetic information for many viruses have been documented, however, there remain a number of viruses of which little or nothing is known. NGS has provided opportunities to conduct research into understanding the entire complement of viruses present in any given sample, whether they are known or unknown. This has enabled scientists to establish a more comprehensive understanding into disease etiology and potentially reduce the negative effects that viruses present during infection.

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Chapter 3

Identification and distribution of multiple virus infections in grapevine leafroll diseased vineyards

3.1. Introduction

Performed research, described within this chapter, was executed in collaboration with the Agriculture Research Council (ARC) and excludes sample collections and total RNA extractions performed in the year 2012 as well as any additional statistical analysis.

In excess of 70 pathogens infect grapevine of which 63 are of viral origin, this represents the highest number of pathogens detected in a single crop (Martelli, 2014; Martelli *et al*., 2012). Grapevine leafroll disease (GLD) is one of the major viral diseases affecting grapevine and is present in most wine-growing regions of the world. *Grapevine leafroll-associated virus* 3 (GLRaV-3) variants are the primary causative agents of GLD and are seen commonly within South African diseased vineyards. GLRaV-3 variants are, however, not the sole infectious agents in a majority of GLD instances; viruses belonging to the families *Flexiviridae*, *Betaflexiviridae*, *Tymoviridae*, *Endornaviridae*, and other *Closteroviridae* are commonly found, together, in GLD vines (Table 4.3).

To date, six GLRaV-3 variant groups exist that comprise 13 complete or near complete genomes (Chooi *et al.,* 2013a; Chooi *et al.,* 2013b; Bester *et al.,* 2012a; Gouveia *et al.,* 2011; Sharma *et al.,* 2011; Jooste *et al.,* 2010; Maree *et al.,* 2008), which share between 99.57% and 62.70% sequence identity. The identity, relative variant group and accession numbers as well as the genome size of each isolate can be found in Table 2.2. Six full genome sequences, representing four genetic variant groups of GLRaV-3, have been published in South African studies. These variant groups include: group I (represented by isolate 621), group II (represented by isolates GP18 and 623), group III (represented by isolate PL-20) and group VI (represented by isolates GH11 and GH30) (Bester *et al.,* 2012a; Jooste *et al.,* 2010; Maree *et al.,* 2008). Recently, an additional two GLRaV-3 isolates were detected, namely: GTG10 (Goszczynski, 2013) and GH24 (GenBank: KM058745). It was determined, based on sequence comparisons of a 305 nt section of the HSP70h gene of representatives of GLRaV-3 variant groups, that isolates GTG10 and GH24 are the same genetic variant, however, are grouped outside of the variant group VI cluster.

Research performed on the genetic variability of GLRaV-3 from various countries around the world, including: South Africa (Goszczynski, 2013; Bester *et al.,* 2012a; Jooste *et al.,* 2011; Jooste *et al.,* 2010), the USA (Seah *et al.,* 2012; Sharma *et al.,* 2011; Wang *et al.,* 2011; Fuchs *et al.,* 2009), New

Zealand (Chooi *et al.,* 2013a; Chooi *et al.,* 2013b), China (Farooq *et al.,* 2012) and India (Kumar *et al.,* 2012), support the understanding of GLD etiology and contribute towards the development of diagnostics and systems in which to manage such diseases. Surveys based on the relative abundance of GLRaV-3 variants within GLD infected vines have been studied extensively. The distribution of GLRaV-3 variants differs with each vineyard; studies in Napa Valley (USA) detected group I and III variants at higher frequencies (Sharma *et al.,* 2011) while in Chinese vineyards group I genetic variants were most prevalent (Farooq *et al.,* 2012). In an earlier South African study, group II GLRaV-3 variants were predominantly detected (Jooste *et al.*, 2011), while in Portuguese vineyards variant group I and II were detected with a higher relative abundance compared to other variant groups (Gouveia *et al.,* 2011).

Research into GLD-associated viruses (GLRaVs) has been conducted in many regions of the world, including: the USA, New York (Fuchs *et al.,* 2009), California (Sharma *et al.,* 2011), Washington (Martin *et al.,* 2005); Tunisia (Mahfoudhi *et al.,* 2008); Chile (Fiore *et al.,* 2008); and Turkey (Akbas *et al.,* 2007). These surveys determined mixed viral infections in single plants as a common occurrence.

Coetzee *et al*. (2010) performed a metagenomic study focusing on the viral population of a South African vineyard in 2010. Plant material from 44 diseased Merlot grapevines, in a single vineyard, was extracted and pooled. Next-generation sequencing was performed on extracted dsRNA and results identified the presence of several grapevine-infecting viruses of which GLRaV-3 was the best represented, followed by *Grapevine rupestris stem pitting-associated virus* (GRSPaV), *Grapevine virus A* (GVA) and *Grapevine virus E* (GVE) (Coetzee *et al.,* 2010).

The etiology of GLD has not been resolved completely and the effects of viral populations on symptom expression are not known. This study represents a comprehensive survey conducted on South African vineyards with focus on determining the composition of viral populations in GLD grapevines collected across the main wine producing regions of South Africa. The distribution of mixed and single virus infections as well as GLRaV-3 variant status of sampled grapevine will also be discussed.

3.2. Materials and Methods

3.2.1. Sampling

A widespread survey was conducted in the wine growing regions of the Western Cape province, South Africa. The samples were collected from 3 regions: the Coastal-, Cape South Coast- and

Breede River Valley regions. A total of 315 (171 red cultivar and 144 white cultivar) grapevines were sampled from 29 farms. In 2012, 125 samples were collected while 190 grapevines were sampled in the year 2013. Most of the collections were done in the Coastal region that included farms in the Darling, Klein Karoo, Paarl, Stellenbosch, Swartland, Tulbagh and Wellington districts. Sampling in the Breede river valley occurred in the Breedekloof and Worcester farms, while Cape South coast sampling was done in the Walker Bay district. A selection of 15 different red-fruited cultivars and 10 different white-fruited cultivars were sampled that included the economically important cultivars Chardonnay, Sauvignon blanc, Cabernet Sauvignon, Merlot and Shiraz. The vineyards selected for the study were all previously used for propagation material by the grapevine industry, but lost their "mother block" status in the 2008/2009 growing season (for plants selected in the 2012 survey) and in the 2010/2011 growing season (for plants selected in the 2013 survey) due to GLD infection of more than 3%, based on symptom expression. These vineyards were selected to collect data for newly infected plants, assuming that the infection was transmitted from surrounding vineyards. Petioles and canes from four infected plants were randomly sampled per vineyard. In red-fruited cultivars, leafroll symptoms were easily visible and sampling was performed to include a variety of leafroll symptoms, ranging from plants displaying severely downward rolling leaves with dark red coloration to the plants with milder leafroll symptoms. In white-fruited cultivars the physical symptoms of GLD was not always distinctive, with the exception of Chardonnay plants where clear downward rolling of the leaves was observed. In white-fruited cultivar blocks, sampling was performed randomly and in some cases based on abnormalities, *i.e.* yellowing of grapevine leaves.

3.2.2. GLRaV-3 variant status

Petioles and phloem scrapings of the collected grapevines were stored at -80°C. Total RNA was extracted from 0.2 g of plant tissue using a modified CTAB (2% [w/v] CTAB, 2.5% [w/v] PVP-40, 100 mM Tris-HCL [pH8], 2 M NaCl, 25 mM EDTA [pH8] and 3% [v/v] β-mercaptoethanol) method (White *et al*., 2008). Total RNA quality and integrity was evaluated spectrophotometrically (Nanodrop 1000) and with gel electrophoresis (1% [w/v] Agarose-TAE).

To differentiate between GLRaV-3 variants in plants and to establish the distribution of single and mixed infections, a one-step, reverse transcription, quantitative PCR high-resolution melting curve (RT-qPCR-HRM) assay was used (Bester *et al*, 2012b). Between 100 ng and 200 ng of purified total RNA was used as template in the RT-qPCR-HRM assay that was performed on a Qiagen Rotor-Gene Q instrument. An RT-PCR assay was developed and used for the specific detection of the GLRaV-3 variant similar to isolate GH24 (GenBank: KM058745).

In order to perform the variant identification, the confidence interval method described in Bester *et al*. (2012b) was applied, with the exception of the variant group I interval. By including more data points from the 2013 survey, this confidence interval was refined. All variant group I infections from 2013 were confirmed by screening the samples with the multiplex RT-PCR described in Bester *et al.* (2012b). The melting temperature of the confirmed variant group I infections could as a result be added to the existing melting curve data points. New variant group I confidence intervals were calculated for both LR3.HRM4 and LR3.HRM6 primer pairs using the extended data point database.

3.2.3. RT-PCR diagnostics

Primers were either designed through the selection of a conserved region of multiple aligned sequences (CLC Main Workbench V.6.8.4) extracted from GenBank, or selected from publications and databases (Table 3.1). Positive controls for *Grapevine virus F* (GVF), *Grapevine leafroll-associated virus* 1 (GLRaV-1), -2 (GLRaV-2), -3 (GLRaV-3), *Grapevine leafroll-associated virus* 4 -like (GLRaV-4), and GRSPaV were obtained from the collection at ARC-Plant Protection Research Institute, Pretoria, South Africa. GLRaV-3 isolate GH24, as well as positive controls for GVA and GVE were obtained from the plant collection at the Department of Genetics, Stellenbosch University, South Africa. No positive controls for *Grapevine virus B* (GVB) and *Grapevine virus D* (GVD) could be sourced, results using the primer pairs developed for the detection of these viruses will therefore not be discussed. A 200 mg portion of each sample was pulverised in a mortar and pestle using liquid nitrogen, and total RNA extracted following an adapted CTAB method (White *et al*., 2008).

Complementary DNA (cDNA) was synthesised by adding 200 ng of total RNA of each sample to 0.3 µl of random hexamers (Promega) and incubating for 5 minutes at 65°C before placing on ice for 2 minutes to complete the primer annealing reaction. A solution containing 1X AMV RT Buffer (Thermo Scientific), 5 U AMV reverse transcriptase (Thermo Scientific), 10 mM dNTPs (Thermo Scientific) and dH_2O was then added to the 5 µl primer annealing reaction and incubated for 60 minutes at 48°C to produce cDNA.

A PCR mixture containing 1X KAPA *Taq* buffer A (KAPA Biosystems), 10 mM dNTPs (Thermo Scientific), 20 mM forward primer (Integrated DNA Technologies), 20 mM reverse primer (Integrated DNA Technologies), 2 µl 5X Cresol loading dye (30% sucrose; 125 mg cresol red dye), 1 U KAPA *Taq* DNA polymerase (KAPA Biosystems), 2 µl cDNA and dH_2O was used to detect each of the viruses in the respective samples. The primer pairs had varying PCR cycle conditions

(Table 3.2); all included an initial denaturation step at 94°C for 3 minutes and a final extension step at 72°C for 7 minutes.

Table 3.1: List of primers used in RT-PCR and RT-qPCR-HRM assays. GenBank accessions used for primer design in this study are listed.

Target Virus:	Primer Pairs:	Sequence (5'- 3'):	Amplicon Size (bp):	Reference/ Genbank Accession Numbers:
GVA	GVA-P-F-7038 GVA-P-R-7273	AGG TCC ACG TTT GCT AAG CAT CGT CTG AGG TTT CTA CTA	238	MacKenzie, 1997
GVB	GVB_26/6.F GVB_26/6.R	ATG TCC CAG GTC TCA AGG AG TTG TCC AGA CTT AAC TTG GGT G	815	This study (KJ524452.1; GU733707.1)
GVD	GVD_26/6.F GVD_26/6.R	TGC ATT CTC GCC TCT TTC CCC GTT TCA CGA ACT CTA AC	251	This study (JQ031716.1; JQ031715.1; Y07764.1)
GVE	GVE_1_Diag_1_7055F GVE_1_Diag_1_7251R	ATG ATT TGA TGC TCA GTC ACA GG GGG TTC TTA TGG CCT GCT TA	213	This study (GU903012.1; JX402759.1; AB432910.1; NC_0011106.1)
GVF	GVF_PRIMER_F GVF_PRIMER_R	TTGGGAGTGGAGGATCTGTA ATGGGCAGTCTGGGTCTATC	217	This study (JX105428.1; NC_018458.1)
GLRaV-1	LR1_HSP70-417F LR1_HSP70-737R	GAG CGA CTT GCG ACT TAT CGA GGT AAA CGG GTG TTC TTC AAT TCT	320	Osman *et al.*, 2007
GLRaV-2	LR2-P24-F-SacI LR2-P24-R-XbaI	ATGAGCTC ATGAGGGTTATAGTGTCTC ATTCTAGA TTAACATTCGTCTTGGAGTTCG	617	This study (JX559644.1; NC_007448.1; DQ286725.2; AY881628.1)
GLRaV-3*	LR3.HRM4.F LR3.HRM4.R	TAA TCG GAG GTT TAG GTT CC GTC GGT TCG TTA ACA ACA A	226	Bester *et al.*, 2012b
GLRaV-3*	LR3.HRM6.F LR3.HRM6.R	GTC ACC AGG TGT TCC AAA C AAC GCC CTG TAT GTC TCC TCT C	305	Bester *et al.*, 2012b
GLRaV-4-like	GLRaV-4.like.F GLRaV-4.like.R	ATG GCA TTG TCT GCG ACT AG TAA ACA CAG ACA TGG GAG TAG C	354	This study (NC_016416.1; NC_016081.1; AY297819.1)
GRSPaV	RSP13-F RSP14-R	GATGAGGTCCAGTTGTTTCC ATCCAAAGGACCTTTTGACC	338	Meng *et al.*, 1999.
GH24-like	CB19_72F CB19_1267R	GCG AAG ACG GAT ACT GTA TCG ATA CAC GAC CCC TAT ATC AGC CG	1195	Unpublished
GTG10	GLRaV-3-GTG10-HSP70-F GLRaV-3-GTG10-HSP70-R	TAA AGT TCG ATA GCG GGG GC CGG CTG AGA CCG TAG ATG AC	146	This study (KC731554.1; KC731553.1)

* Primers used in the one step RT-qPCR-HRM assays.

Table 3.2: PCR cycle condition for the detection of other Clostero-, Viti- and Fovea viruses.

Primer Pair:	Initial Denaturation:		Denaturation:		Annealing:		Extension:		Cycles:	Final Extension:	
	Temp. (°C):	Duration (sec.):	Temp. (°C):	Duration (sec.):	Temp. (°C):	Duration (sec.):	Temp. (°C):	Duration (sec.):		Temp. (°C):	Duration (sec.):
GVA	94	180	94	30	56	30	72	50	35	72	420
GVB					56						
GVD					58						
GVE					60						
GVF					56						
GLRaV-1					58/56				10 + 25		
GLRaV-2					58/56				10 + 25		
GLRaV-3					58				35		
GLRaV-4					62/60				10 + 25		
GTG10					60						
GRSPaV					58				35		
GH24					55			60			

3.3. Results and Discussion

3.3.1. GLRaV-3 variants status

Initial screening of the samples detected the presence of four GLRaV-3 variant groups: I, II, III and VI. Results from the additional RT-PCR assay also confirmed the presence of the new variant, GH24, similar to isolate GTG10 (Goszczynski, 2013). Positive isolates hereafter were referred to as GH24-like.

3.3.2. Adjusted HRM confidence intervals

Twenty-one plants were identified with the RT-qPCR-HRM assay to be potentially infected with only GLRaV-3 variant group I. The multiplex RT-PCR confirmed 15 of these variant group I infections. More than one melting point temperature per sample was generated due to duplex reactions. As a result an additional 29 (LR3.HRM4) and 36 (LR3.HRM6) melting curve data points were generated. After the re-calculation of the 95% melting point confidence interval, using the 2.5 and 97.5 percentiles, the confidence interval for variant group I was adjusted. The confidence interval for LR3.HRM4 was re-calculated as 83.22°C to 84.05°C and for LR3.HRM6 as 84.82°C to 85.90°C (Table 3.3).

Table 3.3: Descriptive statistics and re-calculation of variant group I HRM confidence intervals

Primer Pair:	Number of Data Points		Min		Max		Mean		Temperature Range between Upper and Lower Limit:	
	Bester et al., 2012	Adjusted	Bester et al., 2012	Adjusted	Bester et al., 2012	Adjusted	Bester et al., 2012	Adjusted	Bester et al., 2012	Adjusted
LR3.HRM4	31	60	83.20	83.20	82.98	84.10	83.60	83.68	0.78	0.90
LR3.HRM6	27	63	84.78	84.78	85.42	86.08	85.03	85.33	0.64	1.30
	2.5 th Percentile		**97.5 th Percentile**		**Interquartile Range (IQR) (75 % - 25 %)**					
	Bester et al., 2012	Adjusted	Bester et al., 2012	Adjusted	Bester et al., 2012	Adjusted				
LR3.HRM4	83.22	**83.22**	84.08	**84.05**	0.43	0.37				
LR3.HRM6	84.79	**84.82**	85.39	**85.90**	0.09	0.52				

3.3.3. Distribution of GLRaV-3 variants in vineyards

Single and mixed variant infections were detected in the 315 plants screened. The significance of the chi-square test will be indicated in the results description.

Single variant infections

In total, 119 of the 315 tested plants had single variant infections. Variant groups II and VI were found to be the most prevalent, occurring at frequencies of 47.06% and 37.82% in 119 singly infected plants, respectively. Single infections of group I and GH24-like were recorded in 6.72% and 7.56% of single virus infected plants, respectively. The distribution of single variant infections was detected in each of the districts (Figure 3.1.A). Variant group II single infections occurred predominantly in the Coastal regions, including Stellenbosch, Swartland, Tulbagh, Wellington, Paarl, and Darling. In the Breede River Valley region, variant group VI was marginally more frequent in the Breedekloof district, while in the Worcester district only one plant with a variant group VI infection was detected. In the Cape South Coast region, the Walker Bay district, variant group VI was the only single variant infection detected in five plants (Figure 3.1.A).

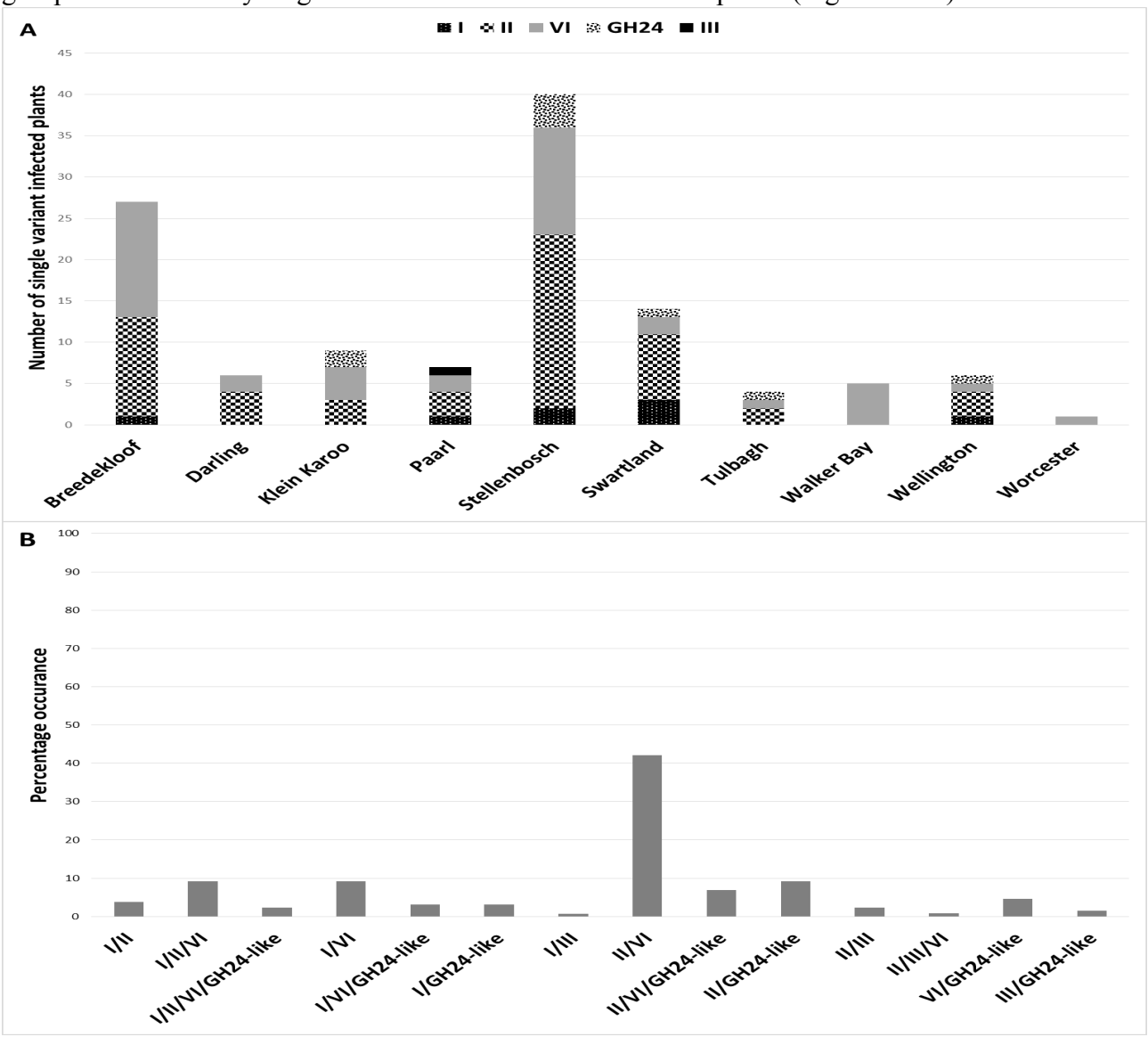

Figure 3.1: Bar graphs representing the distribution and occurrence of single and mixed virus infections. A) Distribution of single variant infections per district. B) Percentage occurrence of 14 mixed variant combinations in vineyards with variant groups II/VI as the most prevalent.

Mixed variant infections

As expected, a high number of plants tested positive for mixed variant infections. Fourteen variant combinations were detected in 130 plants. In total, 56.25% of plants collected in the Breede River Valley region were infected with multiple variants. In the Cape South Coast region 36.36% of plants had multiple variant infections and in the Coastal region 34.51% of plants were mixed infected. The proportion of mixed variant infections was more than 20% higher in the Breede River Valley region. The variant group II/VI combination was the most frequent combination, detected in 43% of the infected plants. Mixed infections of variant group I/II/VI, I/VI and the II/GH24-like combinations were identified in 9% of the plants. The third most frequent variant combination was the II/VI/GH24-like combination, occurring in 6.92% of the plants (Figure 3.1.B).

The total distribution of GLRaV-3 variants in red- and white-fruited cultivars were analysed in the three regions (Figure 3.2.A). Group VI variants occurred predominantly in the Walker Bay district in the Cape South Coast region (Figure 3.2.A) as well as in the Breede River Valley region. Variant group II dominated in the Coastal region. No difference between the infections of GLRaV-3 variants in red- and white-fruited cultivars was detected (Figure 3.2.B). The newly identified GH24-like variant was detected in all the regions and had a high infection percentage in white cultivars in the South Coast region. In the Coastal region all five variants were detected, including variant group III from Paarl district. Although the chi-square test was not significant, variant group III was only detected in the Coastal region. The overall distributions of variants are seen in Figure 3.3.

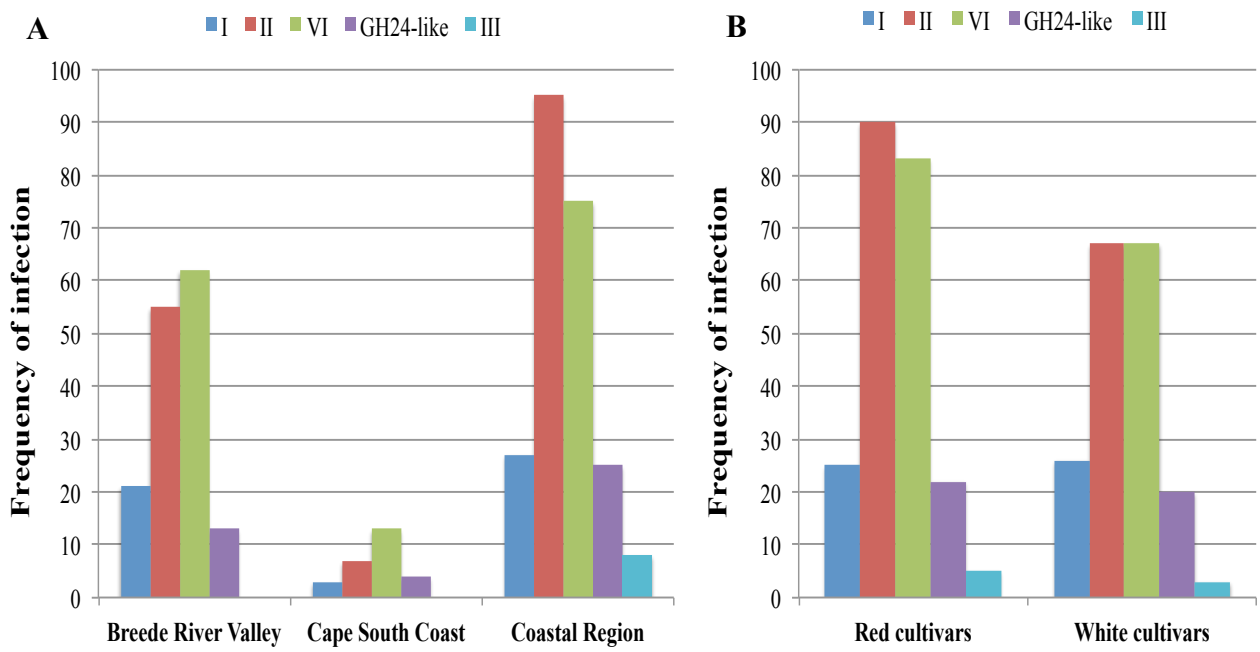

Figure 3.2: Bar graphs of the infection frequency between regions of the Western Cape and between red and white cultivars. A) The total GLRaV-3 variant distribution per region. B) The total GLRaV-3 distribution in white and red cultivars.

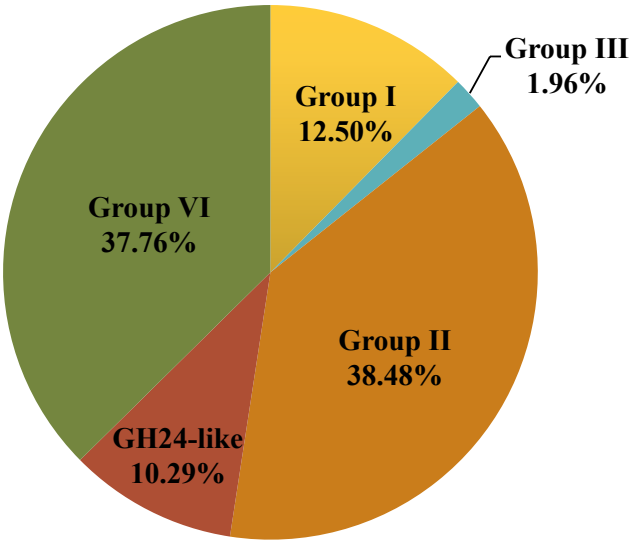

Figure 3.3: Pie chart representing the distribution of individual GLRaV-3 variant percentage infection detected in vineyards.

Detection methods were developed to test for five GLRaV-3 variant groups. Four variant groups: I, II, III, and VI, were detected in the one step RT-qPCR-HRM assay. Another variant, GH24 (GH24-like), was detected with a specific RT-PCR. Single and mixed variant infections were detected in plants showing GLD symptoms. Plants with single infections were detected for all variant groups with group II and VI being the most prevalent. A clear regional distribution of plants with single infected variants was seen with variant group II dominating in the Darling, Paarl, Stellenbosch, Swartland, Tulbagh and Wellington districts. Variant group VI was detected prevalently in the Breede River Valley region, including the Breedekloof and Worcester districts, as well as in the Cape South Coast region (Figure 3.2.A). The overall distribution of the different GLRaV-3 variants showed similar distribution in red- and white-fruited cultivars as shown in Figure 3.2.B. The study highlighted the importance of testing for viruses in white-fruited cultivars. White-fruited cultivars harbour a wide range of viruses although symptoms are not expressed on plants.

The detection of plants with mixed variant infections was expected. A total of 14 mixed variant combinations were detected in 130 of the tested plants. The variant group II/VI combination was detected as the primary mixed infection in all regions and was well distributed between cultivars. In a previous study, the predominant occurrence of variant group II was shown in 10 mother blocks in South African vineyards (Jooste *et al*., 2011). Results of this earlier study also showed the spread of variant group II to be faster in a specific disease cluster compared to variant group III (Jooste *et al*., 2011). This fact may explain the faster spread of variant group II per row in a vineyard. At the time of the earlier study, variant group VI had not yet been identified and the spread of this variant was not investigated. The widespread detection of variant group II and VI in this survey suggest that these two variants are transmitted more effectively to adjacent plants in a disease cluster. A

study is underway to test the transmission efficiency of GLRaV-3 variants. Reports from other countries did not show the dominant presence of variant group II and VI in plants. In China, group I was the most prevalent variant group identified (Farooq *et al*., 2012), while in the Napa Valley both variant group I and III were the most common variants detected (Sharma *et al*., 2011). In a limited survey in New Zealand, variant group I and VI were the most frequently detected variant groups (Chooi *et al*., 2013b). It is interesting to note that variant group III was found in high numbers in the Napa Valley, but in South African vineyards this variant was detected in only eight plants, representing less than 2% of the tested plants. The overall distribution of isolates similar to the recently described isolate GH24 (GH24-like) was just over 10%. GH24-like was detected significantly in red- and white-fruited cultivar vines in the Breede River Valley, Cape South Coast and Coastal regions.

More GLRaV-3 variants have been identified and recently described. Comparison of different variant groups showed two main phylogenetic clades of the virus (Maree *et al*., 2013): the clade that include isolates from groups I-V and the clade containing the group VI and group VI-like (NZ2) isolates. A revision of the second phylogenetic clade, group VI and group VI-like, should be done to include the other divergent variants detected, *i.e.* GH24 and GTG10. In future, full-length coat protein sequences, and preferably full genome sequences, of all newly identified GLRaV-3 variants should be compared to clarify the phylogenetic position of these variants.

3.3.4. Virus populations in GLD-affected plants

Virus-specific primers with appropriate positive controls were used in RT-PCR reactions to detect GVA, GVE, GVF, GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-4–like viruses and GRSPaV. There were 50 plants with single virus infections of GLRaV-2, GLRaV-3, GLRaV-4, GVE, GVF, and GRSPaV. GLRaV-3 was a single infection in 29 of these plants and GRSPaV was found as a single infection in 13 plants.

Most of the plants tested were infected with multiple viruses. Mixed virus infections were found in 251 plants, representing 36 combinations of seven grapevine viruses, indicating that proportions are significantly different. A summary of the mixed virus combinations can be seen in Table 3.4. The most prevalent mixed virus infection, GLRaV-3/GVE, was detected in almost 28% of mixed infected plants. The next most prevalent mixed virus combinations were GLRaV-3/GVE/GRSPaV, GLRaV-3/GRSPaV and GLRaV-3/GVA that occurred between 8% and 10% in plants.

The distribution of these viruses per region is shown in Figure 3.4. GLRaV-3 was detected predominantly in all three regions. Other closteroviruses, GLRaV-1, GLRaV-2 and GLRaV-4-like

viruses, were detected in low frequencies in all regions, except GLRaV-2 detected in 19 plants in the Coastal region. The *Foveavirus*, GRSPaV, was detected between 16% and 22% in all regions in both red- and white-fruited cultivar plants. The vitiviruses screened for, GVA, GVE, and GVF, were present in all regions. GVE was found to be the most common *Vitivirus* in all regions. The recently discovered GVF virus (Al Rwahnih *et al*., 2012) was detected in all three regions. The virus distribution was independent of region, meaning the virus distribution of the different viruses in the three regions followed the same pattern.

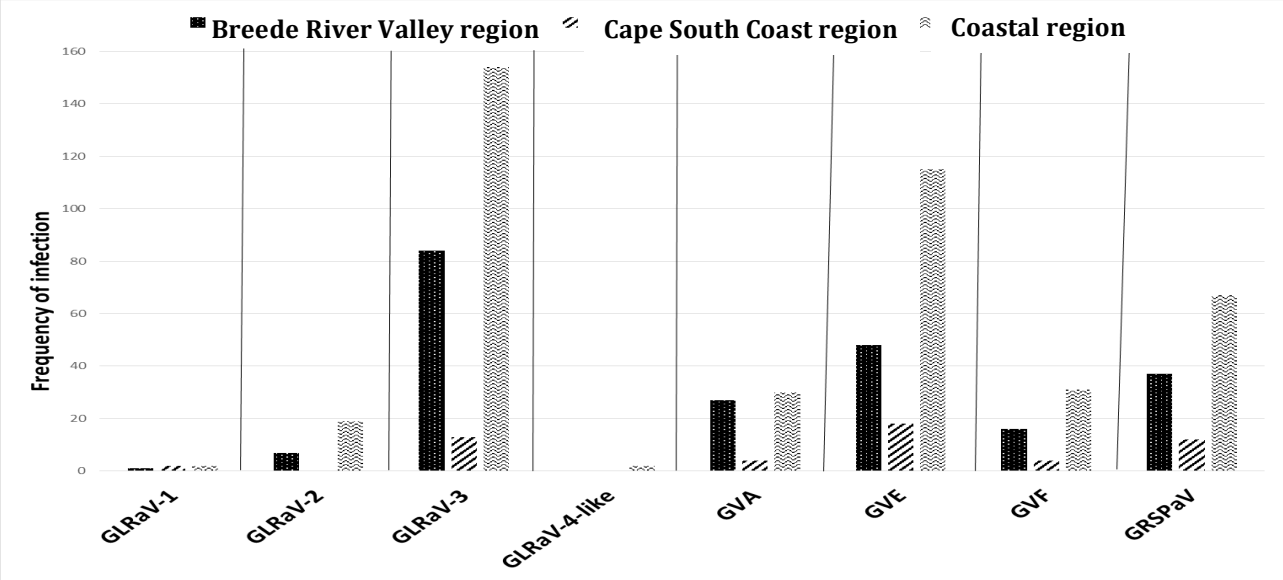

Figure 3.4: Distribution of viruses in three regions of the Western Cape including the Breede river valley region, Cape south coast region and the Coastal region ($c^2_{(df=14)}$=22.56 P=0.0678).

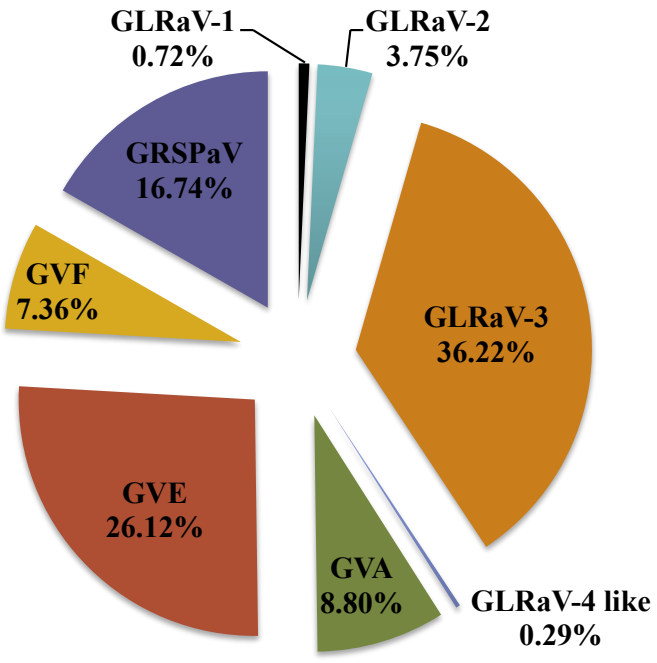

Figure 3.5: Pie chart illustration of the grapevine virus percentage distribution of detected viruses in vineyards and signifying the differences in proportion between viruses.

Table 3.4: Mixed virus infections detected in 270 plants of the survey. The four main virus combinations are highlighted.

Mixed virus combination	Number of infected plants	% infection
GLRaV-1/GLRaV-3/GRSPaV	2	0.8
GLRaV-2/GLRaV-3	3	1.2
GLRaV-2/GLRaV-3/GRSPaV	1	0.4
GLRaV-3/GRSPaV	20	7.97
GVA/GLRaV-3	21	8.37
GVA/GLRaV-3/GLRaV-4	1	0.4
GVA/GLRaV-3/GRSPaV	5	1.99
GVA/GVE/GLRaV-3	14	5.58
GVA/GVE/GLRaV-3/GRSPaV	8	3.19
GVA/GVE/GVF	2	0.8
GVA/GVE/GVF/GVRaV-2/GLRaV-3	1	0.4
GVA/GVE/GVF/GVRaV-2/GLRaV-3/GRSPaV	1	0.4
GVA/GVE/GVF/GLRaV-3	4	1.59
GVA/GVE/GRSPaV	1	0.4
GVA/GVF/GLRaV-3	2	0.8
GVA/GVF/GLRaV-3/GRSPaV	1	0.4
GVE/GLRaV-1	2	0.8
GVE/GLRaV-2	2	0.8
GVE/GLRaV-2/GLRaV-3	5	1.99
GVE/GLRaV-2/GLRaV-3/GRSPaV	5	1.99
GVE/GLRaV-2/GRSPaV	2	0.8
GVE/GLRaV-3	70	27.89
GVE/GLRaV-3/GRSPaV	26	10.36
GVE/GVF	1	0.4
GVE/GVF/GLRaV-1/GLRaV-3	1	0.4
GVE/GVF/GLRaV-2/GLRaV-3	2	0.8
GVE/GVF/GLRaV-2/GRSPaV	1	0.4
GVE/GVF/GLRaV-3	8	3.19
GVE/GVF/GLRaV-3/GRSPaV	6	2.39
GVE/GVF/GRSPaV	1	0.4
GVE/GRSPaV	13	5.18
GVF/GLRaV-2/GLRaV-3	1	0.4
GVF/GLRAV-2/GLRaV-3/GRSPaV	1	0.4
GVF/GLRaV-3	8	3.19
GVF/GLRaV-3/GRSPaV	5	1.99
GVF/GRSPaV	4	1.59

The total distribution of detected viruses is summarised in Figure 3.5. GLRaV-3 was predominantly found in the plants tested in the study, followed by GVE and GRSPaV. The same RNA samples were analysed for the presence of other *Clostero*-, *Viti*- and *Foveaviruses*. Results were obtained for the presence of GVA, GVE, GVF, GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-4-like and GRSPaV. GLRaV-3, GVE, GVA and GRSPaV were detected in a previous deep sequencing analysis of a

South African vineyard (Coetzee *et al*., 2010). A widespread survey was conducted in this study and GLRaV-3 was found to be the primary virus detected in the plants, occurring in 87% of sampled grapevines. A less than 4% infection rate was observed for other closteroviruses, namely: GLRaV-1, GLRaV-2 and GLRaV-4-like viruses. The *Vitivirus*, GVE, was detected in significant percentages (58%) in these plants and GVA and GVF at 19% and 17% respectively. The *Foveavirus*, GRSPaV, was the third most prevalent virus detected in this study, detected in 37% of plants. The detection of the different viruses was based on primers designed in previous studies (for the detection of GVA, GLRaV-1, GLRaV-3, and GRSPaV) and primers designed based on sequence data deposited in GenBank (for the detection of GVE, GVF, GLRaV-3 GH24-like, GLRaV-2 and GLRaV-4-like viruses). Detection is limited to the accuracy and sensitivity of the primers used and we present the results based on this fact.

Most plants (80%) tested positive for mixed virus infections and only 9.2% of plants were singly infected with GLRaV-3. The infection with multiple viruses has a direct effect on the symptom expression of infected plants. Plants showing a typical GLD symptom might be infected with GLRaV-3 and one to four other viruses leading to the mild or severe leafroll symptoms observed. Mixed virus populations occur more frequently in South African vineyards compared to results from the Napa Valley. There, 81% of tested plants had single infections of GLRaV-3 (Sharma *et al*., 2011), a much higher relative abundance of single infected plants when compared to South African vineyards. This is also the case with the GLRaV-3 variants, where 41% of plants were infected with multiple GLRaV-3 variant groups in South African vineyards opposed to 22% multiple infected plants in the Napa Valley study (Sharma *et al*., 2011). In a survey for GLRaV-1, GLRaV-2 and GLRaV-3 conducted in the Finger Lakes vineyards in New York, single infections occurred in 10%, 3% and 15% of plants, respectively; mixed infections of these viruses affected only 3.6% of plants (Fuchs *et al*., 2009). Although the presence of only three viruses was studied in the Finger Lakes district, the low percentage of mixed infected plants were in contrast to what we observe in South African vineyards. A study from Turkey showed a higher prevalence of GLRaV-1 in their vineyards compared to GLRaV-3 (Akbas *et al.*, 2007). The Turkish study noted that mixed infections are common in vines affected by leafroll-associated viruses and detected mixed infections between different GLRaVs. A GLRaV based survey in Tunisia showed that 45.8% of plants tested had mixed multiple infections (Mahfoudhi *et al*., 2008). GLRaV-3 was the most widespread virus, contributing to 76.3% of the total viruses detected in Tunisian vineyards. The GLRaV-4-like viruses (GLRaV-5, -6) had the second highest count followed by GLRaV-1, GLRaV-2, and GLRaV-7, occurring with less than 10% infection (Mahfoudhi *et al*., 2008). In Chile, a low percentage infection was reported for GLRaVs and a high relative abundance of GLRaV-2 reported in their

vineyards (Fiore *et al*., 2008). It is clear that in certain parts of the world certain viruses occur predominantly. The influence of mealybug vectors in the transmission efficiency of GLRaV-3 variants and other virus populations needs to be studied. This confirms the importance of detecting all possible variants of viruses in vineyards, especially when screening planting material distributed by industry to producers.

The viral population per cultivar was identified (Figure 3.6). White-fruited cultivar grapevines, namely Chardonnay and Chenin blanc, were found to contain an average of 2.67 different viruses per infected sample; where-as red-fruited cultivars, namely Cabernet Sauvignon, Merlot and Shiraz, were found to contain an average of 2.55 different viruses per sample. In total an average of 2.61 different viruses was found to infect individual diseased grapevines. The viral population within grapevines was determined per age of grapevine (Figure 3.7) and the average population calculated at 2.55 different viruses. 10-year old grapevines were found to contain, on average, less viruses (2 different viruses per sample), while higher average populations of viruses (3.2 different viruses per sample) were detected in 16-year old grapevines.

Based on the data generated for virus populations in various cultivars and grapevine ages, it was determined that no significant differences exist between viral populations in grapevines of varying age and cultivar. A uniform distribution of viruses was seen between dissimilar grapevines, suggesting that not one type of cultivar or age of grapevine is preferred in terms of virus infection and replication. Instead, the frequency of a virus in grapevines relies purely on the transmission of that virus and the relationship shared among viral populations.

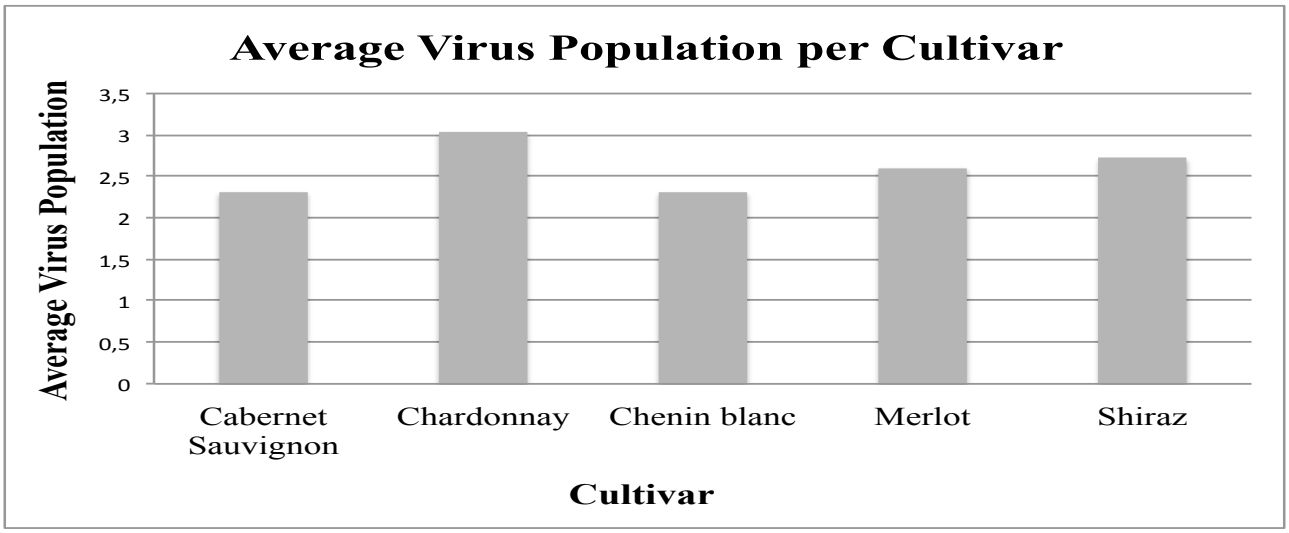

Figure 3.6: Average virus population per cultivar where cultivars are representative of the five most economically important in South Africa. Virus populations range between 2.31 and 3.03 different viruses per sample.

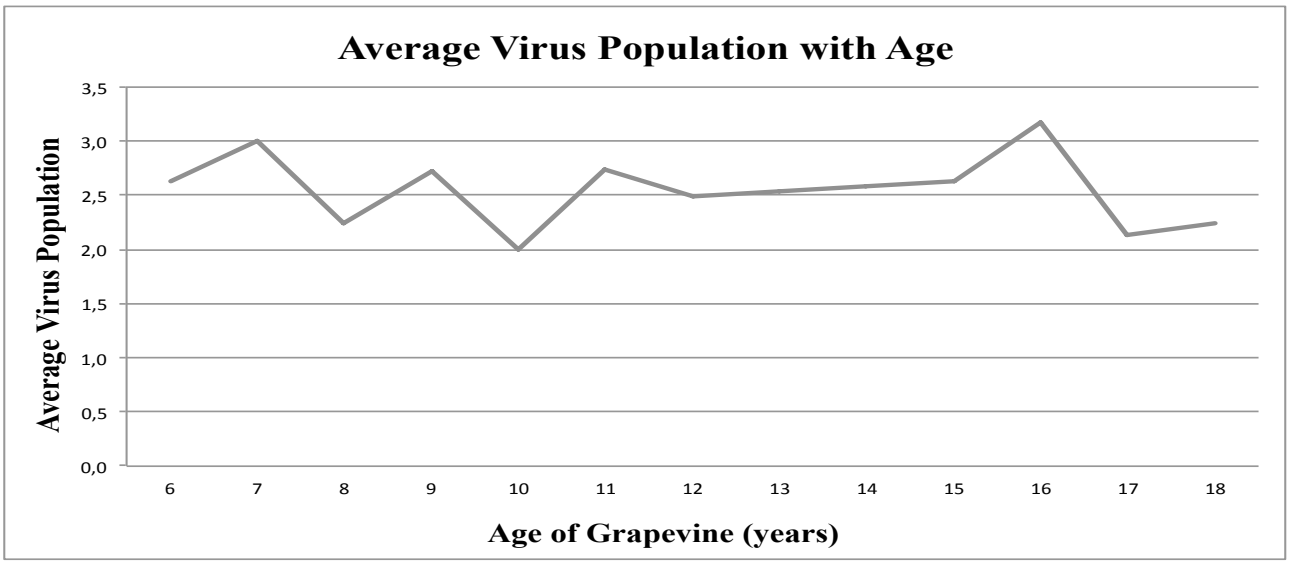

Figure 3.7: Average virus population per age of grapevine sampled. Ages of grapevines range from 6 to 18 years and average virus population ranges from 2 to 3.2 different viruses.

3.4. Conclusion

In this chapter, the distribution of GLRaV-3 variants and other grapevine infecting viruses were presented. We confirmed that GLRaV-3 is the predominant virus in South African vineyards associated with plants showing GLD symptoms. We targeted GLD-affected plants based on symptom expression; however, not all grapevines displayed clear symptomatology, especially in the case of certain white cultivars. With the high relative abundance of GLRaV-3 in these plants we strongly suggest, and confirm previous reports, that GLRaV-3 is the main causative agent of GLD in South African vineyards. Overall, in comparison to other *Clostero-, Viti-, and Foveaviruses*, GLRaV-3 was the most abundant of the detected viruses and was detected in 87% of sampled vines. This confirms the importance of GLRaV-3 in South African vineyards and the significance of detecting all variants of the virus. This study demonstrated the complexity of virus infections in South African vineyards and contributed to the knowledge for the detection of viruses and variants of viruses.

3.5. References

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Chapter 4

Determination of the virus diversity associated with grapevine leafroll disease using next-generation sequencing

4.1. Introduction

The viruses and fungi that infect grapevine are the most devastating of the infectious agents that infect grapevine (Martelli, 2014) and contribute to an extensive decrease in fruit yield and vigor (Martelli and Boudon-Padieu, 2006; Ferreira *et al.*, 2004). No means of natural resistance against these pathogens exists in grapevine, adding additional pressure to an already stressed industry (Oliver and Fuchs, 2011; Fisher *et al.,* 2004; Yamamoto *et al.*, 2000). The etiology of grapevine diseases is not well documented, making studies, like this, important in identifying the relationships shared within viral populations in an attempt to better understand grapevine diseases.

To understand the etiology of a disease, the complete population of pathogens in a host needs to be characterized and methods capable of detecting these populations need to be performed in an unbiased manner. Technologies, like microarray analyses, are capable of detecting numerous viruses simultaneously; however require known sequence information of the viruses tested for (Boonham *et al.,* 2007). Next-generation sequencing (NGS), executed in a metagenomic approach, allows for the simultaneous detection of viral populations without requiring prior knowledge of the viral sequences present in a sample (Adams *et al.,* 2009). Through the ligation of universal adaptors to nucleic acid sequences retrieved from environmental samples, libraries can be constructed to create a dataset of sequences derived from known viruses, novel virus variants as well as entirely new viruses (Adams *et al.,* 2009). Recently, NGS was used for the detection of viral populations in grapevines (Espach, 2013; Al Rwahnih *et al.,* 2011; Coetzee *et al.,* 2010; Adams *et al.,* 2009). Espach *et al.* (2012) and Al Rwahnih *et al.* (2011) detected a population of mycoviruses in grapevine samples by using NGS, in a metagenomic approach (Espach, 2013; Al Rwahnih *et al.,* 2011). Both studies made use of dsRNA as template for library preparation preceding NGS, and detected novel viruses belonging to the families *Chrysoviridae, Endornaviridae, Hypoviridae, Narnaviridae, Partitiviridae* and *Totiviridae.* Coetzee *et al.* (2010) identified a novel chrysovirus with the second most reads in 44 pooled grapevines in their metagenomic NGS study.

Mixed viral infections within single grapevines are a common occurrence; Thompson *et al.* (2014) made use of a macroarray multiplex platform to detect multiple viruses in single grapevine samples. They found 53% of tested grapevines to have mixed infections with viruses from the families *Betaflexiviridae, Closteroviridae, Secoviridae* and *Tymoviridae* (Thompson *et al.,* 2014). Research

performed in the USA (Sharma *et al.,* 2011; Fuchs *et al.,* 2009; Martin *et al.,* 2005), Chile (Fiore *et al.,* 2008) and Turkey (Akbas *et al.,* 2007) found mixed viral infections, in single GLD symptomatic plants, to be a common occurrence.

Grapevine Leafroll disease is the most economically devastating of the grapevine virus diseases and has been reported to contribute up to 60% loss in yield of fruit (Rayapati *et al.,* 2008). The grapevine leafroll-associated viruses (GLRaVs) are the collective infectious agents commonly found in GLD symptomatic grapevines, where *Grapevine leafroll-associated virus* 3 (GLRaV-3) (genus *Ampelovirus,* family *Closteroviridae*) is the primary causative agent. Six variant groups of GLRaV-3 have been identified and their presence in diseased grapevines, as either single or mixed infections, varies between plants (Chooi *et al.,* 2013b; Farooq *et al.,* 2012; Jooste *et al.,* 2011; Sharma *et al.,* 2011). Other viruses commonly infecting grapevine are of the families *Closteroviridae, Betaflexiviridae* and *Secoviridae* (Le Maguet *et al.,* 2012; Golino *et al*., 1992). The interaction of GLRaVs, within diseased grapevines, in affecting plant physiology and symptomatology is not well documented and further research with this focus needs to tale place.

Samples were selected based on typical GLD symptoms and were subjected to NGS with either the Illumina MiSeq platform (for samples processed in 2013) or the Illumina HiSeq 2500 platform (for samples processed in 2014). Results were analyzed with the focus of determining the complexity of viruses within GLD vines.

4.2. Materials and Methods

4.2.1. Plant material

Canes were collected from 17 diseased grapevines from eight different farms in the Western Cape province, South Africa. A selection of four rootstocks, six white- and seven red-fruited cultivars were selected that represented 10 different cultivars of grapevine. The cultivar and location for each sample can be located in Table 4.1. Grapevines were selected based on typical GLD physical symptoms for red-fruited cultivars and white-fruited cultivars where applicable, or selected based on previous knowledge of GLD infection (white-fruited cultivars and rootstock plants). Samples were collected during late summer (February and March) for the years 2013 and 2014, when the visual symptoms of GLD were most prominent. Samples collected in 2013 included samples 7-11. Samples collected in 2014 included samples 1-6, and 12-17. Phloem was scraped off the cane material for each sample and used for dsRNA extractions.

Table 4.1: List of samples subjected to next-generation sequencing with respective name, cultivar and the location at which the samples were collected.

Sample Number:	Sample Name:	Cultivar:	Area:
1	1.49.1	Chardonnay	Malmesbury
2	27.16.74	Pinot Noir	Hermanus
3	38.36.31	Carignan	Malmesbury
4	49STOK	Richter '99	Malmesbury
5	BJ3	Richter '99	Stellenbosch
6	BJ4	Richter '99	Stellenbosch
7	GH23	Cabernet Sauvignon	Stellenbosch
8	GH24	Cabernet Sauvignon	Stellenbosch
9	H35	Semillion	Stellenbosch
10	H36	Shiraz	Rawsonville
11	H38	Chenin blanc	Rawsonville
12	V1	Chardonnay	Vredendal
13	V3	Chardonnay	Vredendal
14	V4R	Ramsey	Vredendal
15	V5	Chenin blanc	Vredendal
16	V6	Merlot	Vredendal
17	10.15.14	Shiraz	Wolseley

4.2.2. Double-stranded RNA extractions from grapevine

Double-strand RNA extractions were performed using an adapted cellulose affinity chromatography method (Morris and Dodds, 1979). For each sample, 20 g of phloem scrapings were pulverized in liquid nitrogen using a mortar and pestle and 112.2 ml of extraction buffer added (45 ml 2X STE [1 X STE (100 mM NaCl, 50 mM Tris-base, 1 mM Na_2EDTA at pH 6.8)], 15 ml 10% [w/v] SDS, 25 ml STE-saturated phenol, 25 ml chloroform, 1.2 ml Bentonite [40 mg/ ml] and 1 ml β-mercaptoethanol) and shaken at 135 rpm for 30 minutes at room temperature. Samples were then centrifuged at 16 000 x*g* for 20 minutes at 22^{o}C. The supernatant was collected and adjusted to 16% EtOH [v/v] with absolute ethanol and 3 g of cellulose powder (MN 2100) was added. The total volume was adjusted to 300 ml with 16% EtOH 1X STE [v/v] and shaken at 135 rpm for 45 minutes at room temperature. The cellulose, containing dsRNA, was allowed to settle and the liquid poured off. The cellulose suspension was run through a 13 mm chromatography column. The column was washed with 100 ml 16% EtOH 1X STE [v/v] and the dsRNA eluted from the cellulose with 42 ml 1X STE. A combination of 500 mg of cellulose (MN 2100) and 8 ml absolute EtOH was added to the elution and the volume of the mixture adjusted to 200 ml with 16% EtOH 1X STE [v/v]. The mixture was shaken at 135 rpm for 60 minutes at room temperature. The mixture was then run through a 9 mm chromatography column. The column was washed with 50 ml 16% EtOH

1X STE [v/v] and the dsRNA eluted with 9 ml 1X STE. After the addition of 0.9 ml 3M NaOAc (pH 5.5) and 25 ml absolute ethanol [v/v], the elution was stored at -20°C overnight. The precipitated dsRNA was centrifuged at 16 000 x*g* for 80 minutes at 4°C, washed with 70% EtOH [v/v] and resuspended in 50 µl reverse osmosis water (RO-H_2O). The dsRNA suspension was subjected to RNase and DNase treatment through which 12.5 µl 10X DNase buffer (Promega), 2.5 µl DNase RQ1 (Promega) and 5 µl RNase T1 (Roche) were added to the suspension and subsequently incubated at 37°C for 30 minutes using an Applied Sciences thermal cycler 2720. A combination of 250 µl phenol, 240 µl chloroform, 10 µl isoamylalcohol and 430 µl 1X STE were added to the dsRNA suspension, mixed thoroughly and centrifuged at 16 000 x*g* for 10 minutes at 4°C. The supernatant was removed and added to 480 µl chloroform and 20 µl isoamylalcohol, mixed thoroughly and centrifuged at 16 000 x*g* for 10 minutes at 4°C. The supernatant was removed, 2.5X absolute EtOH and 10% 3M NaOAc was added and mixed before storing at -20°C for 2 hours. A fraction (20%) of the total volume was then removed and added to a new tube. Both tubes were centrifuged at 19 500 x*g* for 60 minutes at 4°C after which the liquid was drained and the pellets washed with 70% EtOH [v/v]. A combination of 20 µl 1X TE (1 M Tris-base, 500 mM Disodium Salt Dihydrate), 2 µl 3M NaOAc and 50 µl absolute EtOH was added to the tube containing 80% of the total volume and stored at -20°C to be sent for library preparation and NGS. A total of 15 µl RO-H_2O and 5X Cresol loading dye (30% sucrose; 125 mg cresol red dye) was added to the tube containing 20% of the total volume and the dsRNA separated in a 1% [w/v] TAE (40 mM Tris-base, 1 mM Na_2EDTA and 0.1% [v/v] glacial acetic acid at pH 8.0) agarose gel stained with ethidium bromide (EtBr). The quality and quantity of the dsRNA was evaluated through visualization on a 1% [w/v] TAE Agarose gel stained with EtBr. All 17 dsRNA samples were selected for sequencing (Table 4.1) at the Agricultural Research Council Biotechnology Platform (ARC-BP), Pretoria.

4.2.3. Library preparation and next-generation sequencing

Library preparations were performed using an adapted protocol for dsRNA as input RNA that followed an Illumina TruSeq RNA sample preparation protocol (Supplementary Protocol S.1). Samples processed in the year 2013, namely: 7, 8, 9, 10 and 11 were subjected to sequencing using an Illumina MiSeq platform while samples processed in the year 2014, that include: 1-6 and 12-17 were sequenced using Illumina's HiSeq 2500 platform. All samples were sequenced as 100-nucleotide paired-end reads.

4.2.4. Sequence analysis and pre-processing

Short read datasets generated for each sample were processed separately. Generated reads were evaluated for quality using FastQC (http:// bioinformatics.babraham.ac.uk/projects/fastqc/).

Individual sequencing adaptors were identified and trimmed from the reads using Cutadapt v1.6 (Google Code) (Supplementary Table S.7). Nucleotides on the 5'-ends of the datasets were trimmed of unevenly distributed nucleotides to remove potentially incorporated sequencing errors and improve the quality of reads for *de novo* assemblies. The 3' region of the dataset sequences whose mean quality score was less than 28 were trimmed to improve the overall quality of datasets to ensure more accurate read-mappings and *de novo* assemblies. Trimming of datasets was performed using the fastx_trimmer command of FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Datasets were then filtered for quality using the fastq_quality_filter command of FASTX_Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/); the minimum quality score to keep was set at Q20 (-q 20) and the minimum percent of bases with Q20 Phred quality score[Q] was set at 96% (-p 96) (Figure 4.2). Through filtering datasets for Q20 Phred scores it was assured that 99% of bases were called correctly, further improving the quality of reads for subsequent analyses. Reads shorter than 20 nucleotides in length were removed from the dataset.

Datasets that had been quality trimmed and filtered were uploaded into CLC Genomics Workbench 7 (CLC Bio) for subsequent analysis. The datasets were imported as fastq Illumina files with Sanger/ Illumina pipeline 1.9 base encoding. Reads were aligned to the complete set of *Vitis vinifera* chromosomes (19 chromosomes) as well as the *Vitis vinifera* chloroplast and mitochondrial genomes (Supplementary Table S.2). Mapping parameters to host genomes specified that the reference genomes would not be masked, read alignment was set to default (mismatch cost: 2; insertion cost: 3; deletion cost: 3; length fraction: 0.7; similarity fraction: 0.7) with global alignment and random mapping of non-specific matches; un-mapped reads were collected and used for further analysis. Data sets hereafter will be referred to as "high-quality reads".

Read-mapping

High-quality reads were mapped to 124 reference genomes of grapevine-infecting viruses and viroids, obtained from the NCBI GenBank database (Supplementary Table S.3). Parameters of the read-mappings included no masking of the reference genomes, read alignment costs for mismatches, insertions and deletions were set at 2, 3 and 3 respectively; reads that mapped to references required that 90% of the read length match the reference and that 90% of read nucleotides match those of the reference. Reads were utilized in a global alignment with random mapping of non-specific matches and the results were then saved.

[Q] Phred quality scores are allocated to each sequence base call and are logarithmically related to the base-calling error probability. A Phred score of Q20 implies that the probability of an incorrect base call is 1% therefore base calling accuracy is 99%.

De novo assembly

De novo assemblies of NGS reads were performed in CLC Genomics Workbench 7 (CLC Bio) through CLC bio's *de novo* assembly algorithm which functions through the use of de Bruijn graphs, a method also used by other *de novo* assemblers such as Velvet (Zerbino *et al.,* 2009; Zerbino and Birney, 2008), ALLPATHS (Butler *et al.,* 2008) and Euler-SR (Chaisson and Pevzner, 2008). First, read sequences were assembled into simple contig sequences at which point the reads were mapped back to the simple contig sequences as a reference, by doing so the coverage levels of individual contigs was established. Parameters for each *de novo* assembly included: a default word size and bubble size of 20 and 50, respectively; a minimum contig length of 200; global alignment with reads mapping back to contigs using default settings (mismatch cost: 2, insertion cost: 3, deletion cost: 3, length fraction: 0.5 and similarity fraction: 0.8); and update contigs was selected. Contigs created through *de novo* assemblies were selected based on the criteria that they had an average coverage of greater than 50X or were 1 kb in length and subjected to BLAST (Basic Local Alignment Search Tool) searches with both blastn (searches a nucleotide database with a nucleotide query) and tblastx (searches a translated nucleotide database using a translated nucleotide query) analysis against the NCBI non-redundant database with the use of Blast2GO (Conesa *et al.,* 2005). To ensure that high confidence matches were selected the E-value cut-off was set at 1.0E-6. Contigs were classified according to their highest identity. Figure 4.1 illustrates the workflow followed in processing and analyzing the NGS data obtained.

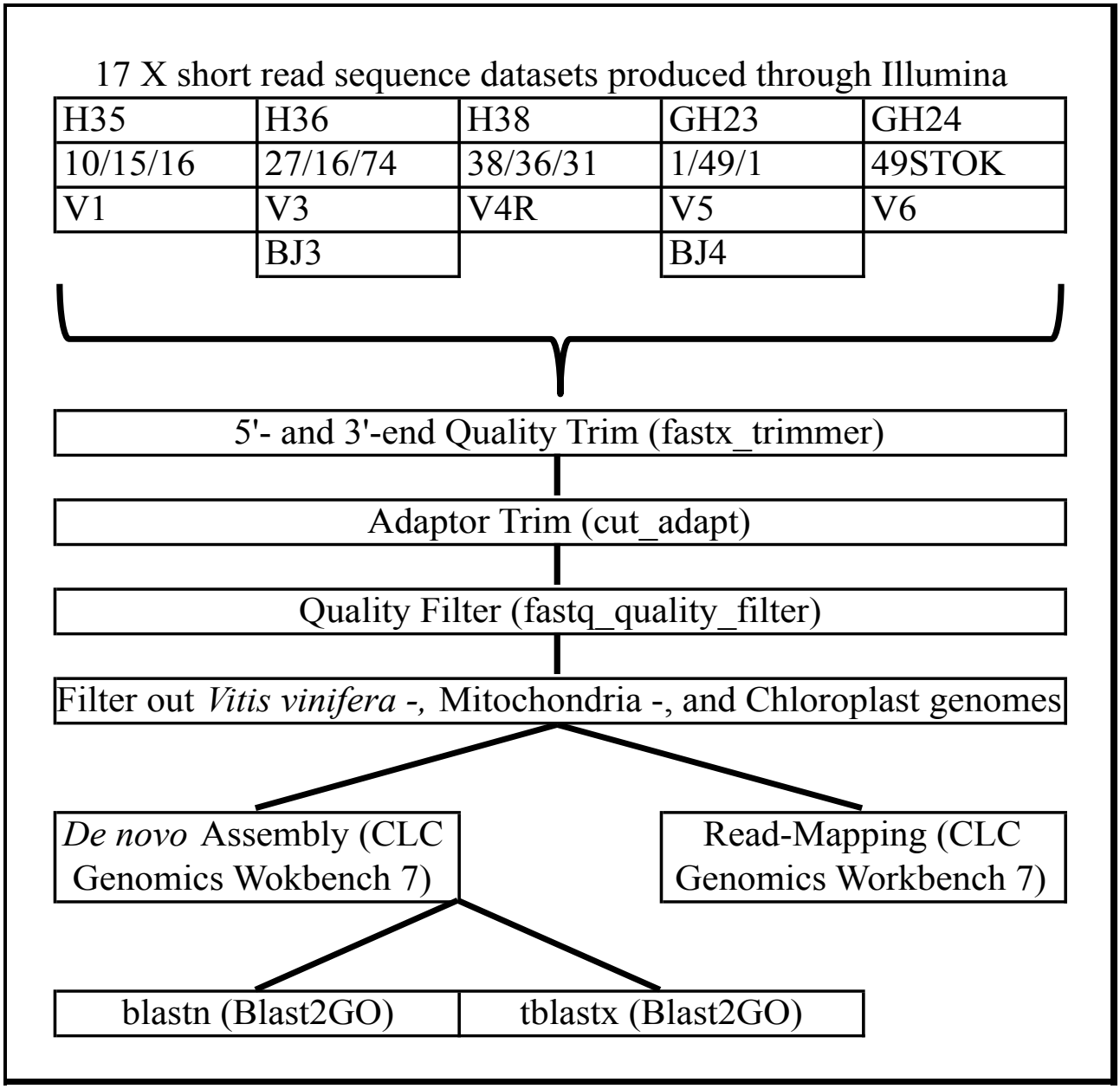


Figure 4.1: Graphical representation of the bioinformatic workflow followed in processing the Illumina short read data using the FASTX-Toolkit package, CLC Genomics Workbench 7 and Blast2GO. This workflow was implemented with each of the 17 read datasets.

4.3. Results and Discussion

4.3.1. Plant material and double-stranded RNA extractions

A selection of multiple grapevine cultivars helped to establish a cultivar-based sensitivity to virus infection. dsRNA samples selected for subsequent deep sequencing had an inferred (comparison to GeneRuler 1 kb DNA ladder) concentration of greater than 20 ng, visualized on a 1% (w/v) agarose TAE gel, to ensure better quality library preparation and sequencing. No differences in dsRNA extraction efficiency and inferred concentrations for each sequenced sample were observed.

4.3.2. Illumina sequencing and pre-processing

Approximately 31 gigabases of data were generated through NGS (Table 4.2). Short read datasets, generated for each sample, were visualized using FastQC (http://bioinformatics.babraham.ac.uk/projects/fastqc/) and trimmed and filtered for quality with FASTX_Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) (Figure 4.2). Once the data had been trimmed of sequencing adaptors and reduced quality sequence sections as well as filtered for high quality reads, between 39.6% and 81.0% of the total reads, per sample, were discarded (Table 4.2).

In total, 46.65% of nucleotides produced through NGS were discarded after quality trimming and filtering. The respective losses of nucleotides for each Illumina platform used can be seen in Figure 4.3, where the Illumina Miseq sequencer lost 25% less data than the HiSeq sequencer during the initial quality trimming and filtering process, indicating greater sequencing efficiency in the Illumina MiSeq sequencing platform. Regardless, a large amount of sequencing data was lost due to insufficient quality, which could be on account of substandard sequencing and extraction practices as well as sample handling.

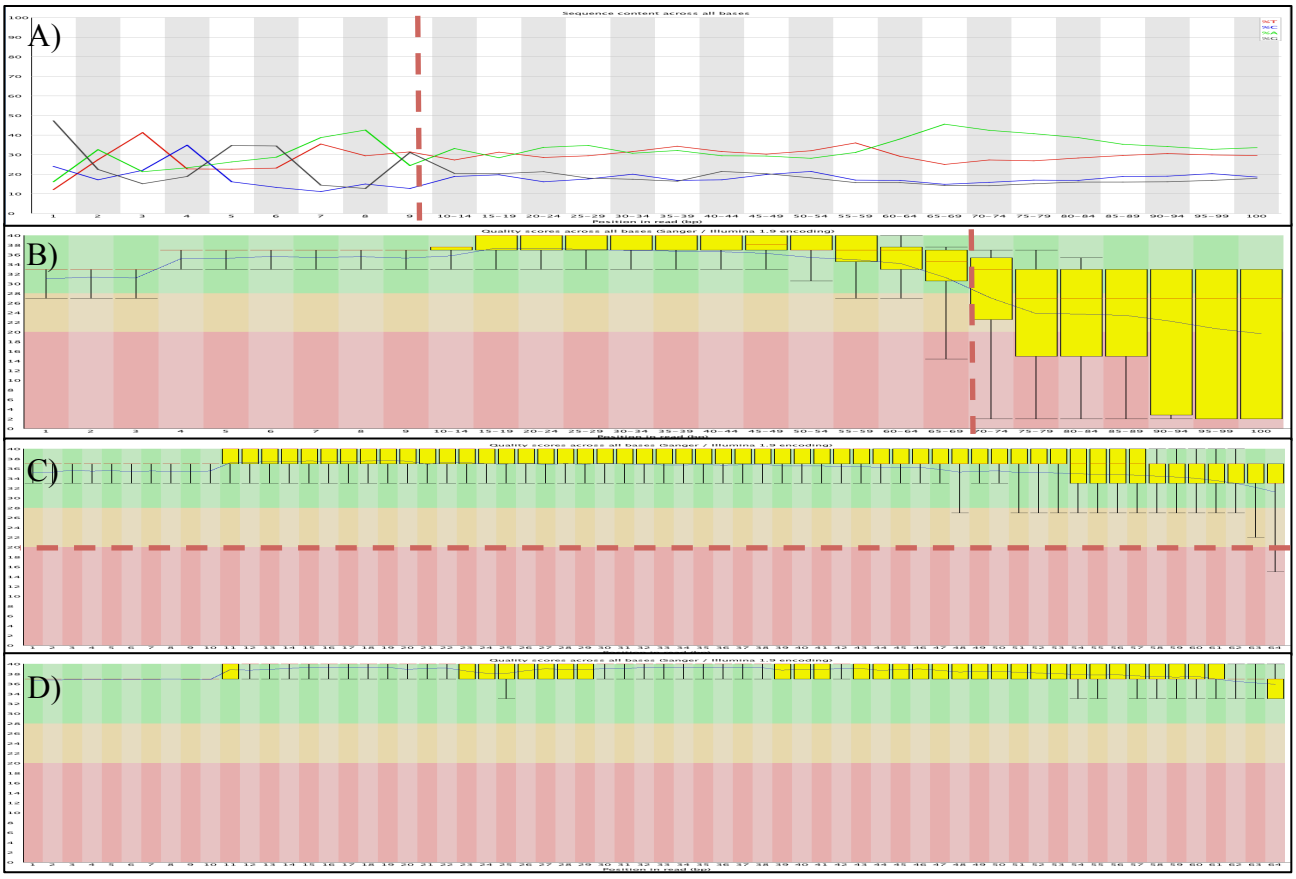


Figure 4.2: Graphs illustrating the quality of the sample 4 (49STOK) sequence dataset, generated in FastQC. A) The percentage nucleotide composition per base, used to trim nucleotides from the 5'-end. B) The average quality score (Phred scores), used to trim nucleotides from the 3'-end. Blue line indicates mean quality score across dataset C) A minimum Phred score of Q20 was used for filtering. D) The sequence dataset quality following trimming and filtering. Checkered red lines display thresholds at which trimming and filtering occurred.

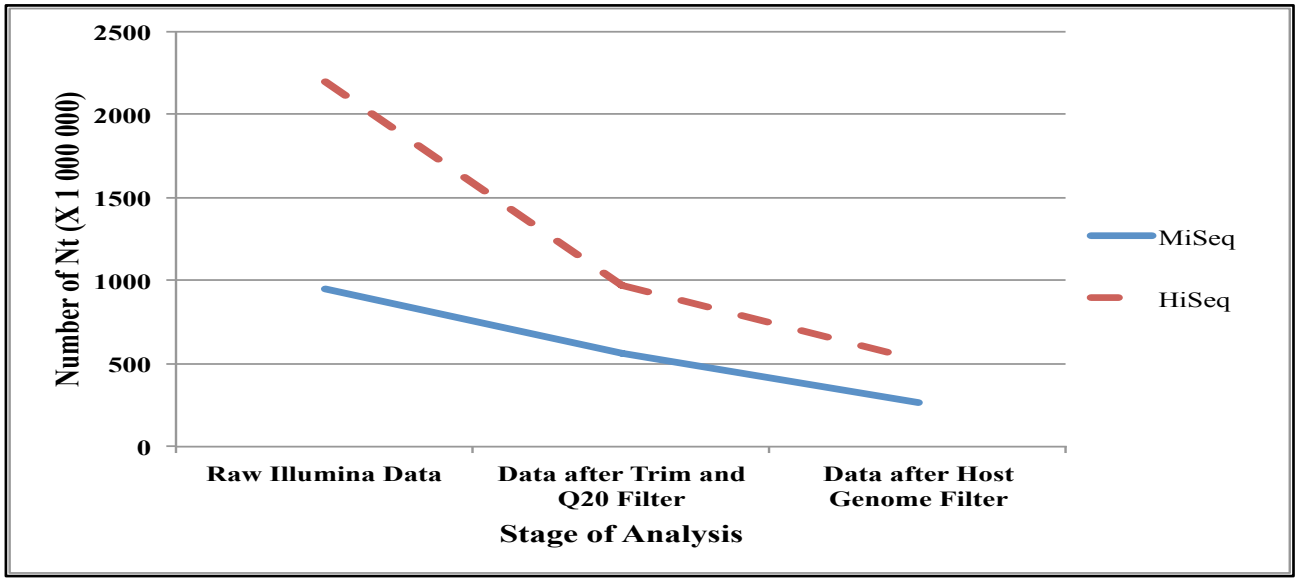


Figure 4.3: Line graph displaying the loss in sequencing data through the trimming and filtering process for each Illumina platform used, namely: MiSeq (2013 samples) and HiSeq 2500 (2014 samples).

Table 4.2: Table displaying the nucleotides generated through NGS for each sample, the nucleotides remaining after trimming and filtering for Q20 as well as filtering of *V. vinifera* genome sequences (percentage nucleotides remaining in brackets) and the total percentage of nucleotides lost through the quality trimming and filtering processes.

Sample Number:	Output (Gb):	Illumina Nt:	Nt after Q20 Filter/ (%):	Nt after Read-Mapping/ (%):	Total Nt Discarded (%):
1	1.49	1 485 733 800	308 275 947/ (20.8)	189 592 067/ (61.5)	87.2
2	0.88	879 073 800	173 310 988/ (19.7)	125 934 927/ (72.7)	85.7
3	2.24	2 241 305 000	1 300 448 836/ (58.0)	487 433 827/ (37.5)	78.3
4	3.56	3 561 818 800	1 828 296 372/ (51.3)	1 573 627 535/ (86.1)	55.8
5	2.51	2 513 532 000	476 657 766/ (19.0)	369 204 150/ (77.5)	85.3
6	2.63	2 632 451 800	1 590 475 027/ (60.0)	651 082 521/ (41.0)	75.3
7	2.58	2 580 219 397	1 522 329 444/ (59.0)	788 799 711/ (51.8)	69.4
8	0.95	948 053 661	559 351 660/ (59.0)	65 750 496/ (11.8)	93.1
9	0.46	461 955 729	272 553 880/ (59.0)	155 250 096/ (60.0)	66.4
10	0.22	215 134 427	126 929 312/ (59.0)	97 986 320/ (77.2)	54.5
11	0.53	525 086 997	309 801 328/ (59.0)	220 141 376/ (71.1)	58.1
12	2.14	2 140 140 400	1 277 088 322/ (59.7)	493 206 390/ (38.6)	77.0
13	1.84	1 836 598 400	1 085 870 839/ (59.1)	243 880 121/ (22.5)	86.7
14	2.20	2 201 147 600	748 338 994/ (34.0)	464 116 339/ (62.0)	78.9
15	3.94	3 940 633 200	2 139 374 475/ (54.3)	1 206 398 861/ (56.2)	69.4
16	1.25	1 247 294 800	402 735 760/ (32.3)	260 120 627/ (64.6)	79.1
17	1.64	1 644 256 600	366 321 918/ (22.3)	203 294 359/ (55.5)	87.6

4.3.3. Read-mappings and analyses

Between 54.5% and 93.1% of the total reads were removed after trimming and filtering for quality and against *Vitis vinifera* genomes, to enrich for viral reads (Table 4.2). High-quality reads were mapped to 124 reference genomes (Supplementary Table S.3), comprised of grapevine-infecting pathogens and a table drawn indicating the prevalence of identified pathogens in each sample (Table 4.3). A total of 100,751,463 reads, representing 52.8% of the total reads remaining after

quality trimming and filtering (Table 4.4), were mapped to reference genomes and detected a total of 300 viruses across 17 samples. Figure 4.3 illustrates the loss of nucleotide data for each of the Illumina sequencers used. 78.86% and 68.30% of the nucleotide data was lost after pre-processing of NGS data using Illumina HiSeq and MiSeq sequencers, respectively. 10.56% less sequencing data was discarded from the Illumina MiSeq platform based on sequencing quality (Figure 4.3).

DNase and RNase treatment was performed consistently on each dsRNA sample. Through the removal of DNA and RNA, the quality of dsRNA samples sent for sequencing was increased; however, a large amount of NGS data, assumedly produced from dsRNA, was not mapped to grapevine-infecting viruses (Table 4.4). Possible reasons for this occurrence include: stringent mapping parameters to reduce non-specific mappings, suboptimal DNase and RNase treatment, contamination of dsRNA sample after treatment of DNA and RNA and contamination of samples during library preparation and NGS.

Read mapping results for each of the 17 datasets were pooled and GLRaV-3 was found to be the most prevalent virus, comprising 31.29% of the total mapped reads. GLRaV-3 variant groups VI and II were the most commonly detected variants, contributing 22.16% and 8.23% of the total mapped reads, respectively. Members of the genus *Vitivirus* contributed to 0.55% of all detected viruses, GVE being the best represented *Vitivirus* with 0.47% contribution. Reads mapping to *Grapevine endophyte endornavirus* (GEEV) were detected in all samples and contributed towards 6.6% of all mapped reads. Three families of viruses contributed towards greater than 1% of the identified viruses, they are: *Closteroviridae, Betaflexiviridae* and *Endornaviridae*, the remaining viruses were identified at lower percentages.

The average coverage of reference genomes and the fraction of reference covered per virus for each sample can be seen in Table 4.3. The average coverage of each virus reference and the fraction of the reference covered give a good indication of the presence and titre of an identified virus. Results that display a high read count with a low fraction of reference covered indicate the mapping of reads to conserved regions of the reference sequence, as can be seen for GLRaV-3 variant group III viruses in samples 4, 6, 13 and 14 (Table 4.3). This may be a possible indication of the presence of alternative isolate representatives of the viruses mapped or an indication of abundance of specific subgenomic RNAs. The stringent parameters set for read-mappings would exclude the mapping of reads to references with less than 90% nucleotide similarity, disallowing the detection of genetically variable isolate genomes. On the other hand, viruses detected with a high percentage of sequence covered and a low read count, as for viroids belonging to the family *Pospiviroidae* and certain *Vitivirus* infections, can be accounted to relatively small genomes, uniform replication across all

open reading frames (ORFs) as well as better quality reads mapping to regions across the reference genomes.

Supplementary Table S.4 displays the fraction of the reference covered for each virus per sample and is a good indicator for the confidence of virus presence. GLRaV-3 variant groups I, II and VI as well as viroids of the family *Pospiviroidae,* were detected with a high fraction of the reference covered. Therefore, there is a high confidence in the presence of these viruses in their respective samples. References of viruses from the families *Secoviridae*, *Geminiviridae*, *Tymoviridae*, *Tombusviridae*, *Bunyaviridae* and certain members of the families *Betaflexiviridae* and *Virgaviridae* were detected with less than 10% of the reference covered. An average of two of each of these viruses was detected across all of the samples. Further validation, through RT-PCR, of these viruses needs to be completed in order to determine their presence.

Samples were considered to have positive virus infection based on certain criteria. The criteria stating that reads mapping to individual viruses cover greater than 10% of the reference genome with greater than 10X average coverage. Viral populations were calculated using the aforementioned criteria and ranged between zero and nine virus species per sample. Sample number 3 and 6 had the highest diversity of infection with nine viruses each, while no viruses were confidently detected in samples 2 and 16. Rootstocks were found to contain the highest average virus population with 5.5 different viruses per sample, followed by white-fruited cultivars (3.8 viruses per sample) and red-fruited cultivars (2.8 viruses per sample). On average, four different viruses were detected in each sample with confident virus calling.

Samples were selected based on physical symptom expression of GLD; red-fruited cultivars exhibit a more pronounced phenotype compared to white-fruited cultivars and rootstocks. Interesting to note, samples 2 and 16 had no positive virus calling and originated from Pinot Noir and Merlot, respectively. The expression of GLD symptoms with these red-fruited cultivars is attributable to low titres of GLRaV-3, indicating that a low virus titre is adequate for the expression of GLD symptoms in grapevine. Moreover, the higher levels of virus diversity in white-fruited cultivars and rootstocks may indicate a potential masking of visual expressions established through relationships shared between different virus species. Such a relationship may include *Grapevine-associated narnavirus* (GNV) that was only detected in white-fruited cultivars and rootstocks and potentially GLRaV-2 (Table 4.3), detected predominantly in rootstocks with a single finding in a red-fruited cultivar (Carignan). Further research into the association of distinctive viruses and subsequent physical expression in differing grapevine cultivars needs to be performed to establish viral relationships that could reduce the negative effects of GLD.

Table 4.3: Table outlining the average coverage (Ave. Cov.) and the fraction of the reference genome (Frac. Ref. Cov.) covered for each sample against viruses from the families *Closteroviridae, Betaflexiviridae, Pospiviridae, Endornaviridae* and *Narnaviridae,* generated through read-mappings. Values in bold represent viruses with confident identification.

Sample Number:	Description:	Cultivar:	Grapevine Infecting Pathogen and Corresponding Family																													
			Closteroviridae														*Betaflexiviridae*								*Pospiviridae*						*Endornaviridae*		*Narnaviridae*	
			GLRaV-2		GLRaV-3 (I)		GLRaV-3 (II)		GLRaV-3 (III)		GLRaV-3 (IV)		GLRaV-3 (VI)		GLRaV-3 (GH24)		GVA		GVE		GVF		GRSPaV		Hop Stunt Viroid		Yellow Speckled		Yellow Speckled		GEEV		GNV	
			Ave. Cov.	Frac. Ref. Cov.	Ave. Cov.	Frac. Ref. Cov.	Ave. Cov.	Frac. Ref. Cov.	Ave. Cov.	Frac. Ref. Cov.	Ave. Cov.	Frac. Ref. Cov.	Ave. Cov.	Frac. Ref. Cov.	Ave. Cov.	Frac. Ref. Cov.	Ave. Cov.	Frac. Ref. Cov.	Ave. Cov.	Frac. Ref. Cov.	Ave. Cov.	Frac. Ref. Cov.	Ave. Cov.	Frac. Ref. Cov.	Ave. Cov.	Frac. Ref. Cov.	Ave. Cov.	Frac. Ref. Cov.	Ave. Cov.	Frac. Ref. Cov.	Ave. Cov.	Frac. Ref. Cov.	Ave. Cov.	Frac. Ref. Cov.
1	1/49/1	Chardonnay	0.09	0.01	1.33	0.14	**16**	**0,19**	0.20	0.02			3.78	0.12					0.32	0.10	0.01	0.01	0.15	0.11							0.62	0.31		
2	27/16/74	Pinot Noir	0.03	0.01	1.40	0.11	9.60	0.18	0.18	0.02			2.12	0.11			0.01	0.01	0.26	0.10	0.02	0.02	0.05	0.04							0.91	0.40		
3	38/36/31	Carignan	**1165**	**1**	**225**	**0,33**	**27096**	**1**	74.79	0.08	0.51	0.08	**1E+06**	**1**	0.03	0.01	**11**	**0,66**	**198**	**0,97**			0.05	0.03	**117**	**1**	**1424**	**1**	0.18	0.18	0.23	0.16		
4	49STOK	Richter '99	0.04	0.02	**16411**	**1**	**29592**	**1**	**1780**	**0,11**	1.29	0.17	**7806**	**1**	0.31	0.02	**28**	**0,97**	**741**	**1**	0.27	0.18	0.15	0.11	**25**	**0,94**	1.34	0.50			**12950**	**1**	0.13	0.13
5	BJ3	Richter '99	0.07	0.01	4.60	0.25	**3249**	**0,81**	0.97	0.05	0.48	0.13	4.21	0.16					5.39	0.39			0.19	0.12			0.48	0.12			0.57	0.30		
6	BJ4	Richter '99	0.02	0.01	**937**	**0,32**	**66591**	**1**	**172**	**0,13**	**10**	**0,25**	1.08	0.12	0.09	0.01	**138**	**0,96**	0.05	0.05	0.01	0.01	**1889**	**1**	**12,00**	**0,81**	**47**	**1**	**19**	**1**	0.18	0.13		
7	GH23	Cab. Sav.					0.05	0.03					0.05	0.02	**21880**	**1**															0.07	0.03		
8	GH24	Cab. Sav.					0.07	0.03							**7507**	**1**									0.73	0.18					0.09	0.05		
9	H35	Semillion	0.02	0.01	**1712**	**0,9**	0.50	0.06	0.24	0.01			**565**	**0,92**			0.08	0.02	0.01	0.01	0.14	0.11					**21**	**0,96**			0.07	0.05		
10	H36	Shiraz	0.01	0.00	0.60	0.10	**43**	**0,14**					**18**	**0,13**					0.71	0.06							0.17	0.17			0.01	0.01		
11	H38	Chenin Blanc	0.01	0.01	**158**	**0,44**	**1209**	**0,6**	0.23	0.01			**378**	**0,61**			0.03	0.03	2.66	0.15							**11**	**0,86**			0.22	0.14		
12	V1	Chardonnay	0.23	0.04	0.40	0.08	7.50	0.19	0.03	0.01			**22644**	**1**			0.03	0.02	0.40	0.10	0.08	0.03	0.03	0.03	7.16	0.81	1.40	0.55	**13**	**0,71**	0.56	0.13	**11**	**0,7**
13	V3	Chardonnay	0.03	0.01	**9927**	**1**	**7494**	**1**	**252**	**0,11**	5.74	0.18	**3299**	**1**	0.01	0.01	**30**	**0,98**	**1398**	**1**	**94**	**0,71**	0.06	0.03	4.2	0.77	3.12	0.93			1.22	0.46	3.13	0.55
14	V4R	Ramsey	0.05	0.02	**1353**	**0,93**	**10114**	**0,93**	88	0.07	0.46	0.14	**7277**	**0,88**	0.02	0.00	0.24	0.18	0.18	0.07	**38**	**0,43**	0.19	0.09	0.45	0.45	1.99	0.44			0.76	0.33	0.52	0.28
15	V5	Chenin Blanc	0.05	0.02	**148**	**0,33**	**1E+05**	**1**	49	0.07	0.94	0.17	6.09	0.23	0.01	0.01	0.02	0.02	**2019**	**1**	**128**	**0,6**	0.03	0.03	3.81	0.79	8.9	0.96	**34**	**0,87**	0.21	0.13	4.05	0.76
16	V6	Merlot	0.03	0.02	0.90	0.12	6.90	0.16	0.06	0.02			1.73	0.12			0.03	0.03	0.11	0.08	0.01	0.01	0.04	0.04							0.31	0.23		
17	16/15/14	Shiraz	0.06	0.01	1.36	0.13	**10**	**0,14**	0.20	0.02			2.69	0.14			0.02	0.02	0.26	0.11	0.04	0.04	0.05	0.04			0.19	0.19			0.59	0.35		

Table 4.4: Table depicting the percentage of total reads mapped for each sample to reference genomes during the read-mapping process as well as the total number of reads remaining after quality trimming and filtering.

Sample Number:	Total Reads:	Mapped Reads:	Percentage of Reads Mapped (%):
1	5715240	6826	0,119
2	3192973	4405	0,138
3	18602935	11581763	62,258
4	27121822	17792469	65,602
5	8914237	999449	11,212
6	22760310	18249348	80,181
7	9172090	4706118	51,309
8	2853489	2385590	83,603
9	1213840	550190	45,326
10	728523	16236	2,229
11	1674079	427620	25,544
12	17642034	5769627	32,704
13	15591971	5742202	36,828
14	11994784	5420584	45,191
15	30483476	27091189	88,872
16	6356502	2991	0,047
17	6734818	4856	0,072

4.3.4. De novo assemblies and analyses

High-quality reads were assembled into contigs using the *de novo* assembly function in CLC Genomics Workbench 7 (CLC Bio). A total of 50.7 million reads were used to construct 37 382 contigs with a total length of 12.65 million nucleotides (Table 4.5). Sample numbers 10 and 14 had almost four times as many contigs constructed compared to the remaining samples. The average N50 and average calculated contig lengths were 400 and 392, respectively. Sample number 13 had the highest N50 value of 756 base pairs. Percentages of matched reads ranged between 2.27% and 95.88% per sample, with an average of 44.22% across all of the samples. The data generated through *de novo* assemblies per sample is highly variable. This is an indication of inconsistent sequencing quality and/ or variable levels of virus infection within each sample.

Table 4.5: Table illustrating the *de novo* assembly characteristics as well as features of the contigs created per sample.

Sample Number:	Length:		Contig Count:	Total Length:	Matched Reads:	Total Reads Assembled (%):
	N50[z]	Average:				
1	257	267	2739	730737	143022	4.22
2	271	282	2139	603578	65474	2.87
3	434	468	344	161034	2532592	36.35
4	557	502	729	365605	12380502	53.06
5	254	271	741	200713	159194	2.27
6	375	408	596	243451	7264946	78.37
7	324	336	2559	860565	855466	9.66
8	514	460	539	247729	3208395	63.76
9	521	517	809	418307	2042208	95.88
10	349	331	10664	3526097	238475	25.15
11	523	512	803	411292	2237148	94.96
12	492	469	176	82586	5896215	86.64
13	756	565	223	126057	3198852	91.42
14	330	334	11831	3950184	4260484	57.70
15	316	380	312	118536	5571768	32.60
16	274	291	879	255696	550739	13.33
17	257	271	1299	351761	121015	3.46

[z] N50 is a statistic based on the median of contig lengths, with preference to longer conitgs, in a set of sequences. It is the length at which 50% of sequence bases are included into contigs that equal or exceed N50 lengths.

A total of 1 334 contigs, generated through *de novo* assemblies, were selected for subsequent BLAST analysis. Criteria for contig selection included that contigs have a greater than 50X average coverage or that contig length was greater than 1 kb. These criteria were selected to increase the confidence in correct virus identification through BLAST analysis and aid in the detection of full-length virus genomes.

Table 4.6 and Figure 4.4 display the distribution of *de novo* assembly data categorized as grapevine viruses, bacteria, viroids, fungi, other viruses and other or unidentified groups after BLAST searches. NGS reads were assembled into contigs and determined that a greater percentage of reads originated from grapevine viruses representing 83% of the total reads subjected to BLAST searches, followed by fungi (6%) and then bacteria (5%). Similar results were obtained through blastn and tblastx searches indicating a good consistency with nucleotide as well as protein searches.

Table 4.6: Illustration of the reads that were classified, per sample, into each category after Blast2GO analyses with blastn (n) and tblastx (t-x) functions.

Read Distribution per Category														
	Grapevine Virus:		Bacteria:		Viroid:		Other Virus:		Fungi:		Other:		Unidentified:	
Sample:	n	t-x	n	t-x	n	t-x	n	t-x	n	t-x	n	t-x	n	t-x
1	682	682	39093	39093							6963	5218	4497	6242
2	547	547	19564	19564							248	248		
3	1286272	1286013						259	365	365				
4	7648714	7556672	560170	551897			29461	419317	2399625	2531583	769953	363605	600600	585449
5	92592	92592	46547	46547							463			463
6	6813573	6834300	328283	335590				1001	26200	25612	24669	19398	39332	16156
7	514980	514980	21638	23224							77993	60241	3676	19842
8	2703450	2703450	23124	23124							113695	112652	2644	3687
9	1120093	1117376	2105	2105	299	299		61			23902	23841		2717
10	17778	17778	8938	8632					193	193	86620	90487	31534	27205
11	1453518	1453653	5799	4468					2045	2288	346718	25569	286067	608169
12	5643173	5643173	82120	79358				5910	960	960	53891	50489	571	825
13	1261100	1261100												
14	1257569	1257569	10133	10133										
15	3961148	3961148	103627	104227	210	210			798	798	3164	2564	928	928
16			499136	499136				197			7919	7415	2445	2752
17			41794	41794							40546	40546	3313	3313

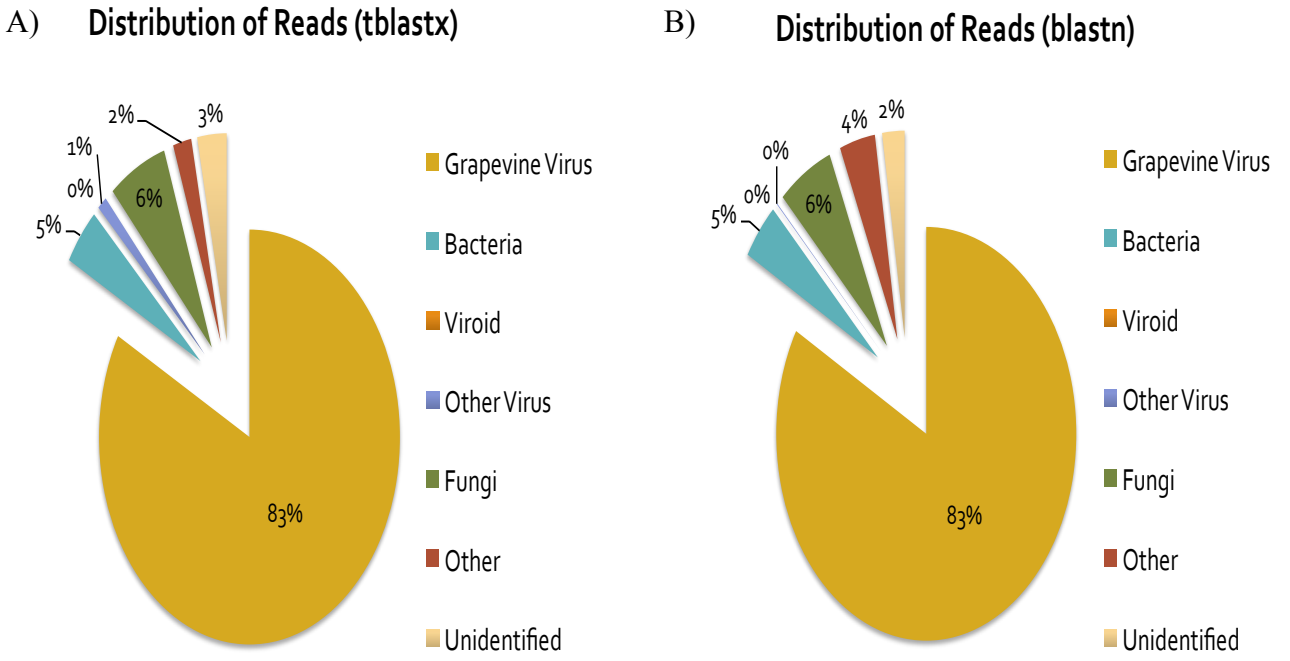
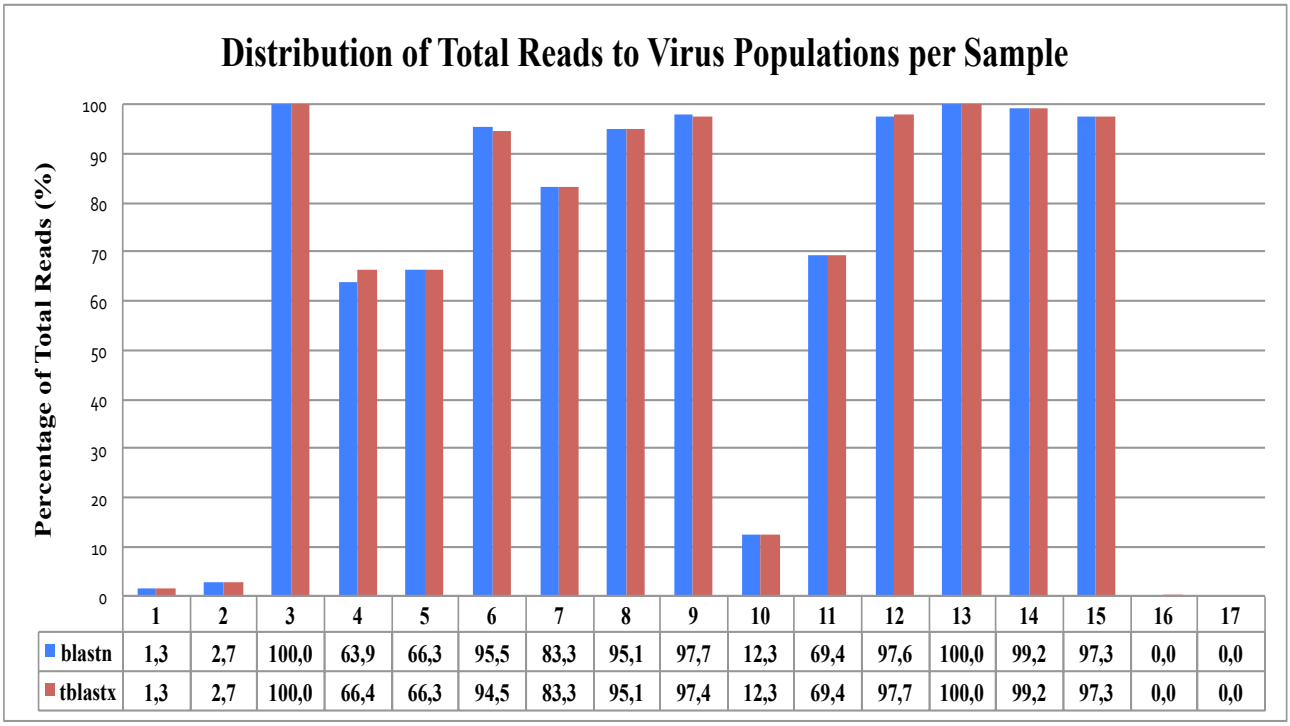


Figure 4.4: Graphical representations of the distribution of *de novo* assembly data to different categories of organisms, determined through Blast2GO. A) Distribution of *de novo* assembled reads using the tblastx function of Blast2GO. B) Distribution of *de novo* assembled reads using the blastn function of Blast2GO.

An average of 83% of *de novo* data was found to be of viral origin. Figure 4.5 displays the distribution of reads, used to construct contigs, to virus populations per sample. 100% of the reads used in *de novo* assemblies for samples 3 and 13 originated from viral populations while no viral contigs were constructed from samples 16 and 17, both red-fruited cultivars. This indicates that either the sequence data had too poor quality to assemble longer contigs or further signifies the low quantity of viral infection needed for visual symptoms to be evident in red-fruited GLD plants.



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
blastn	1,3	2,7	100,0	63,9	66,3	95,5	83,3	95,1	97,7	12,3	69,4	97,6	100,0	99,2	97,3	0,0	0,0
tblastx	1,3	2,7	100,0	66,4	66,3	94,5	83,3	95,1	97,4	12,3	69,4	97,7	100,0	99,2	97,3	0,0	0,0

Figure 4.5: Bar chart displaying the percentage distribution of total reads assigned through blastn (blue shade) and tblastx (red shade) searches (Blast2GO) to grapevine viruses per sample.

GLRaV-3 was found to be the best-represented virus detected in *de novo* assemblies, comprising 97.5% of the total viral population detected (Figure 4.6). Of the GLRaV-3 variant groups, GLRaV-3 variant group I was detected at the highest frequency with 120 and 109 contigs matched through blastn and tblastx searches, respectively. The results, however, may be misleading due to conserved region matches across GLRaV-3 variant groups. Furthermore, the genome of GLRaV-3 isolate GH24 is not present in online databases. GLRaV-3 variant group II was detected with the second highest prevalence; however, was identified with the greatest average coverage across all variants. The primary infectious agents of the pooled viral population across all samples was found to be viruses from the family *Closteroviridae* followed by members of the family *Betaflexiviridae* (Figure 4.6). *Grapevine rupestris stem pitting-associated virus* (GRSPaV) was identified as the most prevalent member of the family *Betaflexiviridae* followed by GVE and GVA. This finding supports the theory that GLRaV-3 is the primary causative agent of GLD and that the vitiviruses are a common occurrence in GLD-affected grapevines (Le Mageut *et al.,* 2012; Pietersen, 2004).

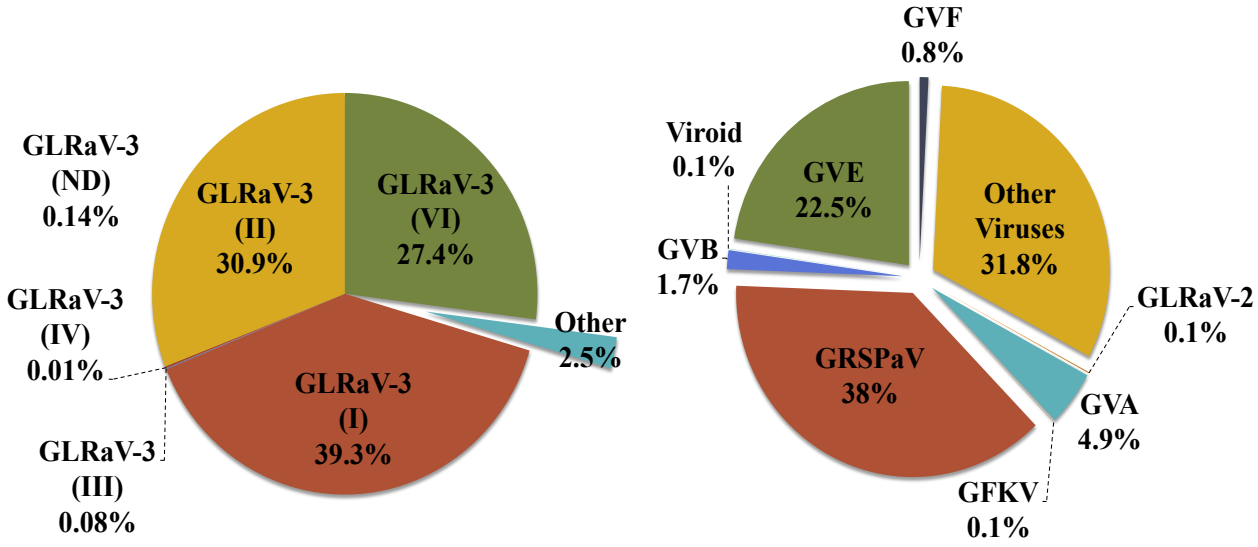


Figure 4.6: Chart representations of the pooled viral population within sampled grapevines, established through *de novo* assemblies. A) Percentage distribution of GLRaV-3 variant groups (denoted in brackets), as the primary causative agents of GLD. B) Viruses detected at lower frequencies whose percentage distribution comprises "Other" (1A).

Table 4.7: *De novo* assembly characteristics displaying the total contig count and coverage of the pooled viruses and viroids from all sampled grapevines.

Virus Coverage						
Virus/ Viroid:	**Contig Count:**		**Total Coverage:**		**Average Coverage:**	
	blastn	**tblastx**	**n**	**t-x**	**n**	**t-x**
GLRaV-3(I)	120	109	1211020	1185278	10091,84	10874,11
GLRaV-3(II)	4	12	10237359	107280,9	2559340	8940,079
GLRaV-3(III)	2	3	2488,842	221,4699	1244,421	73,8233
GLRaV-3(IV)	0	1	0	50,61727	0	50,61727
GLRaV-3(V)	0	0	0	0	0	0
GLRaV-3(VI)	5	9	65354,61	73259,46	13070,92	8139,939
GLRaV-3(ND)	3	0	4206,384	0	1402,128	0
GLRaV-2	1	1	28,19147	28,19147	28,19147	28,19147
GVA	15	19	2175,47	4808,681	145,0313	253,0885
GVB	3	1	1056,515	105,2845	352,1716	105,2845
GVE	7	7	8867,722	8867,722	1266,817	1266,817
GVF	2	1	1655,88	29,14797	827,94	29,14797
GFKV	2	0	24,42819	0	12,21409	0
GRSPaV	9	10	13993,47	15808,7	1554,83	1580,87
Viroid	2	2	158,0679	158,0679	79,03395	79,03395
Other Viruses	7	27	5023,564	16695,34	717,652	618,3459

Grapevine virus F (GVF) was detected for the first time in South African vineyards through *de novo* assemblies performed on samples 13 and 15. GVF was represented by three contigs constructed from 13 675 reads. Subsequent read-mapping results revealed 53 386 reads matched to the GVF reference genome. Sample 15 was selected and a draft genome constructed from mapped reads. Primer pairs were designed to span the complete genome, which was validated by direct Sanger sequencing. Chapter 5 of this thesis focuses on determining the complete genome sequence of GVF, isolated from sample 15. Interestingly, contigs constructed from *Propionibacterium acnes* were also identified in six samples and comprised almost 1.5% of the total bacterial contigs

generated. This result supports the finding of an interkingdom transfer of bacteria from humans to grapevine (Campisano *et al.,* 2014).

4.4. Conclusion

In this study, the viromes of 17 GLD symptomatic grapevines were investigated. It was determined that there is an average of four different viruses per sample, with rootstock and white-fruited cultivars hosting more diverse populations of viruses (Table 4.3). The most abundant virus was GLRaV-3, particularly GLRaV-3 variant group II representatives, supporting the hypothesis that GLRaV-3 is the primary causative agent of GLD in South Africa (Pietersen, 2004). Through read mapping it was determined that viruses from the family *Closteroviridae* are the most prevalent in GLD-affected vines, comprising up to 31% of the mapped reads, while 0.6% of the total mapped reads originated viruses of the genus *Vitivirus*. Although 76% of the raw data generated was lost through quality trimming and filtering, NGS functioned effectively in identifying grapevine-infecting viruses and was capable of identifying a new isolate of GVF. Further research, with focus on viral populations and associations between virus species, needs to commence to broaden the understanding of GLD etiology and assist in reducing the negative effects of this disease.

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Chapter 5

Detection of *Grapevine virus F* in South African vineyards through *de novo* assemblies

5.1. Introduction

Originally the vitiviruses were classified as members of the family *Flexiviridae* (Adams *et al.,* 2005), based on protein structure phylogenetics as well as morphological and genome similarities to other virions within the family (Martelli *et al.,* 2007). Viruses of the family *Flexiviridae* have recently been assigned into three different families. These are namely *Alphaflexiviridae, Betaflexiviridae* and *Gammaflexiviridae*, and all belong to the order *Tymovirales*. The family *Betaflexiviridae* comprises of viruses belonging to the genera *Foveavirus*, *Trichovirus* and *Vitivirus* (Martelli *et al.,* 2007). Currently, nine distinctive viruses belonging to the genus *Vitivirus* have been identified. They are denoted as *Grapevine virus A* (GVA), *Grapevine virus B* (GVB) (Goszczynski *et al.,* 1996), *Grapevine virus D* (GVD) (Martelli *et al.,* 2007), *Grapevine virus E* (GVE) (Nakaune *et al.,* 2008), *Grapevine virus F* (GVF) (Al Rwahnih, 2012), *Heracleum latent virus* (HLV) (Bem and Murant, 1979), *Mint virus 2* (MV2) (Tzanetakis *et al.,* 2007) and *Actinidia virus A* and *B* (Blouin *et al.,* 2012).

The viral particles of the vitiviruses are flexuous, filamentous and non-enveloped and range between 725-785 nm in length with a diameter of 12 nm (Figure 5.1). The nucleocapsid is cross-banded and diagonally striated (Conti *et al.,* 1980). The vitiviruses have a monopartite, positive-sense single-stranded RNA genome that ranges between 7.3 kb and 7.6 kb (Boccardo and d'Aquilio, 1981). Members of the grapevine infecting vitiviruses (GVA, GVB, GVE and GVF) share between 31% and 49% nucleotide identity in the polymerase-coding gene (ORF1), and between 40% and 70% nucleotide identity in the coat protein gene (ORF4) (Al Rwahnih, 2012). Table 5.1 outlines genome sizes, respective protein molecular masses of the ORFs present in these viruses and the associated accession numbers.

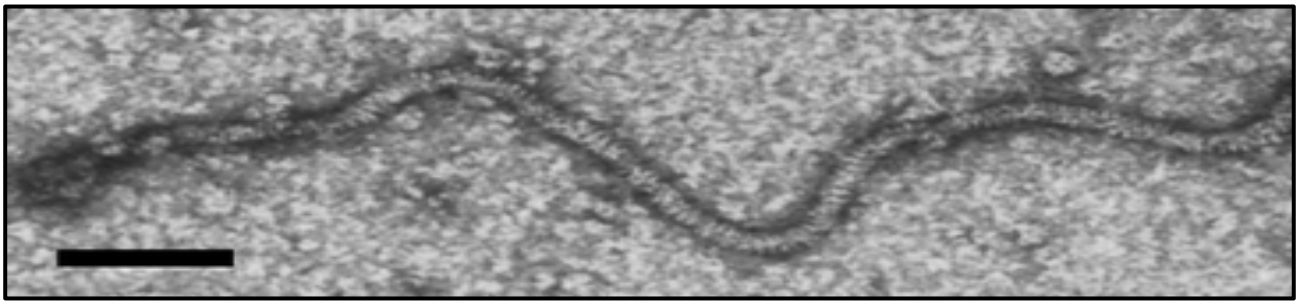

Figure 5.1: Electron micrograph of a GVA virion particle stained in uranyl acetate. The bar positioned on the bottom left corner of the image represents a length of 100 nm (Martelli *et al.,* 2011).

Table 5.1: Genome sizes, sequence accession numbers and protein molecular weights for five members of the genus *Vitivirus*. Adapted from du Preez *et al.* (2011).

Virus:	Genome Size (nt):	Molecular Weights (kDa):					Genome Status:	Accession Number:
		ORF1	ORF2	ORF3	ORF4	ORF5		
GVA	7471	194	19.8	31	21.5	10	Complete	DQ855082.2
GVB	7599	195	20	36.5	21.6	14	Complete	NC_003602.1
GVD	963	-	-	-	17.7	10.5	Partial	Y07764.1
GVE	7571	192.2	21.4	29.2	21.8	12.5	Complete	KF588015.1
GVF	7551	196.9	17.9	30.4	21.7	12.3	Complete	JX105428

The grapevine infecting vitiviruses are common pathological agents of *Vitis vinifera,* which is their natural host. Some have been associated with the Rugose wood complex, a disease associated with pitting and grooving under the bark of grapevines (Martelli, 1993). GVA and GVB are associated with Kober Stem Grooving and corky bark disease, respectively; however, symptoms are usually only visible on grafted vines (Weber *et al.,* 2002). GVE, GVD and GVF have not been proven to cause disease in grapevine. In a majority of instances, the vitiviruses remain in diseased grapevines as latent viruses, their disease symptoms are not well understood. The primary mean of transmission of vitiviruses is through the foregut of insects; namely *Cavariella*, *Heliococcuc*, *Neopulvinaria*, *Ovatus*, *Parthenolecanium*, *Planococcus* and *Pseudococcus* (La Notte *et al.,* 1997; Garau *et al.,* 1995; Engelbrecht and Kasdorf, 1987; Rosciglione *et al.,* 1983). Simultaneous transmission of vitiviruses and GLRaVs has been documented. A study by Hommay *et al.* (2008) identified the simultaneous transmission of GVA and *Grapevine leafroll-associated virus*-1 (GLRaV-1) through the scale insect *Parthenolecanium corni* to *Vitis vinifera*. This supports the notion of transmission interaction between distinctive viruses. Le Maguet *et al.* (2012) supported this concept by identifying *Phenacoccus aceris* as an insect vector for GVA and GVB and the *Ampelovirus* genus. Vitiviruses are common in GLD symptomatic grapevines (Chapter 3 of this thesis); however, their contribution, if any, towards the disease is not well documented or understood. As GLD is considered the most economically devastating grapevine virus disease, it is necessary to identify and understand the relationships between each infectious agent (Maree *et al*., 2013).

NGS provides an accurate and sensitive approach to identify known and novel viruses in diseased plants. With the application of bioinformatics, NGS has been used in the discovery of new genetic variants (Pabinger *et al.,* 2013; Kumar *et al.,* 2012; Marroni *et al.,* 2012; Deschamps and Campbell, 2009); however, it is prone to errors and requires validation. Direct Sanger sequencing identifies

long adjacent DNA sequences using sequence specific primers and is an efficient tool in the validation of novel virus variants detected through NGS (Park *et al.,* 2014).

Little is known about the effect that members of the genus *Vitivirus* have on GLD. Associations between vitiviruses and other grapevine viruses have been reported (La Maguet *et al.,* 2012; Hommay *et al.,* 2008) and found to occur commonly in GLD symptomatic grapevines (Chapter 3 of this thesis). Currently, one complete GVF genome has been reported (Al Rwahnih *et al*., 2012). The following discussion reports on the first discovery of a GVF isolate in South Africa with the use of NGS and the validation of the whole genome with conventional Sanger sequencing.

5.2. Materials and Methods

5.2.1. Plant material

A total of 15.9% of the screened plants tested positive for GVF in the survey discussed in Chapter 3 (Table 3.4). Of the 17 deep sequenced samples, discussed in Chapter 4, 11 showed reads mapping to GVF isolate AUD46129 (JX105428.1) (Table 4.3). Sample V5 (sample number 15) (Table 4.1) was selected for subsequent analysis based on the quantity of read data produced and was attained from the cultivar Chenin blanc in Vredendal, in the Western Cape province, South Africa.

5.2.2. Next-generation sequencing and post-sequencing analysis

Double-stranded RNA (dsRNA) was extracted from 17 grapevine samples and sent for library preparation (Adapted Illumina TruSeq RNA sample preparation protocol) and subjected to next-generation sequencing using either an Illumina HiSeq 2500 or MiSeq platform (Agricultural Research Council Biotechnology Platform, Onderstepoort, RSA). The subsequent process of trimming and filtering to increase the quality of short read datasets is explained in Chapter 4. Reads were mapped to the whole genome sequence of GVF isolate AUD46129 (JX105428.1), using CLC Genomics Workbench 7 (CLC Bio). Mapping parameters included no masking of the reference genome, a global alignment with default settings (mismatch cost: 2; insertion cost: 3; deletion cost: 3; length fraction: 0.7; similarity fraction: 0.7) and random mapping of non-specific matches. A flow diagram illustrating the processing of NGS data can be seen in Figure 4.1. To determine the variability between GVF sequences generated per sample, read datasets were mapped to GVF isolate AUD46129 and consensus sequences extracted with low coverage regions filled with ‘N’ ambiguity symbols. Conflicts were resolved by voting for the best nucleotides, using quality scores. Sample V5 was selected for further analysis based on the quality and variability of sequence data generated. The consensus sequence (draft sequence) from the V5 reads mapping to GVF isolate

AUD46129 (JX105428.1) was used to create a full-length sequence on which 14 primer sets were designed, using Oligo Explorer software 1.1.2, to produce 15 overlapping amplicons for direct Sanger sequencing (Table 5.3).

5.2.3. Total RNA extraction and RT-PCR

A positive control for GVF was obtained from a collection at ARC-Plant Protection Research Institute in Pretoria, South Africa. A total of 200 mg of each sample was pulverized with liquid nitrogen using mortar and pestle and the total RNA extracted using an adapted CTAB (2% [w/v] CTAB, 2.5% [w/v] PVP-40, 100 mM Tris-HCL [pH8], 2 M NaCl, 25 mM EDTA [pH8] and 3% β-mercaptoethanol) method (White *et al*., 2008). Complementary DNA (cDNA) was synthesised by adding 200 ng of total RNA of each sample to 0.3 µl of random hexamers (Promega) and incubating at 65°C for 5 minutes before placing on ice for 2 minutes to complete the primer annealing reaction. A mixture containing 1X AMV RT Buffer (Thermo Scientific), 5 U AMV reverse transcriptase (Thermo Scientific), 10 mM dNTPs (Thermo Scientific) and dH_2O was then added to the 5 µl primer annealing reaction and incubated for 60 minutes at 48°C to produce cDNA.

A PCR mixture containing 1X KAPA *Taq* buffer A (KAPA Biosystems), 10 mM dNTPs (Thermo Scientific), 20 mM forward primer (Integrated DNA Technologies), 20 mM reverse primer (Integrated DNA Technologies), 2 µl 5X cresol, 1 U KAPA *Taq* DNA polymerase (KAPA Biosystems), 2 µl cDNA and dH_2O was used to amplify each of the GVF sequence fragments. The primer pairs had varying PCR cycle conditions (Table 5.2), all included an initial denaturation step at 94°C for 3 minutes and a final extension step at 72°C for 7 minutes.

Table 5.2: PCR primers used for the amplification of the complete genome of GVF isolate V5; included are the primer names, primer sequences (5' to 3' orientation), the PCR product length with each primer used, primer lengths and melting temperatures for each of the primers.

	Primer Number:	Primer Names:	Sequence (5'-3'):	Primer Length (bp):	Amplicon Size (bp):	Tm (°C):
Primer Pairs	1	GVF_F_27-929	CTA TAC GTG CAC AAA ACC TC	20	902	55.7
	2	GVF_R_27-929	TCC TCC GTG ATG GTA GAA C	19		58.4
	3	GVF_F_757-1755	TGG TAC CCT GAC GGA AAA G	19	998	60.9
	4	GVF_R_757-1755	CAT TGC TCG CTC ATC TGT C	19		60.5
	5	GVF_F_1517-2426	TAG CAC CGA TCA CGA GGA TTC	21	909	64.7
	6	GVF_R_1517-2426	GCT CTG TTT GCC TGG ATA CTC	21		61.2
	7	GVF_F_2801-3738	AAG GTC ATA CGG GTA TTC TGC	21	937	60.2
	8	GVF_R_2801-3738	CTC TTC ATA CTC CTC GGT GTT GT	23		62.3
	9	GVF_F_3514-4468	GTA ATC GTG GCA TTT ACT CG	20	954	58.1
	10	GVF_R_3514-4468	GCT CCC TCA ACT TTC TCT C	19		56.4
	11	GVF_F_4243-5118	AAG AGG CTG AAC AAA AGT GC	20	875	59.5
	12	GVF_R_4243-5118	CTG GGC GTC AAT GTC TAT G	19		60.1
	13	GVF_F_4818-5663	CTT TGC TGG GGA TGA TAT GTA CG	23	845	64.7
	14	GVF_R_4818-5663	CCT TCC TTA CTC ACC ACG ATT G	22		63
	15	GVF_F_5400-6221	GTT GGG AGT GGA GGA TCT GTA C	22	821	62.1
	16	GVF_R_5400-6221	TCA GGT CGG CAG TGG ATA TG	20		64.4
	17	GVF_F_6133-6944	CGT GAT TGG GAG TAT GGT TG	20	811	61.2
	18	GVF_R_6133-6944	CTG CGG TTC TTT ATT GCC	18		59.2
	19	GVF_F_5965-6946	TGT GGA GAT GGA GAT AAT GGA C	22	981	61.1
	20	GVF_R_5965-6946	ACC TGC GGT TCT TTA TTG CC	20		63.5
	21	GVF_F_6713-7485	TAA GGT TAG GGG TGT AGA TGC	21	772	58.1
	22	GVF_R_6713-7485	CAC GGG TCA TAT AAC TTC AG	20		55.5
	23	GVF_F_803_1812	ACC CGT GGC TGC TAA CTA CAT C	22	1009	64.7
	24	GVF_R_803_1812	GAC CTC TGA CAC CCT TTC TTG C	22		64.6
	25	GVF_F_2122_4296	AAA TGC TAT CTG CCT GGT TC	20	2174	59.1
	26	GVF_R_2122_4296	ATC GCT CCT TAT GTT GTG C	19		58.7
Terminal Primers	27	GVF-5'_END	GAA AAA TTG ATT TAG TTT CCG AC	23	400	48.8
	28	GVF-3'_END	AAC AGG GGG TAC ACC TG	17	809	54.0

Table 5.3: PCR cycle conditions for each of the primary primer pairs used in the amplification of the complete GVF isolate V5 genome.

Primer Pair:	Initial Denaturation:		Denaturation:		Annealing:		Extension:		Cycles:	Final Extension:	
	Temp. (°C):	Duration (sec):	Temp. (°C):	Duration (sec):	Temp. (°C):	Duration (sec):	Temp. (°C):	Duration (sec):		Temp. (°C):	Duration (sec):
GVF_F_27-929 GVF_R_27-929	94	180	94	30	55	30	72	55	35	72	420
GVF_F_757-1755 GVF_R_757-1755					58			60			
GVF_F_1517-2426 GVF_R_1517-2426					58			55			
GVF_F_2801-3738 GVF_R_2801-3738					58			60			
GVF_F_3514-4468 GVF_R_3514-4468					55			60			
GVF_F_4243-5118 GVF_R_4243-5118					58			55			
GVF_F_4818-5663 GVF_R_4818-5663					60			55			
GVF_F_5400-6221 GVF_R_5400-6221					60			50			
GVF_F_6133-6944 GVF_R_6133-6944					58			50			
GVF_F_5965-6946 GVF_R_5965-6946					58			60			
GVF_F_6713-7485 GVF_R_6713-7485					54			50			
GVF_F_803_1812 GVF_R_803_1812					60			60			
GVF_F_2122_4296 GVF_R_2122_4296					56			120			
GVF-5'_END GVF_R_27-929					50			55			
GVF_F_6713-7485 GVF-3'_END					50			55			

5.2.4. Sanger sequencing and amplicon assembly

Amplified PCR products were visualized under ultraviolet light, excised and purified using a Zymoclean™ Gel DNA Recovery Kit protocol (Zymo research). Purified PCR products were then sent to the Central Analytical Facilities (CAF), at Stellenbosch University, for direct Sanger sequencing. Sequencing results were analyzed in CLC Main Workbench 6.8.4 (CLC Bio) and evaluated for correct base calling before continuing with further analysis. Sequences showing clear base calling were assembled to the GVF isolate V5 draft consensus genome, constructed in CLC Genomics Workbench 7 (CLC Bio). Parameters of the assembly specified that the nucleotide conflicts be resolved by voting based on quality and a minimum read length of 50 bp to be set for assembly with low alignment stringency. The consensus sequence generated through the alignment of PCR amplicons to the GVF isolate V5 draft genome was extracted and named GVF isolate V5 (KP114220).

5.2.5. Genetic variability

Read datasets for 17 samples (Table 4.1) were trimmed of low quality nucleotides and filtered of low quality reads and host genomes. The reads from each dataset were mapped to GVF isolate V5 in CLC Genomics Workbench 7 (CLC Bio). Parameters specified that there should be no masking of the reference genome; alignments were done globally with default settings and non-specific matches be mapped randomly. Consensus sequences for each sample were extracted; regions with low coverage were filled with 'N' ambiguity symbols and conflicts were resolved by voting based on quality scores. Table 5.6 shows the alignment characteristics of the read datasets to GVF isolate V5 and GVF isolate AUD46129. The completed genome sequence of GVF isolate V5 was translated and uploaded into Open Reading Frame Finder (NCBI) to determine the location of and proteins encoded by each of the ORFs present in the genome. The molecular weights of each ORF were determined by taking the average calculation produced through four online websites (encorbio.com/protocols/Prot-MW.htm; expasy.org/compute_pi; protcalc.sourceforge.net/; bioinformatics.org/sms/prot-mw.html). ORFs from GVF isolate V5 and GVF isolate AUD46129 were compared through multiple alignments in CLC Main Workbench 6.8.4 (CLC Bio) on an amino acid and nucleotide level. GVF partial sequences (Supplementary Table S.6), representing the replicase gene of 34 isolates, were downloaded from GenBank and aligned to GVF isolate V5 using CLC Main Workbench 6.8.4 (CLC Bio), to determine the genetic variability between various GVF isolates.

5.3. Results and Discussion

5.3.1. Next-generation sequencing and sample selection

Double-stranded RNA was extracted from 17 GLD symptomatic grapevines (Table 4.1) and sequenced on an Illumina sequencing platform (ARC-BP). Read datasets, for each sample, were trimmed and filtered to improve the quality of the read data (Figure 4.1 and Figure 4.2) and mapped to GVF isolate AUD46129 (JX105428.1). Of the 17 samples that had undergone NGS, 15 were found to contain traces of GVF (Table 5.4). Samples 13 and 15 were found to contain the highest number of reads mapping to GVF isolate AUD46129 with approximately 26 000 and 20 000 reads, respectively. Furthermore, samples 13 and 15 were both found to cover an excess of 90% of the GVF reference genome with an approximated average coverage of 4.5 times more than any other sample (Table 5.4).

Consensus sequences were extracted from each mapping to give full draft genomes with low coverage areas filled with 'N' ambiguity symbols. A multiple alignment of the extracted consensus sequences revealed that samples 13 and 15 share 88.17% and 90.15% nucleotide identities to GVF isolate AUD46129, respectively (Table 5.4).

Table 5.4: Mapping and alignment characteristics of NGS reads to reference genome GVF isolate AUD46129 (JX105428.1). Nucleotide and read count, mean read length, fraction of the reference covered and average coverage are displayed for read-mapping characteristics. Percentage identity, determined through multiple alignments, for each sample to GVF isolate AUD46129 is shown.

Sample:		Mapping Characteristics:					Alignment Characteristcs:
Number:	Name:	Nucleotide Count:	Read Count:	Mean Read Length:	Fraction of Ref Covered:	Average Coverage:	Identity (%):
1	1491	14132	273	51.77	0.5	1.81	73.72
2	271614	7354	140	52.53	0.44	0.97	74.90
3	383631	-	-	-	-	-	-
4	49STOK	315449	4766	66.19	0.48	41.79	87.32
5	BJ3	6680	128	52.19	0.32	0.86	76.35
6	BJ4	32230	464	69.46	0.28	4.23	80.57
7	GH23	516	6	86.00	0.03	0.06	87.60
8	GH24	11737	206	56.98	0.31	1.49	75.55
9	H35	1112	11	101.09	0.11	0.14	84.81
10	H36	3072	48	64.00	0.27	0.40	78.07
11	H38	3096	42	73.71	0.09	0.41	85.69
12	V1	3962	55	72.04	0.13	0.51	85.94
13	V3	1837700	26376	69.67	0.91	240.88	88.17
14	V4R	-	-	-	-	-	-
15	V5	1413208	20146	70.15	0.90	186.86	90.15
16	V6	12051	194	62.12	0.49	1.56	71.12
17	161514	27679	531	52.13	0.77	3.58	69.07

The fraction of the reference genome covered is proportional to the amount of data produced (read count) (Table 5.4) and is an indication of GVF titre. The percentage identity of aligned consensus sequences to GVF isolate AUD46129 is an indication of the variability of each sample to the reference. Based on the values generated for fraction of reference covered and percentage identity to reference genome (GVF isolate AUD46129), samples 13 and 15 were selected as the final candidates in selecting a draft genome for further RT-PCR analysis. Sample 15 was selected as the

final sample for subsequent analysis based on a lower level of virus infection, which may potentially assist in understanding the effects of GVF infection in grapevine.

5.3.2. RT-PCR and Sanger sequencing

In total, 28 primers (Table 5.3) were designed to cover the complete GVF isolate V5 NGS-generated draft genome in 14 overlapping amplicons under varying PCR conditions (Table 5.2). PCR primers were used in combination (Table 5.3) to amplify regions ranging between 772 and 2 174 nucleotides of the complete GVF genome. Additional primer combinations were used to increase the coverage and accuracy of the sequences aligning to GVF isolate V5. In total 82 sequences were assembled to the GVF isolate V5 draft genome. The complete genome spanned 7 539 nucleotides with polyadenylation at the 3'-end that was determined with the 3'-end primer (Table 5.3, primer number 28). The genome sequence was extracted and named GVF isolate V5 (KP114220).

5.3.3. Genetic variability

High-quality reads from 17 read datasets were mapped to GVF isolate V5 (Table 5.5). In total, 3.16 million nucleotides, amounting to 45 417 reads, were mapped to the GVF isolate V5 genome with a 27.8X average coverage. An average of 40.5% of the GVF isolate V5 genome was covered over the 15 samples that had between 67.66% and 100% nucleotide identities in multiple alignments created with each consensus. Based on calculations of the quality and quantity of data produced, it is evident that high levels of variation exist between GVF in samples. Reads from sample 13 covered 98% of the GVF isolate V5 genome and shared 86.92% nucleotide identity. Reference genome GVF isolate AUD46129 (JX105428.1) covered the entire length of GVF isolate V5 and shared 88.96% nucleotide identity. In a multiple alignment of a 287 nucleotide region of the replicase gene of 35 GVF representatives (Supplementary Table S.6), GVF isolates shared between 79.44% and 100% nucleotide identity and an average of 90.28% nucleotide identity to GVF isolate V5. In a read-mapping comparison of consensus sequences created from GVF isolate V5 and AUD46129 (Table 5.6) it was determined that a greater number of total reads were mapped to GVF isolate AUD46129, with a total of 53 386 reads compared to 45 416 reads mapping to GVF isolate V5. A greater average percentage of the reference genome covered, across all GVF positive samples, was seen with GVF isolate V5 with 40.46% of the reference covered (Table 5.6).

Table 5.5: Comparison of the next-generation read mapping characteristics of each GVF positive sample to GVF isolate AUD46129 and GVF isolate V5. Outlined is the read count of mapped reads for each sample as well as the fraction of the GVF isolates covered.

Sample:		Read Count:		Fraction of Ref Covered:	
Number:	Name:	AUD46129	V5	AUD42169	V5
1	1491	273	389	50	55
2	271614	140	92	44	32
4	49STOK	4766	831	48	56
5	BJ3	128	108	32	29
6	BJ4	464	481	28	31
7	GH23	6	6	3	4
8	GH24	206	80	31	23
9	H35	11	5	11	7
10	H36	48	52	27	30
11	H38	42	9	9	12
12	V1	55	59	13	14
13	V3	26376	22588	91	98
15	V5	20146	20084	90	98
16	V6	194	178	49	50
17	161514	531	454	77	68

Table 5.6: Mapping and alignment characteristics of NGS reads and GVF isolate AUD46129 to reference genome GVF isolate V5 (KP114220). Nucleotide and read count, mean read length, fraction of the reference covered and average coverage are displayed for read-mapping characteristics. Percentage identity and a calculated variation quality for each sample to GVF isolate V5 are outlined under alignment characteristics.

Sample:		Mapping Characteristics:					Alignment Characteristcs:
Number:	Name:	Nucleotide Count:	Read Count:	Mean Read Length:	Fraction of Ref Covered:	Average Coverage:	Identity (%):
1	1491	19772	389	50.83	0.55	2.5	71.53
2	271614	4955	92	53.86	0.32	0.64	73.74
3	383631	-	-	-	-	-	-
4	49STOK	55906	831	67.28	0.56	7.33	85.34
5	BJ3	5733	108	53.08	0.29	0.73	78.08
6	BJ4	33492	481	69.63	0.31	4.39	79.80
7	GH23	516	6	86	0.04	0.07	85.45
8	GH24	4531	80	56.64	0.23	0.59	76.78
9	H35	592	5	118.40	0.07	0.08	84.97
10	H36	3328	52	64.00	0.3	0.42	74.55
11	H38	1256	9	139.56	0.12	0.17	84.76
12	V1	4250	59	72.03	0.14	0.56	86.51
13	V3	1573646	22588	69.67	0.98	208.2	86.92
14	V4R	-	-	-	-	-	-
15	V5	1412654	20084	70.34	0.98	187.19	100
16	V6	11183	178	62.83	0.5	1.46	70.14
17	161514	23842	454	52.52	0.68	3.09	71.31
JX105428	GVF isolate AUD46129	7551	1	7,551	1	1	88.96

There is significant genetic variation between GVF isolates. GVF isolate V5 shares 88.96% nucleotide identities to the sole complete genome reference on GenBank records (JX105428.1) and 87.94% nucleotide identity to the near complete GVF sequence generated for sample 13. In a multiple alignment of partial replicase genes of 35 GVF representatives, between 77.94% and 100% nucleotide identities were identified. The comparisons of consensus sequences generated through GVF isolate V5 and GVF isolate AUD46129 (Table 5.6) further demonstrates the variation between potential GVF isolates. The variable number of reads aligning to each genome and the fraction of

reference covered indicates a high level of dissimilarity between GVF species in each sample. Taking into account the quantity of data generated (read count), the greater percentage of reference covered illustrates the similarity of the sample specific GVF isolate to each reference isolate. It is evident that GVF isolate V5 is genetically variable from GVF isolate AUD46129, and that other potential GVF isolates, located in South African vineyards, may have greater genetic variability to known GVF isolates.

The complete genome sequence of GVF isolate V5 was translated and ORFs determined through ORF Finder (NCBI). It was determined that GVF isolate V5 contains five ORFs that share a similar genome organization to other *Vitivirus* species (du Preez *et al.,* 2011). Through the comparison to existing characterized genes, it was determined that ORF1 encoded a replicase gene that contains methyltransferase, AlkB, Viral helicase and RNA-dependent RNA polymerase conserved domains. ORFs 2 to 5 encoded a hypothetical hydrophobic protein, a viral movement protein, a coat protein and RNA-binding protein domains, respectively. The average molecular weight, of four separate calculations (Table 5.7), for each ORF was computed. ORF 1 to 5, of GVF isolate V5, shared 94.79%, 87.50%, 94.12%, 98.99% and 94.55% amino acid identity and 87.09%, 85.42%, 91.79%, 94.30% and 94.85% nucleotide identity to the GVF isolate AUD46129 ORFs, respectively. The average amino acid similarity of all ORFs was calculated as 93.99%, indicating that ORFs within GVF isolate are highly conserved, especially the viral coat protein encoded by ORF4.

A multiple alignment of the amino acids comprising the full replicase gene of *Vitivirus* representatives confirmed the phylogenetic relationship between members of the *Vitivirus* genus (Figure 5.2.B). Figure 5.2.B indicates the relationship between GVA and GVF, where their evolutionary progress is more uniform to that of GVE and then GVB and GVD. This may be due to the prevalence of these viruses in certain diseases and the association that vitiviruses may share with one another during infection and transmission (La Maguet *et al.,* 2012; Hommay *et al.,* 2008). Little is known about the etiology of GLD and other grapevines diseases. Further research of these diseases will be beneficial in gaining knowledge into the physiology and progression of grapevine infecting viruses.

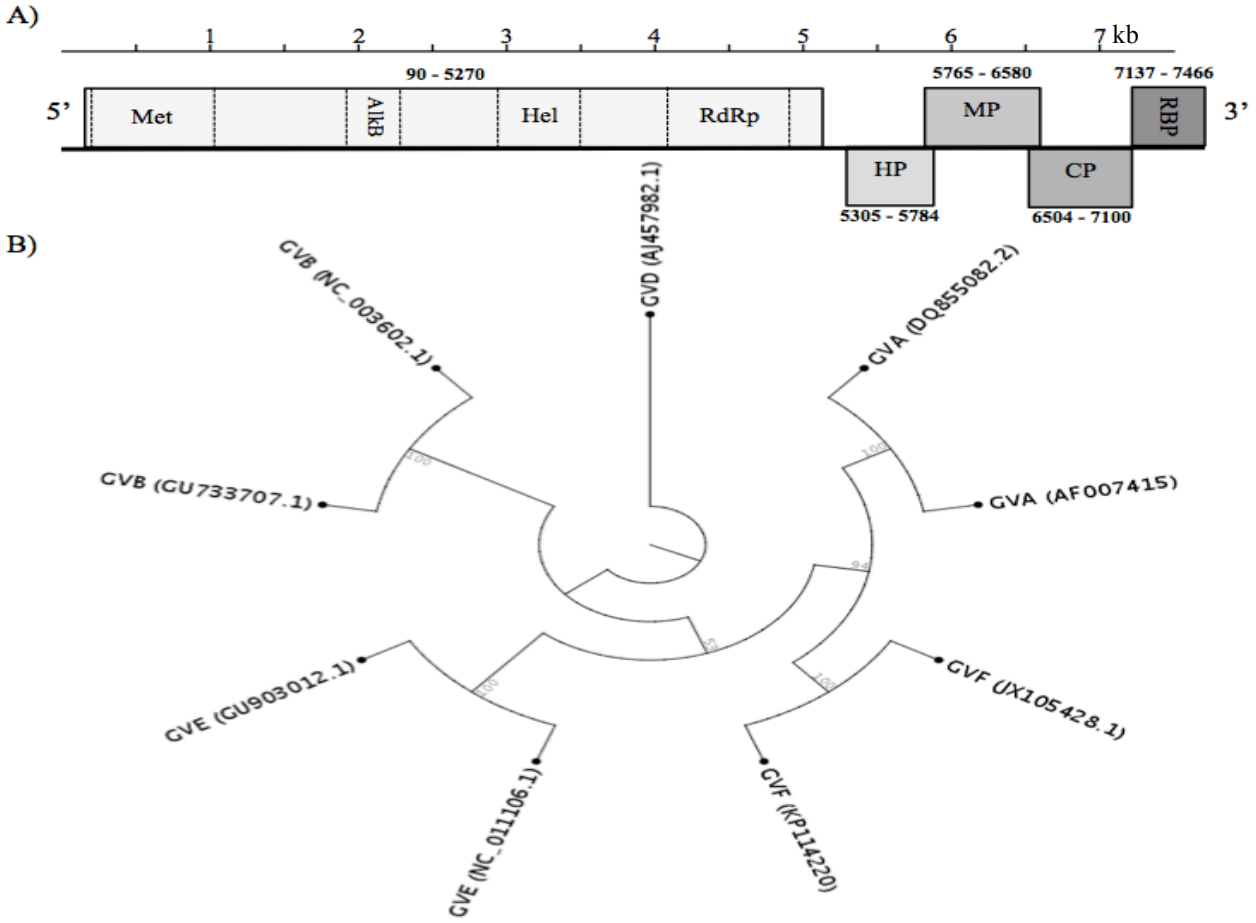

Figure 5.2: Schematic representations of the scaled size and organization of the GVF isolate V5 genome and the phylogenetic relationship shared between *vitiviruses*. A) Relative sizes drawn to scale, and protein domains encoded by ORFs present in the GVF isolate V5 genome. ORF1 encodes the conserved domains of methyltransferase (Met), a member of the Fe (II)-oxoglutarate-dependent dioxygenase superfamily (AlkB), RNA Helicase1 (Hel) and RNA-dependent RNA polymerase (RdRp). ORF2 to ORF5 encode a hypothetical protein (HP), movement protein (MP), coat protein (CP) and RNA-binding protein (RBP), respectively. B) Circular cladogram, of an unrooted tree of *Vitiviruses* using the neighbor-joining algorithm and a bootstrap replicate of 1000 for full length replicase genes, with the exception of the GVD representative. Values on branches represent bootstrap percentages.

Table 5.7: Molecular weights and amino acid numbers of the five open reading frames present in the GVF isolate V5 genome. Molecular weight was calculated as an average from calculations taken from four online calculators, namely: web.expasy.org/cgi-bin/compute_pi/pi_tool (1), bioinformatics.org/sms/prot_mw.html (2), encorbio.com/protocols/Prot-MW.htm (3) and protcalc.sourceforge.net/cgi-bin/protcalc (4).

ORF:	Amino Acids:	Molecular Weights (kDa):				
		1	2	3	4	Average:
1	1727	197.05	197.07	197.05	197.05	197.06
2	160	17.80	17.80	17.80	17.80	17.80
3	272	30.33	30.33	30.33	30.33	30.33
4	199	21.71	21.71	21.71	21.71	21.71
5	110	12.47	12.48	12.49	12.47	12.48

5.4. Conclusion

In this chapter we report on the identification of a new GVF isolate, namely V5 (KP114220), found in the Western Cape province, South Africa. The complete genome spans 7 539 nucleotides with polyadenylation at the 3'-end. The genome shares 88.96% nucleotide similarity to existing complete genome sequences in GenBank records and between 87.50% and 98.99% amino acid

identities in the five encoded protein domains. NGS was effective in initially detecting GVF within sampled grapevines and in determining the prevalence and degree of infection within each sample. Direct Sanger sequencing was used to finalize the genome by filling in gaps of missing read data and to validate the sequences generated through NGS. Multiple alignments of replicase genes and complete consensus sequences determined that a number of genetically variable GVF isolates exist in South African vineyards and vineyards around the world. Further research in the detection and characterization of GVF and other vitivirus species may aid in understanding the effect that these viruses play in grapevine diseases. Further research may assist in establishing an association between different virus types that could potentially reduce the negative effects of grapevine diseases.

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Chapter 6

Conclusion

6.1. Conclusion and future prospects

Grapevine-infecting viruses pose a great threat to the global viticulture industry. Grapevine leafroll disease (GLD) is the most widespread of the grapevine diseases and is economically the most devastating. Present research into GLD is mostly centered on epidemiology and the development of viral diagnostic techniques. Viruses from the families *Closteroviridae* and *Betaflexiviridae* are commonly detected in GLD vines, however, *Grapevine leafroll-associated virus* 3 (GLRaV-3) is the primary contributing agent to the disease. Molecular techniques such as PCR (Mullis *et al.,* 1986) and Sanger sequencing (Sanger *et al.,* 1977) function efficiently in detecting known viruses but are time consuming and costly for large scale experiments. The development of next-generation sequencing (NGS), in 2005, revolutionized molecular biology and viral diagnostics. It is capable of producing large amounts of data in a relatively short period of time and is able to detect novel viruses and variants of viruses. Metagenomics coupled with NGS has provided the ability to identify the virome of an environmental sample, such as a grapevine. It also provides an unbiased technique in identifying all dsRNA species, including those with low titre or novel. This research study focused on identifying the viral populations in GLD vines through RT-PCR and NGS and determining the efficiency of NGS as a diagnostic tool for plant viral diseases. The sequencing of a South African isolate of *Grapevine virus F* (GVF) is also described.

A widespread survey of GLD-affected grapevines was performed in the Western Cape province, South Africa. Virus specific primers were designed to screen 11 viruses using RT-PCR. GLRaV-3 variant groups were distinguished with high-resolution melt (HRM) curve analysis coupled with real-time RT-PCR. Closteroviruses that were detected comprised of 41% of all identified viruses. Viruses from the family *Betaflexiviridae* comprised the remaining 59% of detected viruses. The most frequently identified viruses were from the genus *Ampelovirus*; GLRaV-3 variant group II contributing 38.48% to the combined GLRaV-3 population and GLRaV-3 variant group VI contributing 37.76%. No differences were evident in the number of viruses infecting red- and white-fruited cultivars or in grapevines of varying age.

GLD-affected grapevines were selected based on symptomology; however, not all grapevines displayed clear visual symptomology, particularly in certain white-fruited cultivars. A high incidence of GLRaV-3 was detected throughout the sample set, confirming previous reports that GLRaV-3 is the primary causative agent of GLD in South African vineyards (Pietersen, 2004).

Further research on the viral complexes within GLD-affected grapevines is recommended, especially in white-fruited cultivars where symptomology is masked in a majority of infected grapevines.

Seventeen GLD-affected grapevines were selected for NGS, comprising seven red-, six white-fruited cultivar and four rootstock plants. Either the Illumina MiSeq or Hiseq 2500 NGS platforms were used and generated in excess of 190 million reads. After trimming and filtering for quality and *Vitis vinifera* sequences, 52.8% of the total reads were assembled into contigs through *de novo* assemblies and mapped to grapevine-infecting viruses and viroids. GLRaV-3 was the best represented virus detected through *de novo* assemblies, comprising 97.5% of the analyzed contigs, followed by *Grapevine rupestris stem pitting-associated virus* (GRSPaV) and *Grapevine virus E* (GVE). GLRaV-3 variant group I, representing 39.3% of the total assembled contigs, was found to be the best represented variant followed by GLRaV-3 variant group II viruses, representing 30.9% of the total contigs analyzed. Read-mappings revealed similar results, where each NGS sample was infected with GLRV-3 variant group II, confirming previous studies that indicated high occurrence of GLRaV-3 variant group II in South African vineyards (Jooste *et al*., 2011; Coetzee *et al*., 2010; Jooste *et al*., 2010). On average, four viruses were detected in each sample with rootstock vines and white-fruited cultivars hosting larger populations of viruses compared to red-fruited cultivars. Two red-fruited cultivar samples were identified with a low coverage of GLRaV-3, indicating that a low titre of infection is adequate for GLD symptom expression. This study further confirms the hypothesis that GLRaV-3 is the main causative agent of GLD and states that NGS functions as an efficient tool in the detection of grapevine-infecting viruses.

A widespread survey detected *Grapevine virus F* (GVF) in 16% of the sampled grapevines. Further studies identified GVF in 15 of 17 deep-sequenced samples through read-mapping to a GVF reference (Chapter 5 of this thesis). One sample, namely V5, was selected for subsequent sequencing based on the amount of data generated and the fraction of the reference covered. A draft genome was created and primers designed to span the complete GVF genome. Through direct sequencing the complete genome sequence was identified and validated. The GVF isolate V5 genome encoded five open reading frames (ORFs) that share a similar genome organization to other vitiviruses. The complete genome spanned 7 539 nucleotides with polyadenyltation at the 3' end and shared 89.11% nucleotide identity to existing GVF genomes. A multiple alignment of partial replicase genes of 35 GVF representatives revealed that isolates shared between 74.99% and 100% nucleotide identity to GVF isolate V5. This is the first report of a complete GVF genome in South African vineyards and the second globally. Further research into the latency of GVF in GLD-affected vines and the potential association that GVF shares with other grapevine-infecting viruses

needs to take place to fully understand the etiology of grapevine diseases and the role of GVF in diseased vines.

This study furthered the understanding of GLD etiology and supports the hypothesis that GLRaV-3 is the primary causative agent of the disease. Furthermore, the complex of viruses affecting GLD symptomatic grapevine was investigated and indicated that large populations of viruses exist within GLD-affected vines. Their role in the onset and expression of GLD symptoms needs further investigation. We found NGS to be an efficient tool in detecting various virus types and determining the prevalence of viruses within each sample. Here we define our research through expanding the knowledge of GLD etiology by investigating the populations of viruses in GLD-affected grapevines and identifying a new isolate of GVF found in GLD-affected vines.

Future research should investigate the complex of viruses affecting GLD vines, particularly those in white-fruited cultivars. Performing NGS on a larger sample size would be most beneficial in such investigations. The role of latent viruses in disease association and the transmission of these viruses across grapevine hosts would be of interest. Research into pathogen interactions should be considered with regard to disease symptom expression and the reduction of the negative effects of virus infection. Correct management of *Vitis vinifera* vineyards is important in maintaining virus infection and helping to establish a sustainable virus population for future virus association and evolution studies.

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Supplementary data

Table S.1: List of available bioinformatic tools with the corresponding compatible platform and Internet sources for additional information. Adapted from Zang *et al.* (2011).

Program Name:	Platform:	Website (URL):
De novo assembly		
Abyss	Illumina	http://www.bcgsc.ca/platform/bioinfo/software/abyss
ALLPATHS	Illumina	http://www.broadinstitute.org/science/programs/genome-biology/crd
AMOScmp	Roche	http://sourceforge.net/projects/amos/files/
ARACHNE	Roche	http://www.broadinstitute.org/science/programs/genome-biology/crd
CAP3	Roche	http://pbil.univ-lyon1.fr/cap3.php
Consensus/SeqCons	Roche	http://www.seqan.de/downloads/projects.html
Curtain	Illumina/Roche/ABI	http://code.google.com/p/curtain/
Edena	Illumina	http://www.genomic.ch/edena
Euler-SR	Illumina/Roche	http://euler-assembler.ucsd.edu/portal/?q=team
FuzzyPath	Illumina/Roche	ftp://ftp.sanger.ac.uk/pub/zn1/fuzzypath/fuzzypath_v3.0.tgz
IDBA	Illumina	http://www.cs.hku.hk/~alse/idba/
MIRA/MIRA3	Illumina/Roche	http://chevreux.org/projects_mira.html
Newbler	Roche	roche-applied-science.com/
Phrap	Illumina/Roche	http://www.phrap.org/consed/consed.html#howToGet
RGA	Illumina	http://rga.cgrb.oregonstate.edu/
QSRA	Illumina	http://qsra.cgrb.oregonstate.edu/
SHARCGS	Illumina	http://sharcgs.molgen.mpg.de/
SHORTY	ABI	http://www.cs.sunysb.edu/~skiena/shorty/
SOAPdenovo	Illumina	http://soap.genomics.org.cn
SOPRA	Illumina/ABI	http://www.physics.rutgers.edu/%7Eanirvans/SOPRA/
SR-ASM	Roche	http://bioserver.cs.put.poznan.pl/sr-asm-short-reads-assembly-algorithm
SSAKE	Illumina/Roche	http://www.bcgsc.ca/platform/bioinfo/software/ssake
Taipan	Illumina	http://sourceforge.net/projects/taipan/files/
VCAKE	Illumina/Roche	http://sourceforge.net/projects/vcake
Velvet	Illumina/Roche/ABI	http://www.ebi.ac.uk/%7Ezerbino/velvet

Read-mapping

BFAST	Illumina/ABI	http://sourceforge.net/apps/mediawiki/bfast/index.php?title=Main_Page
Bowtie	Illumina/Roche/ABI	http://bowtie-bio.sourceforge.net
BWA	Illumina/ABI	http://bio-bwa.sourceforge.net/bwa.shtml
CoronaLite	ABI	http://solidsoftwaretools.com/gf/project/corona/
CABOG	Roche/ABI	http://wgs-assembler.sf.net
ELAND/ELAND2	Illumina/ABI	http://www.illumina.com/
EULER	Illumina	http://euler-assembler.ucsd.edu/portal/
Exonerate	Roche	http://www.ebi.ac.uk/~guy/exonerate
EMBF	Illumina	http://www.biomedcentral.com/1471-2105/10?issue=S1
GenomeMapper	Illumina	http://1001genomes.org/downloads/genomemapper.html
GMAP	Illumina	http://www.gene.com/share/gmap
gnumap	Illumina	http://dna.cs.byu.edu/gnumap/
ICON	Illumina	http://icorn.sourceforge.net/
Karma	Illumina/ABI	http://www.sph.umich.edu/csg/pha/karma/
LAST	Illumina	http://last.cbrc.jp/
LOCAS	Illumina	http://www-ab.informatik.uni-tuebingen.de/software/locas
Mapreads	ABI	http://solidsoftwaretools.com/gf/project/mapreads/
MAQ	Illumina/ABI	http://maq.sourceforge.net
MOM	Illumina	http://mom.csbc.vcu.edu/
Mosaik	Illumina/Roche/ABI	http://bioinformatics.bc.edu/marthlab/Mosaik
mrFAST/mrsFAST	Illumina	http://mrfast.sourceforge.net/
MUMer	ABI	http://mummer.sourceforge.net/
Nexalign	Illumina	http://genome.gsc.riken.jp/osc/english/dataresource/
Novocraft	Illumina	http://www.novocraft.com/
PerM	Illumina/ABI	http://code.google.com/p/perm/
RazerS	Illumina/ABI	http://www.seqan.de/projects/razers.html
RMAP	Illumina	http://rulai.cshl.edu/rmap
segemehl	Illumina/Roche	http://www.bioinf.uni-leipzig.de/Software/segemehl/
SeqCons	Roche	http://www.seqan.de/projects/seqcons.html
SeqMap	Illumina	http://biogibbs.stanford.edu/*jiangh/SeqMap/

SHRiMP	Illumina/Roche/ABI	http://compbio.cs.toronto.edu/shrimp
Slider/SliderII	Illumina	http://www.bcgsc.ca/platform/bioinfo/software/slider
SOCS	ABI	http://solidsoftwaretools.com/gf/project/socs/
SOAP/SOAP2	Illumina/ABI	http://soap.genomics.org.cn
SSAHA/SSAHA2	Illumina/Roche	http://www.sanger.ac.uk/Software/analysis/SSAHA2
Stampy	Illumina	http://www.well.ox.ac.uk/~marting/
SXOligoSearch	Illumina	http://synasite.mgrc.com.my:8080/sxog/NewSXOligoSearch.php
SHORE	Illumina	http://1001genomes.org/downloads/shore.html
Vmatch	Illumina	http://www.vmatch.de/
Visualization		
FastQC	Illumina/ABI	http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/
PIQA	Illumina	http://bioinfo.uh.edu/PIQA
ShortRead	Illumina/Roche	http://bioconductor.org/packages/2.6/bioc/html/ShortRead.html
TileQC	Illumina	http://www.science.oregonstate.edu/~dolanp/tileqc

Table S.2: *Vitis vinifera* sequences retrieved from GenBank databases, used to filter host genome sequences from read datasets.

Reference Description:	Accession Number:
Vitis vinifera chromosome 1	NC_012007
Vitis vinifera chromosome 2	NC_012008
Vitis vinifera chromosome 3	NC_012009
Vitis vinifera chromosome 4	NC_012010
Vitis vinifera chromosome 5	NC_012011
Vitis vinifera chromosome 6	NC_012012
Vitis vinifera chromosome 7	NC_012013
Vitis vinifera chromosome 8	NC_012014
Vitis vinifera chromosome 9	NC_012015
Vitis vinifera chromosome 10	NC_012016
Vitis vinifera chromosome 11	NC_012017
Vitis vinifera chromosome 12	NC_012018
Vitis vinifera chromosome 13	NC_012019
Vitis vinifera chromosome 14	NC_012020
Vitis vinifera chromosome 15	NC_012021
Vitis vinifera chromosome 16	NC_012022
Vitis vinifera chromosome 17	NC_012023
Vitis vinifera chromosome 18	NC_012024
Vitis vinifera chromosome 19	NC_012025
Vitis vinifera mitochondrion genome	NC_012119
Vitis vinifera chloroplast genome	NC_007957

Table S.3: Organisms with corresponding Family and GenBank accession number. Used as reference genomes in read-mappings. Adapted from Martelli (2014).

Organism:	**Family:**	**Accession Number:**
Grapevine Fleck Virus	*Tymoviridae*	NC_003347.1
Tomato Black Ring Virus	*Secoviridae*	NC_004439.1
Blueberry Latent Virus	*Partitiviridae*	NC_014593.1
Arabis Mosaic Virus	*Secoviridae*	NC_001495.1
Raspberry Bushy Dwarf Virus	*Idaeovirus**	NC_003739.1
Grapevine Pinot gris Virus	*Betaflexiviridae*	NC_015782.1
Strawberry Latent Ringspot Virus	*Secoviridae*	NC_006964.1
Grapevine Rupestris Stem Pitting-associated Virus	*Betaflexiviridae*	AF026278.1
Cucumber Mosaic Virus	*Bromoviridae*	FJ268744.1
Tobacco Necrosis Virus A	*Tombusviridae*	AY546104.1
Artichoke Italian Latent Virus	*Secoviridae*	X87254.1
Tomato Spotted Wilt Virus	*Bunyaviridae*	JF960237.1
Tobacco Ringspot Virus	*Secoviridae*	U50869.1
Grapevine Bulgarian Latent Virus	*Secoviridae*	NC_015492.1
Blueberry Leaf Mottle Virus	*Secoviridae*	U20621.1
Diaporthe Ambigua Virus 1	*Sordariomycetidae*	AF142094.1
Tomato Ringspot Virus	*Secoviridae*	NC_003840.1
Cherry Leafroll Virus	*Secoviridae*	NC_015414.1
Grapevine Vein Clearing Virus	*Caulimoviridae*	NC_015784.2
Grapevine Rupestris Vein Feathering Virus	*Tymoviridae*	AY706994.1
Peach Rosette Mosaic Virus	*Secoviridae*	AF016626.1
Carnation Mottle Virus	*Tombusviridae*	NC_001265.1
Sowbane Mosaic Virus	*Sobemovirus*	HM163159.1
Grapevine Algerian Latent Virus	*Tombusviridae*	NC_011535.1
Grapevine Deformation Virus	*Secoviridae*	NC_017939.1
Tobacco Necrosis Virus D	*Tombusviridae*	NC_003487.1
Grapevine Red Blotch associated Virus	*Geminiviridae*	NC_022002.1
Grapevine Angular Mosaic Virus	*Bromoviridae*	AY590305.1
Grapevine Syrah Virus	*Tymoviridae*	NC_012484.1
Heracleum Latent Virus	*Betaflexiviridae*	X79270.1
Raspberry Ringspot Virus	*Secoviridae*	NC_005266.1
Petunia Asteroid Mosaic Virus	*Tombusviridae*	AY500881.1
Tomato Mosaic Virus	*Virgaviridae*	AF155507.2
Grapevine Fanleaf Virus	*Secoviridae*	KC900162.1
Alfalfa Mosaic Virus	*Bromoviridae*	NC_001495.1
Broad Bean Wilt Virus1	*Comoviridae*	NC_005290.1
Broad Bean Wilt Virus2	*Comoviridae*	EF528584.1
Grapevine Berry Inner Necrosis Virus	*Tymoviridae*	NC_015220.1
Hungarian Chrome Mosaic Virus	*Secoviridae*	X15346.1
Grapevine Red Globe Virus	*Tymoviridae*	AF521977.1
Grapevine-associated Narnavirus 1	*Narnaviridae*	GU108590.1
Grapevine associated Narnavirus-1 Ctg157	*Narnaviridae*	GU108586.1
Botryotinia Fuckeliana Partitivirus Seg1	*Partitiviridae*	AM491609.1

Botryotinia Fuckeliana Partitivirus Seg2	*Partitiviridae*	AM491610.1
Botryotinia Fuckeliana Partitivirus Seg3	*Partitiviridae*	AM491611.1
Ophiostoma Quercus Partitivirus	*Partitiviridae*	AM111099.1
Australian Grapevine Viroid	*Pospiviroidae*	NC_003553.1
Grapevine Yellow Speckled Viroid 1	*Pospiviroidae*	NC_001920.1
Grapevine Yellow Speckled Viroid 2	*Pospiviroidae*	FJ597947.1
Citrus Exocortis Viroid	*Pospiviroidae*	S67442.1
Hop Stunt Viroid	*Pospiviroidae*	NC_001351.1
Grapevine Viroid Clone Syrah	*Pospiviroidae*	AF462154
Grapevine Viroid Clone Cari2	*Pospiviroidae*	AF462155
Grapevine Endophyte Endornavirus	*Endornaviridae*	NC_019493.1
Chalara Endornavirus	*Endornaviridae*	GQ494150.1
Grapevine-associated Mycovirus 1	*unclassified*	GU108587.1
Grapevine-associated Mycovirus 2	*unclassified*	GU108600.1
Grapevine-associated Mycovirus 3	*unclassified*	GU108601.1
Grapevine Virus A (92/778)	*Betaflexiviridae*	AF441234.2
Grapevine Virus A (JP98)	*Betaflexiviridae*	AF441235.2
Grapevine Virus A (P163)	*Betaflexiviridae*	DQ855088.1
Grapevine Virus F	*Betaflexiviridae*	JX105428
Grapevine Virus D	*Betaflexiviridae*	YO7764.1
Grapevine Virus E	*Betaflexiviridae*	NC_011106.1
Grapevine Virus E (SA94)	*Betaflexiviridae*	GU903012.1
Grapevine Virus B	*Betaflexiviridae*	NC_003602.1
Rosellinia Necatrix Megabirnavirus 1	*Megabirnavirus*	AB512282.1
Rosellinia Necatrix Megabirnavirus 2	*Megabirnavirus*	AB512283.1
Discula Destructiva Virus 1 seg 1	*Partitiviridae*	AB316992.1
Discula Destructiva Virus 1 seg 2	*Partitiviridae*	AF316993.1
Discula Destructiva Virus 1 seg 3	*Partitiviridae*	AF316994.2
Discula Destructiva Virus 1 seg 4	*Partitiviridae*	AF316995.1
Discula Destructiva Virus 2 seg 1	*Partitiviridae*	AY033436.1
Discula Destructiva Virus 2 seg 2	*Partitiviridae*	AY033437.1
Fragaria Chiloensis Cryptic Virus	*Partitiviridae*	NC_009519.1
Raphanus Sativus Cryptic Virus 3 seg 1	*Partitiviridae*	FJ461349.1
Raphanus Sativus Cryptic Virus 3 seg 2	*Partitiviridae*	FJ461350.1
Rosa Multiflora Cryptiv Virus seg 1	*Partitiviridae*	EU024675.1
Rosa Multiflora Cryptiv Virus seg 2	*Partitiviridae*	EU024676.1
Rosa Multiflora Cryptiv Virus seg 3	*Partitiviridae*	EU024677.1
Vicia Cryptic Virus	*Partitiviridae*	EU371896.1
Black Raspberry Cryptic Virus (CRUB)	*Partitiviridae*	EU082132.1
Grapevine-associated Chrysovirus 1	*Chyrsoviridae*	GU108588.1
Grapevine-associated Chrysovirus 2	*Chyrsoviridae*	GU108589.1
Grapevine-associated Chrysovirus 3	*Chyrsoviridae*	GU108596.1
Grapevine-associated Chrysovirus 4	*Chyrsoviridae*	GU108597.1
Cryphonectria Nitschkei Chrysovirus 1	*Chyrsoviridae*	GQ290652.1
Verticillium Chyrsovirus 1 seg 1	*Chyrsoviridae*	HM004067.2
Verticillium Chyrsovirus 1 seg 2	*Chyrsoviridae*	HM004068.2
Verticillium Chyrsovirus 1 seg 3	*Chyrsoviridae*	HM004069.2
Verticillium Chyrsovirus 1 seg 4	*Chyrsoviridae*	HM004070.2

Aspergillus Fumigatus Chrysovirus seg 1	*Chyrsoviridae*	FN178512.1
Aspergillus Fumigatus Chrysovirus seg 2	*Chyrsoviridae*	FN178513.2
Aspergillus Fumigatus Chrysovirus seg 3	*Chyrsoviridae*	FN178514.1
Aspergillus Fumigatus Chrysovirus seg 4	*Chyrsoviridae*	FN178515.1
Fusarium Oxysporum Chyrsovirus 1	*Chyrsoviridae*	EF152346.1
Aster Yellows Witches Broom Phytoplasma	*Acholeplamataceae*	NC_007716.1
Aster Yellows 16S rRNA	*Acholeplamataceae*	JQ675713.1
Maryland Aster Yellows Phytoplasma	*Acholeplamataceae*	DQ837760.1
Grapevine Leafroll-associated Virus 1	*Closteroviridae*	NC_016509.1
Grapevine Leafroll-associated Virus 2	*Closteroviridae*	DQ286725.2
Grapevine Leafroll-associated Virus 2 isolate 3138/07	*Closteroviridae*	JX559644.1
Grapevine Leafroll-associated Virus 2 isolate 93/955	*Closteroviridae*	AY881628.1
Grapevine Leafroll-associated Virus 3 isolate 621	*Closteroviridae*	GQ352621.1
Grapevine Leafroll-associated Virus 3 isolate GP18	*Closteroviridae*	EU259806.1
Grapevine Leafroll-associated Virus 3 isolate PL20	*Closteroviridae*	GQ352633.1
Grapevine Leafroll-associated Virus 3 var. grp. IV	*Closteroviridae*	HQ401015.1
Grapevine Leafroll-associated Virus 3 isolate CI817	*Closteroviridae*	EU344894.1
Grapevine Leafroll-associated Virus 3 var. grp. V	*Closteroviridae*	HQ401016.1
Grapevine Leafroll-associated Virus 3 isolate GH30	*Closteroviridae*	JQ655296.1
Grapevine Leafroll-associated Virus 3 isolate NZ2	*Closteroviridae*	JX220899.2
Grapevine Leafroll-associated Virus 3 isolate GH24	*Closteroviridae*	KM058745
Grapevine Leafroll-associated Virus 4	*Closteroviridae*	NC_016416.1
Grapevine Leafroll-associated Virus 5	*Closteroviridae*	NC_016081.1
Grapevine Leafroll-associated Virus 6	*Closteroviridae*	NC_016417.1
Grapevine Leafroll-associated Virus 7	*Closteroviridae*	NC_016436.1
Grapevine Leafroll-associated Virus 9	*Closteroviridae*	AY297819.1
Grapevine Chrome Mosaic Virus	*Secoviridae*	NC_003622.1
Grapevine Asteroid Mosaic-associated Virus	*Tymoviridae*	AJ249357.2
Grapevine Anatolian Ringspot Virus	*Secoviridae*	NC_018383.1
Tobacco Mosaic Virus	*Virgaviridae*	NC_001367.1
Penicillium Chrysogenum Virus seg 1	*Chyrsoviridae*	NC_007539.1
Penicillium Chrysogenum Virus seg 2	*Chyrsoviridae*	NC_007540.1
Penicillium Chrysogenum Virus seg 3	*Chyrsoviridae*	NC_007541.1
Penicillium Chrysogenum Virus seg 4	*Chyrsoviridae*	NC_007542.1

Table S.4: Fraction of the reference covered for each virus determined per sample through read-mapping. Viruses illustrated in red had a greater than 80% reference coverage, viruses represented in green had less that 10% coverage of the reference.

Sample:	Year Sampled:	Cultivar:	Area:	Number of Reads (X 1 000 000)	GLRaV-2 (CG)	GLRaV-2 (3138)	GLRaV-2 (93|955)	GLRaV-3_1	GLRaV-3_2	GLRaV-3_3	GLRaV-3_4	GLRaV-3_5	GLRaV-3_6 (GH30)	GLRaV-3_6 (NZ2)	GLRaV-3_6 (GH24)	GLRaV-4	GLRaV-6	GLRaV-9	GRSPaV	Grapevine Fleck Virus	GVA_1	GVA_2	GVA_3	GVB	GVE	GVF	GEV	Hop Stunt	Australian Grapevine	Grapevine Yellow Speckled 1	Grapevine Yellow Speckled 2	Grapevine Viroid Clone Cari2	Grapevine-associated Narnavirus-1	Alfalfa Mosaic Virus	Aster Yellows WitchesBroom Phytoplasma	Aster Yellows 16S	Grapevine Anatolian Ringspot Virus	Grapevine Bulgarian Latent Virus	Grapevine Deformation Virus	Grapevine Fanleaf Virus	Grapevine Pinot Gris Virus	Grapevine Red Blotch-associated Virus	Grapevine Syrah Virus	Petunia Asteroid Mosaic Virus	Raspberry Ringspot Virus	Grapevine Rupestris Vein Feathering Virus	Tobacco Mosaic Virus	Tomato Blackring Virus	Tomato Mosaic Virus	Tomato Spotted Wilt Virus
GH23	2012	Cabernet Sauvignon	Stellenbosch	4.71																																											
GH24	2012	Cabernet Sauvignon	Stellenbosch	2.40																																											
38.36.31	2013	Carignan	Malmesbury	18.60																																											
V6	2013	Merlot	Vredendal	6.36																																											
27.74.16	2013	Pinot Noir	Hermanus	3.19																																										
10.15.14	2013	Shiraz	Wolseley	6.73																																										
H36	2012	Shiraz	Rawsonville	0.95																																										
1.49.1	2013	Chardonnay	Malmesbury	5.71																																									
V1	2013	Chardonnay	Vredendal	17.64																																									
V3	2013	Chardonnay	Vredendal	15.59																																									
H38	2012	Chenin Blanc	Rawsonville	2.35																																									
V5	2013	Chenin Blanc	Vredendal	30.48																																									
H35	2012	Semillion	Stellenbosch	2.13																																									
V4R	2013	Ramsey	Vredendal	11.99																																									
49STOK	2013	Richter '99	Malmesbury	27.12																																									
BJ3	2013	Richter '99	Stellenbosch	8.91																																									
BJ4	2013	Richter '99	Stellenbosch	22.76																																									

Table S.5: List of chemicals used for dsRNA and total RNA extractions with corresponding solution components, concentrations, pH, molecular formula, supplier and catalogue number.

Chemicals Table:							
Solution:	**Components:**	**Concentration:**	**pH:**	**Molecular Formula:**	**Molecular Weight (g/mol):**	**Supplier:**	**Catalogue Number:**
10 X STE Buffer*	Sodium Chloride	100 mM	6.8	NaCl	58.44	Merck (Saarchem)	1.06404.0500
	Trizma Base	50 mM		$C_4H_{11}NO_3$	121.14	Sigma-Aldrich	SLBB0099V
	Disodium Salt Dihydrate	1 mM		$C_{10}H_{14}N_2Na_2O_8.2H_2O$/ Na_2EDTA	372.24	Merck (Saarchem)	SAAR2236020EM
CTAB Buffer	CTAB	2%	8.0	$C_{19}H_{42}BrN$	364.46	Merck (Saarchem)	1569500EM
	Polyvinylpyrrolidone (PVP-40)	2.5%		C_6H_9NO	111.14	Sigma-Aldrich	BCBD7485V
	Trizma Base	100 mM		$C_4H_{11}NO_3$	121.14	Sigma-Aldrich	SLBB0099V
	Sodium Chloride	2 M		NaCl	58.44	Merck (Saarchem)	1.06404.0500
	Disodium Salt Dihydrate	25 mM		$C_{10}H_{14}N_2Na_2O_8.2H_2O$/ Na_2EDTA	372.24	Merck (Saarchem)	SAAR2236020EM
1 X TE Buffer	Trizma Base	1 M	7.6	$C_4H_{11}NO_3$	121.14	Sigma-Aldrich	SLBB0099V
	Disodium Salt Dihydrate	0.5 M		$C_{10}H_{14}N_2Na_2O_8.2H_2O$/ Na_2EDTA	372.24	Merck (Saarchem)	SAAR2236020EM
50 X TAE Buffer	Trizma Base	2 M	8.0	$C_4H_{11}NO_3$	121.14	Sigma-Aldrich	SLBB0099V
	Disodium Salt Dihydrate	50 mM		$C_{10}H_{14}N_2Na_2O_8.2H_2O$/ Na_2EDTA	372.24	Merck (Saarchem)	SAAR2236020EM
Sodium Lauryl Sulphate (SDS)		10%	7.2	$(CH_3(CH_2)_{10}CH_2OSO_3)$	288.38	Merck (Saarchem)	SAAR5823610EM
Bentonite	Montmorillonile	40 mg/ml			-	Sigma-Aldrich	MKBF9623V
2-Mercaptoethanol		-		C_2H_6OS	78.13	Sigma-Aldrich	BCBF9538V
Sodium Acetate (NaOAc)		3 M	5.5	CH3COONa.3H2O	136.08	Merck (Saarchem)	SAAR5821010EM
Chloroform		-		$CHCl_3$	119.38	Merck (Saarchem)	SAAR1595040LC
Phenol		-		C_6H_6O	94.11	Merck (Saarchem)	SAAR4971000EM
Absolute Ethanol		96%		CH_3CH_2OH	46.07	Merck (Saarchem)	SAAR2233540LP
Cellulose	Cellulose Powder MN2100	-			-	Macherey-Nagel	512142911
Agarose		-			-	Sigma-Aldrich	SLBD2504V
Lithium Chloride Anhydrous		8 M		LiCl	42.39	Merck (Saarchem)	SAAR3945320EM
Hydrochloric Acid		32%		HCl	36.46	Merck (Saarchem)	SAAR3063040LP
Sodium Hydroxide		10 M		NaOH	40	Merck (Saarchem)	5823200EM
Ethidium Bromide							
16% EtOH 1 X STE	Absolute Ethanol	16%		CH_3CH_2OH	46.07	Merck (Saarchem)	SAAR2233540LP
	10 X STE	1 X		See Above *			
Dnase Buffer	Trizma Base	400 mM		$C_4H_{11}NO_3$	121.14	Promega	M198A
	Magnesium Sulphate Heptahydrate	100 mM		$MgSO_4.7H_2O$	246.48		
	Calcium Chloride Dihydrate	10 mM		$CaCl_2.2H_2O$	147.02		
Dnase RQ1		1 U/µl				Promega	M610A
Rnase T1		100 000 U/ml				Roche	10109193001
Acetic Acid Glacial	Assay	MIN. 99.8 %		CH_3COOH	60.05	Kimix	2789 1/46

Table S.6: GVF isolate representatives of the replicase gene with corresponding lengths and GenBank accession numbers. Used for multiple alignments with GVF isolate V5.

Sequence Description:	Length (bp):	Accession Number:
Grapevine virus F isolate 90/0246 replicase gene, partial cds	288	KF892543.1
Grapevine virus F isolate ARM 12 V25 replicase gene, partial cds	313	KF719053.1
Grapevine virus F isolate K4-77 replicase gene, partial cds	313	KF719052.1
Grapevine virus F isolate K10-13 replicase gene, partial cds	313	KF719051.1
Grapevine virus F isolate K9-21 replicase gene, partial cds	313	KF719050.1
Grapevine virus F isolate ARM 13 V49 replicase gene, partial cds	313	KF719049.1
Grapevine virus F isolate ARM 12 V22 replicase gene, partial cds	313	KF719048.1
Grapevine virus F isolate ARM 14 V22 replicase gene, partial cds	313	KF719047.1
Grapevine virus F isolate GLRV1-GH7 replicase gene, partial cds	313	KF719046.1
Grapevine virus F isolate K1-2 replicase gene, partial cds	313	KF719045.1
Grapevine virus F isolate K3-76 replicase gene, partial cds	313	KF719044.1
Grapevine virus F isolate K9-17 replicase gene, partial cds	313	KF719043.1
Grapevine virus F isolate K1-11 replicase gene, partial cds	313	KF719042.1
Grapevine virus F isolate K1-10 replicase gene, partial cds	313	KF719041.1
Grapevine virus F isolate K3-21 replicase gene, partial cds	313	KF719040.1
Grapevine virus F isolate GLRV2RG-GH7 replicase gene, partial	313	KF719039.1
Grapevine virus F isolate K15-3 replicase gene, partial cds	313	KF719038.1
Grapevine virus F isolate K9-70 replicase gene, partial cds	313	KF719037.1
Grapevine virus F isolate K4-1 replicase gene, partial cds	313	KF719036.1
Grapevine virus F isolate K3-35 replicase gene, partial cds	313	KF719035.1
Grapevine virus F isolate ARM 9 V7 replicase gene, partial cds	313	KF719034.1
Grapevine virus F isolate K2-36 replicase gene, partial cds	313	KF719033.1
Grapevine virus F isolate K14-5 replicase gene, partial cds	313	KF719032.1
Grapevine virus F isolate ARM 8 V43 replicase gene, partial cds	313	KF719031.1
Grapevine virus F isolate K7-30 replicase gene, partial cds	313	KF719030.1
Grapevine virus F isolate K7-67 replicase gene, partial cds	313	KF719028.1
Grapevine virus F isolate K11-42 replicase gene, partial cds	313	KF719029.1
Grapevine virus F isolate K3-72 replicase gene, partial cds	313	F719027.1
Grapevine virus F isolate K5-56 replicase gene, partial cds	313	KF719026.1
Grapevine virus F isolate ARM 9 V13 replicase gene, partial cds	313	KF719025.1
Grapevine virus F isolate ARM 8 V13 replicase gene, partial cds	313	KF719024.1
Grapevine virus F isolate ARM 14 V4 replicase gene, partial cds	313	KF719023.1
Grapevine virus F isolate K10-68 replicase gene, partial cds	313	KF719022.1
Grapevine virus F isolate AUD46129, complete genome	7551	JX105428.1

Table S.7: Oligonucleotide adaptors for Truseq RNA and DNA Sample Prep Kits.

TruSeq Adapters:	Sequence (5' - 3'):
Universal	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
Index 1	GATCGGAAGAGCACACGTCTGAACTCCAGTCACATCACGATCTCGTATGCCGTCTTCTGCTTG
Index 2	GATCGGAAGAGCACACGTCTGAACTCCAGTCACCGATGTATCTCGTATGCCGTCTTCTGCTTG
Index 3	GATCGGAAGAGCACACGTCTGAACTCCAGTCACTTAGGCATCTCGTATGCCGTCTTCTGCTTG
Index 4	GATCGGAAGAGCACACGTCTGAACTCCAGTCACTGACCAATCTCGTATGCCGTCTTCTGCTTG
Index 5	GATCGGAAGAGCACACGTCTGAACTCCAGTCACACAGTGATCTCGTATGCCGTCTTCTGCTTG
Index 6	GATCGGAAGAGCACACGTCTGAACTCCAGTCACGCCAATATCTCGTATGCCGTCTTCTGCTTG
Index 7	GATCGGAAGAGCACACGTCTGAACTCCAGTCACCAGATCATCTCGTATGCCGTCTTCTGCTTG
Index 8	GATCGGAAGAGCACACGTCTGAACTCCAGTCACACTTGAATCTCGTATGCCGTCTTCTGCTTG
Index 9	GATCGGAAGAGCACACGTCTGAACTCCAGTCACGATCAGATCTCGTATGCCGTCTTCTGCTTG
Index 10	GATCGGAAGAGCACACGTCTGAACTCCAGTCACTAGCTTATCTCGTATGCCGTCTTCTGCTTG
Index 11	GATCGGAAGAGCACACGTCTGAACTCCAGTCACGGCTACATCTCGTATGCCGTCTTCTGCTTG
Index 12	GATCGGAAGAGCACACGTCTGAACTCCAGTCACCTTGTAATCTCGTATGCCGTCTTCTGCTTG
Index 13	GATCGGAAGAGCACACGTCTGAACTCCAGTCACAGTCAACAATCTCGTATGCCGTCTTCTGCTTG
Index 14	GATCGGAAGAGCACACGTCTGAACTCCAGTCACAGTTCCGTATCTCGTATGCCGTCTTCTGCTTG
Index 15	GATCGGAAGAGCACACGTCTGAACTCCAGTCACATGTCAGAATCTCGTATGCCGTCTTCTGCTTG
Index 16	GATCGGAAGAGCACACGTCTGAACTCCAGTCACCCGTCCCGATCTCGTATGCCGTCTTCTGCTTG
Index 18	GATCGGAAGAGCACACGTCTGAACTCCAGTCACGTCCGCACATCTCGTATGCCGTCTTCTGCTTG
Index 19	GATCGGAAGAGCACACGTCTGAACTCCAGTCACGTGAAACGATCTCGTATGCCGTCTTCTGCTTG
Index 20	GATCGGAAGAGCACACGTCTGAACTCCAGTCACGTGGCCTTATCTCGTATGCCGTCTTCTGCTTG
Index 21	GATCGGAAGAGCACACGTCTGAACTCCAGTCACGTTTCGGAATCTCGTATGCCGTCTTCTGCTTG
Index 22	GATCGGAAGAGCACACGTCTGAACTCCAGTCACCGTACGTAATCTCGTATGCCGTCTTCTGCTTG
Index 23	GATCGGAAGAGCACACGTCTGAACTCCAGTCACGAGTGGATATCTCGTATGCCGTCTTCTGCTTG
Index 25	GATCGGAAGAGCACACGTCTGAACTCCAGTCACACTGATATATCTCGTATGCCGTCTTCTGCTTG
Index 27	GATCGGAAGAGCACACGTCTGAACTCCAGTCACATTCCTTTATCTCGTATGCCGTCTTCTGCTTG

Protocol S.1: Illumina TruSeq RNA Sample Preparation (Adapted protocol, truncated for dsRNA as input RNA)

DAY 1

1) Fragment dsRNA

- Pellet dsRNA at 12 000g for 60'
- Wash pellet with 70% EtOH (be careful not to loose the pellet, it will be very small ~invisible)
- Air dry pellet for 20' @ RT
- Add 19.5ul of EPF and gently pipette up and down 6 times. (EPF contains random hexamers for RT priming and serves as 1st strand cDNA synthesis rxn buffer). *(*EPF back to -20C)*
- Transfer the entire rxn to a PCR tube (beads included, according to HT protocol). Tubes are still labeled "RBP".

Incubate RFP

- Tube to PCR machine (program 94C for 8min, 4C hold).

*(*The use of a PCR machine is essential as the primers need to bind - flash freezing will not work for this step)*

- When it reaches 4C, give it a quick flick down and proceed immediately to 1st strand cDNA synthesis.

2) Synthesize First Strand cDNA

Preparation

- Remove from -20C: FSM (thaw at RT and then keep at 4C)
- Remove from -20C: SuperScript II Reverse Transcriptase (SRT) (keep at 4C)
- Add 50ul of SuperScript II Reverse Transcriptase to the FSM (First Strand Master Mix) tube. Mix gently, but thoroughly, centrifuge briefly. Aliquot into 50ul stocks (6 rxns aliquots) and keep at -20C. In future will only need to work with the aliquots.

- PCR machine pre-programmed:
  - 25C for 10min
  - 42C for 50min
  - 70C for 15min
  - 4C hold

Make CDP

- No plate magnet – so stick the "RBP" PCR tube to the magnet with a piece of tape. RT (laminar flow), 5min.
- Without removing the tube from the magnet, carefully transfer (2x) 8,5ul of the supernatant (fragmented and primed mRNA) to a new PCR tube. Tubes are labeled "CDP".
- Add 8ul of FSM-SRT mix and gently pipette up and down 6 times.

*(*FSM-SRT back to -20C)*

Incubate 1 CDP

- Tube to PCR machine (program 25C for 10min, 42C for 50min, 70C for 15min, 4C hold).
- When it reaches 4C, give it a quick flick down and proceed immediately to 2nd strand cDNA synthesis.

3) Synthesize Second Strand cDNA

Preparation

- Remove from -20C: SSM and RSB (thaw at RT and then keep at 4C).
- Remove from 4C: AMPure XP beads and Absolute EtOH (in a box labeled TruSeq RNAseq 4C box). These reagents are used at RT.
- Freshly prepare 80% EtOH.
- PCR machine (programmed 16C for 1h, 25C hold).

Add SSM

- Add 25ul of SSM to the "CDP" PCR tube and gently pipette up and down 6 times. *(*SSM back to -20C)*

Incubate 2 CDP

- Tube to PCR machine (program 16C for 1h, 25C hold)

Clean up CDP

- AMPure XP beads and RSB must be at RT. Vortex AMPure XP beads well before use.
- Transfer the entire rxn to a 1.5ml tube. Tubes are still labeled "CDP".
- Add 90ul of AMPure XP beads and gently pipette up and down 10 times.
- RT (Hybex 25C), 15min.
- Tube to magnet RT (laminar flow), 5min.
- Remove and discard 135ul of the supernatant. Do not disturb the pellet. Some liquid (±2,5ul) will remain in the tube.
- Leave the tube on the magnetic stand during the 80% EtOH washes.
- 1) Add 200ul of freshly prepare 80% EtOH without disturbing the beads.
- 1) Incubate at RT (laminar flow) for 30 sec.
- 1) Remove and discard all of the supernatant. Do not disturb the pellet.

- 2) Add 200ul of freshly prepare 80% EtOH without disturbing the beads.
- 2) Incubate at RT (laminar flow) for 30 sec.
- 2) Remove and discard all of the supernatant. Do not disturb the pellet. Try to remove all of the residual EtOH from the bottom of the tube, as it will contain impurities.
- Leaving the tube on the magnet, incubate at RT (laminar flow), 15min.
- The beads must be dry before continuing.
- Remove the tube from the magnet.
- Add 52,5ul of RSB and gently pipette up and down 10 times.
- RT (Hybex 25C), 2min.
- Tube to magnet RT (laminar flow), 5min.
- Transfer 50ul of the supernatant to a new 1.5ml tube. Tubes are labeled "IMP".

SAFE STOPPING POINT Store at -20C for up to 7 days.

DAY 2

4) Perform End Repair

Preparation

- If decide to run controls in the sample, prepare all of them before starting. Dilute 1ul (CTE, CTA, CTL) in 99ul of RSB, mix well and spin down. Refer to manual for details on this procedure.
- If decide not to use controls, replace with the same volume of RSB. In the protocol denoted here the controls were excluded.
- Remove from -20C: ERP and RSB (thaw at RT and then keep at 4C).
- Remove from -20C: The "IMP" tube (thaw at RT and then keep at 4C).
- Remove from 4C: AMPure XP beads and Absolute EtOH (in a box labeled TruSeq RNAseq 4C box). These reagents are used at RT.
- Freshly prepare 80% EtOH.
- Heating block to 30C.
- Hybex microsample incubator to 25C.

Make IMP

- Add 10ul of RSB (or diluted CTE control) to the "IMP" tube.
- Add 40ul of ERP and gently pipette up and down 10 times.

*(*ERP back to -20C)*

Incubate 1 IMP

- Tube to 30C heating block, 30min.

*(*Change the heating block temperature to 37C for next step)*

Clean Up IMP

- AMPure XP beads and RSB must be at RT. Vortex AMPure XP beads well before use.
- Add 160ul of AMPure XP beads and gently pipette up and down 10 times.
- RT (Hybex 25C), 15min.
- Tube to magnet RT (lab bench), 5min.
- Remove and discard 127,5ul (x2) of the supernatant. Do not disturb the pellet. Some liquid (±2,5ul) will remain in the tube.
- Leave the tube on the magnetic stand during the 80% EtOH washes.

- 1) Add 200ul of freshly prepare 80% EtOH without disturbing the beads.
- 1) Incubate at RT (lab bench) for 30 sec.
- 1) Remove and discard all of the supernatant. Do not disturb the pellet.
- 2) Add 200ul of freshly prepare 80% EtOH without disturbing the beads.
- 2) Incubate at RT (lab bench) for 30 sec.
- 2) Remove and discard all of the supernatant. Do not disturb the pellet. Try to remove all of the residual EtOH from the bottom of the tube, as it will contain impurities.
- Leaving the tube on the magnet, incubate at RT (lab bench), 15min.
- The beads must be dry before continuing.
- Remove the tube from the magnet.
- Add 17,5ul of RSB and gently pipette up and down 10 times.
- RT (Hybex 25C), 2min.
- Tube to magnet RT (lab bench), 5min.
- Transfer 15ul of the supernatant to a new 1.5ml tube. Tubes are labeled "ALP".

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5) Adenylate 3' Ends

Preparation

- Remove from -20C: ATL and RSB (thaw at RT and then keep at 4C).
- If stored, remove from -20C: The "ALP" tube (thaw at RT and keep at 4C).
- Heating block to 37C.
- Hybex microsample incubator to 25C.

Add ATL

- Add 2,5ul of RSB (or diluted CTA control) to the "ALP" tube.
- Adjust pipette to 30ul and gently pipette up and down 10 times.
- Add 12,5ul of ATL. Do not mix.

*(*ATL back to -20C)*

Incubate 1 ALP

- Tube to 37C heating block, 30min.

*(*Change the heating block temperature to 30C for next step. NB it will be difficult to reach 30C in such a short time!)*

- Remove the tube and proceed immediately to Ligate Adapters.

6) Ligate Adapters

Preparation

- Remove from -20C: STL and RNA Adapter Index tubes (AR001-AR012, depending on the required index) (thaw at RT and then keep at 4C).
- Remove from 4C: AMPure XP beads and Absolute EtOH (in a box labeled TruSeq RNAseq 4C box). These reagents are used at RT.
- Freshly prepare 80% EtOH.
- Heating block to 30C.
- NB: Leave LIG at -20C until it is used and then return immediately to -20C.

Add LIG

- Add 2,5ul of LIG to the "ALP" tube.

*(*LIG immediately back to -20C)*

- Add 2,5ul of RSB (or diluted CTL control).
- Add 2,5ul of RNA Adapter Index to each sample as required.

*(*RNA Adapter Index back to -20C)*

- Adjust the pipette to 40ul and gently pipette up and down 10 times.

Incubate 2 ALP

- Tube to 30C heating block, 10min.

*(*After this step the heating block can be switched off)*

- To RT (lab bench).

Add STL

- Add 5ul of STL and gently pipette up and down 10 times.

*(*STL back to -20C)*

Clean Up ALP 1

- AMPure XP beads and RSB must be at RT. Vortex AMPure XP beads well before use.
- Add 42ul of AMPure XP beads and gently pipette up and down 10 times.
- RT (Hybex 25C), 15min.
- Tube to magnet RT (lab bench), 5min.
- Remove and discard 79,5ul of the supernatant. Do not disturb the pellet. Some liquid (±2,5ul) will remain in the tube.
- Leave the tube on the magnetic stand during the 80% EtOH washes.
- 1) Add 200ul of freshly prepare 80% EtOH without disturbing the beads.
- 1) Incubate at RT (lab bench) for 30 sec.
- 1) Remove and discard all of the supernatant. Do not disturb the pellet.
- 2) Add 200ul of freshly prepare 80% EtOH without disturbing the beads.
- 2) Incubate at RT (lab bench) for 30 sec.
- 2) Remove and discard all of the supernatant. Do not disturb the pellet. Try to remove all of the residual EtOH from the bottom of the tube, as it will contain impurities.
- Leaving the tube on the magnet, incubate at RT (lab bench), 15min.
- The beads must be dry before continuing.
- Remove the tube from the magnet.
- Add 52,5ul of RSB and gently pipette up and down 10 times.
- RT (Hybex 25C), 2min.
- Tube to magnet RT (lab bench), 5min.
- Transfer 50ul of the supernatant to a new 1.5ml tube. Tubes are labeled "CAP".

Clean Up ALP 2

- Vortex AMPure XP beads well before use. Then add 50ul of AMPure XP beads and gently pipette up and down 10 times.
- RT (Hybex 25C), 15min.
- Tube to magnet RT (lab bench), 5min.
- Remove and discard 95ul of the supernatant. Do not disturb the pellet. Some liquid (±2,5ul) will remain in the tube.
- Leave the tube on the magnetic stand during the 80% EtOH washes.
- 1) Add 200ul of freshly prepare 80% EtOH without disturbing the beads.

- 1) Incubate at RT (lab bench) for 30 sec.
- 1) Remove and discard all of the supernatant. Do not disturb the pellet.
- 2) Add 200ul of freshly prepare 80% EtOH without disturbing the beads.
- 2) Incubate at RT (lab bench) for 30 sec.
- 2) Remove and discard all of the supernatant. Do not disturb the pellet. Try to remove all of the residual EtOH from the bottom of the tube, as it will contain impurities.
- Leaving the tube on the magnet, incubate at RT (lab bench), 15min.
- The beads must be dry before continuing.
- Remove the tube from the magnet.
- Add 22,5ul of RSB and gently pipette up and down 10 times.
- RT (Hybex 25C), 2min.
- Tube to magnet RT (lab bench), 5min.
- Transfer 20ul of the supernatant to a new 0,2ml PCR tube. Tubes are labeled "PCR".

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7) Enrich DNA Fragments

Preparation

- Remove from -20C: PMM and PPC (thaw at RT and then keep at 4C).
- Remove from -20C: The "PCR" tube (thaw at RT and then keep at 4C).
- Remove from 4C: AMPure XP beads and Absolute EtOH (in a box labeled TruSeq RNAseq 4C box). These reagents are used at RT.
- Freshly prepare 80% EtOH.
- Hybex microsample incubator to 25C.
- PCR machine pre-programmed:
    - 98C for 30sec
    - 15 cycles of:
        - 98C for 10sec
        - 60C for 30sec
        - 72C for 30sec
    - 72C for 5min
    - 4C hold

Make PCR

- Add 5ul of PPC to the "PCR" tube.
- Add 25ul of PMM. Adjust the pipette to 40ul and gently pipette up and down 10 times. *(*PPC* and *PMM back to -20C)*

Amp PCR

- Tube to PCR machine. (Pre-programmed: 98C for 30sec; 15 cycles of 98C for 10sec, 60C for 30sec, 72C for 30sec; 72C for 5min; 4C hold).

Clean Up PCR

- AMPure XP beads and RSB must be at RT. Vortex AMPure XP beads well before use.
- Transfer the entire reaction to a 1.5ml tube.
- Add 50ul of AMPure XP beads and gently pipette up and down 10 times.
- RT (Hybex 25C), 15min.
- Tube to magnet RT (lab bench), 5min.
- Remove and discard 95ul of the supernatant. Do not disturb the pellet. Some liquid (±2,5ul) will remain in the tube.

- Leave the tube on the magnetic stand during the 80% EtOH washes.
- 1) Add 200ul of freshly prepare 80% EtOH without disturbing the beads.
- 1) Incubate at RT (lab bench) for 30 sec.
- 1) Remove and discard all of the supernatant. Do not disturb the pellet.
- 2) Add 200ul of freshly prepare 80% EtOH without disturbing the beads.
- 2) Incubate at RT (lab bench) for 30 sec.
- 2) Remove and discard all of the supernatant. Do not disturb the pellet. Try to remove all of the residual EtOH from the bottom of the tube, as it will contain impurities.
- Leaving the tube on the magnet, incubate at RT (lab bench), 15min.
- The beads must be dry before continuing.
- Remove the tube from the magnet.
- Add 32,5ul of RSB and gently pipette up and down 10 times.
- RT (Hybex 25C), 2min.
- Tube to magnet RT (lab bench), 5min.
- Transfer 30ul of the supernatant to a new 1.5ml tube. Tubes are labeled “TSP1”.
- This is the final prepared library.

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